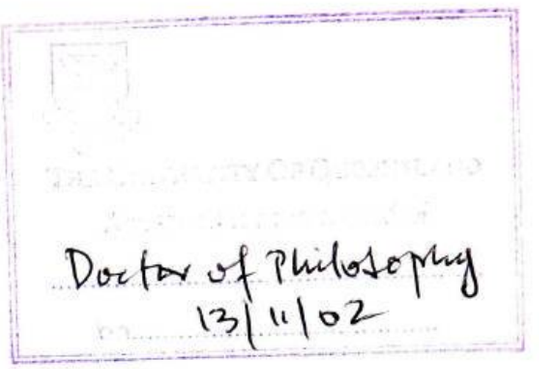

Doctor of Philosophy
13/11/02

ANTIGENIC AND STRUCTURAL ANALYSIS OF THE NS1 GLYCOPROTEIN OF DENGUE VIRUS

Thesis submitted for the degree of Doctor of Philosophy

Department of Microbiology and Parasitology

School of Molecular and Microbial Sciences

University of Queensland

by

Cheryl Bletchly B. Sc. (Hons)

on

8th May, 2002

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is cited in the text.

I give consent for this copy of my thesis, when deposited in the University of Queensland Library, to be available for photocopying and loan by staff and students of this University only, 3 years after the award of the degree.

C. Bletchly 8.5.2002

Cheryl Bletchly Date

ACKNOWLEDGMENTS

Although the collation of a thesis is largely an individual effort a PhD is not possible without the assistance of numerous people.

I would like to acknowledge Professor John Mackenzie as principal supervisor of this project and in particular my co-supervisor, Dr Paul Young for his enormous help and support throughout this PhD and for managing to obtain continued financial support for this work.

Dr. Bruce Innis (formerly of AFRIMS, Bangkok, Thailand) kindly supplied us with the dengue patient sera on which Chapter 3 is based, and thanks also goes to Dr Jenny Martin, 3D Centre, UQ for her advice and for allowing us to use her facilities to set up our crystal screens.

Thanks also to my friends and colleagues at QHSS, Public Health Virology for being so supportive during my monumental struggle to "write up" while working full-time, especially David and Ina for generously offering to read this thesis and for helpful suggestions and advice, and to Greg, who has been an inspiration.

A big thank you must also go to my parents, who have always valued education and encouraged me to continue studying, and who have made many sacrifices to make that possible.

Last but not least, thanks to Paul who listened, encouraged, laughed, understood, read (and read again!), agreed, disagreed, supported and put it all in to perspective.

PUBLICATIONS

Young PR, **Bletchly C** and Mackenzie J (1993) Antigenic and structural analysis of the dengue virus glycoprotein, NS1. Proceedings of the 6th Symposium on Arbovirus Research in Australia 138-142.

Young PR, Hilditch PA, **Bletchly C** and Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue protein NS1 in the sera of infected patients. J Clin Micro **38**, 1053-1057.

Jacobs MG, Robinson PJ, **Bletchly C**, Mackenzie JM and Young PR (2000) Dengue virus nonstructural protein 1 is expressed in a glycosylphosphatidyl-inositol-linked form that is capable of signal transduction. The FASEB J **14**, 1603-1610.

Chan LCL, Young PR, **Bletchly C** and Reid S. (Accepted 2002) Using a Statistically-Optimised Fed-Batch Regime to Improve Production of a Low-Yielding Baculovirus-Expressed Dengue Protein. J Virol Methods.

CONFERENCE ABSTRACTS ACCEPTED FOR PRESENTATION

A Model for the Antigenic Structure of the Dengue Virus Glycoprotein, NS1. P. Young and **C. Bletchly**. Presented at Second Asia-Pacific Congress of Medical Virology, Bangkok, November 1991. (Also published in "Virus Diseases: The Global Challenge to Health for All.")

A Model for the Antigenic Structure of the Dengue Virus Glycoprotein, NS1. **C. Bletchly** and P. Young. Presented at ASMNZMS Conference, Sydney, July 1992.

Antigenic Structure of the Dengue Virus Glycoprotein, NS1. P.R. Young, **C. Bletchly** and A. Doubrovsky. Presented at the Third International Positive Strand RNA Symposium, Florida, September 1992.

Antigenic and Structural Analysis of the Dengue Virus Glycoprotein, NS1. P. Young, **C. Bletchly** and J. Mackenzie. Presented at the 6th Arbovirus Symposium, Brisbane, December 1992.

The Dengue Virus Non-Structural Glycoprotein, NS1: Structure Predictions. P. Young, **C. Bletchly**, J. Mackenzie and A. Doubrovsky. Presented at the IXth International Congress of Virology, Glasgow, August 1993.

Antigenic and structural analysis of the dengue virus nonstructural glycoprotein, NS1. **C. Bletchly** and P.R. Young. Presented at the IVth International Positive Strand RNA Virus Symposium, Utrecht, The Netherlands, May 1995.

Large-scale production of recombinant baculovirus expressed dengue virus NS1 protein. **C. Bletchly**, H. Leblois and P. Young. Presented at the Australian Society for Microbiology Annual Scientific Meeting, Christchurch, New Zealand, September 1996.

Dengue virus NS1 trafficking in the presence of secretory pathway inhibitors. **C. Bletchly** and P.R. Young. Presented at the Australian Society for Microbiology Annual Scientific Meeting, Adelaide, Australia, September, 1997.

Glycosyl-phosphatidylinositol (GPI) anchoring of dengue virus NS1 mediates signal transduction in the presence of NS1 - specific antibodies. P. Young, M. Jacobs, P. Robinson, **C. Bletchly** and J. Mackenzie. Presented at the XIth International Congress of Virology, Sydney, Australia, August, 1999.

ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
APS	ammonium persulfate
ATCC	American Type Culture Collection
BSA	bovine serum albumin
CMC	carboxymethyl cellulose
CPE	cytopathic effect
cpm	counts per minute
CTL	cytotoxic T-lymphocyte(s)
DAB	3,4,3',4',-tetra-aminobiphenyl hydrochloride
DSS	dengue shock syndrome
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GPI	glycosyl phosphatidylinositol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC	intracerebral
IFN	interferon
IP	intraperitoneal
JEV	Japanese encephalitis virus
mA	milliAmps

MAb	monoclonal antibody
MEM	minimal essential media
mNS1	membrane-associated NS1
m.o.i.	multiplicity of infection
MVE	Murray Valley encephalitis
NK	natural killer
NP40	nonidet P40
OPD	o-phenylenediamine
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS.T	phosphate buffered saline containing 0.5% Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.i.	post infection
PMSF	phenylmethylsulfonyl-fluoride
RdRp	RNA dependent RNA polymerase
RIP	radio immune precipitation
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sNS1	secreted NS1
TEMED	tetramethylene-diamine
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane

TX-100	triton X-100
WHO	World Health Organization
YF	Yellow fever

ABSTRACT

The dengue virus protein NS1, is the first non-structural protein to be translated in the dengue virus genome. It is glycosylated, which is unusual for a viral non-structural protein and exists as an oligomer in its native form. The function of NS1 is still largely unknown however there is evidence that it is involved in RNA replication. It is also capable of inducing an immune response in experimental animals, with antibodies to NS1 being able to confer passive protection against lethal virus challenge. The work that comprises this thesis sought to expand our understanding of NS1 by exploring the human immune response to dengue virus infection and the role of NS1 in pathogenesis, and to analyze in detail the antigenicity and structure of the protein by building on our existing data.

Both the human immune response to NS1 and the circulation of NS1 in the serum of dengue virus infected patients was examined. An antigen capture ELISA was first developed for the detection of dengue virus NS1 based on the selection of monoclonal and polyclonal antibodies as the probe and capture reagents respectively. The limit of detection for NS1 with the method developed was 4 ng / mL for dengue-2 virus infections and 15 ng / mL for other dengue serotypes detected with a cross - reactive monoclonal antibody (MAb) probe. Sera were obtained from dengue virus infected patients in Thailand and screened for the presence of NS1 and antibody to NS1. Examination of clinical samples demonstrated that the assay was able to detect NS1 with minimal interference from serum components at the test dilutions routinely used, suggesting that it could form the basis of a useful additional diagnostic test for dengue virus infection. The presence of high levels of secreted NS1 in the sera of patients experiencing secondary dengue virus infections in addition to high NS1 antibody concentrations, suggests that NS1 may be a significant contributor to the formation of circulating immune complexes that are hypothesized to play an important role in the pathogenesis of severe dengue disease.

Although NS1 has been reported to be immunogenic, little detailed information

exists regarding the antigenic epitopes on the native dimeric form of the protein. In this thesis, using a panel of anti - NS1 MAbs in a competitive ELISA, five major antigenic domains and several sub - domains were defined for the secreted form of NS1. Together with PEPSCAN data obtained previously in this laboratory, important protective and antigenic epitopes in the primary and tertiary sequence of NS1 were mapped. Using NS1 derived from both mammalian (Vero) and insect (mosquito) cells, and in both soluble and membrane - associated forms, it was found that the membrane-associated NS1 produced in Vero cells lacked one important antigenic and protective epitope. This may explain differences noted in protection of lethally challenged mice by the different forms of NS1. Partial enzymatic digestions of purified NS1 combined with N-terminal sequencing of the protein fragments obtained and an analysis of their reactivity with the panel of MAbs, provided further insights into the antigenic structure of NS1 at the amino acid sequence level. Detergent phase separation experiments carried out on these proteolytic fragments have identified that the hydrophobic character of NS1 resides in the N-terminal half of the molecule. All of this data has collectively enabled the construction of a structural model for NS1 that is presented in Chapter 4.

Progress was made toward identifying the parameters required for crystallization of dengue-2 NS1 with the expectation that a crystal structure would assist in defining the function of the protein. As the amount of NS1 secreted from dengue virus infected cells is not produced in sufficient quantities to purify for crystallography studies, NS1 was expressed as a recombinant protein in the baculovirus expression system. Pulse-chase experiments defined the optimum conditions for expression of recombinant secreted NS1 from *Spodoptera frugiperda* cells. Recombinant NS1 was immunoaffinty purified and found experimentally to resemble the authentic protein in terms of cellular localization, secretion, oligomerization, glycosylation and antigenicity (using the antigenic epitopes defined in Chapter 4). Cross-linking and electron microscopy studies provided support for the hexameric structure of both native and recombinant NS1. The isoelectric point of NS1 was determined to be 5.7 and concentrated protein (from 5 - 7 mg / mL) was used in crystallography trials. Both microcrystals and crystals were obtained under specific buffer conditions after ten weeks and nine months of incubation respectively. Unfortunately, these crystals

did not diffract and are therefore not useful in collating structural information. However, the successful establishment of a purification regime for NS1 has been achieved and is an important step towards successful crystallization and ultimately structure resolution.

TABLE OF CONTENTS

CHAPTER 3:

NS1 IN HUMAN SERA

CHAPTER 4:

EPITOPE MAPPING OF THE NS1 GLYCOPROTEIN OF DENGUE-2 VIRUS

CHAPTER 5:

TOWARDS THE CRYSTALLIZATION OF NS1: BACULOVIRUS EXPRESSION OF DENGUE-2 VIRUS NS1

CHAPTER 1

INTRODUCTION

1.1 FLAVIVIRUSES

(a) General properties and classification

In 1984 the International Committee for the Nomenclature of Viruses voted to make the *Flaviviridae* (from the Latin *flavus*, meaning yellow) a separate family (Westaway *et al*, 1986). Within this family there are currently three genera: the flaviviruses, the pestiviruses and hepaciviruses (which includes hepatitis C virus) (Ruggli and Rice, 1999) that show no serological cross-reactivity. These three groups have shared properties including a lipid envelope and a single stranded positive sense RNA genome containing a single open reading frame (ORF). Within the flaviviruses, eight serological sub-groups are recognized based on cross neutralization tests (Monath and Heinz, 1986; Calisher *et al*, 1989). Yellow fever (YF) virus is the prototype virus of the flavivirus group that comprises 68 members. Nucleotide sequence analysis of different flaviviruses has shown that they are closely related and have evolved from a common ancestor (Gould *et al*, 1985; Deubel *et al*, 1986; Rice *et al*, 1986; Hahn *et al*, 1988).

The majority of flaviviruses are human or veterinary pathogens (Westaway *et al*, 1985). Approximately 70% of these viruses are transmitted by infected mosquitoes or ticks to vertebrate hosts (Schlesinger and Schlesinger, 1990) and are therefore termed arboviruses (**ar**thropod-**bo**rne viruses). The significant spread of these viruses over the previous few decades has been a consequence of the disruption of the vector-vertebrate equilibrium (Chambers *et al*, 1990) with worldwide eradication considered unlikely due to their maintenance in animal reservoirs and transovarial transmission in arthropods (LeDuc, 1989). Combined, these viruses are a major public health problem although the dengue viruses are considered to be the most significant with up to 100 million infections occurring annually (Halstead, 1988;

Jacobs and Young, 1998) and over two billion people at risk of dengue infection in tropical areas (WHO, 1999).

(b) Historical Background

The study of flaviviruses began with the discovery by Walter Reed and colleagues in 1900 that the disease yellow fever was caused by a filterable agent and transmitted to humans by mosquitoes (Kelly, 1907). For more than two hundred years, yellow fever was one of the great plagues of the world (Strode, 1951; Theiler and Downs, 1973; WHO, 1971; WHO, 1986) that decimated populations and paralysed industry and trade (Tomori, 1999). The tropical and subtropical regions of the Americas were subjected to devastating epidemics and serious outbreaks were recorded in Spain, France, England and Italy (Strode, 1951). A large outbreak of yellow fever occurred in Ethiopia between 1960 and 1962 with 15 000 - 30 000 estimated deaths (Monath *et al*, 1980; WHO 1986). Similar outbreaks still occur in Africa (Anon, 1994) primarily in areas contiguous with rainforest regions (Soper, 1936) where jungle yellow fever is enzootic (Tomori, 1999).

Dengue fever is a very old disease with the earliest record of dengue-like illness found in a Chinese encyclopaedia of disease symptoms and remedies first published during the Chin dynasty (265-420 AD) (Gubler, 1999). Dengue was the second human disease (after yellow fever) whose etiology was identified as a "filterable virus" (Ashburn and Craig, 1907). Early authors have assumed that dengue virus was spread with the slave trade from East Africa to the Carribean, with an extensive outbreak occurring in the West Indies between 1827 and 1828 (Brown, 1977). During the 19th and 20th centuries, outbreaks have occurred on all continents and on the islands of the South Pacific and in the Carribean (Brown, 1977; Gratz and Knudsen, 1996). This extensive spread has continued to the present day. The first outbreak of DSS may have occurred in 1897 in Queensland (Hare, 1898). Dengue was a major cause of death in northern Australia until the beginning of World War I (Lumley and Taylor, 1943). During the last century major epidemics outside of the South East Asian region, where it has been endemic for more than 50 years, were recorded in the southern US (1920,1922) (Chandler and

Rice, 1923), Australia (1925 - 26, 1942, 1954 - 55) (McCallum and Dwyer, 1927), South Africa (1926 - 1927) (Kokernut *et al*, 1956), Greece (1927 - 28) (Copanaris, 1928), Japan (1942 - 45) (Yamada *et al*, 1943) and the Carribean (1963 - 69) (Downs, 1964). Although classical dengue is endemic in the Carribean, an outbreak in Cuba in 1981 with over 340 000 cases and 156 recorded deaths was the first outbreak in the Americas of the disease in its haemorrhagic form (Kouri *et al*, 1986). The Pacific phases of World War II brought the disease to prominence when dengue fever debilitated US army combat forces with the transport of previously unexposed personnel into endemic areas (Gilbertson, 1945; Gubler, 2002). This was the stimulus for the investigation of the disease and its causative agent(s). The major factors contributing to the expansion of the distribution of dengue viruses include the expanding urban population and environment (Gubler, 1999), an increase in vector density and the ease and frequency of rapid air travel allowing the daily movement of viremic individuals (Gubler and Clark, 1995; Gratz and Knudsen, 1996). Dengue viruses have since spread to many of the tropical countries of the world including Asia, Oceania, Africa, the Americas and Australia (Gubler, 2002). Significantly, several outbreaks of dengue fever have been documented in north Queensland over the last ten years (Mackenzie JS *et al*, 1998).

Hotta and his colleagues established three Swiss albino mouse-adapted strains of the virus from dengue epidemics in Japan in 1943 and 1944 (Kimura and Hotta, 1944). All four serotypes were subsequently adapted for growth in mice (Hammon *et al*, 1960; Meiklejohn *et al*, 1952). This was a very significant milestone in dengue virus research allowing the circumvention of humans as the only available host and facilitated subsequent developments in the propagation and quantitation of the viruses in *in vitro* cell cultures.

1.2 DISEASE AND TRANSMISSION

(a) Flaviviruses

Flaviviruses replicate in a wide range of vertebrate and invertebrate hosts

(Karabatsos, 1980; Kuno, 1982). The vector species of mosquitoes or ticks appear to be restricted for individual viruses (Monath, 1986). Humans are usually an incidental host in the arthropod-vertebrate cycle that maintains the viruses in nature (Monath and Heinz, 1996). The incubation period for vertebrates is several days and involves a short viremia. The pathogenesis and tissue tropism of flaviviruses vary from silent infections, fever with malaise, through to jaundice, haemorrhage and encephalitis (Monath and Heinz, 1996). Yellow fever (YF), Japanese encephalitis (JE), Saint Louis encephalitis (SLE), tick-borne encephalitis (TBE), Murray Valley encephalitis (MVE), West Nile (WN) and Kyasanur Forest Disease viruses are all important agents of regional endemic or epidemic disease (Monath, 1986). Recent topical flavivirus infections include West Nile virus encephalitis, due to an outbreak in New York (Lanciotti *et al*, 1999), and the recent emergence of Japanese encephalitis virus in northern Australia (Hanna *et al*, 1999).

(i) Dengue

Pathogenesis

Dengue fever (DF) can lead to the less common but potentially fatal dengue haemorrhagic fever (DHF) with or without dengue shock syndrome (DSS) (Gibson *et al*, 1988). In 1999 dengue and DHF were the second most important tropical infectious disease (after malaria), with over half the world's population (2.5 billion) living in areas at risk for dengue transmission (Gubler, 1999; 2002). It has been estimated that each year there are 50 - 100 million cases of dengue (Clarke, 2002), 500 000 hospitalizations and 25 000 deaths (WHO, 1999). There are four dengue serotypes that cause these disease epidemics of major proportions worldwide. They can be distinguished via complement fixation, haemagglutination inhibition and neutralization assays (Smithburn, 1954; Sabin and Schlesinger, 1954; Russell and Nisalak, 1967). The mosquito, *Aedes aegypti* is the primary vector for dengue virus transmission in urban environments (Simmons *et al*, 1931; Shope, 1980) although *Aedes albopictus* is also able to spread the disease (Gubler, 1988). Mosquitoes remain chronically infected for life (Gubler, 1999) and produce high levels of infectious virus particles in their salivary glands (Whitfield *et al*, 1973).

Zoonotic cycles of dengue virus transmission involving monkeys and forest *Aedes* sp. (*Ae. albopictus*, *Ae. polynesiensis* and other members of the *Ae. scutellaris* group) (Gubler and Meltzer, 1999) have been reported in Malaysia (Rudnick *et al*, 1967) and the west coast of Africa (Roche *et al*, 1983). The presence of the virus in the invertebrate host doesn't result in any apparent disease and replication in invertebrate cell culture shows demonstrably less cytopathic effect than in mammalian cell lines (Mussgay *et al*, 1975). When the mosquito takes a subsequent blood meal, virus is injected into the human along with salivary fluids (Gubler, 1999). The virus enters the bloodstream and is spread via lymphatics and peripheral blood mononuclear cells (PBMC), especially monocytes which support dengue virus replication. Virus can be isolated from the peripheral blood of infected patients during the febrile phase of the illness (Scott *et al*, 1980). Gubler *et al* (1981) reported that there was no difference in the amount of circulating virus in mild and severe disease however Vaughn *et al* (2000) recently demonstrated by mosquito inoculation, that for dengue-1 and dengue-2 infected patients a direct correlation existed between high viremia titres and disease severity. Viral antigens have been identified in mononuclear phagocytes in the skin, thymus, lymph nodes, Kupffer liver cells and the spleen (Bhamarapravati, 1981).

Dengue fever (DF) is the most common result of a dengue virus infection, a self-limiting flu-like illness. In a typical patient, symptoms commence abruptly after a two to seven day incubation period. This illness in humans is characterized by high fever, headache, pains in various parts of the body which may range from mild to severe, a generalized mottled rash, lymphadenopathy and leukopenia (Simmons *et al*, 1931; Siler *et al*, 1926; Shope, 1980). These initial symptoms are followed by bone pain of increasing severity (thus dengue is often referred to as "break-bone fever"), anorexia, vomiting, weakness and prostration. Respiratory symptoms are also common in children at this stage. These disease manifestations generally last from three to seven days whereupon the patient recovers without any complications (Shope, 1980). Treatment is supportive and includes bed rest, antipyretics and analgesics together with electrolyte replacement. Uncomplicated, primary dengue in a previously healthy individual is rarely, if ever, fatal. However the more severe complications of DHF and DSS which are characterized by haemorrhage and shock

(Cohen and Halstead, 1966; Halstead, 1980) are potentially life-threatening. WHO has categorized DHF / DSS into four clinical grades based on symptomatology (Table 1.1). Grade I is the mild febrile form of the disease without haemorrhagic symptoms. Shock is the only criteria that differentiates grade IV from grade III. The molecular basis of the pathogenesis of DF and DHF / DSS has yet to be fully elucidated.

Table 1.1 World Health Organization criteria for classification of DHF patients.

Grade of disease	Signs and symptoms
I	Fever accompanied by non-specific constitutional symptoms with a positive tournique test as the only haemorrhagic manifestation.
II	Same as grade I, except with spontaneous haemorrhagic manifestations.
III	Circulatory failure manifested by rapid, weak pulse with narrowing of the pulse pressure (< 20 mm Hg) or hypotension.
IV	Profound shock with undetectable blood pressure and pulse.

Modified from Henchal and Putnak (1990).

At the onset of symptoms DHF and DSS are indistinguishable from DF, however several days later there is a rapid deterioration. The clinical features of DHF include plasma leakage, bleeding and liver involvement as demonstrated by hepatomegaly and elevated serum transaminase levels (Rothman and Ennis, 1999). Plasma leakage is caused by a diffuse increase in capillary permeability (Kalayanarooj *et al*, 1997). As a consequence, fluid supplementation is required to prevent the circulating blood volume from dropping and causing shock. Bleeding manifestations are variable, ranging from a positive tourniquet test to life-threatening gastrointestinal haemorrhage (Gubler, 2002).

Antibody-dependent enhancement

Both type-specific and cross-reactive antibody responses are elicited in individuals following natural infection (Scott et al, 1972). Neutralizing antibodies against the flavivirus envelope proteins are generated and have been shown to confer long-lasting immunity against homologous serotypes of dengue (Sangkawibhaet et al, 1984; Kaufman et al, 1987), JE (Kimura-Kuroda and Yasui, 1988; Mason et al, 1989), and YF (Gould et al, 1986). In most cases, passive protection has been correlated with the ability of these anti-E antibodies to neutralize the virus in vitro. Kaufman et al (1989) have demonstrated that passive protection can also be produced with prM specific monoclonal antibodies that exhibit weak or undetectable neutralizing activity in vitro. The ability of structural protein specific MAbs to protect experimental animals from infection is consistent with the conventional hypothesis that structural protein antibodies attenuate viral infection by blocking virus binding and/or entry to target cells.

Despite protection against homologous infection, secondary infection with a heterologous strain can in some cases result in the potentially life threatening manifestations of DHF or DSS (Halstead, 1981). A number of prospective studies provide independent support for this hypothesis (Burke *et al*, 1988; Sangkawibhaet *et al*, 1984; Thein *et al*, 1997). The mortality rate of patients suffering haemorrhagic shock syndrome may be as high as 2 to 10% (Halstead, 1988) and affects predominantly young indigenous children (Rehle, 1989), those who have had a previous dengue infection and infants born to immune mothers (Halstead, 1980; Kliks *et al*, 1989; Halstead, 1981; Burke *et al*, 1988). Infection with a third serotype, does not appear to be related to the occurrence of overt disease (Fischer and Halstead, 1970) but rather a subclinical infection as repeated cases of DSS have not been reported (Halstead, 1981). Although it has been postulated that there is a role for dengue specific T cells in the pathogenesis of DHF / DSS, such specific T cell memory responses do not explain these complications when they occur during primary infections (Kurane *et al*, 1989b; Rothman and Ennis, 1999).

Host factors may also contribute to the severity of DHF / DSS. During the dengue

epidemic in Cuba in 1981, chronic diseases (eg. bronchial asthma, diabetes mellitus and sickle cell anemia) were additional risk factors contributing to DHF / DSS (Terval-Lopez, 1991). Significantly more Caucasians than those of African descent developed DHF (Bravo *et al*, 1987) indicating that a genetic factor may be responsible for these observed differences. In 1946, in Madagascar, dengue disease was reported more frequently among Europeans than among Africans (Cullinan, 1946). Likewise it has been demonstrated that DHF / DSS is more prevalent amongst females than males at, and after, the age of four years. It is thought that this reflects a more vigorous immune response in females rather than differences in exposure to infected mosquitoes (Halstead, 1980; Vaughn *et al*, 2000). Nutritional status may also be a contributing factor (Halstead, 1980; Vaughn *et al*, 2000), as good nutrition has an adverse effect on the outcome of DHF / DSS. In countries where the urban poor are clinically undernourished, DHF / DSS is predominantly a disease of the middle and upper classes. This epidemiological data reinforces the potential of a vigorous immune response to potentiate disease. Differences between viral strains are also important in determining the incidence of DHF in a susceptible community (Rico-Hesse *et al*, 1997).

A single autosomal-dominant locus in mice has been shown to confer resistance to flavivirus infection (Brinton, 1986; Sangster, 1993). Flaviviruses can replicate in these resistant mice however the spread of infection is slower and peak viremias are considerably lower than in susceptible mice. This indicates that a specific host gene may alter flavivirus replication competence. Characterization of this gene in humans may add further evidence to epidemiological data that suggests that the genetic constitution of the host may contribute to dengue virus resistance.

No specific receptors for the flaviviruses have been identified to date, apart from the generalized binding to glycosaminoglycans (GAGs) (Chen *et al*, 1997) however these molecules are likely to be co-receptors. Another unidentified high affinity receptor may be required to trigger endocytosis (Jacobs and Young, 1998), such as the recently reported 74 kDa protein in Vero cells (de Jesus Martinez-Barragan and del Angel, 2001). It has been shown that antibodies which bind to the virion have the ability to enhance replication of heterologous virus in cells of lymphoid lineage

in vitro (Daughaday *et al*, 1981). The phenomenon of antibody-dependent enhancement (ADE) has been demonstrated for viruses of various taxonomic groups (Gotoff *et al*, 1994; Weiss and Scott, 1981). Ferguson *et al* (1999) studied the effect of ADE on the transmission dynamics of multistrain pathogen populations by mathematical modelling and concluded that ADE acts to induce cyclical epidemics of all cocirculating strains where in its absence only one or a subset would persist.

Entry of antibody-virus complexes into cells has been reported for a range of flaviviruses (Halstead, 1981; Phillpotts *et al*, 1985; Gollins and Porterfield, 1985). The enhancement of virus production in these cells is due to an increased number of infected cells in the presence of antiviral antibodies (Hotta *et al*, 1984). Experimental evidence has demonstrated the role of antibody enhancement of infection (100 - 1 000 fold increase in titre) with all four dengue serotypes (Morens and Halstead, 1990). Antibody concentration dependent patterns of enhanced virus production was observed in murine macrophages infected with each of the four dengue serotypes, pre-incubated with either immune monkey sera, human immune cord blood sera, or dengue monoclonal antibodies (MAbs) directed to flavivirus glycoprotein specific envelope (E) epitopes (Morens and Halstead, 1990). It has been reported that the best dengue-2 enhancing MAb was dengue-4 derived, further supporting the theory of heterologous serotype enhancement of dengue virus infections. Monkeys infected with dengue-2 virus developed higher levels of viremia if they had actively or passively acquired antibody to heterologous dengue virus than if they had no such antibody (Halstead *et al*, 1993; Halstead, 1979). Antibody enhanced binding of dengue virus to human platelets has been reported by Wang *et al* (1995) and is consistent with epidemiological and experimental data linking preexisting host antibodies to an increased risk of DHF / DSS. The exacerbation of dengue infection by sequential infections indicates that it would be unwise to vaccinate individuals unless protection against all four dengue serotypes can be conferred simultaneously (Rosen, 1989).

In contrast to the theory that DHF / DSS has an imunological basis it has been proposed that dengue virus variants of increased pathogenic potential cause DHF /

DSS (Rosen, 1977; Hammon, 1973) based on patients who acquired dengue with no evidence of a previous dengue infection. The high mutation rate of RNA viruses is an established fact (Holland *et al*, 1982) and combined with alternative replication in two different hosts (ie. vertebrates and arthropods) strong selection pressure for virus variants is likely to exist (Igarashi, 1984). Although it has not yet been determined whether these variants alter the virulence of dengue viruses, higher replication of virus strains isolated from DHF patients in macrophage lines has been observed (Kliks *et al*, 1989). Nevertheless, disease severity is likely to be a result of a combination of various factors in each individual.

Complement activation

A role for complement activation in the pathogenesis of dengue virus infection, particularly plasma leakage in DHF, has been implicated in several published reports (Bokisch *et al*, 1973; Sobel *et al*, 1975; Ruangirachuporn *et al*, 1979; Cornain *et al*, 1987; Suvatte, 1987; Malasit, 1987; Rothman and Ennis, 1999). Decreased levels of C3, C4 and C5 in the sera of patients with DHF was reported as long ago as 1973, with an increased metabolism of C3 and C1q being most evident in patients with clinical grades III and grade IV disease (see Table 1.1) (Bokisch *et al*, 1973). Elevated plasma levels of C3a and C5a (products of complement activation) have been observed in DHF patients (Malasit, 1987) with maximal levels of C3a appearing at the time of defervesence and correlating with the severity of disease. These reports are consistent with the hypothesis that complement activation is likely to play a contributory role in plasma leakage (Rothman and Ennis, 1999).

Many studies have also reported circulating immune complexes in the sera of DHF patients, although the presence of dengue virus or dengue virus antigens in these complexes has never been demonstrated conclusively (Ruangirachuporn *et al*, 1976; Sobel *et al*, 1975; Rothman and Ennis, 1999). The complement pathway may be activated by these immune complexes in secondary infections and explain the massive complement depletion that is reported as a hallmark of dengue virus infection (Halstead, 1988; Jacobs and Young, 1998).

Cell-Mediated Immunity

To enable the design of a safe and effective vaccine and to understand the pathogenesis of DSS, it is of paramount importance to determine which dengue virus proteins induce protective T cell responses and which responses may lead to DHF / DSS.

Antibody may eliminate virus infected cells by antibody-dependent, cell-mediated cytotoxicity (ADCC). ADCC has been reported to lyse infected cells *in vitro* although an *in vivo* role has yet to be conclusively demonstrated (Kurane *et al*, 1984; 1986). Other cell-mediated immune responses have been found to be effective in the lysis of virus infected cells *in vitro* including macrophages, natural killer cells and cytotoxic T cells. Cellular effector functions have been evaluated in dengue virus infections by several groups (Hober *et al*, 1996; Anderson *et al*, 1997; Chang and Shaio, 1994; Shaio *et al*, 1995) demonstrating that dengue virus infection of human monocytes (particularly in the presence of enhancing antibodies), induces the production of vasoactive factors.

Production of IFNγ (interferon-gamma) by CD4+ CTL (cytotoxic T-lymphocytes) (Kurane *et al,* 1989b; 1989c) leads to an increase in F_c receptor expression on monocytes (Guyre *et al,* 1983), which results in a greater number of monocytes infected by virus-antibody complexes (Kotny *et al*, 1988). These monocytes may in turn be susceptible to lysis by class I and class II CTL, culminating in the release of vasoactive factors such as complement components (Bokish *et al*, 1973) and vasoactive cytokines such as tumour necrosis factor (TNF). T cells may also play a role in DHF / DSS by producing interleukin - 2. Interkeukin - 2 augments NK (natural killer) cell activity and proliferation and NK cells can mediate ADCC (Kurane *et al*, 1984) and produce IFNγ and tumour necrosis factor (TNF) possibly further contributing to DHF / DSS. Dengue virus infection of THP-1 cells (a monocytic cell line) or exposure to inactivated dengue virus has also been shown to induce TNFα production (Hober *et al*, 1996). Chang and Shaio (1994) found that IL-1β production (but not TNFα) by primary monocytes was enhanced when infected with dengue virus in the presence of enhancing antibody. More recently Anderson

et al (1997) demonstrated that tissue culture supernatants from primary human monocytes infected with dengue virus, activated endothelial cells *in vitro* and that this effect could be suppressed by antibody to TNFα. A prospective study of Thai children with DHF showed elevated levels of IFNγ, TNFα and soluble TNF receptors (sTNFRs) in patients with DHF and appeared to be associated with the severity of the dengue illness (Green *et al*, 1999). Collectively these studies indicate that infection of human monocytes induces the production of vasoactive factors, especially in the presence of enhancing antibodies.

Given that T lymphocyte responses to peptide antigens demonstrate MHC haplotype preference, candidate vaccines will need to induce protective immune responses in a wide variety of individuals possessing different histocompatibility antigens. The potential concern in using vaccines which elicit responses to the virion surface proteins because of the enhancement phenomenon could be significant, as a vaccine that failed to induce immunity, or that waned over time could lead to serious complications in the event of a natural dengue infection. The absence of non structural proteins from the virion make them candidates for a subunit vaccine approach. It follows therefore, that the elucidation of which non structural proteins are recognized by cross reactive T cells, and which are targets for CTL mediated clearance, will be invaluable in vaccine design strategies.

1.3 VIRION MORPHOLOGY AND ASSEMBLY

The three structural proteins that comprise the flavivirus virion are encoded in the 5' end of the genome (Figure 1.1A). Flaviviruses have a central isometric ribonucleoprotein core composed of a single stranded positive sense RNA genome complexed with a single species of capsid protein. This protein is a highly basic polypeptide that is not conserved among flaviviruses (Schlesinger and Schlesinger, 1990). Surrounding the nucleocapsid is a lipid bilayer derived from host cell membranes in which is embedded the virus specific membrane (M) and envelope (E) proteins (Russell *et al*, 1980) that protect the genome from extra-cellular nucleases (Figure 1.1B). Virions are spherical and have a diameter of approximately 40 - 50 nm (Figure 1.1C i) (Brinton, 1986) with a dense 30 nm core

(see Figure 1.1C ii). Structural organization of the E protein (as demonstrated by the crystal structure of the TBE E protein (Rey *et al*, 1995)) and subsequent work by the same group (Stiasny *et al*, 2001) suggests an icosahedral core with the rod-like E protein homodimers forming a lattice-like network on the external surface (Rey *et al*, 1995; Ferlenghi *et al*, 2001). Smith *et al* (1970) reported 7 nm ring shaped structures on the surface of dengue virus particles which likely represent higher order oligomeric complexes of these dimers (Figure 1.1C ii and iii).

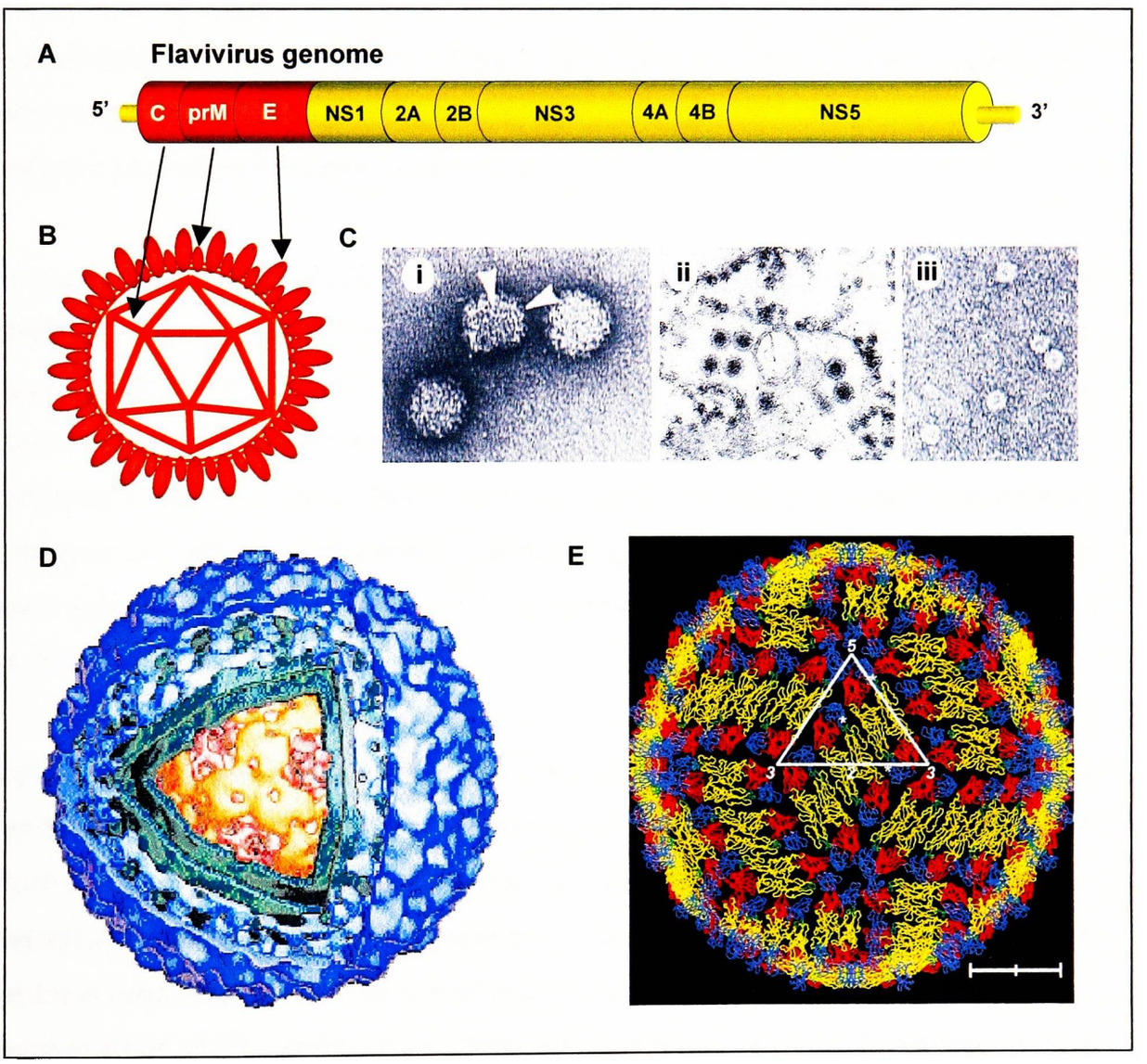


Figure 1.1 **Dengue virus morphology. A, Genome organization of the flaviviruses with structural proteins in red and non-structural proteins in yellow. B, Schematic of the virion structure showing icosahedral core (comprising the C protein and virion genomic RNA) and prM and E proteins embedded in the lipid bilayer. C, Electron micrographs of i) dengue virions with arrow heads highlighting ring-like structures on the virion surface, ii) thin section of dengue 2 virus infected Vero cells (Matsumara and Hotta, 1971), and iii) isolated ring structures (comprised of prM and E) from the virion surface. D, Dengue virus cryoEM reconstruction showing different layers in the virion. E, Postulated arrangement of dengue E homodimers on the virion surface (Kuhn *et al*, 2002).**

The structure of the dengue virion has recently been resolved to 2.4Å by cryo-electron microscopy (Figure 1.1D and E) (Kuhn *et al*, 2002). The surface of the dengue virion also reveals a lattice like network of E homodimers, however the arrangement of these is different to those reported for the TBE recombinant subviral particle (Ferlenghi *et al*, 2001). Kuhn *et al* (2002) suggested that this difference may simply reflect the different fusogenic states of the particles under study. The dengue virion is labile at 4°C for extended periods, upon freeze-thawing, as a result of pH changes and, due to the lipid envelope, is sensitive to organic solvents and detergents (Russell *et al*, 1980). Two virion forms have been characterized although they are indistinguishable morphologically: intracellular virions contain a precursor glycoprotein prM, and an extracellular form with the processed M protein (Shapiro *et al*, 1972).

Virion entry appears to utilize clathrin coated pits on the cell surface (Gollins and Porterfield, 1986a). Later they are found in prelysosomal vesicles where acid-catalyzed membrane fusion is proposed as the mechanism of release for the nucleocapsid (Gollins and Porterfield, 1986a; 1986b; Heinz *et al*, 1994). At low pH, a conformational change in the E protein occurs exposing a fusogenic domain (Roehrig *et al*, 1989), which is thought to be a highly conserved loop at the distal tip of each subunit of the E homodimer (Stiasny *et al*, 1996; Stiasny *et al*, 2001; Allison *et al*, 2001).

Cell tropism for flaviviruses is still poorly understood. Viral entry is thought to be by an endoctyic mechanism following virus binding as described above (Gollins and Porterfield, 1985), although evidence for an alternative plasma membrane penetration process has been presented (Hase *et al*, 1989). The cell surface receptor is presumed to involve the envelope glycoprotein E (Guirakhoo *et al*, 1991; Anderson *et al*, 1992; Heinz *et al*, 1994; Marianneau *et al*, 1996). Anderson *et al* (1992) showed that the degree of binding of the envelope protein correlated with cell susceptibility to dengue virus and suggested that cell tropism is determined in several cell types by the presence of an E protein receptor. Chen *et al* (1997) reported that heparan sulfate is the dengue-2 receptor in Vero cells, although the ability of dengue virus to replicate in cells of both vertebrate and invertebrate cell

lines indicate that more than one receptor could bind dengue viruses. The significance of heparan sulfate in both dengue and yellow fever virus binding has been confirmed by Germi *et al* (2002). A region between ^{284}E and ^{310}E was proposed as being the binding domain (Chen *et al*, 1997). It has also been suggested however, that heparan sulfate may simply play a role in concentrating virus particles at the cell surface allowing more effective interaction with some other surface molecule(s) (Putnak *et al*, 1997; Jacobs and Young, 1998).

Ultrastructural studies indicate that morphogenesis occurs in association with intracellular membranes (Monath, 1990). Studies have consistently observed mature virions first within the lumen of the ER (Hase *et al*, 1989a; Ishak *et al*, 1988; Ko *et al*, 1979). Budding intermediates and cytoplasmic nucleocapsids have rarely been observed indicating that the assembly process occurs rapidly. Virions tend to accumulate within disorderly arrays of membrane bound vesicles prior to transportation through the secretory pathway and final release from the cell (Mackenzie *et al*, 2001).

1.4 GENOME STRUCTURE AND ORGANIZATION

The flavivirus genome consists of single stranded positive sense RNA that is nearly 11 kilobases long (Rice *et al*, 1986). The RNA has a type 1 cap at the 5' end (m^7GpppAmpNp), (Wengler, 1978) and is not polyadenylated at the 3' terminus in dengue and other mosquito borne flaviviruses (Vezza *et al*, 1980). Cells infected with flaviviruses contain no subgenomic RNA (Boulton and Westaway, 1977; Naeve and Trent, 1978; Westaway, 1980). The viral RNA is the only mRNA which serves as messenger for all viral structural and nonstructural proteins (Rice, 1990). Complete sequences of the cDNAs of all four serotypes of dengue virus have been determined: dengue-1 (Mason *et al*, 1987b; Fu *et al*, 1992), dengue-2 (Mackow *et al*, 1987; Zhao *et al*, 1986; Hahn *et al*, 1988; Gualano *et al*, 1998; Irie *et al*, 1989), dengue-3 (Osatomi and Sumiyoshi, 1990; Yuan *et al*, AF3217645), and dengue-4 (Deubel *et al*, 1986). This has revealed the presence of an open reading frame (ORF) which encodes at its 5' end the three structural proteins: the capsid (or core) protein (C), the precursor (prM) of the membrane protein (M) and the envelope

protein (E). The nonstructural proteins are encoded in the remaining two thirds of the genome (Figure 1.1A). Flanking the long ORF are 5' (95 - 132 base pairs in length) and 3' (114 - 624 base pairs in length) noncoding regions. Flavivirus gene products are synthesized as one long polypeptide with co-translational proteolytic processing by both cellular and viral proteases (Rice *et al*, 1986; Westaway, 1987).

The non-coding regions at the 5' and 3' ends of the flavivirus genome form complex secondary structures (Brinton *et al*, 1986; Zhao *et al*, 1986; Brinton and Dispoto, 1988) and have been shown to function in the regulation of translation, RNA replication and encapsidation (Brinton *et al*, 1986; Zhao *et al*, 1986; Rice *et al*, 1986a, Hahn *et al*, 1987; You and Padmanabhan, 1999; You *et al*, 2001; Khromykh *et al*, 2001). The length of these regions vary among the flaviviruses however they all contain putative stem and loop structures (Brinton *et al*, 1986; Zhao *et al*, 1986; Brinton and Dispoto, 1988). Interestingly, the variation in the non-coding regions between the mosquito and tick borne flaviviruses has been suggested may reflect transmission by the different insect vectors (Hahn *et al*, 1987). A sequence motif conserved among all flaviviruses and located at the 3' terminus has been postulated by Mandl *et al* (1993) to act as a recognition signal for a viral or cellular transcription factor. A role in RNA replication for conserved complementary (cyclization) sequences in the 5' and 3' terminal regions of Kunjin flavivirus RNA has been shown by Khromykh *et al* (2001) by mutating the base pairing either separately or simultaneously to restore complementarity with a replicon RNA. None of the RNAs with separate mutations in only 5' or 3' cyclization sequences were able to replicate after transfection, whereas RNA with compensatory mutations in both cyclization sequences was replication competent. These cyclization sequences have also been shown for dengue virus (You and Padmanabhan, 1999; You *et al*, 2001).

(a) Genome Replication

Virions are taken up in coated pits with subsequent fusion of virion and cellular membranes occurring within minutes. Following fusion, the viral RNA is presumably released from endosomes and translation commences in association with the rough

endoplasmic reticulum (ER). A long latent period follows (12 - 16 hours) during which input virion RNA acts as mRNA to initiate synthesis of proteins such as the RNA-dependent RNA polymerase (NS5) and other proteins necessary for viral replication. At this time RNA synthesis can be detected in infected cells (Leary and Blair, 1983) and cell extracts have been shown to have RNA dependent RNA polymerase activity *in vitro* (Chu and Westaway, 1985; 1987).

Three RNA species have been detected in infected cells: a 20 - 28S partially double stranded replicative intermediate (RI), a 20 - 22S completely double-stranded replicative form (RF) and a 40 - 44S positive-strand genomic RNA (Stollar *et al*, 1967; Trent *et al*, 1969; Cleaves *et al*, 1981; Wengler *et al*, 1978). The nature of these species was supported by reports from Cleaves *et al* (1981) and Chu and Westaway (1985) that demonstrated that the RI is RNase sensitive and the RF is RNase resistant.

After translation of the incoming genomic mRNA, RNA is synthesized asymmetically in a semiconservative manner with the double stranded replicative form serving as a recycling template on which only one nascent strand is synthesized. After the latent period, RNA of positive polarity is the major product of viral RNA synthesis (Westaway, 1987; Gong *et al*, 1998). This RNA can then be used either for translation of structural and nonstructural polypeptides, minus strand synthesis, or be encapsidated into virions (Westaway, 1987).

Translation is initiated close to the 5' end of the genome, as indicated by homology of predicted open reading frames in the nucleotide sequence and N-terminal sequencing of translation products. The sequence of translation is NH_2 - C - prM (M) - E - NS1 -NS2A - NS2B - NS3 - NS4A - NS4B - NS5 - COOH. (Rice *et al*, 1985; Biedrzycka *et al*, 1987).

(b) Co-translational Processing

Proteolytic cleavage sites were identified by N-terminal sequence analysis of the final protein products (Speight and Westaway, 1989b; Chambers *et al*, 1989;

Biedrzycka *et al*, 1987) and from alignment of homologous sequences. Cell-free and *in vivo* studies have also been used to study flavivirus polyprotein processing. (Svitkin *et al*, 1981; Markoff, 1989; Nowak *et al*, 1989). Translation takes place in association with the rough ER with some proteins being translocated into the lumen and others remaining localized on the cytoplasmic side. No carboxy terminal post-translational processing has been reported to occur (Speight and Westaway, 1989a, Wright *et al*, 1989) although recent evidence from the Young laboratory has suggested that a proportion of dengue virus NS1 undergoes glycosylphosphatidyl-inositol (GPI) addition at the C-terminus (Jacobs *et al*, 2000).

The processing of core (C) involves removal of the initial methionine from the amino terminus by a cellullar methionine aminopeptidase (Coia *et al*, 1988; Nowak *et al*, 1989; Rice *et al*, 1986b; Boege *et al*, 1983), although this is not universal among flaviviruses (Bell *et al*, 1985). Regions of hydrophobicity and hydrophilicity within core are conserved (Mandl *et al*, 1998). A C-terminal hydrophobic region acts as a signal sequence for translocation of prM into the lumen of the ER. Cleavage at this site has been implicated in the efficient generation of the amino terminus of prM (Lobigs, 1993; Stocks and Lobigs, 1995). This subsequent cleavage, converts core from a membrane-anchored intracellular form to its virion form and has been shown to be an essential component of virion maturation (Lobigs, 1993; Yamshchikov and Copans, 1993) and the flavivirus life cycle (Amberg and Rice, 1999; Lee *et al*, 2000). Virion core had been commonly considered to be free in the cytoplasm of infected cells, but evidence suggests that if is in fact anchored in the ER membrane by a structurally conserved, internal hydrophobic domain (Markoff *et al*, 1997). It thus takes up a hairpin conformation with positively charged regions amino- and carboxy-terminal to this anchor accessible to interact with RNA in the cytoplasm. Cleavage of prM occurs immediately after the sequence motif Arg - X - Arg / Lys - Arg (where X is variable) (Randolph *et al*, 1990). prM cleavage was first postulated to be mediated by the host enzyme furin (Hallenberger *et al*, 1992) and has subsequently been confirmed for TBE by Stadler *et al* (1997). The cleavage of prM from E occurs rapidly as no higher molecular weight precursors have been identified (Chambers *et al*, 1990c) although a C - prM precursor has been reported in cell-free translation systems (Svitkin *et al*, 1981; Markoff, 1989; Nowak *et al*,

1989) and in dengue-2 infected mosquito cells (Murray *et al*, 1993). It has been postulated that the inefficiency of the C / prM cleavage in mosquito cells may be a consequence of the lower incubation temperature required for these cells (Murray *et al*, 1993).

At the C-terminus of prM are two hydrophobic domains separated by a single basic residue (Rice *et al*, 1985). The first is likely to anchor prM in the virion envelope (Nowak *et al*, 1989; Wright *et al*, 1989) whereas the second acts as a signal sequence for translocation of E across the ER (Coia *et al*, 1988; Gruenberg and Wright, 1992).

The N termini of prM, E, NS1 and NS4B follow predicted signalase cleavage sites (von Heijne, 1984) contributed by the C-terminal hydrophobic regions of anchored C, prM, E and NS4A respectively. A signal sequence also precedes the N-terminus of NS4B, suggesting that this hydrophobic protein is processed in association with membranes of the ER. The N-terminus of NS2A follows a cleavage site defined by the sequence (Val - X - Ala; X = Ser, Thr, Gln, Asp, Asp). Although this fulfills the rule for a signalase (von Heijne, 1984), sequence analysis indicates that the site lacks a requisite upstream hydrophobic region, thus the source and specificity of the protease responsible for cleavage between NS1 and NS2A remains to be determined. A role for NS2A in the NS1 - NS2A cleavage is suggested by studies with dengue-4 vaccinia recombinants in which progressive C-terminal deletions of the NS2A region generated uncleaved but glycosylated NS1 - NS2A related proteins (Falgout *et al*, 1989). It has also been shown that efficient cleavage at the dengue-4 virus NS1 - NS2A junction of the viral polypeptide requires the N-terminal hydrophobic signal of NS1 and downstream NS2A in *cis* (Falgout *et al*, 1989). Deletion analysis has shown that a minimum of eight amino acids at the C-terminus of NS1, preceding NS2A, is also required for efficient cleavage at this junction (Hori and Lai, 1990). It was also shown that in the 12 flavivirus amino acid sequences analysed there was a degree of homology within this octapeptide sequence, and a conserved sequence was derived: Leu/Met-Val-Xaa-Ser-Xaa-Val-Xaa-Ala. Substitutions at each of these amino acids were analysed by Pethel *et al* (1992) and tended to indicate a possible structural requirement around this region, which

may or may not include the hydrophobic region downstream. This is discussed in more detail in Chapter 1.5b.

Cleavage sites generating the N termini of NS2B, NS3, NS4A and NS5 are highly conserved among flaviviruses and there does not appear to be any obligate order for processing in the non-structural region (Lin *et al*, 1993; Preugshat and Strauss, 1991). The flavivirus encoded protease (NS2B/NS3) has been shown to mediate cleavage at di-basic amino acid residues (Lys-Arg, Arg-Arg or Arg-Lys) or occasionally between Gln-Arg (dengue-2 and dengue-4 NS3). These sites are usually flanked by short chain amino acids (Rice *et al*, 1988).

1.5 VIRAL PROTEINS

(a) Structural Proteins

The Capsid Protein (C)

The C protein present in virions is a small (12 - 14 kDa) highly positively charged protein (due to a high proportion of lysine and arginine residues) that forms the structural component of the nucleocapsid. Rice *et al* (1985) suggested that the likely function of this positive charge is to partially stabilize the negative charges of the RNA. There is a hydrophobic stretch of uncharged amino acids in the middle of the C protein, which is conserved among flaviviruses (Rice *et al*, 1985; Dalgarno *et al*, 1986) and a conserved amino terminal hydrophilic region (Mandl *et al*, 1988). It is likely that as the charged residues of the C protein complex with the genomic RNA, a nucleoprotein is formed with the external surface consisting of the hydrophobic portion of the C protein. As a result of this surface hydrophobocity the nucleocapsid may act as a membrane vesicle and move along a lipid gradient in the cytoplasm towards the membrane (Hase *et al*, 1989).

Two epitopes have been identified on the dengue-4 virus capsid protein which are recognized by a serotype-specific (between amino acids 47 and 55) and serotype cross-reactive (between amino acids 83 and 92) human CD4+ cytotoxic T-

lymphocyte clone (Gagnon *et al*, 1996). This was the first report that the capsid protein is a target of an antiviral T-cell response in dengue virus infections.

The pre Membrane Protein (prM)

The prM protein is a glycoprotein precursor (18 - 19 kDa) to the structural protein M (8 kDa). This precursor undergoes a delayed cleavage to form M and the N-terminal pr segment (Shapiro *et al*, 1972) that is secreted (Murray *et al*, 1993). M and prM are found on extracellular and intracellular virions respectively, and thus this cleavage is presumably linked to virus maturation and release. Inhibitory effects of weak bases and lysosotrophic amines on prM cleavage have been demonstrated, and this has suggested a specific role for prM in maintenance of the conformation of the E protein during virus passage through acidified sorting compartments (Randolph *et al*, 1990), since flaviviruses are generally inactivated at low pH.

The N-terminal pr segment is predominantly hydrophilic, containing six conserved cysteine residues all of which participate in disulfide bridging (Norwak and Wengler, 1987). Glycosylation of prM presumably has an important role in virion assembly and release as serologically related viruses show position conservation of N-linked glycosylation sites (Wright *et al*, 1980; Markoff, 1989; Ruiz-Linares *et al*, 1989).

Monoclonal antibodies against prM of dengue virus can be protective against challenge with both homologous and heterologous virus (Kaufman *et al*, 1989; Takegami *et al*, 1982).

The Envelope Protein (E)

The E protein is the major virion envelope protein (50 - 60 kDa) (a type I membrane protein) and displays both the virus haemagglutinin activity and the major neutralization epitopes (Gould *et al*, 1990). It is also involved in a number of additional viral functions such as receptor binding and low pH induced membrane fusion (Gollins and Porterfield, 1986; Summers *et al*, 1989). It has been

demonstrated that infection with West Nile virus involves an endosomal acid catalyzed fusion process (Gollins and Porterfield, 1986) and it has been suggested that the conserved region from amino acid residues 98 - 111 is the fusion sequence, based on similarities with other viral fusion domains (Roehrig *et al*, 1990). This hypothesis has been supported by the finding that a monoclonal antibody that recognizes the TBE E protein fusion sequence is able to block fusion (Volkova *et al*, 1999) and recent experimental evidence using site-directed mutagenesis confirms the role of this domain in TBE E-mediated fusion (Allison *et al*, 2001). The E proteins of most of the flaviviruses are glycosylated as shown by the incorporation of labeled sugars (Shapiro *et al*, 1971; Westaway, 1975) and its reactivity with concancalin A (Stohlman *et al*, 1976; Clegg, 1982). Notable exceptions are some isolates of Kunjin virus (Wright, 1982) and West Nile virus (Wengler *et al*, 1985). Evidence exists for both simple and complex glycans (Winkler *et al*, 1987; Zhao *et al*, 1987) with apparent host specific differences. The dengue-2 E protein is glycosylated at ^{67}Asn and ^{153}Asn (Smith and Wright, 1985; Deubel *et al*, 1986; Irie *et al*, 1989). A mutation that eliminated the glycosylation site in the dengue-2 E protein led to a higher pH threshold for fusion (Guirakhoo *et al*, 1993), consistent with the oligosaccharide participating in oligomerization. SDS denaturation resistance was lost when the protein was deglycosylated indicating that the carbohydrate side chain contributed to the stability of the molecule (Winkler *et al*, 1987). For the E protein, correct folding is probably required for eliciting a protective immune response since E protein antigens produced in *E. coli* (Mason *et al*, 1989), and the authentic protein produced under denaturing conditions (Wengler and Wengler, 1989) failed to induce neutralizing antibodies.

The genome sequence of the E glycoprotein of all the flaviviruses examined, show that each of the twelve cysteines forming six disulphide bridges are perfectly conserved in the E ectodomain (Deubel *et al*, 1986; Nowak and Wengler, 1987; Chambers *et al*, 1990a). Combined with virtually superimposable hydrophilicity profiles it is likely that all flavivirus E proteins share a common structure. There is however a high degree of diversity at antigenically important sites which exist without impairing its principal functions (Heinz, 1986).

Structural models for E were originally proposed by Nowak and Wengler (1987) and Mandl *et al* (1989) prior to the publication of the crystal structure for the trypsin released ectodomain of E derived from TBE (Rey *et al,* 1995). The crystallization of the E protein has revealed that the E dimer is a rod shaped head-to-tail oligomer of 170Å that is anchored in the bilayer at each end. The slight curvature of the dimer suggested that these proteins do not form projections as previously reported (Murphy, 1980) but lie parallel to the virion surface (Rey *et al*, 1995). The active fusogenic form has been shown to be a trimer (Allison *et al*, 1995) that forms by reorganization of the virion surface upon exposure to low pH. As noted above, the conserved nature of the disulfide bridges suggests that the three-dimensional structure of other flavivirus E proteins may be similar to that of TBE. This likely homology was used by Kuhn *et al* (2002) in mapping the position of the dengue virus E protein on the surface of the dengue virion structure resolved by cryo-electron microscopy (Figure 1.1D).

Three antigenic domains (A, B and C) have been described within the E protein (Heinz, 1986) and correspond well to the three structural regions identified by Rey *et al* (1995) as domains II, III and I respectively. Domain A contains flavivirus cross-reactive epitopes along the putative fusion domain (amino acids 98 - 111). Regions postulated to modulate pathogenicity have been localized to domain B (amino acids 300 - 395) which is dependent on an intact disulfide bridge. Lobigs *et al* (1990) and Rey *et al* (1995) proposed that this domain may be involved in tissue tropism and receptor recognition due to published reports for various flaviviruses including JE (Cecilia and Gould, 1991; Hasegawa *et al*, 1992), Louping Ill (Jiang *et al*, 1993; Gao *et al*, 1994), TBE (Holzmann *et al*, 1989) and dengue-2 viruses (Hiramatsu *et al*, 1996; Sanchez and Ruiz, 1996). Domain C (amino acids 132 - 177), while not a highly conserved domain, is known to contain a conserved glycosylation site (Guirakhoo *et al*, 1989).

(b) NON STRUCTURAL PROTEINS

The Non Structural Protein, NS1

The gene product NS1 follows E in the polyprotein sequence. It encodes at its C-terminus, a hydrophobic signal sequence that functions as a translocation signal for entry of NS1 into the ER (Rice *et al*, 1985; Biedrzycka *et al*, 1987; Speight *et al*, 1988). Dengue-2 NS1 is a glycoprotein with a molecular weight of 46K and contains two N-linked glycans (Smith and Wright, 1985). Processing of NS1 involves a host signalase cleavage at the amino terminus and a unique cleavage at the carboxy terminus (Speight *et al*, 1988; Chambers *et al*, 1989, Falgout *et al*, 1989; Nowak *et al*, 1989). The enzyme responsible for NS1/NS2A cleavage is unknown, but there is strong evidence that it is a host-encoded enzyme that acts within the ER and requires a consensus sequence motif of eight amino acids (L/M-V-X-S-X-V-X-A) at the carboxy terminus of NS1 (Hori and Lai, 1990) as described previously. Site directed mutagenesis experiments confirmed that the amino acids at positions -8, -7, -5, -3 and -1 were important for cleavage. The lines of evidence suggesting that the enzyme responsible for cleavage of NS1/NS2A resides within the ER; are that it is not abrogated by brefeldin A (Falgout and Markoff, 1995), which blocks membrane traffic from the ER to the Golgi (Klausner *et al*, 1992) and, isolated dog pancreas microsomes, which contain membrane organelles with properties of the ER (Walter and Blobel, 1983), can effect NS1-NS2A cleavage (Falgout and Markoff, 1995). Furthermore, disruption followed by releasing of these microsomes removes the lumenal contents yet does not affect NS1-NS2A cleavage, implying a membrane-bound localization for the putative host protease which cleaves NS1-NS2A (Falgout and Markoff, 1995).

The various unique characteristics of the NS1 protein will be described later in this Chapter (Section 1.6).

The Non Structural Protein, NS2A

The NS2A protein is a relatively small (25 kDa) hydrophobic protein which migrates anomalously on SDS-PAGE gels at approximately 20 kDa (Coia *et al*, 1988; Chambers *et al*, 1989). The function of this protein is unknown. Studies by Wengler *et al* (1990) on West Nile virus infected cells showed that NS2A remained attached to membranes despite high stringency washes and interestingly, NS2A has been

found associated with fractions containing RDRP activity in Kunjin virus infected cells (Chu and Westaway, 1992). Mackenzie *et al* (1998) suggested that NS2A may be involved in targeting the replication complex to cytoplasmic membranes as binding assays indicated that it associates strongly with NS3, NS5 and NS1, which were localized to separate sides of the membrane. As previously described, NS2A has been implicated in the processing of NS1 (Chambers *et al*, 1989). Two forms of NS2A have been observed for some flaviviruses, including YF, JE, KUN and MVE, but not dengue virus NS1. The higher molecular weight form of NS2A referred to as NS1', was demonstrated to be a fusion protein with NS1 (Mason *et al*, 1987).

The Non Structural Protein, NS3

NS3 is the second largest viral protein (68-70 kDa) encoded by dengue viruses. It is highly conserved among flaviviruses (Rice *et al*, 1986) and is hydrophilic. The protease function of NS3 has been experimentally verified (Chambers *et al*, 1990; Preugschat *et al*, 1990; Wengler *et al*, 1991; Pugachev *et al*, 1993). Sequence comparisons suggest that it comprises three separate enzymatic functions; serine protease (Chambers *et al*, 1990; Preugschat *et al*, 1990; Falgout *et al*, 1990) as well as a nucleotide triphosphatase and helicase activity) thought to be involved in nucleotide cofactor binding and hydrolysis (Gorbalenya *et al*, 1989; Wengler and Wengler, 1991) hence a role for NS3 in viral RNA replication was postulated. Processsing of the 2A-2B, 2B-3, 3-4A and 4B-5 sites is catalyzed by a two component viral proteinase (NS2B/NS3) (Bazan and Fletterick, 1989; Gorbalenya *et al*, 1989). RNA-stimulated NTPase activity has been demonstrated for purified NS3 of yellow fever (Warrener *et al*, 1993), West Nile (Wengler and Wengler, 1991) and dengue-2 viruses (Li *et al*, 1999). The RNA triphosphatase activity is thought to aid in the formation of the cap structure at the 5' end of the flavivirus genomic RNA (Wengler and Wengler, 1993). These properties therefore suggest a cytoplasmic location for NS3. Westaway *et al* (1997) showed Kunjin virus NS3 associated with double-stranded RNA within vesicle packets. The recently published crystal structure of the proteolytic domain of dengue-2 NS3 suggests that heterodimerization with NS2B may be involved in the specificity of substrate binding (Krishna-Murthy *et al*, 1999; 2000). Detailed *in vitro* kinetics, and preliminary

inhibitors were recently reported for a recombinant form of NS3 co-expressed with its co-factor NS2B (Leung *et al*, 2001).

Antibodies to NS3 have been shown to play a role in the immune response to dengue virus infection (Tan *et al*, 1990) with antibodies detectable in dengue patient sera by western blot analysis. In addition, Kurane *et al* (1989) have reported that NS3 is a major target of a CTL mediated immune response.

The Non Structural Proteins, NS2B, NS4A and NS4B

These small proteins, like NS2A, are poorly conserved among flaviviruses (Rice *et al*, 1986; Mandl *et al*, 1989) but contain similar structural features consisting of multiple hydrophobic domains which could potentially span membranes. It has been postulated that they may serve to anchor the viral replicase to cellular membranes (Chambers *et al*, 1989). NS2B and NS4B are readily identifiable in infected cells, but NS4A (16 kDa) has been definitively identified only for Kunjin virus by Speight and Westaway (1989). In Kunjin virus infected cells, NS2A and possibly NS4A have been shown to be associated with "heavy" membranes isolated by sedimentation in sucrose gradients (Chu and Westaway, 1992). NS4A binds strongly to the replicase components NS5, NS3 and NS1 as well as the other protease component NS2B (Mackenzie *et al*, 1998, Westaway *et al*, 1997a; 1997b). Coupled with its localization at the putative site of RNA replication (vesicle packets), and proteolytic processing (the precursor membranes and paracrystalline arrays), it seems likely that NS4A is involved with both processes. It may act by anchoring these complexes to the membrane (Mackenzie *et al*, 1998; Rice, 1996) although biochemical data on the topology of this protein are lacking. No post-translational modifications of these proteins are known, despite the presence of a conserved potential N-linked glycosylation site in the C-terminal portion of NS4B (Lee *et al*, 1990). Evidence from Lindenbach and Rice (1999) indicate that NS4A is involved in the process of RNA replication possibly through a direct interation with NS1. NS4A is produced via cleavage at its N- and C-termini by the viral serine protease NS2B-NS3. Both cleavage events appear to be delayed or regulated as the putative NS4A precursors NS3-4A and NS4A-4B have been identified in flavivirus infected cells

(Chambers *et al*, 1990c; Svitkin *et al*, 1981; Cahour *et al*, 1992; Lobigs, 1992; Zhang *et al*, 1992; Preuschat *et al*, 1990).

NS4B shows as little as 31% homology with other flaviviruses (Coia *et al*, 1988) and contains six hydrophobic regions that contribute to its sedimentation with the membrane fraction of infected West Nile virus cell lysates (Wengler *et al*, 1990). However, in dengue-2 and Kunjin viral extracts, NS4B has been reported to be trypsin sensitive and therefore unprotected by membranes (Cauchi *et al*, 1991). NS4B has been shown to be localized to the nucleus by immunoelectron microscopy (Westaway *et al*, 1997a) which is unusual as NS4B does not contain a known nuclear localization signal. It is possible that these proteins form membrane components of the viral replication complexes and could be involved in membrane localization of NS3 and NS5 via protein-protein interactions (Westaway *et al*, 1999).

The Non Structural Protein, NS5

NS5 is the largest (103 - 104 kd) and most highly conserved of all flavivirus proteins (Mandl *et al*, 1989; Rice, 1996). C-terminal sequence data for Kunjin virus indicate that the protein extends to the predicted end of the ORF (Speight and Westaway, 1989). NS5 is a basic protein and does not contain any long hydrophobic domains. The role of NS5 as the viral RNA-dependent RNA polymerase (RDRP) was originally predicted based on sequence homology with known RDRPs (Koonin 1991; Poch *et al*, 1989). This has since been shown by Khromykh *et al* (1998; 1999a; 1999b) through complementation studies. The generation of its N-terminus by cleavage in the cytoplasmic compartment (presumably by NS3 or an alternative protease) suggests that NS5 is located in the cytoplasm, although it is associated with membranes (Cauchi *et al*, 1991; Chu and Westaway, 1992). A highly conserved domain in NS5 (YF residues 521 - 675), contains a sequence motif (Rice *et al*, 1985; 1986) which is present in nonstructural proteins from a number of positive-strand RNA viruses and is believed to play a role in RNA-dependent RNA synthesis (Kamer and Argos, 1984; Rice *et al*, 1986). NS5 is also a putative methyltransferase which is involved in the formation of the RNA cap (Chambers *et al*, 1990a; Tan *et al*, 1996; Koonin, 1993). Motifs are also present for substrate

binding, RNA binding, catalytic activity and formation of secondary structure of RdRps (Koonin, 1991; O'Reilly and Kao, 1998).

The conserved coding sequences in the N-terminal half of NS5 have a possible role in RNA replication by initiating formation of the replication complex. There is a requirement for an association of NS5 with other non-structural proteins (Khromykh *et al,* 1999a) that is thought to be due to binding of NS3 to the N-terminal half of NS5 during replication complex assembly. The replication complex then attaches to the adjacent 3'UTR on completion of translation and moves to the proposed membrane attachment site involving NS4A and NS1 (Khromykh *et al*, 1999b).

1.6 THE NS1 PROTEIN

NS1 was first identified in flavivirus infected suckling mouse brain extracts and was originally referred to as the soluble complement fixing (SCF) antigen. (Russell *et al*, 1970). Since then it has been recognized as being important for its immunogenicity and in being unusual as a glycosylated nonstructural protein. NS1 is located in various regions in infected cells: in association with membranes in the cytoplasm (Stohlman *et al*, 1975; 1978), at the cell surface in association with the plasma membrane (Cardiff and Lund 1976; Gould *et al*, 1985; Westaway and Goodman, 1987) and in infected culture supernatants as a secreted species (Winkler *et al*, 1989; Mason 1989; Desprès *et al*, 1991) (Figure 1.2A). NS1 has also been shown in the perinuclear region of infected cells and as cytoplasmic foci in immunofluorescence studies (Ng *et al*, 1992; Ng and Corner, 1989), initially suggesting a role in virus assembly and release (Rice *et al*, 1986b; Mason 1989).

The dengue NS1 protein is translated from 1056 nucleotides into 352 amino acids that encode a protein backbone of 40 kDa (Deubel *et al*, 1988). NS1 nucleotide sequence data for various flaviviruses has been compared and a high degree of homology exists (Mackow *et al*, 1987; Mandl *et al*, 1989). Cysteine residues in flavivirus NS1 have been found to be totally conserved in both number and position suggesting a common structural organization (Rice *et al*, 1986; Gibson *et al*, 1988; Wang *et al*, 2001). There are a number of amino acid substitutions and deletions

between the NS1 proteins although the majority are semi-conservative thus maintaining the overall hydrophilic nature of the protein. Amino acid conservation also suggests preservation of potential antigenic sites and serological data also supports this view. NS1 lacks a membrane anchoring domain at the amino or carboxy termini (Speight *et al*, 1988; Wright *et al*, 1989) despite the fact that it has been shown to be membrane-associated (Cardiff and Lund, 1976; Schlesinger *et al*, 1990) by ultracentrifugation (Stohlman *et al*, 1975) and immunochemical studies (Cardiff and Lund, 1976; Gould *et al*, 1976; Gould *et al*, 1985; Westaway and Goodman, 1987). A portion of NS1 has been reported to be anchored to cellular membranes via a glycosyl phosphatidylinositol (GPI) anchor in some cell types (Jacobs *et al*, 2000). Crystallographic structural resolution of NS1 has yet to be reported and is the basis of studies in Chapter 5 of this thesis.

(a) Oligomerisation of NS1

NS1 exists as both a monomer (46 kDa) and a dimer (86 kDa) in flavivirus infected cells (Winkler *et al*, 1988; Chambers *et al*, 1989; Mason, 1989). The dengue-2 NS1 dimer was first identified when infected cell lysates were left unboiled prior to SDS-PAGE (Winkler *et al*, 1988). A homodimeric structure was proposed based on both carbohydrate and monomerization analyses (Winkler *et al*, 1988). Although the dengue NS1 dimer is heat sensitive, it is resistant to reducing agents (Winkler *et al*, 1988). It should be noted however that the NS1 dimer of JE virus is partially sensitive to reducing agents (Fan and Mason, 1990). A hexameric form of secreted TBE NS1 has been described from column chromatography studies (Crooks *et al*, 1990) and from gel filtration experiments of dengue-1 NS1 (Flamand *et al*, 1999). The sensitivity of the hexameric form of NS1 to detergents has meant that most studies have focused on the detergent stable dimeric form of the native oligomeric structure. The secreted form of NS1 would appear to be a hexamer. The oligomeric state of the membrane-associated form remains to be determined as purification strategies usually involve the use of detergents.

The oligomer is thought to represent the functional form of the protein (Winkler *et al*, 1988) and has been reported to be more immunogenic in mice than its

monomeric counterpart (Falconar and Young, 1990). Flavivirus NS1 dimers have been detected in both insect and mammalian cells however Flamand *et al* (1992) have reported that the process of dimerization takes considerably longer in mosquito cells. The types of interactions involved in the formation and maintenance of the dimer are not well defined (Pryor and Wright, 1993) although they are non-covalent in nature.

Studies using recombinant cDNA demonstrate that NS1 (preceded by its ER translocation signal sequence) forms dimers in the absence of any other viral protein (Parrish *et al*, 1991; Pryor and Wright, 1993; 1994; Leblois and Young, 1995), implying that NS1 itself contains all the essential information for dimerization. Maintenance of the dimer may only require one half of the protein, since a truncated dimer was isolated following trypsin digestion of membrane-associated NS1 (Cauchi *et al*, 1991). However sequence requirements for dimer formation and maintenance are not necessarily the same. Dimer formation has been addressed using recombinant, mutagenized cDNA expression and it seems that the carboxy-terminal end of the protein is important in dimer formation, and more so than the amino-terminal end. Deletion of the last 79 amino acids of NS1 abolishes dimer formation (Parrish *et al*, 1991), either because this region is directly involved in dimer formation or because the conformation of distant sites required for dimer formation is altered. Substitution within the conserved octapeptide C-terminal sequence did not prevent dimer formation (Parrish *et al*, 1991), consistent with its role as a specific cleavage recognition sequence. However the importance of the other carboxy-terminal amino acids has been supported by further work. Substitution of any of the last three cysteine residues, located within the carboxy-terminal 40 amino acids of the protein, prevents dimer formation whereas substitutions of the first three cysteine residues do not (Pryor and Wright, 1993). Moreover, mutation of a small hydrophobic domain near the C-terminus, but not similar domains near the N-terminus or in the middle of the protein, prevents dimer formation (Pryor and Wright, 1993). Exact constraints on the C-terminal portion of the protein with respect to dimerization have yet to be defined.

In all experimental systems, dimerization is a critical step in NS1 maturation. In

common with many other glycoproteins, mutations which prevent assembly into dimers also disrupt transport to the cell surface and secretion. In contrast, Hall *et al* (1999) reported that a cDNA clone of Kunjin virus with a single amino acid substitution ($proline_{250}$ to leucine) in NS1, ablated dimerization but still allowed replication to occur and for NS1 to be secreted in the monomer form. However this observation could be explained by the dimeric form of NS1 being highly unstable with this mutation and therefore difficult to detect by SDS-PAGE analysis. Virus replication was also shown to be slower, with reduced virulence in mice (Hall *et al*, 1999).

Dimerization of NS1 occurs in the ER prior to transport to the Golgi where processing of some of the N-linked glycans occurs (Winkler *et al*, 1989, Desprès *et al*, 1991) (Figure 1.2A). The conversion of monomers to dimers has been related to the membrane-association of NS1. It appears that the NS1 protein exists temporarily in a hydrophilic form before becoming associated with the membrane. In pulse-chase experiments, the dengue-2 NS1 dimer appeared after 20 - 40 minutes, suggesting that this transformation to the more hydrophobic membrane associated form was possibly due to dimerization and represented the functional form of the protein (Winkler *et al*, 1988; 1989). Winkler *et al* suggested that dimerization could expose hydrophobic domains on the surface of the molecule or that the process of dimerization was connected to post translational modification (eg. fatty acid acylation, carbohydrate interaction, or the attachment of a phosphatidylinositol anchor which could explain the membrane association) (Sefton and Buss, 1987). Flaviviruses within the JE serocomplex, which includes JE, MVE, Kunjin and West Nile viruses, express an additional form of NS1, designated NS1' due to a carboxy terminal extension of the polypeptide backbone (Mason, 1989). Other flaviviruses such as dengue-2, YF and Kokobera viruses however do not appear to produce NS1'.

The kinetics of secretion are different for different flaviviruses. TBE virus NS1 is secreted in 45 minutes (Lee *et al*, 1989) compared to two hours for JE virus NS1 (Mason, 1989) and YF virus (Desprès *et al*, 1991). In contrast, NS1 secretion has not been observed for either JE virus-infected or dengue virus-infected C6/36 cells

(Mason, 1989). The inability of insect cells to further process complex sugars may account for this observation (Heish and Robbins, 1984). Secretion from infected mammalian cells is not however inhibited by tunicamycin or mutated glycosylation sites (Pryor and Wright, 1994; Winkler *et al*, 1989; Desprès *et al*, 1991).

(b) Glycosylation of NS1

Multiple forms of NS1 have been identified for different flaviviruses which have been shown to be generated by variable glycosylation and / or differences in the cleavage of the polypeptide backbone (Mason *et al*, 1987; Winkler *et al*, 1988). Different viral strains as well as different host cells have also been shown to contribute to this heterogeneity. As a consequence, the molecular weight of NS1 varies from 45 - 53 kDa. In dengue virus infected mammalian cells, two forms of NS1 are detected: a membrane bound (mNS1) (Cardiff and Lund, 1976) and an extracellular form (sNS1) (Russell *et al*, 1970). Both species of NS1 have two N-linked glycosylation sites on each molecule (Smith and Wright, 1985) and each have the same polypeptide backbone as revealed by endoglycosidase digestion (Mason *et al*, 1987; Winkler *et al*, 1988; Young, 1990). However, the molecular weight of sNS1 is some 3-5kDa larger that that of mSN1. In addition, sNS1 migrates on SDS-PAGE as a diffuse band between 53 - 57kDa while mNS1 runs as a sharp band of 49 kDa. The difference in molecular weight profiles between sNS1 and mNS1 derives from the fact that the secreted form of NS1 has one of its high mannose linkages trimmed and processed into a complex carbohydrate moiety (Winkler *et al*, 1998; Mason, 1989; Jacobs *et al*, 1992; Young, 1990) inferring transit through the Golgi apparatus (Lee *et al*, 1989). Winkler *et al* (1989) suggested that this indicates a precursor-product relationship between the two species however Mason (1989) demonstrated in pulse-chase radiolabelling experiments that mNS1 accumulated within infected cells suggesting that while mNS1 is the first species expressed, both the high-mannose and complex carbohydrate forms are potentially functional end-products. The exact nature of the cell-surface associated NS1 species has yet to be conclusively identified.

The glycosylation of NS1 is noteworthy as it is unusual for an RNA virus to possess

a glycosylated non structural protein. There is some degree of heterogeneity between glycosylation of NS1 amongst the flaviviruses. NS1 of all four dengue serotypes, together with the NS1 of JE and YF viruses, have two conserved glycosylation sites at ^{130}Asn and ^{207}Asn (Rice *et al*, 1985; Mackow *et al*, 1987; Hahn *et al*, 1988; Irie *et al*, 1989). NS1 derived from WN, KUN, MVE and SLE viruses possess a third glycosylation site at ^{175}Asn (Coia *et al*, 1988; Trent *et al*, 1987; Castle *et al*, 1986; Dalgarno *et al*, 1986, Blitvich *et al*, 1999). Mandl *et al* (1989) have shown for TBE virus that NS1 possesses three glycosylation sites however only ^{207}Asn is conserved with the mosquito borne flaviviruses.

There is evidence that glycosylation is important for the biological activity of NS1 as well as its structural integrity. Mutagenesis of the glycosylation sites in infectious clones can alter neurovirulence (Pletnev *et al*, 1993; Muylaert *et al*, 1996) and ablation of the first glycosylation site reduced the rate of viral RNA accumulation, providing evidence of a role for NS1 in RNA replication (Muylaert *et al*, 1996). Removal of glycosylation sites on the dengue virus NS1 via mutagenesis was also shown to reduce the stability of the native dimeric form of the protein (Pryor and Wright, 1994).

(c) Role of NS1 in infection and immunity

In addition to the envelope (E) protein and prM, NS1 has been associated with antibody-mediated protective immunity. As noted above, NS1 exists in cell associated (Westaway, 1987), cell surface (Schlesinger *et al*, 1985) and extracellular forms (non-virion associated) (Mason, 1989; Smith and Wright, 1985; Winkler *et al*, 1988) and does not form part of the virion. Consequently, antibodies raised against NS1 have no neutralizing or haemagglutination inhibition activity, although some are able to fix complement (Schlesinger *et al*, 1985; Smith and Wright, 1985; Gibson *et al*, 1988; Falconar and Young, 1991).

Schlesinger *et al* (1985) demonstrated that anti-NS1 MAbs afforded passive protection to adult mice against lethal intra-cerebral (IC) challenge with the YF 17D-204 vaccine . Of the five anti-NS1 MAbs tested, two were able to confer protection.

Significantly these protective MAbs had high complement-fixing activities whereas the three non-protective MAbs had little or no detectable complement fixing activity. This observation suggests that protection was most likely mediated by antibody binding to cell surface expressed NS1 and complement mediated lysis of infected cells. It was also shown that active immunization with NS1 led to solid protection against lethal IC challenge with YF 17D-204 virus (Schlesinger *et al,* 1985). NS1 immunization was also demonstrated to protect rhesus monkeys (the natural host of YF) from challenge.

NS1 related protective immunity has also been investigated for dengue-2 virus. Active immunization of mice with dengue-2 NS1 conferred protection against challenge with the homologous dengue-2 virus but not heterologous challenge with dengue-1 virus (Schlesinger, 1987), despite the fact that common antigenic determinants exist on the NS1 protein (Russell *et al*, 1970). Synergistic effects of non-protective and partially protective antibodies were investigated by Henchal *et al* (1988). Although prolonged survival or reduced mortality occurred with most MAb combinations, increased morbidity was observed with the administration of two anti-NS1 MAbs. It was thought that this could be due to mutual competition on overlapping binding sites (Henchal *et al*, 1987) and / or unknown dynamic changes in antigen conformation. In light of more recent findings (Falconar, 1997) it is possible that these MAbs may have had direct deleterious effects on vascular integrity

During human dengue virus infection, NS1 is a well established target of humoral immune responses. The protective capacity of anti-NS1 antibody and the presence of substantial amounts of similar antibody in convalescent sera from dengue fever and dengue haemorrhagic fever patients (Falker *et al*, 1973) suggest a role for complement fixing, anti-NS1 antibodies in the protective immune response to dengue virus infection. Other workers have found no apparent correlation between the complement fixing ability of individual MAbs and protective capacity (Henchal *et al*, 1988; Young and Falconar, 1990), suggesting other immune mechanisms for example, ADCC (antibody-dependent cell-mediated cytotoxity), may also be involved in recognizing NS1 on the surface of infected cells.

(d) NS1 function

Although the precise function of NS1 is still unclear, a role for NS1 in virion assembly, transport and/or release was initially postulated because of its glycosylated nature and the fact that it was secreted with kinetics that mirrored that of the structural glycoproteins prM and E (Rice *et al*, 1986; Lee *et al*, 1989; Mason, 1989). More recent reports from Mackenzie *et al* (1996), and genetic analyses by Muylaert *et al* (1996; 1997) and Lindenbach and Rice (1997; 1999) however suggested a role for NS1 in viral RNA replication despite its extracytoplasmic localization. A function for YF NS1 in early replication was demonstrated with a temperature sensitive mutant produced by a single mutation in NS1. RNA accumulation occurred at the nonpermissive temperature only after establishment of infection at a permissive temperature (Muylaert *et al*, 1997). Viral RNA accumulation, growth in culture and *in vivo* neurovirulence have all been shown to be impaired for viruses containing mutations of either or both N-linked glycosylation sites of yellow fever NS1 (Muylaert *et al*, 1998) indicating that NS1 requires translocation into the ER and subsequent carbohydrate linkage for function. Not surprisingly, a version of NS1 lacking its signal sequence also did not function in *trans* complementation (Moorman *et al*, unpublished data from Lindenbach and Rice, 1999).

NS1 appears to function in a flavivirus-specific manner as dengue-2 virus NS1 did not productively interact with YF virus replication machinery (Lindenbach and Rice, 1999). Complementation with the heterologous dengue-2 NS1 gene was not successful except following a mutation in NS4A with a change at residue 42 of an asparagine to tyrosine (Lindenbach and Rice, 1999) demonstrating for the first time a genetic interaction between NS1 and NS4A. It was further proposed that this translated to a direct interaction between the lumenal NS1 and the transmembrane NS4A that was pivotal to the function and/or stability of the replication complex (Figure 1.2C). These results are supported by previous cryo-EM studies showing that both NS1 and NS4A are localized to vesicle packets (Mackenzie *et al*, 1996; Mackenzie *et al*, 1998; Westaway *et al*, 1997b) (Figure 1.2B).

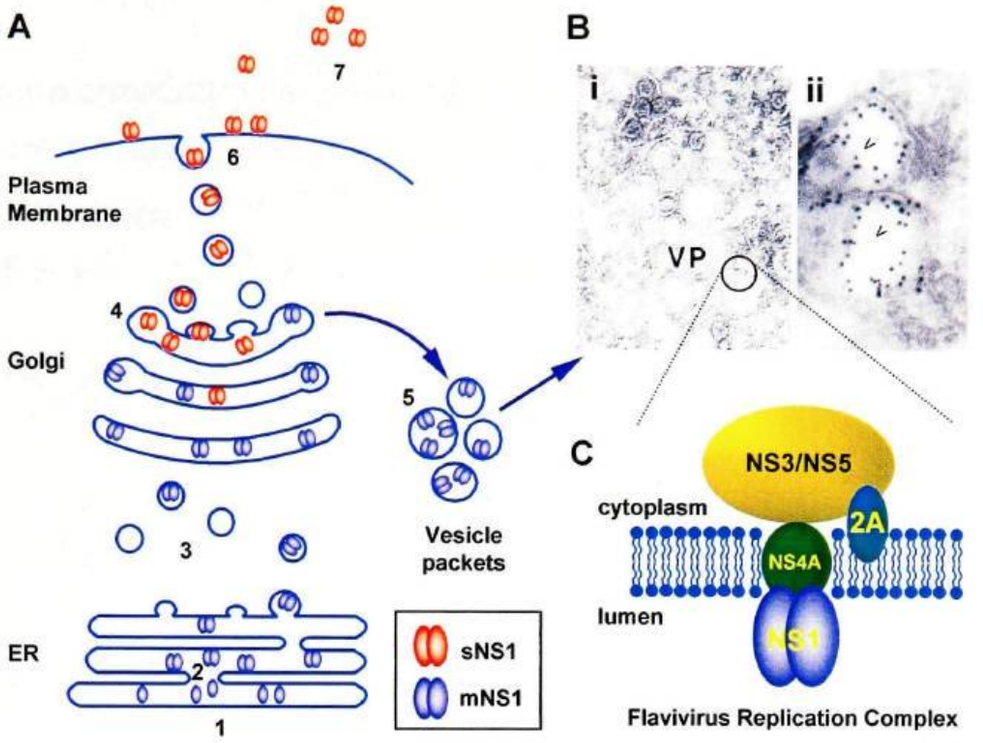


Figure 1.2 **Maturation pathway of the dengue virus protein, NS1. A, NS1 is first expressed in the endoplasmic reticulum (ER) as a hydrophilic monomeric species that is rapidly glycosylated at two sites (1). Dimerization occurs within 10-20 minutes and is associated with the acquisition of hydrophobicity and membrane-association (2). Dimeric NS1 traffics through the secretory pathway to the Golgi (3) where, for a portion of NS1, the carbohydrate moiety at ^{130}Asn is trimmed and processed to a complex form, generating sNS1 (4). mNS1 associates with (or is involved in the formation of) vesicle packets derived from virally modified *trans* Golgi and functions to assist in viral RNA replication (5). sNS1 is transported to the cell-surface (6) and/or secreted (7). B (i), thin-section electron microscopy of dengue virus induced vesicle packets (VP) in Vero cells, (ii), cryo-immuno electron microscopy of vescicle packets immuno-gold labeled with anti-NS1 MAbs (Mackenzie *et al*, 1996). C, Schematic of a hypothetical model showing the composition and architecture of the flavivirus replication complex in association with the VP membrane (Khromykh *et al*, 1999b).**

Further evidence was provided in biochemical assays using a recombinant NS4A fused to glutathione-S-transferase. When bound to glutathione beads, the fusion protein was found to retain several virus-specific proteins including NS1, NS2A, NS3, NS3-4A and NS5 (Mackenzie *et al*, 1998). Although it is unusual for a nonstructural glycoprotein to localize within vesicles and to be required for RNA replication, it has been hypothesized that NS1 may play a structural role in assembling the components of the viral replicase through its relationship with cellular membranes. It may also use protein-protein interactions directly to assemble replicase components although the involvement of other replicase

components cannot be excluded with current evidence.

An unknown enzymatic function for NS1 and the regulation of another viral or host component by NS1, have all been postulated by Lindenbach and Rice (1997). Further work is required to clarify these issues, as the precise role that NS1 plays in RNA replication remains to be fully characterized.

1.7 SCOPE OF THIS THESIS

The aim of this thesis was to more clearly define the antigenic structure of dengue virus NS1. Although many studies have been undertaken on the E glycoprotein antigenicity and structure, detailed equivalent information for NS1 is currently unavailable. The fact that NS1 does not form part of the virion and is unable to contribute to the phenomenon of ADE, together with the fact that it is able to induce a protective immune response in both humans and animals makes it a potential component of dengue vaccine studies. To this end, the identification of antigenic regions of the protein were a priority. Where possible, attempts have been made to relate antigenic regions to that of a speculative tertiary model of NS1. The human immune response to NS1 during dengue virus infection was also investigated using immunoassays. This study was aimed primarily at determining specific anti-NS1 antibody responses and NS1 levels in the serum of infected patients and to assess if there was any correlation with disease severity and these parameters. As the function of NS1 is still unknown, crystallography studies were undertaken to obtain a clearer understanding of function based on structural homology with other proteins. A purification regime was developed for recombinant NS1 expressed in the baculovirus system, to allow the production of high concentrations of protein. This recombinant protein was rigorously tested prior to crystallography trials to ensure that it maintained the native conformation of the protein in regard to oligomerization, glycosylation and antigenicity. Preliminary crystals generated in this study have formed the basis of ongoing studies. Taken together, this thesis undertakes to increase our knowledge on the antigenicity, structure and human immune response to dengue virus NS1.

CHAPTER 2

MATERIALS AND METHODS

2.1 TISSUE CULTURE

(a) Maintenance of cell lines

Vero cells were split with 1% trypsin / versene at a ratio of 1:10 in M199 medium (Life Technologies) with 5% foetal calf serum (Life Technologies) and supplemented with sodium hydrogen bicarbonate (0.176% final concentration), 100 U / mL penicillin and streptomycin (Life Technologies) and incubated at 37°C in an atmosphere of 5% CO_2. For maintenance, Vero cells were incubated in the same media with a decrease in foetal calf serum to 2%.

C6/36 cells (*Aedes albopictus*) were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI medium (Life Technologies) with 5% foetal calf serum and supplemented with antibiotics at a final concentration of 100 U / mL penicillin and streptomycin and 1% glutamax (Life Technologies). C6/36 cells were incubated at 28°C and were split at a ratio of 1:10 twice weekly.

Spodoptera frugiperda (*Sf*9) cells were purchased from Invitrogen and maintained primarily in TC100 medium (Life Technologies) supplemented with 10% foetal calf serum (Life Technologies) with the addition of 0.1 mg / mL kanamycin and 100 U / mL penicillin and streptomycin. For serum free conditions SF900 II medium (Life Technologies) was used and supplemented with 0.1 mg / mL kanamycin (Life Technologies) and 100 U / mL penicillin and streptomycin (Life Technologies). Serum free adapted Sf9 cells were a gift from Dr. Steve Reid from the Chemical Engineering Department, University of Queensland. *Sf*9 cells were incubated at 28°C either on a shaking platform at 120 rpm or in magnetic spinner bottles (Techne).

Serum free adapted *Sf*9 cells were maintained with a strict splitting regime. The suspension cultures were adjusted to a concentration of 4 X 10^5 cells / mL at each split and were never allowed to reach a density of greater than 4 X 10^6 cells / mL. Cultures were infected at an m.o.i. of 1 - 10 at a cell density of 2 X 10^6 cells / mL.

All cell lines used were routinely screened for the presence of *Mycoplasma* sp. using the Gen-Probe Rapid Detection System (BioMediQ) or an in-house PCR assay.

(b) Propagation of Dengue Virus

Dengue type 2 virus (PR159 or New Guinea-C strain) was grown either in Vero cells at 37°C or C6/36 cells at 28°C. Cell monolayers were infected at an m.o.i. of 0.01 - 1 in serum free media. The monolayers were incubated for one hour at their respective temperatures to allow virus adsorption after which the final concentration of foetal calf serum was adjusted to 2%. The tissue culture supernatants were harvested and clarified at approximately four days post infection or when cytopathic effect (CPE) was evident. Virus stocks were titred by plaque assay, aliquotted and stored at -70°C.

(c) Dengue virus plaque assay

Vero cell monolayers were grown in 24 well plates (Nunc). When the cells were 90% confluent, 200 µL of serially diluted virus was added in serum free M199 media and incubated for one hour at 37°C in an atmosphere of 5% CO_2. The inoculum was removed by aspiration and M199 media containing 1.5% (final concentration) carboxymethyl cellulose (CMC) (BDH) was overlaid on to the cells. The plates were incubated undisturbed for a minimum of seven days. 1.0 mL of 20% formalin was added to each CMC containing well and incubated for 30 minutes. The formalin was removed, the plate washed and then 0.2% crystal violet dissolved in PBS was added to each well and left for 30 minutes. The plates were then washed well under running water, air dried and the plaques counted.

(d) Preparation of polyethylene glycol (PEG) pellets

At four days p.i., tissue culture supernatants were clarified at 4 000 rpm for 15 minutes. The pellet was discarded and the supernatant was adjusted to a final concentration of 0.4 M NaCl and 7% PEG 6 000 (BDH) and mixed overnight at 4°C. The mixture was centrifuged at 13 000 rpm for 30 minutes and the pellet resuspended in 1.0 mL PBS. Samples were stored at -20°C.

(e) Baculovirus plaque assay / plaque purification

Monolayers of *Sf*9 cells in 35 X 10 mm dishes (Nunc) were prepared, the tissue culture supernatant removed and serial dilutions of virus diluted in TC100 was added (250 µL / dish). The virus was allowed to adsorb for one hour before being removed. Two mLs of a 1:1 mixture of 3% low gelling temperature (LGT) agar and AcNPV-PBS (150 mM NaCl, 25 mM KCl, 5 mM Na_2HPO_4, 1 mM KH_2PO_4) was gently overlaid onto the cells and allowed to set. 1.0 mL of TC100 + 10% FCS was then added to the surface of the agar and the cells were subsequently incubated at 28°C in a humid environment for a minimum of four days. A 1.0 mL solution of 0.03% neutral red (Life Technologies) diluted in AcNPV-PBS was added to each dish and incubated for one hour at 28°C. The solution was then removed, the outer edges of the dishes sealed in parafilm, inverted and incubated for 24 hours. Plaques were either counted (for plaque assay) or individually picked using a pasteur pipette and added to 200 µL of serum free TC100. The plaque picks were then stored at 4°C overnight to allow elution of the virus and 100 µL was subsequently added to *Sf*9 cells in 24 well dishes (Nunc) to amplify the virus. All recombinant baculoviruses were plaque purified three times. At each stage culture supernatants and cell extracts were screened by an immuno-dot blot assay for protein production using NS1-specific monoclonal antibodies. Those showing maximal secretion were chosen for further plaque purification.

(f) Immunofluorescence

The tissue culture supernatant of infected cell monolayers in individual wells of 24

well plates was removed, the cells were washed and then resuspended in PBS. Aliquots (30 µL) were dispensed onto immunofluorescence slides and allowed to air dry thoroughly. Alternatively, infected cells monolayers were grown on glass coverslips in 24 well plates, with the coverslips being taken through subsequent immuno-staining. If required, the cells were fixed in ice cold acetone for 15 minutes. Polyclonal serum or MAb was added to each spot in a 100 µL volume of 0.01% Evans Blue and incubated in a humid chamber at 37°C for 30 minutes. The cells were subsequently washed by immersion in a stirring bath of PBS for five minutes. This was repeated three times. The secondary probe was added at a 1:200 dilution in PBS and incubated at 37°C for 30 minutes in a humid chamber. Cells were washed as previously described and mounted with glycerol saline (9:1 glycerol : PBS pH 8.6 containing 2.5% 1,4-diazabicyclo(2.2.2)octane) under a glass coverslip. Fixed and stained coverslips were inverted into glycerol saline on a glass microscope slide. The edges were sealed with Depex (BDH) and the slides were viewed under an immunofluorescence microscope.

2.2 ANTIBODIES

(a) Polyclonal rabbit anti-NS1

Polyclonal rabbit anti-NS1 was prepared by subcutaneous injection of rabbits with dengue-2 virus NS1 protein immuno-affinity purified from infected Vero supernatants. Fifty µg of NS1 was mixed with an equal volume of complete Freund's adjuvant and injected at multiple subcutaneous sites. Two injections were given two weeks apart with a third injection (in incomplete Freund's) four weeks later. Blood was collected four weeks after the third injection, allowed to clot and serum was stored in aliquots at -70°C, or at -20°C, mixed 1:1 with sterile glycerol.

(b) Monoclonal antibodies

The anti-NS1 monoclonal antibodies (MAbs) used in this study were selected from an extensive panel produced and described by Falconar and Young (1991). Their characteristics are described in Table 2.1.

BALB/c mice were hyperimmunized with immunoaffinity purified dimeric sNS1 from dengue-2 virus (PR159) and splenocytes were fused with the SP2/0 Ag14 plasmacytoma cell line. After cloning twice by limiting dilution, high titre stocks were obtained by ascitic fluid production in syngeneic mice.

TABLE 2.1 : Characteristics of anti-NS1 monoclonal antibodies used in this study

Monoclonal antibodies	linear epitope[1]	NS1 Antibody Specificity	Passive protection[2] (%)
1C6.3	-	Flavivirus cross-reactive	14
1A12.3	^{113}YSWKTWGKA^{121}	Flavivirus cross-reactive	50
4H3.4	^{111}LRYSWKTWG^{119}	Flavivirus cross-reactive	13
3D1.4	^{113}YSWKTWGKA^{121}	Flavivirus cross-reactive	30
3A5.4	^{111}LRYSWKTWG^{119}	Flavivirus cross-reactive	30
5H10.3d	-	D2 specific	13
1G5.4d	-	D2 specific	50
1H7.4	^{25}VHTWTEQYK^{33}	D2 specific	<6
5H4.4	^{25}VHTWTEQYK^{33}	D2 specific	10
2C9.4	-	D2 specific	10
4D4.4	-	D2 specific	10
5B9.3	-	Flavivirus cross-reactive	<6
5C7.4	-	D2 specific	20
5H5.4	^{301}TTASGKLIT^{309}	D2/D4 specific	20
1G5.3	^{299}RTTTASGKL^{307}	Flavivirus cross-reactive*	<6
5H4.3	-	Flavivirus cross-reactive*	33
5F10.3	-	Flavivirus cross-reactive*	17
1E2.3	-	D2/D4 specific	50
5H6.3	-	D2/D4 specific	38
5B6.3	-	D2/D4 specific	17
2A5.1	-	D2/D4 specific	60
5B5.3	-	D2/D3/D4 specific	38

1. Linear epitope specifity was described in Falconar *et al* (1994).
2. Passive protection data as reported in Young *et al* (1990).

* Recognizes dengue-2 and dengue-4 and encephalitic flaviviruses (Young *et al*, 1990)

2.3 MOLECULAR CLONING

(a) NS1 gene constructs

Total cytoplasmic RNA was extracted 24 hours post-infection from dengue-2 virus infected C6/36 cells (m.o.i. of 10). The NS1 and NS2A genes were copied as cDNA using reverse transcriptase and primers located 3' of NS2A and was subsequently amplified by PCR using specific primers outlined in Table 5.1. The amplified products were cloned into the *Bam*H1 site of plasmid Bluescript II SK (+/-) (Stratagene). Nucleotide sequences at the 5′ and 3′ end of the inserts were verified by dideoxynucleotide sequencing (Sanger *et al*, 1977) using Sequenase (USB).

(b) Construction of recombinant baculoviruses

The NS1 inserts were removed from plasmid Bluescript SK by *Bam*H1 digestion and ligated to a *Bam*H1, dephosphorylated derivative of the transfer vector pAcYM1 (Matsuura *et al*, 1987). The derived recombinant transfer vectors were characterised by restriction enzyme digest analyses. *Spodoptera frugiperda* cells were transfected with a mixture of recombinant transfer vector DNA (1 μg) and AcRP23-LacZ baculovirus linearized DNA (50 ng) using Lipofectin (Boehringer) (Kitts *et al*, 1990). Recombinant viruses were originally identified on the basis of their white plaque phenotype after staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). To confirm that NS1 protein was being expressed from the different constructs, infected cell extracts were analysed by immunoblotting with an NS1 specific MAb. Recombinant viruses were plaque purified three times before amplification of the virus stocks. Primary, secondary and tertiary virus stocks were grown, titred and stored at -70°C for long term storage or at 4°C (for working stocks).

2.4 ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

(a) NS1 capture ELISA

Immulon 4 ELISA plates (Dynatech) were coated with 50 µL of a 1:1500 dilution of rabbit anti-NS1 polyclonal sera in coating buffer (0.15 M Na_2CO_3, 0.35 M $NaHCO_3$, pH 9.6) overnight at 4°C. This antibody was removed and the plates were blocked with PBS containing 1% gelatin (type A from porcine skin, Bloom 60) (Sigma) and incubated at room temperature for one hour. The sample to be quantitated for NS1 (either purified preparations, controls or patient serum samples) were serially diluted in PBS.0.25% gelatin and incubated at 37°C for one hour. Plates were then washed with PBS containing 0.5% Tween (PBS.T) and an anti-NS1 MAb was added at a 1:1000 dilution in PBS.0.25% gelatin. After a one hour incubation at 37°C the plates were washed as before and a 1:1000 dilution of peroxidase-conjugated goat anti-mouse IgG (Jackson Immunochemicals) was added and incubated as previously described. The plates were then washed and developed using standard o-phenylene diamine (OPD) substrate methodology. The reaction was halted by the addition of 25 µL of 2 M H_2SO_4 and the optical densities were read at a wavelength of 492 nm (MR5000, Dynatech). This method is shown diagrammatically in Figure 3.1.

(b) ELISA detection of NS1-specific antibodies in patient serum samples

Immulon 4 microtitre plates were coated with 50 µL per well of a 1 µg / mL stock of immunoaffinity purified secreted NS1 (sNS1) and incubated overnight at 4°C. After washing the plate four times with PBS.T, 150 µL per well of PBS.1% gelatin (Sigma) was added and incubated at room temperature for one hour.

Serum samples were diluted in PBS.T.0.25% gelatin (usually 1:5, 1:10 and 1:50) and incubated at 37°C for one hour. Following four washes with PBS.T a 1:1000 dilution of peroxidase conjugated goat anti-human IgG was added and incubated at 37°C for one hour. After washing with PBS.T the plates were developed using standard OPD methodology as above.

(c) Competition ELISA

The competition assays were based on a method described by Lopez *et al* (1986) with slight modification. Each competition experiment was done in quadruplicate and 50 µL was used per well, unless specified otherwise. Immulon 4 microtitre plates (Pacific Diagnostics) were coated with 10 µg of Protein A (Pharmacia) in coating buffer overnight at 4°C. After washing the plates with PBS.T, 150 µL of blocking solution (PBS containing 1% gelatin) was added to each well and left at room temperature for one hour. The plates were washed with PBS.T and then 18 µg of rabbit anti mouse IgG (H & L) in PBS.T / 0.25% gelatin was allowed to bind to the Protein A for one hour at 37°C. Following four washes with PBS.T, the primary MAb (diluted 1 / 100 in PBS.T / 0.25% gelatin) was added and incubated at 37°C for one hour. Plates were washed as before and immunoaffinity purified ^{35}S-methionine / cysteine-labelled NS1 (2 000 cpm / well) was combined with a 1/10 dilution of the secondary MAb in PBS.T / 0.25% gelatin, and immediately added to the plate and incubated at 37°C for one hour. The plate was then washed four times with PBS.T followed by the addition of lysis buffer (2% SDS, 1% 2-mercaptoethanol in SDS - PAGE sample buffer) to each well. After ten minutes incubation at room temperature the lysis buffer was removed individually from each well, added to 1.0 mL of scintillation fluid (Optiphase HiSafe II, Wallac) and counted in a β-scintillation counter (LKB). A set of positive controls were run with each set of experiments and binding was expressed as a percentage of NS1 binding in the absence of competing antibody ie.

$$\frac{\text{average cpm in the presence of competing antibody}}{\text{average cpm in the absence of competing antibody}} \times 100 = \% \text{ binding}$$

Between 0 - 30% binding was defined as strong competition, 31 - 75% was defined as partial competition, 76 - 150% as no competition and greater than 150% as indicating enhanced binding.

2.5 PROTEIN ANALYSIS

(a) Immunoaffinity purification of NS1

(i) Preparation of the immunoaffinity column

A protein A column was washed sequentially with 50 mLs of 40 mM diethylamine (DEA) in PBS, 0.1 M citric acid pH 2.5, 2 M urea pH 8 and high salt binding buffer (1.5 M glycine / 3 M NaCl pH 8.63). The latter was used because the monoclonal antibody (5B9.3) employed to generate the column was of the IgG1 subclass. Ascites (20 mLs) was clarified at 10 000 rpm for 60 minutes and filtered through a 0.45 μm filter. NaCl was added to give a final concentration of 3 M and glycine to 1.5 M. The pH was adjusted to 9.0. The protein A was then added to the ascites solution and mixed overnight at 4°C on a rotating mixer before being poured into a column and washed with 50 mLs of high salt binding buffer. Antibody was eluted with 0.1 M citric acid pH 3 and twenty 0.5 mL fractions were collected. Protein assays were performed (with a BCA kit (Pierce)) and the six peak fractions were collected.

A 20 mg / mL sodium periodate solution was added to the peak antibody fractions at a 1:10 dilution and mixed at room temperature for sixty minutes. The oxidizer was removed using an Econo-Pac 10DG desalting column and six peak fractions (0.5 mL each) were collected. After washing to remove isopropanol, affi-gel Hz (Bio-Rad) was added to the peak fractions and mixed on a rotary shaker overnight at room temperature. The gel slurry was poured into a chromatography column and washed with PBS and subsequently stored in PBS containing 0.02% sodium azide.

(ii) Protein production

Vero cells were infected at an m.o.i. of 0.5 and incubated for four days prior to immunoaffinity purification. C6/36 cells were infected with the same regime for two days and *Sf*9 cells were infected with recombinant baculovirus at an m.o.i. of 1 for 24 hours. For ^{35}S - labelled NS1, cells were then labelled overnight with 50 μCi / mL

of *trans* ^{35}S-methionine / cysteine (ICN) in minimal essential media (MEM) deficient in methionine and cysteine (ICN) for Vero and C6/36, and methionine and cysteine free SF900II for *Sf*9 cells. Both cell monolayers and media were harvested separately and membrane-associated (mNS1) and secreted forms of NS1 (sNS1) were immunoaffinity purified according to the method of Falconar and Young (1990). In summary, infected cell culture supernatants were clarified by centrifugation and adjusted to contain 5 mM EDTA, 0.02% NaN_3, 1 mM PMSF and 1 mM benzamidine HCl.

Cell monolayers were washed with ice cold PBS (pH 7.4) and scraped into ice-cold non-denaturing harvest buffer (HB; 0.005 M EDTA pH 8, 0.01 M Tris pH 7.4, 0.15 M NaCl, with protease inhibitors). TX-100 was added to give a final concentration of 2% (v/v) and the cells were solubilized with slow mixing on a magnetic stirrer overnight at 4°C. The detergent insoluble fraction was removed by centrifugation at 13 000 rpm at 4°C. The TX-100 concentration in the cell lysates was reduced to 0.5% by the addition of ice-cold HB. All subsequent steps were performed at 4°C.

The immunoaffinity column was prepared for use by equilibration of the column with HB containing 0.5% TX-100 and the cell lysate and culture media fractions were passed through the column separately. The column was then washed with HB containing 0.1% TX-100 and the bound NS1 was eluted by reverse flow using 20 mM DEA (diethylamine) in HB with 0.1% TX-100. Half mL fractions of the NS1 containing eluate were collected and neutralized with 40 µL of 1 M Tris-HCl pH 6.0. For secreted NS1 the immunoaffinity purification was the same as that stated above, except for the omission of detergent from the procedure.

Eluted protein was analyzed for purity by western blotting and silver staining. Protein concentration was determined using a Lowry modified BCA (bicinchoninic acid) reagent (Pierce) with BSA protein standards.

(b) Centricon purification of NS1

Routinely, centricon-30 (Amicon) and microcon-100 concentrators (Amicon) were

used to concentrate protein solutions according to the manufacturer's instructions. Briefly, protein solution (2 mL maximum volume) was added to the sample reservoir. The centricon concentrator was placed in an SS34 rotor and centrifuged at 5000g at 4°C in 15 minutes intervals, until the desired concentration was achieved as determined by the amount of filtrate generated. The concentrator assembly was then disassembled and the filtrate retained for analysis (if required). A retentate cup was then placed over the sample reservoir and the assembly was inverted and centrifuged at 1000g for 2 minutes at 4°C to collect the retentate.

(c) Cross-linking

A protein solution for cross-linking was diluted in 0.2 M NaCl / 50 mM Hepes with a range of BS^3 (*Bis* (Sulfosuccinimidyl) Suberate) (Pierce) concentrations (usually 0 - 20 mM) and incubated at room temperature for two hours. The samples were then boiled in sample buffer containing DTT (5 mM) and run on pre-cast 4-20% SDS - PAGE gradient gels (Bio-Rad) at 4°C prior to silver staining or western blotting.

(d) Enzymatic Digestions

(i) Trypsin and *Staphylococcus aureus* V8 protease

Routinely, 0.2 µg of immunoaffinity purified sNS1 in 5 µL, was digested with 100 µg / mL trypsin or *Staphylococcus aureus* V8 protease in SDS - PAGE sample buffer for two hours at 37°C. Preparations were either boiled in the presence of 0.12% SDS or left untreated prior to digestion. Digests were solubilized with disruption buffer containing SDS, without a reducing agent, (unless otherwise stated) and boiled (unless otherwise stated) before separation on a 10% SDS - PAGE gel.

(ii) Endoglycosidase digestions

Protein to be analysed was adjusted to 0.4% SDS and boiled for five minutes unless otherwise specified. An equal volume of 2X reaction buffer was added to the supernatant: for endoglycosidase H (Roche Molecular Biochemicals) the buffer

consisted of 0.2 M sodium citrate pH 5.5 and for endoglycosidase F (Roche Molecular Biochemicals) the buffer contained 0.1 M sodium phosphate pH 6.8, 0.1 M EDTA, 1% triton X-100. For endoglycosidase F and H, 0.05 units and 0.001 units were used respectively for 10µL reactions. Samples were digested at 37°C for three hours. An appropriate volume of 5X SDS sample buffer was then added and the digested samples were analysed by SDS - PAGE and western blotting.

(e) Immunoblotting

All protein preparations were electrophoresed through 10% discontinuous SDS - PAGE gels (0.375 M Tris-HCl pH 8.8, 0.1% SDS) with a 3.5% stacking gel (0.125 M HCl pH 6.8, 0.1% SDS) (Laemmli, 1970) using a disruption buffer containing SDS, without a reducing agent, unless otherwise stated. Proteins were transferred to 0.2 micron nitrocellulose membranes (Schleicher & Schuell) in transfer buffer (0.5 M Tris, 0.4 M glycine pH 8.4, 20% methanol) at 160 mA per gel for 20 minutes on a semidry western blotter (Satroblot II, Sartorius). Blots were probed with various MAbs (Table 2.1) as previously described (Young, 1989). The membrane was dried prior to blocking for two hours in PBS.T containing 3% skim milk powder (Carnation or Diploma) (PBS.T.MP). Blots were probed with an appropriate antibody diluted in PBS.T.MP and incubated for a minimum of one hour at room temperature. The membrane was then given three 10 minute washes in PBS.T followed by the addition of the secondary antibody (usually peroxidase-conjugated goat anti-mouse IgG (H & L) (Jackson Immunochemicals)) diluted in PBS.T.MP and incubated at room temperature for one hour.

(i) Enzyme substrate detection

Following the same washing procedure the proteins were visualized by the addition of 30 mg of chloronaphthol (Sigma) and 10 mg of DAB (BDH) each dissolved in 5 mL of methanol, added to 40 mL of PBS and 10 µL of hydrogen peroxide (Young, 1989).

(ii) Chemiluminescence detection

Alternatively, proteins were detected by chemiluminescence (ECL PLUS, Amersham Pharmacia Biotech) after the final wash in PBS.T following the manufacturer's instructions. Briefly, the primary antibody was diluted 1:5000 in PBS.T and added to the membrane, sealed in a plastic bag and incubated for one hour at room temperature. The membrane was washed three times, for five minutes each and the secondary antibody was diluted 1:10 000 in PBS.T and incubated for one hour at room temperature. The membrane was washed as before and given a final rinse in PBS. Supplied solutions A and B were allowed to come to room temperature and combined in a 40:1 ratio in a sufficient volume to allow 100 μL / cm^2 of membrane (generally 2.5 mL total volume for a mini-gel blot). Excess PBS was drained from the membrane and placed protein side up on a sheet of plastic. The mixed detection reagents were added to the membrane and incubated for 5 minutes in the dark at room temperature. Excess detection reagent was drained from the membrane and placed protein side down, on to a piece of plastic, folded to seal the edges and air bubbles smoothed out. The blot was exposed to X-ray film in an X-ray cassette and exposed initially for 15 seconds and subsequently for up to two minutes depending on the intensity of the signal after developing the first exposure.

(iii) N-terminal amino acid sequencing

Protease digested NS1 was separated on a preparative 10% SDS - PAGE gel, transferred to 0.2 μm PVDF membrane (Bio-Rad) in a 10 mM CAPS blotting buffer (pH 11) containing 10% methanol, and stained with 0.1% amido black. Appropriate bands were excised with a scalpel and submitted to an external facility for N-terminal sequencing.

(f) Triton X-114 (TX-114) phase separation of proteins

The method followed was that of Bordier (1981). Briefly a sucrose cushion (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.06% TX-114 (Sigma), 6% sucrose, 0.003%

bromophenol blue) was added to a 0.5 mL microcentrifuge tube and 200 µL (ie. 150 µL NS1 and ¼ volume of 4X TX-114 buffer (40 mM Tris-HCl pH 7.4, 600 mM NaCl, 4% TX-114)) of the test sample was slowly layered on top of the cushion. The tube was then incubated at 30°C for three minutes before being centrifuged at 2000 rpm for three minutes at room temperature. The aqueous phase (upper layer) was removed to a fresh microcentifuge tube containing 1 µL of TX-114 on ice and subsequently loaded back onto the same sucrose cushion. The tube was reincubated at 30°C for three minutes and centrifuged at 2000 rpm for three minutes at room temperature. The supernantant was collected in a fresh microcentrifuge tube on ice containing 4 µL of TX-114 and incubated at 30°C and recentrifuged. This supernatant was collected as the aqueous phase. The detergent phase was collected from underneath the sucrose layer. Volumes and TX-114 concentrations were adjusted to give concentrations of salt and surfactant content. Samples were analysed by western blotting.

(g) Two dimensional gel electrophoresis

Isoelectric focusing was performed using a Mini-PROTEAN II 2D cell (Bio-Rad) following the manufacturer's instructions. Proteins were subsequently visualized by silver staining.

(i) Tube gel preparation

Briefly, capillary gel tubes were set up in a vertical position in a casting tube which was sealed at the base with parafilm. Monomer solution was prepared (See Appendix 2.1) and each tube was filled to ¾ of the length of the tube. After polymerization of the solution the blue striped end of the tubes were attached to the sample reservoirs with tubing connectors and the bottom of each tube was washed with lower buffer. A gel tube was inserted into each of the 16 positions in the tube adaptor. Each sample reservoir was filled with upper chamber buffer (Appendix 2.1) and lower chamber buffer (Appendix 2.1) was added to the base. With a magnetic stirring bar in the base and with the apparatus sitting on a magnetic stirrer, the capillary tubes were pre-electrophoresed successively at 200V for 10 minutes,

300V for 15 minutes and 400V for 15 minutes. The buffers were then discarded and fresh buffers added to the sample reservoirs.

(ii) Sample preparation, loading and isoelectric focusing

An equal volume of first dimension sample buffer (Appendix 2.1) was added to the sample and incubated at room temperature for 15 minutes. With a hamilton syringe, the sample was underlaid at the base of the sample reservoir. The sample was then overlaid with 20 - 40 μL of sample overlay buffer (Appendix 2.1). Gels were run at 500V for 10 minutes followed by 750V for 3.5 hours.

(iii) The Second Dimension

To remove the tube gels from the capillary tubes a 1.0 mL syringe was added to the white end of a tube gel ejector. This was then attached to the end of a capillary tube and the gel gently extruded on to a piece of parafilm. The gel tube was then positioned lengthwise on to the top of a mini-protean gel (prepared as described previously) prepared with a two-dimensional gel comb. Two dimensional gel markers (Bio-Rad) were run concomitantly. The resulting gels were then silver stained.

(h) Staining of SDS-PAGE gels

(i) Silver staining

Gels were run as previously described and fixed in a solution of 30% ethanol and 10% acetic acid for one hour. The silver stain procedure is a modification of that of Blum *et al* (1987). Gels were rinsed three times with 30% ethanol for 20 minutes each followed by two rinses of 15 minutes in distilled water. The gel was then sensitized by the addition of a 0.25 g / L sodium dithionite solution for one minute, followed by two one minute washes in distilled water. The gel was then stained with a solution of 0.2% silver nitrate and 1mM formaldehyde for 30 minutes and washed for one minute in distilled water. Protein bands were then visualised with the

addition of a developing solution (6% sodium carbonate, 6 mM formaldehyde, 20 μM sodium thiosulphate) for five to ten minutes. Finally the reaction was stopped with the addition of 3.5% acetic acid and the gel washed in distilled water. For storage the gels were dried on to 3MM filter paper (Whatman) using a gel dryer (Bio-Rad).

(ii) Coomassie blue staining

Gels were fixed for one hour in 30% methanol, 10% acetic acid and then stained with 0.006% Coomassie Brilliant Blue G-250 (Bio-Rad), 10% acetic acid for two hours. Destaining was performed in 10% acetic acid for approximately two hours and fresh destain solution was added as required.

(i) Pulse-chase experiments

Prior to labelling 3 X 10^5 *Sf*9 cells were added to 35 x 10 mm dishes (Nunc), allowed to attach and form a monolayer and infected with a recombinant baculovirus at an m.o.i. of either 1 or 10. At 24 hours post infection (p.i.) the cell monolayers were washed twice with methionine / cysteine deficient SF900 II (Gibco) and then starved in the same media for 30 minutes. The cells were then pulse labelled for one hour with 50 μCi / mL of *trans* ^{35}S methionine / cysteine (ICN Biomedicals). After the pulse period, the monolayers were washed and chased in "cold" media with an excess (10 mM) of methionine and cysteine for various periods.

(j) Analysis of baculovirus expressed protein by radio-immuno precipitations (RIP)

Tissue culture fluids were harvested in the presence of protease inhibitors (10 mM pefabloc (Boehringer), 10 μg / mL aprotinin (Bayer), 2 μg / mL leupeptin (Sigma), 2 μg / mL benzaminidine (Sigma) and 10 μg / mL Nα-p-tosyl-L-lysine chloro-methyl ketone (TLCK) (Sigma)). Subsequent to the addition of protease inhibitors the samples were clarified by centrifugation at 2 000g for 15 minutes at 4°C and the

supernatant was used for further analysis. Cell monolayers were rinsed with ice cold AcNPV phosphate buffered saline (AcNPV-PBS: 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and incubated on ice for 20 minutes in 500 µL RIPA buffer (10 mM HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% triton X-100, 0.1% SDS) containing protease inhibitors. The *Sf*9 monolayers were then scraped from the dish into the RIPA buffer and clarified by centrifugation for 20 minutes at 13 000 g at 4°C. An appropriate volume of 4X RIPA buffer with protease inhibitors was added to the tissue culture supernatant so that the buffer conditions would mirror that of the cell extract.

Equal volumes (200 µL) of the cell extract and cell culture supernatant were added to 300 µL of RIPA buffer and precleared with 25 µL of 30% Protein A sepharose (Pharmacia) hydrated in RIPA buffer, and mixed for one hour at 4°C. The samples were then centrifuged at 10 000g for five minutes and the pellet was discarded before adding 3 µL of an anti-NS1 monoclonal antibody and mixing the samples overnight at 4°C. A 25 µL aliquot of a 30% suspension of protein A sepharose was added to the samples and incubated at 4°C for one hour. The sepharose was then pelleted by centrifugation, rinsed twice with RIPA buffer containing 0.5 M NaCl and finally with RIPA buffer. Bound protein was released by boiling the sepharose in 50 µL of dissociation buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.001% bromophenol blue) and resolved on a 10% SDS - PAGE gel. Gels were fixed (30% ethanol, 10% acetic acid) for one hour, soaked in Amplify (Amersham) for 15 minutes, dried and autoradiographed.

(k) Factorial screen for crystallography trials

(i) Hanging drop vapour diffusion method

Twenty-four well plates were set up with 1.0 mL of each Hampton factorial solution in the base. The composition of each solution is listed in Appendix 5.1. The rim of each well was greased with vaseline and glass coverslips were prepared with one microlitre of the concentrated protein solution together with one microlitre of the factorial solution. The coverslip was then inverted over the well and gently pressed

to ensure that the well was completely sealed. Duplicate plates were set up and incubated at 4°C and 20°C respectively.

Plates were observed using an inverted microscope, on completion of the set up procedure and weekly thereafter.

(I) Electron Microscopy

Proteins were diluted in PBS to concentrations of the order of 0.01 - 0.001 mg / mL. Where cross-linking was performed, prior to dilution 10% gluteraldehyde (Fluka) was added to the PBS to achieve a final concentration of 1% gluteraldehyde. Droplets of approximately 3 μL of this solution were applied to thin carbon film on 400-mesh copper grids that had been glow-discharged in nitrogen for 30 seconds. After one minute the excess protein was drawn off, followed by washing in 5 μL of PBS, then 2 - 3 droplets of negative stain (2% uranyl acetate or 2% uranyl formate) was added. Uranyl acetate was obtained from Agar; uranyl formate was prepared according to the method of Russell and Hyder (1976). The grids were air-dried and then examined at 80 kV in a JEOL 100B transmission electron microscope at a magnification of 100 000X, or in a JEOL 2000FX transmission electron microscope at 120 kV and a magnification of 67 000X. Electron micrographs were recorded on Kodak SO-163 film and developed in undiluted Kodak D19 developer. The electron-optical magnification was calibrated for both microscopes by comparison with the width of Tobacco Mosaic Virus (180Å) imaged under these conditions. This work was performed by Dr. Lynne Lawrence, BRI, Melbourne.

APPENDIX 2.1

TWO DIMENSIONAL GEL BUFFERS

SOLUTIONS FOR THE FIRST DIMENSION

First Dimension Acrylamide stock

Acrylamide / bis	28.38 g
30% T / 5.4% C	1.62 g

The above chemicals were made to 100 mL with water, filtered and stored at 4°C in the dark.

10% Triton X-100 stock (w/v) solution

10 mL TX-100 was added to 90 mL distilled water and deionized overnight with 5g of AG 501-X8 ion exchange resin (BioRad)

First Dimension Sample Buffer

9.5 M Urea	5.7 g
2.0% triton X-100	2 mL of 10% stock
5% β-mercaptoethanol	0.5 mL
1.6% 5 / 7 Ampholyte	400 µL
0.4% 3 / 10 Ampholyte	100 µL

The above components were diluted to a final volume of 10 mL and warmed in a water bath to dissolve urea. Aliquots were stored at -70°C.

First Dimension sample overlay buffer

9 M urea	5.41 g
0.8% 5 / 7 Ampholyte	200 µL
0.2% 3 / 10 Ampholyte	100 µL
0.05% Bromophenol Blue	500 µL

The above components were diluted to 10 mL with distilled water and warmed in a water bath (maximum of 45°C) to dissolve urea. Aliquots were stored at -70°C.

Upper Chamber Buffer

degassed 100 mM NaOH

Lower Chamber Buffer

degassed 10 mM H_3P0_4

First Dimension gel monomer solution

9.2 M urea	5.5 g
4% acrylamide	1.33 mL first dimension acrylamide stock
20% triton X-100	2.0 mL 10% triton X-100 solution
1.6% 5 / 7 Ampholyte	400 µL
0.4% 3 / 10 Ampholyte	100 µL
0.01% ammonium persulfate	10 µL of 10% solution
0.1% TEMED	10 µL

This solution was made fresh (without the polymerization reagents) and warmed to dissolve the urea. The solution was then degassed for a minimum of 15 minutes before adding the TEMED and APS.

SOLUTIONS FOR THE SECOND DIMENSION

SDS Sample Equilibration buffer

0.0625 M Tris-HCl, pH 6.8	12.5 mL of 0.5 M Tris-HCl pH 6.8 stock
2.3% (w/v) SDS	23 mL 10% SDS solution (w/v)
5.0% (v/v) β - mercaptoethanol	5 mL
10% glycerol (w/v)	8 mL
0.05% Bromophenol Blue	2.5 mL
Distilled Water	49 mL

CHAPTER 3

NS1 IN HUMAN SERA

Publications arising from this Chapter:

Young PR, Hilditch PA, **Bletchly C** and Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. Journal of Clinical Microbiology **38** (3) 1053 - 1057.

3.1 INTRODUCTION

NS1 was first described in the early 1970s as a soluble complement fixing (SCF) antigen in infected cell cultures (Brandt *et al*, 1970; Cardiff *et al*, 1971) and was recognized as an important immunogen in infections of humans (Russell *et al*, 1980), and experimentally infected mice (Brandt *et al*, 1970). As NS1 does not from part of the virion, antibodies directed against it have no neutralizing or haemagglutination-inhibiting activity (Gibson *et al*, 1988). Protection by antibodies to NS1 is thought to involve immune recognition of NS1 on the surface of infected cells, leading to complement-mediated lysis and/or antibody dependent cell cytotoxicity or ADCC (Schlesinger *et al*, 1987). It should also be noted that the onset of hypovolemic shock in DHF patients is preceded by extensive activation of the serum complement system (Russell *et al*, 1969; Memoranda, 1973).

Despite the fact that the dengue virus non-structural protein NS1 has been found to be able to induce a partially protective immune response in experimental animal models (Schlesinger *et al*, 1985; Henchal *et al*, 1988), and for anti-NS1 MAbs to confer passive protection, few studies have examined in detail, the human immune response to NS1 in dengue virus infection (Falker *et al*, 1973; Kuno *et al*, 1990; Churdboonchart *et al*, 1991; Alcon *et al*, 2002). In contrast, the antigenic regions of the flavivirus E protein have been comprehensively studied (Henchal *et al*, 1985; Mandl *et al*, 1989; Guirakhoo *et al*, 1989; Hall *et al*, 1990; Tsekhanovskaya *et al*, 1993; Seif *et al*, 1995; Roehrig *et al*, 1998; Serafin and Aaskov, 2001; Beasley and

Aaskov, 2001) and serotype specific and cross-reactive determinants have been reported (Henchal *et al*, 1982; Megret *et al*, 1992; Iacono-Connors *et al*, 1996; Roehrig *et al*, 1998). There is also extensive information available in the literature regarding the human immune response to the E protein in flavivirus infections (Shyu *et al*, 1997; AbuBakar *et al*, 1997; Valdés *et al*, 2000; Roehrig *et al*, 2001; Wang *et al*, 2001).

The most prominent feature of DHF is plasma leakage (Kurane and Ennis, 1994). Microscopic studies at autopsy show swelling of capillary endothelial cells and capillary edema in soft tissues. Surprisingly though, the tissue damage observed in pathological studies is not very severe compared to the severity of illness (Bhamarapravati, 1989). Dengue virus antigens are not detected in vascular endothelial cells and patients with DHF recover quickly by replacement of lost plasma with electrolyte solutions (Nimmannitya, 1987). It has been suggested that this plasma leakage is due to an increase in vascular permeability arising through a malfunction in vascular integrity, rather than direct viral mediated destruction of endothelial cells (Kurane *et al*, 1991b; Kurane and Ennis, 1992). Henchal *et al* (1987) speculated that NS1 may stimulate an anamnestic response during human secondary dengue infections and that high levels of anti-NS1 may contribute to the pathogenesis of DSS through the formation of immune complexes.

If the proposal that NS1 be included as part of a sub unit vaccine to prevent dengue virus infections is to be considered, more information is required in relation to NS1 in naturally acquired human dengue virus infection. Limited information is currently available regarding the human dengue NS1-specific antibody responses and a better understanding of the immune response to NS1 and its role in protective immunity and / or pathogenesis of DHF / DSS is required (Shu *et al*, 2000). This section of the study therefore was designed to establish an assay that was able to quantitatively assess the level of NS1 in the serum of infected individuals and to relate these findings to disease severity. Previous studies in this area have been inconclusive due to the difficulties inherent in comparing results obtained using different methodologies, different flaviviruses, different viral proteins and different

stages of flavivirus infections. We aimed therefore to establish a sensitive capture ELISA using an existing panel of MAbs (Falconar and Young, 1991), and to screen a relatively large series of patient sera that included acute and convalescent sera, primary and secondary sera and samples from patients suffering different clinically defined grades of dengue virus infection. Furthermore, the induced antibody response to NS1 was also investigated in order to examine the postulated role of antibody to NS1 in pathogenesis originally suggested by Falker *et al* (1973).

3.2 RESULTS

(a) NS1 Capture ELISA

An NS1 antigen capture ELISA was developed to detect and quantitate NS1. This assay was in the form of a double sandwich ELISA in which the NS1 protein was captured by a polyclonal antibody immobilized on a microtitre plate. This polyclonal rabbit anti-NS1 is able to bind all four serotypes of NS1, with a subsequent specific MAb used as a selective probe for individual serotypes (Figure 3.1). Various capture and detection parameters were investigated. Patient serum samples were tested for the presence of circulating NS1 using the optimized ELISA. A preliminary screen was performed at a dilution of 1/10 to detect positive samples followed by titration and quantification.

(b) Development of the assay

(i) Selection and optimization of capture and detection antibodies

In order to establish a sensitive capture ELISA for the dengue virus NS1 glycoprotein, a cross-reactive rabbit polyclonal antisera raised against immunoaffinity purified sNS1 was generated and used to capture NS1 from all four dengue virus serotypes. A panel of MAbs listed in Table 3.1 including both cross-reactive and type-specific epitopes were also trialled as capture probes. Using any of the MAbs either in combination or alone as capture antibodies resulted in a

decreased signal and so the rabbit polyclonal antibody was chosen for all subsequent studies. Previous chequerboard analyses of a dilution series of the rabbit antisera against the full panel of MAbs and using a standard concentration of immunoaffinity purified NS1, established an optimum dilution of 1:1500 for the polyclonal antibody.

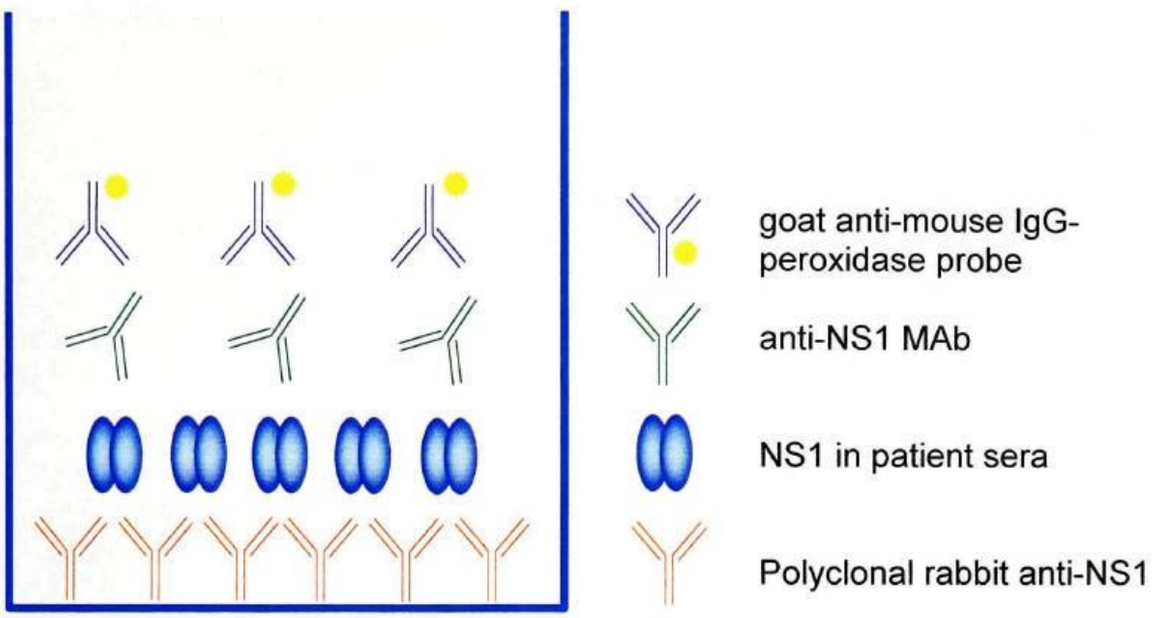


Figure 3.1 **Schematic diagram of NS1 capture ELISA. Polyclonal rabbit anti-NS1 was used to coat the wells of an ELISA plate. Diluted patient serum samples were then added and allowed to bind. Non-specific serum components were removed by washing and an NS1 specific monoclonal antibody (MAb) probe was then added. The MAb was detected with a goat anti-mouse IgG-peroxidase probe.**

Figure 3.2 shows the results of using these MAbs as detection probes in the analysis of a range of dilutions of the immunoaffinity purified secreted form of NS1. The graph clearly indicates that the dengue-2 specific MAbs (5H4.4, 1H7.4, and 2C9.4) clustered as a group and showed the highest binding profile. Peptide binding studies (Falconar *et al*, 1994) and the competition analyses described in the subsequent chapter (Chapter 4) have mapped these MAbs to the same epitope (^{25}VHTWTEQYK^{33}). The cross - reactive MAbs (3D1.4, 3A5.4 and 4H3.4) also clustered together reflecting their common epitope specificity (^{111}LRYSWKTWGKA^{121}) (Falconar *et al*, 1994). Although these MAbs are less efficient as detection probes for dengue-2 NS1, they should be able to be utilized for the analysis of NS1 produced by each of the other dengue virus serotypes.

Table 3.1 **NS1 specific monoclonal antibodies used as detection probes in the NS1 capture ELISA**

Monoclonal antibody[a]	ELISA titre[b]	Linear epitope[c]	Cross-reactivity with other dengue serotypes[d]			
			DEN1	DEN2	DEN3	DEN4
1H7.4	6.0	+	-	+++	-	-
2C9.4	5.4	+	-	++	-	-
5H4.4	5.4	+	-	+++	-	-
5H6.3	4.6	-	-	+++	-	+
5B5.3	3.5	-	-	++	+++	+
4H3.4	5.3	+	+++	+++	+++	+++
3D1.4	5.2	+	+++	+++	+++	+++
3A5.4	5.7	+	+++	+++	+++	+++

a All monoclonal antibodies used in this study are of the IgG1 isotype and are described in detail in Falconar and Young (1991).
b Titres are expressed as $\log_{10}$ 50% end-points as measured against purified NS1 coated ELISA plates.
c Reactivity of the MAbs with linear epitopes was determined by dot blot analysis against reduced and carboxy-methylated, purified NS1. PEPSCAN analysis has identified the location of these epitopes and is reported in Falconar *et al*, 1994. See text for details.
d Cross-reactivities were determined by immunoblot analysis of SDS-PAGE separated SDS lysates of Vero cells infected with the dengue virus serotype prototypes, DEN1 (Hawaii), DEN2 (NGC), DEN3 (H87) and DEN4 (H241).

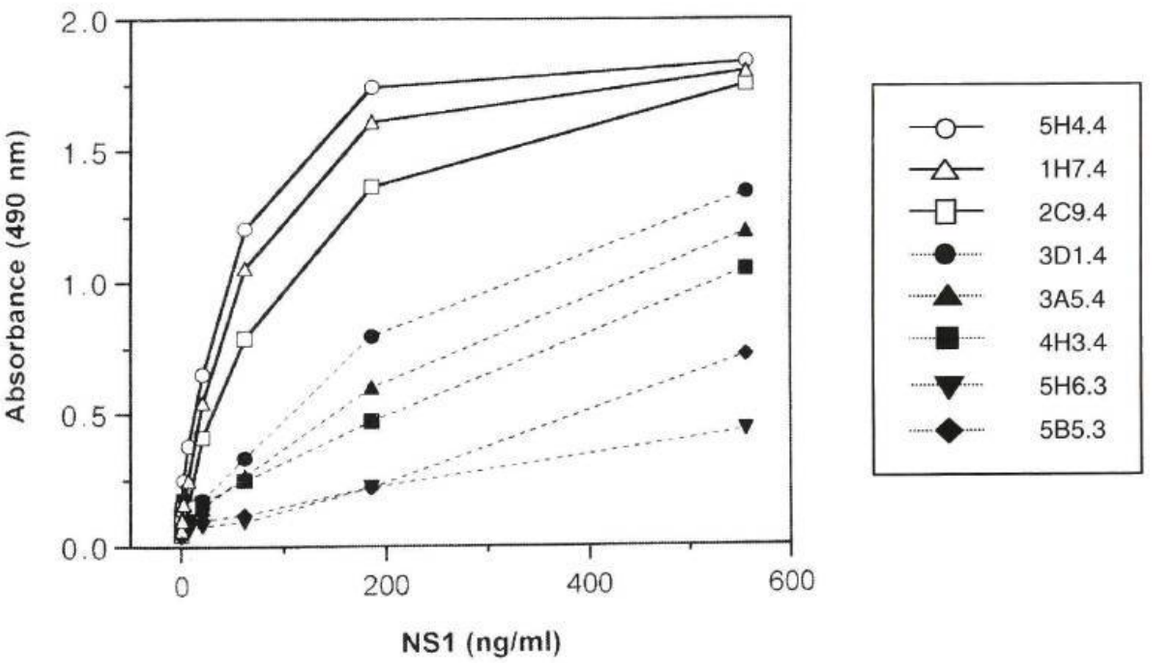


Figure 3.2 **Reactivity of a panel of monoclonal antibodies against serial dilutions of immunoaffinity purified NS1 in the capture ELISA. NS1 was captured with a 1:1500 dilution of rabbit anti-NS1 polyclonal antisera coated onto ELISA plates and subsequently probed with the monoclonal antibodies at a dilution of 1:1000.**

Due to the identification of synergistic interactions between specific MAbs in the competition analyses (described in Chapter 4), the effect of various combinations of MAbs on the detection sensitivity of the assay was examined. No improvement in detection was observed with these mixtures of MAbs when compared with the MAb 1H7.4 alone, therefore this MAb was selected for further assessment.

(ii) Sensitivity and reproducibility of the assay

To determine the sensitivity of the NS1 capture assay, replicates of serially diluted NS1 of known concentration were probed with MAb 1H7.4 (Figure 3.3). Both PBS and pooled normal human sera were used as diluents in order to determine the effect that the presence of serum may have on detection sensitivity. Figure 3.3 shows that the effect of normal human serum components on the capture and detection of NS1 was minimal at a dilution of 1 in 5 and negligible at 1 in 10. As the subsequent analysis of patient sera was carried out at dilutions equal to, or greater than 1 in 10, it was assumed that serum at these dilutions had little effect on detection sensitivity.

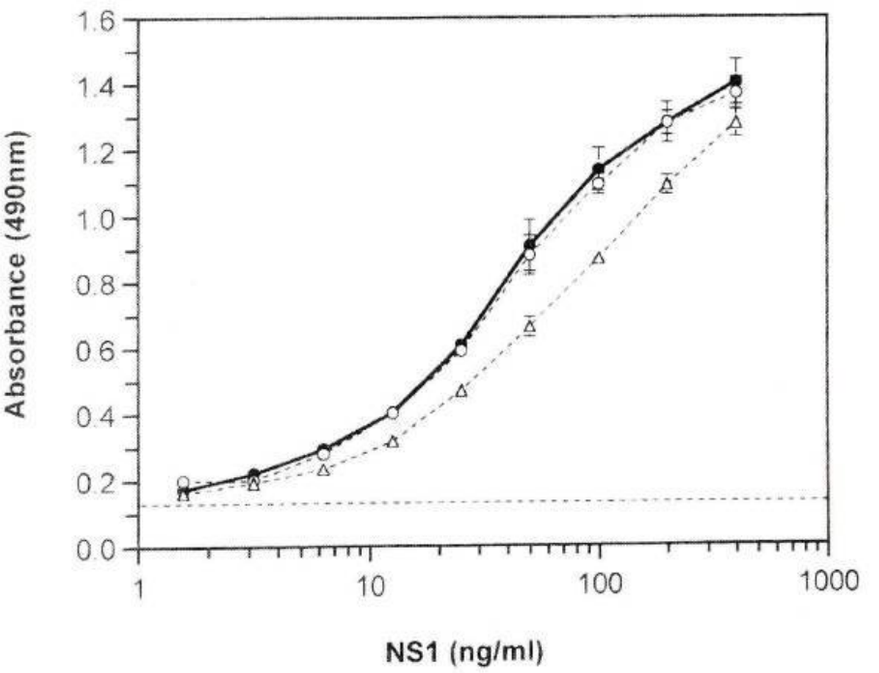


Figure 3.3 **Effect of human serum components on the detection of NS1 in the capture ELISA. Purified secreted NS1 was serially diluted in the absence (●) or presence of normal human serum at a dilution of 1 in 5 (△) and 1 in 10 (○). Data points represent the mean ± SD for four replicates. The dashed line is the mean for the negative control samples (OD_{490} 0.122 ± 0.012).**

The assay was considered positive if a sample gave an OD reading that was 3 standard deviations (0.036 OD_{492} units) above the mean OD value for negative control samples (0.122 $\pm$ 0.012). The limit of detection of NS1 using these criteria was approximately 4 ng / mL when the MAb 1H7.4 was used, however this was reduced to 15 ng / mL when the cross - reactive probe 3D1.4 was utilized.

The reproducibility of the assay was examined with multiple replicates assessed within the same test and by repeated testing over a two month period. Not surprisingly, the coefficient of variation (CV) results varied considerably depending on the level of NS1 present in the samples. In subsequent analyses of patient test sera, samples were titrated and estimates of NS1 levels were determined by comparison of absorbance readings of appropriately diluted fractions with a standard curve derived from a titration series of purified NS1, within the range 20 to 200 ng / mL. At a concentration of 100 ng / mL, the coefficient of variation for 12 replicates tested in the same assay was 4% and the test-to-test coefficient of variation, using 16 replicates was 12%.

(iii) Comparison of type-specific and group-reactive monoclonal antibodies in the detection of secreted NS1 of all four dengue virus serotypes

In order to examine the reactivity in the ELISA capture assay of NS1 derived from each of the four dengue virus serotypes, clarified tissue culture supernatants harvested from infected cell monolayers at peak CPE were tested (ranging from five to nine days depending on the serotype). The supernatant fractions were tested in the capture assay using either the type-specific (1H7.4), or group-reactive (3D1.4) monoclonal antibodies as detection probes (Figure 3.4). The specificity of the 1H7.4 probe for dengue 2 virus NS1 is clearly demonstrated in Figure 3.4A, as is the increased detection sensitivity of the MAb (approximately four fold) compared with MAb 3D1.4 (Figure 3.4B). Comparison with a standard curve performed in parallel and prepared with serial dilutions of purified dengue 2 virus NS1 of known concentration indicated that between 5 - 7 μg / 10^6 cells of NS1 is secreted from

dengue-2 virus infected Vero cells under these conditions. A time course analysis of NS1 secretion from infected cells, paralleled virus titres as determined by plaque assay (PR Young, personal communication) suggesting that it may be possible to use secreted NS1 as a surrogate marker for viral infection.

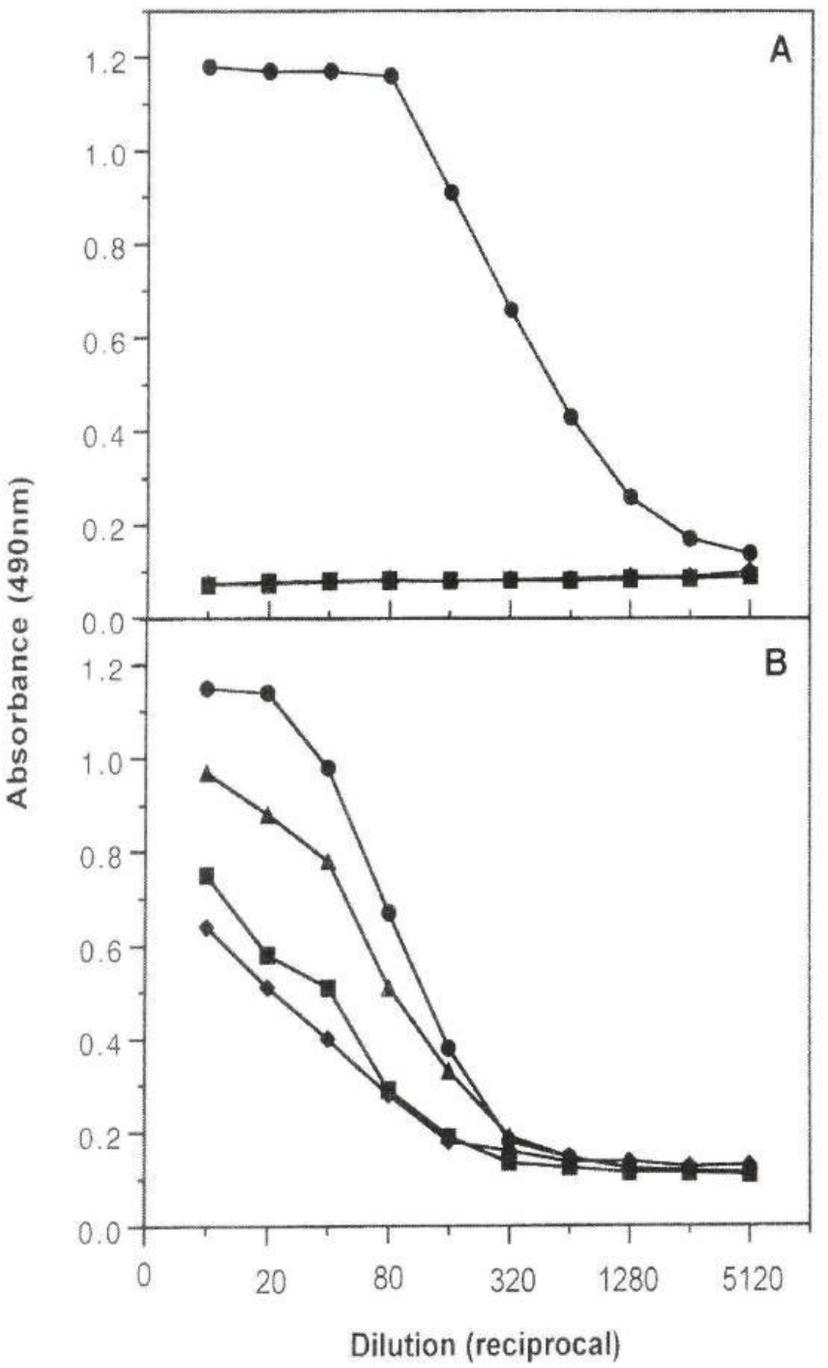


Figure 3.4 **Capture ELISA detection of NS1 secreted from Vero cells infected with each of the four dengue virus serotypes. A, type-specific (1H7.4) and B, group-reactive (3D1.4) monoclonal antibodies were used as the detection probes for tissue culture supernatants from Den1 (Hawaii◆), Den2 (NG-C●), Den3 (H81■) and Den4 (H241▲) infected cells.**

It is not known whether 3D1.4 recognizes the NS1 species derived from each serotype with equal avidity, however it is interesting to note that the reactivity profile demonstrated in Figure 3.2 reflects the antigenic pairings of the two known serotype subgroups, dengue 2/4 and dengue 1/3. It is also likely that the different levels of detection are in part due to the binding characteristics of the rabbit polyclonal capture antibody which was prepared by immunization with purified dengue-2 virus NS1 (refer to Chapter 2.2a). Differences in growth kinetics in Vero cells between the four dengue serotypes will also contribute to the varying levels of antigen detected in this assay. Currently, the assay employing 3D1.4 as the detection probe can only provide estimates of NS1 derived from all four serotypes. Purified preparations of each serotype will need to be generated to establish appropriate standard curves.

(iv) Detection of NS1 in patient sera

In order to test the suitability of the assay for quantifying NS1 in clinical samples, a panel of sera from dengue virus infected patients was examined. Serum samples were serially diluted and processed in the ELISA capture assay (using 1H7.4 as the detecting antibody) in parallel with a titration of immunoaffinity purified sNS1 as a standard. Absorbance readings of those dilutions that fell within the straight-line portion of the standard curve (10 - 100 ng / mL) were used to calculate the serum NS1 concentration.

(c) EVALUATION OF SERUM SAMPLES

A total of 589 serum samples from 304 different patients were obtained from colleagues at AFRIMS, Bangkok and were assayed for the presence of NS1 using the NS1 antigen capture ELISA. A large proportion of the serum samples were paired sera. Appendix 3.1 gives information on each serum sample according to the patient's age, sex, infecting serotype (by HAI) and grade of disease and is arranged in numerical order. A clinical diagnosis of DHF was assigned following the World Health Organization clinical definition based on the presence of plasma leakage

and thrombocytopenia (WHO, 1986; Table 1.1). Control serum samples from both outpatients and inpatients were supplied with the serum panel. Not all assays were performed on all serum samples due to the limitation of the volumes available. Several tables presenting identical information will be included in this results section that are derived from the original table (Appendix 3.1) but are sorted according to relevant characteristics for clearer interpretation of the results.

(i) Analysis of serum samples from dengue infected patients

Most notably, NS1 was not detected in the serum of the 30 patients with primary dengue infection (Table 3.2) indicating that NS1 is either absent, or is present in concentrations below the level of detection of the assay. Given that NS1 is secreted from all infected mammalian cells the latter is the most likely possibility. Three primary infected patients did however have detectable levels of antibody to NS1 in both S1 and S2 sera suggesting that NS1 may be complexed to antibody and is therefore undetectable by the anti-NS1 probe. In patients with the more severe form of the disease (secondary infections with DHF grades III and IV) (Refer Table 1.1 and Table 3.3) the results show detectable levels of NS1 in a high proportion of the S1 sera, along with detectable anti-NS1 antibody. In the convalescent (S2) sera NS1 was rarely detectable while antibody to NS1 was usually at higher levels than for S1 sera. As the S2 sera were usually collected during convalescence some two weeks after the first bleed, viraemia and NS1 production would have long ceased. Indeed the detection of NS1 in any of the S2 sera is of interest as it may indicate either prolonged infection or continuing NS1 circulation.

Interestingly, Appendix 4.1 also shows that two negative control inpatients, who had been admitted for reasons other than flavivirus infection showed detectable levels of NS1 in their S1 serum samples of 5.1 and 1.3 µg / mL. These NS1 concentrations are low compared to some of the other values determined but may indicate that these patients had a low level dengue infection at the time of admission that went undiagnosed.

Table 3.2 **Data for patients with primary infections (n=30)**

SERUM #	S1	SEROTYPE	DHF GRADE	NS1 ug/mL	NS1 [Ab] *	S2	NS1 ug/mL	NS1 [Ab] *	1° / 2°
41	M3132	2	1	0	2.36	M3158	0	2.52	1
1	M0519	1	1	0	NT	M0577	0	NT	1
2	M0582	1	1	0	NT	M0638	0	NT	1
3	M0736	1	1	0	NT	M0802	0	NT	1
4	M1580	1	1	0	NT	M1594	0	NT	1
5	M2822	1	2	0	NT	M2870	0	NT	1
6	M2994	1	2	0	NT	M3019	0	NT	1
11	M6848	1	2	0	NT	M7120	0	NT	1
12	M7172	1	2	0	NT	M7200	0	NT	1
15	M4837	2	2	0	NT	M4866	0	NT	1
175	M10996	3	2	0	NT	M11057	0	NT	1
183	M7878	1	2	0	NT	M7890	0	NT	1
187	M4215	1	2	0	NT	M4246	0	NT	1
209	M4183	1	2	0	NT	M4212	0	NT	1
213	M3165	1	2	0	NT	M3166	0	NT	1
13	M0926	1	3	0	NT	M0960	0	NT	1
48	M6829	1	3	0	NT	M7061	0	NT	1
181	M5631	1	3	0	NT	M5665	0	NT	1
190	M11327	2	3	0	NT	M11428	0	NT	1
193	M5196	1	3	0	NT	M5254	0	NT	1
176	M1795	3	4	0	NT	M1811	0	NT	1
43	M3010	2	DF	0	2.32	M3055	0	2.47	1
42	M0022	2	DF	0	2.40	M0090	0	2.58	1
10	M6795	3	DF	0	NT	M7114	0	NT	1
17	M0138	1	DF	0	NT	M0162	0	NT	1
18	M0379	1	DF	0	NT	M0380	0	NT	1
19	M1673	1	DF	0	NT	M1709	0	NT	1
20	M1739	1	DF	0	NT	M1755	0	NT	1
177	M0177	3	DF	0	NT	M0201	0	NT	1
178	M0723	3	DF	0	NT	M0749	0	NT	1

* reciprocal $\log_{10}$ end point titrations NT = Not Tested

As described earlier, the capture assay used the type-specific MAb 1H7.4 and as such, only detected dengue 2 virus NS1. The data shown in Table 3.3 highlights this serotype-specificity with detectable levels of NS1 only being found in patients with HAI confirmed dengue 2 virus infections. Interestingly, there was a single exception to this, with NS1 being detected at quite high levels in a patient diagnosed by HAI to be infected with dengue 3 virus (Table 3.3, S1 #M9210). Either the patient was dually infected or the HAI data provided an incorrect diagnosis. The possibility of dual infection with more than one serotype has been documented previously using RT-PCR (Laille *et al*, 1991). Intriguingly, this patient also had the highest level of detected NS1 in their convalescent sera (Table 3.3, S2#M9273) which may indicate an ongoing persistent infection.

Table 3.3 Data for patients with secondary dengue infections

SERUM #	S1	SEROTYPE	DHF GRADE	NS1 ug/mL	NS1 [Ab] *	S2	NS1 ug/mL	NS1 [Ab] *	1° / 2°
45	M0650	2	1	0	3.91	M0668	0	4.32	2
44	M0634	2	1	0	5.03	M0646	0	5.04	2
21	M0651	2	1	0	NT	M0725	0	NT	2
22	M0787	2	1	0	NT	M0810	0	NT	2
23	M1909	2	1	0	NT	M2017	0	NT	2
24	M2059	2	1	0	NT	M2018	0	NT	2
182	M7203	3	1	0	NT	M7229	0	NT	2
180	M7161	2	1	11.5	3.17	M7199	0	4.87	2
174	M4462	2	1	46.5	2.87	M4686	0	4.92	2
49	M0155	2	2	0	2.32	M0173	0	4.4	2
51	M1647	2	2	0	4.09	M1678	0	5.79	2
50	M0290	2	2	0	4.64	M0291	0	5.79	2
25	M0136	2	2	0	NT	M0160	0	NT	2
28	M0516	2	2	0	NT	M0517	0	NT	2
71	M5803	3	2	0	NT	M6427	0	NT	2
184	M7946	3	2	0	NT	M8035	0	NT	2
	M9244	3	2	0	NT	M9368	0	NT	2
27	M0204	2	2	20	2.91	M0238	0	4.25	2
	M9210	3	2	44	1.95	M9273	39	2.06	2
26	M0140	2	2	61	2.26	M0164	0	4.88	2
52	M1664	2	2	10	2.81	M1665	0	5.80	2
53	M2826	2	2	4.6	3.24	M2897	0	5.80	2
16	M6768	3	3	0	NT	M6808	0	NT	2
30	M0626	2	3	0	NT	M0662	0	NT	2
31	M0683	2	3	0	NT	M0747	0	NT	2
32	M1648	2	3	0	NT	M1679	0	NT	2
47	M4286	3	3	0	NT	M4306	0	NT	2
185	M1839	3	3	0	NT	M1942	0	NT	2
210	M4401	3	3	0	NT	M4659	0	NT	2
214	M3672	3	3	0	NT	M3778	0	NT	2
57	M0570	2	3	15	3.28	M0790	0	5.43	2
58	M0581	2	3	37.5	3.11	M0627	0	4.23	2
56	M0481	2	3	48.5	3.32	M0491	4.5	3.95	2
29	M0021	2	3	54	2.09	M0497	0	3.15	2
55	M0270	2	3	23	3.57	M0297	0	5.12	2
54	M0032	2	3	43.5	3.29	M0091	0	5.83	2
60	M1690	2	4	0	4.4	M1722	0	5.47	2
33	M0309	2	4	0	NT	M0310	0	NT	2
35	M2859	2	4	0	NT	M2899	0	NT	2
43	M2583	2	4	0	NT	M2616	0	NT	2
207	M3804	2	4	0	NT	M3891	0	NT	2
61	M1803	2	4	4	4.13	M1847	0	5.75	2
62	M2041	2	4	14	2.60	M2053	0.9	3.87	2
63	M3041	2	4	42	2.87	M3180	0	5.48	2
66	M1820	2	DF	0	2.93	M1853	0	3.63	2
37	M1654	2	DF	0	NT	M1662	0	NT	2
38	M1689	2	DF	0	NT	M1711	0	NT	2
39	M1752	2	DF	0	NT	M1807	0	NT	2
40	M1801	2	DF	0	NT	M1846	0	NT	2
70	M5743	3	DF	0	NT	M5769	0	NT	2
192	M2465	3	DF	0	NT	M2555	0	NT	2
194	M10390	3	DF	0	NT	M10745	0	NT	2
64	M0880	2	DF	3	2.17	M0909	0	3.49	2
65	M1671	2	DF	3	2.58	M1707	0	3.95	2
36	M3315	2	DF	26	2.05	M3655	0	3.42	2
67	M2206	2	DF	46	2.32	M2470	0	2.91	2

Table 3.4 **Data for patients with dengue serotype 2 infections only (n=49)**

SERUM #	S1	SEROTYPE	DHF GRADE	NS1 ug/mL	NS1 [Ab]	S2	NS1 ug/mL	NS1 [Ab]	1° / 2°
43	M3010	2	DF	0	2.32	M3055	0	2.47	1
42	M0022	2	DF	0	2.40	M0090	0	2.58	1
66	M1820	2	DF	0	2.93	M1853	0	3.63	2
37	M1654	2	DF	0	NT	M1662	0	NT	2
38	M1689	2	DF	0	NT	M1711	0	NT	2
39	M1752	2	DF	0	NT	M1807	0	NT	2
40	M1801	2	DF	0	NT	M1846	0	NT	2
64	M0880	2	DF	3	2.17	M0909	0	3.49	2
65	M1671	2	DF	3	2.58	M1707	0	3.95	2
36	M3315	2	DF	26	2.05	M3655	0	3.42	2
67	M2206	2	DF	46	2.32	M2470	0	2.91	2
41	M3132	2	1	0	2.36	M3158	0	2.52	1
45	M0650	2	1	0	3.91	M0668	0	4.32	2
44	M0634	2	1	0	5.03	M0646	0	5.04	2
21	M0651	2	1	0	NT	M0725	0	NT	2
22	M0787	2	1	0	NT	M0810	0	NT	2
23	M1909	2	1	0	NT	M2017	0	NT	2
24	M2059	2	1	0	NT	M2018	0	NT	2
180	M7161	2	1	11.5	3.17	M7199	0	4.87	2
174	M4462	2	1	46.5	2.87	M4686	0	4.92	2
15	M4837	2	2	0	NT	M4866	0	NT	1
49	M0155	2	2	0	2.32	M0173	0	4.4	2
51	M1647	2	2	0	4.09	M1678	0	5.79	2
50	M0290	2	2	0	4.64	M0291	0	5.79	2
25	M0136	2	2	0	NT	M0160	0	NT	2
28	M0516	2	2	0	NT	M0517	0	NT	2
27	M0204	2	2	20	2.91	M0238	0	4.25	2
26	M0140	2	2	61	2.26	M0164	0	4.88	2
52	M1664	2	2	10	2.81	M1665	0	5.80	2
53	M2826	2	2	4.6	3.24	M2897	0	5.80	2
190	M11327	2	3	0	NT	M11428	0	NT	1
30	M0626	2	3	0	NT	M0662	0	NT	2
31	M0683	2	3	0	NT	M0747	0	NT	2
32	M1648	2	3	0	NT	M1679	0	NT	2
57	M0570	2	3	15	3.28	M0790	0	5.43	2
58	M0581	2	3	37.5	3.11	M0627	0	4.23	2
56	M0481	2	3	48.5	3.32	M0491	4.5	3.95	2
29	M0021	2	3	54	2.09	M0497	0	3.15	2
55	M0270	2	3	23	3.57	M0297	0	5.12	2
54	M0032	2	3	43.5	3.29	M0091	0	5.83	2
60	M1690	2	4	0	4.4	M1722	0	5.47	2
33	M0309	2	4	0	NT	M0310	0	NT	2
35	M2859	2	4	0	NT	M2899	0	NT	2
43	M2583	2	4	0	NT	M2616	0	NT	2
207	M3804	2	4	0	NT	M3891	0	NT	2
	M0474	2	4	0	4.31	M0575	0	5.15	
61	M1803	2	4	4	4.13	M1847	0	5.75	2
62	M2041	2	4	14	2.60	M2053	0.9	3.87	2
63	M3041	2	4	42	2.87	M3180	0	5.48	2

Data from patients infected with dengue-2 only are shown in Table 3.4 and all subsequent analyses refer to only this data set. Nine patients (n=19) with grade III and IV DHF dengue-2 virus infections showed detectable NS1 in their S1 sera, however the S2 sera (n=19) showed only two samples with detectable NS1 levels of 4.5 μg / mL and 0.9 μg / mL. Figure 3.5B shows that levels of NS1 antibody generally increase more markedly between S1 and S2 sera for DHF than for DF patients. Figure 3.5A conversely shows that the levels of NS1 antigen detected decreases in the S2 sera for all grades of DHF and DF. Taken together these results most likely reflect the fact that the levels of NS1 present at the time the S1 sera were taken correlate with active infection and induce the specific antibody response that is subsequently detected in the S2 sera. The high levels of NS1, coupled with a rapid anamnestic rise in anti-NS1, also suggests the likely generation of immune complexes during this phase of the illness.

In order to test for NS1 that was associated with immune complexes, a number of techniques such as microwave and acid treatment that have been previously reported to successfully dissociate immune complexes (Kestens *et al*, 1991; Miles *et al*, 1993) were analysed for subsequent detection of bound NS1. Each protocol appeared to lead to significant loss of NS1 antigenicity (data not shown). Although immune complex detection was considered an important component of these studies, the available serum samples had been freeze-thawed on numerous occasions, a procedure which is known to generate non-specific aggregates that are potential sources of error in these assays (Lambert *et al*, 1978), and so these studies were discontinued. Alternative procedures are currently under investigation in collaboration with our colleagues at AFRIMS, Thailand and the University of Massachusetts, USA.

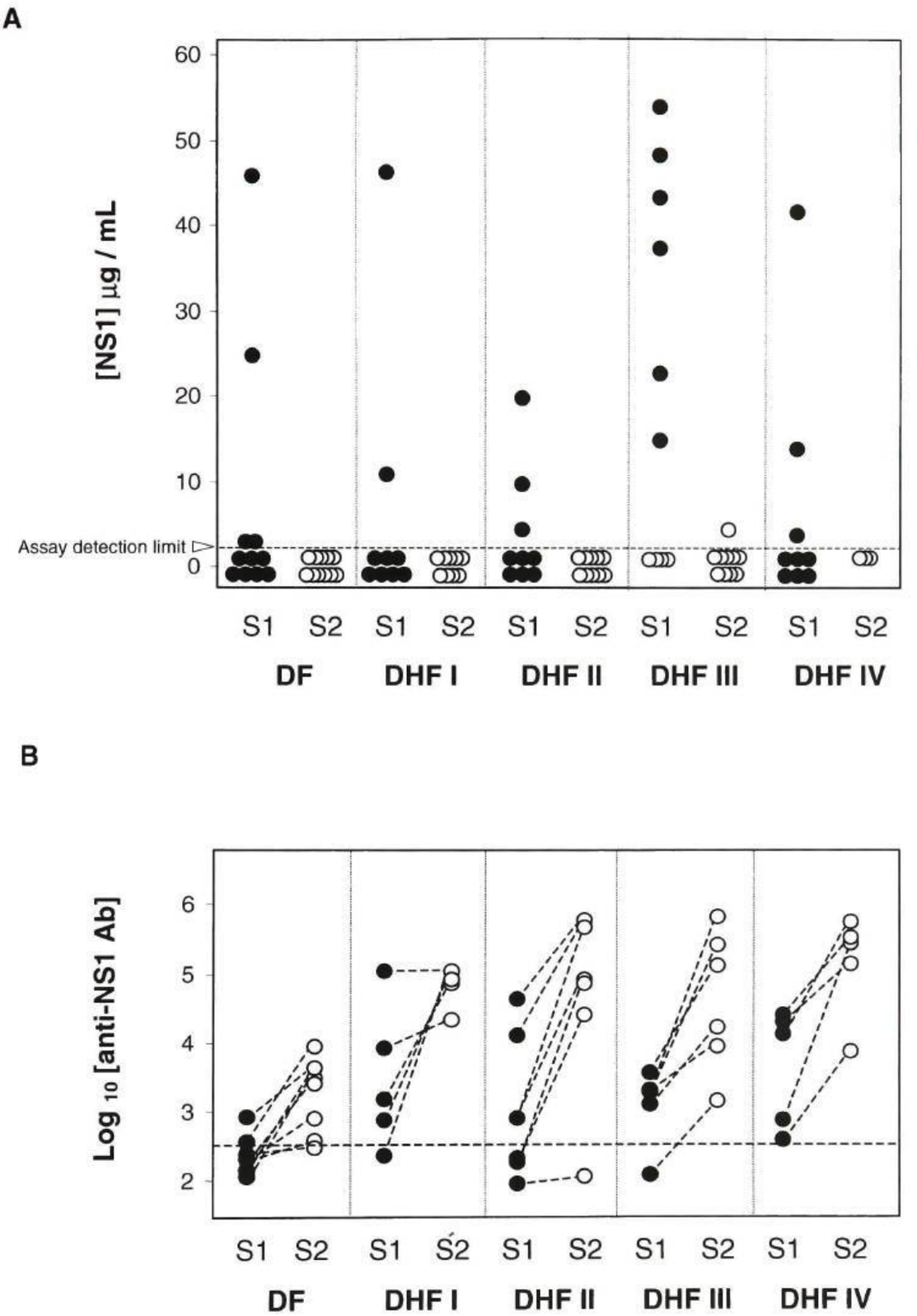


Figure 3.5 **Plots of A, NS1 levels and B, anti-NS1 antibody levels in patient sera correlated with clinically defined grades of dengue disease. Acute and convalescent sera are designated S1 (●) and S2 (○) respectively. Capture assay detection limit is shown in A. Mean of negative control sera titres in the antibody ELISA is shown with a horizontal dashed bar in B.**

Figure 3.6 shows the correlation between DHF grade and both NS1 and anti-NS1 antibody concentration in patient serum. Although the sample size is relatively small, the graph shows that serum taken at convalescence has higher anti-NS1 antibody concentrations with NS1 being undetectable either because levels have waned by this time or because the protein is bound within immune complexes. For serum taken from patients during the acute phase of infection there appears to be a correlation between disease severity and the presence of both detectable NS1 and antibody to NS1.

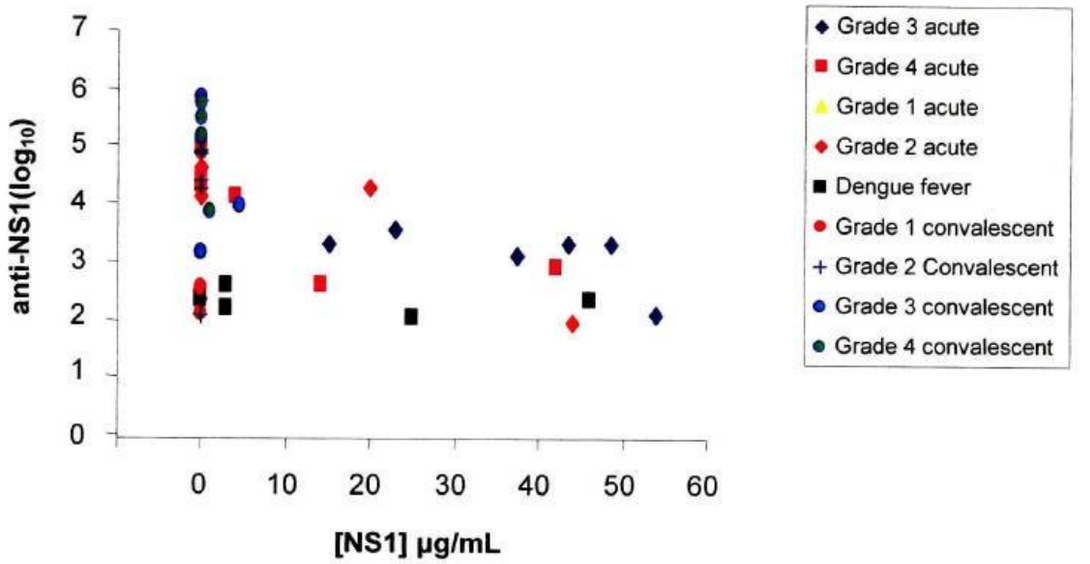


Figure 3.6 **Correlation between clinically defined dengue disease with NS1 concentration and anti-NS1 antibody levels.**

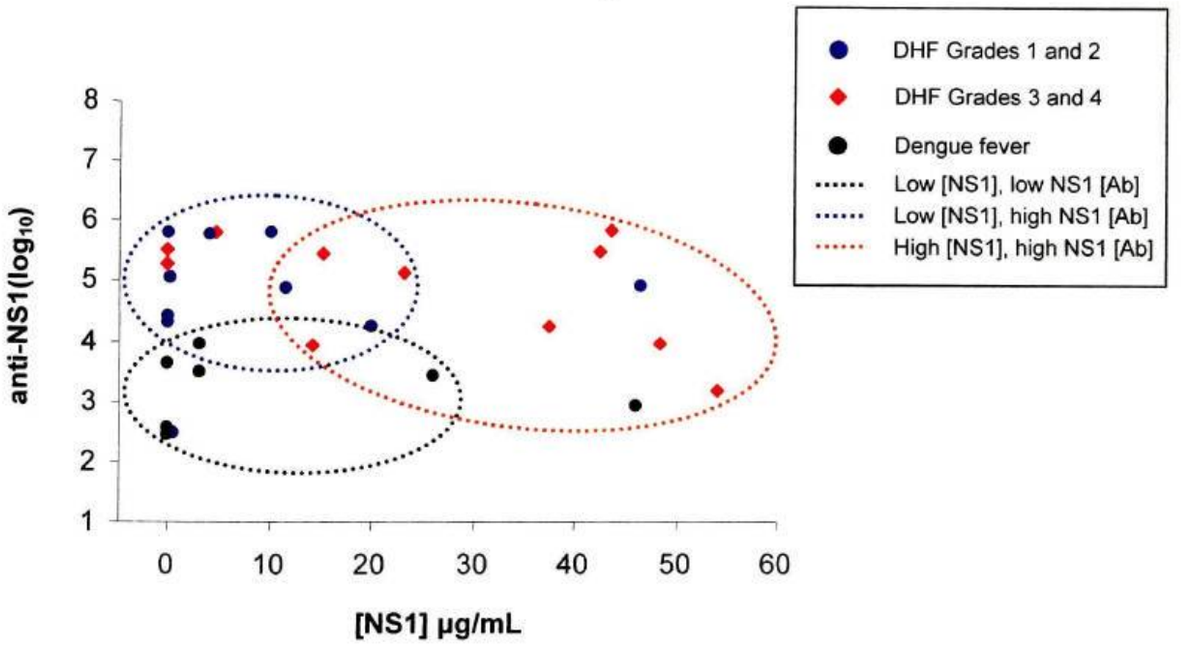


Figure 3.7 **Graphic plot comparing NS1 concentration in sera taken from patients in the acute phase of the disease (S1 sera) with anti-NS1 antibody levels in sera during convalescence (S2 sera) for patients with different grades of disease.**

Vaughn *et al* (2000) have shown in a study of patients in Thailand that those with dengue-2 infection were more likely to present with DHF (66%) than those infected by other virus serotypes. Therefore, taking patients with dengue 2 infections only (as the assay is most sensitive in detecting this serotype), those with the most severe form of the disease (grades III and IV) tended to have high levels of antibody to NS1 in the S2 sera with the concomitant "disappearance" of NS1 (Table 3.4).

A number of reports have suggested that levels of anti-NS1 in patient sera may correlate with disease severity (Chan *et al*, reported in Brandt, 1988; Shu *et al*, 2000). In an attempt to find a correlation between either NS1 levels and/or anti-NS1 and disease severity these parameters were plotted in a number of ways. An analysis of dengue patient sera where acute NS1 concentration is plotted against convalescent anti-NS1 antibody titre (Figure 3.7) reveals that there is a trend for those patients with the more severe forms of dengue infection (grades III and IV) to have high levels of both NS1 and antibody to NS1. Those patients however, with DF infections have lower levels of antigen (in S1 sera) and antibody (in S2 sera) overall. Patients with grade II and III infections have higher levels of anti-NS1 (in S2 sera) and lower levels of NS1 (in S1 sera). It should be pointed out that the graph clearly shows that there are significant outliers to this trend. The limitation of numbers of serum samples from seriously ill patients makes a more thorough analysis difficult. Nevertheless, these results suggest that high levels of both NS1 antigen and antibody to NS1 may be significant markers for patients who progress to the more serious complications of dengue infection. More detailed studies involving the kinetics of appearance of NS1 in patient serum samples taken daily during the course of the acute phase of the disease would be required to provide a clearer picture of any disease association.

(ii) Data from a North Queensland dengue outbreak

Patient serum samples were also obtained from the former State Health Laboratories in Brisbane, from a dengue outbreak in 1995 in North Queensland.

Being from an area where dengue is not endemic, the majority of samples were primary cases. As NS1 was not detected in the serum samples taken from primary patients in Thailand this series was examined for comparison.

The results from the north Queensland outbreak are shown in Table 3.5. Due to the low concentrations of NS1 detected, the accuracy of the quantitation is difficult to verify because the OD values didn't fall within the "straight line" region of the standard curve. Nevertheless the results show that NS1 was able to be detected in some primary infected patients (14/34) at low levels, in contrast to the results obtained from the serum samples from Bangkok. The inability to detect NS1 in these samples is most likely the result of handling issues and sample stability, possibility due to several freeze/thawing cycles which the serum endured or because they were collected late in illness. As can be seen from Table 3.5, NS1 was undetectable in all convalescent serum samples as would be expected when active infection wanes.

Table 3.5 NS1 quantitation results form dengue patients in a North Queensland outbreak

Identifiying Code	Primary/Acute	Secondary By HAI	D2 Isolated	NS1 ug/mL	Convalescent	NS1 ug/mL
27854	+			0.05	29540	ND
29207	+			0.05		
20448	+			0.07		
21842	+		+	0.14		
26910	+			0.84		
29532	+			1.46	33437	ND
26910	+		+	1.64		
26732	+			1.88		
25449	+			1.92	30121	ND
22662	+		+	>2		
25795	+			>2	27514	ND
26680	+			>2	31637	ND
27119	+			>2	30106	ND
27865	+			>2	28808	ND
15231	+		+	ND		
17885	+		+	ND		
19804	+		+	ND		
20362	+		+	ND		
20412	+		+	ND		
22710	+		+	ND		
24106	+		+	ND		
24190	+		+	ND		
25426	+		+	ND		
27119	+		+	ND		
27840	+		+	ND		
28664	+		+	ND		
30112	+		+	ND	30112	ND
31823	+		+	ND		
32157	+		+	ND		
32351	+			ND	33979	ND
33090	+		+	ND		
37832	+		+	ND		
39780	+		+	ND		
41369	+		+	ND		
21010		+		ND		
27836		+		ND		
28115		+		ND		
28672		+		ND		
29516		+		ND		
32022		+		ND		
32349		+		ND		
33984		+		ND		
35374		+		ND		
1/5 NHS		N/A	N/A	ND		

3.3 DISCUSSION

A capture ELISA for the detection of dengue virus NS1 has been developed and described. Screening of a panel of MAbs as detection probes and utilizing rabbit polyclonal anti-NS1 hyperimmune sera as capture antibody, led to the optimization of both type-specific and serotype cross-reactive assays. Despite the availability of an extensive number of MAbs specific for linear and conformational determinants (Falconar and Young, 1991), the majority were ineffective as detection probes. Significantly, only those MAbs specific for linear determinants, one type specific and the other cross-reactive among the dengue viruses, were efficient as detection probes. These linear epitopes have been identified previously through binding of MAbs to a set of overlapping peptides (Falconar *et al*, 1994). Although the amino acid sequence of the peptide defined by the dengue-2 virus specific MAbs (^{25}VHYWYEQYK^{33}) is shared by the other three virus serotypes, the type-specificity of their binding was clearly demonstrated in this study (Figure 3.3). This finding supports the earlier demonstration that amino acids flanking this linear epitope appear to contribute conformational constraints that lead to the epitope specificity seen in the native protein (Falconar *et al*, 1994). The sensitivity of the dengue-2 virus type-specific assay was estimated using the immnoaffinity purified secreted form of NS1. Using this preparation as a reference standard, the assay was able to detect levels of NS1 down to 4 ng / mL. In the second part of this study, levels of NS1 in a panel of patient sera were examined with the aim of assessing the potential of the capture assay to measure NS1 as a surrogate marker of infection.

The detection of NS1 in patient sera demonstrated that it is produced in dengue virus infected individuals and is secreted to circulate in the bloodstream. The significance of the detection of NS1 in the acute phase of secondary but not primary infections in one series of dengue patients, may indicate that in the primary infection, NS1 concentrations may be below the level of detection of the assay or that it is absent altogether. The former appears to be the case given a recent study by Alcon *et al* (2002) which reported detectable NS1 levels in dengue-1 infected

primary patients albeit at low levels (in the range of 0.04 – 2 μg / mL). This study correlates well with our findings on serum taken from patients with primary infection in a North Queensland outbreak in which low levels of NS1 were detected.

The significance of the presence of secreted NS1 for disease outcome is unclear although it has been postulated to contribute to dengue virus pathogenesis (Young *et al*, 2000; Alcon *et al*, 2002; Libraty *et al*, 2002). Early reports in the literature recognized anti-NS1 responses in various flavivirus infections by western blot detection, including Japanese encephalitis virus (Lee *et al*, 1995) and TBE in human sera and spinal fluid (Matveeva *et al*, 1995). Antibody appeared no earlier than eight days post infection and no difference was detected between the immune responses of males and females (Matveeva *et al,* 1995). Although the immunoblot procedure does not provide quantitative data, reports using this procedure have noted that anti-NS1 responses were found exclusively in secondary infections (Falker *et al*, 1973; Kuno *et al*, 1990; Churdboonchart *et al*, 1991; Valdés *et al*, 2000). Anti-NS1 antibody has also been detected more frequently in severe, rather than milder grades of DHF (Chan *et al*, reported in Brandt, 1988). This study has been able to confirm this finding with data showing that the highest overall levels of NS1 antibody was detected in DHF grade III and IV patients (Table 3.4). It has been proposed that antibodies generated during the acute phase of disease may contribute to the pathogenesis of DHF (Shu *et al*, 2000). One possible pathogenic mechanism was postulated by Falconar (1997) who demonstrated that anti-NS1 MAbs produced haemorrhage in mice. The cross-reactivity of these antibodies with fibrinogen, thrombocytes and endothelial cells suggested that antigenic mimicry may contribute to DHF pathogenesis. Further work is required to determine if this phenomenon occurs in naturally acquired dengue infections.

A more obvious candidate for a role in pathogenesis are immune complexes. Immune complexes, formed when antibodies combine non-covalently with their corresponding antigens, may be formed after interaction of antibodies with tissue-fixed antigens or with antigens free in serum (Lambert *et al*, 1978). Immune complexes have been implicated in the pathogenesis of diseases such as chronic

hepatitis, measles, malaria and DHF (Ruangirachuporn *et al*, 1979) and have been reported in the sera of DHF patients with secondary infections (Petchhclai and Saelim, 1978; Theofilopoulos *et al*, 1976; Ruangjirachuporn *et al*, 1979). The level of immune complexes and complement consumption has been shown to correlate well with the clinical severity of dengue disease in several studies (Ruangjirachuporn *et al*, 1979; Sobel *et al*, 1975; Theofilopoulos *et al*, 1976) pointing to a clear role for immune complexes in the pathogenesis of DHF and DSS (Bokish *et al*, 1973; Malasit, 1987; Rothman and Ennis, 1999). Immune complexes have been reported in 80% of DHF patients (n = 80) from day 2 after fever, and up to 3 days prior to shock (Ruangjirachuporn *et al*, 1979). These immune complexes have also been found in biopsy tissues (Boonpucknavig *et al*, 1976a; Boonpucknavig *et al*, 1976b). Under appropriate conditions, circulating immune complexes may be deposited in tissues resulting in complement activation and polymorphonuclear cell-mediated tissue injury (Cochrane and Dixon, 1976), and may also interact with receptors on cells resulting in release of biologically active mediators. For example, complement can cause the lysis of dengue infected mononuclear phagocytes via complement mediated cytolysis, resulting in the release of potent biological mediators which could affect vascular permeability (Bhadki and Kazatchkine, 1990). Additionally, these biological mediators could be released by natural killer (NK) cell activity (Hill *et al*, 1993) and/or antibody dependent cell mediated cytotoxicity (ADCC) (Kurane and Ennis, 1992) that has been demonstrated against dengue virus infected cells *in vitro* (Kurane *et al*, 1984).

Immune complexes were originally postulated by Russel (1971) as the initiating factor in the immunopathological mechanism of shock in DHF although the specific antigen(s) involved was not identified. Several investigators have reported that both the amount of dengue virus antigen circulating as immune complexes and the level of complement consumed, (providing an indication of antigen-antibody complexing) by the classic pathway varies in direct proportion with disease severity (Ruangjirachuporn *et al*, 1989; Malasit, 1987; Suvatte, 1987; Bokisch *et al*, 1973). C3 split products were demonstrated to be present in the sera of DHF grade III and IV patients, and split product appearance correlated with shock (Churdboonchart *et*

al, 1983). There is evidence that activation occurs via both classical and alternate complement pathways, with classical pathway involvement via antigen-antibody complexes possibly the more prominent (Cornain *et al*, 1987).

Huang *et al* (1999) reported that anti-NS1 antibody in patients with primary dengue infection reacted with a peptide corresponding to the first 15 amino acids of NS1. This report is interesting in that it presents an opportunity to examine serum from dengue patients and to study the human immune response to various NS1 epitopes. It would be valuable to be able to utilize a similar competition assay to that described in Chapter 4 of this thesis, to examine which NS1 epitopes are recognized by human anti-NS1 antibody as a basis for vaccine studies.

This study has now identified the secreted form of dengue virus NS1 as a potential candidate for a viral antigen that may form immune complexes (Young *et al*, 2000). The antigen capture ELISA has detected relatively high levels of NS1 in the sera of dengue virus infected patients. It is likely that either free or immune complexed NS1 may become bound by cell populations such as platelets, monocytes and / or endothelial cells. Dengue-specific immune complexes have been identified on the surface of platelets from infected patients (Boonpucknavig *et al*, 1979). It follows that these cells would then become targets of complement mediated lysis leading to the induction of the pronounced thrombocytopenia observed in DHF / DSS (Funuhara *et al*, 1987).

Although this study was unable to generate conclusive data confirming an association of NS1 and anti-NS1 antibody with disease severity it has provided the basis for additional studies. The preliminary data suggested that the serum of patients with severe forms of dengue infection contained both high levels of NS1 antigen in acute sera and high levels of specific antibody in convalescent sera. Subsequent studies in the Young laboratory in collaboration with colleagues in Bangkok and access to samples from a prospective study on DHF patients, found that NS1 levels detected in dengue patient serum correlated with viremia levels and were higher in DHF patients compared to DF. An elevated plasma level of NS1

(≥600 ng / mL) within 72 hours of illness onset identified patients at risk of developing DHF (Libraty *et al*, 2002).

The potential role of NS1 (previously known as the soluble complement fixing antigen, SCF) in immunopathogenesis was first proposed in 1973 (Falker *et al*, 1973) due to the presence of anti-SCF antibodies in sera from patients undergoing secondary infections. This study confirms and considerably extends these and subsequent observations. It has also been suggested that anti-NS1 antibody bound to cell-surface NS1 in a multimeric form may activate complement more efficiently than the monomer, as the affinity of complement binding increases proportionately with immune complex size (Cooper, 1985). This proposal warrants further investigation especially in the light of evidence that native secreted NS1 is a hexameric multimer (Flamand *et al*, 1999; Chapter 5 of this thesis). NS1 may prove to be a candidate antigen that may be pivotal to the formation of immune complexes in secondary infections and is present in sufficient quantities to explain the complement activation seen in DHF / DSS. More primary sera are needed however to examine this issue more clearly as well as kinetic data to look for trends in antigen / antibody production.

APPENDIX 3.1 Individual patient data

SERUM PAIR #	SERA (S1)	SEROTYPE	AGE*	SEX	DHF GRADE	NS1 ug/mL	NS1 Ab titre *	SERA (S2)	NS1 ug/mL	NS1 Ab titre *	Primary or Secondary
1	M0519	1	5/5	F	1	0	NT	M0577	0	NT	1
2	M0582	1	5/9	M	1	0	NT	M0638	0	NT	1
3	M0736	1	4	M	1	0	NT	M0802	0	NT	1
4	M1580	1	4/6	F	1	0	NT	M1594	0	NT	1
5	M2822	1	3/2	F	2	0	NT	M2870	0	NT	1
6	M2994	1	4/4	M	2	0	NT	M3019	0	NT	1
10	M6795	3	5	M	DF	0	NT	M7114	0	NT	1
11	M6848	1	6	F	2	0	NT	M7120	0	NT	1
12	M7172	1	7	M	2	0	NT	M7200	0	NT	1
13	M0926	1	7/8	F	3	0	NT	M0960	0	NT	1
14	M4496	4	7/5	M	ND	0	NT	M4497	0	NT	2
15	M4837	2	6/3	M	2	0	NT	M4866	0	NT	1
16	M6768	3	9/4	M	3	0	NT	M6808	0	NT	2
17	M0138	1	6/3	M	DF	0	NT	M0162	0	NT	1
18	M0379	1	4	M	DF	0	NT	M0380	0	NT	1
19	M1673	1	5/5	F	DF	0	NT	M1709	0	NT	1
20	M1739	1	9/8	F	DF	0	NT	M1755	0	NT	1
21	M0651	2	7/6	F	1	0	NT	M0725	0	NT	2
22	M0787	2	8	M	1	0	NT	M0810	0	NT	2
23	M1909	2	7	M	1	0	NT	M2017	0	NT	2
24	M2059	2	8/11	F	1	0	NT	M2018	0	NT	2
25	M0136	2	9	F	2	0	NT	M0160	0	NT	2
26	M0140	2	4	F	2	61	2.26	M0164	0	4.88	2
27	M0204	2	5/11	F	2	20	2.91	M0238	0	4.25	2
28	M0516	2	9/3	M	2	0	NT	M0517	0	NT	2
29	M0021	2	5/10	F	3	54	2.09	M0497	0	3.15	2
30	M0626	2	5/7	F	3	0	NT	M0662	0	NT	2
31	M0683	2	8/3	M	3	0	NT	M0747	0	NT	2
32	M1648	2	4/6	F	3	0	NT	M1679	0	NT	2
33	M0309	2	4/5	F	4	0	NT	M0310	0	NT	2
35	M2859	2	4/5	F	4	0	NT	M2899	0	NT	2
36	M3315	2	8/3	M	DF	26	2.05	M3655	0	10 3.42	2

SERUM PAIR #	SERA (S1)	SEROTYPE	AGE	SEX	DHF GRADE	NS1 ug/mL	NS1 Ab titre	SERA (S2)	NS1 ug/mL	NS1 Ab titre	Primary or Secondary
40	M1801	2	6/7	F	DF	0	NT	M1846	0	NT	2
41	M3132	2	8/0	F	1	0	2.36	M3158	0	2.52	1
42	M0022	2	3/3	F	DF	0	2.40	M0090	0	2.58	1
43	M2583	2	4/3	M	4	0	NT	M2616	0	NT	2
43	M3010	2	4/6	M	DF	0	2.32	M3055	0	2.47	1
44	M0634	2	5/6	F	1	0	5.03	M0646	0	5.04	2
45	M0650	2	9/11	F	1	0	3.91	M0668	0	4.32	2
46	M1957	NEG OUT	6	F	N/A	0	2.34	M2018	0	2.66	N/A
47	M4286	3	8/1	F	3	0	NT	M4306	0	NT	2
48	M6829	1	5/2	M	3	0	NT	M7061	0	NT	1
49	M0155	2	8/5	M	2	0	2.32	M0173	0	4.4	2
50	M0290	2	3/6	F	2	0	4.64	M0291	0	5.79	2
51	M1647	2	3/0	F	2	0	4.09	M1678	0	5.79	2
52	M1664	2	8/4	M	2	10	2.81	M1665	0	5.80	2
53	M2826	2	5/6	M	2	4.6	3.24	M2897	0	5.80	2
54	M0032	2	6/8	F	3	43.5	3.29	M0091	0	5.83	2
55	M0270	2	6/4	F	3	23	3.57	M0297	0	5.12	2
56	M0481	2	9	M	3	48.5	3.32	M0491	4.5	3.95	2
57	M0570	2	7	M	3	15	3.28	M0790	0	5.43	2
58	M0581	2	7/9	M	3	37.5	3.11	M0627	0	4.23	2
60	M1690	2	5/10	M	4	0	4.4	M1722	0	5.47	2
61	M1803	2	7/10	F	4	4	4.13	M1847	0	5.75	2
62	M2041	2	8/3	F	4	14	2.60	M2053	0.9	3.87	2
63	M3041	2	8	F	4	42	2.87	M3180	0	5.48	2
64	M0880	2	8/10	M	DF	3	2.17	M0909	0	3.49	2
65	M1671	2	9/8	M	DF	3	2.58	M1707	0	3.95	2
66	M1820	2	4/8	M	DF	0	2.93	M1853	0	3.63	2
67	M2206	2	9/8	F	DF	46	2.32	M2470	0	2.91	2
69	M06370	NEG OUT	7	F	N/A	0	-	M06549	0	-	N/A
70	M5743	3	6/10	M	DF	0	NT	M5769	0	NT	2
71	M5803	3	3/11	M	2	0	NT	M6427	0	NT	2
72	M6379	NEG OUT	9	M	N/A	0	NT	M6554	0	NT	N/A
73	M6381	NEG OUT	12	M	N/A	0	NT	M6531	0	NT	N/A

SERUM PAIR #	SERA (S1)	SEROTYPE	AGE	SEX	DHF GRADE	NS1 ug/mL	NS1 Ab titre	SERA (S2)	NS1 ug/mL	NS1 Ab titre	Primary or Secondary
79	M6390	NEG OUT	8	M	N/A	0	NT	M6535	0	NT	N/A
80	M6392	NEG OUT	11	M	N/A	0	NT	M6537	0	NT	N/A
81	M1932	NEG OUT	6	M	N/A	0	NT	M1976	0	NT	2
82	M1934	NEG OUT	7	M	N/A	0	NT	M1968	0	NT	N/A
83	M1944	NEG OUT	7	M	N/A	0	NT	M1980	0	NT	N/A
84	M1946	NEG OUT	12	M	N/A	0	NT	M2006	0	NT	N/A
85	M1947	NEG OUT	10	F	N/A	0	NT	M2003	0	NT	N/A
86	M1949	NEG OUT	6	F	N/A	0	NT	M1992	0	NT	N/A
87	M1951	NEG OUT	10	F	N/A	0	NT	M2036	0	NT	N/A
88	M1952	NEG OUT	9	M	N/A	0	NT	M2007	0	NT	N/A
89	M1955	NEG OUT	10	F	N/A	0	NT	M2011	0	NT	N/A
91	M1958	NEG OUT	10	F	N/A	0	3.38	M2089	0	3.43	N/A
92	M1960	NEG OUT	7	M	N/A	0	NT	M2048	0	NT	N/A
94	M1965	NEG OUT	8	M	N/A	0	NT	M2166	0	NT	N/A
159	M30169	NEG IN		F	N/A	0	NT	M30192	0	NT	N/A
160	M30230	NEG IN	3/11	M	N/A	0	NT	M30265	0	NT	N/A
161	M30206	NEG IN	2	F	N/A	0	NT	M30253	0	NT	N/A
162	M17449	NEG IN	10	F	N/A	0	NT	M18471	0	NT	N/A
163	M16776	NEG IN	10	F	N/A	5.1	2.92	M17918	0	3.69	N/A
164	M16113	NEG IN	10	F	N/A	0	NT	M17655	0	NT	N/A
165	M16673	NEG IN	5	F	N/A	0	NT	M17674	0	NT	N/A
166	M16114	NEG IN	12	F	N/A	0	NT	M17157	0	NT	N/A
167	M30459	NEG IN	5	F	N/A	0	NT	M30449	0	NT	N/A
168	M30389	NEG IN	7	F	N/A	0	NT	M30401	0	NT	N/A
169	M30385	NEG IN	5	M	N/A	0	NT	M30400	0	NT	N/A
170	M30375	NEG IN	6	M	N/A	0	NT	M30386	0	NT	N/A
171	M30382	NEG IN	14	F	N/A	0	NT	M30390	0	NT	N/A
172	M30420	NEG IN		M	N/A	0	NT	M30425	0	NT	N/A
173	M30453	NEG IN	10	M	N/A	1.3	2.22	M40458	0	2.70	N/A
174	M4462	2	6/7	M	1	46.5	2.87	M4686	0	4.92	2
175	M10996	3	9	M	2	0	NT	M11057	0	NT	1
176	M1795	3	4	M	4	0	NT	M1811	0	NT	1
177	M0177	3	7	F	DF	0	NT	M0201	0	NT	1
178	M0723	3	6/11	M	DF	0	NT	M0749	0	NT	1
180	M7161	2	9/0	M	1	11.5	3.17	M7199	0	4.87	2

SERUM PAIR #	SERA (S1)	SEROTYPE	AGE	SEX	DHF GRADE	NS1 ug/mL	NS1 Ab titre	SERA (S2)	NS1 ug/mL	NS1 Ab titre	Primary or Secondary
181	M5631	1	8/11	F	3	0	NT	M5665	0	NT	1
182	M7203	3	3/7	F	1	0	NT	M7229	0	NT	2
183	M7878	1	8	F	2	0	NT	M7890	0	NT	1
184	M7946	3	8/7	M	2	0	NT	M8035	0	NT	2
185	M1839	3	8	M	3	0	NT	M1942	0	NT	2
186	M3302	4	8/9	F	ND	0	NT	M3730	0	NT	2
187	M4215	1	4	M	2	0	NT	M4246	0	NT	1
188	M4221	4	8	M	ND	0	NT	M4230	0	NT	2
189	M6400	NEG OUT	11	F	N/A	0	NT	M6551	0	NT	N/A
190	M11327	2	3/7	M	3	0	NT	M11428	0	NT	1
192	M2465	3	7/6	F	DF	0	NT	M2555	0	NT	2
193	M5196	1	9	F	3	0	NT	M5254	0	NT	1
194	M10390	3	9/1	M	DF	0	NT	M10745	0	NT	2
195	M30008	NEG IN	6	M	N/A	0	2.70	M30013	0	2.70	N/A
196	M30623	NEG IN	13	M	N/A	0	NT	M30632	0	NT	N/A
197	M30654	NEG IN	3	F	N/A	0	NT	M30805	0	NT	N/A
205	M1197	4	7/3	M	ND	0	NT	M1214	0	NT	2
206	M2762	4	8/3	M	ND	0	NT	M2779	0	NT	2
207	M3804	2	5/9	M	4	0	NT	M3891	0	NT	2
209	M4183	1	8	M	2	0	NT	M4212	0	NT	1
210	M4401	3	7/7	F	3	0	NT	M4659	0	NT	2
211	M11504	4	9/1	M	ND	0	NT	M11526	0	NT	2
212	M0292	4	3/8	M	ND	0	NT	M0324	0	NT	2
213	M3165	1	3/6	M	2	0	NT	M3166	0	NT	1
214	M3672	3	3/11	F	3	0	NT	M3778	0	NT	2
215	M2704	JE	11/3	M	N/A	0	NT	M2717	0	NT	N/A
216	M2711	JE	8	F	N/A	0	NT	M2747	0	NT	N/A
	M0474	2	9	M	4	0	4.31	M0575	0	5.15	
	M1962	NEG OUT	6	F	N/A	0	NT	M2041	0	NT	N/A
	M1962	3	8/8	F	DF	0	NT	M2045	0	NT	ND
	M9210	3	7/5	M	2	44	1.95	M9273	39	2.06	2
	M9244	3	4/10	M	2	0	NT	M9368	0	NT	2

NEG OUT: Negative control, outpatient without flavivirus infection N/A: Not applicable M: Male F: Female * Age (x/y) is given in years (x) and months (y)
NEG IN: Negative control, inpatient without flavivirus infection NT: Not tested ND: Not determined

CHAPTER 4

EPITOPE MAPPING OF THE NS1 GLYCOPROTEIN OF DENGUE-2 VIRUS

4.1 INTRODUCTION

The development of effective dengue virus subunit vaccines would clearly be aided by a more comprehensive knowledge of the location and structure(s) of those epitopes on dengue virus proteins that elicit a protective immune response. Epitopes present on dengue viral structural proteins have been extensively studied and reported widely in the literature (Roehrig *et al*, 1983; Henchal *et al*, 1985; Guirakhoo *et al*, 1989; Aaskov *et al*, 1989; Innis *et al*, 1989; Mandl *et al*, 1989a; Megret *et al*, 1992; Jianmin *et al*, 1995; Roehrig *et al*, 1998; 1990; Crill and Roehrig, 2001). However, both *in vitro* and *in vivo* evidence suggests that antibodies raised against structural proteins of one serotype may enhance replication of heterologous dengue virus serotypes in F_c receptor bearing cells (Kliks *et al*, 1989; Morens *et al*, 1987). Consequently, there has been a long-standing interest in the study of the non-structural glycoprotein NS1 as a candidate immunogen for inclusion in vaccine strategies. This interest is based on its demonstrated antigenicity (Russell *et al*, 1980) and the ability of anti-NS1 antibodies to passively protect experimental animals against live virus challenge (Schlesinger *et al*, 1985; Gould *et al*, 1986; Henchal *et al*, 1988; Schlesinger *et al*, 1986,1987; Young and Falconar, 1990); or by active immunization with purified native or recombinant NS1 (Falgout *et al*, 1990; Putnak and Schlesinger, 1990; Falconar and Young, 1991; Qu *et al*, 1993). A sub-unit vaccine based on NS1 has the potential of inducing a protective immune response in the absence of infection-enhancing virion reactive antibodies.

The relationship of epitopes to both the primary and tertiary structure of NS1 is poorly understood (Henchal *et al*, 1987; Chambers *et al*, 1990; Burke and Monath, 2001). Only limited studies defining epitope specificity on the flavivirus

NS1 protein have been published and these have reported variable findings. Using monoclonal antibodies prepared against yellow fever (YF) virus, Gould *et al* (1986) demonstrated that epitopes on NS1 were mainly type specific (ie. YF specific) although Schlesinger *et al* (1985) published results indicating that flavivirus group-reactive determinants were also present. Hall *et al* (1990; 1995) reported that for MVE NS1, the majority of epitopes recognized by monoclonal antibodies were specific to MVE and not flavivirus-group reactive consistent with previous findings (Schlesinger *et al*, 1983; Gould *et al*, 1985; Henchal *et al*, 1987). The production of a large panel of monoclonal antibodies to the dengue-2 virus NS1 by Falconar and Young (1991) revealed MAbs defining a number of dengue virus subgroup, dengue virus complex and flavivirus cross-reactive epitopes on the NS1 protein. One group of MAbs recognized a dengue-2 and dengue-4 virus subgroup epitope and another quite significant group was shown to cross-react with linear and conformational epitopes common to the NS1 of all four dengue virus serotypes and some members of the antigenic group III flaviviruses. A number of dimer-specific NS1 monoclonal antibodies were also identified consistent with a report of a dimer specific anti-NS1 MAb specific for MVE NS1 (Hall *et al*, 1990).

Work in our laboratory examined the reaction of 34 mouse monoclonal antibodies (Falconar and Young, 1991) to 174 overlapping nonameric peptides, representing the complete sequence of dengue-2 virus NS1 (PR159S1) in a PEPSCAN analysis (Falconar *et al*, 1994). This analysis revealed the precise binding sites of eight MAbs from this panel. A potentially significant finding was the identification of a flavivirus cross-reactive, linear epitope involved in protection (Young and Falconar, 1990). A summary of these findings is shown in Table 2.1. Passive protection data in mice had also been obtained previously (Young and Falconar, 1990) (Table 2.1), which showed that some of these anti-NS1 MAbs were able to confer up to 60% passive protection against challenge. With this background of information, the aim of this section of the work was to build a more detailed epitope map for the remaining MAbs on the native dimer form of NS1 and therefore to identify epitopes which may be important in conferring protection. In addition, it was expected that a detailed picture of the

antigenic structure of NS1 may provide insights into the tertiary structure of this protein.

4.2 RESULTS

(a) Monoclonal antibody epitope competition assay

The methodology used to examine monoclonal antibody binding sites has previously been described (Lopez *et al*, 1986). As this protocol does not require any chemical modification of the antibodies and uses immunoaffinity purified NS1 in its native dimer form (as shown by silver stained SDS-PAGE gels and western blot analysis), interactions occurring between antigen and antibody should not be affected by steric hindrance artefacts. Plates were initially coated with protein A which binds to the F_c portion of rabbit anti-mouse IgG which in turn captured the primary anti-NS1 MAb. Fifty microlitres of protein A (0.01 mg / ml) was added per well of a microtitre plate, according to the published method (Lopez *et al,* 1986). Excess competing antibody was then added in the presence of purified labelled [^{35}S]methionine / cysteine NS1. A total of 2000 counts per minute (cpm) of [^{35}S] - NS1 in 50 µL was added per reaction.

To establish optimal conditions for this assay, the effect of different dilutions of the rabbit anti-mouse capture antibody and both primary and competing monoclonal antibodies was first evaluated. The binding of radiolabelled NS1 to four different anti-NS1 monoclonal antibodies captured by varying dilutions of rabbit anti-mouse antibody is shown in Figure 4.1. Replicates were performed in quadruplicate and then averaged. Dilutions of 1/100 and greater, led to a marginal decrease in the amount of radiolabelled NS1 that was bound to the captured MAb, so a 1/50 dilution of the rabbit anti-mouse IgG was chosen for the assay.

Figure 4.2 shows the level of NS1 binding by two different dilutions (1/100 and 1/500) of primary MAb under conditions of competition and non-competition. Bound radioactivity is given as a percentage of the value obtained in the

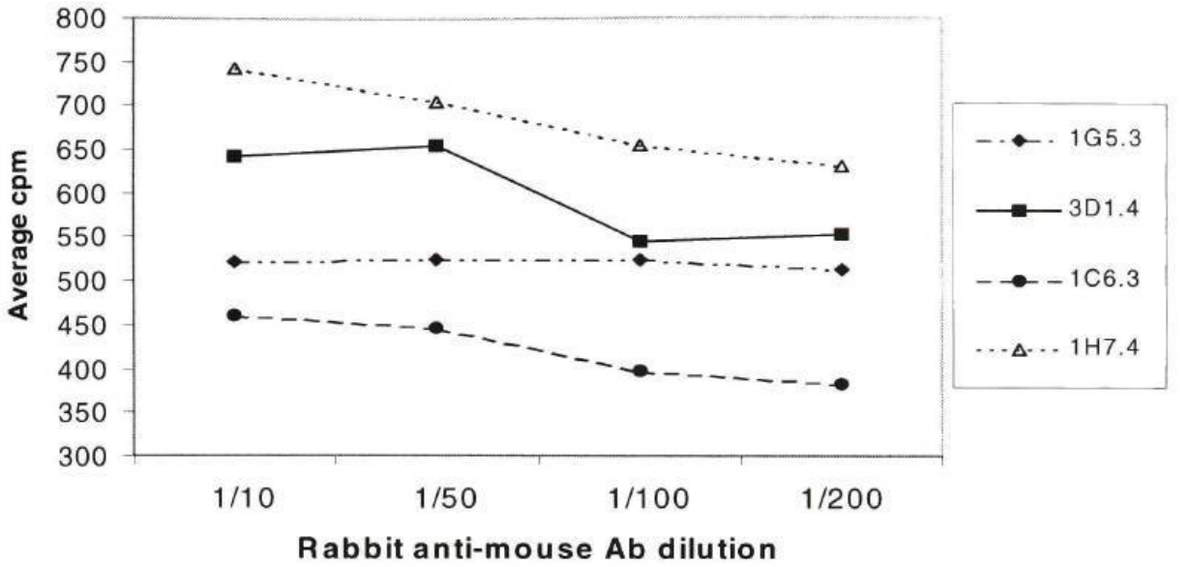
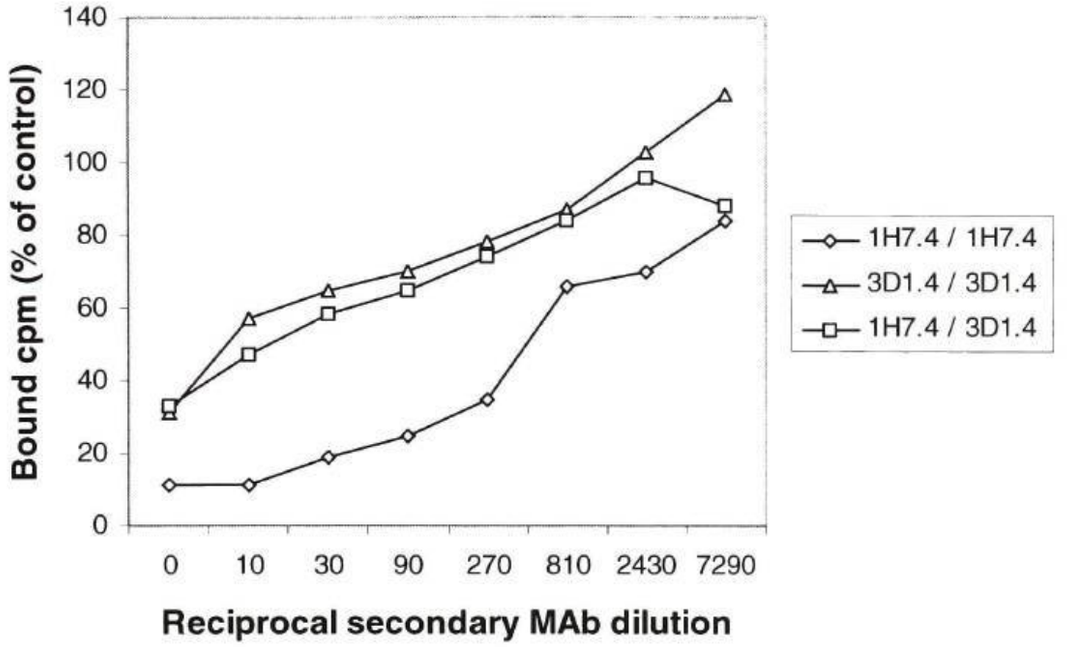


Figure 4.1 **Titration of rabbit anti-mouse IgG antibody against various anti-NS1 MAbs in the presence of 2000 cpm of ^{35}S-methionine/cysteine labeled NS1.**

absence of competitor antibody.

The MAbs 3D1.4 and 1H7.4 were used in these trial experiments in pair-wise crosses as they had previously been shown to bind to topologically distinct linear epitopes (Falconar *et al*, 1994). At a 1/500 dilution (Figure 4.2B), little difference was observed between self and cross-competition (particularly for 3D1.4), while at a 1/100 dilution of primary antibody (Figure 4.2A), 1H7.4 competed strongly with itself for NS1 binding but did not compete with primary binding by 3D1.4. A dilution of 1/100 was therefore chosen for primary MAb binding. Although significant competition for binding by 1H7.4 with itself was seen out to dilutions of 1/810 of secondary antibody, 3D1.4 was not as effective at competitive binding with itself above dilutions of 1/10. Subsequent analyses demonstrated that MAbs binding to the epitope recognized by 1H7.4 exhibited higher binding avidities (see Fig 3.1 and data not shown) than most of the other antibodies in the panel, all of which were required in high concentrations to effectively compete as secondary antibodies. A 1/10 dilution was therefore chosen for all secondary anti-NS1 MAb competition.

A



B

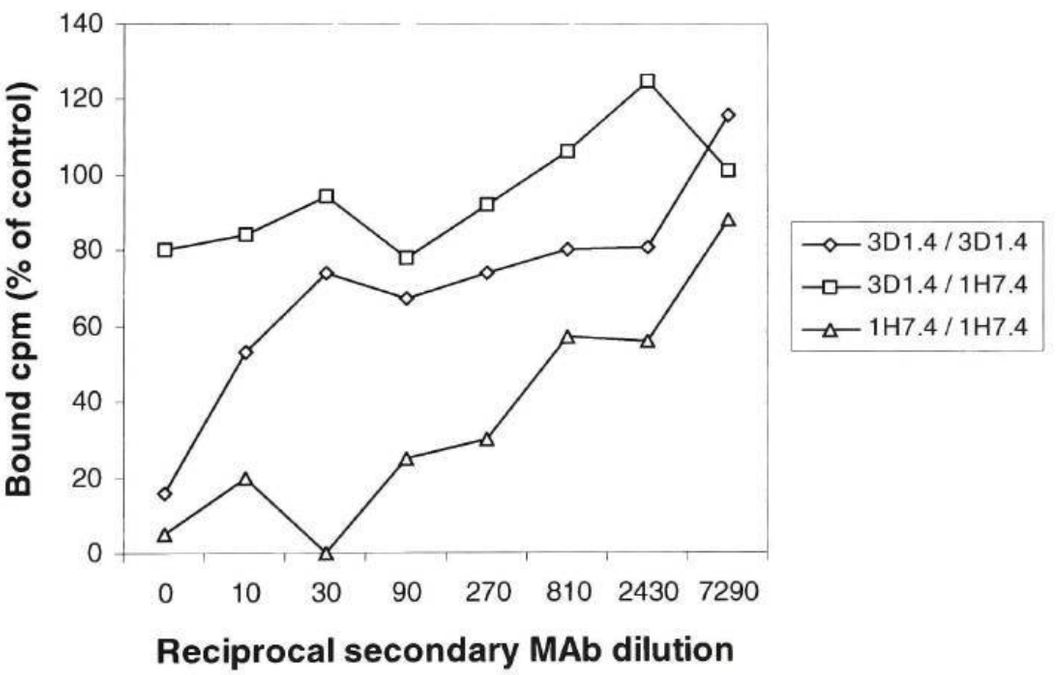


Figure 4.2 **Homologous and cross-competition binding of ^{35}S-NS1 between anti-NS1 MAbs 1H7.4 and 3D1.4 at either 1:100 dilution (A) or 1:500 dilution (B) of the primary MAb. Bound cpm was calculated as a percentage of a control containing no 2° competing MAb. Graph legends refer to 1°/2° MAbs.**

(b) Identification of antigenic domains

A chequerboard competition map of 22 MAbs reacted against metabolically labelled sNS1 immunoaffinity purified from infected Vero cell tissue culture supernatants is shown in Figure 4.3. The original antibody panel chosen comprised 34 MAbs in total, however due to poor primary binding in this assay, twelve MAbs were eliminated from the study in the preliminary screen. With few exceptions, all MAbs showed efficient self-competition (top left to bottom right diagonal in Figure 4.3) thus providing an inbuilt control for the effectiveness of the assay.

(i) Antigenic map for sNS1 derived from Vero cells

The competition map shown in Figure 4.3 reveals five major antigenic domains (labelled A, B, C, D and E) for sNS1 derived from Vero cells, with domains C and D comprising a cluster of overlapping epitopes. Domain B and E define unique sites recognized only by the MAbs 5H10.3d and 5B5.3 respectively. The presence within the A, C and D domains of antibodies that were previously shown to react with defined linear determinants (Falconar *et al*, 1994) enabled the placement of this epitope map in the context of the primary sequence (Figure 4.4A). The topology of epitope distribution on NS1 is shown schematically in Figure 4.4b. Interestingly, the primary binding of several MAbs (5H10.3d, 5B9.3, 5H4.3 and 5F10.3) were found to dramatically enhance the binding of secondary MAbs, particularly in the A domain. The dimer specific MAb 5H10.3d also promotes stronger binding of MAbs recognizing the D3, D4 and D5 epitopes. Enhanced binding of A determinant specific MAbs suggested that this epitope may be further exposed through conformational changes induced by antibody binding to selected epitopes in the non-overlapping domain D. Alternatively, this synergistic binding may simply be due to the stabilization of the native structure of NS1. Of particular note is the binding profile of 5B9.3. As a secondary antibody it successfully competes with all MAbs within the C and D domains. Given that a subset of the C domain antibodies have been shown to react with an epitope in the N-terminal half of NS1 (C1, amino acids 23-33; Falconar *et al*,

1994) while D domain antibodies reacted with epitopes in the C-terminal half (D1, amino acids 249-257 and D2, amino acids 299-307; Falconar *et al*, 1994) this cross-competition indicated that the large overlapping cluster may define a structural domain that comprised the two ends of the same NS1 molecule in close proximity (schematically represented in Figure 4.4B).

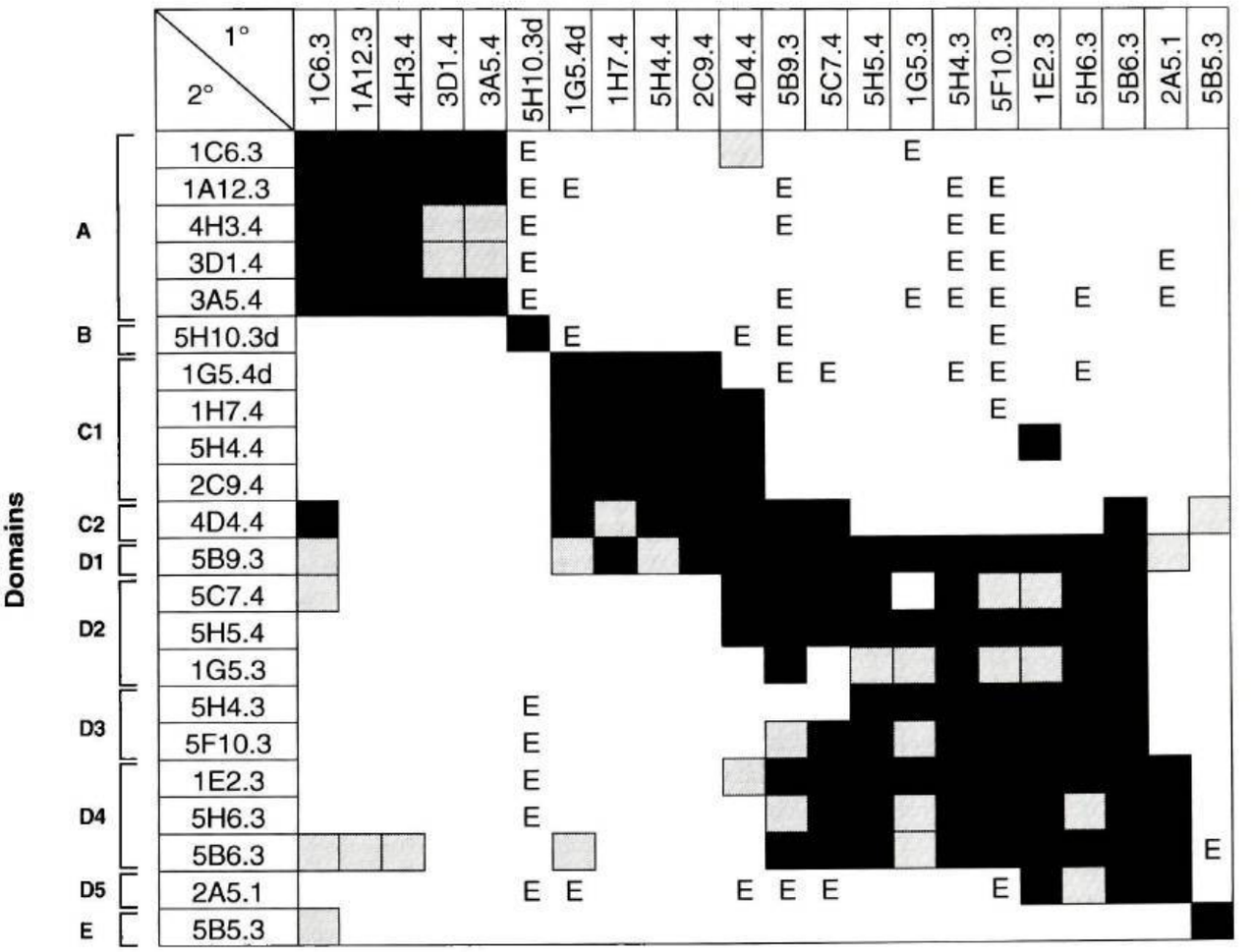


Figure 4.3 **Competition map of captured anti-NS1 MAb binding of ^{35}S-sNS1 from Vero cells in the presence of secondary competitor MAb. Primary MAbs (1°) are listed horizontally and secondary MAbs (2°) vertically.**

- **< 30% binding**
- **30 – 50% binding**
- **51 – 150% binding**
- **E > 151% binding (Enhancement)**

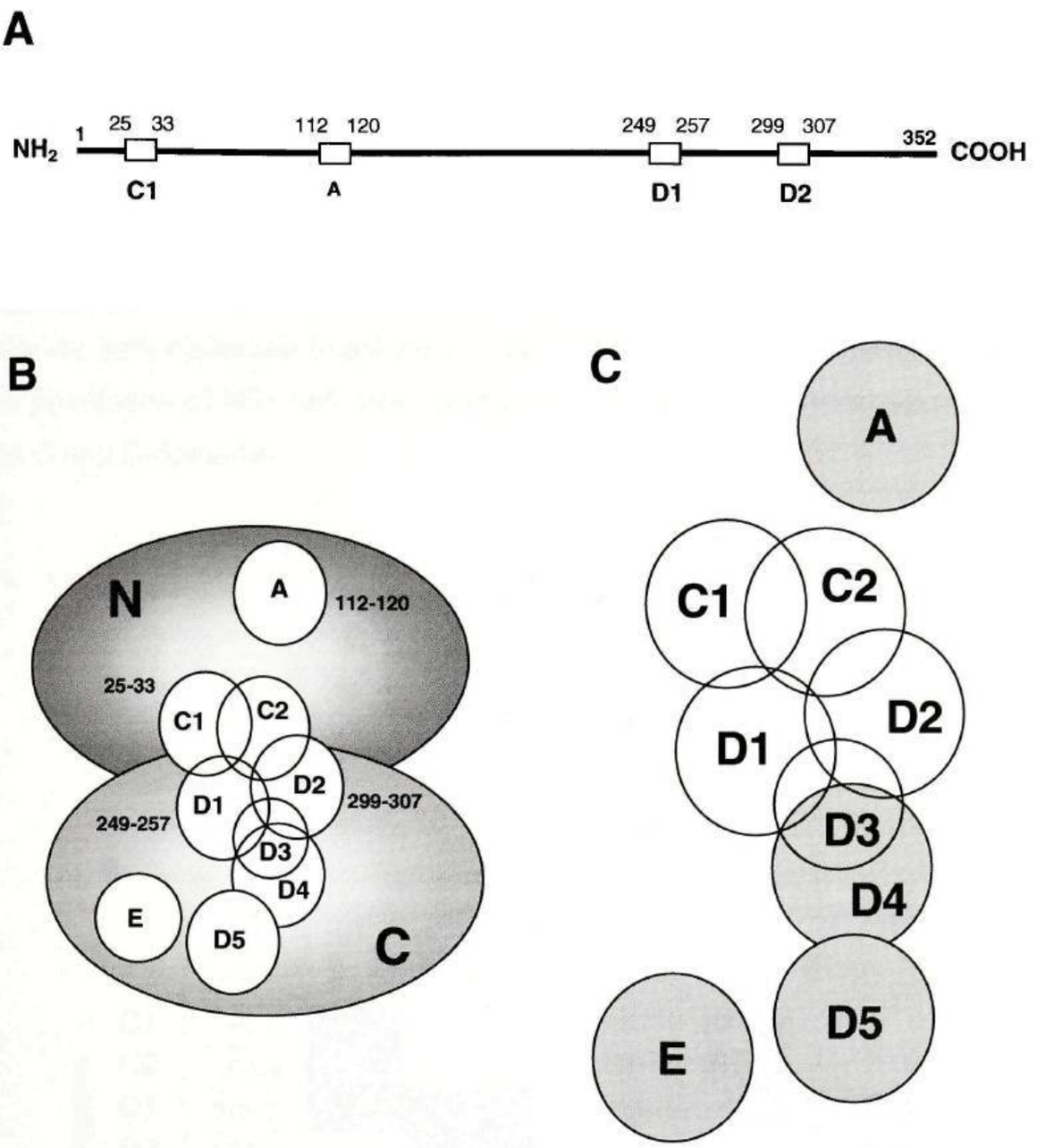


Figure 4.4 **Location of antigenic epitopes on NS1. A, Schematic of the location of linear determinant specific epitopes on the primary sequence of dengue-2 virus NS1; B, topology of overlapping epitopes defined by the competition map; and C, overlapping epitopes with shaded regions signifying epitopes that bound MAbs that were able to provide >30% passive protection in a mouse model (Young and Falconar, 1990). N and C represent the N- and C-terminal halves of the NS1 molecule respectively.**

(ii) Analysis of intramolecular versus intermolecular competition

The competition map shown in Figure 4.3 was determined using native sNS1 in its oligomeric, most likely hexameric form. Therefore, it is possible that the competition observed between epitopes that are spatially distant in the primary sequence (the C1/C2 and D1/D2 epitope clusters) may be the result of either inter- or intra-molecular interactions (see Figure 4.5A). In the former, a head-to-tail orientation of NS1 subunits could give rise to the competition seen between the C and D domains.

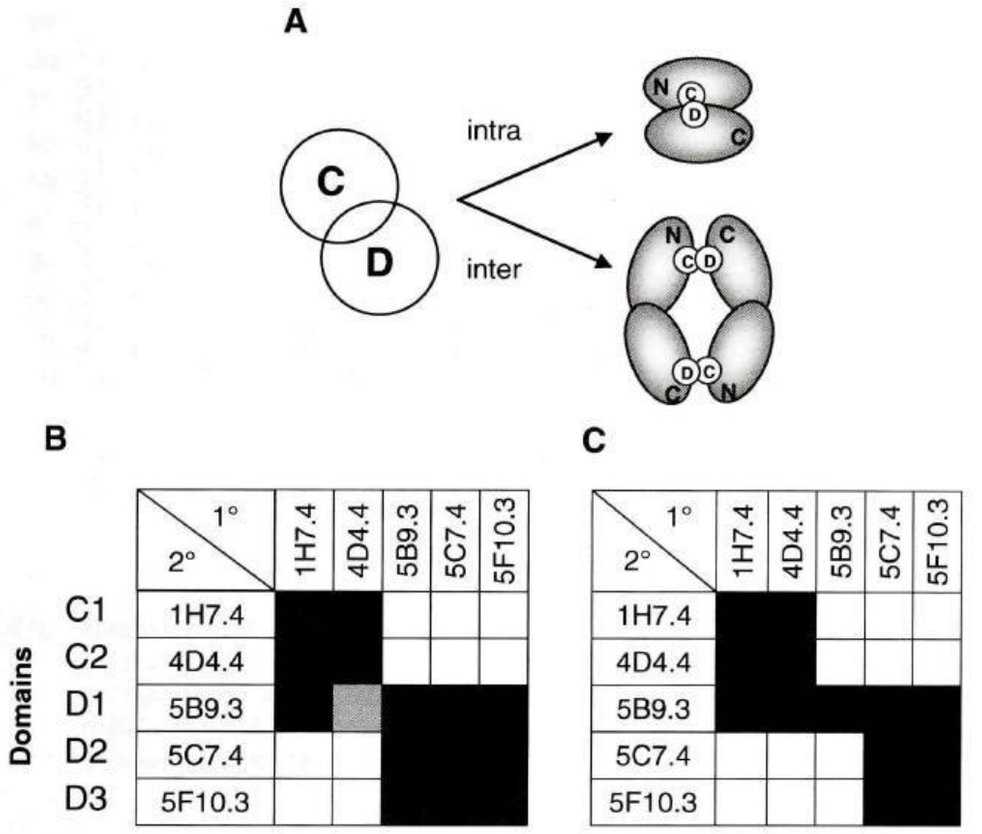


Figure 4.5 **Intra- versus inter-competition between MAbs reacting with the C and D epitopes. A, schematic representing two possible molecular arrangements for NS1 sub-units leading to competition between these two epitopes. N and C indicate the N- and C-terminal halves of the NS1 molecule. Competition maps of B, monomeric sNS1 and C, monomeric mNS1. Solid squares represent > 70% competition, shaded boxes 50-70% competition.**

In order to determine which of these structural forms leads to the observed competition, epitope mapping was carried out for monomeric sNS1. The formation of dimeric NS1 involves non-covalent interactions that may be dissociated by either heat or low pH treatment (Winkler and Winkler, 1989;

Falconar and Young, 1990). Both of these procedures could also lead to denaturation of NS1 and a loss in structural integrity. Therefore, primary binding by the panel of MAbs to heat treated and monomeric NS1 was initially assessed for retention of epitope reactivity. Figure 4.6 shows a histogram of the percentage reduction in primary binding of selected MAbs to immunoaffinity purified, ^{35}S - methionine / cysteine labelled monomeric sNS1 and mNS1 (monomerization was confirmed by western blot analysis).

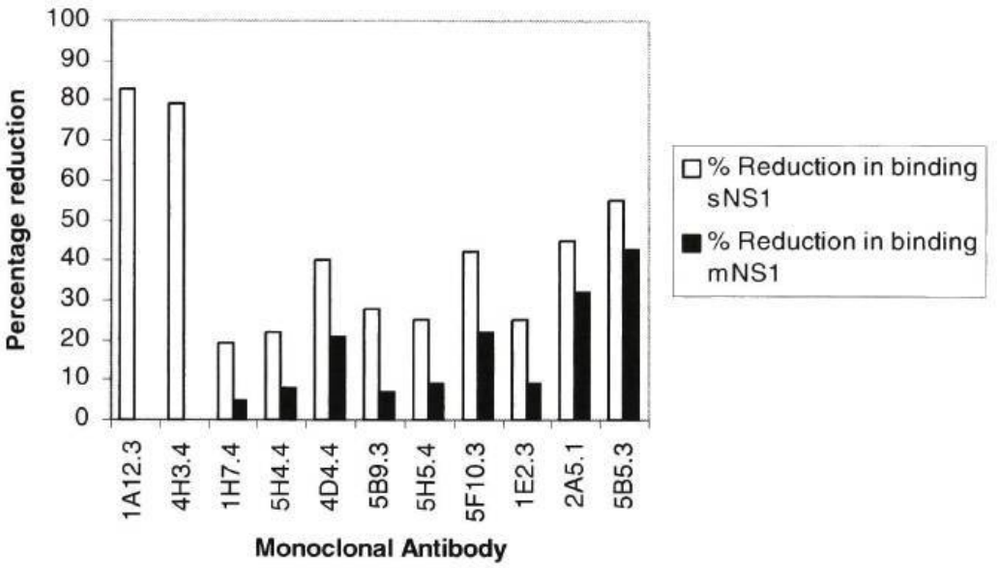


Figure 4.6. Reduction in binding of monomeric ^{35}S-NS1 to representative MAbs of each of the epitope domains. Percentage reduction determined by comparison with binding to dimeric ^{35}S-NS1 run in parallel. Both mNS1 and sNS1 forms were examined. Data represents an average of four replicates.

All MAbs showed a reduction in primary binding although a greater reduction overall was seen in MAb binding to monomeric sNS1. This overall reduction in binding was not unexpected given that binding is measured in terms of total bound ^{35}S - methionine / cysteine label and that dimeric NS1 had been converted to monomeric NS1. Consequently only half as much radioactivity (or one sixth, if the majority of NS1 is in hexameric form) should be expected to bind to the same quantity of MAb. The significant reduction in binding of A determinant specific MAbs (1A12.3 and 4H3.4) however suggested that this epitope may not be exposed in the monomeric form. Regardless of these qualitative differences, the majority of MAbs (with the exception of A domain

specific MAbs) reacted with NS1 in its monomeric form. Given this finding, partial competition maps were generated for monomeric sNS1 and mNS1 to address the question of overlapping epitopes in the N- and C-terminal domains (Figure 4.5B and C). Both maps showed that 5B9.3 (D1) still effectively competed as a secondary MAb with 1H7.4 (C1) indicating that the N- and C-terminal regions of one NS1 molecule may be in close spatial proximity and that the observed competition is occurring intramolecularly. It should be noted however, that the cross-competition between C2 and D2 MAbs seen for the dimeric form of NS1 (Figure 4.3) is lost in the monomeric NS1 form (Figure 4.5B and C).

(iii) Epitope specificity

The strain specific and cross-reactive binding pattern of each MAb in the panel had previously been determined (see Table 2.1; Falconar and Young, 1991). MAbs that shared a similar specificity clustered predominantly into the same epitope groups defined by the competition map (Table 4.1). Epitope A comprised MAbs that recognized NS1 from all four dengue virus serotypes plus members of the encephalitic flaviviruses (eg. JEV, MVE and KUN). C1 and C2 consisted of MAbs that were dengue-2 virus specific and recognized both linear (1H7.4, 5H5.4) and conformational (2C9.4, 4D4.4 and 1G5.4d) determinants. D1, D2 and D3 MAbs primarily recognized epitopes defining dengue-2 and dengue-4 subgroup specificity as well as recognizing NS1 of the encephalitic flaviviruses. D4 and D5 MAbs recognized NS1 only from dengue-2 and dengue-4 virus. The sole MAb recognizing the unique E epitope (5B5.3) reacted with NS1 from dengue-2, dengue-3 and dengue-4 viruses. In summary, this panel of MAbs identified five separate flavivirus reactivity patterns that closely parallelled epitope clusters (Table 4.1; A, C1/C2, D1/D2/D3, D4/D5 and E) that define topologically distinct domains on the NS1 protein.

The protective efficacy of passive administration of these MAbs in a mouse model has been previously determined (Young and Falconar, 1990). The MAbs that provided significant passive protection (>30%) belong to the epitopes shaded in Figure 4.4C and includes one of the D3 epitope MAbs, 5H4.3. As with

Flavivirus cross-reactivity, these MAbs also involve topologically distinct domains.

Table 4.1 Flavivirus cross-reactivity of domain specific MAbs

DOMAIN	FLAVIVIRUS CROSS-REACTIVITY
A	Flavivirus cross-reactive
C1, C2	Dengue-2 specific
D1, D2, D3	Dengue-2 and 4 specific and encephalitic flaviviruses
D4, D5	Dengue-2 and Dengue-4
E	Dengue-2, Dengue-3, Dengue-4

(iv) Antigenic map for mNS1 derived from Vero cells

The epitope profile of immunoaffinity purified NS1 from infected cell lysates (the membrane-associated form, mNS1) was also examined by competition mapping (Figure 4.7). The map showed the same basic pattern of epitope groupings with two major differences. The first was an apparent disruption in the integrity of D domain competition and the second was a failure of any of the A domain specific MAbs to bind to this form of NS1. Intriguingly, it was these MAbs which failed to bind efficiently to the monomeric form of sNS1 (Figure 4.6). The absence of detectable binding of the A specific MAbs to mNS1 occurred despite the fact that these antibodies recognized a linear sequence present in both forms of NS1 and had readily reacted with mNS1 bound to ELISA plates or immunoblots following SDS-PAGE separation. Presumably, these procedures lead to structural modifications that exposed the epitope that is otherwise sequestered in the native solubilized form of mNS1 used in the competition assay. MAbs specific for the A determinant have been shown to provide significant passive protection to mice in lethal virus challenge experiments (shown schematically in Figure 4.4C). It is relevant to note therefore that mice immunized with sNS1 were afforded

much higher levels of protection than those immunized with mNS1 (Young and Falconar, 1990).

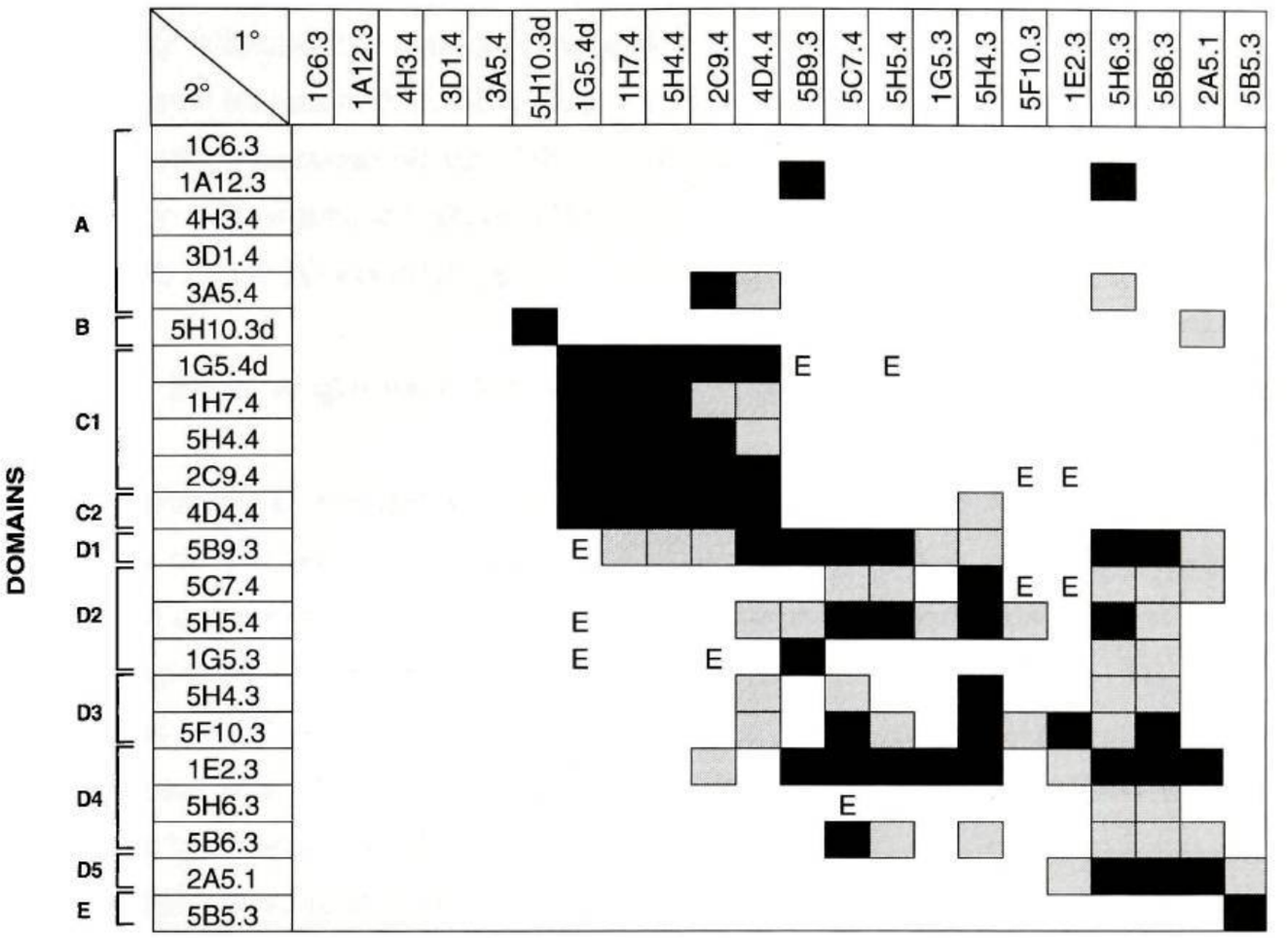


Figure 4.7 **Competition map of captured anti-NS1 MAb binding of ^{35}S-mNS1 from Vero cells in the presence of secondary competitor MAb. Primary MAbs (1°) are listed horizontally and secondary MAbs (2°) vertically.**

< 30% binding
30-50% binding
51-150% binding
E > 151% binding (Enhancement)

The loss in integrity of the epitope map across the D domain may be a consequence of reduced overall binding of the MAbs to mNS1 as a result of detergent solubilization of this membrane-associated species and the continued presence of detergent in the assay. It should be noted that sNS1 was purified and prepared in the absence of detergent (for details refer to 2.5a (iii)). Under these conditions MAbs with higher avidities might effectively block competition by other MAbs recognizing the same epitope. For example, primary binding of the MAbs 5B9.3, 5F10.3 and 1E2.3 all effectively prevented competition by most

secondary binding antibodies while as secondary antibodies, they efficiently competed with most D domain specific MAbs. Alternatively, disruption in the D domain competition may indicate induced conformational changes to a region directly affected by detergent solubilization and membrane dissociation. An additional feature of the mNS1 map that should be noted is the loss of the cross-competition between MAbs of the C2 and D2 domains that was seen for sNS1 (Figure 4.3). Again, this observation may be a direct consequence of alterations in MAb reactivity resulting from detergent solubilization of NS1.

(v) Effect of glycosylation on antigenic maps

The differential availability of the A epitope for MAb binding in the two different forms of NS1 was unexpected. Apart from their localization (cell-associated for mNS1 and extracellular for sNS1) the only other major difference between these two species is their glycosylation profile. Both are glycosylated at two sites, ^{130}Asn and ^{207}Asn. However for mNS1, both of the linked glycans are of the high-mannose type while sNS1 contains one high-mannose and one complex glycan. It has been shown previously that the processing of only one glycosylation site for sNS1 is likely the result of dimerization prior to passage through the Golgi (Young and Falconar, 1990). This assumption is based on the observation that one of the carbohydrate sites is resistant to cleavage by endoglycosidases when NS1 is in its native dimeric form (Young and Falconar, 1990) ^{130}Asn was subsequently identified as the complex glycan linkage site (Young *et al*, 1993; Pryor and Wright, 1994) with ^{207}Asn being the high mannose site which is sequestered following dimerization. As ^{130}Asn is in close proximity to the primary sequence of the A epitope (A specific MAbs have been shown to bind to peptides comprising amino acids 111-121; Figure 4.4A) (Falconar and Young, 1994) it was hypothesized that the differential antibody binding may be the result of conformational constraints or changes in this region induced by two different carbohydrate moieties. In a preliminary analysis, an epitope map was generated for immunoaffinity purified membrane-associated NS1 derived from dengue virus infected C6/36 mosquito cells. Insect cells are not able to add complex terminal sugars to carbohydrate moieties on glycoproteins and as such it was expected

that this species would generate an epitope map that was similar to that of mammalian cell derived mNS1. Figure 4.8 shows the epitope map of a selected subset of MAbs for C6/36 derived NS1 (Figure 4.8B) in comparison with the epitope map for Vero mNS1 (Figure 4.8C) and sNS1 (Figure 4.8A).

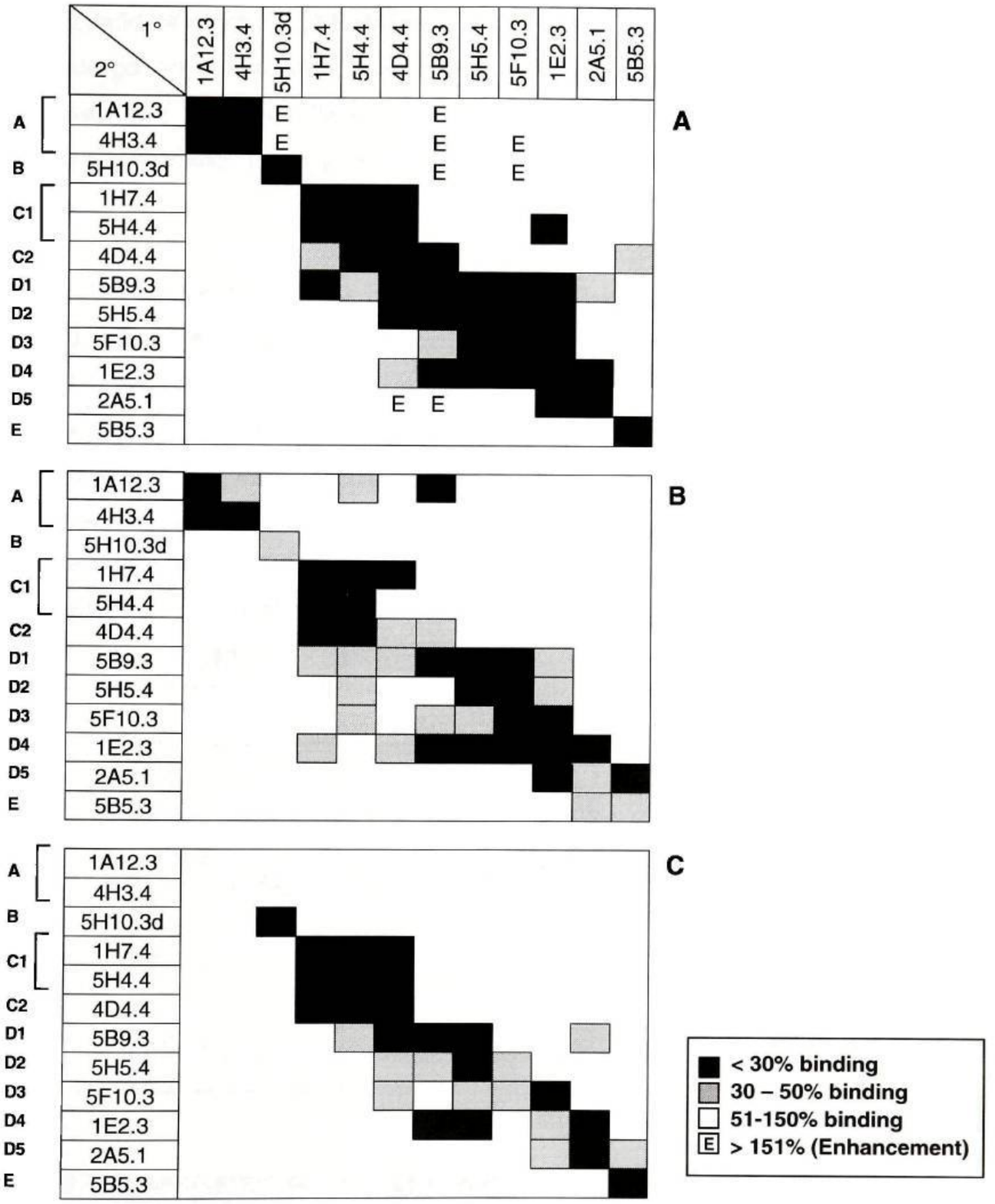


Figure 4.8 **Partial epitope competition maps of A, Vero (mammalian) cell derived sNS1; B, C6/36 (insect) cell derived mNS1; C, Vero cell derived mNS1.**

While the same loss in integrity of the D domain was found with the insect cell derived mNS1, all A specific MAbs were found to readily bind to this species. This result suggested that the presence or absence of complex sugars on this carbohydrate moiety was not sufficient to explain the differential antibody binding. It should be pointed out however that glycoproteins expressed in insect cells do undergo some additional modifications during traffic through the Golgi (Butters *et al*, 1981; Naim and Koblet, 1992) and it may be these modifications that lead to conformational changes in NS1 that resulted in exposure of the A epitope.

To examine the possibility that the lack of recognition of the A epitope in Vero cell derived mNS1 was simply a consequence of steric hindrance by the carbohydrate, ^{35}S - methionine / cysteine labelled immunoaffinity purified NS1 was treated with endoglycosidase F before examining primary binding by the A epitope specific MAb 1A12.3. The MAb 1H7.4 (C1 epitope) which binds efficiently to mNS1, was also examined as a positive control. Prior to the binding analysis, the removal of carbohydrates was confirmed by autoradiography of the digested samples (Figure 4.9).

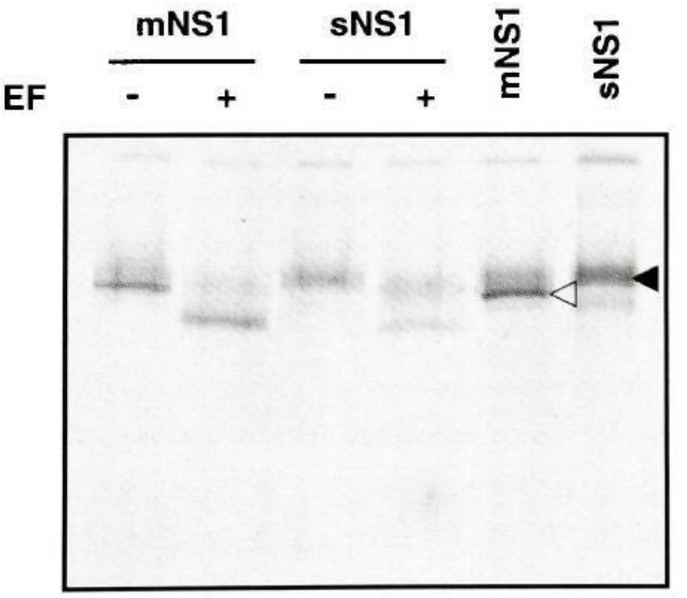


Figure 4.9 **Autoradiograph of endoglycosidase F (EF) treated ^{35}S-sNS1 and ^{35}S-mNS1. Untreated preparations of immunoaffinity purified ^{35}S-met/cys labeled mNS1 (open arrowhead) and sNS1 (solid arrowhead) are shown in the last two lanes. Mock digested and endo F digested samples of both mNS1 and sNS1 are shown in the first four lanes.**

It should be noted that carbohydrate removal was not completely efficient. As dimeric NS1 was digested, the carbohydrate linked to ^{207}Asn is likely to remain intact. Figure 4.10 shows histograms of untreated, mock digested and endoglycosidase F digested radiolabelled mNS1 and sNS1 bound to 1A12.3 (Figure 4.10B) and 1H7.4 (Figure 4.10A).

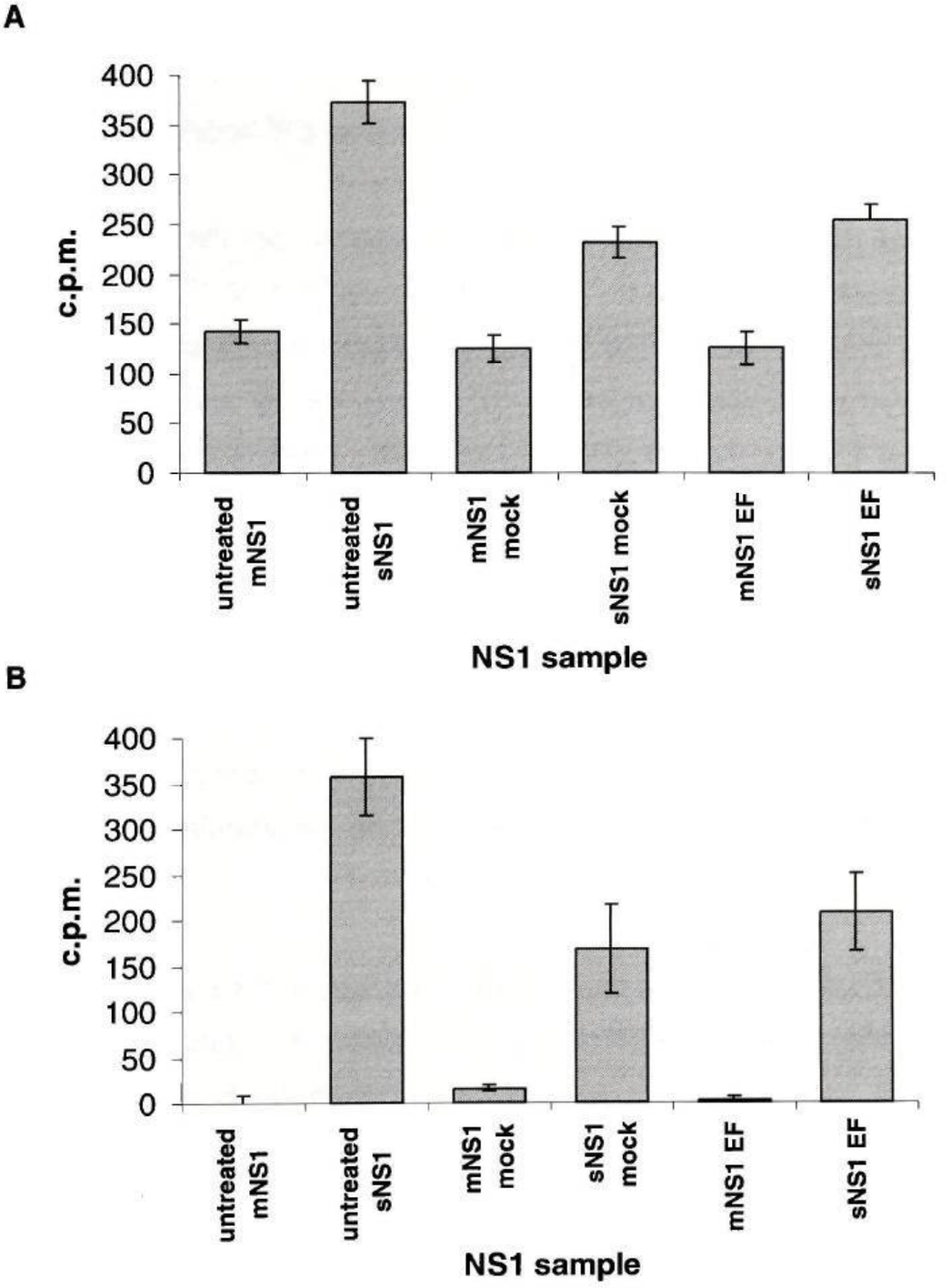


Figure 4.10 **Histograms showing the amount of metabolically labeled and immunoaffinity purified ^{35}S-mNS1 and ^{35}S-sNS1 captured by A, MAb 1H7.4 (C1 epitope) or B, MAb 1A12.3 (A epitope) following mock digestion or endoglycosidase F (EF) digestion. Error bars represent 1 standard deviation (SD) around the mean of four replicates.**

Mock digestion of the NS1 samples reduced overall binding (particularly to sNS1) but removal of the carbohydrate moiety did not modify the binding efficiency of either MAb to mNS1 or sNS1. Collectively these results would suggest that the differential glycosylation of the two forms of NS1 generated in mammalian cells may not be responsible for the differences in A epitope recognition.

(c) Partial Enzyme Digestions

The previous identification of antibody-reactive linear determinants spanning the NS1 sequence $^{112\text{-}120}$A, $^{25\text{-}33}$C1, $^{249\text{-}257}$D1 and $^{291\text{-}307}$D2 (Figure 4.4A) (Falconar *et al*, 1994) provided the opportunity to use MAbs specific for these regions as "site-specific" probes or markers, in the competition maps. As the preceding section showed, competition within specific epitope clusters that included these MAbs helped to identify the binding domains of conformation specific MAbs. In order to provide further insights into the location of these epitope clusters, partial enzyme digestion of purified NS1 was performed with the objective of reliably producing overlapping cleavage fragments that spanned the entire length of the molecule. It was hypothesized that the identification of these fragments by N-terminal sequencing followed by analysis of MAb binding on immunoblots would provide further information on binding sites in the context of the primary sequence.

Several proteases were examined for their ability to generate NS1 fragments by partial digestion that were suitable for SDS-PAGE and immunoblot analysis. These included chymotrypsin, *S. aureus* V8 protease, trypsin and hydroxylamine. Preliminary experimental digestions with each of these proteases individually on immunoaffinity purified sNS1 indicated that of all these reagents, trypsin and *S. aureus* V8 protease (V8) generated the most reproducible cleavage fragments. Both native dimeric NS1 and heat denatured monomeric NS1 were used as substrates. The most reproducible cleavage fragments generated were from trypsin treatment of native dimeric NS1, and V8 digestion of heat-denatured (monomeric) NS1 (data not shown). The kinetics of

digestion were also examined in order to define optimal reaction conditions. Immunoblots of SDS-PAGE separated cleavage fragments from sNS1 treated with 1 µg / mL of trypsin at 37°C over incubation periods ranging from 0 to 60 minutes were prepared (Figure 4.11) and were probed with representative MAbs from different epitope groups; A, 1G5.3 (D2); B, 5B9.3 (D1); C, 1H7.4 (C1) and D, 3D1.4 (A). Two significant observations were the generation of a 25 kDa fragment which was recognized by the D domain specific MAbs 1G5.3 (D2) and 5B9.3 (D1) and the total loss of reactivity by 3D1.4 (A) to any species. Extended cleavage experiments (not shown) demonstrated that the 25 kDa species was relatively stable to further digestion. The differential recognition of this species by the D domain specific MAbs suggested that it was a C-terminal fragment and most likely corresponded to the gp24 trypsin fragment reported by Cauchi *et al* (1991). The loss of 3D1.4 binding is probably the consequence of trypsin cleavage within the exposed epitope as two lysines and one arginine were present in the linear A determinant, ^{112}RYSWKTWGK^{120} (Falconar *et al*, 1994).

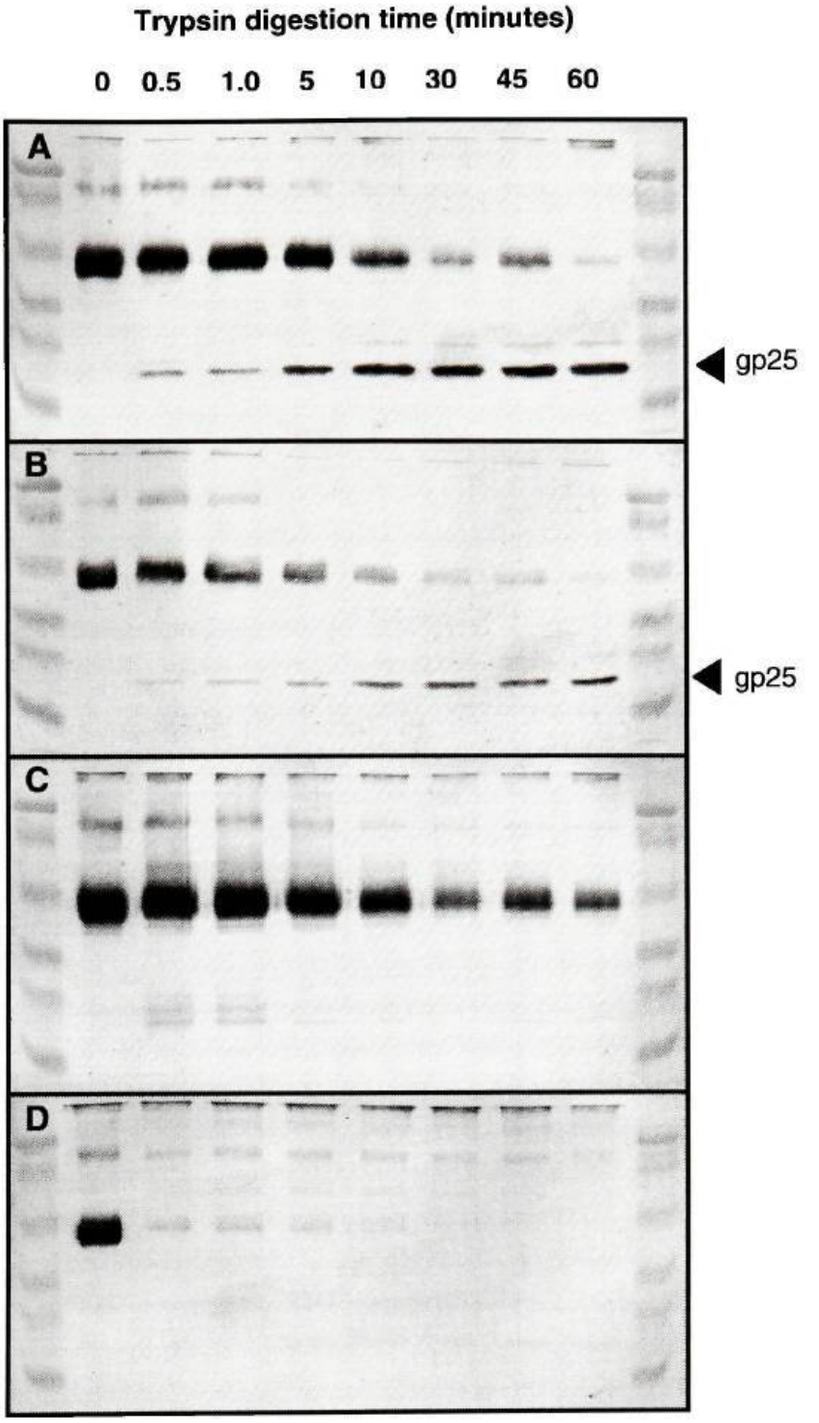


Figure 4.11 **Western blots of immunoaffinity purified NS1 digested with trypsin for various time periods and probed with the anti-NS1 MAbs A, 1G5.3 (D2 epitope); B, 5B9.3 (D1 epitope); C, 1H7.4 (C1 epitope) and D, 3D1.4 (A epitope).**

(i) Identification of high mannose and complex glycosylation sites

The cleavage fragments generated by trypsin digestion of NS1 were further examined by endoglycosidase treatment (Figure 4.12). Both heat denatured and native dimeric NS1 were subjected to either mock (Figure 4.12A and B, lanes 1 and 2 respectively) or trypsin digestion (Figure 4.12A and B, lanes 3 - 8).

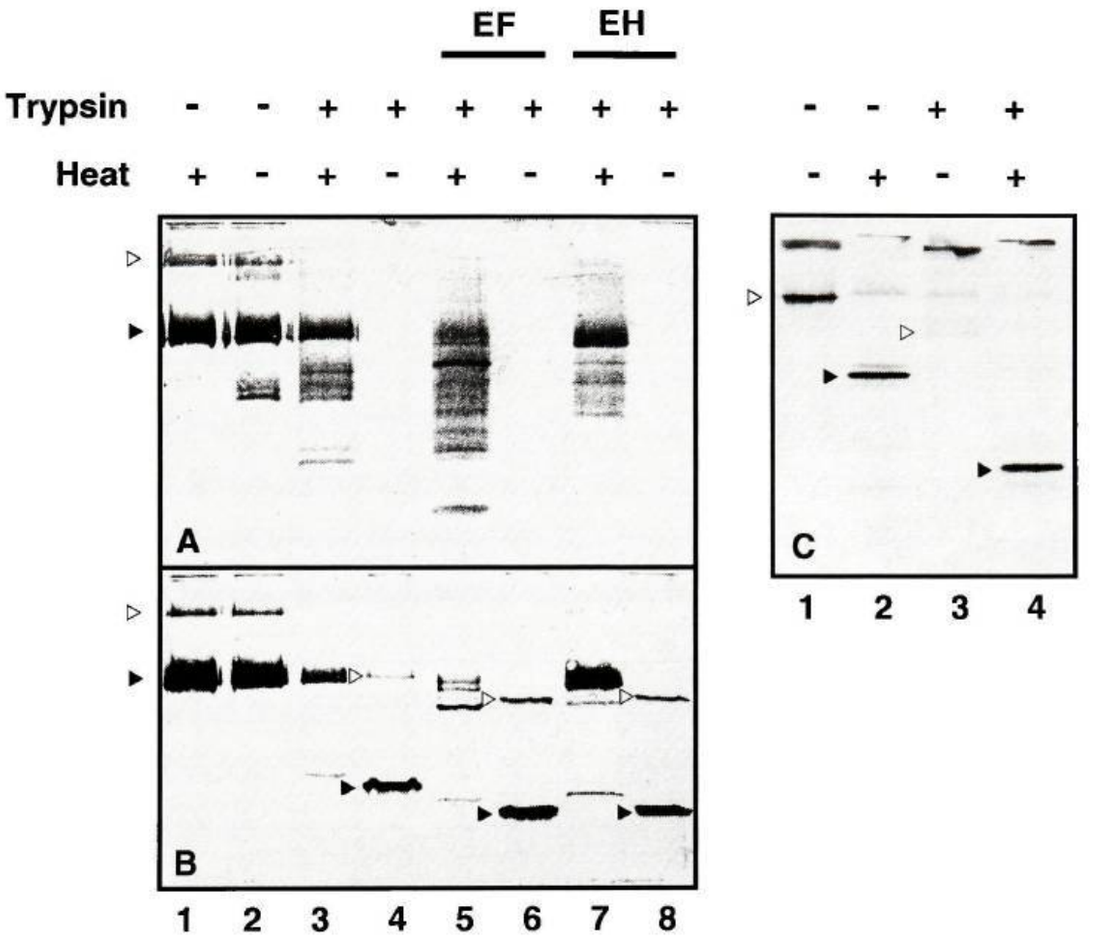


Figure 4.12 **Western blots of immunoaffinity purified sNS1 either heated or left unheated prior to mock digestion or trypsin digestion. A and B, selected samples were further digested with or without the endoglycosidases F (EF) and H (EH). All samples were then boiled prior to SDS-PAGE separation. Blots were probed with the anti-NS1 MAbs 3D1.4 (A) and 1G5.3 (B). C, Western blot probed with 1G5.3 of unheated mNS1 digested with or without trypsin. Samples were then either left unheated or heated prior to SDS-PAGE analysis as indicated. Dimer and monomer forms are shown (open and solid arrowheads respectively).**

The products of trypsin digestion were then treated with either endoglycosidase F (Figure 4.12A and B, lanes 5 and 6) or endoglycosidase H (Figure 4.12A and B, lanes 7 and 8) prior to SDS-PAGE separation and immunoblotting with MAbs

3D1.4 (Figure 4.12A) or 1G5.3 (Figure 4.12B). As identified previously, the gp25 fragment was only generated efficiently when native dimeric NS1 was digested (compare Figure 4.12B and lanes 3 and 4). By contrast, reactivity to 3D1.4 (Figure 4.12A, lane 4) was completely lost following digestion of dimeric NS1. Heat denatured monomeric NS1 as well as a number of cleavage fragments were however recognized by this antibody (Figure 4.12A, lane 3). This result is entirely consistent with the previous finding (see Figure 4.6) that monomeric sNS1 failed to react efficiently with A epitope specific MAbs indicating that this epitope is poorly exposed in the monomer form and is therefore unlikely to be available for trypsin digestion. Both endoglycosidase F and H digestion were found to remove approximately 3 kDa from the gp25 trypsin cleavage fragment (Figure 4.12, lanes 6 and 8 respectively) indicating that this species had one high mannose carbohydrate linkage. Combined with the observation that this species is recognized by MAbs reactive to epitopes in the C-terminal half of NS1, the finding that this species contains only the second carbohydrate linkage site, ^{207}Asn (known to be the linkage site for the high mannose glycan) indicates that this C-terminal fragment is generated by cleavage between the two glycan sites, ^{130}Asn and ^{207}Asn. A somewhat unexpected finding was the removal from what appeared to be full-length NS1 (arrowed in Figure 4.12B lane 4) of 6 - 8 kDa by both endoglycosidase F (lane 6) and endoglycosidase H (lane 8). This molecular weight difference would represent two carbohydrate moieties, both of the high mannose type due to complete cleavage by endoglycosidase H. However this digestion was of sNS1 which contains one of its glycans as the complex type which should be resistant to endoglycosidase H treatment. On closer inspection, the species indicated in Figure 4.12, lane 4 (open arrowhead) is slightly higher in molecular weight than full length NS1 (compare with lanes 1 - 3). As traces of dimeric NS1 can be seen in the undigested tracks (Figure 4.12A and B lanes 1 and 2) it may be inferred that the species migrating at 50 kDa in lane 4 is not full-length NS1 but a dimer of the gp25 fragment. This dimer would contain two high mannose glycans and explain the endoglycosidase digestion result.

To confirm this hypothesis, trypsin digested native dimeric sNS1 was either boiled or left unboiled prior to SDS-PAGE separation and immunoblot analysis

(Figure 4.12C). While the 50 kDa species was seen in the unheated sample, no gp25 was detected (Figure 4.12C, lane 3). Prior heat treatment however resulted in conversion of the 50 kDa species (gp50) into gp25 (Figure 4.12C, lane 4) confirming that gp50 is likely to be a dimer of gp25. Cauchi *et al* (1991) had previously shown that the gp24 trypsin fragment they had identified also existed as a dimer. The identification of this fragment as being derived from the C-terminal half of NS1 confirms previously published evidence that the dimerization domain is in this part of the molecule (Pryor and Wright, 1993; Gruenberg and Wright, 1992; Pryor *et al*, 1998; Hall *et al*, 1999).

Similar kinetic experiments were performed for V8 digestion of heat denatured monomeric NS1 (data not shown) in order to establish optimal reaction conditions. As for trypsin, representative MAbs from four different epitope groups were used to probe immunoblots of partial V8 digestions separated by SDS-PAGE (Figure 4.13). Four differentially recognized cleavage fragments were identified for further analysis (indicated by arrowheads in Figure 4.13); two glycoproteins gp31 and gp20 and two smaller polypeptides with no linked glycans, p13 and p10. Analysis following reduction with 2-mercaptoethanol confirmed that they were single polypeptide fragments (data not shown).

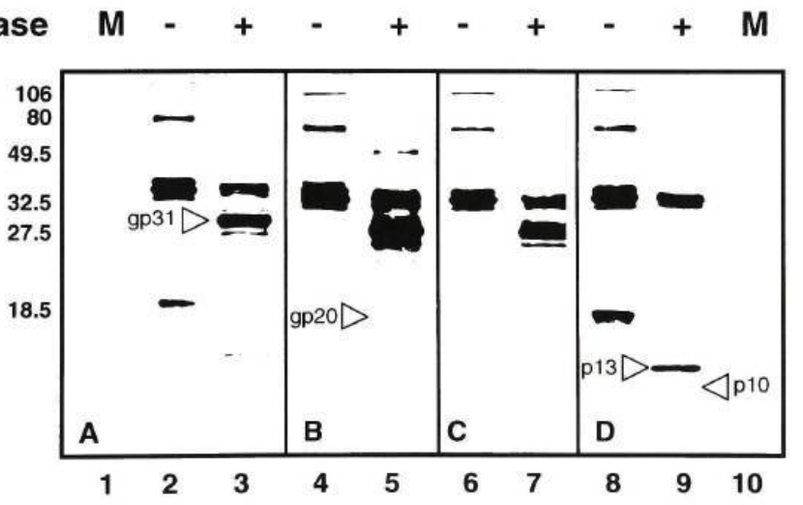


Figure 4.13 **Western blot analysis of V8 protease digested mNS1 probed with the anti-NS1 MAbs (A) 5B9.3, (B) 1H7.4, (C) 3D1.4 and (D) 1G5.3. Cleavage fragments chosen for analysis are arrowed. M refers to protein marker standards.**

The glycosylation status of these species was determined by a series of endoglycosidase H and F digestions (data not shown). Gp31 contains both high mannose and complex carbohydrate moieties and gp20 only glycans of the complex type indicating that gp31 covers the region comprising both glycosylation sites ^{130}Asn and ^{207}Asn while gp20 contains only the first glycosylation site, ^{130}Asn. A 22-25 kDa species was also recognized by MAbs 5B9.3 and 1G5.3 in the mock digested sample (Figure 4.13, lanes 2 and 8 respectively). This fragment has a similar reactivity profile to the gp25 cleavage fragment generated by trypsin digestion, and was often seen in purified preparations of NS1 that had not been frozen immediately following purification or that had been subjected to incubation conditions at room temperature and above. The spontaneous generation of this species has been noted previously for the NS1 protein of dengue virus (Henchal *et al*, 1987) and probably reflects cleavage by contaminating trace amounts of trypsin-like activity in preparations not treated with protease inhibitors.

(ii) N-terminal sequence analysis of proteolytic fragments

In order to precisely define the identity of the five cleavage fragments chosen for analysis, preparative SDS-PAGE gels were run, blotted and then stained as described in section 2.5e (iii). The region of the blot corresponding to the appropriate fragment was cut and the PVDF strips were submitted (to the Protein Facility, QIMR) for N-terminal sequence analysis. Figure 4.14 shows strip blots of trypsin and V8 protease digested NS1 probed with representative MAbs from the major epitope groups. The five cleavage fragments investigated are indicated by arrows. The five panels at the top right of Figure 4.14 show the N-terminal amino acids identified for these fragments. The N-terminus of gp31 and gp20 is the authentic N-terminus of NS1 while p13 and p10 have ^{239}Ser as their N-terminal amino acid. The trypsin fragment gp25 has ^{173}Glu as its N-terminus, confirming that this is a C-terminal fragment of NS1. Schematics of the various cleavage fragments generated are shown in the bottom panel. The presence of either complex or high mannose carbohydrate moieties linked to ^{130}Asn on gp31 and gp20 depended on whether NS1 had been derived from the secreted or

membrane-associated form respectively (schematically represented in Figure 4.14B).

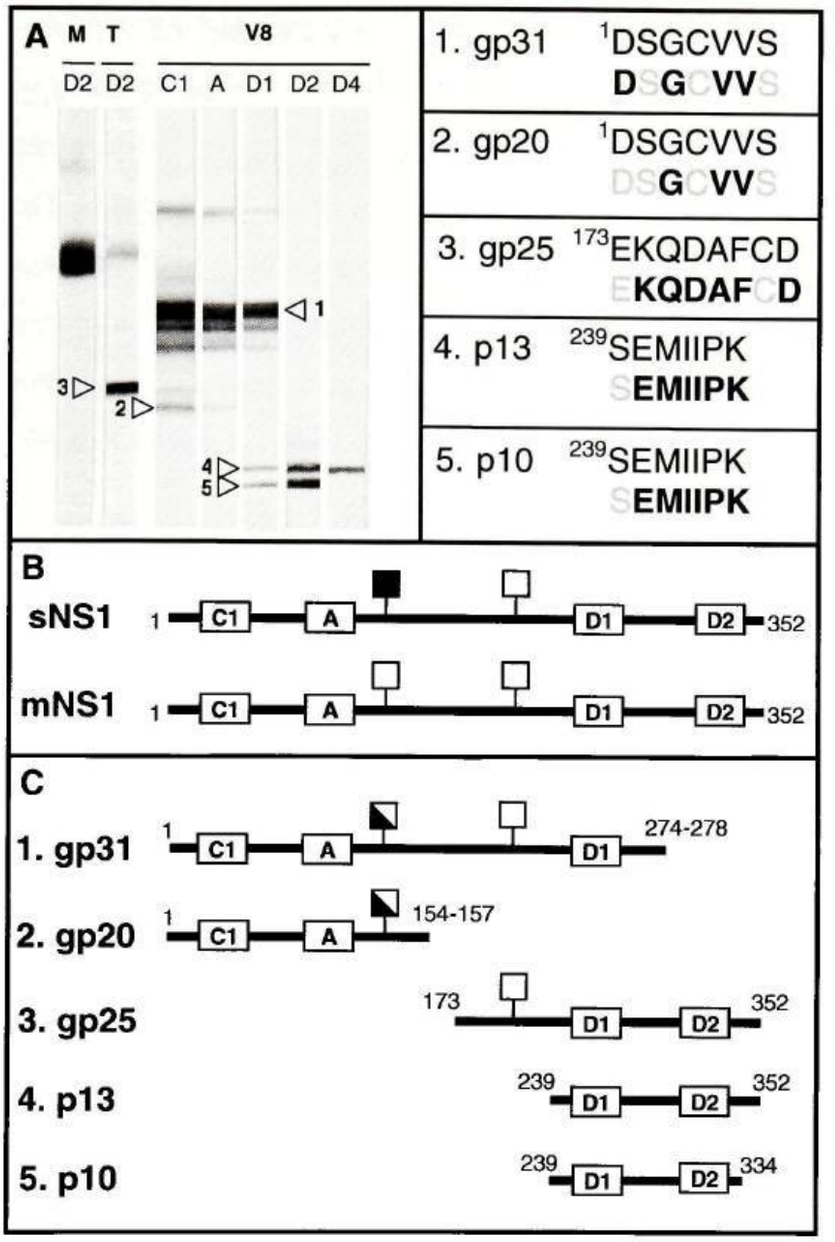


Figure 4.14 **Location of proteolytic cleavage fragments in the context of the primary NS1 sequence. A, left panel; immunoblot strips of SDS-PAGE separated cleavage fragments probed with representative MAbs from epitope groups A (3D1.4), C1 (1H7.4), D1 (5B9.3), D2 (1G5.3), and D4 (1E2.3) showing the different patterns of reactivity observed. A, right panel; N-terminal sequence analysis of the five cleavage fragments identified in the left panel. The primary amino acid sequence of NS1 is shown on top, with amino acids identified by N-terminal sequencing in bold below. B, schematic representation of sNS1 and mNS1. C, schematic representation of cleavage fragments in relation to the full sequence. The positions of linear epitopes are identified by lettered boxes and glycosylation sites by square paddle-pops. Open and filled boxes represent high mannose and complex CHO moieties respectively.**

While the exact C-terminus cannot be defined for any of these fragments from the analyses presented, their molecular weights, location of cysteine residues involved in disulfide bridges, carbohydrate moieties and cleavage site specificity has limited the options and assisted in defining their locations. The C-terminus of gp31 most likely ends at either ^{274}E, ^{276}D or ^{278}D while that of gp20 ends at ^{154}E, ^{156}E or ^{157}D. Both gp25 and p13 have molecular weights that would suggest they possess the true C-terminus while p10 must be truncated at the C-terminus, most likely back to ^{334}E. The reactivity of MAbs known to bind to linear determinants within the primary sequence of NS1 (boxes labelled A, C1, D1 and D3 in the schematics) is entirely consistent with the identity of the fragments represented in Figure 4.14.

Having identified an overlapping set of cleavage fragments, the entire panel of MAbs, including those that are conformation specific, were reacted with strips cut from blots of SDS-PAGE preparative gels of cleavage fragments generated by digestion with either V8 protease or trypsin. Reactivity with specific fragments placed MAb binding within the context of the primary sequence (Table 4.2). In confirmation of the competition maps, epitopes A, C1 and C2 were located in the N-terminal half of the NS1 molecule. Epitopes comprising both the D domain and E epitope spanned the C-terminal third. All D domain specific MAbs and the single representative of the E epitope, 5B5.3 recognized the p13 cleavage fragment generated by V8 digestion. Based on molecular weight, this fragment comprises C-terminal residues from ^{239}S to presumably the true C-terminus, ^{352}A. Significantly, MAbs belonging to the D4 and D5 epitopes did not recognize p10 which had the same N-terminus as p13 but was missing approximately the C-terminal 17 amino acids. The separation of MAbs recognizing these epitopes from the other D domain reactive MAbs is consistent with the competition profile (Figure 4.3), Flavivirus cross-reactivity (Table 4.1) and ability to confer passive protection in a mouse model (Figure 4.4C). The absence of reactivity with p10 could be the result of either the location of the D4 and D5 epitopes in the C-terminal 17 amino acids or alternatively, a conformational change induced in the region ^{239}S to ^{334}E through the loss of this C-terminal fragment that alters the availability of these epitopes for MAb binding.

Domains		V8 digestion				Trypsin
		gp31	gp20	p13	p10	gp25
A	1C6.3	+	+	-	-	-
	1A12.3	+	+	-	-	-
	4H3.4	+	+	-	-	-
	3D1.4	+	+	-	-	-
	3A5.4	+	+	-	-	-
B	5H10.3d	+ / -	-	-	-	-
C1	1G5.4d	+	-	-	-	-
	1H7.4	+	+	-	-	-
	5H4.4	+	+	-	-	-
	2C9.4	+	+	-	-	-
C2	4D4.4	+	-	-	-	-
D1	5B9.3	+	-	+	+	+
D2	5C7.4	-	-	+	+	+
	5H5.4	-	-	+	+	+
	1G5.3	-	-	+	+	+
D3	5H4.3	-	-	+	+	+
	5F10.3	-	-	+	+	+
D4	1E2.3	-	-	+	-	+
	5H6.3	-	-	+	-	+
	5B6.3	-	-	+	-	+
D5	2A5.1	-	-	+	-	+
E	5B5.3	-	-	+	+	+

Table 4.2 **Reactivity of individual MAbs with immunoblots of V8 protease and trypsin digested NS1 cleavage fragments.**

Reactivity of MAbs belonging to epitopes D1, D2 and D3 with the p10 fragment localizes them to the region spanning ^{239}S to ^{334}E.

The reactivity of 5B9.3 (epitope D1) with both gp31 and gp25 confirmed that these were overlapping fragments. Furthermore, the reactivity of the conformational epitope specific MAb 4D4.4 (C2) with gp31 and not gp25, located this MAb to the N-terminal half of the molecule. This finding lends further support to the suggestion that the cross-competition identified in the epitope map between these two MAbs (see Figure 4.3) as well as the MAbs from the C1 and D2 epitopes, was not simply the result of distant conformational effects but true

overlapping competition representing spatially adjacent sites on the same molecule.

(d) Triton X-114 (TX-114) phase extractions of NS1

Having identified the location of the cleavage fragments in the primary sequence, it was thought that they may be used to provide further insight into the region of NS1 responsible for membrane association. TX-114 phase separation experiments have previously shown that flavivirus NS1 is first expressed as a monomeric hydrophilic species (Winkler *et al*, 1989). This was consistent with a sequence that suggested a predominantly hydrophilic protein with few hydrophobic regions (Figure 4.15).

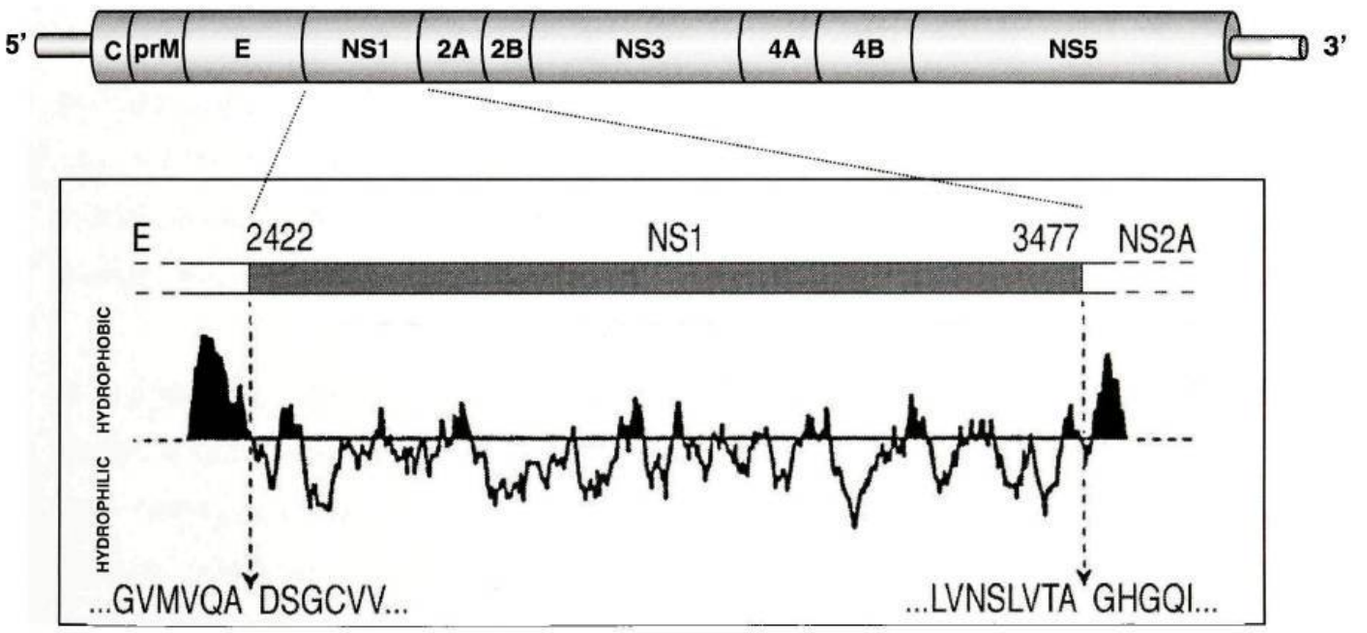


Figure 4.15 Relative hydrophobicity plot of dengue virus NS1. Schematic of the dengue virus genome is shown above. The boxed panel below highlights the region of the genome encoding NS1 (numbers refer to nucleotides from the 5' end of the genome) and a hydrophobicity plot of the translated sequence. The amino acid sequence of the cleavage site between E/NS1 and NS1/NS2A are indicated below.

Oligomerization occurs within 20 minutes in the ER (Winkler *et al*, 1989) with an associated transition to hydrophobic character (Winkler *et al*, 1989; Mason, 1989; Despres *et al*, 1991; Flamand *et al*, 1992; Leblois and Young, 1995). Although the nature of this induced hydrophobicity has been extensively investigated, no clear picture has emerged (Lindenbach and Rice, 2001). Our laboratory has identified cell-surface associated NS1 that is linked to a GPI anchor (Jacobs *et al*, 2000) however these studies showed only a small percentage of membrane - associated NS1 was anchored in this way. It is assumed that for the majority of mNS1, oligomerization induces a conformational change that exposes hydrophobic residues capable of interacting stably with membranes. This possibility is supported by the results presented earlier in this chapter which provide clear evidence for a change in the conformation of NS1 between its secreted and membrane-associated forms in the differential exposure of the A determinant and the disruption of D domain integrity.

In order to investigate the hydrophobic character of NS1, immunoaffinity purified preparations of both sNS1 and mNS1 were examined by TX-114 phase separation (Figure 4.16). Both native oligomeric NS1 (sNS1, lanes 2 and 3; mNS1, lanes 6 and 7) and heat treated monomeric NS1 (sNS1, lanes 4 and 5; mNS1, lanes 8 and 9) were examined.

In agreement with results previously reported (Fan and Mason, 1990; Mason, 1989; Winkler, 1989) the dimeric form of NS1 partitions into both the aqueous and detergent phases (sNS1, lanes 2 and 3; mNS1, lanes 6 and 7) indicating a "partial" hydrophobic character. Monomeric NS1 in contrast, partitions almost exclusively into the aqueous phase confirming the suggestion that hydrophobicity was induced only following oligomerization (sNS1, lanes 4 and 5; mNS1, lanes 8 and 9). The fraction of NS1 for both forms that remained dimeric despite heat treatment, partitioned into both phases, thereby providing an internal control. It was noted that a trace amount of monomeric mNS1 was variably found in the detergent phase in repeated experiments, while monomeric sNS1 was never found in this fraction. As a subset of NS1 has previously been

shown to be GPI-anchored (Jacobs *et al*, 2000) it is possible that this small fraction of mNS1 may represent this post-translationally modified form.

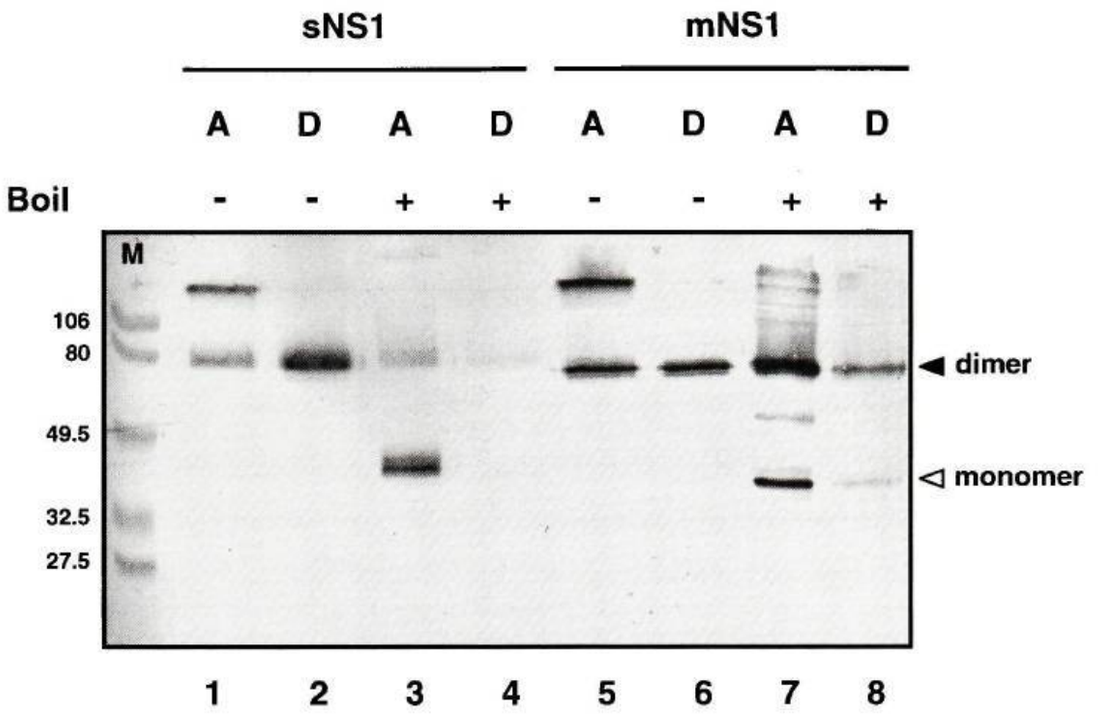


Figure 4.16 Immunoblot analysis of TX-114 detergent phase separation of dimeric and monomeric sNS1 and mNS1. Immunoaffinity purified sNS1 and mNS1 were either boiled or left untreated prior to TX-114 phase separation. Aqueous (A) and detergent (D) phases were then separated by SDS-PAGE, the gel was blotted and then probed with the MAb 3D1.4. Dimer and monomer forms are arrowed. Protein standard markers (M) are as indicated.

To attempt to identify the region of NS1 responsible for imparting this hydrophobic character, the products of both V8 protease and trypsin digestion were subjected to TX-114 phase separation. Immunoblots of SDS-PAGE separated digests were probed with the A epitope MAb, 3D1.4 (Figure 4.17A, V8 digest) and the D3 epitope MAb, 1G5.3 (Figure 4.17B, trypsin digest).

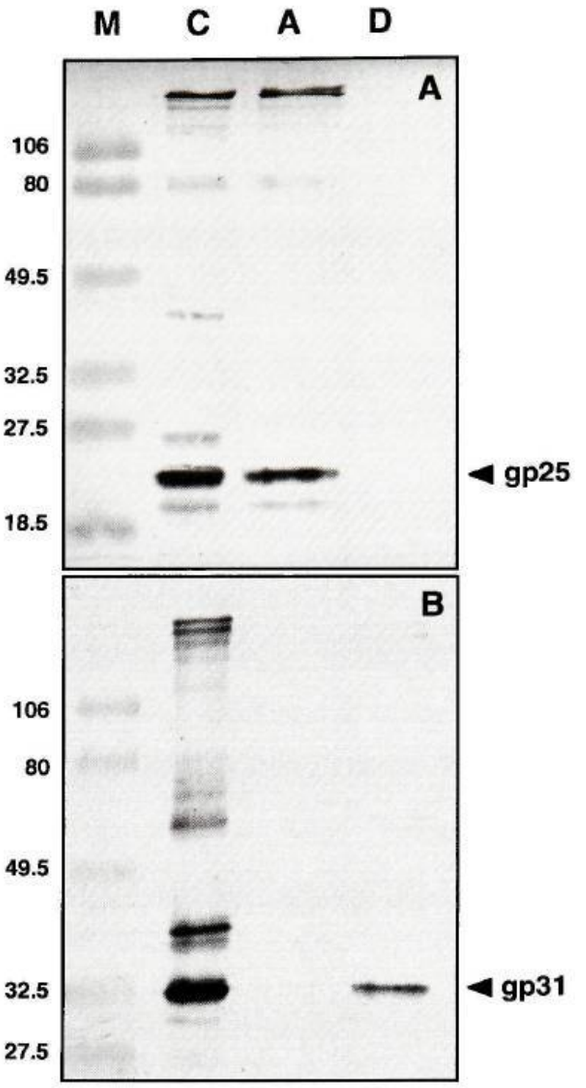


Figure 4.17 **Immunoblot analysis of TX-114 detergent phase separation of A, trypsin digested dimeric mNS1 and B, V8 protease digested monomeric mNS1. Samples were either subjected to TX-114 phase separation resulting in aqueous (labeled A) and detergent (labeled D) phase fractions or left as an untreated control (labeled C). Samples were separated by SDS-PAGE, the gel was blotted and then probed with the MAbs 1G5.3 (A) or 3D1.4 (B). Cleavage fragments are indicated by arrows to the right of the figures. Molecular weights of protein standard markers (M) are as indicated to the left.**

The gp25 trypsin fragment (Figure 4.17A) and the gp31 V8 protease fragment (Fig 4.17B) were clearly seen. The partitioning of gp31 predominantly into the detergent phase for mNS1 (Figure 4.17B) and gp25 into the aqueous phase (Figure 4.17A) indicated that the hydrophobic character acquired by NS1 would appear to reside in the N-terminal half of the molecule (amino acids 1-172).

4.3 DISCUSSION

(i) Antigenic structure and epitope organization of dengue virus NS1

Five antigenic domains and up to ten distinct epitopes on the dengue virus NS1 glycoprotein have been identified in competition assays using a panel of dengue virus cross-reactive, dengue-2 virus specific and flavivirus cross-reactive MAbs. Antigenic maps for three different species of immunoaffinity purified dengue-2 NS1 were generated; secreted and membrane-associated NS1 (sNS1 and mNS1 respectively) derived from dengue virus infected mammalian (Vero) cells and mNS1 from insect (C6/36) cells. A similar antigenic profile was observed for all three species (Figure 4.8) with some important differences being noted. The most significant difference was the lack of recognition by A determinant specific MAbs of Vero cell derived mNS1. This linear epitope, localized to amino acids 112-120 in the primary sequence (Falconar *et al*, 1994) is known to react with flavivirus cross-reactive MAbs, many of which have also been shown to confer passive protection in a mouse model (Young *et al*, 1994). The differential display of this epitope on different NS1 species might explain the finding reported earlier (Young and Falconar, 1990) that greater protection from lethal dengue challenge was observed for mice immunized with sNS1 than for those immunized with mNS1. The second major difference between the antigenic maps is the breakdown in the integrity of competition in the D domain cluster of epitopes for mNS1 derived from either mammalian or insect cells by comparison with the sNS1 D domain (see Figure 4.8).

MAb reactivity with proteolytic digests (Figure 4.14) as well as PEPSCAN analyses (Falconar *et al*, 1994) have shown the D domain epitope cluster to be localized to the C-terminal half of the molecule while the A and C epitopes are located in the N-terminal half (see Figure 4.4). The observation that a number of C domain specific MAbs (C1 and C2) competed with those reactive for the D domain (D1 and D2) suggests that these epitopes may be in close proximity in the native folded structure. The possibility that the observed competition was a result of inter-molecular interactions rather than intra-molecular association was

investigated by comparing competition maps of both dimeric and monomeric forms. Although competition between C2 (4D4.4) and D2 (5H4.4) MAbs was lost in the monomeric form of sNS1, the one-way competition between D1 (5B9.3) and C1 (1H7.4) was retained for both mNS1 and sNS1. It is difficult to interpret the significance of the loss of the C2-D2 competition under these conditions particularly in light of the absence of competition between these two epitopes in dimeric mNS1 (Figure 4.7), and the significantly reduced primary binding of the C2 specific MAb, 4D4.4 following monomerization (Figure 4.6). It is possible that the treatments employed to generate monomers (heating) and mNS1 (detergent solubilization) may be responsible for this altered reactivity and that only native oligomeric sNS1 is providing an accurate picture of MAb competition. The one-way competition seen by 5B9.3 as a secondary MAb may be a consequence of (a) displacement of the bound antibody by a more avid species recognizing the same epitope, (b) positional effects where antibody binding blocks reactivity to an adjacent site or (c) conformational changes induced by binding to a positionally distant epitope that affects binding sites elsewhere in the polypeptide (Henchal *et al*, 1987). The first explanation can be discounted given that these MAbs recognize different, sequence defined binding sites. However the latter two possibilities cannot be excluded for this MAb. Nevertheless, the two-way cross-competition between epitopes C2 and both D1 and D2 for dimeric sNS1, suggests that competition may indeed be a direct result of recognition of spatially adjacent sites. This finding has been incorporated into the structural model presented at the end of this Chapter (Figure 4.20). Final resolution of the spatial arrangement of these epitopes will only be revealed by the three-dimensional structural determination of NS1.

Two previously defined characteristics of the panel of MAbs, flavivirus cross-reactivity and the ability to confer passive protection were placed in the context of the epitope map (Table 4.1 and Figure 4.4C) (Falconar and Young, 1991; Young and Falconar, 1990). Not surprisingly, these MAb phenotypes clustered reasonably well with epitope groupings, suggesting that they were associated with defined, topologically distinct domains. Five flavivirus cross-reactivity patterns previously identified (Falconar and Young, 1991) (Table 4.1) clustered

into separate domains that comprise either single or groups of epitopes. The ability to confer passive protection in a mouse model also clustered into particular domains with MAbs from epitope groups A, D3, D4, D5 and E, providing protection from a lethal virus challenge when passively administrated to mice (Figure 4.4C and Young and Falconar, 1990). It is interesting to note that MAbs belonging to the large overlapping cluster of epitopes, C1, C2, D1 and D2 do not provide passive protection in this model. Given that protection by NS1-specific antibody is thought to be primarily mediated by antibody dependent cell cytotoxicity (ADCC) and/or complement mediated lysis of infected cells (Henchal *et al*, 1988) accessibility of antibody to NS1 on the infected cell surface would be a pre-requisite for providing the protective effect. Although the MAbs comprising this epitope cluster were able to bind to cell-surface NS1 in immunoflorescence studies (data not shown) it is still possible that binding orientation is not optimal for effective ADCC or complement mediated lysis.

One of the most intriguing observations in this study was the considerable variation in the reactivity of A determinant specific MAbs to the multiple forms of NS1 analysed. Under the same conditions, reactivity of the remaining panel of MAbs remained fairly consistent. These variable reactivity patterns included the loss of binding to mNS1 purified from Vero cells (Figure 4.7) but not for insect cell derived mNS1 (Figure 4.8B). A epitope specific MAbs also failed to react with NS1 that had been monomerized by heat treatment (Figure 4.6). This differential exposure of the A epitope in native and monomer forms was further supported by their different susceptibility to trypsin digestion (Figure 4.12A). The presence of two lysines and one arginine residue within the linear sequence previously defined as being recognized by these MAbs (Falconar *et al*, 1994) ensured cleavage within the epitope and therefore loss of MAb reactivity only if the epitope was exposed. In addition, primary binding of several D domain specific MAbs to sNS1 was shown to significantly enhance subsequent binding by A epitope reactive MAbs (Figure 4.3). This synergistic binding indicates a conformational dependency between the two halves of the molecule and provides further support for the spatial juxtaposition of N- and C-terminal regions of NS1 suggested by the overlapping C and D epitopes (Figure 4.4B). In

summary, the variability in reactivity of A determinant specific MAbs suggests a degree of flexibility in the structure of the domain encompassing the A epitope, with altered orientation in each form of NS1 determining how efficiently this epitope is presented for MAb binding.

Digestion of proteolytic fragments with endoglycosidases H and F formally confirmed earlier findings (Young *et al*, 1993; Pryor and Wright, 1994) that in the secreted form of NS1, it is only the carbohydrate linkage site at ^{130}Asn that undergoes trimming and complex processing in the Golgi. Presumably the presence of only high-mannose residues at ^{207}Asn reflects the sequestration of this site in the oligomeric form of the protein and hence protection from subsequent modification during passage through the Golgi. Given that ^{130}Asn is only ten amino acids C-terminal to the A epitope (^{112}R-K^{120}; Falconar *et al*, 1994) the possibility was investigated that the altered binding profiles of the A epitope specific MAbs for mNS1 and sNS1 were the result of differences in the glycosylation status of this site. However, endoglycosidase digestion of both mNS1 and sNS1, failed to modify MAb binding thereby demonstrating that the differential reactivity was not a simple consequence of steric hindrance by the different carbohydrate moieties (Figure 4.10). Nevertheless, it is still possible that trimming and processing of carbohydrate residues at ^{130}Asn may induce irreversible conformational changes that affect exposure of the A epitope. Binding profiles of NS1 produced in cells treated with tunicamycin or from recombinant constructs with this carbohydrate site removed would need to be performed to conclusively address this possibility.

The N-glycosylation site ^{207}Asn is conserved in all mosquito-borne flaviviruses sequenced to date. The functional and structural significance of this finding remains to be fully elucidated, however the conservation of this site does suggest that glycosylation plays an important role in NS1 function. Previous studies have provided data that supports this hypothesis (Pryor and Wright, 1994; Pletnev *et al*, 1993). It has been shown that the stability of the native oligomeric form of NS1 is largely dependent on the carbohydrate moiety attached to this site (Pryor and Wright, 1994) and that recombinant viruses

lacking this carbohydrate are significantly attenuated (Pletnev *et al*, 1993; Pryor *et al*, 1998). As noted above, removal of the carbohydrate residue at ^{207}Asn substantially destabilizes the oligomeric integrity of NS1 while removal of the carbohydrate at ^{130}Asn does not (Pryor and Wright, 1994). However, if both carbohydrate residues are removed (recombinant G3 in Pryor and Wright, 1994) dimer integrity is significantly recovered suggesting that while the carbohydrate at ^{207}Asn does indeed stabilize the oligomeric form, the carbohydrate at ^{130}Asn may also play a role by providing a countering destabilizing effect.

Apart from the glycosylation profile, the other defining characteristic that differentiates mNS1 from sNS1 is membrane-association. A simple explanation for the absence of the A epitope on mNS1 may be that this epitope is buried in a region that is involved in membrane-association, whereas in the secreted form it is exposed. The finding that it is the N-terminal half of NS1 that harbours hydrophobic properties (Figure 4.17) is consistent with this hypothesis. Indeed, the association of the A epitope with a region of hydrophobic character is supported by a number of other observations made in this study. Oligomeric sNS1 is partially hydrophobic as determined by TX-114 phase separation experiments and is recognized by A determinant specific MAbs (Figure 4.16). Conversion to a monomeric species by heat treatment renders sNS1 hydrophilic, implying that hydrophobic domains are buried in this form of the protein. This conversion is paralleled by a loss of reactivity by the A epitope MAbs (Figure 4.6). It is interesting to note that although monomeric full-length NS1 is hydrophilic, the monomeric N-terminal gp31 cleavage fragment does exhibit hydrophobic properties (Figure 4.17). Presumably the loss of the C-terminal fragment no longer allows this N-terminal hydrophobic domain to be buried. The association between C-terminal and N-terminal domains has been discussed previously in the context of the epitope map and this hydrophobic interaction may be reflecting the same association. The possibility that the C-D epitope interaction is hydrophobic in nature may also explain the dramatic loss in integrity of the D domain competition map for detergent solubilized mNS1 (Figure 4.7).

Numerous studies have already been reported that have sought to define the antigenicity of flavivirus NS1 at the epitope level. These have utilized synthetic peptides, recombinant deletion constructs, phage display and MAb epitope mapping (Henchal *et al*, 1987; Putnak *et al*, 1988, Mason *et al*, 1990; Yao *et al*, 1995; Garcia *et al*, 1997; Huang *et al*, 1999; Wu *et al*, 2001; Bugrysheva *et al*, 2001). The current study is the most comprehensive to date having identified the binding profile of 22 individual MAbs and generated a competition map that places the binding of these MAbs in the context of the primary amino acid sequence.

The competition map presented by Henchal *et al* (1987) and derived from the reactivity of nine different MAbs with dengue-2 virus NS1 has some striking similarities with the competition map described in this Chapter. Although eight of the competition MAbs used by Henchal *et al* (1987) were dengue-2 virus specific, two main overlapping epitope clusters were identified that are similar to the C and D domains recognized here. One of their MAbs, 47-10/10 is involved in one-way competition similar to our 5B9.3 and it is this MAb that unites the two major domains as overlapping clusters. The similarity between these two MAbs is strengthened by the fact that 47-10/10 was also shown to recognize a spontaneously generated 22 kDa fragment, suggesting that this MAb, like 5B9.3, recognizes an epitope in the C-terminal half of the molecule. This was also the only MAb that showed any cross-reactivity, in this case with JEV. It is significant to note that MAbs defining the epitopes D1, D2 and D3 in the current study also recognized JEV in addition to dengue-2, dengue-4 and other members of the encephalitic flaviviruses.

Putnak *et al* (1988) cloned and expressed deletion constructs of NS1 from the New Guinea C strain of dengue-2 virus in *E. coli* to study the antigenic domains of the protein. Both the N- and C-terminal regions of NS1 were found to be antigenic when reacted with dengue-2 mouse polyclonal sera and dengue-2 MAbs respectively. This correlates with our C and D domains. Conversely, a rabbit polyclonal antiserum against purified NS1 reacted more strongly with carboxy-terminal than amino-terminal derived proteins. This was interpreted as

being due to differences in antigen presentation or it could be explained by differences in immunodominance in different species (Putnak *et al,* 1988).

In contrast to the studies of Putnak *et al* (1988), subsequent findings by Mason *et al* (1990) with *E. coli* expressed dengue-1 virus NS1, found that monoclonal antibodies reacted with determinants clustered at the amino-terminus (amino acids 57 - 126). The reason for this discrepancy most likely lies in the methods used to generate and select monoclonal antibodies (Mason *et al,* 1990). The determinants described by Mason *et al* (1990) between amino acids 57 - 126 correlate with our A determinant which specifically recognizes the region between amino acids 111 - 121 (See Table 2.1 (Falconar *et al*, 1994)). Due to the small number of anti-NS1 MAbs used by Mason *et al* (five in total, only one of which cross reacted with dengue-2 NS1) it is likely that they too, are seeing a subset of the large range of epitopes recognized in the current study.

The identification of epitope clusters along the full-length of the protein has also provided an opportunity to assess likely correlates in the characteristics of MAbs and epitopes previously reported by others. The current work primarily builds on the identification by PEPSCAN analysis (Falconar *et al*, 1994) of the binding sites of a limited set of MAbs that were originally recognized as reacting with linear determinants (Falconar and Young, 1991). Of the five epitopes reported in that original study, four were found to map topologically to their expected sites as suggested by the specific peptide sequence each had been show to recognize. However one epitope, recognized by the MAb 5H4.3, did not fall into the expected epitope grouping following competition analysis nor did it recognize the relevant peptide fragment that would correlate with the originally defined epitope (referred to as 24A, comprising amino acids ^{61}T-K^{69}) (Falconar *et al*, 1994). The competition map placed this MAb into the D3 epitope group with reactivity to the p10 cleavage fragment thereby identifying its binding site to be within the amino acids ^{275}M-E^{334}. It can be inferred that the peptide originally identified in the PEPSCAN analysis was operating as a mimotope of the true, conformationally dependent binding site. This finding also helps to explain why in the earlier studies this MAb failed to show the sub-complex reaction profile with peptides

that was seen with native NS1 when synthetic peptides derived from the amino acids 61-69 of all four serotypes were examined (Falconar *et al*, 1994).

More recently, the focus of epitope investigations has been on the use of synthetic peptides and the application of phage display. Garcia *et al* (1997) generated a series of synthetic peptides based on the hydrophilic profile of NS1. The five peptides generated were derived from regions of predicted hydrophilicity and reacted with sera taken from dengue-4 virus infected patients. Four of the five reacted preferentially with infected patient sera when compared to controls. Interestingly, the one peptide that failed to react, corresponded exactly to the sequence recognized in dengue-2 virus by our MAb 5B9.3. Whether the lack of reactivity to this region by human sera is specific only to dengue 4 virus will require further study but it is relevant to note that 5B9.3 is known to cross-react with dengue-4 virus NS1. One of the reactive peptides consisted of a seventeen amino acid region derived from the C-terminus of NS1. This region corresponds to the site recognized by MAbs defining the D4 and D5 epitopes, indicating that human and murine immune responses target this region. Wu *et al* (2001) reports the identification of a B-cell epitope on dengue-1 virus NS1 through the use of phage display and selection with MAb 15F3-1. Interestingly, the epitope region identified corresponds precisely with our A epitope although the authors do not refer to the prior identification of this epitope in an earlier paper from our laboratory (Falconar *et al*, 1994). The majority of sera taken from dengue-1 virus infected patients responded to a peptide corresponding to this region, thereby identifying the A determinant as a B cell epitope recognized in the human response to dengue virus infection.

(ii) Structural Model of Dengue-2 NS1

Figure 4.18 is a schematic representation of the primary structure of the NS1 protein of dengue-2 virus summarizing the locations of antigenic determinants, known disulfide bridges, biophysical features and structural characteristics of this protein that have been determined in the current study and reported previously.

There are twelve cysteine residues in the NS1 protein of flaviviruses and these are totally conserved amongst all members sequenced to date. These residues form intra-chain disulfide bonds and the level of conservation suggests a common structure throughout the flavivirus family. The positions of these residues are indicated in Figure 4.18 with the disulfide bonds indicated for those that have been biochemically identified to date for MVE NS1 (Blitvich *et al*, 2001). Earlier work by Pryor and Wright (1993) had employed site-directed mutagenesis of cysteine pairs to identify the location of disulfide bridges. They reasoned that the mutation of naturally paired cysteines would have a less disruptive effect on dimer formation than pairs of cysteines not normally involved in forming disulfide bridges. All pairwise combinations were assessed with ^{316}Cys11, and it was found that only with the associated mutation of ^{313}Cys10, were dimers formed. It is possible therefore that ^{313}Cys10 and ^{316}Cys11 also form a disulfide bridge although biochemical or structural data is required to confirm this hypothesis.

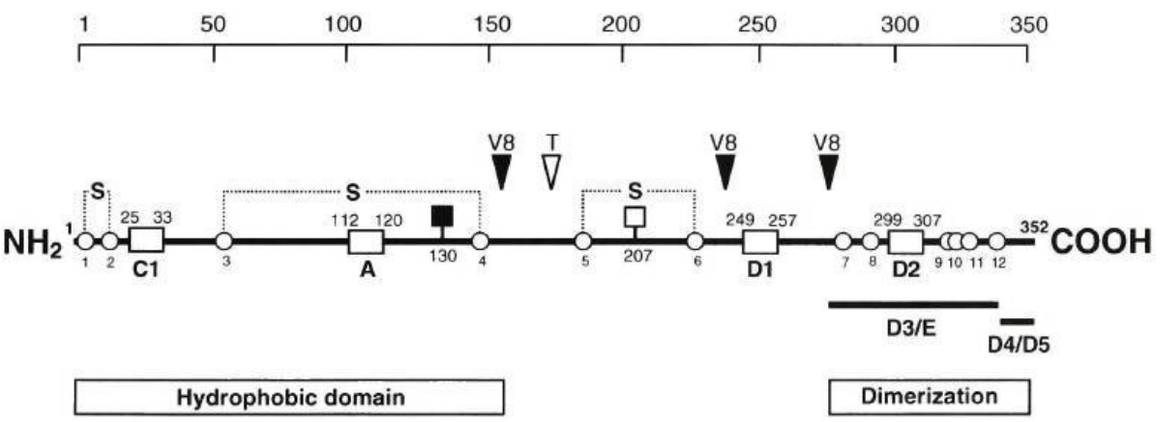


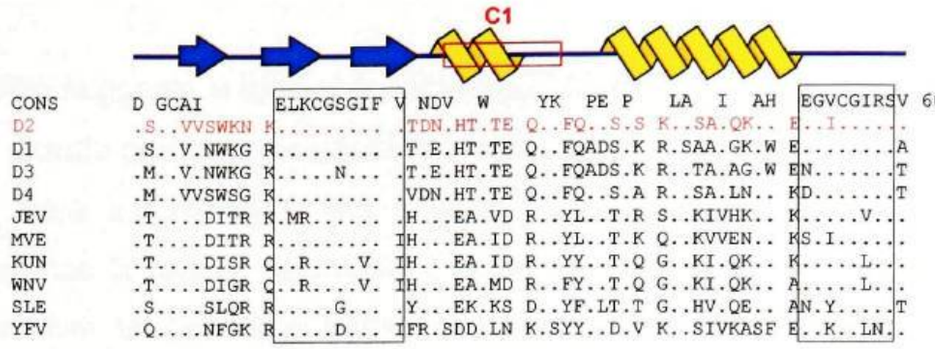
Figure 4.18 **Schematic of the primary structure of dengue 2 virus NS1. Relative positions of cysteine residues are indicated with open circles, and the known disulphide bridges are shown. Linear sequence epitopes are indicated by the open boxes with the binding domains of conformation specific epitopes identified by solid bars. Glycosylation sites are marked with square paddle-pops (high mannose, open box; complex, filled box) and the sites of V8 protease and trypsin cleavage are indicated with arrow heads. The regions identified to have hydrophobic properties and to be involved in dimerization are also shown.**

The partial proteolytic digestions performed in the current study are consistent with the disulfide bridges identified by Blitvich *et al* (2001). V8 protease and trypsin digestion between residues ^{143}Cys4 and ^{179}Cys5 and between ^{223}Cys6 and ^{280}Cys7 without the formation of disulfide-linked species indicate that

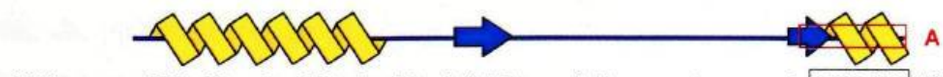
cysteines ^{4}Cys1 to ^{143}Cys4 are involved in disulfide bridges, as are cysteines ^{179}Cys5 and ^{223}Cys6. Although the disulfide bridges formed by the remaining six cysteines await confirmation, a number of possibilities are suggested by existing data. As discussed above, cysteine residues ^{313}Cys10 and ^{316}Cys11 are likely to be linked by disulfide bonds (Pryor and Wright, 1993) while disulfide bridges between ^{280}Cys7 and ^{291}Cys8, ^{312}Cys9 and ^{313}Cys10, and ^{316}Cys11 and ^{329}Cys12 have been excluded by Blitvich *et al* (2001) based on peptide fragments identified by HPLC. These findings leave only the paired residues between ^{280}Cys7, ^{291}Cys8, ^{312}Cys9 and ^{329}Cys12 to be defined. Figure 4.18 also shows the binding sites for the majority of epitopes examined in this study. D domain epitopes are seen to cluster in the C-terminal third of NS1 with the binding location of the conformational epitopes D3, D4, D5 and E defined by their interaction with peptide fragments (summarized in Figure 4.14). The dimerization domain has been localized to the C-terminus of NS1 through a number of independent studies. These include the expression of truncated recombinants (Parrish *et al*, 1991) and the disruption of dimerization following site-directed mutagenesis of cysteine residues and selected hydrophobic residues within this region (Pryor and Wright, 1993). The identification in the current study of a dimeric trypsin cleavage fragment representing the C-terminal half of the molecule is consistent with these observations. Finally, TX-114 phase separation experiments performed on selected protease cleavage fragments have localized the hydrophobic character of NS1 to a site within the N-terminal half of the molecule.

In order to provide further insights into the structural organization of the NS1 protein, a secondary structure model was built for data generated by the structure prediction algorithm PHD (Rost and Sander, 1994a; 1994b; Rost, 1996). This web based program uses multiple aligned protein sequences as input to estimate the probability of secondary-structure motifs and solvent accessibility. Figure 4.19 shows a consensus of the final secondary structure predictions generated, with helices (graphic spirals), β sheets (open arrows), and coils (straight lines) indicated above a subset of representative aligned sequences (of a total of 27) obtained from the protein database and used in the

analysis. The locations of the defined linear epitopes A, C1, D1 and D2 are shown, as are areas of high amino acid sequence conservation amongst the flaviviruses (open boxes). A particularly high level of sequence homology can be seen in the C-terminal fifty amino acids, perhaps reflecting the location in this region of the functionally important dimerization domain (Parrish *et al*, 1991; Pryor and Wright, 1993).

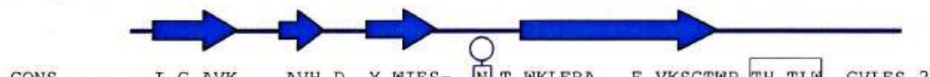



```
CONS      D GCAI      ELKCGSGIF V  NDV  W    YK  PE P   LA  I  AH   EGVCGIRSV 60
D2        .S..VVSWKN K.........  .TDN.HT.TE Q..FQ..S.S K..SA.QK.. E..I.....
D1        .S..V.NWKG R.........  .T.E.HT.TE Q..FQADS.K R.SAA.GK.W E........A
D3        .M..V.NWKG K.....N...  .T.E.HT.TE Q..FQADS.K R..TA.AG.W EN.......T
D4        .M..VVSWSG K.........  .VDN.HT.TE Q..FQ..S.A R..SA.LN.. KD.......T
JEV       .T....DITR K.MR......  .H...EA.VD R..YL..T.R S..KIVHK.. K.....V...
MVE       .T....DITR R.........  IH...EA.ID R..YL..T.K Q..KVVEN.. KS.I.....
KUN       .T....DISR Q..R....V.  IH...EA.ID R..YY..T.Q G..KI.QK.. K.....L...
WNV       .T....DIGR Q..R....V.  IH...EA.MD R..FY..T.Q G..KI.QK.. A.....L...
SLE       .S....SLQR R.....G...  .Y...EK.KS D..YF.LT.T G..HV.QE.. AN.Y.....T
YFV       .Q....NFGK R.....D...  IFR.SDD.LN K.SYY..D.V K..SIVKASF E..K..LN..
```

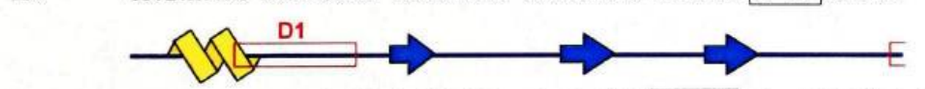



```
CONS       RLEH MW   I  ELN  L  EN  DLSVVV     G Y      L        L YGWK WGK 120
D2        T...NL..KQ .TS...HI.S ..EVK.TIMT GDIK.IMQVG KRS.RPQPTE .H.S..T...
D1        T...NI..KQ .SN...HI.L ..DMKFT... GDVS.ILAQG KKMIRPQPME HK.S..S...
D3        T.M.NLL.KQ .AN...YI.W ..NIK.T... GDIT.VLEQG KRT.TPQPME .K.S..T...
D4        T...NV..KQ .TN...YV.W .GGH..T..A GDVK.VLTKG KRA.TPPVSD .K.S..T...
JEV       T....Q..EA VRD...VL.K ..AV...... NKPV.R.RSA PKR.SMTQEK FEM...A...
MVE       N.F..Q..ES VRD...AL.K ..AI...... EKQK.M.RAA PNR.RLTVEE .DI...A...
KUN       S....Q..EA VKD...TL.K ..GV...I.. EKQE.M.KSA PRR.TATTEK .EI...A...
WNV       S....Q..EA .KD...TL.K ..GV...... EKQN.M.KAA PKR.AATTEK .EM...A...
SLE       S....L..EN .QR...AIFE D.EI...... QEDPKY.KRA PRR.KKLEDE .D....K...
YFV       DS...E..RS RAD.I.AIFE ..EV.I.... QDPKNV.QRG THPFSRIRDG .Q....T...
```

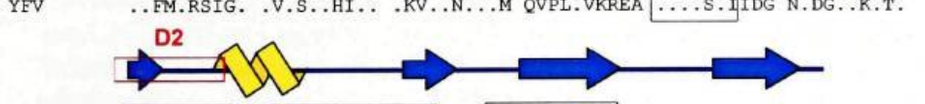
```
CONS         F PEL N  TF IDGPET  ECR   RAW NS EVEDFGF G   T  WL   RE  T  CD 180
D2        AKMLST..H. Q..L......  A...NTN... ..L...Y.. .VFT.NI..R L..KQDAF..
D1        AKIIGADVQ. T..I....N.  P...DNQ... .IW...Y.. .IFT.NI..K L.DSY.QV..
D3        AKIVTA.TQ. SS.I....N.  P...SAS... .VW...Y.. .VFT.NI..K L..MY.QL..
D4        AKI.T..AR. S..L....D.  S...NER... ..L...Y.. .MFT.NI.MK F..GSSEV..
JEV       SIL.A...A. S..VV.....  K...DEH... ..I.I..... .ITS.RV..K I..ES.DE..
MVE       SLL.AA..A. S..VV.....  A...NSK... ..F.I..... .ITS.RV..K L..EN.SE..
KUN       SIL.A...A. N..V......  K...TQN... .NL....... .LTS.RMF.R V..SN.TE..
WNV       SII.A...A. N..V......  E...TAN... ..M....... .LTS.RMF.R I..TN.TE..
SLE       TLFVE.R.G. N..VV.....  K...TAN... ..FK...... .MVF.QL..T I..EN.TE..
YFV       NLV.S.GRK. GS.I...KSR  K...FSN.V. ..FQI.E..T .VFT.RVYMD AVFEY.ID..
```



```
CONS         I G AVK    AVH D  Y WIES- N T WKLERA   E VKSCTWP TH TLW  GVLES 239
D2        SKLMSA.I.D NR...A.MG. .....AL.D. ..I.K.SFI. ....H..KS. ...SN.....
D1        HRLMSA.I.D SK...A.MG. .....EK.E. ...A..SFI. ..T.I..KS. ...SN.....
D3        HRLMSA...D ER...A.MG. .....QK.GS ....K.SLI. ..T....KS. ...SN.....
D4        HRLMSA.I.D QK...A.MG. .....SK.Q. .QI.K.SLI. ..T.L..K.. ...SN.....
JEV       GA.I.T...G HV...S.LS. .....RY.D. ......VFG. .......E.. ...GD..E..
MVE       ST.I.T...G NH...S.LS. .....GL.G. ......IFG. .......E.. ...GDA.E.T
KUN       SK.I.T...N NL.I.S.LS. .....RF.D. ......VLG. .......E.. ...GD.....
WNV       SK.I.T...N NM...S.LS. .....GL.D. ......VLG. .......E.. ...GD.....
SLE       SA.I.T.I.G DR...S.LS. .....KK.E. .Q....VMG. .......E.. ...GD..V..
YFV       GS.L.A..NG KKSA.GSPTF .MG.HEV.G. .MIHTLEALD Y.E.E..L.. .I-GTS.E..
```




```
CONS       EMIIP   AG P S HN RPG Y TQT GPW   G  E DFDY CPGTTVT  E  CG RGPS R 299
D2        ..V..KNF.. .V.Q..N... .H...A...H L.KL.M...F .E....VVT. D..N....L.
D1        .....KIYG. .I.Q..Y... .F...A...H L.KL.L...L .E....VVD. H..N....L.
D3        D....KSL.. .I.Q..Y... .H...A...H L.KL.L..N. .E....VIT. N..T....L.
D4        Q.L..KSY.. .F.Q..Y.Q. .A...V...H L.KL.I..GE .......IQ. D.DH....L.
JEV       .L...HTI.. .K.K..R.E. .K..NQ...D ENGIVL.... ....K..IT. D..K....V.
MVE       .L...VTL.. .R.K..R.E. .KV.VQ...D EEDIKL.... .......VS. H..K....V.
KUN       DL...ITL.. .R.N..R... .K..SQ...D E.RV.I.... .......LS. S..H...AT.
WNV       DL...ITL.. .R.N..R... .K..NQ...D E.RV.I.... .......ISD S.EH...AA.
SLE       .....VTLG. .K.H..K.N. .H...K...S E.EITL.... .......VT. H..N..A.L.
YFV       ..FM.RSIG. .V.S..HI.. .KV..N...M QVPL.VKREA ....S.IIDG N.DG..K.T.
```




```
CONS       TTT SGKLIT  WCCRSCTLP PLRE  EDGC WYGMEIRP     E  LV S  V A 352
D2        ...A.....  E.........  ...VRG.... ........LK EK.EN..S.L .T.
D1        ...VT..T.H E.........  ....KG.... ........VK EK.EN..K.M .S.
D3        ...V.....H E.........  ....YMG.... ........IN EK.ENM.K.L .S.
D4        ...A....V. QM......M.  ....LG.... ........LS EK.ENM.K.Q .T.
JEV       ...D.....  D.......S..  ....RT.N.. ........VR HD.TT..R.Q .D.
MVE       ...D....V. D.........  ....TTAS.. .....T..MK HD.ST..K.R .Q.
KUN       ...E.....  D.........  ....YQTDN. ........QR HD.KT..Q.Q .N.
WNV       ...E.....  D.........  ....QT.N.. ........TR HD.KT..Q.R .N.
SLE       ...A....V. D.......S..  ....YTTK.. ........VK EE.AK..K.R .T.
YFV       S..D...V.P E.......M.  .VS.HGS... ..P.....RK TH.SH..R.W .T.
```

The prediction suggests a high level of helical content in the N-terminal third of NS1 with β sheets and coils comprising the remainder. The secondary structure predictions were incorporated into a tertiary structure model along with the known disulphide linkages, suspected overlapping epitopes and cleavage sites that were shown to generate stable sub-domains (Figure 4.20). The model suggests that disulfide linkages may be found between ^{280}Cys7-^{329}Cys12 and ^{291}Cys8-^{312}Cys9 but linkages between ^{280}Cys7-^{312}Cys9 and ^{291}Cys8-^{329}Cys12 are equally possible.

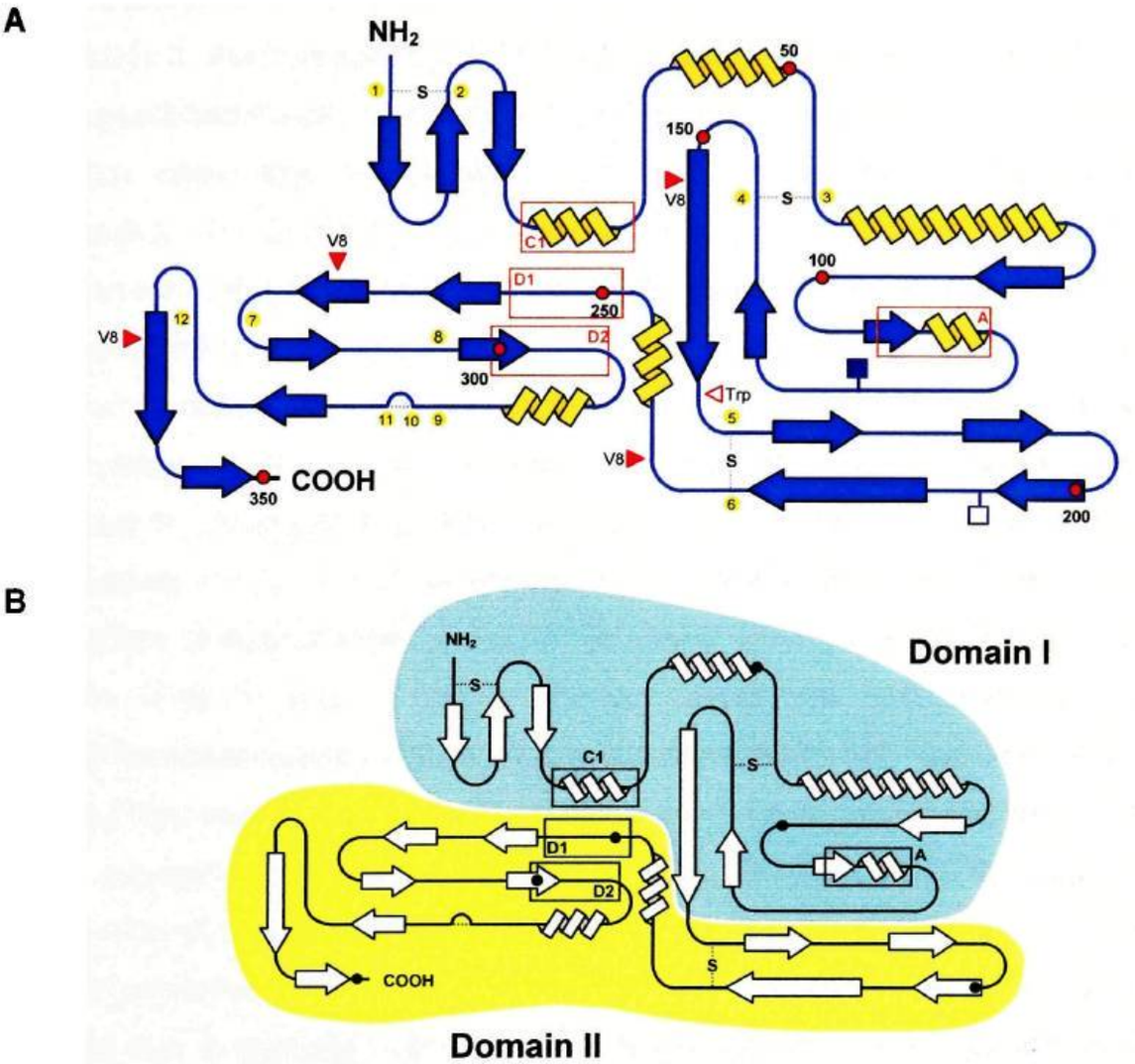


Figure 4.20 **Tertiary structure model of dengue virus NS1. A, Positions of cysteines and disulphide bridges are shown, as are linear sequence epitopes (open boxes). Epitopes C1, D1 and D2 are shown in close association based on competition mapping. Predicted secondary structure elements are schematically drawn as spirals (α-helices), arrows (β-sheets) and lines (coils) and glycosylation sites are indicated by paddle-pops. Sites of proteolytic digestion are shown with arrowheads (V8 protease, filled; trypsin, open). B, Two domains of NS1, based on the stability of cleavage fragments, are highlighted.**

The cleavage of mature oligomeric mNS1 and sNS1 by trypsin at ^{172}R to yield a stable C-terminal oligomeric fragment that is resistant to further digestion and maintains its dimeric configuration, suggests that NS1 may comprise two distinct domains. These have been highlighted in Figure 4.20 and termed domains I and II. The presence of the carbohydrate moiety at ^{207}Asn within this fragment is consistent with its protection in the dimeric form from further trimming and processing despite passage through the Golgi.

The N-terminal third of the molecule was examined for regions that may contribute to the hydrophobicity of this part of the molecule (Figure 4.17). Three of the predicted helices in this region were found to be amphipathic in nature and in each case, the hydrophobic residues involved were highly conserved, suggesting an important functional role. One of the predicted helices forms a component of the A epitope. Given the failure of A determinant specific MAbs to recognize the membrane-associated form of NS1, it is tempting to speculate that membrane-association may be the consequence of the differential exposure of this epitope. It is also known that the hexameric form of secreted NS1 is sensitive to detergent treatment (Flamand *et al*, 1999; own observations). In considering these two observations it is possible that dimerization and the acquisition of hydrophobic character for newly synthesised NS1 commits each dimeric form to one of two pathways; either the hydrophobicity induces membrane-association or alternatively, an association with two other dimers via hydrophobic interactions. This implies that membrane-associated NS1, in its role as a component of the replication complex, is dimeric in its mature form. Maturation of the secreted form however involves the generation of hexameric NS1. Furthermore, the association of mNS1 with membranes via a hydrophobic domain that is spatially near the glycosylation site at ^{130}Asn may help to explain the lack of processing of this moiety despite traffic through the Golgi. This hypothesis is currently under investigation in the Young laboratory.

CHAPTER 5

TOWARDS THE CRYSTALLIZATION OF NS1: BACULOVIRUS EXPRESSION OF DENGUE-2 VIRUS NS1

5.1 INTRODUCTION

Although the function of NS1 is yet to be fully defined, several recent publications provide evidence that NS1 is involved in RNA replication (Chu and Westaway, 1992; Mackenzie *et al*, 1996; Muylaert *et al*, 1996; 1997; Lindenbach and Rice; 1997; 1999). Earlier reports however, suggested that NS1 played a role primarily in virus assembly and maturation due to its presence on the cell surface and its association with the E protein through the secretory transport pathway (Rice *et al*, 1986b; Mason 1989).

Since 1992 it has been known that NS1 cosediments in sucrose gradients with heavy membrane fractions extracted from infected cells which have an associated RNA-dependent RNA polymerase activity (Chu and Westaway, 1992). However, the first report that suggested a direct role for NS1 in RNA replication was shown by immunofluoresence and cryo-immuno electron microscopy (Mackenzie *et al*, 1996). This study demonstrated the co-localization of NS1 and viral double-stranded RNA replicative intermediates with virus modified cellular vesicles. Genetic studies have provided further evidence implicating NS1 in viral RNA replication (Muylaert *et al*, 1996; 1997; Lindenbach and Rice, 1997; 1999). Although the precise role of NS1 in RNA replication remains to be identified it has been postulated that NS1 is structurally involved in the assembly of the components of the viral replicase (Mackenzie *et al*, 1996; Lindenbach and Rice, 1997).

The elucidation of the correct three dimensional structure of dengue virus NS1

should provide further insights into the function of this glycosylated non-structural protein. As has been outlined in Chapter 4 of this thesis, and in the absence of a functional assay, a detailed antigenic structure of the dengue-2 virus NS1 was obtained which will serve as a basis for interpretation of tertiary structural information. The aim of this section of the thesis was to express and purify sufficient NS1 for preliminary crystallization studies.

NS1 from a range of sources is potentially suitable for crystallography. It is imperative that protein destined for crystallography is of high concentration and purity. Mammalian cells (for example, Vero cells), produce both a secreted (sNS1) and membrane-associated form of NS1 (mNS1). The secreted form contains both a high-mannose and complex type glycan suggesting transport through the Golgi apparatus (Lee *et al*, 1989), whereas the membrane-associated form contains only high-mannose linkages on its two N-linked glycosylation sites (Lee *et al*, 1989). In agreement with findings by Mason (1989) we have found that insect cells (eg. C6/36 mosquito cells) produce only a relatively small amount of the secreted form of NS1 however a membrane-associated form is produced in higher concentration than that seen in Vero cells. Of the various forms of NS1 available, the membrane-associated form derived from C6/36 cells is produced in the highest amounts. The use of the membrane-associated form could be problematic however in terms of extraction and purification of the protein from the infected cell. Furthermore the recent publication from our laboratory outlining evidence for the anchoring by glycosylphosphatidyl inositol (GPI) of a portion of NS1 to cellular membranes (Jacobs *et al*, 2000) also suggests that membrane associated NS1 would likely be comprised of a heterogeneous population of protein species.

GPI anchored proteins are derived from nascent forms that contain two signal peptides, both of which are removed during processing in the ER. Removal of an amino terminal signal peptide occurs through the action of a signal peptidase (Blobel and Dobbestein, 1975; Lingappa *et al*, 1977) and the carboxy-terminal signal peptide targets the protein to a putative GPI transamidase in the ER. Covalent attachment of the GPI moiety to an internal residue (the ω site) occurs

with the concomitant release of the carboxy terminal signal peptide. This mature (GPI linked) form of the protein is subsequently transported through the secretory pathway to the plasma membrane where it is anchored solely through the fatty acids that are components of the GPI anchor. The precise role of GPI anchoring has yet to be determined although it has been implicated in signal transduction (Robinson *et al*, 1989; Su *et al*, 1991; Robinson, 1997) and confers on proteins the ability to transfer between the plasma membranes of different cells (Ilangumaran *et al*, 1996). It has been shown that GPI-anchored proteins that are transferred remain functional (Medof *et al*, 1985) and retain the capacity for signal transduction (van den Berg, 1995).

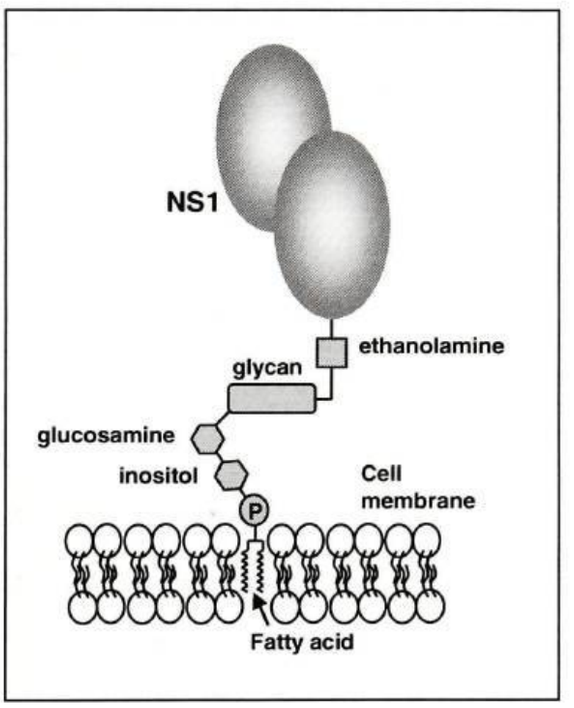


Figure 5.1 **A schematic representation of the components of the glycosylphosphatidyl inositol (GPI) anchor showing oligomeric NS1 attached to a cellular membrane.**

It is envisaged that non-GPI anchored NS1 would be a more appropriate protein for crystallography because of the lack of this complex and highly glycosylated species (See Figure 5.1). In natural dengue infections most of the NS1 protein is retained within the infected cell, either in the ER or at the cell surface, with a proportion being secreted (Winkler *et al*, 1988; Mason, 1989; Post *et al*, 1991). Previous work by Post *et al* (1991) reported that 35 - 45% of YF NS1 was secreted from infected

mammalian cells. Recombinant baculovirus expression of JE virus NS1 reported recombinant protein levels corresponding to 6% of the total cell protein content (Flamand *et al*, 1995) with the majority of NS1 being retained intracellularly.

The aim of the work described in this chapter was to express non - GPI anchored dengue-2 virus NS1 in the baculovirus expression system by using a range of constructs introducing mutations at or near the ω site. Once expressed, recombinant protein would be purified and concentrated for use in crystallography trials.

As with the E protein (Heinz *et al*, 1991), all twelve cysteines are conserved in the NS1 protein of the flaviviruses. Thus a common structural organization of NS1 can be assumed. A crystal structure of NS1 should provide insights into the functional roles of this protein as well as providing an additional target for drug design.

5.2 RESULTS

(a) Design and generation of recombinant baculoviruses

All mutants were based on a construct encoding an N - terminal signal sequence of 28 amino acids derived from the carboxy terminus of E, which is sufficient for the translocation of the NS1 protein into the ER (Falgout *et al*, 1989). This coding sequence was preceded by a consensus Kozak motif (ACC) and a methionine residue to provide an efficient translation initiation site (Kozak, 1984). Sequence analysis of this isolate of dengue-2 virus (PR159 strain) has been previously reported by Leblois and Young (1994).

Four different constructs were made which are shown schematically in Figure 5.2. The first construct, Ac.NS1, contained the signal sequence and the entire nucleotide coding sequence of NS1. For the mutant constructs, the 3' - ends were modified. Ac.NS1Δ9 and Ac.NS1.F were designed with the intention of preventing GPI anchoring by removing or ablating the ω site. Ac.NS1Δ9 construct had the last

nine amino acids removed from NS1 whereas Ac.NS1.F had the terminal amino acid substituted to a phenylalanine residue which has been shown to be a non-acceptor residue for GPI anchoring (Udenfriend and Kodukula, 1995; Eisenhaber *et al*, 1998).

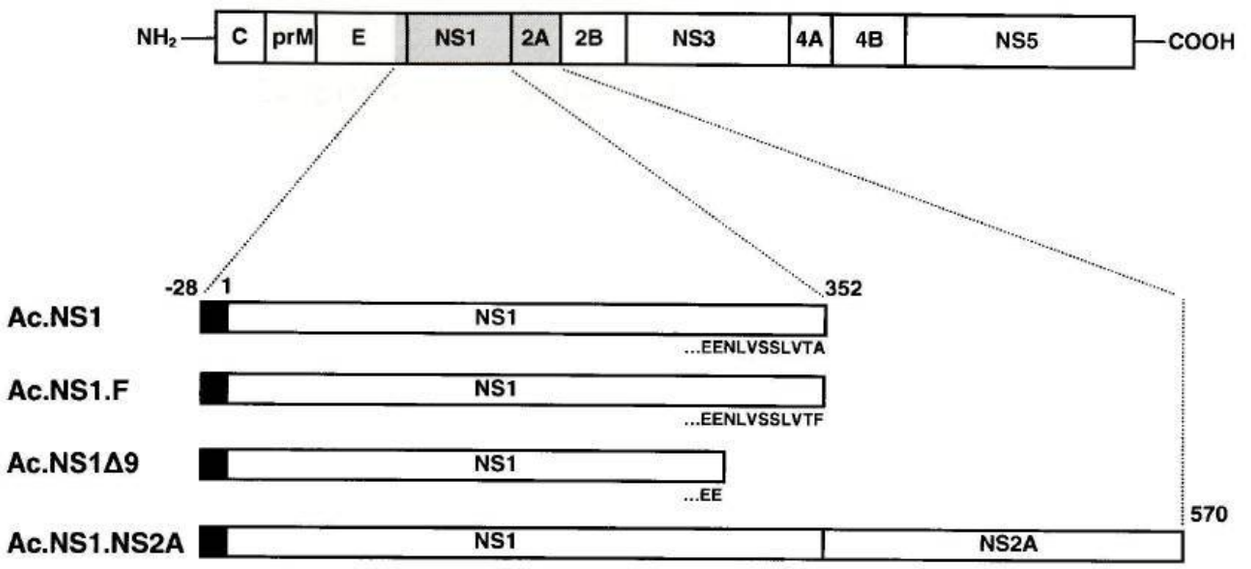


Figure 5.2 Schematic representation of the dengue virus genome and of constructs made for recombinant baculovirus expression of NS1 and modified NS1.

PCR primers were designed to amplify NS1 from the beginning of the signal sequence to the carboxy terminal VTA site or to the desired substitution or deletion. These are listed in Table 5.1. The sequence of each construct was confirmed prior to the expression of recombinant proteins.

Table 5.1 Sequence of oligonucleotides used to amplify NS1 and produce mutant constructs.

NS1 CONSTRUCTS		PRIMER SEQUENCE (5' – 3')
NS1	Forward	GACGGATCCACCATGAATTCACGCAGCACCTCA
NS1	Reverse	GCTGGATCCCTAGGCTGTGACCAAGGAGTTGAC
NS1.F	Forward	GACGGATCCACCATGAATTCACGCAGCACCTCA
NS1.F	Reverse	GGCCGGATCCCTAGAATGTGACCAAGGAGTTGAC
NS1Δ9	Forward	GACGGATCCACCATGAATTCACGCAGCACCTCA
NS1Δ9	Reverse	GGCCGGATCCCTACTCTTCTTTCTCTTTCAATGG
NS1.NS2a	Forward	GACGGATCCACCATGAATTCACGCAGCACCTCA
NS1.NS2a	Reverse	GGCCGGATCCGATATCCTACCTTTTCTTGCTGGTTCTTGA

NS1.NS2a contained the full length NS1 and NS2a as it had been reported that NS2a was required for the correct processing of NS1 (Chambers *et al*, 1989), and high molecular weight forms of NS1 containing regions of NS2a, had been identified in YF (Chambers *et al*, 1990) and JE virus infected cells (Mason *et al*, 1987; Mason, 1989).

The recombinant baculoviruses were constructed by standard molecular biology techniques as described by Kitts *et al* (1990; 1993). The various NS1 constructs were sub-cloned into a baculovirus transfer vector (pAcYM1) (Matsuura, 1987) and then co-transfected with *Bsa*B1 linearized AcRP23-LacZ baculovirus DNA using Lipofectin (Life Technologies) into *Spodoptera frugiperda* (*Sf*) cells. The supernatants were harvested at 48 hours post-transfection and used to infect fresh *Sf* cells. After three successive plaque purifications where recombinants were selected on their ability to secrete high levels of NS1 (by immuno-slot blot analysis), high titred stocks were obtained.

(b) Biochemical characterization of recombinant derived NS1

For a recombinant protein to be considered suitable for crystallography experiments it needs to be ascertained that the expressed protein resembles authentic native protein. To this end the known characteristics of NS1 such as dimerization, secretion, glycosylation and antigenicity were examined.

(i) Secretion

The four recombinant baculoviruses were used to infect *S. frugiperda* cells at an equivalent m.o.i. of one. Tissue culture supernatants and cell extracts were harvested to compare expression levels. The autoradiograph in Figure 5.3 clearly shows that the expression levels in the Ac.NS1.F, Ac.NS1Δ9 and Ac.NS1.NS2a recombinant viruses were markedly less that that of Ac.NS1. Ac.NS1 and Ac.NS1.F produced recombinant protein of a molecular weight of 50 kD, whereas Ac.NS1Δ9 produced a doublet probably due to incomplete glycosylation (Figure 5.3, open arrow). The Ac.NS1.NS2a recombinant protein was present as both the full-

length and processed forms in the cell extract although only the processed NS1 was secreted.

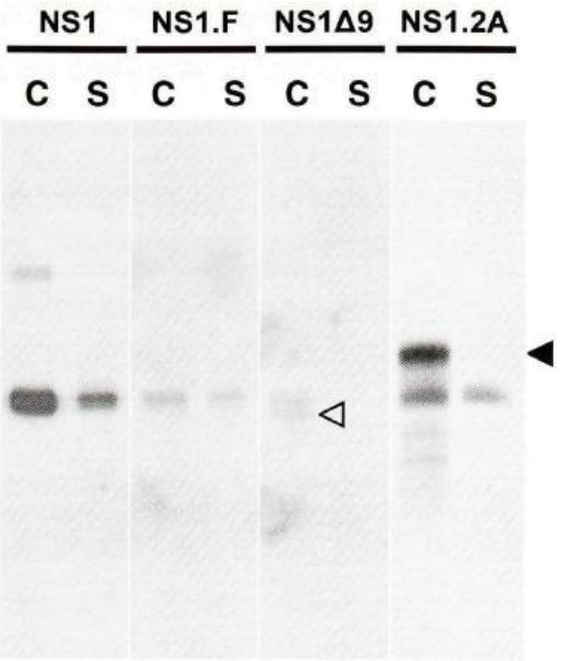


Figure 5.3 **Autoradiograph of cell extracts (C) and supernatants (S) from *Sf* cells infected with each recombinant baculovirus at an moi of 1 and immuno-precipitated with the anti-NS1 MAb, 1H7.4. Cells were pulse labeled for one hour and chased overnight. The open arrow indicates incomplete carbohydrate processing of the NS1Δ9 expressed product; and the solid arrow, the incomplete cleavage of the NS1.NS2A precursor.**

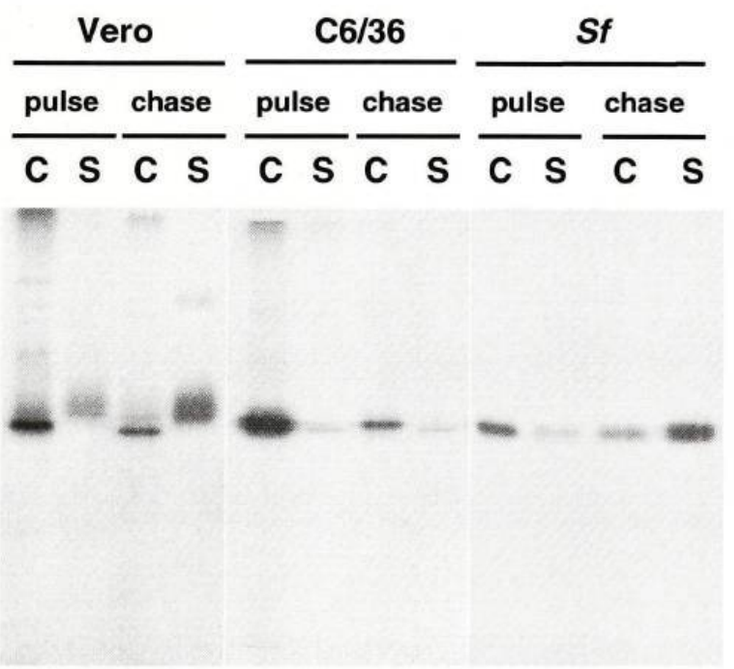


Figure 5.4 **Autoradiograph of cell extracts (C) and supernatants (S) from cells infected with dengue 2 virus (Vero and C6/36 cells) or Ac.NS1 (*Sf* cells) and immuno-precipitated with the anti-NS1 MAb, 1H7.4. Cells were pulse labeled for three hours and harvested immediately (pulse) or chased overnight (chase).**

A pulse-chase experiment shown in Figure 5.4 compared the expression levels of the recombinant protein in *S. frugiperda* cells with that of dengue-2 virus infected cells. As has been reported previously (Mason, 1989), C6/36 cells showed very little secreted NS1 although comparatively they produced the highest level of cell-associated NS1. Infected Vero cells produced levels of NS1 similar to that of the recombinant, with the secreted form being of a higher molecular weight due to complex glycosylation. The results of the recombinant protein chase experiment clearly showed the recombinant protein being chased into the supernatant after 24 hours.

As it was our intention to proceed towards crystallography, maximal secretion levels were required to maintain the viability of this approach and thus the Ac.NS1 baculovirus recombinant was chosen as the basis of further study.

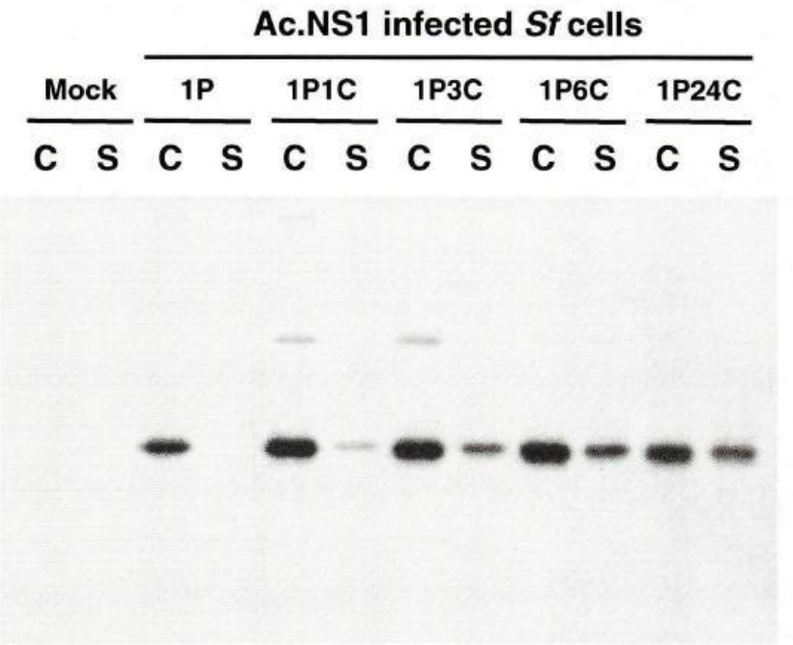


Figure 5.5 **Autoradiograph of cell extracts (C) and supernatants (S) from *Sf* cells infected with Ac.NS1 at an moi of 1 and immuno-precipitated with the anti-NS1 MAb, 1H7.4. Cells were pulse labeled for one hour and chased for 1, 3, 6 and 24 hours as indicated.**

S. frugiperda cells were infected with recombinant virus (Ac.NS1) at an m.o.i. of 1 to examine the kinetics of secretion over a 24 hour period. Cell extracts and tissue culture supernatants were assayed for the presence of secreted NS1 via

radioimmunoprecipitation of labelled cells with an NS1 specific monoclonal antibody. After a one hour pulse at 24 hours post - infection, NS1 is readily detectable within the cell however secretion had not yet occurred to a detectable level (Figure 5.5). After a further one hour chase NS1 became evident in the supernatant, albeit at low levels. Secretion continued to increase up to 24 hours post pulse, however the amount of cell-associated NS1 remained relatively constant and did not appear to chase out of the cell even after 24 hours.

(ii) Cellular localization

Recombinant NS1 was also present on the surface of infected *Spodoptera frugiperda* cells as shown by immunofluorescence in unfixed cells (Figure 5.6A and C), and in the cytoplasm of acetone fixed cells (note the large nuclei of *Sf* cells) (Figure 5.6B). The ability of the recombinant protein to be both secreted and membrane-associated reflects the characteristics of native NS1 produced in dengue virus infected mammalian cells. These results are in agreement with that of Flamand *et al* (1992) for JEV NS1, and Leblois and Young (1995) for dengue-2 NS1, demonstrating that recombinant NS1 can be transported to the plasma membrane in the absence of any other viral protein and also that NS1 alone contains all of the sequence requirements for membrane-association and secretion.

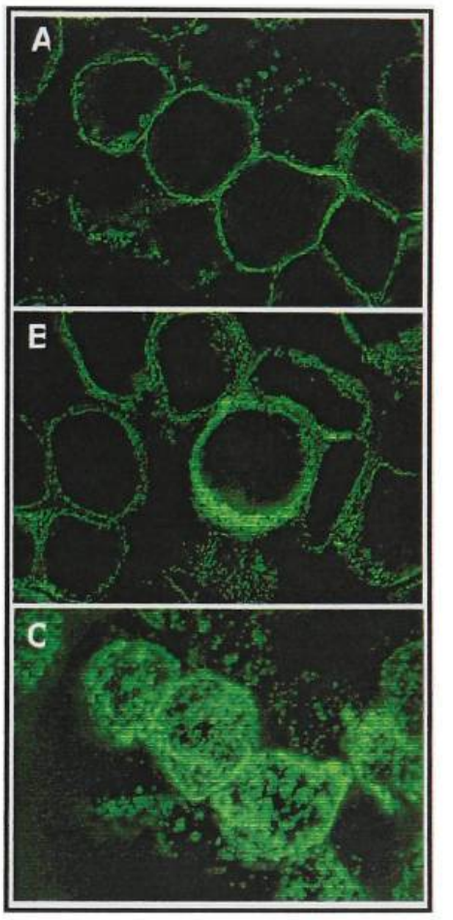


Figure 5.6 Immunofluorescence confocal microscopy of Ac.NS1 infected *Sf* cells. Infected cells were either left unfixed (A and C) to examine cell surface expression of NS1 or acetone fixed (B) prior to staining with the anti-NS1 monoclonal antibody 1H7.4. Panel C represents a computer reconstruction of the infected cell surface using a Z-series sequence of confocal images.

(iii) Glycosylation and dimerization

On expression of wild-type dengue virus proteins in infected cells, monomeric NS1

undergoes a rapid conversion to a dimeric form and is glycosylated at two N-linked sites. The glycosylation profile of recombinant derived NS1 was examined by endoglycosidase digestion. The western blot presented in Figure 5.7 shows the effect on immunoaffinity purified recombinant NS1 of digestion with both endoglycosidase F and endoglycosidase H.

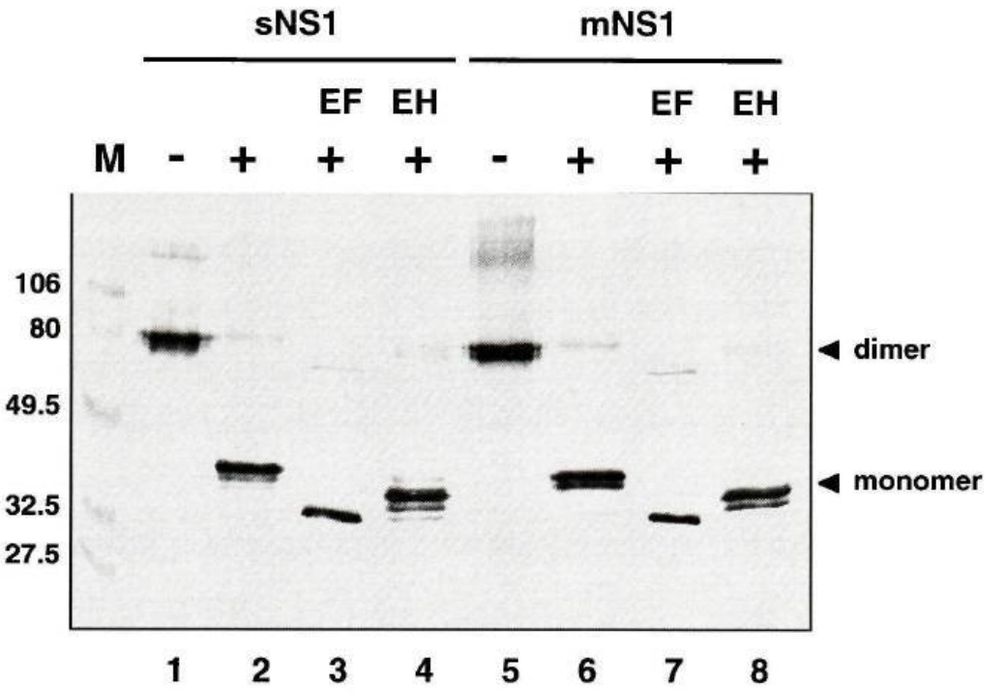


Figure 5.7 **Western blot of immunoaffinity purified recombinant NS1 from infected cells (mNS1) or supernatants (sNS1). NS1 was digested with either endoglycosidase F (EF) or endoglycosidase H (EH), and either boiled (+) or not boiled (-) prior to running a 10% SDS-PAGE gel. The western blot was probed with the anti-NS1 MAb 1H7.4.**

The difference in migration between the samples digested with the two endoglycosidases, and mock treated NS1 is consistent with the presence of two glycosylation sites in the recombinant protein and is similar to that observed for authentic NS1 (see Chapter 4.2b (v)). Endoglycosidase F digestion removes both carbohydrate moieties revealing an identical polypeptide backbone for both sNS1 and mNS1. The apparently anomalous migration of the marker proteins was a consistent feature of these pre-stained standards. They were not routinely used for molecular weight determination. Both secreted and membrane-associated recombinant NS1 treated with endoglycosidase H exhibited an intermediate molecular weight when compared to the mock treated and endoglycosidase F treated NS1 (Fig 5.7, lanes 4 & 8). This indicated that one of the two polymannose

sugars on both forms of NS1 was trimmed to a trimannosyl core that was endoglycosidase H resistant (Tarentino and Maley, 1975). This modification most likely represents the fully processed high-mannose glycan identified in baculovirus infected *S. frugiperda* cells (Jarvis and Summers, 1989). Addition of complex carbohydrates that occurs in mammalian cells, does not appear to occur in insect cells due to the absence of the requisite glycosyltransferases (Butters and Hughes, 1981; Jarvis and Finn, 1995). The finding that baculovirus recombinant expressed mNS1 was partially resistant to endoglycosidae H digestion is at variance with observations of NS1 expressed by dengue virus infection with both mammalian and insect cells. For native mNS1, endoglycosidase H digestion resulted in the complete removal of carbohydrate moieties suggesting that this form of NS1 had not undergone any further processing beyond the linkage of two polymannose sugars in the ER. This discrepancy between recombinant and native forms may be the result of different pathways of maturation. In flavivirus infected cells, NS1 is functionally retained in association with vesicles that are the sites of viral RNA replication and therefore are not likely to pass through the Golgi where processing occurs. The maturing recombinant NS1 in contrast undergoes no such retention.

Figure 5.7 also demonstrates that both recombinant secreted and membrane-associated NS1 formed dimers as is seen for authentic NS1, and this dimer was non-covalent in nature being converted to the monomeric form by heating.

(iv) Antigenicity

An antigenic map of both recombinant membrane-associated and secreted Ac.NS1 was constructed in the same way as for NS1 purified from dengue virus infected cells which was described previously in Chapter 4. Representative MAbs from each antigenic group (A, B, C1, C2 etc) were used in competition analyses and the resulting maps are shown in Figure 5.8. The high degree of correlation between the competition maps for both the recombinant secreted (Figure 5.8B) and recombinant membrane-associated NS1 (Figure 5.8C) with native secreted NS1 (Figure 5.8A) derived from dengue infected Vero cells (complete map shown in Figure 4.3), indicated that antigenically the proteins were very similar. This probably reflected

the correct processing and folding of the protein to maintain recognition by both linear and conformational MAbs. Preservation of structural integrity within the dimeric form of recombinant NS1 is promising in the absence of a functional *in vitro* assay. An interesting observation from this data was that in contrast to membrane-associated NS1 derived from Vero cells, A domain specific MAbs recognize membrane-associated recombinant protein. In this regard, the competition profile of the two membrane-associated NS1 preparations from insect cells (both *Sf* and C6/36 cells; - compare Figure 5.8C and Figure 4.8B respectively) are similar. As discussed in Chapter 4, this may be a consequence of a similar glycosylation profile leading to a particular protein conformation.

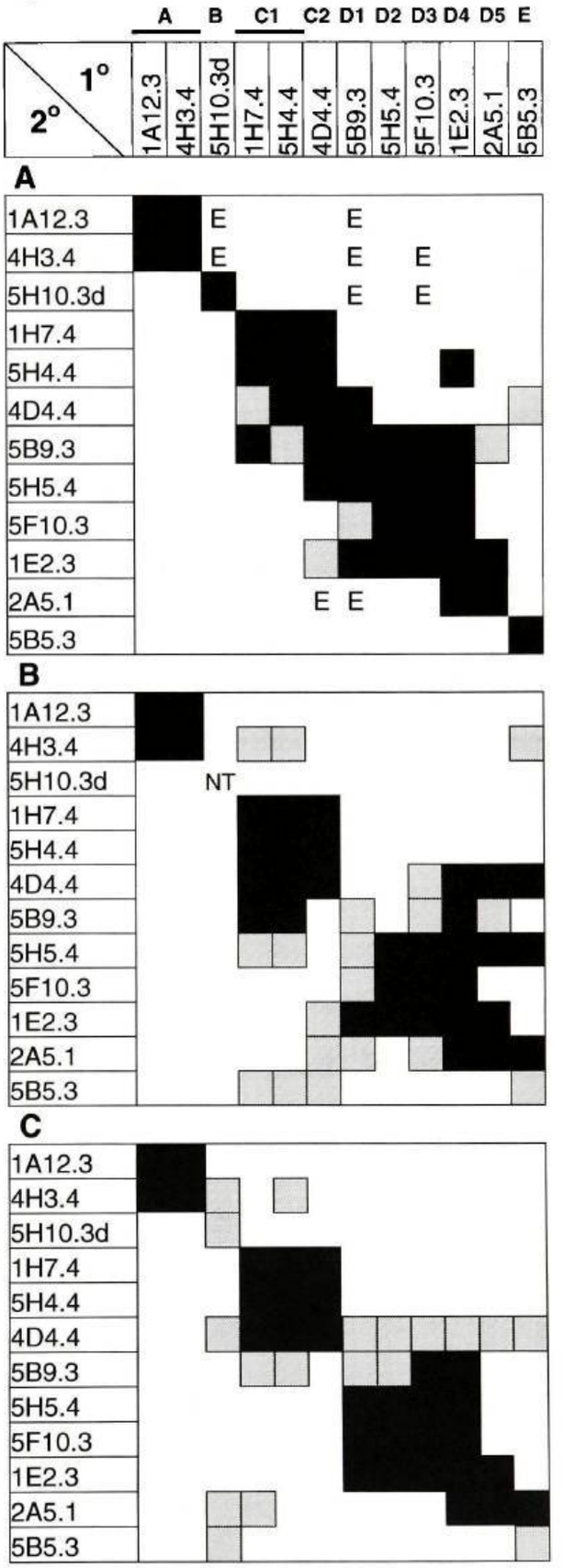


Figure 5.8 Antigenic competition maps of A, Vero-derived sNS1; B, *Sf*-derived recombinant sNS1; C, *Sf*-derived recombinant mNS1. Shading represents competition between MAbs of 0-30% (clear boxes), 31-50% (grey boxes) and 51-150% (black boxes). E, Enhanced binding (>150% binding). NT, Not tested. Letters on top of the figure refer to epitope domains identified in Chapter 4.

Protection experiments in female Balb/c mice were performed to compare the immunogenicity of immunoaffinity purified recombinant NS1 to immunoaffinity purified native NS1. Equal doses of NS1 (as determined by

protein concentration) afforded equal protection against challenge, to that seen with native protein (Qu and Young, unpublished observations). These experiments thus further confirmed that recombinant NS1 behaves immunogenically and antigenically in a similar way to native protein.

(c) Towards the crystallization of NS1

Previous attempts in our laboratory to purify large amounts of native protein for crystallography have proven difficult due to the comparatively low concentrations of NS1 produced in dengue virus infected cells. As has been described in the preceding section of this chapter, a recombinant protein has been expressed that appeared to structurally and antigenically resemble the authentic protein. This recombinant protein has been shown to be glycosylated and to dimerize while also maintaining the ability to be either secreted or to become membrane-associated in the infected cell. Most importantly, the previously identified antigenic domains within the native dimer form of dengue-2 virus NS1 have also been retained. It was therefore considered likely that the crystal structure of this protein would accurately reflect that of the native protein and we therefore proceeded towards crystallization of recombinant NS1.

(i) Two-dimensional gel analysis

Two-dimensional gels were performed to allow comparison between the native and recombinant NS1 protein. Membrane-associated ^{35}S - labelled NS1 derived from dengue-infected C6/36 cells was electrophoresed on a two-dimensional gel and four different isoelectric points (pI) from 5.5 - 5.8 were obtained (Figure 5.9A). This corresponded to four different isoforms of membrane-associated NS1 of very similar molecular weight. To confirm that these bands were indeed NS1 and not artefacts, the gel was exposed to X-ray film. As can be seen from Figure 5.9B the bands in both figures correspond with each other. This result confirmed our initial hypothesis that the membrane-associated form of NS1 would not be ideal for crystallography studies as revealed by the four different pIs. Secreted and

recombinant NS1 were also focussed on a two dimensional gel (Figure 5.9C) and the isoelectric point was determined to be approximately 5.7. This is the first report of a pI value for dengue virus NS1.

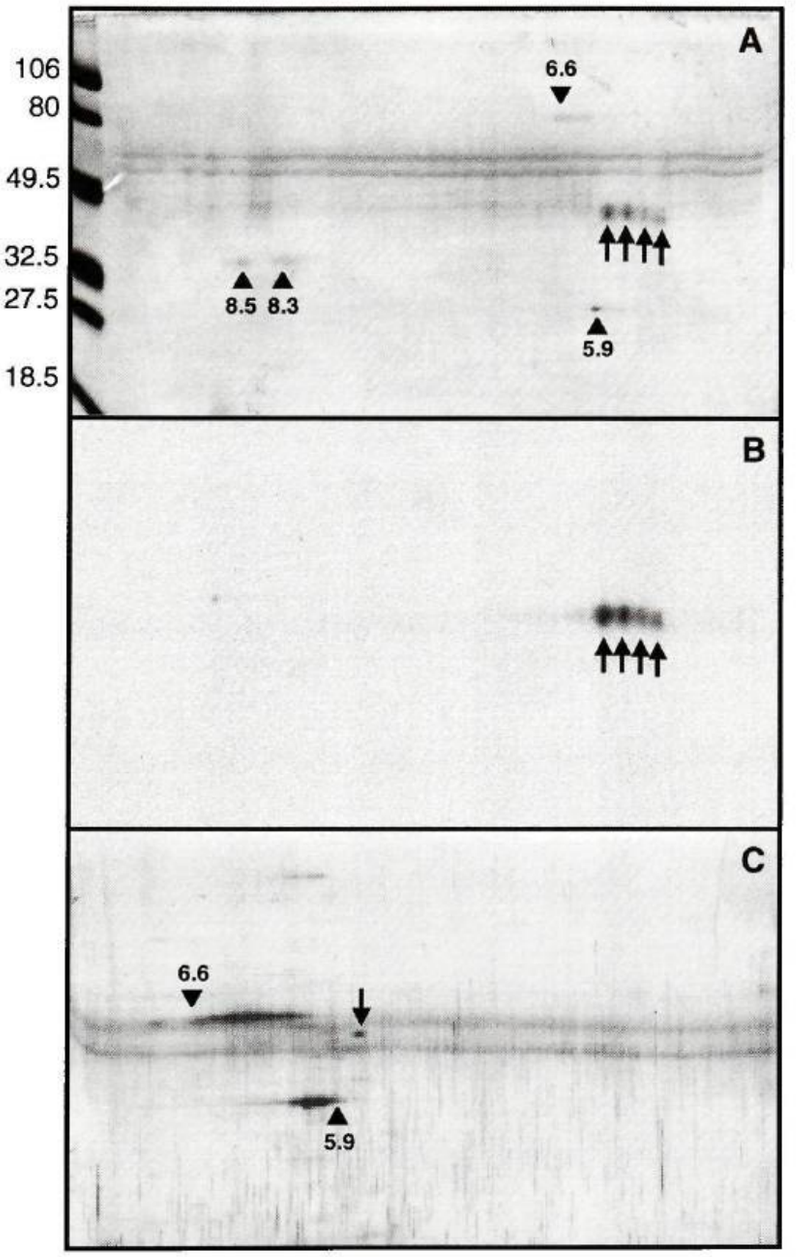


Figure 5.9 **Two dimensional gels. (A) Silver stained gel of ^{35}S-mNS1 purified from dengue virus infected C6/36 cells or (C) recombinant sNS1. Arrows indicate NS1 isoforms. B is an autoradiograph of A. pI markers are indicated with arrow heads.**

(ii) Pulse-chase analysis of the recombinant protein

Pulse-chase experiments were performed at different m.o.i.s to determine the optimum time points for the harvest of recombinant protein from infected cells. Figure 5.10 is an autoradiograph of radio-immunoprecipitations showing a comparison between m.o.i.s of 1 and 10 and the amount of protein produced in a three hour pulse. It is clear that the majority of protein was produced at 24 hours with an m.o.i. of 10 although protein production rapidly waned after 24 hours probably due to cell death. However at the m.o.i. of 1, protein production was maximal at 48 hours and was still detected up to 96 hours post-infection.

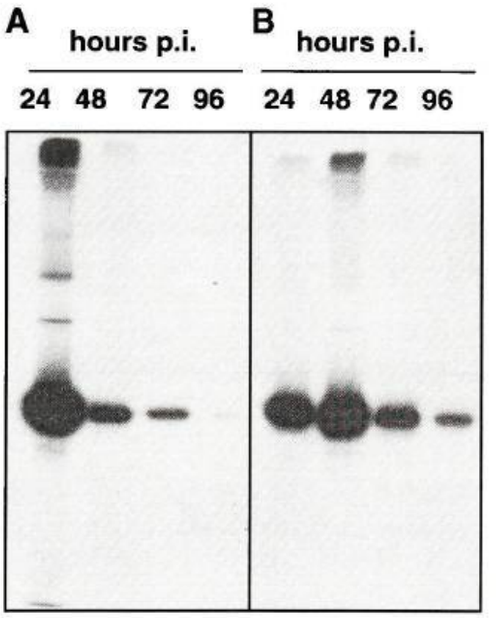


Figure 5.10 **Autoradiographs of radio-immunoprecipitations of supernatants from ^{35}S-labelled Ac.NS1 infected *Sf* cells. Supernatants were harvested at 24, 48, 72 and 96 hours after infection (p.i.) at an m.o.i. of 10 (A) or 1 (B).**

An m.o.i. of 1 was chosen with harvests taken at 48 hours for subsequent experiments.

(iii) Purification of recombinant NS1

The development of efficient protocols for immunoaffinity column purification of NS1 has been described previously by this laboratory (Falconar and Young, 1990).

In this project, initial attempts at purification were performed using tissue culture supernatants harvested from infected *Sf* cells grown in TC100 media (Life Technologies) containing 10% FCS. An SDS-PAGE silver stain profile of the fractions eluted from a typical immunoaffinity purification is shown in Figure 5.11. In addition to the purified NS1 a high level of contaminating protein bands were seen.

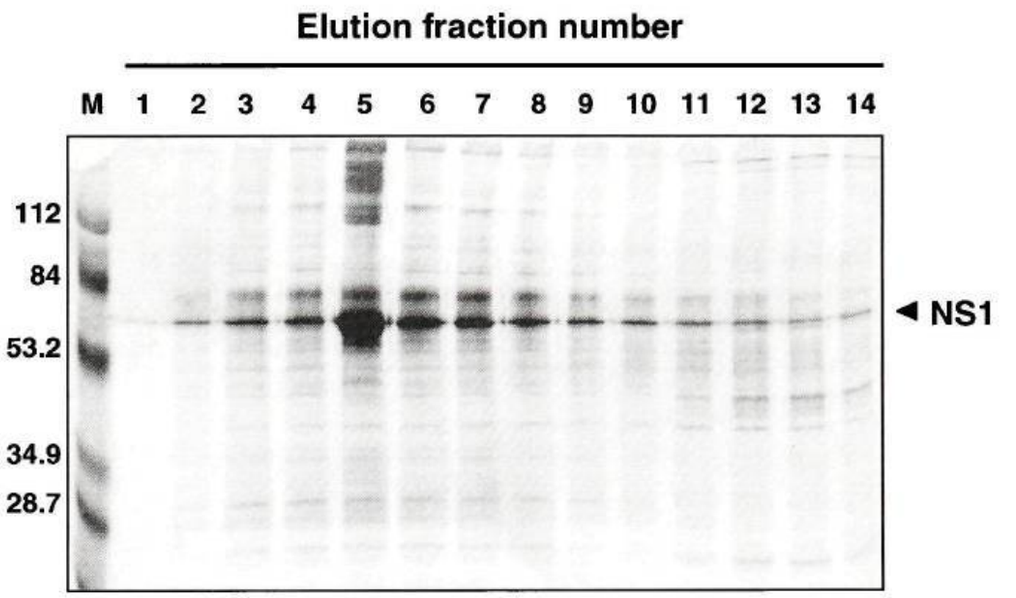


Figure 5.11 **10% SDS-PAGE silver stained gel of samples from each immunoaffinity column elution fraction (10 μL/track) purified from Ac.NS1 infected *Sf* cell supernatants grown in TC100 media with 10% FCS.**

Variable levels of contaminating protein was seen in all individual purifications, with none yielding NS1 of sufficient purity for crystallization. Given the possibility that a contributing factor to this contamination may be due to the high concentration of FCS in the medium we converted to serum free media (SF900II, Life Technologies) with *Sf* cells that had been previously adapted to serum free conditions. (Kindly provided by Dr Steve Reid's laboratory, Chemical Engineering Department, University of Queensland, St. Lucia, Brisbane). Subsequent purifications from 500 mL cultures gave significantly cleaner elution profiles as shown in Figure 5.12. The two gels shown are representative examples of numerous purifications done over the course of this study.

[Subsequent to the finding that serum free cultures were able to provide cleaner elution profiles a problem was experienced with the supplier, who had changed the media formulation. Numerous problems were encountered in terms of cell growth and recombinant protein production, and as time was a critical factor, media conditions for the final purifications reverted to TC100 with 10% FCS.]

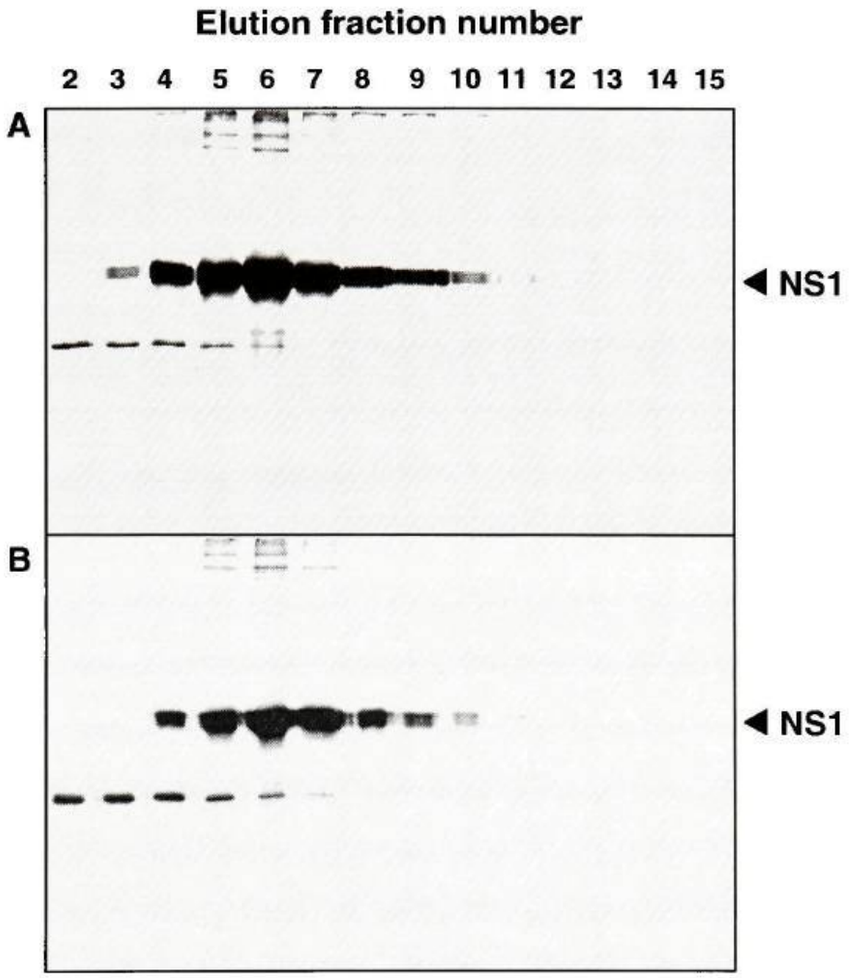


Figure 5.12 **10% SDS-PAGE silver stained gels of immunoaffinity column elution fractions from two different batches of Ac.NS1 infected cell supernatants grown in SF900II media.**

(d) Oligomeric forms of NS1

The dimeric form of NS1 was originally detected by Winkler *et al* (1988) for dengue 2 virus when infected cell lysates were left unboiled prior to analysis by SDS-PAGE. Crooks *et al* (1990) further showed that TBE secreted NS1 was likely to be hexameric. This group also suggested that the efficiency with which NS1 acts as an

effective immunogen, may in part be due to the native form existing as a higher order oligomeric structure (Crooks *et al*, 1994). Indeed the dimeric form of dengue-2 NS1 had been shown to be more immunogenic than the monomer (Young and Falconar, 1991), and sNS1 was more protective than mNS1 (Young and Falconar, 1991). The oligomerization of NS1 secreted from cells infected with a defective adenovirus containing the gene coding for NS1 alone was examined by column chromatography (Crooks *et al*, 1994). Primarily pentameric and hexameric forms were identified, with a small proportion of material being consistent with decameric and dodecameric oligomers. Flamand *et al* (1999) have recently confirmed that dengue-1 virus NS1 secreted from Vero cells is indeed a hexamer form. This study has been able to support these findings and to verify that both the native dengue-2 NS1 and recombinant baculovirus-expressed NS1 are also hexameric.

(i) Chemical cross-linking

Immunoaffinity-purified NS1 was used in cross-linking experiments to determine whether evidence could be found for a higher oligomeric form of recombinant NS1 that reflected that found for native NS1. Figure 5.13A shows the results of cross-linking both native and recombinant NS1 with BS^3. SDS - PAGE analysis clearly shows that with increasing concentrations of BS^3, both forms of NS1 resolve as higher oligomeric species. All intermediates were observed (labelled in Fig 5.13A) with the highest oligomeric form detected having a molecular weight of approximately 300 kDa, consistent with a hexamer.

(ii) Electron microscopy

NS1 protein derived from both dengue-2 infected Vero cells and from recombinant baculovirus infected *S. frugiperda* cells were immunoaffinity purified and examined by electron microscopy as described in Section 2.5 (I). Figure 5.13B and C shows negative stain electron micrographs of recombinant baculovirus-derived, and native NS1 respectively. (Electron microscopy was performed by Dr. Lynne Lawrence, BRI, Melbourne). These preparations were shown to be composed principally of monodispersed particles with a size range from 10 - 12 nm (solid arrow heads).

The range of particle sizes suggested some breakdown of the higher oligomeric forms probably into tetramer and/or dimer configurations (4 - 6 nm: open arrow heads). However the size of the larger particles was consistent with a molecular weight of between 300 and 350 kDa or six times the molecular weight of sNS1. At high magnification (Figure 5.13D and E) these particles exhibited discernible higher order structure when viewed along apparent points of symmetry.

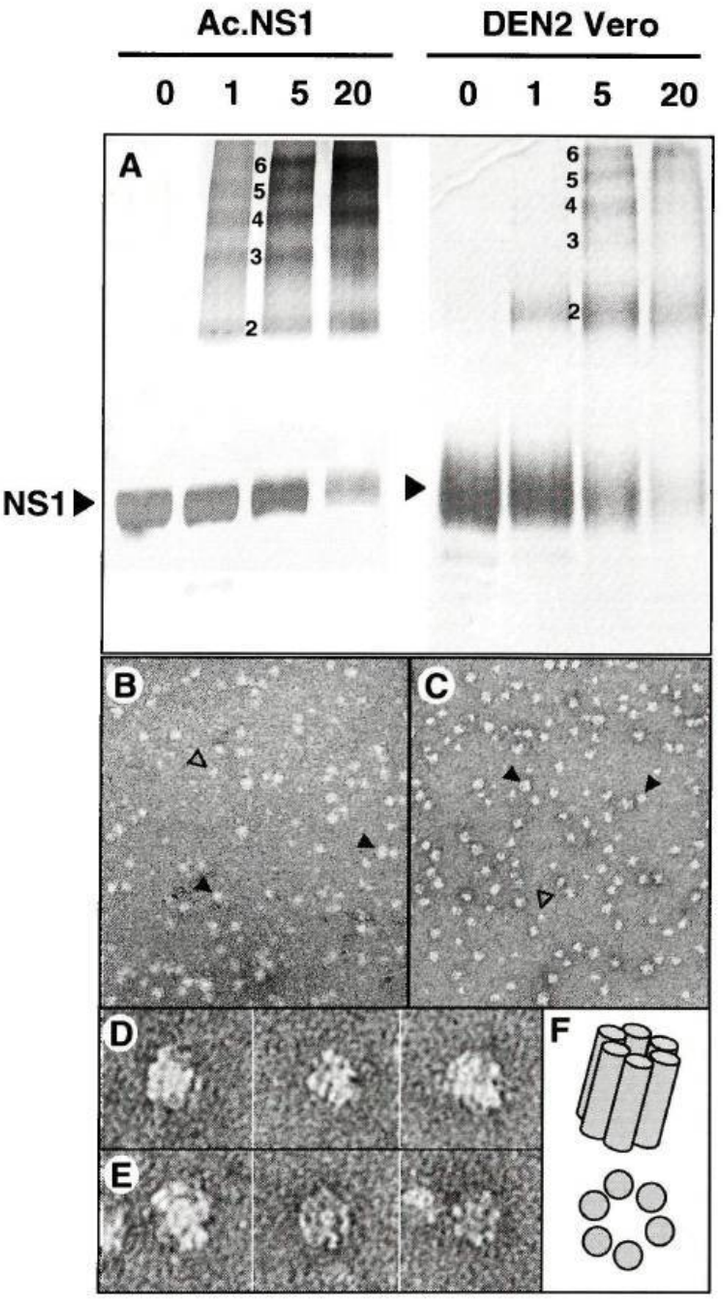


Figure 5.13 **Five-20% gradient SDS-PAGE silver stained gel of immunoaffinity purified recombinant NS1 and native dengue-2 (Vero derived) NS1 cross-linked with BS^3 at concentrations of 1, 5 and 20 mM (A). Negative stain electron micrographs of recombinant sNS1 (B) and infected Vero cell derived native dengue-2 sNS1 (C) and at higher magnification (D and E respectively). Schematic representation of D and E (F).**

The predominant structural form appeared to be a "bundle of rods" seen either from side on (Fig 5.13D) or end on (Fig 5.13E), which conceivably represent the assembly of three stable dimeric units (schematically represented in Figure 5.13F).

(iii) Freeze / Thawing

During the course of this study, aliquots of immunoaffinity purified recombinant NS1 had been routinely stored at -80°C. Freeze - thawing however is known to potentially disrupt protein-protein interactions with subsequent deleterious effects on structural analyses (J.Martin, personal communication). Although dimeric forms of NS1 appear to be highly stable, the fragility of the hexameric form was examined.

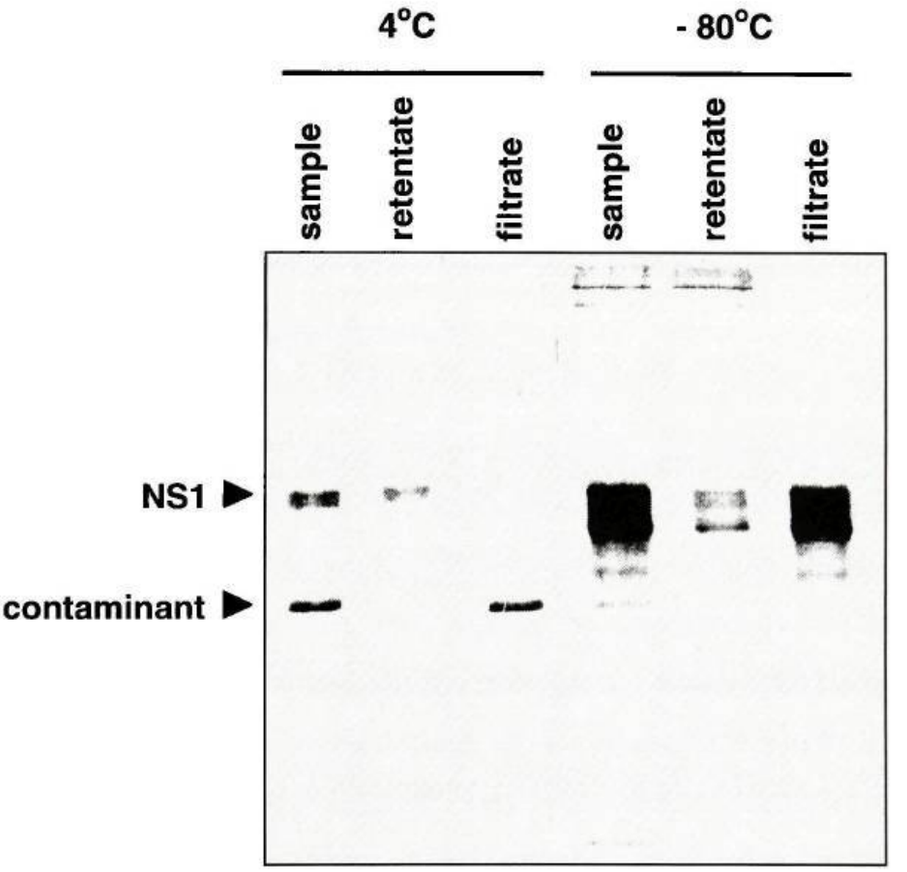


Figure 5.14 Analysis of retentate and filtrate samples of recombinant NS1 processed through a 100k MW filter (centricon 100) and run on a 10% SDS-PAGE silver stained gel. Samples were immunoaffinity purified and stored at either 4°C or -80°C prior to analysis.

Both fresh (stored for less than three days at 4°C after immunoaffinity purification)

and frozen samples of purified NS1 were examined for retention or passage through a 100k molecular weight cut-off filter (centricon 100). The results shown in Figure 5.14 demonstrate that on freeze / thawing, the higher oligomeric forms of NS1 are broken down sufficiently to enable them to pass through the 100k molecular weight cut-off filter and appear in the flow-through, whereas very little NS1 remains in the retentate (compare tracks 6 and 5 respectively). In contrast, purified NS1 stored at 4°C remained in the retentate with some protein being lost during the procedure. The lower band in lane 1 represents contaminating serum albumin (BSA) derived from the 10% foetal calf serum in the tissue culture supernatant and co-purified with NS1 on the immunoaffinity column (see elution fractions in Figure 5.12). This contaminating species was completely removed during the centricon 100 filtration as shown by the analysis comparing lanes 2 and 3, offering a convenient additional purification of NS1.

(e) Crystallography trials

(i) Factorial Crystal screens

Several crystallography trials were performed using Hampton incomplete factorial screens I and II (See Appendix 5.1). Each screen is composed of a buffer set, and each individual buffer contains a combination of various salts, buffers and precipitants (Tables 5.2 and 5.3). The first trial involved the use of NS1 purified in the presence of 0.1% Triton X-100 detergent. Peak fractions from three immunoaffinity purifications were pooled and concentrated with a centricon 30 concentrator. The protein concentration was assayed and was determined to be approximately 3 mg / mL. Factorial crystal screens I and II were set up according to the hanging drop vapour diffusion method (McPherson, 1989) (Described in Chapter 2.5k (i)). At the completion of the procedure, the drops were observed and over 90% of wells showed phase separation of the components which suggested that the triton X-100 (TX-100) detergent (which had not been removed during the centricon procedure), was the likely cause. The drops also appeared to spread and

did not show the type of surface tension that is usually observed. It was therefore decided to avoid the use of detergent in future purification procedures to alleviate this problem.

The second crystallography trial was comprised of protein from several immunoaffinity purifications performed in the absence of detergent (shown in Figures 5.12A and B) that had been concentrated with a centricon 100 and stored at 4°C. Surprisingly, it can be seen that cleaner elution profiles were obtained with the omission of TX-100 and FCS in the media. The final protein concentration obtained was approximately 5 mg / mL (determined by the BCA protein assay) with some precipitation of the protein being observed at this concentration. Coomassie blue SDS - PAGE analysis of this preparation (Figure 5.15) shows the purity and concentration of the final protein obtained.

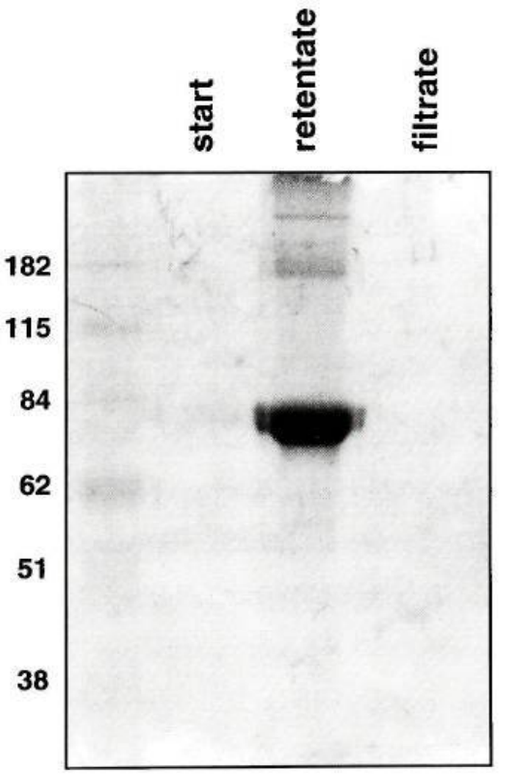


Figure 5.15 Coomassie blue stained 10% SDS-PAGE gel of 5 μL of a 1.5 mL pool of recombinant NS1 (start) and 5 μL of a 200 μL retentate after processing through a centricon 30 concentrator. The filtrate represents 5 μL from the 1.3. mL filtrate.

Hampton factorial crystal screens I and II (the components of which are listed in Appendix 5.1) were set up in trays and incubated at room temperature (20°C) or at 4°C. Examination of the trays revealed a common feature in those wells where precipitation and / or crystals were observed after ten weeks. A high molecular weight of polyethylene glycol (PEG) as the precipitant usually resulted in precipitation occurring and gave an indication of the next type of screen required. Two of the most interesting observations however were of microcrystals in crystal screen I (well 41) which contained 0.1 M Hepes pH 7.5, 10% v/v 2-propanol and 20% w/v PEG 4000, and in crystal screen II (well 45) which had very small log shaped micro-crystals (shown in Figure 5.16A) and contained 0.01 M nickel (II) chloride hexahydrate, 0.1 M Tris pH 8.5 and 20% polyethylene glycol monomethyl ether 2000. These crystals grew after three months at 20°C however they were only 0.05 mm long on the longest axis and therefore not large enough to use for X-ray diffraction studies. This preliminary screen had therefore obtained its objective which was to narrow down the type of conditions where the protein was starting to crystallize or precipitate.

Continued observation of these crystal screen wells over nine months led to the observation of a larger crystal of triangular shape (1.2 mm in its longest dimension (Figure 5.16B)) that grew under the conditions of 0.1M Tris pH 8.5, 20% ethanol at 20°C (well 44, crystal screen II). Unfortunately this crystal failed to diffract which may have been due to the age of the crystal (Dr Tracy Arakaki, 3D Centre, UQ, personal communication). Achieving growth of a reasonably sized crystal however is indicative of a pure and highly concentrated protein preparation that, with further work should yield a suitable candidate for efficient X-ray diffraction studies.

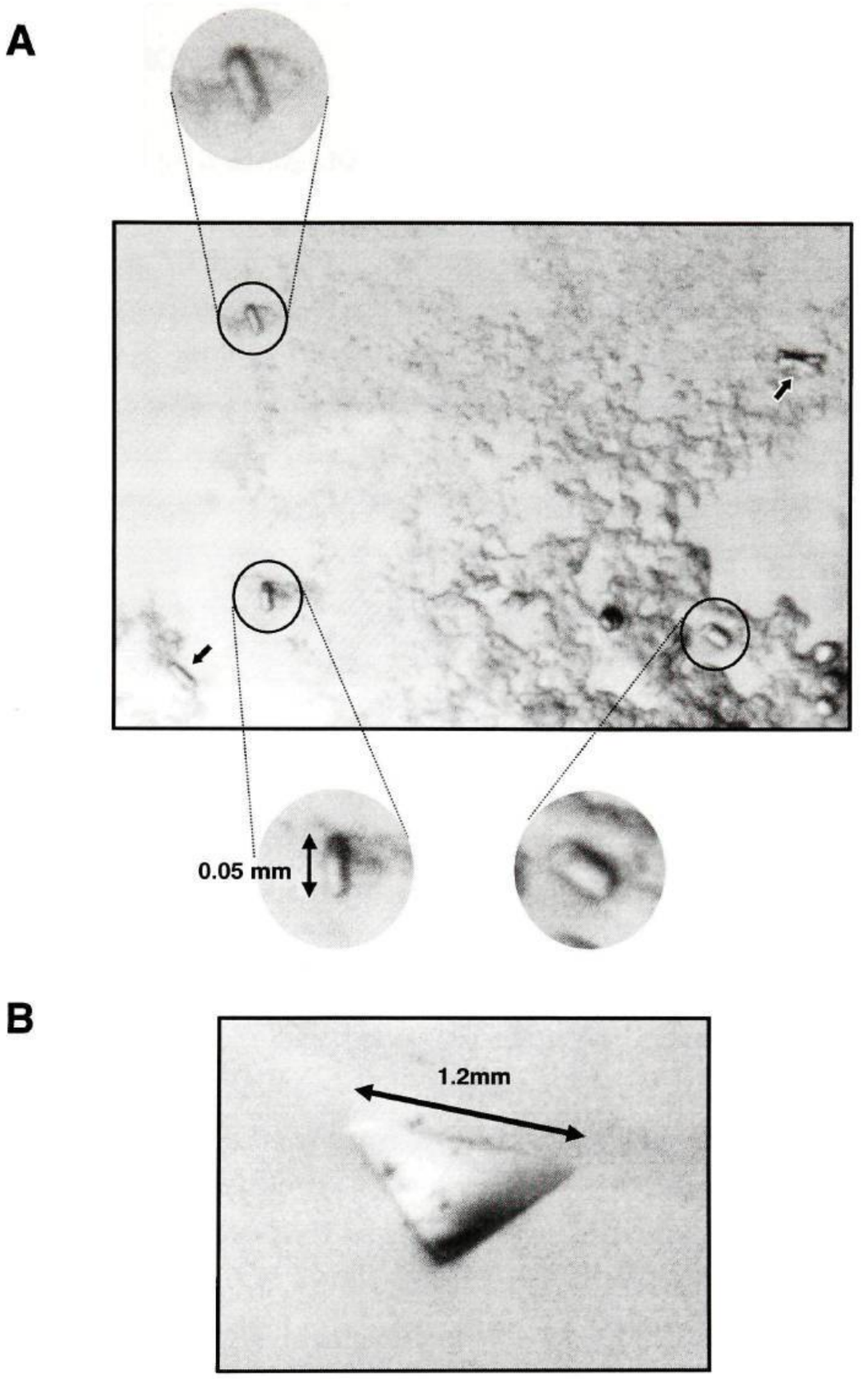


Figure 5.16 Microcrystals grown in 0.01 M nickel (II) chloride hexahydrate, 0.1 M Tris pH 8.5 and 20% polyethylene glycol monomethyl ether 2000 (A) and 0.1M Tris pH 8.5, 20% ethanol (B) and observed after three months and nine months respectively.

5.3 DISCUSSION

Recombinant NS1 from various flaviviruses including JE (McCown *et al*, 1990; Flamand *et al*, 1992), Kunjin (Khromykh *et al*, 1996), dengue-2 (Qu *et al*, 1993; Leblois and Young, 1995), dengue-4 (Eckels *et al*, 1994), MVE (Blitvich *et al*, 1999), Louping Ill (Venugopal *et al*, 1994) and YF (Desprès *et al*, 1991) viruses have been successfully expressed in the baculovirus expression system. In accordance with the findings described in this chapter, recombinant baculovirus expressed JEV NS1 exhibited the correct molecular weight, secretion, glycosylation and dimerization pattern however the majority of the protein accumulated in the ER, where it formed insoluble aggregates (Flamand *et al*, 1992). Yellow fever NS1, expressed with the envelope protein in *Sf* cells exhibited similar size, folding and antigenicity to the authentic protein (Desprès *et al*, 1991). In contrast YF proteins E and NS1, when expressed together did not produce detectable amounts of NS1 in the extracellular medium (Desprès *et al*, 1991). This discrepancy has been postulated to be due to the presence of the E protein in the construct which associates with NS1 in a compartment preceding the Golgi in the secretory transport pathway and slows its passage to the surface of the cell (Flamand *et al*, 1992).

Other groups have successfully used baculovirus expression systems for the production of recombinant flavivirus NS1. Baculovirus expression of Louping Ill (LI) virus NS1 resulted in an expressed protein which reacted with LI specific MAbs and polyclonal antisera in immunofluorescence studies (Venugopal *et al*, 1994). It was expressed on the surface of cells but was not secreted from the cell. In agreement with our results with baculovirus expressed dengue-2 virus NS1, LI NS1 showed the same antigenic reactivity as the native NS1 protein (Venugopal *et al*, 1994) confirming that the baculovirus expression system is able to faithfully reproduce the expressed protein as with other flaviviruses. We have further confirmed this observation for recombinant dengue-2 NS1 with our extensive competition map analysis (Figure 5.8). Representative MAbs from the five antigenic domains identified in Chapter 4, were all recognized by recombinant dengue-2 NS1 with the pattern of cross competition being very similar between these domains. As the

available anti-NS1 monoclonal antibodies (22 in total) had corresponding epitopes along the whole length of the NS1 molecule this antigenic analysis is more extensive than those performed in other laboratories. Thus we are confident that this protein closely resembles the authentic secreted form of NS1.

Previous reports have suggested that the flavivirus NS1 may be hexameric (rather than dimeric) in its native form (Crooks *et al,* 1990; Crooks *et al,* 1994) with recent convincing evidence also being reported for dengue-1 virus NS1 produced in Vero cells (Flamand *et al,* 1999). We were able to confirm these observations for dengue-2 virus NS1 derived from infected Vero cells, and also for our recombinant NS1 derived from *Spodoptera frugiperda* cells by chemical cross-linking studies and by negative stain electron microscopy. Taken together, these findings indicate that our recombinant protein is a suitable surrogate for authentic NS1.

The results described in this chapter clearly indicate that the recombinant NS1 protein produced by baculovirus expression, and purified and concentrated using the methods described should be suitable for further crystallography studies. Two-dimensional gel analysis of the recombinant protein determined that it comprised a single isoform with an iso-electric point (pI) of approximately 5.7 when compared with the marker controls, further demonstrating its suitability for crystallization. Along with the native NS1 analysis this is the first report describing the iso-electric point for flavivirus NS1. Disc gel electrophoresis analysed by the Ferguson plot showed that each of the dengue NS1 serotypes are charge isomers although the charge differences appeared to be small (McCloud *et al,* 1970). Somewhat surprisingly, micro-crystals formed in the incomplete factorial crystal screens in a buffer pH range of 7.5 - 8.5. Proteins are thought to crystallize around their pI because they are least soluble at that point however there is no hard and fast rule and this discrepancy is not considered unusual (Dr Jenny Martin, 3D Centre, UQ, personal communication).

Analysis of two different m.o.i.s and the corresponding level of NS1 production by pulse-chase labelling, showed that although NS1 was secreted maximally at 24

hours at an m.o.i. of 10, for continuous NS1 secretion it was more appropriate to infect at an m.o.i. of 1 and to collect supernatant harvests every 24 hours up to 72 hours. In this way the infected cell was able to secrete more NS1 before cell death occurred and less virus stock is utilized as inoculum. The high levels of secretion obtained from the baculovirus expression system meant that the protein could be purified exclusively from the tissue culture supernatants thereby simplifying the purification procedure by not having to isolate protein from cell extracts. In addition, it was noted that while all secreted NS1 was oligomeric in nature a significant proportion of membrane-associated NS1 remained monomeric. Presumably the latter represents misfolded protein retained in the ER as a consequence of over-expression in this system. Furthermore, omission of TX-100 detergent from the purification procedure was found to be necessary to prevent phase - separation in hanging drops. Although micro-crystals were obtained in the absence of detergent, the failure to form suitable crystals for X-ray diffraction analysis could be due to a level of microheterogeneity arising from the different states of oligomerization / aggregation during the crystallization process (McPherson *et al*, 1986). This was also evident from the electron microscopy observations. The addition of a neutral detergent such as β - octyl-glucoside has been shown by McPherson *et al* (1986) to give more reproducible and rapid crystal growth at the expense of microcrystals especially for glycoproteins. This is a parameter that needs further investigation however these experiments were difficult due to the expense of this detergent. Significantly, this detergent at 0.5% was successfully used in the purification of the TBE envelope protein (Heinz *et al*, 1991) from which a crystal structure was derived with a protein concentration of 6 mg / mL (Rey *et al*, 1995). Preliminary studies have indicated that detergent exchange (TX-100 for octyl-glucoside) could be made on the immunoaffinity column and further studies are underway to test the products of this exchange. PEG was found to be an important constituent of buffers in which crystals / microcrystals were observed. Interestingly, PEG 8000 was a component of the buffer conditions required for crystallization of the TBE E protein (Heinz *et al*, 1991). The use of serum free media (SF900II, Life Technologies) was also found to be preferential to medium containing foetal calf serum. Despite ultimately having to continue the study with TC100 + 10% FCS due to supplier problems, we were able

to obtain microcrystals of 0.05 mm in length and a crystal of 1.2 mm on its longest axis, from protein produced in TC100 media. Ideally crystals need to be of the order of 0.5 mm before they can be used in X-ray diffraction studies (Dr. Tracy Arakaki, 3D Centre, UQ, personal communication). It was demonstrated in section 5diii that one cycle of freeze / thawing was able to disrupt the higher oligomeric forms of NS1. Purified protein destined for crystallization trials was therefore stored at 4°C prior to use and was never permitted to go through any cycles of freeze / thawing.

Endoglycosidase analysis (Maley *et al*, 1989) of the recombinant protein demonstrated that both of the N-glycosylation sites were utilized when expressed in *S. frugiperda* cells, in accordance with the reports of other researchers (Flamand *et al*, 1992; Qu *et al*, 1993). The maintenance of glycosylation of the recombinant NS1 may be relevant to the preservation of the overall structure of the protein as the loss of the carbohydrate moiety on the second glycosylation site of NS1 has been reported by other researchers to significantly decrease dimer stability and secretion of the protein (Pryor and Wright, 1994; Flamand *et al*, 1999). Additionally, previous experiments by Muylaert *et al* (1996) and Pletnev *et al* (1993) showed that removal of the complex-type oligosaccharide correlated with a decrease in mouse neurovirulence. This may imply that the carbohydrate structure may be significant in the formation of the mature secreted form of NS1 (Flamand *et al*, 1999). Crooks *et al* (1994) also commented that the increased level of glycosylation seen in secreted NS1 appeared to assist stabilization of the dimer. Because of these findings examination of a deglycosylated form of NS1 for structural studies was not considered to be an option.

As with other RNA viruses (Harrison, 1989), such as poliovirus (Hogle *et al*, 1989), rhinovirus and mengovirus (Rossman *et al*, 1987) and other viral glycoproteins (Laver *et al*, 1990; Weis *et al*, 1988; Wiley and Skehel, 1987; Rey *et al*, 1995) the resolution of the three dimensional structure of the dengue virus NS1 will have a significant impact on the elucidation of the functional properties of this molecule as well as potentially providing a target for rational drug design.

APPENDIX 5.1

Table 5.2 Components of Hampton Factorial Crystal Screen I buffers

TUBE	SALT	BUFFER	PRECIPITANT
1	0.02 M Ca Chloride	0.1 M Na Acetate pH 4.6	30% v/v 2-methyl-2,4-pentanediol
2	None	None	0.4 M K / Na Tartrate
3	None	None	0.4 M NH4 Phosphate
4	None	0.1 M Tris HCl pH 8.5	2.0 M NH4 Sulfate
5	0.2 M Na Citrate	0.1 M Na Hepes pH 7.5	30% v/v 2-methyl-2,4-pentanediol
6	0.2 M Mg Chloride	0.1 M Tris HCl pH 8.5	30% w/v PEG 4000
7	None	0.1 M Na Cacodylate pH 6.5	1.4 M Na Acetate
8	0.2 M Na Citrate	0.1 M Na Cacodylate pH 6.5	30% v/v 2-propanol
9	0.2 M NH4 Acetate	0.1 M Na Citrate pH 5.6	30% w/v PEG 4000
10	0.2 M NH4 Acetate	0.1 M Na Acetate pH 4.6	30% w/v PEG 4000
11	None	0.1 M Na Citrate pH 5.6	1.0 NH4 Phosphate
12	0.2 M Mg Chloride	0.1 M Na Hepes pH 7.5	30% v/v 2-propanol
13	0.2 M Na Citrate	0.1 M Tris HCl pH 8.5	30% v/v PEG 400
14	0.2 M Ca Chloride	0.1 M Na Hepes pH 7.5	28% v/v PEG 400
15	0.2 M NH4 Sulfate	0.1 M Na Cacodylate pH 6.5	30% w/v PEG 8000
16	None	0.1 M Na Hepes pH 7.5	1.5 M Li Sulfate
17	0.2 M Li Sulfate	0.1 M Tris HCl pH 8.5	30% PEG 4000
18	0.2 M Mg Acetate	0.1 M Na Cacodylate pH 6.5	20% PEG 8000
19	0.2 M NH4 Acetate	0.1 M Tris HCl pH 8.5	30% v/v 2-propanol
20	0.2 M NH4 Sulfate	0.1 M Na Acctate pH 4.6	25% w/v PEG 4000
21	0.2 M Mg Acetate	0.1 M Na Cacodylate pH 6.5	30% v/v 2-methyl-2,4-pentanediol
22	0.2 M Na Acetate	0.1 M Tris HCl pH 8.5	30% w/w PEG 4000
23	0.2 M Mg Chloride	0.1 M Na Hepes pH 7.5	30% v/v PEG 400
24	0.2 M Ca Chloride	0.1 M Na Acetate pH 4.6	20% v/v 2-propanol
25	None	0.1 M Imidazole pH 6.5	1.0 M Na Acetate
26	0.2 M NH4 Acetate	0.1 M Na Citrate pH 5.6	30% v/v 2-methyl-2,4-pentanediol
27	0.2 M Na Citrate	0.1 M Na Hepes pH 7.5	20% v/v 2-propanol
28	0.2 M Na Acetate	0.1 M Na Cacodylate pH 6.5	30% w/v PEG 8000
29	None	0.1 M Na Hepes pH 7.5	0.8 M K / Na Tartrate
30	0.2 M NH4 Sulfate	None	30% w/v PEG 8000
31	0.2 M NH4 Sulfate	None	30% w/v PEG 4000
32	None	None	2.0 M NH4 Sulfate
33	None	None	4.0 M Na Formate
34	None	0.1 M Na Acetate pH 4.6	2.0 M Na Formate
35	None	0.1 M Na Hepes pH 7.5	1.6 M Na / K Phosphate
36	None	0.1 M Tris HCl pH 8.5	8% w/v PEG 8000
37	None	0.1 M Na Acetate pH 4.6	8% w/v PEG 4000
38	None	0.1 M Na Hepes pH 7.5	1.4 M Na Citrate
39	None	0.1 M Na Hepes pH 7.5	2% v/v PEG 400 & 2.0 M NH4 Sulfate
40	None	0.1 M Na Citrate pH 5.6	20% v/v 2-propanol & 20% w/v PEG 4000
41	None	0.1 M Na Hepes pH 7.5	10% v/v 2-propanol & 20% w/v PEG 4000
42	0.05 M K Phosphate	None	20% w/v PEG 8000
43	None	None	30% w/v PEG 1450
44	None	None	0.2 M Mg Formate
45	0.2 M Zn Acetate	0.1 M Na Cacodylate pH 6.5	18% w/v PEG 8000
46	0.2 M Ca Acetate	0.1 M Na Cacodylate pH 6.5	18% w/v PEG 8000
47	None	0.1 M Na Acetate pH 4.6	2.0 M NH4 Sulfate
48	None	0.1 M Tris HCl pH 8.5	2.0 M NH4 Phosphate
49	1.0 M Li Sulfate	None	2% w/v PEG 8000
50	0.5 M Li Sulfate	None	15% w/v PEG 8000

Table 5.3 Components of Hampton Factorial Crystal Screen II buffers

TUBE	SALT	BUFFER	PRECIPITANT
1	2.0 M Sodium chloride	None	10% w/v PEG 6000
2	0.01 M Hexadecyltrimethylammonium Bromide	None	0.5 M Sodium Chloride, 0.01 M Magnesium Chloride hexahydrate
3	None	None	25% v/v Ethylene Glycol
4	None	None	35% v/v Dioxane
5	2.0 M Ammonium Sulfate	None	5% v/v iso-Propanol
6	None	None	1.0 M Imidazole pH 7.0
7	None	None	10% w/v Polyethylene Glycol 1000 10% w/v Polyethylene Glycol 8000
8	1.5 M Sodium Chloride	None	10% v/v Ethanol
9	None	0.1 M Sodium Acetate trihydrate pH 4.6	2.0 M Sodium Chloride
10	0.2 M Sodium Chloride	0.1 M Sodium Acetate trihydrate pH 4.6	30% v/v MPD
11	0.01 M Cobaltous Chloride hexahydrate	0.1 M Sodium Acetate trihydrate pH 4.6	1.0 M 1,6 Hexanediol
12	0.1 M Cadmium Chloride dihydrate	0.1 M Sodium Acetate trihydrate pH 4.6	30% v/v Polyethylene Glycol 400
13	0.2 M Ammonium Sulfate	0.1 M Sodium Acetate trihydrate pH 4.6	30% w/v Polyethylene Glycol Monomethyl Ether 2000
14	0.2 M Potassium Sodium Tartrate tetrahydrate	0.1 M tri-Sodium Citrate dihydrate pH 5.6	2.0 M Ammonium Sulfate
15	0.5 M Ammonium Sulfate	0.1 M tri-Sodium Citrate dihydrate pH 5.6	1.0 M Lithium Sulfate monohydrate
16	0.5 M Sodium Chloride	0.1 M tri-Sodium Citrate dihydrate pH 5.6	4% w/v Ethylene Imine Polymer
17	None	0.1 M tri Sodium Citrate dihydrate pH 5.6	35% v/v tert-Butanol
18	0.01 M Ferric Chloride hexahydrate	0.1 M tri-Sodium Citrate dihydrate pH 5.6	10% v/v Jeffamine M-600
19	None	0.1 M tri-Sodium Citrate dihydrate pH 5.6	2.5 M 1,6 Hexanediol
20	None	0.1 M MES pH 6.5	1.6 M Magnesium Sulfate heptahydrate
21	0.1 M Sodium dihydrogen phosphate mono / 0.1 M mono-Potassium dihydrogen Phosphate	0.1 M MES pH 6.5	2.0 M Sodium Chloride
22	None	0.1 M MES pH 6.5	12% w/v Polyethylene Glycol 20,000
23	1.6 M Ammonium Sulfate	0.1 M MES pH 6.5	10% v/v Dioxane
24	0.05 M Cesium Chloride	0.1 M MES pH 6.5	30% v/v Jeffamine M-600
25	0.01 M Cobaltous Chloride hexahydrate	0.1 M MES pH 6.5	1.8 M Ammonium Sulfate
26	0.2 M Ammonium Sulfate	0.1 M MES pH 6.5	30% w/v Polyethylene Glycol Monomethyl Ether 5000
27	0.01 M Zinc Sulfate heptahydrate	0.1 M MES pH 6.5	25% v/v Polyethylene Glycol Monomethyl Ether 550
28	None	None	1.6 M tri-Sodium Citrate dihydrate pH 6.5
29	0.5 M Ammonium Sulfate	0.1 M HEPES pH 7.5	30% v/v MPD
30	None	0.1 M HEPES pH 7.5	10% w/v Polyethylene Glycol 6000, 5% v/v MPD
31	None	0.1 M HEPES pH 7.5	20% v/v Jeffamine M-600
32	0.1 M Sodium Chloride	0.1 M HEPES pH 7.5	1.6 M Ammonium Sulfate
33	None	0.1 M HEPES pH 7.5	2.0 M Ammonium Formate
34	0.05 M Cadmium Sulfate hydrate	0.1 M HEPES pH 7.5	1.0 M Sodium acetate
35	None	0.1 M HEPES pH 7.5	70% v/v MPD
36	None	0.1 M HEPES pH 7.5	4.3 M Sodium Chloride
37	None	0.1 M HEPES pH 7.5	10% w/v Polyethylene Glycol 8000, 8% v/v Ethylene Glycol
38	None	0.1 M HEPES pH 7.5	20% w/v Polyethylene Glycol 10,000
39	0.2 M Magnesium Chloride hexahydrate	0.1 M TRIS pH 8.5	3.4 M 1,6 Hexanediol
40	None	0.1 M TRIS pH 8.5	25% v/v tert-Butanol
41	0.01 M Nickel(II) Chloride hexahydrate	0.1 M TRIS pH 8.5	1.0 M Lithium Sulfate monohydrate
42	1.5 M Ammonium Sulfate	0.1 M TRIS pH 8.5	12% v/v Glycerol anhydrous
43	0.2 M mono Ammonium dihydrogen Phosphate	0.1 M TRIS pH 8.5	50% v/v MPD
44	None	0.1 M TRIS pH 8.5	20% v/v Ethanol
45	0.01 M Nickel(II) Chloride hexahydrate	0.1 M TRIS pH 8.5	20% w/v Polyethylene Glycol Monomethyl Ether 2000
46	0.1 M Sodium Chloride	0.1 M Bicine pH 9.0	30% w/v Polyethylene Glycol Monomethyl Ether 550
47	None	0.1 M Bicine pH 9.0	2.0 M Magnesium Chloride hexahydrate
48	2% v/v Dioxane	0.1 M Bicine pH 9.0	10% w/v Polyethylene Glycol 20,000

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis describes many new characteristics of the dengue virus NS1 glycoprotein in terms of antigenicity and structure, its presence in patient sera and also provides a basis for the purification and screening conditions required to ultimately determine the crystal structure of the molecule.

6.1 NS1 in dengue patient sera and its role in pathogenesis

The development of an antigen capture ELISA for the detection of NS1 in patient sera has described for the first time an assay for the detection and quantitation of NS1 in the serum of dengue patients (Young *et al*; 2000). NS1 was able to be detected in dengue patients experiencing primary and secondary infections with the concentration of NS1 increasing in secondary infections. Specific antibody to NS1 was detected in dengue patients also with increased concentrations being detected in secondary infections. These combined results have hinted at a correlation between relatively high levels of NS1 in acute sera in combination with high levels of antibody to NS1 in the convalescent sera of patients with severe grades of disease (grades III and IV). On the basis of this work, a collaborative study between this laboratory and AFRIMS in Bangkok, Thailand, has utilized the NS1 capture ELISA to examine NS1 levels in dengue infected children in serum taken daily during the course of the illness. The results showed that NS1 levels greater than or equal to 600 ng / mL on presentation was an excellent prognostic marker for the subsequent development of DHF (Libraty *et al*, 2002). Utilizing the assay to determine which dengue patients are at risk of developing DHF, using NS1 as a marker, should be extremely beneficial in terms of clinical management and disease outcome.

The pathogenesis of dengue haemorrhagic fever / dengue shock syndrome has

remained largely unexplained. Many of the sequelae including plasma leakage, complement depletion and thrombocytopenia are not accounted for in the models suggested by Kurane and Ennis (1994) and Halstead (1998). Any proposed mechanism must account for all the clinical manifestations of the disease, especially the lack of pathology in major organs observed at autopsy and the rapid recovery, without significant sequelae for survivors. These observations suggest that these clinical manifestations are mediated by the local release of quick acting biological mediators capable of causing severe illness with little resulting structural injury. Although our initial results are preliminary in their findings we have hypothesized that the presence of NS1 immune complexes may contribute to the pathogenesis of dengue disease. Immune complexes have been reported in DHF patients (Theofilopoulos *et al*, 1976) and other researchers have reported that there is a direct correlation between the level of immune complexes and complement consumption in severe disease (Bokisch *et al*, 1976a, Malasit, 1987; Rothman and Ennis, 1999; Boonpucknavig *et al*, 1976b). We have suggested that it is circulating NS1 immune complexes that are able to be deposited in tissues resulting in complement activation and tissue injury. Complement may also lead to lysis of dengue infected mononuclear phagocytes via complement-mediated cytolysis, resulting in the release of potent biological mediators that could affect vascular permeability (Bhadki and Kazatchkine, 1990). NS1 immune complexes may also interact with cellular receptors resulting in the release of biologically active mediators through natural killer cell activity or ADCC (Hill *et al*, 1993; Kurane *et al*, 1984; Jacobs and Young 1998). Despite the evidence for the pivotal role of immune complexes and complement it does not explain the disease manifestations in infants undergoing primary infection who have low antibody levels. This scenario provides evidence for mechanisms elicited by cell-mediated immunity in pathogenesis. Although progress is being made towards understanding individual aspects of dengue pathogenesis, further work is required to analyse how each immune mechanism interplays in contributing to severe DHF and DSS especially with the increasing number of outbreaks occurring throughout the world.

6.2 The antigenicity and structure of NS1

Five antigenic domains and up to ten distinct epitopes on NS1 have been identified, described and putatively mapped to the tertiary structure of the protein. This is the first time that such a comprehensive antigenic map has been presented for dengue virus NS1. Other groups have described antigenic epitopes for NS1 of various flaviviruses (Henchal *et al,* 1987; Putnak *et al*, 1988; Mason *et al*, 1990; Yao et al, 1995; Garcia *et al*, 1997; Huang *et al*, 1999; Hall *et al*, 1990; Wu *et al*, 2001; Bugrysheva *et al*, 2001) however the large panel of anti-NS1 monoclonal antibodies previously produced in this laboratory (Falconar and Young, 1991) have enabled the collation of this extensive map. By examining the monoclonal antibody competition profile for different forms of NS1 derived from different cell lines, several differences were noted. The lack of recognition by MAbs of an important protective epitope on membrane-associated NS1 derived from Vero cells (the A epitope) is the likely reason for the previous observation by Falconar and Young (1990) that sNS1 generated higher levels of protection to lethally challenged experimental animals than mNS1 derived from dengue infected Vero cells. The lack of reactivity by MAbs to the epitope was found not to be a consequence of steric hinderance by an adjacent carbohydrate moiety although we did not discount the possibility that it could be due to conformational effects of carbohydrate addition and / or modification. To definitively address these possibilities would require further experimentation, either with the glycosylation inhibitor tunicamycin, or with recombinant constructs. Another possible reason for the differential recognition of the A domain, is that in mNS1, the region encompassing the A epitope is involved in membrane association and may therefore be hidden within the tertiary structure. TX-114 phase extraction experiments with proteolytic fragments supports this theory as the N-terminal third of the molecule was shown to exhibit hydrophobic properties.

A structure prediction algorithm suggested that the three alpha-helices present in the N-terminal region of flavivirus NS1 are amphipathic in nature. The hydrophobic residues comprising these helices are highly conserved implying a

functional role. Coincidently, one of these helices forms a component of the A epitope which was found to be differentially displayed in secreted and membrane-associated forms and between oligomeric forms. On this basis it is speculated that this hydrophobic domain may determine the fate of newly synthesized NS1 (Figure 6.1).

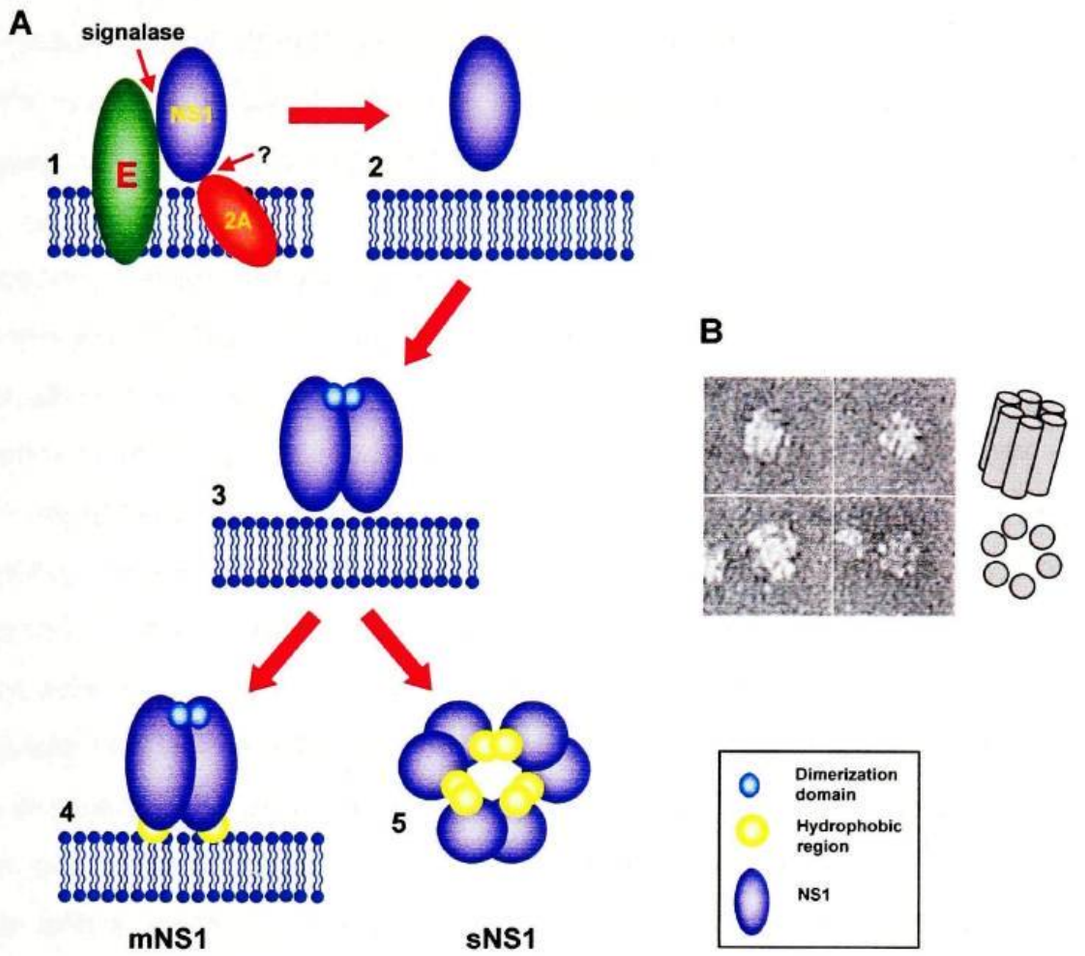


Figure 6.1 **Hypothetical model for the formation of mNS1 and sNS1. A, Schematic diagram highlighting key stages in the intra-cellular maturation pathway of NS1. NS1 is expressed in the context of a polyprotein which is post-translationally cleaved to yield the individual viral proteins. Signalase cleavage at the N-terminus and cleavage by an as yet unidentified ER resident host-cell protease at the C-terminus yields full-length NS1 (1). Reflecting its hydrophilic profile, this species is found initially as a monomeric soluble protein (2). Dimerization occurs within 10-20 minutes after synthesis via the interaction between a domain (light blue sphere) located in the C-terminal region of the protein. Dimerization is accompanied by the acquisition of a hydrophobic character which the present study has suggested is located in the N-terminal region (yellow sphere) of the protein. This induced hydrophobicity commits the dimer to either membrane-association (4) or association with other dimers (5) in order to stabilize the exposed hydrophobic domain. B, Electron micrographs of purified sNS1 highlighting apparent correlates of the hypothetical sNS1 structure viewed either from the side (top panel) or end on (bottom panel).**

Once NS1 is cleaved from NS2A in the ER by a resident protease, it is essentially a soluble monomeric hydrophilic protein. After dimerization has occurred through interaction of a domain at the highly conserved C-termini, the dimer acquires a hydrophobic character that this thesis has shown to be localized to the N-terminal third of the molecule. It is the induction of this hydrophobicity that we speculate may commit the molecule to membrane-association and its subsequent role as a component of the replication complex. In this way the hydrophobic region is stabilized by membrane-association, implying that the functional form of NS1 within the infected cell may be dimer. As NS1 levels increase to concentrations higher than that required for RNA replication, it is tempting to speculate greater chance of dimer interactions may predominate thereby stabilizing the exposed hydrophobic domain through association with two other dimers (Figure 6.1) to generate the secreted hexameric form of NS1. This hypothesis implies that the hexameric form of NS1 may simply be the result of over-expression and serves as a way of removing unwanted levels of protein from the cell. This would in turn explain why hexameric forms are readily generated by NS1 over-expressed in the baculovirus expression system as shown in Chapter 5 of this thesis. The possibility that the secreted form of NS1 also has a functional role either *in vitro* or *in vivo* cannot be discounted. It is proposed that future work examine some of these possibilities by mutating the conserved amino acids in the hydrophobic region and assaying the expressed protein for oligomer formation, secretion and hydrophobicity. It would also be critical to determine which oligomeric form of NS1 exists on cellular membranes however these experiments are inherently difficult, as detergent solubalization is usually required to purify the protein away from the membranes.

Evidence from the competition analyses that C and D domain reactive MAbs were able to compete with each other, although they are known to be located distally in the primary sequence suggests that these regions may be in close proximity in the native folded structure. This was also supported by results indicating that intermolecular competition occurs between these epitopes. Some of the data presented here is also consistent with the possibility that the

interaction between the C and D domains is hydrophobic in nature. This may explain the decrease in integrity of these domains for mNS1 that had been prepared by detergent solubalization. Further evidence is provided by a lack of passive protection provided by MAbs in the C1, C2, D1 and D2 domains, which suggests that their orientation on, or near, the infected cell surface is not optimal to provide effective ADCC or complement-mediated lysis to confer such protection.

A structural model for NS1 was provided in Figure 4.20 by collating the data from this study. We now await the three dimensional resolution of the crystal structure of native NS1 as a basis on which to compare our model.

It would also be interesting in future work to apply the competition analysis to serum from dengue patients to determine which NS1 epitopes antibody responses are directed to. In terms of vaccine development it would be invaluable to know whether specific epitope responses vary between individuals and according to the severity of subsequent disease.

6.3 Recombinant baculovirus expressed NS1 and its suitability for crystallography studies.

Recombinant baculovirus-expressed secreted NS1 was immunoaffinity purified and, in the absence of a functional assay was assessed for antigenic and biochemical integrity. It was found to resemble native NS1 in terms of cellular localization, secretion, oligomerization, glycosylation and antigenicity. The extensive antigenic map that had been established for native NS1 in Chapter 4, was invaluable in analyzing the structure of recombinant NS1 and determining that antigenically, the expressed protein had retained authentic antibody reactivity. This was reinforced with protection experiments using this recombinant protein that suggested that it may be suitable for further vaccination studies. Cross-linking and electron microscopy studies provided support for the reported hexameric structure of both native and recombinant sNS1. Crystal screens were performed with purified recombinant NS1 and several crystal

structures were obtained. Although too small for diffraction analysis the approximate buffer conditions for crystallography and methodology for purifying protein have been determined.

Future attempts at NS1 protein purification for crystallography should ideally be carried out in serum free media and protein fractions stored at 4°C as freeze / thawing was found to be detrimental to the hexameric structure. Nevertheless recombinant protein produced in serum containing media (due to supply problems) did ultimately give some crystal structures. Due to time limitations, further crystal screens could not be performed in this study but future work should focus on production of recombinant secreted NS1 from large-scale cultures of *Spodoptera frugiperda* cells. A recent collaborative study between ourselves and the Chemical Engineering Department, UQ has optimised fed-batch cultures in the presence of an insect moulting hormone to increase recombinant NS1 production (Chan *et al*, 2002). The application of this technique to recombinant NS1 production is likely to increase the yields and concentration of expressed protein and assist in the successful production of crystals suitable for structural analysis. It is envisaged that solving the crystal structure of NS1 will shed some light on the elusive biological function of the dengue-2 virus glycoprotein, NS1 and allow verification of the accuracy of our proposed structural model.

REFERENCES

Aaskov JG, Geysen HM and Mason TJ (1989) Serologically defined linear epitopes in the envelope protein of dengue-2 (Jamaica strain 1409). Arch Virol **105**, 209-221.

AbuBakar S, Azmi A, Mohamed-Saad N, Shafee N and Chee HY (1997) Antibody responses of dengue fever patients to dengue 2 (New Guinea C strain) viral proteins. Malays J Pathol **19**, 41-51.

Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V and Flamand M (2002) Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Micro **40**, 376-381.

Allison SL, Schalich J, Stiasny K, Mandl CW, and Heinz FX (2001) Mutational evidence for an internal fusion peptide in flavivirus envelope E. J Virol **75**, 4268-4275.

Allison SL, Schalich J, Stiasny K, Mandl CW, Kunz C and Heinz FX (1995) Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by acidic pH. J Virol **69**, 695-700.

Amberg SM, Nestorowicz A, McCourt DW and Rice CM (1994) NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: *in vitro* and *in vivo* studies. J Virol **68**, 3794-3802.

Amberg SM and Rice CM (1999) Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. J Virol **73**, 8083-8094.

Anderson R, King AD and Innis BL (1992) Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. J Gen Virol **73**, 2155-2159.

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

Anonymous. (1994) Yellow fever virus surveillance in Western Africa. WHO Wkly Epidem Rec **69**, 93-95.

Arias CF, Preugschat F and Strauss JH (1993) Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology **193**, 888-899.

Ashburn PM and Craig CF (1907) Experimental investigations regarding the etiology of dengue fever. J Infect Dis **4**, 440-475.

Barboni E, Pliego Rivero B, George AJT, Martin SR, Renouf DV, Hounsell EF, Barber PC and Morris RJ (1995) The glycophosphatidylinositol anchor affects the conformation of Thy-1 protein. J Cell Science **108**, 487-497.

Bartholomeusz AJ and Wright PJ (1993) Synthesis of dengue virus RNA *in vitro*: initiation and the involvement of proteins NS3 and NS5. Arch Virol **128**, 111-121.

Bazan JF and Fletterick RJ (1989) Detection of dengue trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology **171**, 637-639.

Beasley DW and Asskov JG (2001) Epitopes on the dengue 1 virus envelope protein recognized by neutralizing IgM monoclonal antibodies. Virology **20**, 447-458.

Bell JR, Kinney RM, Trent DW, Lenches EM, Dalgarno L and Strauss JH (1985) NH_2-terminal amino acid sequences of structural proteins of three flaviviruses. Virology **143**, 224-229.

Bhadki S and Kazatchkine MD (1990) Pathogenesis of dengue: an alternative hypothesis. Southeast Asian J Trop Med Public Health **21**, 652-657.

Bhamarapravati N (1981) Dengue haemorrhagic fever. Hotta S (Ed) ICMR, Kobe 207-214.

Bhamarapravati N (1989) Hemostatic defects in dengue hemorrhagic fever. J Inf Dis (Suppl 4) S826-S829.

Biedrzycka A, Cauchi MR, Bartholomeusz A, Gorman JJ and Wright PJ (1987) Characterization of protease cleavage sites involved in the formation of the envelope glycoprotein and three nonstructural proteins of dengue virus type 2, New Guinea C strain. J Gen Virol **68**, 1317-1326.

Blackwell JL and Brinton MA (1997) Translation elongation factor-1 alpha interacts with the 3' stem-loop region of West Nile genomic RNA. J Virol **71**, 6433-6444.

Blitvich BJ, Scanlon D, Shiell BJ, Mackenzie JS and Hall RA (1999) Identification and analysis of truncated and elongated species of the flavivirus NS1 protein. Virus Research **60**, 67-79.

Blitvich BJ, Scanlon D, Shiell BJ, Mackenzie JS, Pham K and Hall RA (2001) Determination of the intramolecular disulfide bond arrangement and biochemical identification of the glycosylation sites of the nonstructural protein NS1 of Murray Valley encephalitis virus. J Gen Virol **82**, 2251-2256.

Blobel G and Dobberstein B (1975) Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chain on membrane-bound ribosomes on murine myeloma. J Cell Biol **67**, 835-851.

Blum H, Beier H and Goss HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8**, 93-99.

Boege U, Heinz FX, Wengler G and Kunz C (1983) Amino acid composition and amino-terminal sequences of the structural proteins of a flavivirus, European Tick-borne encephalitis virus. Virology **126**, 651-657.

Bokish VA, Top FH, Russell PK, Dixon FJ and Muller-Eberhard HJ (1973) The potential pathogenic role of complement in dengue haemorrhagic fever syndrome. N Engl J Med **289**, 996-1000.

Boonpucknavig V, Bhamarapravati N, Boonpucknavig S, Futrakul P and Tanphaichitr P (1976a) Glomerular changes in dengue haemorrhagic fever. Arch Pathol Lab Med **100**, 206-212.

Boonpucknavig S, Bhamarapravati N, Nimmannitya S, Phalavadhtana A and Siripont J (1976b) Immunofluorescent staining of the surfaces of lymphocytes in suspension from patients with dengue haemorrhagic fever. Am J Path **85**, 37-48.

Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem **256**, 1604-1607.

Boulton RW and Westaway EG (1976) Replication of the flavivirus Kunjin: proteins, glycoproteins, and maturation associated with cell membranes. Virology **69**, 416-430.

Bost B and Sander C (1993) Prediction of protein secondary structure at better than 70% accuracy. J Mol Biol **232**, 584-599.

Bost B and Sander C (1994) Combining evolutionary information and neural networks to predict protein secondary structure. Proteins **19**, 55-77.

Brandt WE (1988) Current approaches to the development of dengue vaccines and related aspects of the molecular biology of flaviviruses. J Inf Dis **157**, 1105-1111.

Bravo JR, Guzman MG and Kouri GP (1987) Why dengue haemorrhagic fever in Cuba? 1. Individual risk factors for dengue haemorrhagic fever / dengue shock syndrome (DHF/DSS). Trans Roy Soc Trop Med Hyg **81**, 816-820.

Bray M, Zhao B, Markoff L, Eckels K, Chanock R and Lai C-J (1989) Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against dengue virus encephalitis. J Virol **63**, 2853-2856.

Brinton MA (1986) Replication of flaviviruses. In "Togaviridiae and Flaviviridiae" (S. Schlesinger and MJ Schlesinger Eds.) pp 327-374. Plenew, New York/London.

Brinton MA and Dispoto JH (1988) Sequence and secondary structure analysis of the 5'-terminal region of the flavivirus genome RNA. Virology **162**, 290-299.

Brown AWA (1977) Yellow fever, dengue and dengue haemorrhagic fever. In: A World Geography of Human Disease. Hove M (Ed.) Academic Press pp 271-317.

Bugrysheva JV, Matveeve VA, Dobrikova EY, Bykovskaya NV, Korobova SA, Bakhvalova VN abd Morozova OV (2001) Tick-borne encephalitis virus NS1 glycoprotein duting acute and persistent infection of cells. Virus Res **76**, 161-169

Bukowski JF, Kurane I, Lai C-J, Bray M, Falgout B and Ennis F (1989) Dengue virus-specific cross-reactive CD8+ human cytotoxic T lymphocytes. J Virol **63**, 5086-5091.

Burke T (1968) Dengue haemorrhagic fever: a pathological study. Trans R Soc Trop Med Hyg **5**, 682-692.

Burke DS and Monath TP (2001) Flaviviruses In: Fields Virology Vol 1 pp 1043-1125. Knipe DM and Howley PM (Eds) Philadelphia: Lippincott Williams and Wilkins.

Burke DS, Nisalak A, Johnson DE and Scott, RM (1988) A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg **38**, 172-180.

Butters TD and Hughes RC (1981) Isolation and characterization of mosquito cell membrane glycoproteins. Biochem Biophys Acta **640**, 655-671.

Butters TD, Hughes RC and Vischer P (1981) Steps in the biosynthesis of mosquito cell membrane glycoproteins and the effects of tunicamycin. Biochim Biophys Acta **640**, 672-686.

Cahour A, Falgout B and Lai CJ (1992) Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junction is mediated by viral proteinase NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J Virol **66**, 1525-1542.

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE and Porterfield JS (1989) Antigenic relationships between flaviviruses as detected by cross-neutralization tests with polyclonal antisera. J Gen Virol **70**, 37-43.

Cauchi MR, Henchal EA and Wright PJ (1991) The sensitivity of cell-associated dengue virus proteins to trypsin and the detection of trypsin-resistant fragments of the non-structural protein NS1. Virology **180**, 659-667.

Cardosa MJ, Gordon S, Hirsch S, Springer TA and Porterfield JS (1986) Interaction of West Nile virus with primary murine macrophages: role of cell activation and receptors for antibody and complement. J Virol **57**, 952-959.

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

Castle E, Nowak T, Leidner U, Wengler G and Wengler G (1985) Sequence analysis of the viral core protein and the membrane-associated proteins V1 and NV2 of the flavivirus West Nile virus and of the genome sequence for these proteins. Virology **145**, 227-236.

Catanzaro PJ, Brandt WE, Hogrefe WR and Russell PK (1974) Detection of dengue cell-surface antigens by peroxidase-labeled antibodies and immune cytolysis. Infect Immunol **10**, 381-388.

Cardiff RD and Lund JK (1976) Distribution of dengue-2 antigens by electron immunocytochemistry. Infect Immun **13**, 1699-1709.

Cecilia D and Gould EA (1991) Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. Virology **181**, 70-77.

Chambers TJ, Hahn CS, Galler R and Rice CM (1990a) Flavivirus genome organization, expression and replication. Ann Rev Microbiol **44**, 649-688.

Chambers TJ, McCourt DW and Rice CM (1989) Yellow fever virus proteins NS2A, NS2B and NS4B: identification and partial N-terminal amino acid sequence analysis. Virology **169**, 100-109.

Chambers TJ, McCourt DW and Rice CM (1990c) Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. Virology **177**, 159-174.

Chambers TJ, Nestorowicz A, Amberg SM and Rice CM (1993) Mutagenesis of the yellow fever virus NS2b protein: effects on proteolytic processing, NS2B-NS3 complex formation and viral replication. J Virol **67**, 6797-6807.

Chambers TJ, Weir RC, Grakoui A, McCourt DW, Bazan JF, Fletterick RJ and Rice CM (1990b) Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. Proc Natl Acad Sci USA **87**, 8898-8902.

Chan C, Young PR, Bletchly C and Reid S (Accepted March 2002) Production of the baculovirus-expressed Dengue virus glycoprotein NS1 can be dramatically improved with optimised regimes for fed-batch cultures and the addition of the insect moulting hormone, 20-hydroxyecdysone. J Virol Methods.

Chandler AC and Rice L (1923) Observations on the etiology of dengue fever. Am J Trop Med Hyg **3**, 233-262.

Chang DM and Shaio MF (1994) Production of interleukin-1 (IL-1) and IL-1 inhibitor by human monocytes exposed to dengue virus. J Infect Dis **170**, 811-817.

Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ and Marks RM (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nature Medicine **3**, 866-871.

Chu PW and Westaway EG (1985) Replication strategy of Kunjin virus: evidence for recycling role of replicative form RNA as template in semiconservative and asymmetric replication. Virology **140**, 68-79.

Chu PWG and Westaway EG (1992) Molecular and ultrastructural analysis of heavy membrane fractions associated with the replication of Kunjin virus RNA. Arch Virol **124**, 177-191.

Churdboonchart V, Bhamarapravati N, Peampramprecha S and Sirinavin S (1991) Antibodies against dengue virus proteins in a primary and secondary dengue hemorrhagic fever. Am J Trop Med Hyg **44**, 481-493.

Churdboonchart V, Bhamarapravati N and Futrakul P (1983) Crossed immunoelectrophoresis for the detection of split products of the third complement in dengue hemorrhagic fever I. Observations in patients' plasma. Am J Trop Med Hyg **32**, 569-576.

Clarke T (2002) Break-bone fever. Nature **416**, 672-674.

Cleaves GR, Ryan TE and Schlesinger RW (1981) Identification and characterization of type 2 dengue virus intermediate and replicative form RNAs. Virology **111**, 73-83.

Clegg JCS (1982) Glycoprotein detection in nitrocellulose transfers of electrophoretically separated protein mixtures using Concavalin A and peroxidase: Application to arenavirus and flavivirus proteins. Anal Biochem **127**, 389-394.

Cohen SN and Halstead SB (1966) Shock associated with dengue infection. I. Clinical and physiologic manifestations of dengue hemorrhagic fever in Thailand, 1964. J Pediatrics **68**, 448-456.

Cochrane CG and Dixon FJ (1976) Antigen-antibody complex induced disease. Textbook of Immunopathology. 2nd Ed. Miescher PA and Mueller-Eberhard HJ (Eds) New York: Grune and Stratton **1**, 137-156.

Coia G, Parker MD, Speight G, Byrne ME and Westaway EG (1988) Nucleotide and complete amino acid sequences of Kunjin virus: Definitive gene order and characteristics of the virus-specified proteins. J Gen Virol **68**, 1-21.

Cooper NR (1985) The classical complement pathway: activation and regulation of the first complement component. In: Advances in Immunology. Kunkel HG and Dixon FJ (Eds) New York: Academic Press 151-216.

Copanaris P (1928) L'épidémie de dengue en Grèce au cours de l'été 1928. Bull Internat Hyg Pub **20**, 1590-1601.

Copeland CS, Zimmer KP, Wagner KR, Healey GA, Mellman I and Helenius A (1988) Folding, trimerization, and transport are sequential events in the biogenesis of Influenza virus hemagglutinin. Cell **53**, 197-209.

Cornain S, Ikeuchi H and Sumarmo (1987) Immunological changes and recovery in patients with dengue heamorrhagic fever. SE Asian J Trop Med Pub Hlth **18**, 340-345.

Cosgriff M (1991) Mechanisms of disease in Hantavirus infection: pathophysiology of haemorrhagic fever with renal syndrome. Rev Inf Dis **13**, 97-107.

Crill WD and Roehrig JT (2001) Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J Virol **75**, 7769-7773.

Crooks AJ, Lee JM, Dowsett AB and Stephenson JR (1990) Purification and analysis of infectious virions and native non-structural antigens from cells infected with tick-borne encephalitis virus. J Chromatogr **502**, 59-68.

Crooks AJ, Lee JM, Easterbrook LM, Tinofeev AV and Stephenson JR (1994) The NS1 protein of tick-borne encephalitis forms multimeric species upon secretion from the host cell. J Gen Virol **75**, 3453-3460.

Cullinan EB (1946) Medical disorders in East Africa. Trans Roy Soc Trop Med Hyg **39**, 353-368.

Dalgarno I, Trent DW, Strauss JH and Rice CM (1986) Partial nucleotide sequence of the Murray Valley encephalitis virus genome: competition of the encoded polypeptides with yellow fever virus structural and nonstructural proteins. J Mol Biol **187**, 309-323.

Daughaday CC, Brandt WE, McCown JM and Russell PK (1981) Evidence for two mechanisms of dengue virus infection of adherent human monocytes: trypsin-sensitive virus receptors and trypsin-resistant immune complex receptors. Inf Imm **32**, 469-473.

De Jesus Martinez-Barragan JJ and del Angel R (2001) Identification of a putative coreceptor on Vero cells that participates in dengue 4 virus infection. J Virol **75**, 7818-7827.

De Madrid AT and Porterfield JS (1974) The flaviviruses (group B arboviruses): a cross-neutralization study. J Immunol **23**, 91-96.

Desprès P, Girard M and Bouloy M (1991) Characterization of yellow fever virus proteins E and NS1 expressed in Vero and *Spodoptera frugiperda* cells. J Gen Virol **72**, 1331-1342.

Deubel V, Kinney RM, Esposito JJ, Cropp CB, Vorndam AV, Monath TP and Trent D (1988) Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. J Gen Virol **69**, 1921-1929.

Deubel V, Kinney RM and Trent DW (1986) Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype. Virology **155**, 365-377.

Deubel V, Kinney RM and Trent DW (1988) Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology **165**, 234-244.

Downs WG (1964) Human infections with arboviruses in Trinidad, W. I. Am J Trop Med Hyg **13**, 337-382.

Eisenhaber B, Bork P and Eisenhaber F (1998) Sequence properties of GPI-anchored proteins near the ω - site: constraints for the polypeptide binding site of the putative transamidase. Prot Eng **11**, 1155-1161.

Elder JH and Alexander S (1982) Endo-β-N-Acetylglucosaminidase F: Endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. PNAS **79**, 4540-4544.

Falconar AK (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol **142**, 897-917.

Falconar AK and Young PR (1990) Immunoaffinity purification of native dimer forms of the flavivirus non-structural glycoprotein, NS1. J Virol Methods **30**, 323-332.

Falconar AKI and Young PR (1991) Production of dimer-specific and dengue virus group cross-reactive monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol **72**, 961-965.

Falconar AKI, Young PR and Miles MA (1994) Precise location of sequential dengue virus subcomplex and complex B cell epitopes on the nonstructural-1 glycoprotein. Arch Virol **137**, 315-326.

Falgout B, Chanock R and Lai C-J (1989) Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J Virol **63**, 1852-1860.

Falgout B and Markoff L (1995) Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. J Virol **69**, 7232-7243.

Falgout B, Pethel M, Zhang Y-M and Lai C-J (1991) Both nonstructural proteins NS2b and NS3 are required for the proteolytic processing of Dengue virus nonstructural proteins. J Virol **65**, 2467-2475.

Falgout B, Bray M, Sclesinger JJ (1990) Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus non structural protein NS1 protects against lethal dengue virus encephalitis. J Virol **64**, 4356-4363.

Falker WA, Diwan AR and Halstead SB (1973) Human antibody to dengue soluble complement-fixing (SCF) antigens. J Immunol **111**, 1804-1809.

Fan W and Mason PW (1990) Membrane association and secretion of the Japanese encephalitis virus NS1 protein from cells expressing NS1 cDNA. Virology **177**, 470-476.

Ferguson N, Anderson R and Gupta S (1999) The effect of antibody-dependent enhancement on the transmission dynamics and persistence of multiple strain pathogens. PNAS **96**, 790-794.

Ferguson MAJ and Williams AF (1988) Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Ann Rev Biochem **57**, 285-320.

Ferlenghi I, Clarke M, Ruttan T, Allison SL, Schalich J, Heinz FX, Harrison SC, Rey FA and Fuller SD (2001) Molecular organization of a recombinant subviral particle from tick-borne encephalitis virus. Mol Cell **7**, 593-602.

Fischer DB and Halstead SB (1970) Observations related to pathogenesis of dengue hemorrhagic fever. Yale J Biol Med **42**, 329-349.

Flamand M, Chevalier M, Henchal E, Girard M and Deubel V (1995) Purification and renaturation of Japanese encephalitis virus nonstructural glycoprotein NS1 overproduced by insect cells. Prot Exp Purif **6**, 519-527.

Flamand M, Deubel V and Girard M (1992) Expression and secretion of Japanese encephalitis virus nonstructural protein NS1 by insect cells using a recombinant baculovirus. Virology **191**, 826-836.

Flamand M, Megret F, Mathieu M, Lepault J, Rey FA and Deubel V (1999) Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol **73**, 6104-6110.

Fu J, Tan B-H, Yap E-H, Chan Y-C and Jan YH (1992) Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275-90). Virology **188**, 953-958.

Funahara Y, Ogawa K, Fujita N and Okuno Y (1987) Three possible triggers to induce thrombocytopenia in dengue virus infection. SE Asian J Trop Med Pub Hlth **18**, 351-355.

Gagnon SJ, Zeng W, Kurane I and Ennis FA (1996) Identification of two epitopes on dengue 4 virus capsid protein recognized by a serotype-specific and a panel of serotype cross-reactive human CD4+ cytotoxic T-lymphocyte clones. J Virol **70**, 141-147.

Gao GF, Hussain MH, Reid HW and Gould EA (1994) Identification of naturally occurring monoclonal antibody escape variants of louping ill virus. J Gen Virol **75**, 609-614.

Garcia G, Vaughn DW and Del Angel RM (1997) Recognition of synthetic oligopeptides from nonstructural proteins NS1 and NS3 of dengue-4 virus by sera from dengue virus-infected children. Am J Trop Med Hyg **56**, 466-470.

Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RWH, Zarski JP and Drouet E (2002) Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. Virology **292**, 162-168

Gibson CA, Schlesinger JJ and Barrett ADT (1988) Prospects for a virus non-structural protein as a subunit vaccine. Vaccine **6**, 7-9.

Gilbertson WE (1945) Sanitary aspects of the control of the 1943-1944 epidemic of dengue fever in Honolulu. Am J Pub Hlth **35**, 261-270.

Gollins SW and Porterfield JS (1985) Flavivirus infection enhancement in macrophages: an electron microscope study of viral cellular entry. J Gen Virol **66**, 1969-1982.

Gollins SW and Porterfield JS (1986a) pH-dependent fusion between the flavivirus West Nile and liposomal model membranes. J Gen Virol **67**, 157-166.

Gollins SW and Porterfield JS (1986b) The uncoating and infectivity of the flavivirus West Nile on interaction with cells: effects of pH and ammonium chloride. J Gen Virol **67**, 1941-1950.

Gong YH, Trowbridge R, Macnaughton TB, Westaway EG, Shannon AD and Gowans EJ (1996) Characterization of RNA synthesis during a one-step growth curve and the replication mechanism of bovine viral diarrhoea virus. J Gen Virol **77**, 2729-2736.

Gong Y, Shannon A, Westaway EG and Gowans EJ (1988) The replicative intermediate molecule of bovine viral diarrhoea virus contains multiple nascent strands. Arch Virol **143**, 399-404.

Gorbalenya AE, Donchenko AP, Koonin EV and Blinov VM (1989) N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. Nucleic Acids Res **17**, 3889-3897.

Gorbalenya AE, Koonin EV, Donchenko AP and Blinov VM (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res **17**, 4713-4729.

Gotoff R, Tamura M, Janus J, Thompson J, Wright P and Ennis FA (1994) Primary influenza A virus infection induces cross-reactive antibodies that enhance uptake of virus into F_c receptor-bearing cells. J Inf Dis **169**, 200-203.

Gould EA, Buckley A, Barrett ADT and Cammack N (1986) Neutralising (54K) and non-neutralising (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. J Gen Virol **67**, 591-595.

Gould EA, Buckley A, Cammack N, Barrett AD, Clegg JC, Ishak R and Varma MG (1985) Examination of the immunological relationships between flaviviruses using yellow fever virus monoclonal antibodies. J Gen Virol **66**, 1369-1382.

Gould EA and Buckley A (1989) Antibody-dependent enhancement of yellow fever and Japanese encephalitis virus neurovirulence. J Gen Virol **70**, 1605-1608.

Gould EA, Buckley A, Groeger BK, Cane PA and Doenhoff M (1987) Immune enhancement of yellow fever virus neurovirulence for mice: studies of the mechanisms involved. J Gen Virol **68**, 3105-3112.

Gould EA, Buckley A, Higgs S and Gaidamovick S (1990) Antigenicity of flaviviruses. Archives Virology (Suppl 1) 137-152.

Gratz NG and Knudsen (1996) The rise and spread of dengue, dengue haemorrhagic fever and its vectors. WHO

Green S, Kurane I, Edelman R, Tacket CO, Eckels KH, Vaughn DW, Hoke CH Jr and Ennis FA (1993) Dengue-virus specific human CD4+ T-lymphocyte responses in a recipient of an experimental live-attenuated virus type 1 vaccine: Bulk culture proliferation, clonal analysis and presursor frequency determination. J Virol **67**, 5962-5967.

Green S, Vaughn D, Kalayanarooj S, Nimmannitya S, Suntayakorn S, Nisalak A, Lew R, Innis BL, Kurane I, Rothman AL and Ennis FA (1999) Early immune activation in acute dengue is related to development of plasma leakage and disease severity. J Infect Dis **179**, 755-762.

Grun JB and Brinton MA (1987) Dissociation of NS5 from cell fractions containing West Nile virus-specific polymerase activity. J Virol **61**, 3641-3644.

Grun JB and Brinton MA (1988) Separation of functional West Nile virus replication complexes from intracellular membrane fragments. J Gen Virol **69**, 3121-3127.

Gualano RC, Pryor MJ, Cauchi MR, Wright PJ and Davidson AD (1998) Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. J Gen Virol **79**, 437-446.

Gubler DJ (1988) Dengue. Monath TP (Ed) In: The arboviruses: ecology and epidemiology. Vol II Boca Raton, FL:CRC Press 223-260.

Gubler DJ (1994) Prevention and control of epidemic dengue in north Queensland, Australia. Dengue Surveillance Summary **68**, 1-3.

Gubler DJ (1997) Dengue and dengue hemorrhagic fever; Its history and resurgence as a global public health problem. In: Dengue and dengue hemorrhagic fever. Gubler DJ and Kuno G (Eds) pp CAB International, London 1-22.

Gubler DJ (1999) Dengue viruses. In Encyclopaedia of Virology 2nd ed. Granoff A and Webster RG (Eds) Academic Press, San Diego. 375-384.

Gubler DJ (2002) Epidemic dengue / dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends in Microbiology **10**, 100-103.

Gubler DJ and Clark GG (1995) Dengue / dengue hemorrhagic fever: The emergence of a global health problem. Emerging Infectious Dis **1**, 55-57.

Gubler DJ and Metzler M (1999) Impact of dengue / dengue hemorrhagic fever on the developing world. Adv Virus Res **53**, 35-70.

Gubler DJ, Suharyono W, Tan R, Abidin M and Sie A (1981) Viraemia in patients with naturally acquired dengue infection. Bull World Health Org **59**, 623-630.

Guirakhoo F, Heinz FX and Kunz C (1989) Epitope model of tick-borne encephalitis virus envelope glycoprotein E: analysis of structural properties, role of carbohydrate side chain and conformational changes occurring at acidic pH. Virology **169**, 90-99.

Guirakhoo F, Heinz FX, Mandl CW, Holzmann H and Kunz C (1991) Fusion activity of flaviviruses: composition of mature and immature (pr-M containing) tick-borne encephalitis virions. J Gen Virol **72**, 1323-1329.

Guirakhoo F, Hunt AR, Lewis JG and Roehrig JT (1993) Selection and partial characterization of dengue 2 virus mutants that induce fusion at elevated pH. Virology **194**, 219-223.

Guyre PM, Morganerli P and Miller R (1983) Recombinant immune interferon increases immunoglobulin G F_c receptors on cultured human mononuclear phagocytes. J Clin Invest **72**, 393-397.

Hack CE, De Groot ER, Fely-Bersma RJ, Nuijens JH, Strack Van Schijndel RJ, Erenberg-Belmer AJ, Thijs LG and Aarden LA (1989) Increased plasma levels of interleukin-6 in sepsis. Blood **74**, 1704-1710.

Hahn YS, Galler R, Hunkapiller T, Dalrymple JM, Strauss JH and Strauss EG (1988) Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology **162**, 167-180.

Hahn CS, Hahn YS, Rice CM, Lee E, Dalgarno L, Strauss EG and Strauss JH (1987) Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. J Mol Biol **198**, 33-41.

Hall RA, Broom AK, Hartnett AC, Howard MJ and Mackenzie JS (1995) Immunodominant epitopes on the NS1 protein of MVE and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum. J Virol Methods **51**, 201-210.

Hall RA, Khromykh AA, Mackenzie JM, Scherret JH, Khromykh TI and Mackenzie JS (1999) Loss of dimerization of the nonstructural protein NS1 of Kunjin virus delays viral replication and reduces virulence in mice, but still allows secretion of NS1. Virology **264**, 66-75.

Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk HD and Garten W (1992) Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature **360**, 358-361.

Halstead SB (1979) *In vivo* enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Inf Dis **140**, 527-533.

Halstead SB (1980) Immunological parameters of togavirus disease syndromes. In: The Togaviruses. RW Schlesinger (Ed) Academic Press, New York pp 107-173.

Halstead SB (1981) The pathogenesis of dengue: molecular epidemiology in infectious disease. Am J Epidemiol **114**, 632-648.

Halstead SB (1984) Selective primary health care: strategies for the control of disease in the developing world. XI Dengue. Rev Infect Dis **6**, 251-264.

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

Halstead SB (1989) Antibody, macrophages, dengue virus infection, shock and hemorrhage: a pathogenic cascade. Revs Inf Dis II (supplement 4), 831-839.

Halstead SB, O'Rouke EJ and Allison AC (1977) Dengue viruses and mononuclear phagocytosis. II. Identity of blood and tissue leucocytes supporting *in vitro* infection. J Exp Med **146**, 218-229.

Halstead SB, Porterfield JS and O'Rourke EJ (1980) Enhancement of dengue virus infection in monocytes by flavivirus antisera. Am J Trop Med Hyg **29**, 638-642.

Halstead SB, Shotwell H and Casals J (1973) Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. J Infect Dis **128**, 15-22.

Hammon WM (1973) Dengue hemorrhagic fever - Do we know its cause? Am J Trop Med Hyg **22**, 82-91.

Hammon WM, Rudnik A and Sather GE (1960) Viruses associated with epidemic haemorrhagic fevers of the Phillipines and Thailand. Science **131**, 1102-1103.

Hanna JN, Ritchie DA, Phillips DA, Lee JM, Hills SL, van den Hurk AF, Pyke AT, Johansen CA and Mackenzie JS (1999) Japanese encephalitis in north Queensland, Australia, 1998. Med J Aust **170**, 533-536.

Harrison SC (1989) Common features in the design of small RNA viruses. In: Concepts in viral pathogenesis III. Notkins AL and Oldstone MBA (Eds.) Springer, New York 3-19.

Hase T, Summers PL and Eckels KH (1989a) Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. Arch Virol **104**, 129-143.

Hase T, Summers PL, Eckels KH and Putnak JR (1989b) Morphogenesis of flaviviruses. In: Subcellular Biochemistry **15**, 275-305.

Hasegawa H, Yoshida M, Shiosaka T, Fugita S and Kobayashi Y (1992) Mutation in the envelope protein of Japanese encephalitis virus affect entry into cultured cells and virulence in mice. Virology **191**, 158-165.

He R-T, Innis BL, Nisalak A, Usawattanakul W, Wang S, Kalayanarooj S and Anderson R (1995) Antibodies that block attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2. J Med Virol **45**, 451-461.

Heinz FX (1986) Epitope mapping of flavivirus glycoproteins. Advances Virus Res **31**, 103-168.

Heinz FX, Aver G, Stiasny K, Holzmann H, Mandl C, Guirakhoo F and Kunz C (1994) The interactions of the flavivirus envelope proteins: implications for virus entry and release. Arch Virol **9**, 339-348.

Heinz FX, Berger R, Tuma W and Kunz C (1983) A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology **126**, 525-537.

Heinz FX and Kunz C (1980) Chemical crosslinking of tick-borne encephalitis virus and its subunits. J Gen Virol **46**, 301-309.

Heinz FX, Stiansy K, Puschner-Auer G, Holzmann H, Allison S, Mandl CW and Kunz C (1994) Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimic association with the protein prM. Virology **198**, 109-117.

Heish P and Robbins PW (1984) Regulation of asparagine-linked oligosaccaride processing: oligosaccaride processing in *Aedes albopictus* mosquito cells. J Biol Chem **259**, 2375-2382.

Henchal EA, Gentry MK, McCown JM and Brandt WE (1982) Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am J Trop Med Hyg **31**, 830-836.

Henchal EA, Henchal LS and Schlesinger JJ (1988) Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol **69**, 2101-2107.

Henchal EA, Henchal LS and Thaisomboonsuk BK (1987) Topological mapping of unique epitopes of the dengue-2 virus NS1 protein using monoclonal antibodies. J Gen Virol **68**, 845-851.

Henchal EA, McCown JM, Burke DS, Seguin MC and Brandt WE (1985) Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. Am J Trop Med Hyg **34**, 162-169.

Henchal EA and Putnak JR (1990) The dengue viruses. Clin Microbiol Rev **3**, 376-396.

Hilgard P and Stockert R (2000) Heparan sulfate proteoglycans initiate dengue virus infection of hepatocytes. Hepatology **32**, 1069-1077.

Hill AB, Lobigs M, Blanden RV, Kulkarni A and Mullbacher A (1993) The cellular immune response to flaviviruses. Marcel Dekker Inc, New York.

Hiramatsu K, Todano M, Men R and Lai CJ (1996) Mutation analysis of a neutralization epitope on the dengue type 2 virus envelope protein: Monoclonal antibody resistant DEN2 / DEN4 chimeras exhibit reduced mouse neurovirulence. Virology **224**, 437-445.

Hober D, Poli L, Roblin B, Gestas P, Chungue E, Granic G, Imbert P, Pecarere JL, Vergez-Pascal R and Wattre P (1993) Serum levels of tumour necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue infected patients. Am J Trop Med Hyg **48**, 324-331.

Hober D, Shen L, Benyoucef S, De Groote D, Deubel V and Watte P (1996) Enhanced TNFα production by monocyte-like cells exposed to dengue virus antigens. Immunol Lett **53**, 115-120.

Hogle JM, Syed R, Yeates TO, Jacobsen D, Critchlow T and Filman DJ (1989) Structural determinants of serotype specificity and host range in polio virus. In Concepts in viral pathogenesis III. Notkins AL and Oldstone MBA (Eds.) Springer, New York. 20-29.

Hoke CH, Nisalak A, Sangawhipa N, Jatanasen S, Laorakapongse T, Innis BL, Kotchasenee S-O, Gingrich JB, Latendresse J, Fukai K and Burke DS (1988) Protection against Japanese encephalitis by inactivated vaccines. N Eng J Med **319**, 608-614.

Holland JJ, Spindler V, Horodyski F, Grabau E, Nichol S and Venderpol S (1982) Rapid evolution of RNA genomes. Science **215**, 1577-1585.

Holzmann H, Mandl CW, Guirakhoo F, Heinz FX and Kunz C (1989) Characterization of antigenic variants of tick-borne encephalitis selected with neutralizing monoclonal antibodies. J Gen Virol **70**, 219-222.

Hori H and Lai C-J (1990) Cleavage of dengue virus NS1-NS2a requires an octapeptide sequence at the C terminus of NS1. J Virol **64**, 4573-4577.

Hotta H, Wiharta AS and Hotta S (1984) Antibody-mediated enhancement of dengue virus in macrophage cell lines, Mk1 and Mm1 (41802). Proc Soc Exp Biol Med **175**, 320-327.

Huang J-L, Huang J-H, Shyu R-H, Teng C-W, Lin Y-L, Kuo M-D, Yao C-W and Shaio M-F (2001) High level expression of recombinant dengue viral NS1 protein and its potential use as a diagnostic antigen. J Med Virol **65**, 553-560.

Huang JH, Wey JJ, Sun YC, Chin C, Chien LJ and Wu YC (1999) Antibody responses to an immunodominant nonstructural 1 synthetic peptide in patients with dengue fever and dengue hemorrhagic fever. J Med Virol **57**, 1-8.

Iacono-Connors LC, Smith JF, Ksiazek TG, Kelley CL and Schmaljohn CS (1996) Characterization of Langat virus antigenic determinants defined by monoclonal antibodies to E, NS1 and prM and identification of a protective, non-neutralizing preM-specific monoclonal antibody. Virus Res **43**, 125-136.

Igarashi A (1984) A hypothesis on the geographical distribution of arboviruses. Trop Med **26**, 173-179.

Illangumaran S, Robinson PJ and Hoessli DC (1996) Transfer of exogenous glycosylphosphatidyl inositol (GPI)-linked molecules to the plasma membrane surface. Trends Cell Biol **6**, 163-167.

Innis BL, Thirawuth V and Hemachhdha C (1989) Identification of continuous epitopes of the envelope glycoprotein of dengue type 2 virus. Am J Trop Med Hyg **40**, 676-687.

Irie K, Mohan PM, Sasaguri Y, Putnak R and Padmanabhan R (1989) Sequence analysis of cloned dengue virus type 2 genome (New Guinea-C strain). Gene **75**, 197-211.

Ishak R, Tovey DG and Howard CR (1988) Morphogenesis of yellow fever virus 17D in infected cell cultures. J Gen Virol **69**, 325-335.

Jacobs MG, Robinson PJ, Bletchly C, Mackenzie JM and Young PR (2000) Dengue virus nonstructural protein 1 is expressed in glycosylphosphatidyl-inositol-linked form that is capable of signal transduction. The FASEB J **14**, 1603-1610.

Jacobs SC, Stephenson JR and Wilkinson GWG (1992) High-level expression of the tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector: protection elicited in a murine model. J Virol **66**, 2086-1095.

Jacobs SC, Stephenson JR and Wilkinson GWG (1993) Sequence of the NS1 gene of the K23 isolate of tick borne encephalitis virus and identification of conserved motifs. Arch Virol **129**, 279-285.

Jacobs SC, Stephenson JR and Wilkinson GWG (1994) Protection elicited by a replication - defective adenovirus vector expressing the tick-borne encephalitis virus non-structural glycoprotein NS1. J Gen Virol **75**, 2399-2402.

Jacobs MG and Young PR (1998) Dengue: a continuing challenge for molecular biology. Current Opinion Infect Dis **11**, 319-324.

Jarvis DL and Finn EE (1995) Biochemical analysis of the N-glycosylation in baculovirus-infected Lepidopteran insect cells. Virology **212**, 500-511.

Jarvis DL and Summers MD (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus infected cells. Mol Cell Biol **9**, 214-223.

Jiang WR, Lowe A, Higgs S, Reid H and Gould ER (1993) Single amino acid codon changes detected in louping ill virus antibody-resistance mutants with reduced neurovirulence. J Gen Virol **74**, 931-935.

Jianmin Z, Linn ML, Bulich R, Gentry MK and Aaskov JG (1995) Analysis of functional epitopes on the dengue 2 envelope (E) protein using monoclonal IgM antibodies. Arch Virol **140**, 899-913.

Johnson BK and Chanas AC (1981) The potential for the spread of arboviruses into new areas and for their subsequent persistence. Abstr Hyg Comm Dis **56**, 165-180.

Kalayanarooj S, Vaughn DW, Nimmannitya S, Green S, Suntayakorn S, Kunentrasai N, Viramitrachai W, Ratanachu-eke S, Kiatpolpoj S, Innis BL, Rothman AL, Nisalak A and Ennis FA (1997) Early clinical and laboratory indicators of acute dengue illness. J Infect Dis **176**, 313-321.

Kamer G and Argos P (1984) Primary structural comparison of RNA-dependent polymerases from plant, animal, and bacterial viruses. Nucleic Acid Res **12**, 7269-7282.

Karabatsos K (1980) General characteristics and antigenic relationships. In: St. Louis encephalitis. Monath TP (Ed) Washington DC: American Public Health Association 105-158.

Kaufman BM, Summers PL, Dubois DR and Eckels KH (1987) Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg **36**, 427-434.

Kaufman BM, Summers PL, Dubois DR, Housten Cohen W, Gentry MK, Timchak RL, Burke DS and Eckels KH (1989) Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg **41**, 576-580.

Kelly HA (1907) Walter Reed and yellow fever. McClune, Phillips and Co., New York.

Kestens L, Hoofd G, Gigase PL, Deleys R and van der Groen G (1991) HIV antigen detection in circulating immune complexes. J Virol Methods **31**, 67-76.

Khromykh AA, Meka H, Guyatt KJ and Westaway EG (2001) Essential role of cyclization sequences in flavivirus replication. J Virol **75**, 6719-6728.

Khromykh AA, Sedlak PL, Guyatt KJ, Hall RA and Westaway EG (1999a) Efficient *trans*-complementation of the flavivirus kunjin NS5 protein but not of the NS1 protein requires its coexpression with other components of the viral replicase. J Virol **73**, 10272-10280.

Khromykh AA, Sedlak PL and Westaway EG (1999b) *trans*-complementation analysis of the flavivirus Kunjin NS5 gene reveals an essential role for translation of its N-terminal half in RNA replication. J Virol **73**, 9247-9255.

Khromykh AA, Varnavski AN, Sedlak PL and Westaway EG (2001) Coupling between replication and packaging of flavivirus RNA: evidence derived from the use of DNA-based full-length cDNA clones of Kunjin virus. J Virol **75**, 4633-4640.

Khromykh AA, Varnavski AN and Westaway EG (1998) Encapsidation of the flavivirus kunjin relicon RNA by using a complementation system providing Kunjin structural proteins in trans. J Virol **72**, 5967-5977.

Kimura R and Hotta S (1944) On the inoculation of dengue virus into mice. Nippon Igakku **3379**, 629-633.

Kimura T, Kimura-Kuroda J, Nagashima K and Yasui K (1994) Analysis of virus-cell binding characteristics on the determination of Japanese encephalitis virus susceptibility. Arch Virol **139**, 239-251.

Kimura-Kuroda J and Yasui K (1988) Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. J Immunol **141**, 3606-3610.

Kitts PA, Ayres MD and Possee RD (1990) Linearization of baculovirus DNA enhances the recovery of recombinant viral expression vectors. Nucleic Acids Research **18**, 5667-5672.

Kitts PA and Possee RD (1993) A method for producing recombinant baculovirus expression vectors at high frequency. Biotechniques **14**, 810-817.

Klausner RD, Donaldson JG and Lippincott-Schwartz J (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol **116**, 1071-1080.

Kliks SC, Nimmanitya S and Burke DS (1988) Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg **38**, 411-419.

Kliks SC, Nisalak A, Brandt WE, Wahl L and Burke DC (1989) Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg **40**, 444-451.

Ko KK, Igarashi A and Fukai K (1979) Electron microscopic observation on *Aedes albopictus* cells infected with dengue virus. Arch Virol **62**, 41-52.

Kodukula K, Cines D, Amthauer R, Gerber L and Udenfriend S (1992) Biosynthesis of phoshatidylinositol glycan (PI-G) anchored membrane proteins in cell free systems: cleavage of the nascent protein and addition of the PI-G moiety depend on the size of the carboxy-terminal signal peptide. PNAS **89**, 1350-1353.

Kokernut RH, Smithburn KC and Weinburn MP (1956) Neutralizing antibodies to arthropod-borne viruses in human beings and animals in the Union of South Africa. J Immunol **77**, 313-323.

Koonin EV (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol **72**, 2197-2206.

Koonin EV (1993) Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. J Gen Virol **74**, 733-740.

Kouri G, Guzman MG and Bravo J (1986) Hemorrhagic dengue in Cuba: history of an epidemic. Bull PAHO **20**, 24-30.

Kozak (1984) Selection of initiation sites by eucaryotic ribosomes: effect of AUG triplets upstream from the coding sequences for preprocessing. NAR **12**, 3873-3893.

Kries TE and Lodish HF (1986) Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. Cell **46**, 929-937.

Krishna Murphy HM, Clum S and Padmanabhan R (1999) Dengue virus NS3 serine protease: crystal structure and insights into interaction of the active site of substrates by molecular modeling and structural analysis of mutational effects. J Biol Chem **274**, 5573-5580.

Krishna Murphy HM, Judge K, DeLucas L and Padmanabhan R (2000) Crystal structure of dengue virus NS3 protease in complex with a Bowman-Birk inhibitor: implications for flaviviral polyprotein processing and drug design. J Mol Biol **301**, 759-767.

Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay S, Chipman PR, Strauss EG, baker TS, Strauss JH (2002) Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell **108**, 717-725.

Kulkarni AB, Mullbacher A and Blanden RV (1991) *In vitro* T-cell poliferative response to the flavivirus, West Nile. Viral Immunol **4**, 73-82.

Kuno G (1982) Dengue virus replication in a polyploid mosquito cell culture grown in serum-free medium. J Clin Micro **16**, 851-855.

Kuno G, Vornkam AV, Gubler DJ and Gomez I (1990) Study of anti-dengue NS1 antibody by western blot. J Med Virol **32**, 102-108.

Kurane I, Bukowski JF, Lai C-H, Brinton M, Bray M, Falgout B, Zhao B, Ennis FA (1989a) Dengue virus specific human cytotoxic T lymphocytes (CTL). In: 2nd International Symposium on Positive Strand RNA Viruses. Vienna, Austria.

Kurane I, Hebblewaite D, Brandt WE and Ennis FA (1984) Lysis of dengue-virus cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J Virol **52**, 223-230.

Kurane I, Innis BL, Nimmanitya S, Nisalak A, Meager A, Janus J and Ennis FA (1991) Activation of T lymphocytes in dengue virus infections: high levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2 and interferon gamma in sera of children with dengue. J Clin Invest **88**, 1473-1480.

Kurane I, Innis BL, Nisalak A, Hoke C, Nimmanitya S, Meager A and Ennis FA (1989b) Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. J Clin Invest **83**, 506-513.

Kurane I, Meager A and Ennis FA (1989c) Dengue virus specific human T cell clones. Serotype cross reactive proliferation, interferon gamma production, and cytotoxic activity. J Exp Med **170**, 763-775.

Kurane I, Okamoto Y, Dai LC, Zeng LL, Brinton MA and Ennis FA (1995) Flavivirus-cross reactive HLA-DR15-restricted epitope on NS3 recognized by human CD4+ CD8- cytotoxic T lymphocyte clones. J Gen Virol **76**, 2243-2249.

Kurane I, Russell PK, Rothman JF, Ennis FA, Lai C-J, Bray M, Falgout B, Zhao B, Zhang Y-M and Brinton MA (1990) Human T lymphocyte responses to dengue viruses: Recognition of dengue virus proteins by T lymphocytes. Vaccines 131-133.

Lambert PH, Dixon FJ, Zubler RH, Agnello V, Cambiaso C, Casali P, Clarke J, Cowdery JS, MsDuffie C, Hay FC, MacLennan ICM, Masson P, Müller-Eberhard HJ, Penttinen K, Smith M, Tappeiner G, Theofilopoulos AN and Verroust P (1978) A WHO collaborative study for the evaluation of eighteen methods for detecting immune complexes in serum. J Clin Lab Immunol **1**, 1-15.

Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, Mackenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T and Gubler DJ (1999) Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science **286**, 2334-2337.

Laver WG, Air GM, Webster RG and Smith-Gill SJ (1990) Epitopes on protein antigens: misconceptions and realities. Cell **61**, 553-556.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Laille M, Deubel V and Sainte-Marie FF (1991) Demonstration of concurrent dengue 1 and dengue 3 infection in six patients by the polymerase chain reaction. J Med Virol **34**, 51-54.

Leary K and Blair CD (1983) Japanese encephalitis replication; studies on host cell nuclear involvement. Exp Mol Path **36**, 264-270.

Leblois H and Young PR (1994) Sequence of the dengue virus type 2 (strain PR-159) NS1 gene and comparison with its vaccine derivative. Nucleic Acids Research **21**, 1668.

Leblois H and Young PR (1995) Maturation of the dengue-2 virus NS1 protein in insect cells: effects of downstream NS2A sequencs on baculovirus expressed gene constructs. J Gen Virol **76**, 979-984.

LeDuc J (1989) Epidemiology of hemorrhagic fever viruses. Revs Inf Dis II, S730-S735.

Lee E, Fernon C, Simpson R, Weir RC, Rice CM and Dalgarno L (1990) Sequence of the 3' half of the Murray Valley encephalitis virus genome and mapping of the nonstructural proteins NS1, NS3 and NS5. Virus Genes **4**, 197-213.

Lee JM, Crooks AJ and Stephenson JR (1989) The synthesis and maturation of a non-structural extracellular antigen from tick-borne encephalitis and its relationship to the intracellular NS1 protein. J Gen Virol **70**, 335-343.

Lee T, Komiya T, Watanabe K, Aizawa C and Hashimoto H (1995) Immune response in mice infected with the attenuated Japanese encephalitis vaccine strain SA14-14-2. Acta Virologica **39**, 161-164.

Lee E, Stocks CE, Amberg SM, Rice CM and Lobigs M (2000) Mutagenesis of the signal sequence of yellow fever virus prM protein: enhancement of signalase cleavage *in vitro* is lethal for virus production. J Virol **74**, 24-32.

Leung D, Schroder K, White H, Fang NX, Stoermer MJ, Abbenante G, Martin JL, Young PR and Fairlie DP (2001) Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. J Biol Chem **276**, 45762-45771.

Li H, Clum S, You S, Ebner KE and Padmanabhan R (1999) The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. J Virol **73**, 3108-3116.

Liao C, Lin Y, Wu B, Tsao C, Wang M, Liu C, Huang Y, Chen J, Wang J and Chen L (2001) Salicylates inhibit flavivirus replication independently of blocking nuclear factor kappa B activation. J Virol **75**, 7828-7839.

Libraty DH, Young PR, Pickering D, Endy TP, Kalayanaroo S, Green S, Vaughn DW, Nisalak A, Ennis FA and Rothman AL (2002) High levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. Submitted to J Inf Dis.

Lin C, Chambers TJ and Rice CM (1993) Mutagenesis of conserved residues at the yellow fever virus 3/4A and 4B/5 dibasic cleavage sites: effects on cleavage efficiency and polyprotein processing. Virology **192**, 596-604.

Lindenbach BD and Rice CM (1997) *Trans*-complementation of yellow fever virus NS1 reveals a role in early RNA replication. J Virol **71**, 9608-9617.

Lindenbach BD and Rice CM (1999) Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J Virol **73**, 4611-1621.

Lingappa VR, Devillers-Thiery A and Blobel G (1977) Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin. PNAS **74**, 2432-2436.

Littaua R, Kurane I and Ennis FA (1990) Human IgG F_c receptor II mediated antibody-dependent enhancement of dengue virus infection. J Immunol **144**, 3183-3186.

Livingston PG, Kurane I, Dai LC, Okamoto Y, Lai CJ, Men R, Karaki S, Takiguchi M and Ennis FA (1995) Dengue virus-specific, HLA-B35-restricted, human CD8+ cytotoxic T lymphocyte (CTL) clones. Recognition of NS3 amino acids 500-508 by CTL clones of two different serotype specificities. J Immunol **154**, 1287-1295.

Livingston PG, Kurane I, Lai CJ, Bray M and Ennis FA (1994) Recognition of envelope protein by dengue virus serotype-specific human CD4+ CD8- cytotoxic T-cell clones. J Virol **68**, 3283-3288.

Lobigs M (1992) Proteolytic processing of a Murray Valley encephalitis virus non-structural polyprotein segment containing the viral proteinase: accumulation of a NS3-4A precursor which requires mature NS3 for efficient processing. J Gen Virol **73**, 2305-2312.

Lobigs M (1993) Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. PNAS **90**, 6218-6222.

Lobigs M, Usha R, Nestorowicz A, Marshall ID and Weir RC (1990) Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse virulence. Virology **176**, 587-595.

Lopez JA, Guillen M, Sanchez-Fauquier A and Melera JA (1986) An antigen binding assay to determine the specificity of monoclonal antibodies against influenza virus and mapping of epitopes. J Virol Methods **13**, 255-264.

Low MG (1989) The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta **988**, 427-454.

Lumley GF and Taylor FH (1943) Dengue. Sch Public Health Trop Med Serv Publ No 3 Australas Med Publ Co Sydney.

Mackenzie JS, Broom AK, Hall RA, Johansen CA, Lindsay MD, Phillips DA, Ritchie SA, Russell RC and Smith DW (1998) Arboviruses in the Australian region, 1990-1998. Communicable Dis Intell **22**, 93-100.

Mackenzie J, Jones M and Young PR (1996) Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. J Virol Methods **220**, 232-240.

Mackenzie JM, Khromykh AA, Jones MK and Westaway EG (1998) Subcellular localization and some biochemical properties of the flavivirus kunjin nonstructural proteins NS2A and NS4A. Virology **245**, 203-215.

Mackenzie JM, Khromykh AA and Westaway EG (2001) Stable expression of noncytopathic Kunjin replicons simulates both ultrastructural and biochemical characteristics observed during replication of Kunjin virus. Virology **279**, 161-72.

Mackow E, Makino Y, Zhao B, Zhang Y-M, Markoff I, Buckler-White A, Guiler M, Chanock R and Lai C-J (1987) The nucleotide sequence of dengue type 4 virus: analysis of genes coding for non-structural proteins. Virology **159**, 217-228.

Mady BJ, Erbe DV, Kurane I, Fanger MW and Ennis FA (1991) Antibody-dependent enhancement of dengue virus infection mediated by bispecific antibodies against cell surface molecules other than F_c gamma receptors. J Immunol **147**, 3139-3144.

Malasit P (1987) Complement and dengue haemorrhagic fever / shock syndrome. SE Asian J Trop Med Public Health. **18**, 316-320.

Mandl CW, Guirakhoo F, Holzmann H, Heinz FX and Kunz C (1989a) Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis virus as a model. J Virol **63**, 564-571.

Mandl CW, Heinz FX and Kunz CH (1988) Sequence of the structural proteins of tick-borne encephalitis virus (Western subtype) and comparative analysis with other flaviviruses. Virology **166**, 197-205.

Mandl CW, Heinz FX, Stöckl E and Kunz C (1989b) Genome sequence of tick-borne encephalitis virus (Western subtype) and comparative analysis of nonstructural proteins with other flaviviruses. Virology **173**, 291-301.

Mandl CW, Holzmann H, Kunz C and Heinz FX (1993) Complete genomic sequence of Powassan virus: evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. Virology **194**, 173-184.

Markoff L (1989) *In vitro* processing of dengue virus structural proteins: cleavage of the pre-membrane protein. J Virol **63**, 3345-3352.

Markoff L, Falgout B and Chang A (1997) A conserved internal hydrophobic domain mediates the stable membrane integration of the dengue virus capsid protein. Virology **23**, 105-117.

Marianneau P, Megret F, Oliver R, Morens DM and Deubel V (1996) Dengue virus binding to the hepatoma HepG2 and simian Vero cell surfaces differs. J Gen Virol **77**, 2547-2554.

Mason PW (1989) Maturation of Japanese encephalitis virus glycoprotein produced by infected mammalian and mosquito cells. Virology **169**, 354-364.

Mason PW, Dalrymple JM, Gentry MK, McCown JM, Hoke CH, Burke DS, Fournier MJ and Mason TL (1989) Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J Gen Virol **70**, 2037-2049.

Mason PW, McAda PC, Dalrymple JM, Fournier MJ and Mason TL (1987) Expression of Japanese encephalitis virus antigens in *Escherichia coli*. Virology **158**, 361-372.

Mason PW, McAda PC, Mason TL and Fournier MJ (1987b) Sequence of the dengue-1 virus genome in the region encoding the 3 structural proteins and the major nonstructural protein NS1. Virology **161**, 262-267.

Mason PW, Pincus S, Fournier MJ, Mason TL, Shope, R and Paoletti E (1991) Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Virology **180**, 291-305.

Mason PW, Züogel MU, Semproni AR, Fournier MJ and Mason TL (1990) The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*. J Gen Virol **71**, 2107-2114.

Mathew A, Kurane I, Tothman AL, Zeng LL, Brinton MA and Ennis FA (1996) Dominant recognition by human CD8+ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1.2a. J Clin Invest **98**, 1684-1694.

Mathur A, Kamlesh LA and Chaturvedi UC (1983) Host defence mechanisms against Japanese encephalitis virus infection in mice. J Gen Virol **64**, 805-811.

Matsumura T and Hotta S (1971) Electron microscopic observations on the purified dengue virus (type 1). Kobe J Med Science **17**, 85-95.

Matsuura Y, Possee RD, Overton HA and Bishop DH (1987) Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J Gen Virol **68**, 1233-1250.

Matveeva VA, Popova RV, Kvetkova EA, Chernicina LO, Zlobin VI, Puchovskaya NM and Morozova OV (1995) Antibodies against tick-borne encephalitis virus (TBEV) non-structural and structural proteins in human sera and spinal fluid. Immunol Letts **46**, 1-4.

McCallum F and Dwyer JP (1927) Dengue as a cause of death. Med J Australia **1**, 10-15.

McCloud TG, Brandt WE and Russell PK (1970) Molecular size and charge relationships of the soluble complement-fixing antigens of dengue viruses. Virology **41**, 569-572.

McPherson A, Koszelak S, Axelrod H, Day J, Williams R, Robinson L, McGrath M and Cascio D (1986) An experiment regarding crystallization of soluble proteins in the presence of β-octyl glucoside. J Biol Chem **261**, 1969-1975.

McPherson A (1989) Preparation and analysis of protein crystals. 2nd Ed Krieger, Malabar, Fla 96-97.

Medof ME, Kinoshita T, Silber R and Nussenzweig V (1985) Amelioration of lutic abnormalities of paroxysmal nocturnal hemoglobinuria with decay-accelerating factor. PNAS **82**, 2980-2984.

Megret F, Hugnot JP, Falconar A, Gentry MK, Morens DM, Murray JM, Schlesinger JJ, Wright PJ, Young P, Van Regenmortal MHV and Deubel V (1992) Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein. Virology **187**, 480-491.

Meiklejohn G, England B and Lennette EH (1952) Adaption of dengue virus strains in unweaned mice. Am J Trop Med Hyg **1**, 51-58.

Miles SA, Balden E, Magpantay L, Wei L, Leiblein A, Hofheinz D, Toedter G, Stiehm ER, Bryson Y and the Southern California Pediatric AIDS Consortium (1993) Rapid serological testing with immune-complex-dissociated HIV p24 antigen for early detection of HIV infection in neonates. N Eng J Med **328**, 297-302.

Monath TP (1986) Pathobiology of the flaviviruses. Schlesinger S, Schlesinger MJ (Eds) In: The Togaviridae and Flaviviridae. New York: Plenum 375-440.

Monath TP, Craven RB, Adjukiewicz A, Germain M, Francy DE, Ferrara I, Samba EM, N'Jie H, Cham K, Fitzgerald SA, Crippen PH, Simpson DI, Bowen ET, Fabiyi A and Salaun JJ (1980) Yellow fever in the Gambia 1978-1979: epidemiologic aspects with observations on the occurrence of Orungo virus infections. Am J Trop Med Hyg **29**, 912-928.

Monath TP and Heinz FX (1996) Virology 3rd Edition. Fields BN, Knipe DM and Howley PM (Eds) New York: Lippincott-Raven **1**, 961-1034.

Morens DM, Halstead SB and Marchette NJ (1987) Profiles of antibody dependent enhancement of dengue virus type 2 infection. Microb Pathogen **3**, 231-237.

Morens DM and Halstead SB (1990) Measurement of antibody-dependent infection enhancement of four dengue virus serotypes by monoclonal and polyclonal antibodies. J Gen Virol **71**, 2909-2914.

Morens DM, Venkateshan CN and Halstead SB (1987) Dengue 4 virus monoclonal antibodies identify epitopes that mediate immune infection enhancement of dengue 2 viruses. J Gen Virol **68**, 91-98.

Murphy FA (1980) Togavirus morphology and morphogenesis In: The Togaviruses pp 241-316. Schlesinger RW (Ed) New York: Academic Press.

Murray JM, Aaskov JG and Wright PJ (1993) Processing of the dengue virus type 2 proteins prM and C-prM. J Gen Virol **74**, 175-182.

Mussgay M, Enzmann P-J, Horzinek MC and Weiland E (1975) Growth cycle of arboviruses in vertebrate and arthropod cells. Prog Med Virol **19**, 258-323.

Muylaert IA, Chambers TJ, Galler R and Rice CM (1996) Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: effects on virus replication and mouse virulence. Virology **222**, 159-168.

Muylaert IR, Galler R and Rice CM (1997) Genetic analysis of the yellow fever virus NS1 protein: identification of a temperature-sensitive mutation which blocks RNA accumulation. J Virol **71**, 291-298.

Naeve CW and Trent DW (1978) Identification of Saint Louis encephalitis virus mRNA. J Virol **25**, 535-545.

Naim HY and Koblet H (1992) Asparagine-linked oligosaccarides of Semliki Forest virus grown in mosquito cells. Arch Virol **122**, 45-60.

Ng ML, Choo WKP and Ho YL (1992) Detection of flavivirus antigens in purified infected Vero cell plasma membranes. J Virol Methods **39**, 125-138.

Ng ML and Corner LC (1989) Detection of some dengue-2 virus antigens in infected cells using microscopy. Arch Virol **104**, 197-208.

Ng ML, Pedersen JS, Toh BH and Westaway EG (1993) Immunofluorescent sites in Vero cells infected with the flavivirus Kunjin. Arch Virol **78**, 177-190.

Nimmannitya S (1987) Clinical spectrum and management of dengue hemorrhagic fever. Southeast Asian J Trop Med Pub Health **3**, 392-397.

Nowak T, Farber PM, Wengler G and Wengler G (1989) Analyses of the terminal sequences of West Nile virus structural proteins and of the *in vitro* translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. Virology **169**, 365-376.

Nowak T and Wengler G (1987) Analysis of disulfides present in the membrane proteins of the West Nile flavivirus. Virology **156**, 127-137.

Nugent CI, Johnson KL, Sarnow P and Kirkegaard K (1999) Functional coupling between replication and packaging of poliovirus replicon RNA. J Virol **73**, 427-435.

O'Reilly EK and Kao CC (1998) Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. Virology **252**, 287-303.

Osatomi K and Sumiyoshi H (1990) Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176**, 643-647.

Parrish CR, Woo WS and Wright PJ (1991) Expression of the NS1 gene of dengue virus type-2 using vaccinia virus. Dimerization of the NS1 glycoprotein. Arch Virol **117**, 279-286.

Pang T, Devi S, Yeen WP, McKenzie IFC and Leong YK (1984) Induction and characterization of delayed-type hypersensitivity response to dengue virus infection. Inf Imm **43**, 429-431.

Petchclai B and Saelim P (1978) Circulating immune complexes in dengue haemorrhagic fever. Lancet **2**, 638-639.

Pethel M, Falgout B and Lai C-J (1992) Mutational analysis of the octapeptide sequence motif at the NS1-NS2A cleavage junction of dengue type 4 virus. J Virol **66**, 7225-7231.

Phillpotts RJ, Stephenson JR and Porterfield JS (1985) Antibody-dependent enhancement of tick-borne encephalitis virus infectivity. J Gen Virol **66**, 1831-1837.

Pletnev AG, Bray M and Lai CJ (1993) Chimeric tick-borne encephalitis and dengue type 4 viruses: effects of mutations on neurovirulence in mice. J Virol **67**, 4956-4963.

Poch O, Sauvaget I, Delarue M and Tordo N (1989) Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J **8**, 3867-3874.

Porterfield JS (1986) Antibody-dependent enhancement of viral infectivity. Adv Virus Res **31**, 335-355.

Post PR, Carvalho R and Galler R (1991) Glycosylation and secretion of yellow fever virus nonstructural protein NS1. Virus Res **18**, 291-302.

Preugschat F and Strauss JH (1991) Processing of nonstructural proteins NS4a and NS4b of dengue 2 virus *in vitro* and *in vivo*. Virology **185**, 689-697.

Preugschat F, Yao C-W and Strauss JH (1990) *In vitro* processing of dengue virus type 2 nonstructural proteins NS2A, NS2B and NS3. J Virol **64**, 4364-4374.

Pryor MJ, Gualano RC, Lin B, Davidson A and Wright PJ (1998) Growth restriction of dengue virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins. J Gen Virol **79**, 2631-2639.

Pryor MJ and Wright PJ (1993) The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. Virology **194**, 769-780.

Pryor MJ and Wright PJ (1994) Glycosylation mutants of dengue virus NS1 protein. J Gen Virol **75**, 1183-1187.

Pugachev KV, Nomokonova NY, Dobrikova EY and Wolf YI (1993) Site-directed mutagenesis of the tick-borne encephalitis virus NS3 gene reveals the putative serine protease domain of the NS3 protein. FEBS Lett **328**, 115-118.

Putnak JR, Charles PC, Padmanabhan R, Irie K, Hoke CH and Burke DS (1988) Functional and antigenic domains of the dengue-2 virus nonstructural glycoprotein NS-1. Virology **163**, 93-103.

Putnak JR, Niranjan K-T and Innis BL (1997) A putative cellular receptor for dengue viruses. Nature Medicine **3**, 828-829.

Putnak JR and Schlesinger JJ (1990) Protection of mice against yellow fever virus encephalitis by immunization with a vaccinia virus recombinant encoding the yellow fever nonstructural proteins, NS1, NS2a, and NS2b. J Gen Virol **71**, 1697-1702.

Qu X, Chen W, Maguire T and Austin F (1993) Immunoreactivity and protective effects in mice of a recombinant dengue 2 Tonga virus NS1 protein produced in a baculovirus expression system. J Gen Virol **74**, 89-97.

Quereshi AA and Trent DW (1973) Group B arbovirus structural and non-structural antigens. III. Serological specificity of solubilized intracellular viral proteins. Inf Imm **8**, 993-999.

Rabilloud T, Carpentier G and Tarroux P (1988) Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. Electrophoresis **9**, 288-291.

Randolph VB, Winkler G and Stollar V (1990) Acidotrophic amines inhibit proteolytic processing of flavivirus prM protein. Virology **174**, 450-458.

RehleTM (1989) Classification, distribution and importance of arboviruses. Trop Med Parasit **40**, 391-395.

Rey FA, Heinz FX, Mandl C, Kunz C and Harrison SC (1995) The envelope glycoprotein from tick-borne encephalitis virus at 2Å resolution. Nature **375**, 291-298.

Rice CM, Aebersold R, Teplon DB, Pata J, Bell JR, Vorndam AV, Trent DW, Brandriss MW, Schlesinger JJ and Strauss JH (1986a) Partial N-terminal amino acid sequences of 3 nonstructural proteins of 2 flaviviruses. Virology **151**, 1-9.

Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL and Strauss JH (1985) Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science **229**, 726-733.

Rice CM, Strauss EG and Strauss JH (1986b) Structure of the flavivirus genome In "The Togaviridae and Flaviviridae" S. Schlesinger and MJ Schlesinger (Eds) pp 279-326 Plenum, New York/London.

Rice CM and Strauss JH (1990) Production of flavivirus polypeptides by proteolytic processing. Semin Virol **1**, 357-367.

Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mese MTR, Nogueira RMR and da Rosa AT (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology **230**, 244-251.

Robinson PJ (1997) Signal transduction via GPI-anchored membrane proteins. Exp Med Biol **419**, 365-370.

Robinson PJ, Millrain M, Antoniou J, Simpson E and Mellor AL (1989) A glycosophospholipid anchor is required for Qa-2-mediated T cell activation. Nature **342**, 85-87.

Roche JC, Cordellier R, Hervy JP *et al* (1983) Isolement de 96 souches de virus dengue 2 a partir de moustiques captures en Cote d'Ivoire et Haute Volta. Ann Inst Pasteur Virol **134E**, 233-244.

Roehrig JT, Bolin RA and Kelly RG (1998) Monoclonal antibody mapping of the envelope glycoprotein of the dengue-2 virus, Jamaica. Virology **246**, 317-328.

Roehrig JT, Hunt AR, Johnson AJ and Hawkes RA (1989) Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology **171**, 49-60.

Roehrig JT, Johnson AJ, Hunt AR, Bolin RA and Chu MC (1990) Antibodies to dengue-2 virus E glycoprotein synthetic peptides identify antigenic conformation. Virology **177**, 668-675.

Roehrig JT, Mathews JH, Risi PA, Brubaker JR and Hunt AR (1992) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York pp. 277-281.

Roehrig JT, Mathews JH and Trent DW (1983) Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology **128**, 118-126.

Roehrig JT, Staudinger LA, Hunt AR, Mathews JH and Blair CD (2001) Antibody prophylaxis and therapy for flavivirus encephalitis infections. Ann N Y Acad Sci **951**, 286-297.

Rosen L (1977) *The emperor's new clothes revisited*, or reflections on the pathogenesis of dengue haemorrhagic fever. Am J Trop Med Hyg **26**, 337-343.

Rosen L (1989) Disease exacerbation caused by sequential dengue infections: Myth or reality? Revs Inf Dis II, S840-S842.

Rossman MG, Arnold E, Karner G, Kremer MJ, Luo M, Smith TJ, Vriend G, Rueckert RR, Mosser AG, Sherry B, Boege V, Scraba DG, McKinlay MA and Diana GD (1987) Structure and function of human rhinovirus 14 and mengovirus: neutralizing antigenic sites, putative receptor binding site, neutralization by drug binding. p 59-77 In: Positive strand RNA viruses. Brinton MA and Rueckert RR (Eds) Alan R Liss, Inc, New York.

Rothman AL and Ennis FA (1999) Immunopathogenesis of dengue hemorrhagic fever. Virology **257**, 1-6.

Rothman AL, Kurane I, Ennis FA, Lai C-J, Zhang Y-M, Bray M, Falgout B, Zhao B and Schlesinger JJ (1990) Lymphocyte proliferative responses to dengue virus E and NS1 proteins in BALB/C mice. Vaccines 135-138.

Rothman AL, Kurane I, Zhang Y-M, Lai C-J and Ennis FA (1989) Dengue virus-specific T-lymphocyte proliferation: Serotype specificity to recombinant viral proteins. J Virol **63**, 2486-2491.

Rost B (1996) PHD: predicted one-dimensional protein structure by profile based neural networks. Meth Enzym **266**, 525-539.

Rost B and Sander C (1994a) Combining evolutionary information and neural networks to predict protein secondary structure. Proteins **19**, 55-72.

Rost B and Sander C (1994b) Conservation and prediction of solvent accessibility in protein families. Proteins **20**, 216-226.

Ruangjirachuporn W, Boonpucknavig S and Nimmanitya S (1979) Circulating immune complexes in serum from patients with dengue haemorrhagic fever. Clin Exp Immunol **36**, 46-53.

Rudnick A, Marchette NJ and Garcia R (1967) Possible jungle dengue - recent studies and hypotheses. Jpn J Med Sci Biol **20**, 69-74.

Ruggli N and Rice CM (1999) Functional cDNA clones of the *Flaviviridae*: strategies and applications. Adv Vir Res **53**, 183-207.

Ruiz Linares A, Cahour A, Desprès P, Girard M and Bouloy M (1989) Processing of yellow fever virus polyprotein: role of cellular proteases in maturation of the structural proteins. J Virol **63**, 4199-4209.

Russel PK (1991) Immunological mechanisms in the dengue shock syndrome. Progr Immunol **1**, 831-833.

Russell PK, Brandt WE and Dalrymple JM (1980) Chemical and antigenic structure of flaviviruses. In: "The Togaviruses: biology, structure and replication. Schlesinger RW (Ed) pp 503-529 Academic Press, New York.

Russell PK, Chiewsilp D and Brandt WE (1970) Immunoprecipitation analysis of soluble complement-fixing antigens of dengue virus. J Immunol **105**, 838-845.

Russell ER and Hyder ML (1976) The thermal decomposition of uranyl formate and the preparation of ammonium uranyl fromate, $(NH_4)UO_2(COOH)_4$. Inorg Chem Letters **12**, 247-250.

Russell PK and Nisalak A (1967) Dengue virus infection by the plaque reduction neutralization test. J Immunol **99**, 291-296.

Salas-Benito JS and del Angel RM (1997) Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. J Virol **71**, 7246-7252.

Sanchez IJ and Ruiz BH (1996) A single nucleotide change in the E protein of dengue 2 Mexican strain affects neurovirulence in mice. J Gen Virol **77**, 2541-2545.

Sangkawibhaet N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, Phanthumachinda B and Halstead SB (1984) Risk factors for dengue shock syndrome: A prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol **120**, 653-699.

Sangster MY, Heliams DB, Mackenzie JS and Shellam GR (1993) Genetic studies of flavivirus resistance in inbred strains derived from wild mice: evidence for a new resistance allele at the flavivirus resistance locus. J Virol **67**, 340-347.

Schlesinger JJ and Brandriss MW (1981) Growth of 17D yellow fever virus in a macrophage-like cell line U937: Role of F_c and viral receptors in antibody-mediated infection. J Immunol **127**, 659-665.

Schlesinger JJ and Brandriss MW (1983) 17D Yellow fever virus infection of P388D1 cells mediated by monoclonal antibodies: properties of the macrophage F_c receptor. J Gen Virol **64**, 1255-1262.

Schlesinger JJ, Brandriss MW, Cropp CB and Monath TP (1986) Protection against yellow fever in monkeys by immunization with yellow fever virus non-structural protein NS1. J Virol **60**, 1153-1155.

Schlesinger JJ, Brandriss MW, Putnak JR and Walsh EE (1990) Cell surface expression of yellow fever virus non-structural glycoprotein NS1: consequences of interaction with antibody. J Gen Virol **71**, 593-599.

Schlesinger JJ, Brandriss MW and Walsh EE (1985) Protection against 17D Yellow Fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and active immunization with gp48. J Immunol **135**, 2805-2809.

Schlesinger JJ, Brandriss MW and Walsh EE (1987) Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol **68**, 853-857.

Schlesinger JJ and Chapman SE (1999) Influence of the human high-affinity IgG receptor FcγR1 (CD64) on residual infectivity of neutralized dengue virus. Virology **260**, 84-88.

Schlesinger JJ, Foltzer M and Chapman S (1993) The F_c portion of antibody to yellow fever virus NS1 is a determinant of protection against yellow fever encephalitis in mice. Virology **192**, 132-141.

Schlesinger JJ, Putnak JR and Eckels KH (1992) Approaches to flavivirus vaccine development. Biotechnology **20**, 289-307.

Schlesinger S and Schlesinger MJ (1990) Replication of *Togaviridae* and *Flaviviridae*. Virology 697-711.

Schulz IT and Schlesinger RW (1963) Plaque assay of dengue and other group B arthropod-borne viruses under methyl cellulose overlay media. Virology **19**, 40-48.

Scott RM, Nisalak A, Cheamudon U, Seridhoranakul S and Nimmannitya S (1980) Isolation of dengue viruses from peripheral blood leukocytes of patients with haemorrhagic fever. J Inf Dis **141**, 1-6.

Seif SA, Marita K, Matsuo S, Hasebe F and Igarashi A (1995) Finer mapping of neutralizing epitopes(s) on the C-terminal of Japanese encephalitis virus E-protein expressed in recombinant *Escherichia coli* system. Vaccine **13**, 1515-1521.

Serafin IL and Aaskov JG (2001) Identification of epitopes on the envelope (E) protein of dengue 2 and dengue 3 viruses using monoclonal antibodies. Arch Virol **124**, 2469-2479.

Shaio MF, Cheng SN, Yuh YS and Yang KD (1995) Cytotoxic factors released by dengue virus-infected human blood monocytes. J Med Virol **46**, 216-223.

Shapiro D, Brandt WE, Cardiff RD and Russell PK (1971) The proteins of Japanese encephalitis virus. Virology **44**, 108-124.

Shapiro D, Brandt WE and Russell PK (1972) Change involving a viral membrane glycoprotein during morphogenesis of group B arboviruses. Virology **50**, 906-911.

Sheu SY and Lo SJ (1994) Biogenesis of the hepatitis B viral middle (M) surface protein in a human hepatoma cell line: demonstration of an alternative secretion pathway. J Gen Virol **75**, 3031-3039.

Shope RE (1980) Medical significance of togaviruses: an overview of diseases caused by togaviruses in humans and in domestic and wild vertebrate animals. In: The Togaviruses pp 47-82. Schlesinger RW (Ed) New York: Academic Press.

Shu P-Y, Chen L-K, Chang S-F, Yueh Y-Y, Chow L, Chien L-J, Chin C, Lin T-H and Huang J-H (2000) Dengue NS1-specific antibody responses: isotype distribution and serotyping in patients with dengue fever and dengue hemorrhagic fever. J Med Virol **62**, 224-232.

Shyu WR, Wang YC, Chin C and Chen WJ (1997) Assessment of neutralizing antibodies elicited by a vaccine (Nakayama) strain of Japanese encephalitis virus in Taiwan. Epidemiol Infect **119**, 79-83.

Siler JF, Hall MW and Kitchens AP (1926) Dengue: its history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity and prevention. Philippine J Sci **29**, 1-304.

Simmons JS, St. John JH and Reynolds FHK (1931) Experimental studies of dengue. Phillipine J Sci **44**, 1-247.

Smith TJ, Brandt WE, Swanson JL, McCown JM and Buescher EL (1970) Physical and biological properties of dengue-2 virus and associated antigens. J Virol **5**, 524-532.

Smith GW and Wright PJ (1985) Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and *Aedes albopictus* cells. J Gen Virol **66**, 559-571.

Smithburn KC (1954) Antigenic relationships among certain arthropod-borne viruses as revealed by neutralization tests. J Immunol **72**, 376-388.

Sobel AT, Bokisch VA and Müller-Eberhard HJ (1975) C1q deviation test for the detection of immune complexes, aggregates of IgG and bacterial products in human serum. J Exp Med **142**, 139-150.

Soper FL (1936) Jungle yellow fever: new epidemiological entity in South America. Rev Hyg Saude Publica **10**, 107-144.

Speight G, Coia A, Parker MD and Westaway EG (1988) Gene mapping and positive identification of the nonstructural proteins NS2a, NS2b, NS3, NS4b and NS5 of the flavivirus kunjin and their cleavage sites. J Gen Virol **69**, 23-24.

Speight G and Westaway EG (1989a) Carboxy-terminal analysis of 9 proteins specified by the flavivirus Kunjin: evidence that only the intracellular core protein is truncated. J Gen Virol **70**, 2209-2214.

Speight G and Westaway EG (1989b) Positive identification of NS4A, the last of the hypothetical nonstructural proteins of flaviviruses. Virology **170**, 299-301.

Stadler K, Allison SL, Schalich J and Heinz FX (1997) Proteolytic activation of tick-borne encephalitis virus by furin. J Virol **71**, 8475-8481.

Stiasny K, Allison SL, Mandl and Heinz FX (2001) Role of metastability and acidic pH in membrane fusion by Tick-borne encephalitis virus. J Virol **75**, 7392-7398.

Stiasny K, Allison SL, Marchler-Bauer A, Kunz C and Heinz FX (1996) Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol **70**, 8142-8147.

Stocks CE and Lobigs M (1998) Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J Virol **72**, 2141-2149.

Stohlman SA, Eylar OR and Wisseman CC Jr. (1976) Isolation of the dengue virus envelope glycoprotein from membranes of infected cells by concanavalin A affinity chromatography. J Virol **18**, 132-140.

Stohlman SA, Wisseman CL, Eylar OR and Silverman DJ (1975) Dengue virus-induced modifications of host cell membranes. J Virol **16**, 1017-1026.

Stollar V, Schlesinger RW and Stevens TM (1967) Studies on the nature of dengue viruses. III. RNA synthesis in cells infected with type 2 dengue virus. Virology **33**, 650-658.

Strode GK (Ed) (1951) Yellow Fever. New York: McGraw-Hill.

Su B, Waneck GL, Flavell RA and Bothwell AL (1991) The glycosyl phosphatidylinositol anchor is critical for Lys-6A/E-mediated T cell activation. J Cell Biol **11**, 377-384.

Summers PL, Housten Cohen W, Ruiz MM, Hase T and Eckels KH (1989) Flaviviruses can mediate fusion from without in *Aedes albopictus* mosquito cell cultures. Virus Res **12**, 383-392.

Suvatte V (1987) Immunological aspects of dengue haemorrhagic fever: studies in Thailand. SE Asian J Trop Med Pub Health **18**, 312-315.

Svitkin YV, Ugarova TY, Chernovskaya TV, Lyapustin VN, Lashkerich VA and Agol VI (1981) Translation of tick-borne encephalitis virus (flavivirus) genome *in vitro*: synthesis of two structural polypeptides. Virology **110**, 26-34.

Takegami T, Washizu M and Yasui K (1986) Nucleotide sequence at the 3' end of Japanese encephalitis virus genomic RNA. Virology **152**, 483-486.

Tan BH, Fu J, Sugrue RJ, Yap EH, Chan YC and Tan YH (1996) Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. Virology **216**, 317-325.

Tan CH, Yap EH, Singh M, Deubel V and Chan YC (1990) Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus. J Gen Virol **71**, 745-748.

Tarentino AL and Maley F (1975) A comparison of the substrate specificities of endo-β-N-acatylglucosaminidases from *Streptomyces griseus* and *Diplococcus pneumoniae*. Biochem Biophys Res Comm **67**, 455-462.

Tervel-Lopez E (1991) Dengue. A Review. Investigacion Clinica **32**, 201-217.

Theiler M and Smith HH (1937) Use of yellow fever virus modified by *in vitro* cultivation for human immunization. J Exp Med **65**, 787-800.

Theiler M and Downs WG (1973) The arthropod-borne viruses of vertebrates, Yale University Press, New Haven.

Thein S, Aung MM, Shwe TN, Aye M, Zaw A, Aye K, Aye KM and Aaskov J (1997) Risk factors in dengue shock syndrome. Am J Trop Med Hyg **56**, 566-572.

Theofilopoulos AN, Wilson CB and Dixon FJ (1976) The Raji cell radioimmunoassay for detecting immune complexes in human serum. J Clin Invest **57**, 169-182.

Timofeev AV, Ozherelkov SV, Pronin AV, Deeva AV, Karganova GG, Elbert LB and Stephemson JR (1998) Immunological basis for protection in a murine model of tick-borne encephalitis by a recombinant adenovirus vector carrying the gene encoding the NS1 nonstructural protein. J Gen Virol **79**, 689-695.

Tomori O (1999) Impact of yellow fever on the developing world. Adv Virus Res **53**, 5-34.

Trent DW, Swensen CC and Qureshi AA (1969) Synthesis of Saint Louis encephalitis virus ribonucleic acid in BHK 21/13 cells. J Virol **3**, 385-394.

Trirawatanapong T, Chandran B, Putnak R and Padmanabhan R (1992) Mapping of a region of dengue virus type-2 glycoprotein required for binding by a neutralizing monoclonal antibody. Gene **116**, 139-150.

Tsekhanovskaya NA, Matveev LE, Rubin SG, Karavanov AS and Pressman EK (1993) Epitope analysis of tick-borne encphalitis (TBE) complex viruses using monoclonal antibodies to envelope glycoprotein of TBE virus (persulcatus subtype). Virus Res **30**, 1-16.

Udenfriend S and Kodukula K (1995) How glycosylphosphatidylinositol-anchored membrane proteins are made. Annu Rev Biochem **64**, 563-591.

Umenai T, Krzysko R, Bektimirov TA and Assaad FA (1985) Japanese encephalitis: current world status. Wld Hlth Org Bull **63**, 625-631.

Uren MF, Doherty PC, Allan JE (1987) Flavivirus-specific murine L3T4+ T cell clones: Induction, characterization and cross-reactivity. J Gen Virol **68**, 2655-2663.

Valdés K, Alvarez M, Pupo M, Vázquez S, Rodríguez R and Guzmán MG (2000) Human dengue antibodies against structural and nonstructural proteins. Clin Diag Lab Immunol **7**, 856-857.

Van den Berg CW, Cinek T, Hallett MB, Horejsi V and Morgan BP (1995) Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes Ca^{2+}- signaling competent. J Cell Biol **131**, 669-677.

Varnavski AN and Khromykh AA (1999) Noncytopathic flavivirus replicon RNA-based system for expression and delivery of heterologous genes. Virology **255**, 366-375.

Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntaykorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA and Nisalak A (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Inf Dis **181**, 2-9.

Vaughn DW, Hike CJ, Yokasan S, Lachance R, Innis BL, Rice RM and Bhamarapravati N (1996) Testing of a dengue 2 live-attenuated vaccine (strain 16681 PDK 53) in ten American volunteers. Vaccine **14**, 329-336.

Vasconcelos PFC, Costa ZG, Travassos da Rosa ES, Luna E, Rodrigues SG, Barros VLRS, Dias JP, Monteiro HAO, Olivia OFP, Vasconcelos HB, Oliveira RC, Sousa MRS, Barbosa da Silva J, Cruz ACR, Martins EC and Travassos Da Rosa JFS (2001) Epidemic of jungle yellow fever in Brazil, 2000: Implications of climatic alterations in disease spread. J Med Virol **65**, 598-604.

Venugopal K, Reid HW and Gould EA (1994) Tick-borne flavivirus NS1 gene: identification of conserved peptides and antigenic analysis of recombinant louping ill virus NS1 protein. Virus Res **31**, 245-254.

Vezza AC, Rosen L, Repik P, Dalrymple J and Bishop DH (1980) Characterization of the viral RNA species of prototype dengue viruses. Am J Trop Med Hyg **24**, 643-652.

Volkova TD, Vorovitch MF, Ivanov VT, Timofeev AV and Volpina OM (1999) A monoclonal antibody that recognizes the predicted tick-borne encephalitis virus E protein fusion sequence blocks fusion. Arch Virol **144**, 1035-1039.

von Heijne G (1983) Patterns of amino acids near signal sequence cleavage sites. Eur J Biochem **133**, 17-21.

von Heijne G (1984) How signal sequences maintain cleavage specificity. J Mol Biol **173,** 243-251.

von Heijne G (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res **14**, 4683-4690.

von Heijne G (1990) The signal peptide. J Membr Biol **115**, 195-201.

Walter P and Blobel G (1983) Preparation of microsomal membranes for cotranslational protein translocation. Virology **213**, 254-257.

Wang T, Anderson JF, Magnarelli LA, Bushmick S, Wong S, Koski RA and Fikrig E (2001) West Nile virus envelope protein: role in diagnosis and immunity. Ann N Y Acad Sci **951**, 325-327.

Wang P, Geng L, Qin E, Tu M and Zhao W (2001) Determination and analysis of the complete genomic sequence of the dengue type 4 virus B5 strain isolated in China. J Biochem Mol Biol **17**, 148-154.

Wang S, He R, Patarapotikul J, Innis BL and Anderson R (1995) Antibody-enhanced binding of dengue-2 virus to human platelets. Virology **213**, 254-257.

Warrener P, Tamura JK and Collett MS (1993) An RNA-stimulated NTPase activity associated with yellow fever virus NS3 protein expressed in bacteria. J Virol **67**, 989-996.

Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ and Whiley DC (1988) Structure and function of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature **333**, 426-431.

Weiss RC and Scott FW (1981) Antibody-mediated enhancement of disease in feline infectious peritonitis: comparison with dengue haemorrhagic fever. Comparative Immunology, Microbiology and Infectious Diseases **4**, 175-189.

Wengler G, Castle E, Leidner U, Nowak T and Wengler G (1985) Sequence analysis of the membrane protein V3 of the flavivirus West Nile virus and of its gene. Virology **147**, 264-274.

Wengler G, Czaya G, Färber PM and Hegemann JH (1991) *In vitro* synthesis of West Nile virus proteins indicates that the amino-terminal segment of the NS3 protein contains the active centre of the protease which cleaves the viral polyprotein after multiple basic amino acids. J Gen Virol **72**, 851-858.

Wengler G and Wengler G (1989) An analysis of the antibody response against West Nile E protein purified by SDS-PAGE indicates that this protein does not contain sequential epitopes for efficient induction of neutralizing antibodies. J Gen Virol **70**, 987-992.

Wengler G and Wengler G (1991) The carboxy-terminal part of the NS3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. Virology **184**, 707-715.

Wengler G, Wengler G and Gross HJ (1978) Studies on the virus-specific nucleic acids synthesized in vertebrate and mosquito cells infected with flaviviruses. Virology **63**, 1852-1860.

Wengler G, Wengler G, Nowak T and Castle E (1990) Description of a procedure which allows isolation of viral nonstructural proteins from BHK vertebrate cells infected with the West Nile flavivirus in a state which allows their direct chemical characterization. Virology **177**, 795-801.

Westaway EG (1965) The neutralization of arboviruses. II. Neutralization in heterologous virus-serum mixtures with four group B arboviruses. Virology **26**, 528-537.

Westaway EG (1975) The proteins of Murray Valley encephalitis virus. J Gen Virol **27**, 283-292.

Westaway EG (1980) Replication of flaviviruses. In: The Togaviruses pp 531-581. Schlesinger RW (Ed) New York: Academic Press.

Westaway EG (1987) Flavivirus replication strategy. Adv Virus Res **33**, 45-90.

Westaway EG, Brinton MA, Gaidamovich SY, Horzinek MC, Igarashi A, Kaariainen L, Lvov DK, Porterfield JS, Russel PK and Trent DW (1985) Flaviviridae. Intervirology **24**,183-192.

Westaway EG, Khromykh AA, Kenney MT, Mackenzie JM and Jones MK (1997a) Proteins C and NS4B of the flavivirus kunjin translocate independently into the nucleus. Virology **234**, 31-41.

Westaway EG, Khromykh AA and Mackenzie JM (1999) Nascent flavivirus RNA colocalised *in situ* with double-stranded RNA in stable replication complexes. Virology **258**, 108-117.

Westaway EG, Mackenzie JM, Kenney MT, Jones MK and Khromykh AA (1997b) Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA and of NS2B with NS3 in virus-induced membrane structures. J Virol **71**, 6650-6661.

Westaway EG and Goodman MR (1987) Variation in distribution of the three flavivirus specific glycoproteins detected by immunofluorescence in infected Vero cells. Arch Virol **94**, 215-228.

Wiley DC and Skehel JJ (1987) The structure and function of the haemagglutinin membrane glycoprotein of influenza virus. Ann Rev Biochem **56**, 365-394.

Whitfied SG, Murphy FA and Sudia WD (1973) St. Louis encephalitis virus: an ultrastructural study of infection in a mosquito vector. Virology **56**, 70-87.

Whitford WG and Mertz LM (1994) Multiplicity of baculoviral infection and recombinant protein production in Sf9 cells. Focus **18**, 75-76.

Winkler G, Heinz FX and Kunz C (1987) Studies on the flavivirus E proteins and the role of carbohydrate in antigenic structure. Virology **159**, 237-243.

Winkler G, Randolph VB, Cleaves GR, Ryan TE and Stollar V (1988) Evidence that the mature form of the flavivirus non-structural protein is a dimer. Virology **162**, 187-196.

Winkler G, Maxwell SE, Ruemmler C and Stoller V (1989) Newly synthesized dengue-2 nonstructural protein is a soluble protein but becomes partially hydrophobic and membrane associated after dimerization. Virology **171**, 302-305.

World Health Organization (1971) Expert committee on yellow fever, 3rd Rep, WHO Tech Rep Ser **479**, 1056.

World Health Organization (1975) Technical grades for diagnosis, treatment, surveillance, prevention and control of dengue hemorrhagic fever. Bull World Health Org. Geneva

World Health Organization (1985) Arthropod-borne and rodent-borne viral disorders. Techn Rep Ser. 719

World Health Organization (1986) Prevention and control of yellow fever in Africa, WHO Geneva.

Wright PJ (1982) Envelope protein of the flavivirus Kunjin is apparently not glycosylated. J Gen Virol **59**, 29-38.

Wright PJ, Cauchi MR and Ng ML (1989) Definition of the carboxy termini of the three glycoproteins specified by dengue virus type 2. Virology **171**, 61-67.

Wright PJ, Warr HM and Westaway EG (1980) Preliminary characterization of glycopeptides derived from glycoproteins specified by the flavivirus Kunjin. Virology **104**, 482-486.

Wu HC, Huang YL, Chao TT, Jan JT, Huang JL, Chiang HY, King CC and Shaio MF (2001) Identification of B-cell epitope of dengue virus type 1 and its application in diagnosis of patients. J Clin Micro **39**, 977-982.

Yamada M, Iguma J and Oshima M (1943) Discovery of *Aedes aegypti* in mainland Japan. Gunidan Zasshi **359**, 397-398.

Yamshchikov VF and Compans RW (1993) Regulation of the late events in flavivirus protein processing and maturation. Virology **192**, 38-51.

Yao ZJ, Kao MC, Loh KC and Chung MC (1995) A serotype-specific epitope of dengue virus 1 identified by phage displayed random peptide library. FEMS Microbiol Lett **127**, 93-98.

You S, Falgout B, Markoff L and Padmanabhan R (2001) *In vitro* synthesis from exogenous dengue viral RNA templates requires long range interactions between 5'- and 3'-terminal regions that influence RNA structure. J Biol Chem **276**, 15581-15591.

You S and Padmanabhan R (1999) A novel *in vitro* replication system for Dengue virus. Initiation of RNA synthesis at the 3' end of exogenous viral RNA templates requires 5'- and 3'-terminal complementary sequences of the viral RNA. J Biol Chem **274**, 33714-33722.

Young PR (1989) An improved method for the detection of peroxidase-conjugated antibodies on immunoblots. J Virol Methods **24**, 227-236.

Young PR (1990) Antigenic analysis of dengue virus using monoclonal antibodies. Southeast Asian J Trop Pub Health **21**, 646-651.

Young PR, Bletchly C and Mackenzie J (1993) Antigenic and structural analysis of the dengue virus glycoprotein, NS1. Arbovirus Research in Australia 138-142.

Young PR and Falconar AKI (1990) Nonstructural proteins as virus vaccines. Uren M and Blok J (Eds) In: Arbo Research in Australia 62-67.

Young PR, Hilditch PA, Bletchly C and Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue protein NS1 in the sera of infected patients. J Clin Micro **38**, 1053-1057.

Yuan X, Geng L, Li X, Yu M and Qin E (Unpublished) Complete sequence of demgue virus type 3 strain 80-2 isolated from Guangxi China. AF317645: submitted 30 Oct 2000.

Zhang YM, Hayes EP, McCarty TC, Dubois DR, Summers PL, Eckels KH, Chanock RM and Lai C-J (1988) Immunization of mice with a structural protein and nonstructural protein NS1 expressed by baculovirus recombinants induces resistance to dengue virus encephalitis. J Virol **62**, 3027-3031.

Zhang L and Padmanabhan R (1993) Role of protein conformation in the processing of dengue virus type 2 nonstructural polyprotein precursor. Gene **129**, 197-205.

Zhang L, Mohan PM and Padmanabhan R (1992) Processing and localization of dengue virus type 2 polyprotein precursor NS3-NS4A-NS4B-NS5. J Virol **66**, 7549-7554.

Zhao B, Mackow E, Buckler-White A, Markoff L, Chanock RM, Lai C-J and Makino Y (1986) Cloning full length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology **155**, 77-88.

Zhao B, Prince G, Horswood R, Eckels K, Summers P, Channock R and Lai C-J (1987) Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. J Virol **61**, 4019-4022.