INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Characterization of dengue virus envelope protein

by

Runtao He

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Dalhousie University

Halifax, Nova Scotia

August, 1997

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Characterization of dengue virus envelope protein"

by Runtao He

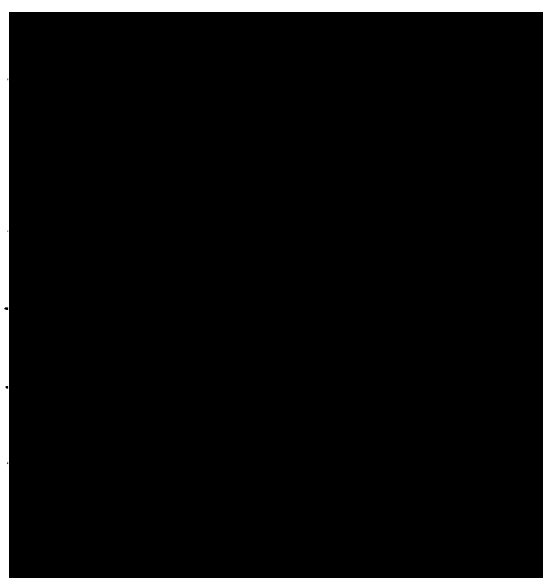
in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: August 29, 1997

External Examiner

Research Supervisor

Examining Committee



DALHOUSIE UNIVERSITY

DATE: September 7, 1997

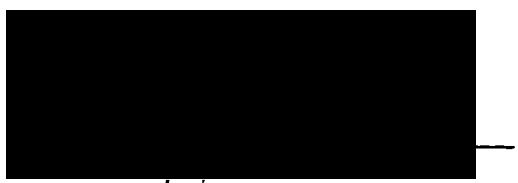
AUTHOR: Runtao He

TITTLE: Characterization of dengue virus envelope protein

DEPARTMENT OR SCHOOL: Microbiology and Immunology

DEGREE: Ph.D. CONVOCATION: Fall YEAR: 1997

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.



Signature of Author

For Songli, Zhong-Zhong and Elizabeth

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

ADE	antibody-dependent enhancement
bp	base pairs
BSA	bovine serum albumin
CP	cell protein
ddH2O	distilled and de-ionized water
E	envelope protein
ECL	enhanced chemiluminescence
Fib	fibronectin
h	hour
HIV	human immunodeficiency virus
Ig	immunoglobulin
Kb	kilobase
kD	kilodalton
MEM	minimal essential medium
min	minute
PAGE	polyacrylamide gel electrophoresis
PAN	post-attachment neutralization
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p.i.	post infection
prM	pre-membrane protein
RT	reverse transcriptase
sec	second
SDS	sodium dodecyl sulfate
TAE	Tris acetate EDTA buffer

ABSTRACT

Dengue virus infection is the leading cause of morbidity and mortality in many tropical countries. The dengue virus envelope protein (E) plays pivotal roles in mediating infection, pathogenesis and host immune response.

We investigated mechanisms of antibody neutralization of dengue virus infection and found that neutralization occurs mainly by blocking E protein-mediated virus attachment to host cells. Dengue-immune sera demonstrated a strong correlation between cell attachment-blocking and neutralization activities. These activities were correlated with levels of E-specific antibodies and involved IgG rather than IgM.

We found that dengue-specific antibody triggers binding of dengue virus to platelets, which might offer a partial explanation for the thrombocytopenia seen in severe dengue disease. Virus binding was mediated by the E protein and involved a platelet component other than Fc receptor.

Dengue virus E protein was found to form complexes with pre-membrane protein (prM). Previous studies on other flaviviruses suggested that this association is important in virus maturation and release. We found that prM-E association not only forms a heterodimer, but also forms higher order molecular structures. We also presented evidence that the site of prM-E association on E protein is in the carboxy terminal anchoring domain, while cell-binding activity resides in a trypsin-releasable ectodomain of the E protein.

One of the barriers of generating recombinant dengue virus vaccine is to find a low-cost and high level expression eukaryotic system. We, for the first time, expressed dengue virus E protein in yeast, which was used in antibody epitope mapping studies and which represents a potentially attractive vaccine candidate against dengue virus.

ACKNOWLEDGMENTS

My heartfelt thanks go to my supervisor Dr. Robert Anderson, for having me in his laboratory and leading me to an appealing career, for his tutelage, thoughtfulness, encouragement and friendship. I also wish to thank members of my supervisory committee, Dr. Spencer Lee, Dr. Tim Lee and Dr. Donald Stoltz for their help and guidance on my research projects. I am very grateful to my wife Songli Wang, for her technical support on my projects, for her devotion to the family and for her faith in my profession. I am indebted to all the other members in our laboratory for their generous assistance and help.

I would like to thank Dr. Jerry Johnston, Dr. Christine Barnes, Dr. Lois Murray, Dr. Roy Duncan and Dr. Melanie Dobson for their helpful comments, discussions and material support. I want to express my gratitude to Kendra Gillis for her technical assistance. I thank members of the Department of Microbiology and Immunology, for all their help throughout my Ph.D program. I also want to thank individuals in the Blood Bank in Victoria General Hospital for their support on my research.

Finally, I would like to thank my parents for their unconditional support, encouragement and and understanding. I am also grateful to all my friends for their friendship and help.

CHAPTER 1

INTRODUCTION

1. FLAVIVIRUS GENOME AND STRUCTURE

Dengue virus is a member of the flavivirus family, which contains three genera: the flaviviruses, the pestiviruses and the hepatitis C viruses (Rice, 1996). These three genera are distinct from each other in their biological properties and serological activities. However, they share the same genome organization and RNA replication strategy. There are four serotypes of dengue virus, from Den-1 to Den-4, which are antigenically related but distinguishable from each other (Rice, 1996).

Flaviviruses are small, enveloped animal viruses containing a single positive strand genomic RNA. The virions are spherical with a diameter of 40-60 nm including an envelope and an envelope spike protein which is usually glycosylated (Chambers et al., 1990a). The nucleocapsid (20-30 nm) is surrounded by a lipid bilayer and consists of core (C) protein and viral RNA. Embedded in the envelope bilayer are the envelope (E) protein and pre-membrane (prM) protein. The extracellular virus particles (mature virions) contain mostly the processed membrane (M) protein, while the intracellular virus particles (immature virions) contain mainly the unprocessed prM protein and are less infectious than mature virions. Maturation occurs by budding of spherical nucleocapsids with icosahedral symmetry through cytoplasmic membranes (Chambers et al., 1990a; Rice, 1996).

Flavivirus genomes contain a single-stranded RNA of about 11kb, which has a type I cap at the 5' end and has no poly (A) at the 3' terminus. The genomic RNA consists mainly of a long open reading frame which contains about 10,000

bases (Chambers et al., 1990a). This open reading frame is flanked by nontranslated regions on both ends.

The first AUG at the beginning of the flavivirus open reading frame serves as the translational initiation codon. However, initiation may also occur at the second in-frame AUG, which is about 12-14 bp downstream (Castle et al., 1985). The virus RNA is translated into a polyprotein which is cotranslationally or posttranslationally cleaved at specific sites by viral or host cell proteases to produce smaller polypeptides. The structural proteins are encoded at the 5' end of the genome, and are followed by the non-structural proteins, encoded towards the 3' end of the virus RNA. The structural proteins are capsid protein (C), pre-membrane protein (prM) and envelope protein (E). The non-structural (NS) proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The gene order of the flavivirus genome is: NH_2-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH (Castle et al., 1985).

The posttranslational processing starts from the capsid (C) protein. The C protein contains a hydrophobic carboxy-terminal sequence that functions as a signal sequence for translocation of pre-membrane (prM) protein into the endoplasmic reticular lumen, where the prM is glycosylated. The carboxy-terminal sequence of the prM serves as a stop-transfer sequence for the prM and also as the leader sequence for the envelope protein. Similarly as for the prM carboxy-terminal sequence, two adjacent hydrophobic sequences at the C terminus of the E protein act both as stop-transfer sequence for the E protein and as leader sequence for the NS-1. After the translocation of prM-E-NS-1 into the endoplasmic reticular lumen, the protein is cleaved into prM, E, and NS-1. The cleavage of C-prM is mediated by the virus-encoded NS 2B+NS3 protease complex (Amberg et al., 1994). The prM is cleaved into M protein right before the virus is released; cleavage is believed to be mediated

by host cell enzymes (Ruiz et al., 1989; Markoff, 1989; Rice, 1996). Most of the cleavages of flavivirus non-structural proteins are mediated by the NS2B-3 protease (Lobigs, 1992; Lin et al., 1993; Amberg et al., 1994). The cleavages of the non-structural proteins have been shown to be very important in the flavivirus replication. Inactivating NS2B-3 or blocking cleavage at certain sites can abolish virus replication (Chambers et al., 1990b; Nestorowicz et al., 1994).

2. FLAVIVIRUS PROTEINS

2.1. The C protein

The flavivirus C protein is a small structural protein with a molecular weight of 11 kD. It is a small basic protein and a major component of the nucleocapsid. The overall sequence homology of C protein among flaviviruses is low. However, the hydrophobic sequences are relatively conserved (Mandl et al., 1988).

2.2. The prM and M proteins

The prM (26 kD) is the precursor of the structural protein M (8kD). PrM and M are found on intracellular and extracellular virions respectively. The prM is cleaved to form M shortly before the virus is released. The amino-terminal portion of prM is released as the so-called pr protein into the extracellular medium (Murray et al., 1993). The association of the prM in the immature virion is believed to protect the immature virus from fusion with the plasma membrane during virus maturation (see Chapter 3).

PrM is also an immune target of flaviviruses; mAbs against the prM protein have been shown to have low neutralizing activities for dengue virus infection (Kaufman et al., 1989). Also, animals immunized with prM/M give rise to some degree of protection (Bray and Lai, 1990). During the replication of some

flaviviruses, the cleavage of prM is not complete, thus allowing the protein to function as a target for neutralizing antibodies (Monath and Heinz, 1996). Furthermore, a recent study showed that prM was involved in CD8+ T-cell-mediated CTL killing (Mathew et al., 1996).

2.3. The E protein

The E protein of flaviviruses is a glycosylated envelope protein with a molecular weight about 55-60 kD. It plays important roles in many important processes such as virion assembly (Nowak et al., 1989), receptor binding (Anderson et al., 1992) and membrane fusion during virus entry and replication (Gollins and Porterfield, 1986; Kimura and Ohyama, 1988; Randolph et al., 1990); also it is a major target for neutralizing antibodies (Della-Porta and Westaway, 1977; Peiris et al., 1982; Hall et al., 1996).

The x-ray structure of a soluble form of the TBE E protein was determined recently (Rey et al., 1995). This soluble form of the E protein was obtained by trypsin cleavage which truncates the protein at residue 395 or 408 (Heinz et al., 1991, 1994). It has been shown that this soluble form of the E protein is a head-to-tail homodimer.

Three structural domains were defined in the crystallography study. Domain I contains about 120 residues in three segments (residues 1-51, 137-189 and 285-302). Domain II is composed of two segments (residues 52-136 and 190-284). The region encompassing residues 98-113 in this domain is conserved in almost all flaviviruses; also, this sequence is hydrophobic and rich in glycine (Rey et al., 1995). It is believed that this fragment is the fusogenic sequence of the flavivirus E protein (Roehrig et al., 1989). Domain III contains residues 303-395. It has an IgC-like

structure which may be recognized by integrins (Bork et al., 1994); also, domain III of some mosquito-borne flavivirus E proteins contains an RGD sequence which is the motif that binds to integrins, suggesting that domain III might be involved in virus-cell binding activities (Rey et al., 1995; Stuart and Gouet, 1995). For most flaviviruses, the E protein contains six disulfide bridges and at least one *N*-glycosylation site which together play important roles in maintaining protein conformation (Nowak and Wengler, 1987; Mandl et al., 1989a; Winkler et al., 1987).

There are two hydrophobic sequence clusters at the carboxy-terminal sequence of the E protein, the first of which acts as a membrane anchor sequence. Structure/function relationships in this region which is around residues 410-485 are less characterized. However, recent studies suggested this region may have other functions other than membrane anchoring. For example, it was suggested that this region may be involved in the prM-E association and E-membrane fusion activities (Heinz et al., 1995; Stuart and Gouet, 1995; also see Chapter 3).

The E protein is the major component of the virion surface and plays an important role in the generation of neutralizing antibodies and induction of other protective immune responses. It has been shown that subviral particles containing the E protein or recombinant E proteins can induce neutralizing antibodies against infections of some flaviviruses, such as dengue (Bray et al., 1989; Feighny et al., 1994), Japanese encephalitis virus (Jan et al., 1993; Konishi et al., 1992) and yellow fever virus (Pincus et al., 1992). Also passive protection experiments using E-specific monoclonal antibodies have shown that these antibodies can inhibit virus infection in animals (Men et al., 1991; Kreil and Eibl, 1997).

2.4. The NS1 protein

The flavivirus NS1 glycoprotein is found both associated with the cell surface and secreted as an extracellular non-virion form (Post et al., 1991). The secretion of NS1 occurs only in mammalian cells and not in mosquito cells (Mason et al., 1989; Post et al., 1991). The natural extracellular form of NS1 is a dimer with a molecular weight of about 90 kD. Mutagenesis studies suggested that the N-terminus of the NS1 is important for the dimerization of NS1 (Pryor and Wright, 1993).

The functions of NS1 in virus replication have not been well characterized. A recent study has shown that NS1 of yellow fever virus may be a virulence determinant, since mutations of NS1 have been shown to decrease virus neurovirulence (Muylaert et al., 1996). Another study demonstrated that NS1 might be involved in flavivirus RNA replication. This study showed that a single alanine substitution for Arg-299, a residue which is conserved among flaviviruses, blocked viral RNA accumulation and abolished infection (Muylaert et al., 1997).

The NS1 protein is expressed on the surface of infected cells (Cardiff and Lund, 1976; Falconar and Young, 1990) and also secreted extracellularly (Jacobs et al., 1992). It has been demonstrated that NS1 can induce immune responses against flavivirus infection, such as dengue (Eckels et al., 1994), Murray valley encephalitis virus (Hall et al., 1996) and TBE virus (Jacobs et al., 1992). The mechanism of anti-NS1 antibody-mediated neutralization is not clear. Since antibodies to NS1 do not react with virions, targets of these antibodies might be flavivirus infected cells expressing NS1 on the surface; and it has been shown that neutralizing activities are dependent on the Fc portion of anti-NS1 antibodies, indicating that the neutralization

activity is mediated by other mechanisms such as complement-mediated cytotoxicity (Schlesinger et al., 1993).

2.5. The NS3 protein

NS3 is the second largest flavivirus protein with a molecular weight of about 68-70 kD. The sequence of this protein is highly conserved among most flaviviruses (Mandl et al., 1989b; Lee et al., 1990). It has been shown that the interaction of NS3 with the hydrophobic NS2B protein gives rise to enzymatic activities. For example, dengue virus NS2B and NS3 forms a stable complex which cleaves NS3 (Arias et al., 1993). The NS2B-3 complex has also been shown to cleave other non-structural proteins, including NS4A and NS4B (Nestorowicz et al., 1994). Other than participating with NS2B, NS3 alone has also been shown to have protease activity which cleaves within an RNA helicase sequence motif of NS3 (Teo and Wright, 1997).

As indicated above, NS3 not only has protease activities, but may also have helicase and RNA triphosphatase activities. Sequence comparison showed that NS3 contains regions of high homology with RNA helicases which are involved in nucleotide cofactor binding and hydrolysis (Arias et al., 1993). It has also been demonstrated that NS3 acts as triphosphatase that is involved in the formation of the cap structure at the 5' end of flavivirus genome (Wengler and Wengler, 1993).

2.6. The NS5 protein

The NS5 protein is the last protein encoded in the flavivirus long open reading frame. It is also the largest and highly conserved viral protein with a molecular weight of about 103-104 kD (Mandl et al., 1989b). NS5 is a basic protein and is believed to be the flavivirus RNA-dependent RNA polymerase. Sequence

analysis showed that NS5 contains a GDD motif which is known as a character of RNA-dependent RNA polymerase of positive-strand RNA viruses (Kamer and Argos, 1984; Rice et al., 1985, 1986). Recent studies with *E. coli* expressed NS5 of dengue 1 virus showed that the protein had RNA-dependent RNA polymerase activities; blocking this protein with NS5 specific antibodies inhibited its polymerase activities (Tan et al., 1996).

Other than RNA-dependent RNA polymerase activities, computer-assisted identification also showed that a sequence motif that is conserved in a number of S-adenosylmethionine (SAM)-utilizing methyltransferases and is implicated in SAM binding was found in the N-terminal portion of NS5 proteins of flaviviruses as well. It was proposed in the same study that the N-terminal domain of NS5 functions as a methyltransferase involved in viral RNA capping, while its C-terminal domain contains the RNA-dependent RNA polymerase activities (Koonin, 1993).

2.7. The NS2A, NS2B, NS4A and NS4B

Functions of these proteins are less well known compared with those of other flavivirus non-structural proteins. The NS2A is a protein with a molecular weight of about 24 kD. It has been shown that the carboxy terminal sequence of this protein may be important for the recognition by cell protease or viral protease which cleaves NS1/2A (Leblois and Young, 1995). NS2B is a protein with a molecular weight of about 27 kD. It contains a highly charged and conserved central region flanked by hydrophobic segments (Falgout et al., 1993). Together with NS3, this protein has been shown to have protease activities which process most structural and non-structural proteins at dibasic sites (Amberg et al., 1994; Chambers et al., 1990a, 1991; Falgout et al., 1991; Jan et al., 1995). Other than protease activities, the

NS2B-3 complex has been demonstrated to participate in RNA localization to cellular membranes (Rice, 1996).

The function of the NS4A protein has not been well characterized. However, the hepatitis C virus NS4A protein facilitates the protease activity of NS3; also it prevents the nuclear accumulation of NS3 in the presence of p53 and increases the cytoplasmic accumulation of NS3 (Vishnuvardhan et al., 1997; Ishido et al., 1997). The NS4B protein has a molecular weight of about 27 kD. The function of this protein is unknown (Rice, 1996).

3. FLAVIVIRUS REPLICATION

In general., the entry of enveloped virus into host cells occurs by two main routes: receptor mediated endocytosis and direct fusion with the plasma membrane. In receptor-mediated endocytosis the virus is internalized at clathrin-coated pits and enveloped within vesicles and then delivered to endosomes where virus-endosomal membrane fusion occurs at low pH (Marsh et al., 1989; Kielian et al., 1990). It is known that orthomyxo-, toga, flavi-, rhabdo-, bunya, and arenaviruses fuse with host cell membranes at low pH (Hernandez et al., 1996). The second route involves direct fusion with the plasma membrane. Following binding to cell surface receptors, virus-cell fusion occurs at neutral pH. Retro-, paramyxo-, herpes-, corona-, hepadna, and poxviruses are known to fuse with cells at neutral pH (Hernandez et al., 1996).

Flaviviruses are thought to enter cells by receptor-mediated endocytosis. There are at least two kinds of receptors that mediate flavivirus entry: Fc receptors, which mediate virus entry in the presence of non-neutralizing or sub-neutralizing antibodies (see antibody dependent enhancement below); and non-Fc receptor(s). It has been shown with West Nile virus that both types of receptors mediate virus entry

by receptor-mediated endocytosis. The uptake of viral particles in coated pits was recorded by electron microscopy (Gollins and Porterfield, 1985). Another similar study showed that Kunjin virus entered Vero cells through the same pathway (Ng and Lau, 1988). However, some flaviviruses may enter host cells via different pathways, for example, electron microscopic observations suggested that Japanese Encephalitis virus entered mosquito cells by a novel pathway involving direct penetration of the plasma membrane followed by dissolution of the viral structure (Hase et al., 1989).

Flavivirus uncoating is pH dependent. It has been demonstrated that at low pH, the uncoating of West Nile virus occurs more rapidly than at neutral pH. Also, increasing the cellular pH with ammonium chloride can block virus uncoating, indicating that flavivirus uncoating occurs in endosomes after the virus is endocytosed (Gollins and Porterfield, 1986).

Flavivirus replication starts with synthesis of a complementary negative strand RNA, which is used as a template to produce additional positive-stranded viral RNA molecules. These positive strands can then be used for translation, synthesis of additional negative strand viral RNA or can be used to form new virions (Westaway et al., 1985; Chambers et al., 1990a). The synthesis of positive strand and negative strand RNAs are regulated in a ratio of these 10:1 (Cleaves et al., 1981). The mechanism for this regulation is not well known; recent studies with Japanese encephalitis virus indicated that NS3, NS5 might be involved in this regulation. It was found that NS3 and NS5 bind to viral plus strand RNA, also both proteins were found to interact with each other and form a protein-protein complex, suggesting that the viral genomic RNA may form a replication complex together with NS3 and NS5; this complex may be involved in Japanese encephalitis virus minus-strand RNA synthesis (Chen et al., 1997).

Electron microscopic studies of flavivirus-infected cells have shown that mature virions are formed first in the endoplasmic reticular lumen (Deubel et al., 1981; Hase et al., 1987; Ishak et al., 1988; Sriurairatna et al., 1973, 1977), then virions are accumulated in membrane-bound vesicles. The endoplasmic reticulum is believed to be involved in the transport of nascent virions through the host secretory pathway to the cell surface where exocytosis occurs (Fan and Mason, 1990; Markoff et al., 1994). Budding of virions at the plasma membrane has also been demonstrated occasionally; however, it does not appear to be a major mechanism of virion formation (Hase et al., 1987; Matsumura et al., 1977; Ohyama et al., 1977; Sriurairatna et al., 1977).

Studies on flavivirus protein synthesis and virion release showed that virus formation starts from the nucleocapsid. The C protein interacts with viral genomic RNA to form a nucleocapsid structure, which later on acquires an envelope structure by budding into the endoplasmic reticular lumen. The prM and E are co-synthesized to ensure proper folding of the E protein. Experimental data indicated that the flaviviruses use oligomerization and prM cleavage to regulate the fusion activities of the E protein (Wengler et al., 1989; Chambers et al., 1990a; Heinz et al., 1994). Later stages in virion maturation include modification of E and prM by trimming and terminal addition of carbohydrates (Chambers et al., 1990a; Mason, 1989; Nowak et al., 1989).

The cytopathic effect of flavivirus infection varies depending on virus and host cell types (Mussgay et al., 1975; Brinton, 1985). In vertebrate cells, dramatic cytopathic changes including vacuolation and proliferation of intracellular membranes may occur (Halstead et al., 1984). For arthropod cells, fusion and syncytium

formation is the most common feature after flavivirus infection. However, the infection is usually not cytopathic to mosquito cells; this explains why mosquitoes can remain chronically infected for life and produce high levels of infectious virus (Varma et al., 1974; Trent et al., 1980).

4. DENGUE DISEASE

4.1. Classic dengue fever

There are two kinds of dengue diseases: The mild disease, or classic dengue fever, and the severe disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DDS). Classic dengue fever develops after 3 to 15 days of incubation. The illness begins with an onset of fever, malaise, headache, retroocular pain, visual disturbances, myalgia, skin rash, nausea and vomiting. The fever usually lasts about 3 to 7 days. However, the fever is usually biphasic, in which high fever and some of the symptoms reoccur after the first phase (Halstead, 1989). Classic dengue fever is a self-limited disease. Patients usually recover in 10 to 14 days.

Classic dengue fever has been investigated histopathologically. The main findings are: swelling of endothelial cells of small vessels, diapedesis of neutrophils, extravasation of red blood cells, perivascular edema, and infiltration of mononuclear cells (Ramirez-Ronda and Garcia, 1994). It is not clear whether direct viral infection, immune complexes, or other mechanisms cause these pathological changes. Dengue viral antigens have also been found following cells of dengue fever patients: liver cells (Rosen et al. 1989), Kupffer cells, mononuclear cells in the skin, pulmonary

macrophages, thymus, lymphatic nodules, spleen, heart, and bone marrow (Halstead, 1989).

4.2. Dengue hemorrhagic fever and dengue shock syndrome

Most people who acquire dengue infection develop mild dengue disease. However, about 1% of dengue infections result in a severe form of dengue disease, dengue hemorrhagic fever/dengue shock syndrome. The onset of the disease is similar as that of classic dengue fever. However, 3 to 4 days after the onset of the disease, patients have signs of: increased vascular permeability, hypovolemia, hemoconcentration, thrombocytopenia, signs of vascular collapse, such as low blood pressure, and hemorrhagic manifestations, such as a positive tourniquet test, skin hemorrhages, epistaxis, and gum bleeding. Shock may develop in the late stages of the disease. If untreated, the case-fatality rate of DHF/DSS can be as high as 40-50%. With supportive treatment, the fatality is about 1-5% (Monath, 1994).

The World Health Organization has classified DHF/DSS into four levels: grade I, fever accompanied by nonspecific constitution symptoms; grade II, spontaneous bleeding in addition to the manifestations of grade I, usually in form of skin and/or other hemorrhages; grade III, circulatory failure manifested by rapid and weak pulse, narrowing of pulse pressure, or hypotension with presence of cold clammy skin and restlessness; and grade IV, profound shock with undetectable blood pressure and pulse (WHO, 1986).

5. HISTORY AND EPIDEMIOLOGY

Dengue infections have emerged as the most important mosquito-borne viral disease in the world. There are about 2.5 billion people at risk of dengue infection,

100 million cases of dengue infections annually, including 250,000 severe dengue cases and 30,000 deaths per year (Patz et al., 1996).

Dengue has been recognized as a clinical disease for more than 200 years. The first dengue epidemic was reported by Benjamin Rush in Philadelphia in 1780 when the epidemic occurred in Asia, Africa and North America. From the eighteenth to nineteenth centuries, dengue caused large epidemics in Asia, Caribbean, North America, Japan and Australia. (Johnson et al., 1967; Erik et al. 1996 Fields).

Until the early 1950's, dengue was considered a mild febrile disease even though rare hemorrhagic and fatal cases occurred. However, in the late 1950's, the first reported epidemics of dengue hemorrhagic fever (DHF) occurred in Southeast Asia (in Philippines in 1954), and DHF became the most important cause of mobidity and mortality of children in that region. (Hammon, 1969). Now severe dengue diseases have become endemic in many southeast Asian and Caribbean countries (Gubler, 1987).

Dengue can also affect to North Americans returning from travel to tropical areas (MMWR, 1996; Duperval et al., 1993). In 1995, 448 Americans who traveled to Mexico, Caribbean islands and other tropical regions were suspected to have dengue infection. Eighty six of them were serologicaly or virologicaly diagnosed as dengue infection. Indigenous transmission of dengue virus was also reported in the US During 1980, 1986 and 1995 in Texas, 63 cases in 1980 and seven cases in 1995 of dengue infection were diagnosed among persons who did not travel outside of the United States (MMWR, 1996).

In addition to North America, other countries with temperate climate are also under increased threat because of increased numbers of travelers who may import tropical diseases and exotic transmission vectors into these areas. These vectors can be adapted to cold climates and transmit the virus to other humans (Kuno, 1995). For example, there are about 50 cases of dengue fever in Sweden each year, attributed to travelers to tropical regions, some of whom suffered severe dengue disease (Wittesjo et al., 1993; Niklasson and Vene, 1996). Also, because of global warming, dengue-transmitting mosquitoes have been found to be a potential threat in high-latitude areas such as the Northern US and France (Knudsen et al., 1996; MMWR, 1996).

Important risk factors for severe dengue disease were proposed in the analysis of Cuban dengue epidemic in 1981, including: 1. age: severe dengue disease mainly affects children, since the majority of adults have been exposed to one or more infections and have developed immunity against infection; 2. gender: adult females are at higher risk than adult males; 3, race: caucasians have higher frequency of having severe dengue disease; 4. health condition: people with chronic diseases, such as sickle cell anemia, bronchial asthma, and diabetes are at higher risk of acquiring severe dengue disease (Bravo et al., 1983, Guzman et al., 1987, Hospedales, 1990).

6. MOLECULAR EPIDEMIOLOGY

Phylogenetic studies and nucleotide sequencing of the E protein have been conducted to investigate dengue virus genotypes in the world. The genotypic classification is useful to determine the origin and spread of epidemics of dengue infection. For example, it was found that a genotype of dengue 1 virus, which was originally found in Thailand, was later found in the Americas where it caused an endemic in 1977 (Guzman et al., 1990; Zulkarnain et al., 1994). As another example, a genotype of dengue 2 virus (New Guinea C strain), isolated in 1944, was found in

Asia and later on introduced to the Americas in 1981. This virus caused an epidemic of severe dengue disease in Cuba. Based on these findings, it appears that new strains of virus may be introduced to new geographical locations by viremic humans or by virus-infected mosquitoes.

7. DENGUE TRANSMISSION VECTORS

Dengue virus is transmitted from human to human by the bite of infected mosquitoes of the Aedes genus. There are four major species of Aedes mosquitoes that can transmit dengue virus: *A. aegypti, A. albopictus, A. triseriatus and A. mediovittatus* (Lifson et al. 1996).

A. aegypti is the most important vector of dengue. The spread of dengue throughout the world can be directly related to the proliferation of this mosquito species. The mosquitoes can only proliferate during high humidity and rain. However, increased urbanization in the modern world allows the mosquitoes to adapt to urban life. They can establish artificial habitats in urban areas, such as water storage containers and flower vases. Female mosquitoes are the principal virus transmitting vectors since they are the ones that feed on human blood (Coleman, 1972; Coleman and McLean, 1973).

A. *albopictus* is a secondary vector for dengue infection. It usually stays in rural areas. However, it may move into urban areas if *A. aegypti* is absent. *A. albopictus* is more susceptible to dengue infection than *A. aegypti.* Also, *A. albopictus* can transmit the virus through transovarial (i.e vertical) transmission (Rosen et al., 1983).

The adult mosquitoes only bite during the day time. A mosquito feeding on a person who is in the first to fifth day of disease symptoms, can transmit the disease to another person. An incubation time of 8 to 11 days is required for the virus to replication in mosquitoes and make the mosquitoes to become infective. Once the mosquito is infected, the infection can persist for the rest of the mosquito's life (about 15 to 65) days. Humans and certain monkeys are the only known reservoir of dengue virus (Rosen et al., 1983).

Mosquitoes generally bite when temperatures are above 20°C (Mclean et al., 1974). For example, the epidemic in Australia stopped when the temperature dropped to 14-15°C (Derrick, 1958). However, recent findings indicated that some mosquitoes can adapt to cold temperatures and transmit dengue viruses (Ward and Burgess, 1993); also, some outdoor breeders have become indoor breeders thus allowing them to transmit the virus even if the outdoor temperatures are low (Watts et al., 1987).

Vector density is also an important factor in causing dengue epidemics. Some reports indicated that there was a correlation between the density of the vector in the environment and outbreak of dengue fever (Gubler, 1989). This finding was further supported by previous observations that vector mosquitoes move very slowly in the urban areas, about 25 meters per 24 h (Morlan and Hays, 1958). It was found that during an outbreak in Puerto Rico, a dengue outbreak in one community did not spread to another community 30 yards away (Neff et al., 1967). Other findings suggested that mosquitoes are genetically controlled to have house-entering and house-staying behaviors (Trpis, 1978), more than 99% of mosquitoes stay in the original home (Trpis and Hausermann, 1986). These data suggest the requirement of

a high density of virus-bearing mosquitoes in the environment in order to cause epidemics of dengue.

For the above reasons, vector control is one of the most important ways to prevent dengue epidemics. For emergency epidemic control, low-volume application of insecticide (such as malathion) has been shown to be effective. Some retrospective studies showed that dengue cases declined after the application of insecticides (Gubler et al., 1989). "Immunization of mosquitoes" is another way of control dengue-transmitting vectors. In one strategy, antisense RNA of dengue virus is cloned into a Sindbis virus vector which is then used to infect mosquitoes. It has been shown that mosquito cells (C6/36) containing a fragment of prM antisense RNA were not infectable by dengue virus (Gaines et al., 1996); also, mosquitoes infected with a Sindbis virus vector containing prM antisense RNA, did not support dengue virus infection (Olson et al., 1996).

8. DIAGNOSIS OF DENGUE INFECTION

Diagnosis of dengue depends on virus isolation or serologic tests. In most cases, virus may be isolated from the blood during the early stage of the disease. Cells from *Toxorhynchites amboinensis* (TRA-284), *Aedes albopictus* cells (C6/36) and *Aedes pseudoscutellaris* (AP-61) are the most widely used for virus recoveries. Cultures are examined by immunofluorescence or countercurrent immuno-electrophoresis with anti-dengue antibodies to detect the virus (Kuno et al., 1985).

Recently, molecular biology techniques for example, reverse transcriptase-polymerase chain reaction (RT-PCR), have also been applied in the diagnosis of dengue virus infection. Using serotype-specific primers, RT-PCR can rapidly and specifically amplify individual serotypes of dengue viruses. It has been demonstrated

that RT-PCR can detect extremely low viremia levels (Deubel et al., 1990; Lanciotti et al., 1992).

9. PATHOGENESIS OF DENGUE DISEASE

Current evidence indicates a strong role of the host immune response in the pathogenesis of dengue disease. Some of the relevant immune factors are discussed below:

9.1. Immune responses to dengue virus infection

9.1.1. Humoral immune responses

Immunoglobulin M (IgM) and immunoglobulin G (IgG) are the major immunoglobulins detected after flavivirus infection (Dittmar et al., 1979). After a primary infection, IgM antibodies are initially produced and then followed by IgG antibodies. Levels of anti-viral IgM may persist for 4-6 months (Gunasegaran et al., 1986). However, in some cases, the IgM response is low or even absent, especially during a first flavivirus infection (Edelman et al., 1973). In contrast, the IgG response, especially during secondary dengue virus infection with a heterologous serotype, is usually strong and maintained at high levels for a longer time (Jianmin et al., 1995).

The immune responses mediated by IgM are different from that of IgG. For example, IgM produced by humans in dengue virus infection does not enhance infection of susceptible host cells, while IgG mediates antibody dependent enhancement (see below) (Halstead, 1977). This may be due to the absence of Fc receptors for IgM on host cells (Jianmin et al., 1995). IgM does not activate the classical complement pathway after it binds to virions (Scott and Russell, 1972; Davis

and, 1989); in contrast, IgG-virion complexes activate Complement through the classical pathway (Scott et al., 1976). It was proposed that IgM may not be able to form the crab-like conformation on the surface of virions required for binding of C1q, or IgM may bind to different epitopes on the dengue virion than IgG (Jianmin et al., 1994). Furthermore, anti-flavivirus IgM does not generally cross react with heterologous flaviviruses; however, anti-flavivirus IgG is highly cross-reactive. (Figueiredo et al., 1989; Holzmann et al., 1996).

Studies with monoclonal IgM and IgG against dengue virus showed that both types of mAbs have neutralizing activities. Suprisingly, neutralizing epitopes recognized by both antibodies were found to be located in the same region of the E protein (residues 298-397) (Megret et al., 1992; Jianmin et al., 1994).

9.1.2. Antibody-dependent enhancement (ADE)

Antibody-dependent enhancement of infection is a phenomenon in which viral infection of susceptible cells is enhanced by the addition of virus-specific antibodies (Morens, 1994). One of the first reports on ADE was by Hawkes (1967) in the 1960's after tissue-culture techniques became available and popularly used. Dilutions of neutralizing immune antisera were incubated with homologous flaviviruses and then used to infect chicken embryo cells. It was found that when neutralizing antisera were diluted, some of them lost neutralizing activity and instead enhanced the virus infection. In 1977 Scott Halstead suggested that there was a correlation between the observed in vitro enhancement and the epidemiology and pathogenesis of dengue disease. This phenomenon was then called antibody-depended enhancement, or ADE (Halstead, 1977). ADE is thought to occur when complexes of virus and non-neutralizing or cross reactive antibodies (cross react with other dengue serotypes) bind to the Fc receptors of monocytes or other Fc receptor

bearing cells via the Fc portion of the antibody, thereby enhancing virus binding and infection, and causing severe dengue disease (Halstead, 1988, Halstead, 1989, Kliks et al., 1988).

As mentioned in this chapter, dengue disease was considered a relatively "benign" disease before the 1950's. Why did it cause epidemics of fatal disease? Epidemiological and statistical studies in Thailand showed that most primary dengue virus infections were benign, while severe dengue disease occurred almost exclusively during secondary infection (Basaca-Sevilla and Halstead, 1966; Halstead et al., 1965; Halstead, 1977). It was also noticed that there was an age prevalence of severe dengue disease with most cases occurring during 7 months to 3-5 years of age. Furthermore, studies indicated that severe dengue disease occurred in infants with circulating anti-dengue antibodies from the mother during primary infection, while in school-aged children during the secondary infection (Halstead, 1969, 1977).

Studies with rhesus monkeys showed that sequential infection with dengue-4 and dengue-2 occasionally caused severe disease (Halstead et al., 1973; Marchette et al., 1973); also, administration of heterologous anti-dengue antisera to monkeys caused significant increase of the virus load (Halstead et al., 1979). In addition, in vitro studies showed that dengue virus infection of human peripheral blood monocytes could be enhanced in the presence of non-neutralizing or sub-neutralizing antibodies (Marchette et al., 1975; 1976; Halstead et al., 1976).

Epidemiological studies on the outbreak of dengue in Cuba in 1977 and the subsequent epidemic in 1981 indicated that no severe dengue cases were found in the first epidemic with dengue-1. However, the second epidemic with dengue-2 resulted in one of the world's largest severe dengue epidemics (Guzman et al., 1984; Bravo et

al., 1987; Kouri et al., 1987). Another finding in the Cuban epidemic studies was that severe dengue cases occurred in infants and older children who had primary dengue infection in the 1977 epidemic. However no cases were found with children aged 1-2 years old, who would not have had pre-existing maternal anti-dengue antibodies (Bravo et al., 1987). All these data suggested that there is a link between pre-existing anti-dengue antibodies and severe dengue disease.

ADE may increase virus load in the patient and may induce increased release of vasodilation factors, such as IL-1 and IL-6, which contribute to the pathogenesis of severe dengue disease (Morens, 1994). There is a rapid response to the treatment of fluid administration, which is different from responses to other shock syndromes. It has been noticed that even for some severe dengue shock syndrome cases, administration of fluids itself without "plasma expanders" is enough to reverse the disease. These findings suggest that there might be large amounts of rapidly-acting vasodilating factors in the circulation of severe dengue patients; and the fluid administration helps to dilute these factors out of the circulation and correct the severe dengue disease.

However, the ADE theory still remains controversial because of the following reasons, 1) So far direct evidence of ADE come only from in-vitro studies. Demonstrating direct ADE in humans has been difficult, because the viremia during the second dengue infection is depressed by the rapid and early immune response. 2) Severe dengue disease can occur (although at very low frequency) in noninfantile individuals with primary dengue infection (Barnes and Rosen, 1974). In addition, a review study of a dengue epidemic in Greece during 1927-1928 indicated that severe dengue cases were found at that time; however, no evidence of sequential infection was found (Rosen, 1986). These data suggested that there might be some other

mechanisms which cause severe dengue disease. 3) Only 1% of individuals who have secondary dengue infection develop severe dengue disease (Halstead, 1992), suggesting that there might be some host factors that contribute to the pathogenesis of severe dengue disease. It has been reported that the prevalence of one HLA-A, two HLA-B, HLA Cw1 and HLA-A29 antigens appeared to be related to the development of DHF/DSS (Chiewsilp et al., 1981; Paradoa-Perez et al., 1987). 4) It has been proposed that strain virulence might be important for the development of severe dengue disease (Kouri et al., 1987). Molecular epidemiology studies have shown that some virus strains are more likely to be related to severe dengue disease than others (Zin et al., 1995; Thant et al., 1996). Another study showed that a single nucleotide change in the E protein gene affects virus virulence in animals (Sanchez and Ruiz, 1996). These results support the hypothesis that viral factors may be important for severe dengue pathogenesis. Nevertheless, as mentioned above, the evidence for ADE as an important risk factor for severe dengue diseases is extremely strong.

In conclusion, evidence from epidemiological., clinical and in vitro studies support the hypothesis that severe dengue disease is dependent on ADE, which occurs during sequential heterologous infection with dengue virus. Additional factors, such as host genetics and viral factors may also contribute to severe dengue disease.

9.1.3. Cellular immune responses

The T-cell responses to some flaviviruses have been shown to involve CD4+, CD8- and CD4-, CD8+ T-lymphocytes. Primary dengue virus infections induce serotype-specific and serotype-cross-reactive, CD4+ and CD8+ memory cytotoxic T lymphocytes; while secondary infections with a virus of a different

serotype and higher virus load can substantially activate these serotype cross-reactive CD4+ and CD8+ memory and CTL T cells (Kurane and Ennis, 1992).

The rapid release of cytokines and chemical mediators caused by activated T cells plays an important role in the pathogenesis of severe dengue disease (Kurane et al., 1992). It has been demonstrated that peripheral blood mononuclear cells (PBMC) from dengue-immune individuals produced higher levels of cytokines than PBMC from non-immune individuals. It was also shown that CD4+ T cells are the dominant cytokine producers, nevertheless, CD8+ cells can also produce low levels of cytokines. It was found that levels of IL-2, IL-4, TNF-α, TNF-β and IFN-γ increased when dengue-immune PBMC were challenged with the virus (Mori et al., 1997). Some of these cytokines are vasodilators and may be involved in the pathogenesis of severe dengue disease.

The CTL response is another important part of cellular immunity against flavivirus infections. Studies with dengue virus showed that both CD4+, CD8- and CD4-, CD8+ T-cells can mediate CTL responses to dengue virus infection. These CTL responses eliminate virus-infected cells from the host (Kurane et al., 1991; Zivny et al., 1995). The predominant source of T-cell recognition peptides seems to be from NS3; however, CD4+, CD8- T-cells recognize different epitopes on NS3 than those recognized by CD4-, CD8+ ones (Kurane et al., 1993; Lobigs et al., 1994; Livingston et al., 1995; Mori et al., 1997).

9.1.4. Complement

Like many other virus infections, Complement is activated in flavivirus infection. As for dengue virus, it has been shown that levels of C1q, C3 and C4 are decreased in severe dengue patients. C3 was shown to be activated by both classical

and alternative pathways during flavivirus infection (Bokisch et al., 1973; Nishioka, 1974). It was shown that the shock and capillary leakage occur at the same time that C3a and C5a reach peak levels (Churdboonchart, 1983. Hospedales, 1990, Malasit, 1987). Nevertheless, the presumed interplay of circulatory immune complexes, complement and other events involved in manifestation of severe dengue disease is still not clear.

9.2. PLATELETS

Thrombocytopenia occurs in severe dengue disease, and contributes to uncontrolled bleeding (Funahara et al., 1987). The direct cause of thrombocytopenia is still not clear. However, there are postulations: 1. Impaired platelet biogenesis. Megakaryocytes are affected during the development of severe dengue disease. Rothwell et al. (1996) found that dengue-2 virus can infect stromal cells. Clinical evidence indicated that bone marrow depression was found during the first 4 days of DHF/DSS (Aung et al., 1975; Putintseva et al., 1986; La-Russa and Innis, 1995). Direct infection of megakaryocytes may damage the cells and shorten the lifespan of the platelets. 2. Immune destruction of platelets. Virus immune complexes have been reported on the surface of platelets isolated from dengue patients (Boonpucknavig et al. 1976). This may allow immune responses to "tag" or lyse platelets, possibly leading to thrombocytopenia.

In conclusion, the pathogenesis of severe dengue disease is not completely understood. However, it is known that both humoral and T cell responses are involved. In secondary infection with a different serotype virus, enhancing antibodies increase the number of infected monocytes. The infected monocytes activate CD4+ memory T cells induced by the primary infection. The activated T cells produce cytokines such as IFNγ and IL2. IFNγ upregulates the expression of Fcγ receptors

and MHC class I and class II molecules. The increased number of Fcγ receptors may in turn enhance the infection of monocytes through ADE. The upregulation of MHC molecules may increase the recognition of dengue virus antigens by CTLs which can lyse dengue infected cells. The lysed cells can release chemical mediators, which may mediate the pathological changes in severe dengue disease. Vasoactive activators such as TNF-α, liberated from either T cells or virus-infected monocytes (Anderson et al., 1997) are also important potential modulators of endothelial cell permeability in severe dengue disease.

10. VACCINE DEVELOPMENT

There is no effective vaccine against dengue virus at the present time. The major difficulties are the existence of four serotypes of dengue virus and the lack of animal models (Halstead, 1988). The current strategies to develop dengue vaccines include using live attenuated dengue virus or recombinant dengue viral proteins as vaccines (Bhamarapravati, 1989, Venugopal., 1994).

Some attenuated vaccine candidates, including single serotype or quadravalent vaccines, have been developed. Clinical trials proved that some vaccines are safe and highly immunogenic. One study in the US showed that people immunized with a live-attenuated dengue 2 vaccine developed neutralizing antibody that persisted for two years (Vaughn et al. 1996). However, large scale studies of the attenuated dengue vaccines in humans are required to confirm the efficacy of the vaccines. In general., attenuated vaccines have some disadvantages: 1) The cost of the vaccine is high. 2) The attenuated strain of the virus may revert to the virulent phenotypes. For dengue virus vaccine, there is an additional concern: ADE.

Some single serotype, live-attenuated vaccines showed induction of neutralizing antibodies (Vaughn et al., 1996). However, these single serotype vaccines may not protect individuals from infection with another serotype, or they may even enhance the infection through ADE. A live-virus vaccine study with dengue-2 virus showed that people who previously received yellow fever (a member of flavivirus family) vaccine were more likely to develop viremia than non-immune individuals, suggesting that heterologous anti-flavivirus antibodies may have enhancing activities (Halstead et al., 1983).

ADE is not only a concern for dengue virus vaccine development, but also a concern for other vaccines including respiratory syncytial virus (RSV) and HIV. Trials of an RSV vaccine conducted in 1960's showed that subsequent natural RSV infection produced more severe disease in vaccinated infants than in non-immune ones (Chin et al., 1969). As for HIV, it has been reported that anti-HIV antisera isolated from patients could enhance the virus infection in vitro, suggesting that enhancing antibodies may contribute to the spread and pathogenesis of HIV in vivo (Homsy et al., 1990); another study showed that AIDS patients were more likely to have enhancing antibodies than those who are asymptomatic HIV-seropositive patients, indicating that enhancing antibodies may increase progression of the disease (Fust et al., 1994).

To overcome the above problems, many dengue vaccine strategies are now focusing on using recombinant dengue virus components. The progress in molecular biology technology, protein chemistry and immunology have made it possible to express recombinant viral proteins used for vaccine production. The main strategy in recombinant vaccines is to express dominant epitopes of viral proteins, which can induce strong immune responses (Ellis, 1996). Several expression systems have been

used to express dengue viral proteins to develop vaccines, including prokaryotic, insect and mammalian systems.

Prokaryotic systems, using E. coli to express dengue virus proteins, were the first to be used for the development of potential dengue vaccines. A study showed that mice immunized with a dengue-2 E and NS1 fusion protein expressed in *E. coli* were protected against dengue virus infection (Srivastava et al., 1995). However, in general., *E. coli* -produced recombinant proteins do not provide highly protective immune responses, because of conformational differences between proteins from prokaryotic systems and mammalian ones. It has been shown that dengue type 1 and type 2 E protein expressed in *E. coli* induced antibodies which reacted with authentic dengue proteins; however this E protein could not induce protective immune responses in mice (Mason et al., 1990). Another study with *E. coli* -expressed yellow fever virus E protein showed that the recombinant protein induced only a partially protective immune response (Cane and Gould, 1988).

The baculovirus expression system using *Autographa californica* nuclear polyhedrosis virus offers advantages in producing high-level expression of foreign genes, especially those that require proteolytic processing, glycosylation or secretion (Luckow and Summers, 1988). It has been demonstrated that a baculovirus expressing a 4 kb dengue-4 virus cDNA sequence encoding from C to NS2A generated E and NS1 proteins similar in size to the authentic proteins. Furthermore, these proteins induced resistance in mice against fatal dengue encephalitis (Zhang et al., 1988). Another study using baculovirus-expressed dengue-2 virus E protein also showed protection of mice against dengue challenge (Feighny et al., 1994; Delenda et al., 1994). A pitfall of using recombinant baculoviruses in vaccine development is the

high cost, which has hampered vaccine development against rabies virus (Prehaud et al., 1989).

Mammalian cell expression systems using mammalian viruses have an advantage that the foreign gene products can be amplified in the host during the virus replication. Adenoviruses, herpesviruses and poxviruses are commonly used mammalian expression vectors. However, even if adenoviruses and herpesviruses have been used to express foreign proteins, their oncogenic potential needs to be fully investigated before they can be used in vaccine developments. Vaccinia viruses do not have oncogenic potential and have been used for expression of several flaviviral structural and non-structural genes including all dengue-4 virus structural proteins and two non-structural proteins, NS1 and NS2A (Zhao et al., 1987; Bray et al., 1989). However, the expression products showed inconsistent results in animal immunization experiments. Vaccinia virus-expressed dengue-4 E protein induced strong resistance to virus infection (Bray et al., 1989); while another similar study with dengue-2 virus E protein showed poor immune responses (Deubel et al., 1988). Reasons for the poor immune responses were not clear; however, it was speculated that insufficient levels of expression, poor conformation and subcellular localization might be responsible. These results suggested that alternative constructs should be considered to enhance the immunogenicity of the expression products (Venugopal and Gould, 1994). A concern about using vaccinia virus in vaccines is that some vaccinia virus strains may give rise to virulent strains (Ehrengut et al., 1975; Tignor et al., 1992). To solve this problem, attenuated vaccinia viruses are being used for vaccines (Tartaglia et al., 1992). Also species-specific recombinant pox viruses based on raccoon poxvirus (Esposito et al., 1988), canary poxvirus (Tartaglia et al., 1992) and so on are also being used. Although these viruses may be incapable of causing

high levels of productive infection in heterologous species, they may produce sufficient proteins to induce immune responses (Venugopal and Gould, 1994).

In addition to the above expression systems, other potential systems for delivering dengue virus proteins have also been considered. For example, virus-like particles of hepatitis B virus have been used to express dengue-2 virus E protein and shown to induce neutralizing antibodies (unpublished result of Chang-GJ, cited in Venugopal and Gould, 1994). In addition, full-length infectious dengue-4 virus cDNA has been cloned and can be genetically engineered to develop dengue vaccines (Lai et al., 1991).

11. SCOPE OF RESEARCH DESCRIBED IN THIS THESIS

Many questions remain concerning the mechanisms of pathogenesis of dengue disease. However, it is clear that the viral E protein plays a key role in pathogenesis and in the induction of both enhancing and neutralizing antibody responses. We have therefore chosen to study the E protein as a tool to investigate both pathogenetic mechanisms as well as potential vaccine strategies against dengue virus infection.

CHAPTER 2

MECHANISMS OF ANTIBODY NEUTRALIZATION OF DENGUE VIRUS INFECTION

1. INTRODUCTION

The host antibody response is an important mechanism of defense against virus infection. Host antibodies are especially important in dengue virus infection since they are known to both neutralize as well as enhance virus infection. Despite the importance of antibody in modulating dengue virus infection, little is known of the mechanisms by which antibody effects its actions. In this chapter, we describe studies aimed at understanding the mechanism of host antibody neutralization of dengue virus.

Many studies have been done to investigate the mechanism of antibody neutralization of viruses. The mechanisms of antibody-mediated neutralization vary depending on the kind of virus infection. Generally, antibody mediates neutralization by one of the following mechanisms: blocking virus-cell attachment, causing virus aggregation and neutralization after virus-cell attachment (Dimmock, 1993).

Antibodies can inhibit virus attachment to the host cell surface by blocking receptor-binding sites or changing conformations of virus attachment proteins. For example, anti-HIV antibodies that blocked the binding site of gp 120 to the CD4 receptor abolished virus infection of T cells (Back et al., 1990). Antibodies against Newcastle disease virus were shown to be able to change the conformation of the attachment protein (HN glycoprotein) and to neutralize virus infection. Two groups of monoclonal antibodies that recognized different domains of the virus attachment

protein were used to study the mechanism of neutralization (each group of antibodies could recognize overlapping epitopes). Both groups were shown to have neutralizing activities. However, one group of antibodies could block virus binding to host cells, while the other group could not, indicating that some neutralizing antibodies may inhibit virus infection by changing the conformation of virus attachment proteins (Iorio and Bratt, 1983; Iorio et al., 1989; Iorio et al., 1991).

Antibodies mediate virus aggregation by crosslinking virus particles and reducing virus infectivity. It has been reported that certain monoclonal antibodies against poliovirus (Brioen et al., 1983), influenza virus (Armstrong et al., 1990) and rhinovirus (Smith et al., 1993) neutralize virus infection by causing virus aggregation. Antibody-mediated virus aggregation is highly dependent on the optimal ratio of antibody and virus, as high concentration of antibody can block the epitopes that are necessary for the formation of aggregates (Lafferty and Oertelis, 1963; Armstrong et al., 1990; Dimmock, 1993).

Furthermore, antibodies can inhibit virus infection after the attachment of virus to host cells via blocking the following steps: 1) endocytosis. Inhibition of virus endocytosis is usually mediated by large immunoglobulin molecules, such as IgA and IgM which interfere with the virus binding to a sufficient number of cell receptor units to initiate endocytosis. It has been shown that secretory IgA can mediate the neutralization of influenza virus infection by blocking the endocytosis of the virus. It was shown that secretory IgA did not completely block virus-cell attachment, but completely inhibited the internalization of the virus (Taylor and Dimmock, 1985). 2) virus fusion. Antibodies can also block virus fusion to the endosomal membrane or plasma membrane by either blocking fusion epitope(s) of virus protein or by blocking the binding of virus to its co-receptor(s) that mediate

fusion. For example, it has been reported that antibodies against rabies virus neutralize virus infection via inhibiting virus fusion with the endosomal membrane (Dietzschold et al., 1987). It has also been shown that antibodies that block the binding of HIV to its co-receptor CXCR4 inhibit virus fusion to the plasma membrane and abolish virus infection (Endres et al., 1996; McKnight et al., 1997). 3) post-primary uncoating. It has been shown that a group of neutralizing antibodies (IgG) against influenza virus infection did not inhibit attachment, internalization, fusion or transport of the genome to the cell nucleus (Possee and Dimmock, 1982; Taylor and Dimmock, 1985; Outlaw and Dimmock, 1991). However, there was no transcription of the virus RNA (Rigg et al., 1989), indicating that the inhibition of virus infection occurred at post uncoating steps. Possee et al (1981) showed that the virion transcriptase activity of neutralized virus was reduced compared with non-neutralized virus suggesting that certain antibodies against influenza virus neutralize infection by somehow inactivating virion transcriptase activity.

Although many studies have been done to study mechanisms of antibody neutralization of some virus infections, there is still a lack of understanding of the neutralization mechanisms for other viruses. For example, the mechanism(s) of antibody neutralization of dengue virus infection is less well studied. It is not clear whether antibodies inhibit virus infection by blocking attachment or by blocking postattachment events.

So far several studies have shown that most monoclonal antibodies against domain III of the dengue virus E protein have neutralizing activities (Roehrig et al., 1990; Trirawatanapong et al., 1992; Megret et al., 1992; Lin et al., 1994). This domain is to be involved in flavivirus-receptor binding (Rey et al., 1995; Stuart and Gouet, 1995; Hiramatsu et al., 1996), suggesting that blocking virus attachment by

anti-dengue antibodies may play a role in the antibody neutralization of dengue virus infection. To investigate this hypothesis, we performed the following study with acute phase dengue patient antisera to examine the ability and mechanism of neutralizing human antisera to interfere with the first measurable event in dengue virus infection.

2. MATERIALS AND METHODS

2.1. Cells and virus

African Green Monkey kidney Vero cells were propagated as monolayer cultures in Eagle's modified minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). The dengue-2 serotype (16681 strain) was used for these studies. This strain was originally isolated from a Thai child with dengue shock syndrome (Halstead and Simasthien, 1970)

2.2. Sera

Mouse monoclonal antibody (mAb) 3H5 against dengue-2 E protein (Gentry et al., 1982) was kindly provided by Dr. A. King, Dept. of Virus Diseases, Walter Reed Army Institute of Research. Mouse antibodies against dengue-2 virus were prepared as ascites fluids from virus-immunized BALB/c mice. Specimens from patients acutely infected with dengue type 2 virus were provided by Dr. B. Innis Department of Virology, AFRIMS, Bangkok Thailand. The specimens came from a case series of patients with suspected dengue infection at the Bangkok Children's Hospital in 1990 (n=1,242). In that case series there were 156 patients viremic with dengue type 2 (as assayed by inoculation into Toxorhynchites mosquitoes) when an acute serum specimen was collected. Of the 156 acute sera, 61 (39%) were retrieved from -20°C storage. For each specimen, the following data on the donor were

available: age and sex, the grade of dengue infection (i.e dengue fever or dengue hemorrhagic fever grade I through 1V, level of anti-dengue IgM and IgG determined by isotype-capture EIA (Innis et al., 1989) and the presence (secondary infection) or absence (primary infection) of circulating anti-dengue antibody at the time of infection. The latter was deduced from the presence or absence of an anamnestic anti-flavivirus antibody response during the donor's hospital course.

2.3. Virus-cell binding

The previously described virus-cell binding assay (Anderson et al., 1992) was modified as follows. Radiolabeled dengue-2 virus was prepared by culturing virus-infected Vero cells from 24-48 h post-inoculation (PI) in the presence of ^{35}S-methionine (100 μCi/ml). Aliquots of harvested culture fluids were mixed with medium and allowed to adsorb at 4°C to Vero cell monolayers in 24-well plates. Following adsorption for various times, monolayers were washed with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Monolayers were washed once more with PBS and solubilized in dissociation buffer for analysis by SDS-PAGE (Laemmli, 1970) using 5-18% polyacrylamide gradient gels. Gels were impregnated with 1M sodium salicylate, dried and fluorographed by exposure to Kodak X-ray film at -70°C.

2.4. Sucrose gradient fractionation of supernatant virus

Clarified culture fluid supernatant from ^{35}S-methionine-labeled dengue-infected Vero cells was overlaid on 10-50% (w/w) sucrose gradients in PBS and centrifuged for 16 h at 45k rpm in a Beckman SW60 rotor. Gradient tubes were pierced from the bottom and drained by gravity into eight microfuge tubes. Aliquots were analyzed by SDS-PAGE and fluorography.

2.5. Virus-cell attachment blocking assays

Clarified culture fluid supernatants were mixed with dilutions of antisera, incubated 1 h at 4°C and added to monolayer cultures of Vero cells in 24-well plates. Final concentrations were: 5 x 10^4 plaque forming units (pfu, as assayed on Vero cells) of radiolabeled virus, 5 x 10^4 Vero cells (multiplicity of inoculation, MOI = 1) and various dilutions of antiserum as indicated in the Results and/or Figures. Following incubation for 1 h at 4°C, unadsorbed material was removed by washing and cell-bound radiolabel was detected by SDS-PAGE fluorography as described above. Levels of radiolabeled E protein were quantitated from the X-ray film by means of an Apple Color One Scanner linked to an Apple Macintosh Centris 610 computer using Scan Analysis software (Release 2.20; Biosoft, Cambridge, U.K.). This method proved an excellent quantitator of relative levels of radiolabeled proteins (Pearson's coefficient of the correlation between densitometric quantitation and known amounts of radiolabeled proteins = 0.96; n = 10, $P < .001$). Maximum binding (cell-bound viral E protein in the absence of antibody) was assigned a value of 100 and all other densitometric quantitations expressed as a percentage of this amount. Blocking activities were then obtained by subtraction from 100.

2.6. Virus neutralization assays

Mixtures of dengue-2 virus and antisera were incubated for 1 h at 4°C and assayed for infectivity by inoculation onto Vero cell monolayers (MOI = 1, using same numbers of viral pfu and cells as described above for the virus-cell attachment blocking assays). After 1h adsorption at 4°C, cells were washed twice with medium and overlaid with agar-containing medium. Plaques were counted after 5 days incubation at 37°C. The percentage of total virus neutralized by each serum sample at a 1:100 dilution was calculated.

Virus neutralization assays were also performed with ^{35}S-methionine. The infected cells were labeled with ^{35}S-methionine at 30 h PI for 12 h. Culture fluids were collected and immunoprecipitated with dengue-immune serum. Samples were analysed on SDS-PAGE and fluorographed. Relative levels of radiolabeled viral proteins were quantified by scanning densitometry as described above.

2.7. Post-attachment virus neutralization assays

Dengue virus was allowed to bind to Vero cells 1 h at 4°C, then serial dilutions of anti-dengue antiserum (#59) were added to the binding samples and incubated for 1 h. Cells were then washed with medium and incubated at 37°C. Infected cells were labeled for 12 h with ^{35}S-methionine at 30 h PI. Culture fluids were immunoprecipitated with dengue immune serum and analysed by 10% SDS-PAGE and fluorography.

2.8. Immunoprecipitation for E-specific antibodies

Supernatant culture fluid from ^{35}S-methionine-labeled dengue-infected Vero cells was mixed with immunoprecipitation (IP) buffer (10 mM Na_2HPO_4 pH 7.2, 0.15 M NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS). Aliquots of patient sera (1:100 final dilution) were added, mixed and let stand overnight at 4°C. Immune complexes were bound to formalin-fixed, protein A-bearing *Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitates were recovered by microcentrifugation and washing with IP buffer and were subsequently analyzed by SDS-PAGE and fluorography. The E protein band was semi-quantitated by scanning densitometry of the X-ray film as described above.

2.9. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were set up using a BioRad Protean-II one-dimensional gel apparatus. Lower (resolving) gels generally consisted of either 10, 12.5 or 5-22% gradients of acrylamide/bisacrylamide (37.5:1w/w) in 0.45M M Tris pH8.8, 2% SDS, 0.33% ammonium persulfate (freshly prepared), 0.05% TEMED. The upper (stacking) gel contained 5.5% acrylamide/bisacrylamide (37.5:1 w/w), 0.125 M Tris pH6.8, 1% SDS, 1% ammonium persulfate and 0.05% TEMED.

3. RESULTS

3.1. Characterization of virus supernatants used for cell-binding

To confirm that the E protein is virus associated, we examined culture fluid from ^{35}S-methionine-labeled dengue-infected Vero cells on a sucrose gradient. Fractions of the gradient were analyzed by SDS-PAGE. As shown in Fig. 1, the E protein was found in fraction 2 (which contained the peak virus infectivity). In addition to E, the other viral structural proteins, prM, M and C (C protein of dengue-2 is generally poorly labeled with ^{35}S-methionine; cf. Randolph et al., 1990; Murray et al., 1993) were found in the same fraction. The protein profile is very similar to a published dengue-2 virus profile (Randolph et al., 1990).

3.2. Binding of intact dengue-2 virus to Vero cells

In order to confirm that our virus cell-binding procedure was detecting binding of intact virus, a time-course study was performed. As shown in Fig. 2, ^{35}S-methionine labeled dengue virus was used for binding to Vero cells. Binding samples were harvested at different time periods, run on SDS-PAGE and subjected to

Figure 1.

E protein is virus-associated. Culture fluid supernatant from ^{35}S-methionine-labeled dengue-infected Vero cells was fractionated on a 10-50% sucrose gradient. Aliquots from each of the eight fractions (1 being the bottom fraction and 8 the top) as well as the total culture fluid supernatant (T) were analyzed by 10-22% fluorographic SDS-PAGE. Virus was observed mainly in fraction 2 as indicated by the virion structural proteins E, prM, M and C. Fib= Fibronectin; (NS1)= non-structural protein 1 dimer

Figure 1.

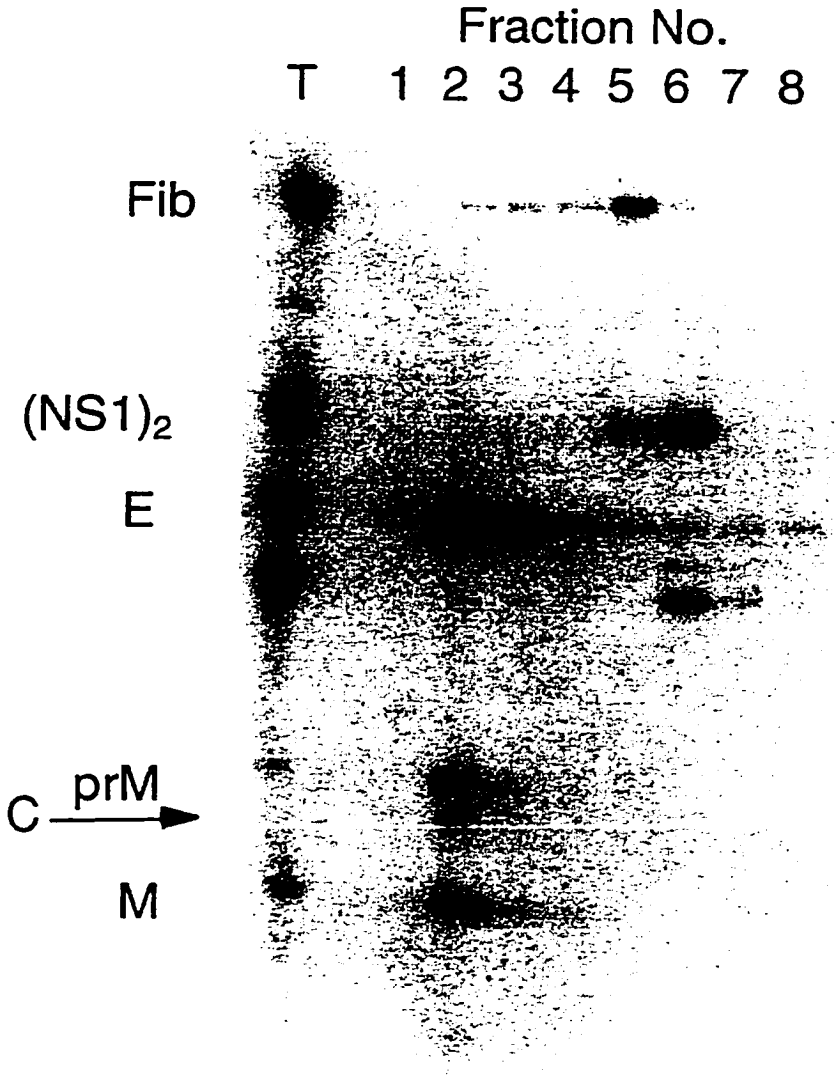
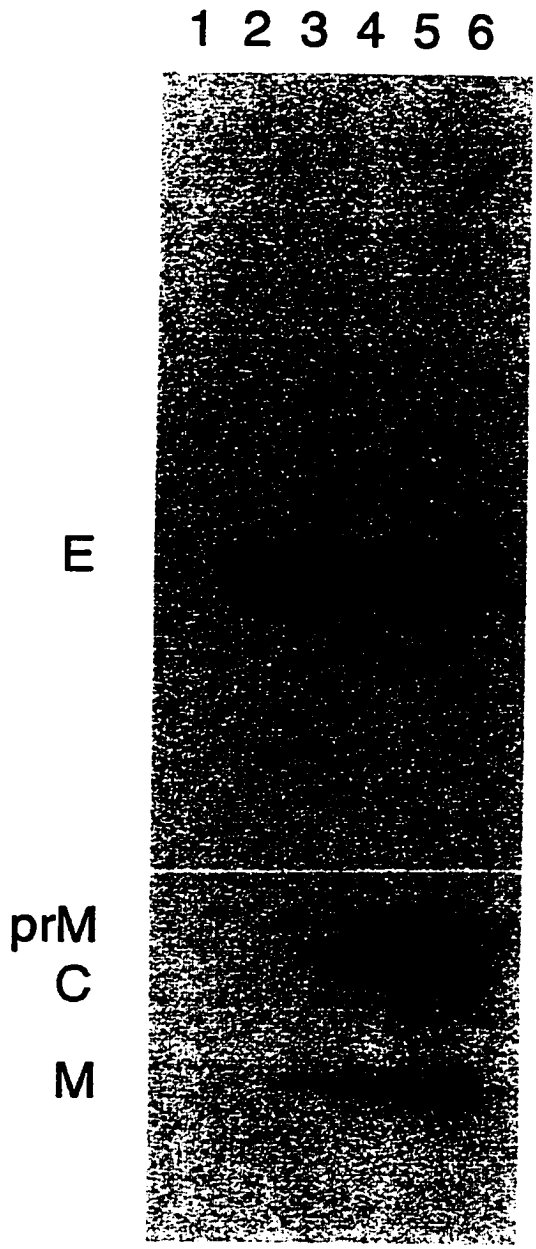


Figure 2.

Time-dependent binding of radiolabeled dengue to Vero cells. Culture fluid supernatant from ^{35}S-methionine-labeled dengue infected Vero cells was incubated with Vero cell monolayers for 0, 5, 15, 30, 60 and 90 min (lanes 1-6, respectively) at 4°C. Washed cell monolayers were solubilized and analyzed by SDS-PAGE and fluorography to reveal binding of radiolabeled virus.

Figure 2.



autoradiography. Virus-binding to Vero cells was observed in a time-dependent manner up to about 60 min. All viral structural proteins (E, M, prM and C) can be visualized in the autoradiograph, indicating binding of intact virus to cells.

3.3. Blocking of virus-cell attachment with mouse monoclonal antibody

We tested the ability of a murine monoclonal anti-dengue-2 neutralizing antibody (3H5) to block binding of virus to Vero cells. The binding site of mAb 3H5 has been partly characterized. This antibody has been shown to be one of the strongest neutralizing antibodies against dengue virus infection (Littaua et al., 1990; Trirawatanapong et al., 1992; Hiramatsu et al., 1996). Incubation of mAb 3H5 with radiolabeled virus was found to block virus-cell binding to a considerable degree (Fig. 3A). However, the blocking of binding to Vero cells was not quite as effective as neutralization (compare Figs. 3A and 3B), indicating that other mechanisms, in addition to blocking of virus-cell binding, may also contribute to the strong neutralization activity of mAb 3H5.

3.4. Blocking of virus-cell attachment with human antisera

In order to test the ability of patient antisera to inhibit virus-cell binding, and to neutralize dengue virus infection, we performed binding and neutralization assays with 61 serum samples at a final dilution of one-tenth the original serum concentration. The results of the virus-cell binding assay, shown in figure 4, showed large differences in the activities of different patient antisera to block virus-cell attachment. Some serum samples (e.g. 59, 510, 1056, 1082) showed strong blocking activity, while others showed weak (e.g. 6, 66) or partial (e.g. 105, 109, 118) ability to block virus-cell binding.

Figure 3.

Blocking of binding of radiolabeled dengue virus to Vero cells by mAb 3H5 (A) and comparison with virus neutralization (B). Conditions of assay for virus binding were as described in Fig. 2, except that aliquots of radiolabeled virus were mixed with dilutions of mAb 3H5, prior to adsorption to Vero cells for 1h at 4°C. Cell attachment blocking and neutralization activities are expressed on a scale (y axis) of 0 to 1.0 with 1.0 representing 100% activity.

Figure 3.

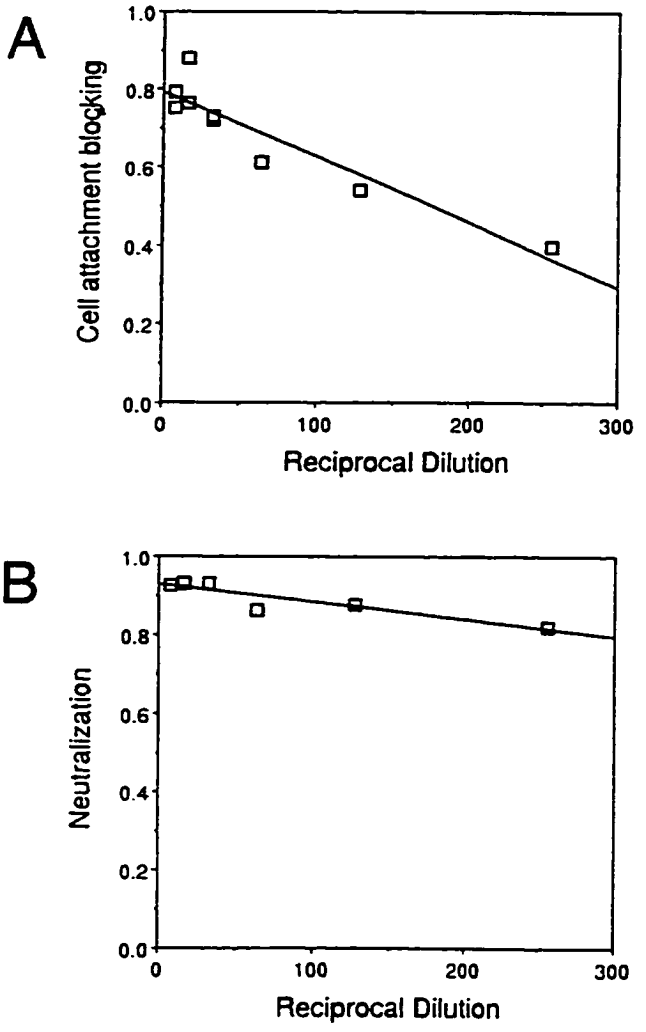
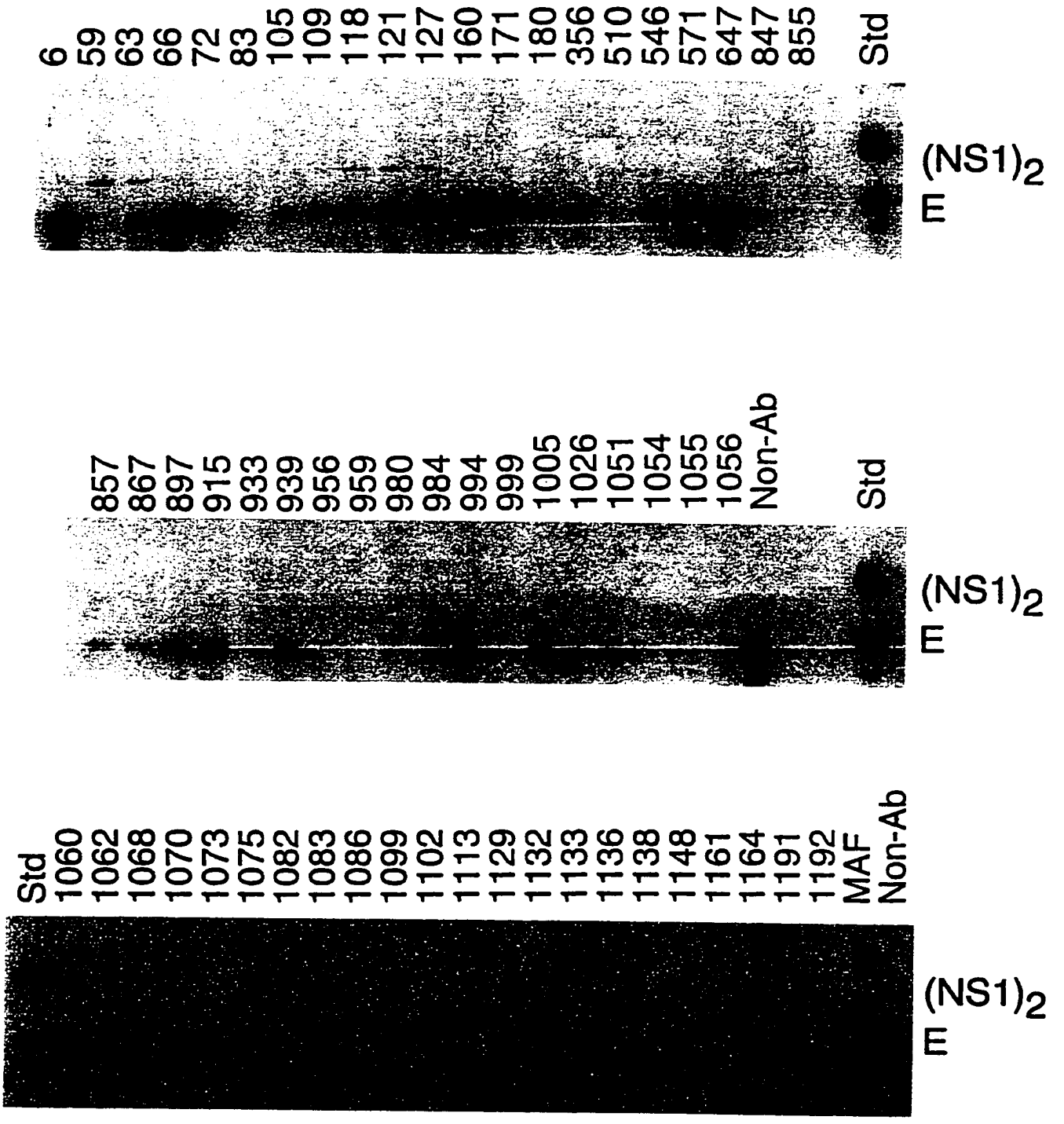

A
Cell attachment blocking
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Reciprocal Dilution
B
Neutralization
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Reciprocal Dilution

Figure 4.

Blocking of binding of radiolabeled dengue virus to Vero cells by patient antisera. Conditions of assay for virus binding were as described in Fig. 2, except aliquots of radiolabeled virus were mixed with 1:10 dilutions of patient antisera, prior to adsorption to Vero cells for 1h at 4°C. Std is a standard immunoprecipitate of the radiolabeled supernatant culture fluid showing the positions of the E and $(NS1)_2$ proteins. For comparison, blocking with mouse anti-dengue ascites fluid (MAF) and no-antibody controls are shown in the last two lanes.

Figure 4.


6
59
63
66
72
83
105
109
118
121
127
160
171
180
356
510
546
571
647
847
855
Std
(NS1)2
E
857
867
897
915
933
939
956
959
980
984
994
999
1005
1026
1051
1054
1055
1056
Non-Ab
Std
(NS1)2
E
Std
1060
1062
1068
1070
1073
1075
1082
1083
1086
1099
1102
1113
1129
1132
1133
1136
1138
1148
1161
1164
1191
1192
MAF
Non-Ab
(NS1)2
E

In order to compare cell-attachment blocking and neutralization activities of the patient sera, assays were performed using final concentration of 1:100 (Appendix 1). Scatterplot and linear regression analysis of the neutralization and cell attachment-blocking data are shown in Fig. 5. For the complete collection of sera, the Pearson's coefficient was 0.90, giving a $P < .001$, indicating a very strong correlation between neutralization and cell attachment blocking activities of patient antisera (Fig. 5A).

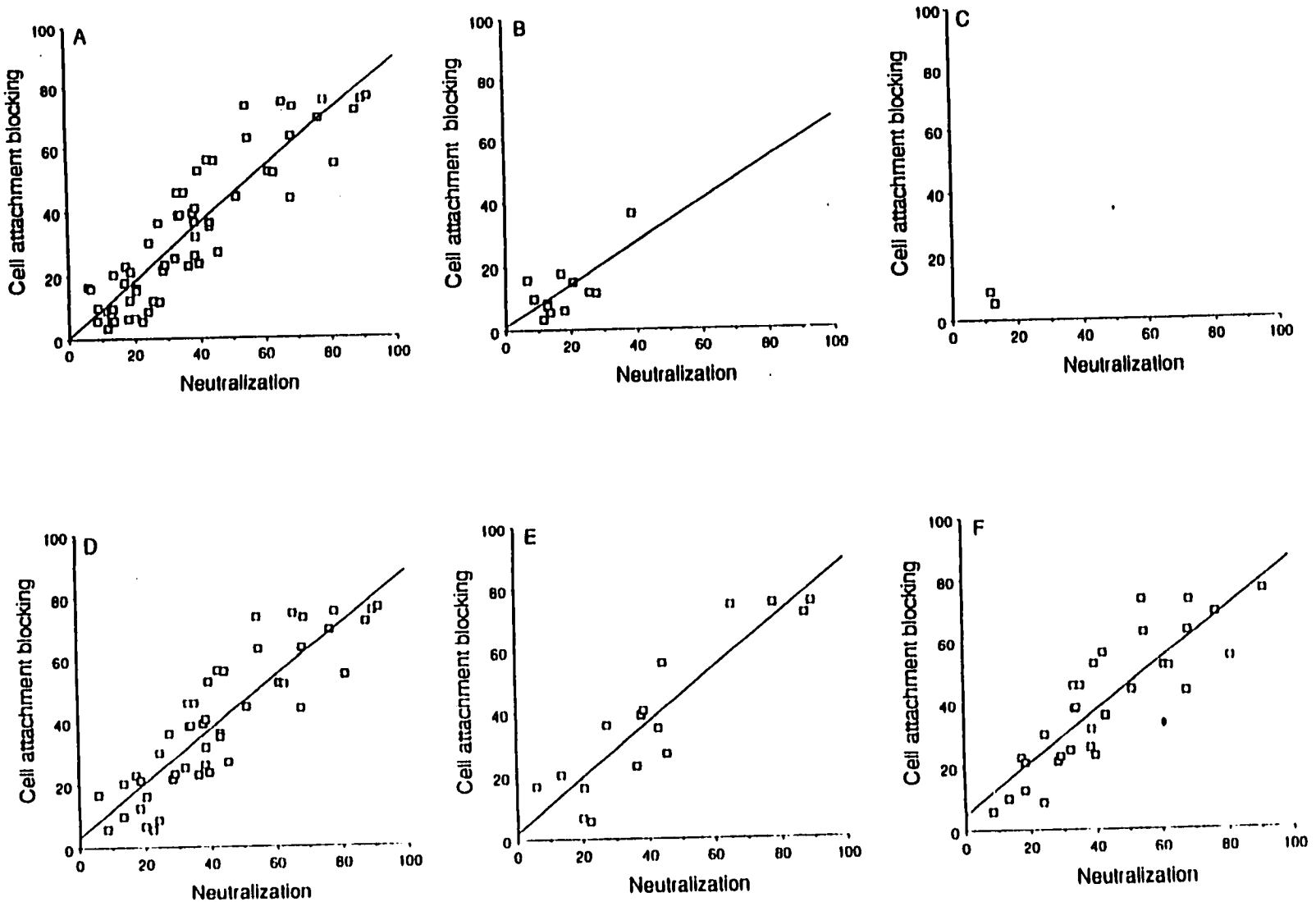
Correlations of subpopulations of patient sera were studied in a similar manner. There were high correlations for infants with primary infection and maternal antibodies (Fig. 5B Pearson's coefficient of .69, $P < .01$) and older children with secondary infection and preexisting antibodies from prior dengue virus infections (Fig. 5D Pearson's coefficient of .88, $P < .001$). The group of older children was further analyzed as two groups according to the level of homologous antibody. Children whose sera were collected prior to developing a detectable homologous antibody response, as defined by low dengue-specific IgM levels of <10 EIA units were selected as group I, and children whose sera were collected after they had mounted a detectable homologous antibody response, as defined by dengue-specific IgM levels >10 EIA units were selected as group II. The Pearson's coefficient for group I was .91, $P < .001$ (Fig. 5E). The Pearson's coefficient for group II was .86, $P < .001$ (Fig. 5F). A final group consisting of two children with primary infection but without maternal or self-derived antibodies showed very low neutralization and cell attachment-blocking activities (Fig. 5C).

Although there is a high degree correlation between blocking of virus binding and neutralization in most groups mentioned above, the values of neutralization are generally higher than the values of cell attachment blocking,

Figure 5.

Scatterplot analyses of neutralization and cell attachment blocking activities of patient sera at 1:100 dilutions. All 61 serum samples (A; Pearson's coefficient = .90, $P < .001$); infants with presumably maternal anti-dengue antibodies; (B; Pearson coefficient = .69, $P < .05$); older children with neither maternal or self-derived preexisting anti-dengue antibodies (C; Pearson's coefficient not calculated from this small group); older children with preexisting, self-derived anti-dengue antibodies (D; Pearson's coefficient = .88, $P < .001$); subgroup of D whose sera were collected before mounting a detectable homologous antibody response (E; Pearson's coefficient = .91, $P < .001$); and subgroup of D whose sera were collected after mounting a detectable homologous antibody response (F; Pearson's coefficient = .86, $P < .001$).

Figure 5.


A
B
C
D
E
F
Cell attachment blocking
Neutralization
0
20
40
60
80
100

suggesting that some other mechanisms, such as post-attachment neutralization, may also play a role in neutralization of virus infection.

To see which type of antibody is important in virus neutralization and virus-cell attachment-blocking activities, we analyzed the correlation between anti-dengue IgM, IgG titer and activities of neutralization (Fig. 6) and blocking of virus-cell attachment (Fig. 7) A strong correlation was observed between the titer of anti-dengue IgG and neutralization (Fig. 6A) (Pearson's correlation coefficient was 0.72 $P<.001$). The correlation between the titer of IgG and cell attachment blocking activities was also very strong (Fig. 7A) (Pearson's coefficient was 0.64 $P< .001$). However, there was no clear correlation between the titer of IgM and neutralization (Fig. 6B) and virus-cell attachment blocking (Fig. 7B).

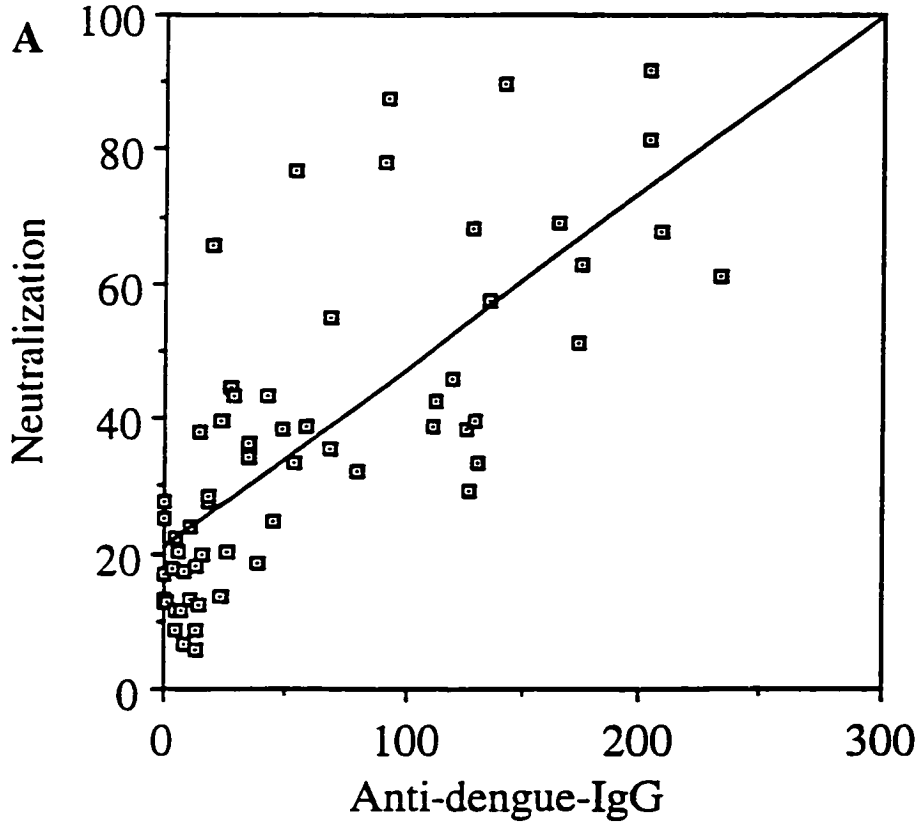
3.5. Post-attachment-neutralization activities of patient antisera

The above results indicated that neutralizing antibodies for dengue blocked infection mainly by inhibiting virus-cell attachment. Nevertheless, it has been shown that antibodies against herpes simplex virus, papillomaviruses, respiratory syncytial virus and HIV have post-attachment neutralization activities (Burioni et al., 1994; Christensen et al., 1995; Osiowy and Anderson, 1995; Armstrong and Dimmock, 1996). We investigated whether patient dengue-immune serum showed post-attachment neutralization activity. The one patient antiserum tested did not show significant post-attachment neutralizing activity (Fig. 8).

Figure 6

Scatterplot analyses of neutralization activities and levels of anti-dengue IgG (A) (Pearson's coefficient = .72, $P < .001$) and IgM (B) (no significant correlation) of patient sera at 1:100 dilutions.

Figure 6


A
100
80
60
40
20
0
Neutralization
0
100
200
300
Anti-dengue-IgG

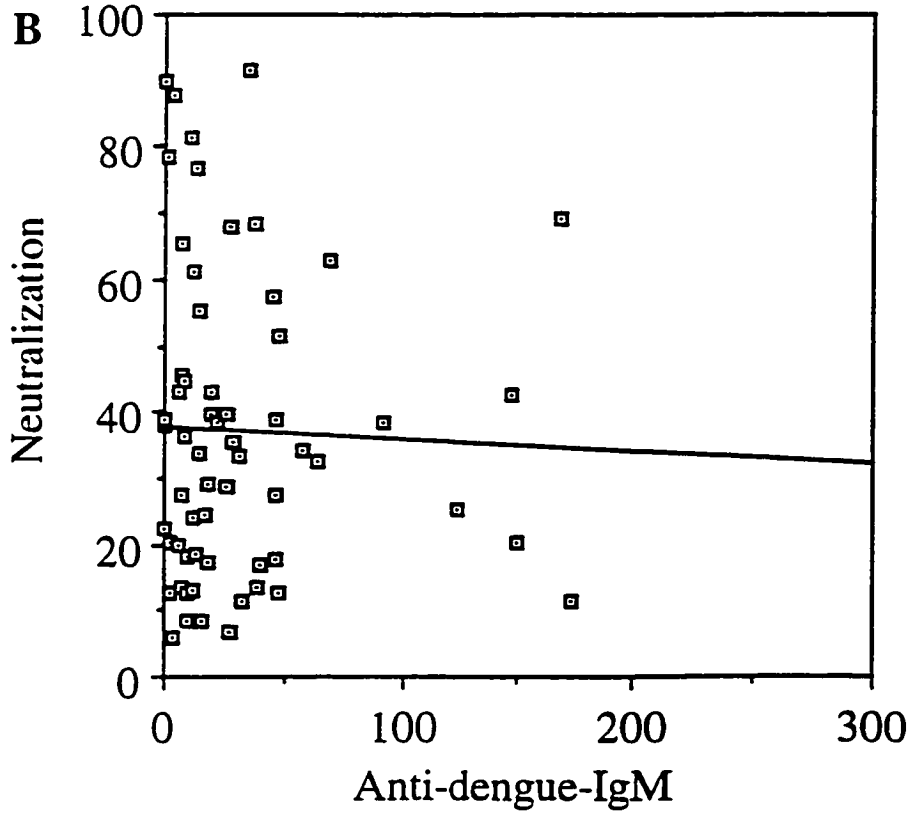

B
100
80
60
40
20
0
Neutralization
0
100
200
300
Anti-dengue-IgM

Figure 7

Scatterplot analyses of cell attachment-blocking activities and levels of anti-dengue IgG (A) (Pearson's coefficient = .63, $P < .001$) and IgM (B) (no significant correlation) of patient sera at 1:100 dilutions.

Figure 7

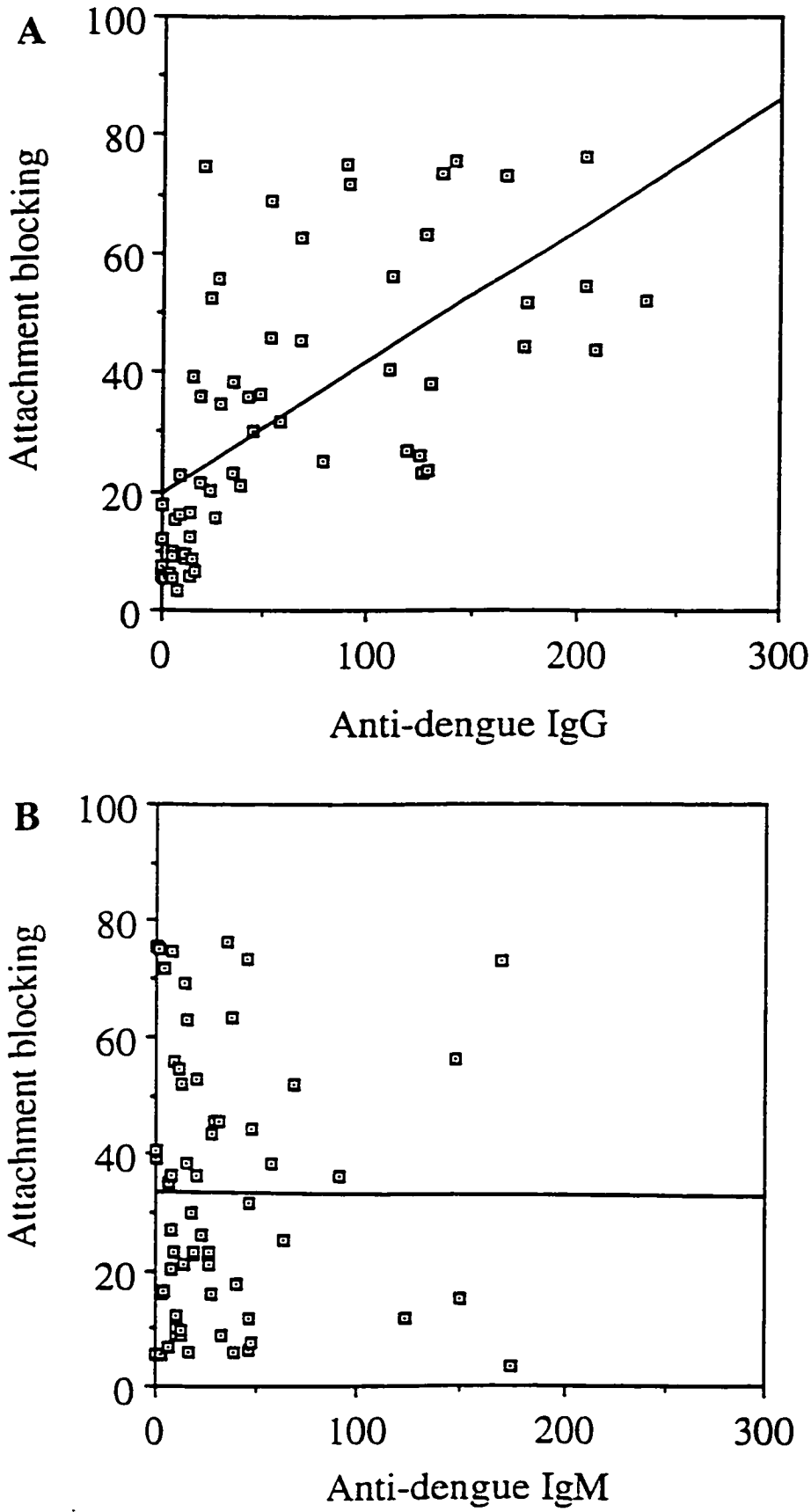
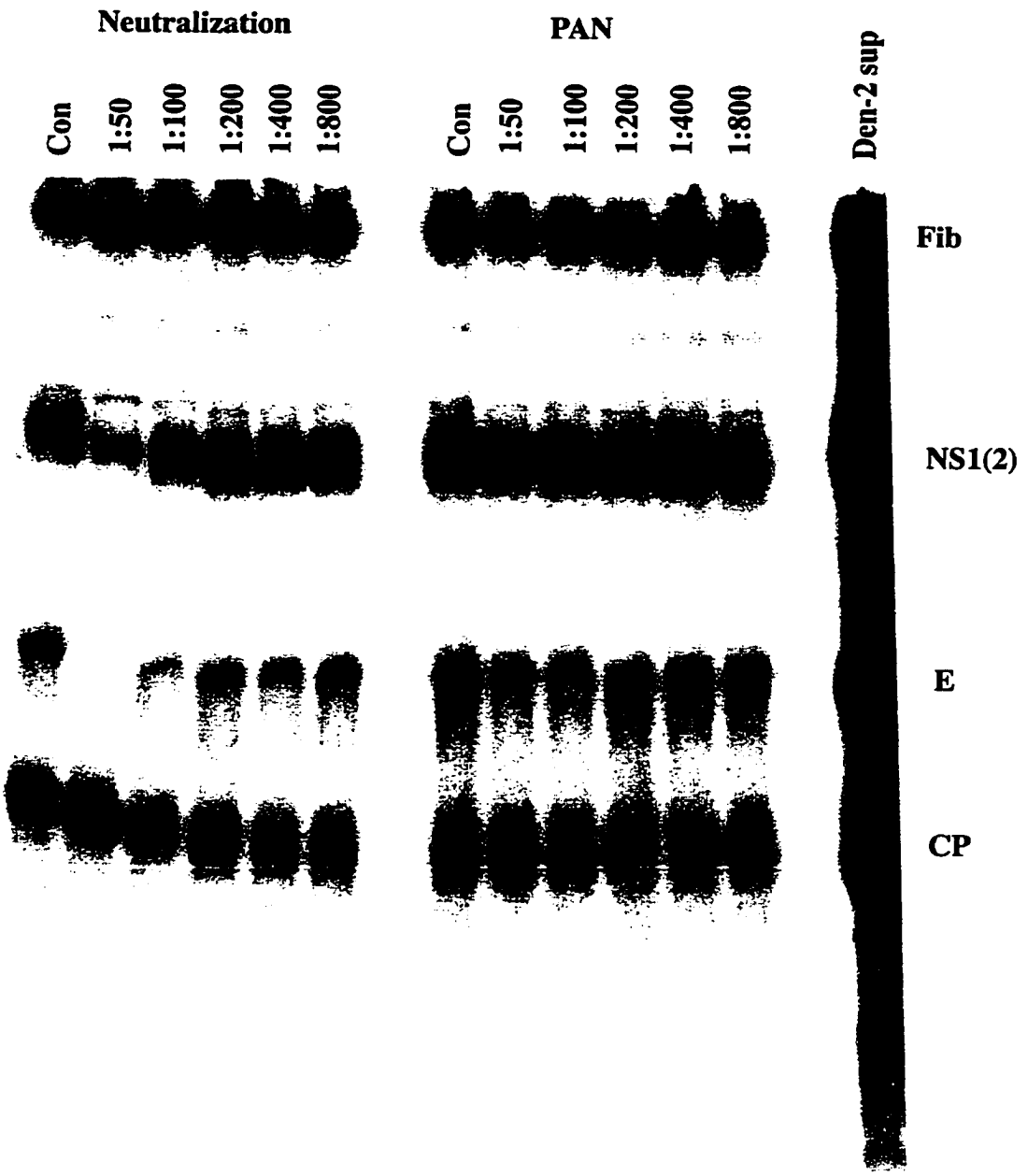

A
100
80
60
40
20
0
Attachment blocking
0
100
200
300
Anti-dengue IgG
B
100
80
60
40
20
0
Attachment blocking
0
100
200
300
Anti-dengue IgM

Figure 8.

Comparison of antibody-mediated neutralization and postattachment neutralization of dengue virus infection. Neutralization was performed by mixing virus with dilutions of dengue-immune serum (#59) for 1 h at 4°C, then adding to Vero cells for 1 h at 4°C. Post-attachment neutralization was performed by adding radiolabeled virus to Vero cells at 4°C, then serial dilutions of dengue-immune antisera were added to the binding samples and incubated for 1 h. Cells were then washed with medium and incubated at 37°C. Infected cells were labeled for 12 h with ^{35}S-methionine at 30 h post infection. Supernatants from infected cells were immunoprecipitated with dengue-immune serum and analyzed on 10% SDS-PAGE and fluorography

Figure 8.


Neutralization
PAN
Con
1:50
1:100
1:200
1:400
1:800
Con
1:50
1:100
1:200
1:400
1:800
Den-2 sup
Fib
NS1(2)
E
CP

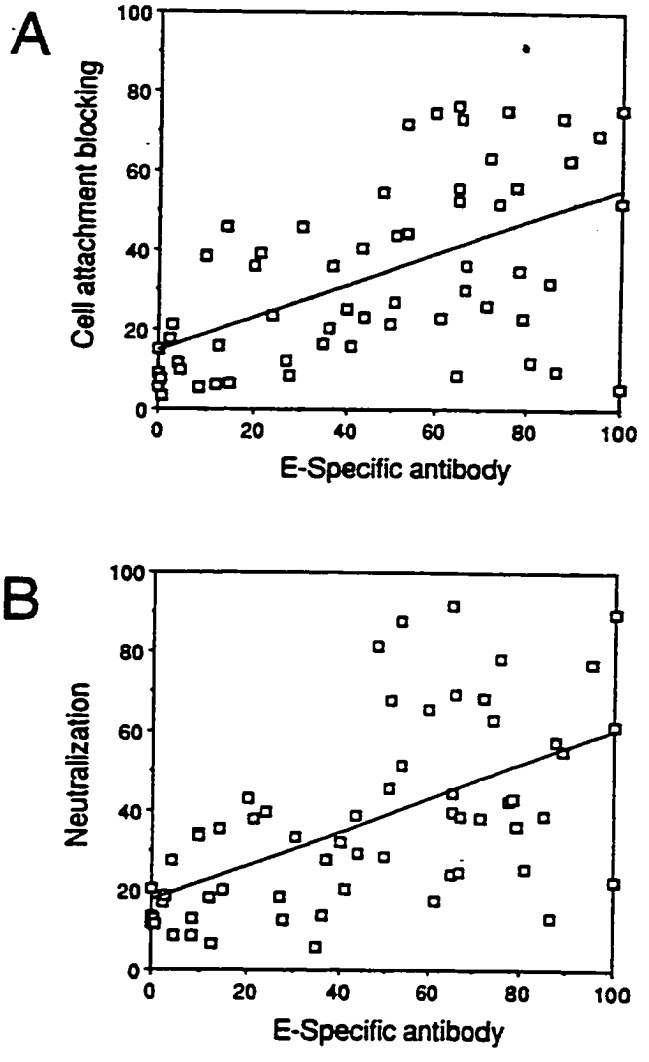
3.6. Correlation of neutralizing and attachment-blocking activities with E-specific antibodies.

The cell-attachment protein on dengue virus is the E protein. Immunoprecipitation analysis was performed in order to semi-quantitate E-specific antibodies. The relative amounts of radiolabeled E protein immunoprecipitated with a 1:100 dilution of patient antiserum were quantitated by scanning densitometry and plotted against cell attachment blocking and neutralization activities as shown in Fig. 9. Patient antisera showed considerable heterogeneity in E-specific antibody responses. Scatterplots indicated a less definitive trend than those observed between blocking and neutralization (Fig. 9). Correlation coefficients were also lower (Pearson's coefficient = 0.586 for blocking vs. E-antibody reactivity, and 0.556 for neutralization vs E-antibody reactivity) but still highly significant ($P<0.01$). The lower correlation coefficients probably reflect a greater degree of experimental error associated with the immunoprecipitation assays.

Figure 9.

Scatterplot analyses of cell attachment blocking (A) and neutralization (B) activities plotted against E-specific antibody reactivities. E-specific antibodies were determined by immunoprecipitation of ^{35}S-methionine-labeled E protein using 1:100 dilutions of patient antisera. Levels of immunoprecipitated radiolabeled E protein were determined by scanning densitometry of SDS-PAGE fluorograms, and are expressed as relative units. Pearson's coefficients for A = 0.586, $P<0.01$, and for B = 0.556, $P<0.01$

Figure 9.


A
Cell attachment blocking
E-Specific antibody
B
Neutralization
E-Specific antibody
0
20
40
60
80
100

4. DISCUSSION

The above studies are the first demonstration that human neutralizing antisera against dengue virus act largely by blocking virus attachment to susceptible cells. The cells used in this study were monkey kidney Vero cells which are a popular cell line for dengue virus propagation. Vero cells do not have Fc receptors, and so bind dengue virus through a still unidentified dengue virus receptor. It will be important to apply the same types of analyses used in this study with Vero cells to other cell types that are thought to be natural host cells in dengue virus infection. It has been reported that macrophage-like cells are major host cells for dengue virus infection (Halstead, 1989; Morens and Halstead, 1990).

Our own unpublished studies have indicated that performing the same kind of assays mentioned above with macrophage-like cell lines, such as U937 or peripheral blood monocytes, is difficult at the present time since levels of binding of radiolabeled dengue virus are much lower than those observed with Vero cells. The reason for the lower amounts of dengue virus binding to these cells may relate to previous speculations that U937 or peripheral blood monocytes may have relatively low levels of dengue virus receptors because these cells produce only low yields of virus in the absence of enhancing antibody (Brandt et al., 1982; Brandt et al., 1979; Daughaday et al., 1981). There are also indications that the expression of dengue virus receptors on U-937 cells is dependent on culture conditions (Daughaday et al., 1981).

Viruses can use different kinds of molecules on the cell surface as receptors. One virus may have more than one receptor on different kinds of cells, as has been shown for HIV (Norkin, 1995). The dengue virus receptor(s) has not yet

been characterized. Whether different kinds of cells in the natural host express different kinds of dengue virus receptors remains to be answered. Studies from our lab (Anderson et al., 1992; and this thesis) demonstrate that the E protein is the cell-binding protein. A recent study using a recombinant chimeric E-IgG protein showed that this soluble form of the E protein was also a major determinant of virus binding to Vero, CHO, glial and human endothelial cells (Chen et al., 1996). However, this study also suggested that macrophage-like cells may express the same kind of receptors as Vero and other cell lines at lower levels, which can mediate dengue virus infection without Fc receptors. Other studies showed that dengue virus can infect human monocytes in the absence of antibodies (Brandt et al., 1979; Daughaday et al., 1981; Kurane et al., 1990; Sittisombut et al., 1995), which implies that blocking of non-FcR mediated binding of dengue virus to macrophage-like cells also blocks infection of these cells. Studies with HIV indicated that two receptors, CD4 and Fc receptor, are required for antibody-dependent enhancement of HIV infection in macrophage-like cells. The enhancement of HIV infection was blocked by anti-CD4 antibody or soluble CD4 (Takeda et al., 1990). Similarly, although dengue virus binding and infection in macrophage-like cells are mainly dependent on Fc receptors. Nevertheless, blocking of virus binding to virus receptor(s) may also affect Fc receptor-mediated binding and infection.

In our study, mAb 3H5 was able to block virus-cell attachment as indicated by >50% blocking at high dilutions up to 1:100. The neutralizing activity of mAb 3H5 was even greater, indicating that blocking of virus-cell attachment by this antibody only partially contributed to the neutralization activity of this antibody. Binding of mAb 3H5 to its epitope may thus also affect virus infection at other levels, such as postattachment and virus aggregation. Another possibility may be of multiple virus binding sites on cellular receptors. Blocking of some sites may not be enough to

completely block productive infection. A small proportion of total virus-binding proteins present on the cell surface may be able to cause productive infection. Evidence from the HIV field, for example, suggests that HIV binds to monocytes via multiple membrane proteins in addition to the CD4. However only binding to CD4 ensures productive infection (Finbloom et al., 1991; Endres et al., 1996).

A high correlation between blocking of dengue-2 virus attachment and blocking virus infection to Vero cells was observed in this study with patient antisera. However, blocking of virus attachment and neutralization was more effective than with mAb 3H5. Most of the antisera showed stronger neutralization activities than blocking of virus binding. This result suggests that even though blocking of virus-cell attachment is the predominant mechanism of antibody neutralization of dengue virus, other mechanisms also contribute.

It has been shown with patient antisera and vaccine studies that human antibodies can react with dengue E, C and nonstructural proteins NS1, NS3 and NS5 (Churdboonchart et al., 1991; Putnak et al., 1996). The E protein of dengue virus is considered to be the major antigen in virus antibody-mediated neutralization. Studies with monoclonal antibodies against the E protein demonstrated neutralization of the virus infection (Putnak et al., 1991; Feighny et al., 1992; Chen et al., 1996). However, antibodies against nonstructural proteins and structural proteins other than E protein can also contribute to neutralization of virus infection (Hahn et al., 1990; Srivastava et al., 1995; Putnak et al., 1996). For example, anti-NS1-E fusion protein neutralized dengue virus infection more effectively than anti-E protein antibody alone. Also, anti-M antibodies have been shown to be able to neutralize dengue virus infection (Kaufman et al., 1989; Srivastava et al., 1995). Despite the above, however, results from the present study support the idea that anti-E protein antibodies

are the most important antibodies in neutralization and blocking of dengue virus attachment. Nevertheless, it would appear from the results shown in Fig. 8, that patients vary considerably with respect to anti-E protein antibody reactivities. Also a strong anti-E response is not always indicative of strong neutralization or virus cell-attachment blocking activities. Semi-quantitative assays of the M protein antibody response were not successful, due to low incorporated levels of ^{35}S-methionine into this protein (see also Feighny et al., 1992; Smucny et al., 1995).

The present study used patient antisera to study the antibody response developed during an active dengue type 2 infection. Data for most antisera (47 out of 61) indicated a secondary dengue type-2 infection (Status designation 2 in Appendix 1; panels D-F in figure 5). Of these 47 patients, 16 were sampled prior to development of an appreciable homologous antibody response (Fig. 5E) while 31 were sampled at a time point at which a homologous antibody response was more evident (Fig. 5F). The results therefore suggest that the initial response (Fig. 5B) consists largely of neutralizing antibodies able to block virus attachment to cellular receptors. This observation is very important to understand the initial recovery phase in response to infection. Future studies are planned to examine the neutralizing and virus-cell attachment blocking activities in convalescent sera in order to determine whether late secondary antibody neutralizes by pre- or post-attachment mechanisms.

Studies of virus neutralization in other systems, as probed by monoclonal or monospecific antibodies, have revealed a number of steps in the virus replication cycle at which inhibition of infection may occur (Dimmock, 1984, 1993; Outlaw and Dimmock, 1991). Normal polyclonal antibody responses are composed of a variety of antibody specificities, which block infection at stages which include both pre- and post-cell-attachment events as well as virus aggregation. The results shown in Fig. 5

suggest an important role for attachment-blocking antibodies in neutralization of dengue virus. They also suggest that a relatively small subset of neutralizing antibodies inhibit infection at a post-attachment step.

The concentration of anti-dengue IgG in antisera correlated highly with neutralization and anti-attachment activities, while the concentration of IgM correlated poorly with neutralization activities of antisera. These results suggested that IgG is the predominant antibody in antisera that blocks virus-cell attachment and neutralizes virus infection. Similar findings have been reported with other viruses, such as influenza virus (Paladino et al., 1995) and Nairovirus (Green et al., 1993).

The phenomenon of postattachment neutralization of viruses is well recognized. For example, Gollins and Porterfield (1986) found that other than blocking virus-cell attachment, there is an alternative neutralization mechanism for West Nile Virus. Roehrig et al (1988) emphasized the concept of postattachment neutralization of virus infection, when they studied the mechanisms of antibody-mediated neutralization with alphaviruses (Roehrig et al., 1988). This mechanism was also found to be one of the neutralization mechanisms with herpes simplex virus, HIV and RSV (Burioni et al., 1994; Armstrong et al., 1996; Osiowy and Anderson, 1995). Other than antibody-mediated blocking of virus-cell attachment, antibody-mediated blocking of the endosomal-viral fusion step has also been reported as a mechanism of virus neutralization. However, we were unable to find that postattachment neutralization contributes significantly to the neutralization of dengue virus by human anti-dengue sera. Nevertheless postattachment neutralization may partly contribute to the neutralizing activities of some mAbs, such as 3H5, for which the neutralization titer cannot be completely accounted for by blocking of virus-cell attachment.

Comprehensive investigations of the neutralizing antibody response have been carried out in infections of human immunodeficiency virus type 1 (HIV-1). The mechanisms of antibody-mediated HIV neutralization involve inhibition of infection both before and after the stage of gp120 binding (Back et al., 1990; Kang et al., 1991; Hariharan et al., 1993; Poignard et al., 1996; Armstrong et al., 1996). Distinct neutralization epitopes on the HIV gp120 have been identified. Several epitopes are known to be involved in virus-cell attachment (Kang et al., 1991; McKeating et al., 1992; Kang et al., 1993; Poignard et al., 1995; Armstrong et al., 1996), while others are known not to be involved in virus-cell attachment (Linsley et al., 1988; Kang et al., 1991; Chamat et al., 1992; Poignard et al., 1996). Antibodies that have strong neutralization activities do not necessarily block the virus-receptor binding. For example, anti-V3-region antibodies have strong neutralization activity, although this region does not contain the virus-receptor binding site (Poignard et al., 1996). Recently such neutralizing antibodies have been shown to block HIV binding to secondary (chemokine) receptors (Choe et al., 1996; Cocchi et al., 1996). Similar kinds of studies can be applied to dengue virus once the virus receptor(s) and virus-cell binding sites are characterized.

In conclusion, even though the locations of cell receptor-binding epitopes on the dengue E protein remain currently uncertain, the results of this study suggest that the primary mechanism of antibody-mediated neutralization of dengue virus is by blocking virus attachment to cell receptors. Structural considerations have suggested that domain III of the flavivirus E protein may be involved in virus-cell attachment (Rey et al., 1995; Hiramatsu et al., 1996). An epitope around 383-393 is a known strong neutralization epitope, and has been speculated to be involved in virus attachment (Hiramatsu et al., 1996). Studies with another flavivirus, Murray Valley

Encephalitis (MVE) virus, suggested that an important determinant of MVE pathogenicity involved an RGD sequence (residues 388-390 of the MVE E protein sequence) (Lobigs et al., 1990). Future identification of cell receptor-binding epitopes on the dengue E protein will help us to better understand the pathogenesis of dengue virus infection, which appears to be strongly influenced by the host immune response.

CHAPTER 3

ANTIBODY-ENHANCED BINDING OF DENGUE-2 VIRUS TO HUMAN PLATELETS

1. INTRODUCTION

Thrombocytopenia is a disease that is characterized by a dramatic decrease in the platelet count in the blood. The disease in humans can be caused by various virus infections, such as HIV (Zon et al., 1987; Cinque et al., 1993; Glatt and Anand, 1995; Sullivan and McDonald, 1995), Epstein-Barr virus (Hugo et al., 1989; Connolly et al., 1994), and most notably dengue virus (Bhamarapravati et al., 1967; Bokisch et al., 1973; Halstead, 1982; Rothwell et al., 1996).

The multifactorial causes of thrombocytopenia produced by virus infections are yet to be completely understood. It has been proposed that the following mechanisms might be involved: 1. Direct virus infection of megakaryocytes (the platelet-producing cells) and/or interaction with platelets. 2. Virus-mediated immune mechanisms that affect megakaryocytes and platelets (Zucker-Franklin, 1994). Direct infection of megakaryocytes by viruses is not common. Some examples are HIV (Zucker-Franklin, 1989; and Friend Leukemia virus (Brown and Axelrad, 1976). Viruses that induce immune-mediated thrombocytopenia are also not numerous, but include HIV (Zon et al., 1987; Coyle et al., 1997), Epstein-Barr virus (Hugo et al., 1989; Connolly et al., 1994), and dengue (Bokisch et al., 1973; Halstead et al., 1982).

In the case of HIV, two mechanisms similar to those described above are possibly involved in thrombocytopenia: impairment of the bone marrow

megakaryocyte compartment by direct virus infection, and a virus-induced autoimmune response (Re et al., 1994; Davis and Zauli, 1995; Karpatkin, 1989; Hegde et al., 1992; Kurtzberg and Stockman, 1994). For example, in situ hybridization with an HIV probe showed that HIV RNA could be detected in patients' megakaryocytes and furthermore, electron microscopy showed that there were morphologic changes (particularly, vacuolization) of HIV infected platelets (Zucker-Franklin et al., 1989). Also, immune complexes were found on platelets from patients infected with HIV (Walsh et al., 1984) and high levels of platelet-associated IgG, IgM and C3C4 were found in HIV patients (Bettaib et al., 1992).

As for thrombocytopenia caused by Epstein-Barr virus (EBV) (Chen et al., 1995), it has been observed clinically that thrombocytopenia may be caused by virus-induced autoimmune responses as well as by increased macrophage hemophagocytosis activities in the bone marrow (eg. Diebold et al., 1976). This hemophagocytosis may involve destruction of virus-infected megakaryocytes by macrophages thereby leading to a decrease in platelet production.

Dengue is probably the best example of a virus which causes thrombocytopenia in humans. The causes of dengue-associated thrombocytopenia are not yet clear. In contrast to the mild thrombocytopenia occasionally seen in HIV and Epstein-Barr virus infections, thrombocytopenia caused by dengue virus infection can be very severe. While mild thrombocytopenia is often found in dengue fever, severe thrombocytopenia is a hallmark feature of DHF/DSS. Platelet levels often drop as low as 60,000/mm^3 in 3-5 days (compared to normal values of 100,000-300,000/mm^3) which may contribute to the hemorrhaging observed in vital organs (Lopez et al., 1978; Bhamarapravati et al., 1989; Kabra et al., 1992; Ibrahim et al., 1995). Several mechanisms may be involved in the thrombocytopenia caused by dengue virus. For

example, studies have shown that dengue virus-antibody complexes occur on the surface of platelets from patients with DHF/DSS, suggesting that platelets may subsequently be destroyed in the reticuloendothelial system (Boonpucknavig et al., 1979). It has also been reported that in spite of low platelet counts, blood levels of thrombopoietin (a regulator of platelet production) are not increased, prompting speculation that dengue virus infection may block the production of thrombopoietin (Putintseva et al., 1986). In addition, dengue-2 virus has been shown to infect stromal cells in human bone marrow (La-Russa and Innis, 1995; Rothwell et al., 1996), which may affect megakaryocyte maturation and the production of platelets.

In the following study, we show the first evidence that dengue virus has the ability to bind to human platelets. Binding occurs only in the presence of virus-specific antibodies. These antibodies may be sera from dengue infected patients or monoclonal antibodies specific for the dengue E protein. The results suggest that virus binding to platelets may play an important role in the pathogenesis of DHF/DSS and support the concept that immune-mediated clearance of platelets may play a role in triggering thrombocytopenia in DHF/DSS.

2. MATERIALS AND METHODS

2.1. Virus

The dengue-2 serotype (16681 strain), which was propagated in Vero cells, was used for this study. This strain was originally isolated from a Thai child with dengue shock syndrome (Halstead and Simasthien, 1970) and was passaged sequentially on monkey kidney LLC-MK2 cells, mosquito C6/36 cells and finally on monkey kidney Vero cells. Radiolabeled dengue-2 virus was prepared by culturing

virus-infected Vero cells from 24-48 h post-inoculation (PI) in the presence of ^{35}S-methionine (100 μCi/ml).

2.2. Antibodies

Mouse monoclonal antibodies (mAb) 3H5, 9D12, 1B7 5F10 (Gentry et al., 1982) were kindly provided by Dr. A. King, Dept. of Virus Diseases, Walter Reed Army Institute of Research. Mouse monoclonal antibody JN120, JN129, JN142, JN179 were kindly provided by Dr. J. Patarapotikul, Mahidol University. For details of mAbs 3H5, 9D12, 1B7 see Henchal et al., (1985), mAb 5F10 see Kaufman et al., (1987) and mAbs JN120, JN129, JN142, JN179 see Pothipunya et al., (1993). All mAbs are neutralizing; mAb 3H5 is dengue-2 type-specific; all other mAbs recognize E protein among the four serotypes of dengue as well as certain other flaviviruses. Mouse antibodies against dengue-2 virus were prepared as ascites fluids from virus-immunized BALB/c mice. Control human antisera were obtained from sero-negative volunteers. Anti-human-platelet and anti-human-red blood cell (RBC) antibodies were prepared in out-bred Swiss Mice. Twenty ml of human platelets or 5 ml RBCs (from the blood bank of Victoria General Hospital., Halifax, Nova Scotia) were washed 3 x with PBS and pelleted. The platelet or RBC pellet was mixed 1:4 with complete Freund's Adjuvant and injected 0.5 ml IP to Swiss mice. The first and second boosts were at two weeks and three weeks after the first inoculation. Ascitic fluid and serum were collected and used as the source of anti-platelet or anti-RBC antibody. Anti-Vero-cell antibody was prepared similarly. Briefly, a 150cm^2 flask of confluent Vero cells were washed 3 x with PBS and scraped off the plastic surface. The cell pellet was mixed with complete Freund's Adjuvant and inoculated into Swiss mice as above.

2.3. Human platelets

Human platelets were prepared as follows: Blood, collected from healthy volunteers, was mixed with one sixth part acid-citrate-dextrose, containing 2.5% trisodium citrate, 2% glucose and 1.5% citric acid. Blood was then centrifuged at 900 rpm for 10 min at room temperature. The supernatant was collected as platelet rich plasma (PRP). To eliminate red cells, the PRP was centrifuged at 2000 rpm for 5 min and the upper 80% PRP transferred to another tube. Platelets were washed in phosphate-buffered saline, pH 7.4 containing bovine serum albumin (1 mg/ml) and 1 mM EDTA.

2.4. Virus-platelet and virus-cell binding

The virus-platelet binding assay was carried out as follows: 1. Binding without anti-dengue antibodies, radio-labeled virus was added to platelets or Vero cells, incubated at 4°C for 10, 20, 40, and 60 min for the time course study. 2. Binding with anti-dengue antibody: radio-labeled virus was mixed with serial dilutions of mAbs and convalescent sera and incubated at 4°C for 1 h. The virus-antibody mixtures were then added to human platelets, RBCs or Vero cell monolayers in 24-well plates for 1 h at 4°C. In some experiments, platelets or convalescent sera were pretreated with anti-FcγRII antibody IV.3 (Medarex) or staphylococcal protein G (Sigma Chemical Co, St. Louis, Mo) respectively. Following virus binding, platelets, RBCs or Vero cells were pelleted and washed with PBS/BSA and solubilized in dissociation buffer for analysis by SDS-PAGE using 5-18% polyacrylamide gradient gels. Gels were impregnated with 1M sodium salicylate, dried and fluorographed by exposure to Kodak X-ray film at -70°C.

3. RESULTS

3.1. Dengue virus binds poorly to platelets in the absence of antibody

In order to determine whether dengue virus binds to normal human platelets, we performed binding assays using ^{35}S-methionine-radiolabeled virus. Radiolabeled virus was added to platelets and allowed to bind for various times at 4°C. Binding to Vero cells was used as a positive control, since Vero cells have been shown to bind dengue virus well (Chapter 2). As shown in Fig. 10, binding of dengue virus to Vero cells occurred in a time-dependent manner. However, binding of the virus to platelets occurred at a much lower level than that to Vero cells. For comparison, high levels of fibronectin were observed to bind to platelets and Vero cells, consistent with previous reports of fibronectin receptors on the surface of platelets and the broad distribution of fibronectin binding protein on cells (Plow and Ginsberg, 1981; Sonnenberg, 1995). Thus, it is evident that normal platelets bind dengue virus relatively poorly.

3.2. Antibody-enhanced binding of dengue virus to platelets

The above assay showed that dengue virus alone binds poorly to human platelets. However, virus binding may be enhanced by antibody by the following mechanisms: 1. Binding of virus-antibody complexes to platelet Fc receptors, or 2. antibody-mediated conformational change of the virus E glycoprotein, allowing virus binding to other molecules on the surface of platelets. To investigate this, we performed a binding assay with radio-labeled dengue virus, human dengue-convalescent antisera and human platelets. Aliquots of dengue-immune antisera and normal., dengue-nonimmune sera were mixed with radiolabeled virus and then added to platelets. As shown in Fig. 11, binding of virus to platelets was increased dramatically in the presence of anti-dengue antisera. No increase was observed in samples with control

antisera, indicating that the enhanced binding is dependent on the presence of dengue-specific antibody. As in our previous study with Vero cells (Chapter 2) virus bound to cells contained the major viral structural proteins E, prM, M and C. This likely indicates binding of a mixture of both "mature" (containing E, M and C) and "immature" (containing predominantly E and prM) virus particles, in which the latter have approximately 1/8 the specific infectivity of the former (Randoph et al., 1990).

3.3. Antibody-enhanced binding of dengue virus to platelets using monoclonal antibodies

To further investigate the mechanism of the observed antibody-enhanced virus binding to platelets, we performed another binding study using a group of eight E-specific mAbs to study their ability to influence virus binding to platelets. With the exception of mAb 3H5, all other mAbs were able to enhance virus binding to platelets. However, the degree of enhanced binding by mAbs was much less than that seen with human dengue-convalescent antiserum (Fig. 12). This result indicated that there may be both enhancing and non-enhancing epitopes on dengue virus E protein that determine the ability of virus-antibody complexes to bind to platelets.

3.4. Virus-antibody binding to platelets cannot be blocked by anti-FcR antibody

Platelets express type II Fcγ receptors (FcγRII) capable of binding antibody (IgG)-antigen complexes (Vancura and Steiner, 1987). The FcγRII on human platelets has the highest affinity for monomeric murine IgG1 followed by IgG2b, whereas monomeric IgG2a binds poorly (Ramasamy et al., 1976; Boltz et al., 1981). In our study, the most effective mAbs for enhancing virus binding to platelets were in fact of the IgG2a subclass (Fig. 12B), which suggested that there was little correlation between degree of antibody-mediated enhanced virus binding to human

Figure 10.

Time course binding of radiolabeled dengue virus to Vero cells and platelets. Culture fluid (T) from ^{35}S-methionine-labeled dengue-2 virus-infected Vero cells was added to Vero cell monolayers (1 x 10^6 cells/sample) or human platelets (5 x 10^8 platelets/sample) and allowed to adsorb for 10, 20, 40, 60 or 90 min at 4°C. Cells/platelets were washed three times, solubilized in dissociation buffer and analyzed by SDS-PAGE (10% polyacrylamide) fluorography. Abbreviations: Fib, fibronectin; CP, unknown secreted cellular protein from Vero cells; E and $(NS1)_2$ are dengue virus-specified glycoproteins. Virion binding is indicated by the radiolabeled E protein. The smaller virion proteins, M, C and prM, migrate near the dye front and are not well resolved in this 10% gel system.

Figure 10.

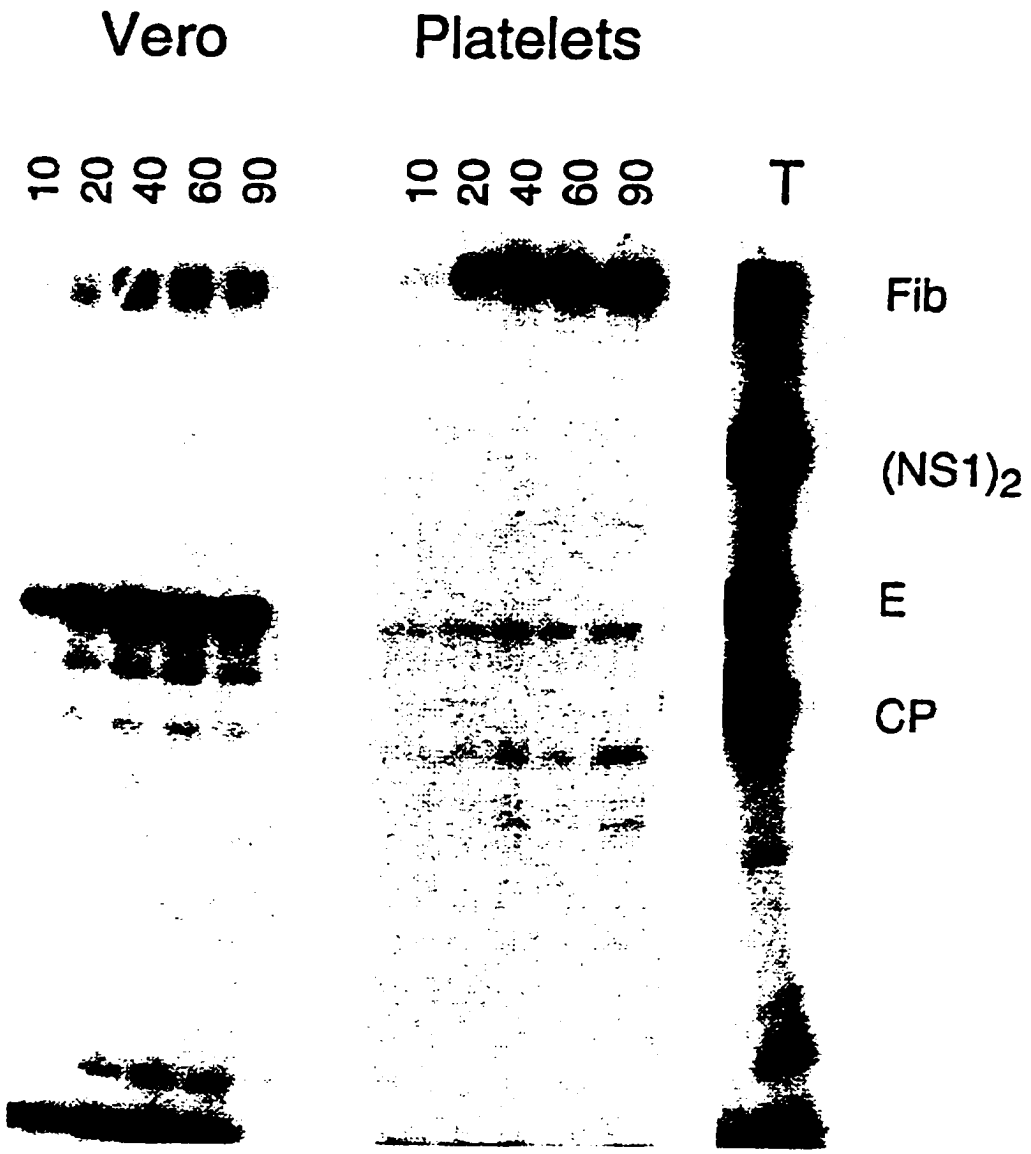
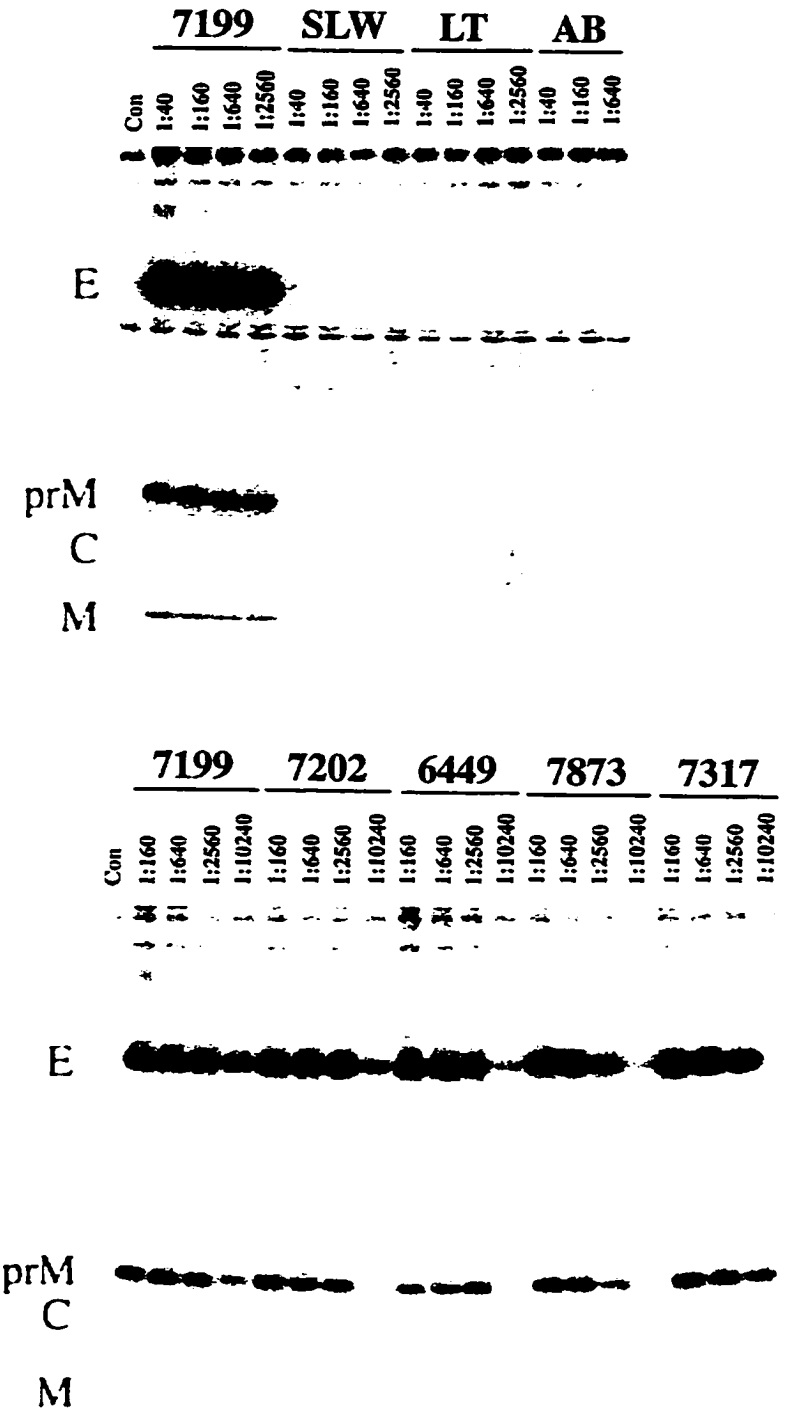


Figure 11.

Antibody-enhanced binding of radiolabeled dengue virus to platelets. Convalescent antiserum samples from patients recovering from dengue-2 infection (labeled 7199, 7202, 6449, 7873 and 7317) or from normal., dengue-nonimmune individuals (AB, SLW, LT) were used (convalescent sera had dengue-2 plaque reduction neutralization titers (PRNT) greater than 1:640; titers for normal sera were below 1:10). Culture fluid from ^{35}S-methionine-labeled dengue virus-infected Vero cells was mixed with dilutions of the respective serum samples for 1 h at 4°C, then added to platelets and incubated a further 1 h at 4°C. Platelets were washed, solubilized and analyzed by SDS-PAGE (10-22% gradient polyacrylamide) fluorography. Abbreviations as in legend to Fig.10, except M and prM are the membrane and membrane precursor proteins, respectively.

Figure 11.


7199
SLW
LT
AB
Con
1:40
1:160
1:640
1:2560
1:40
1:160
1:640
1:2560
1:40
1:160
1:640
1:2560
1:40
1:160
1:640
E
prM
C
M
7199
7202
6449
7873
7317
Con
1:160
1:640
1:2560
1:10240
E
prM
C
M

platelets and IgG subclass of the respective antibody (Fig. 12B). Contrary to this result, antibody-dependent enhancement of dengue virus infection in erythroleukemic K562 cells which bear the same FcγRII as platelets has been reported to show dependence on IgG subclass. For example mAb 3H5 (IgG1) but not mAb 4G2 (IgG2a) was shown to mediate antibody-dependent enhancement of dengue-2 virus infection in K562 cells, which express FcγRII receptor as well (Littaua et al., 1990). These differences suggest that the mechanism of antibody-dependent enhanced virus binding to human platelets may be different from other cells which bear Fc receptors.

The question of dependence on platelet Fc receptors for antibody-enhanced virus binding was further investigated using a monoclonal antibody against FcγRII: mAb IV.3. This monoclonal antibody is known to block ligand binding to FcγRII receptors on macrophage-like cells. Platelets were pretreated with mAb IV.3. before being used in the binding assay. As a control the same concentration of mAb IV.3 was used to pretreat K562 cells. Virus-antibody mixture was then added to platelets and K562 cells. As shown in Fig. 13B, IV.3. completely blocked antibody-enhanced binding of dengue virus to K562 cells. However, IV.3. had little effect on antibody-enhanced virus binding to platelets (Fig. 13A), suggesting that (unblocked) Fc receptor is not required for antibody-dependent binding of dengue virus to platelets.

We also treated the convalescent antiserum with staphylococcal protein G Protein G binds to the Fc portion of IgG and is known to interfere with IgG/FcR interactions. Protein G was shown to have little effect on antibody-enhanced binding of virus to platelets but completely blocked antibody-dependent virus binding to K562 cells (data not shown). This further supports the idea that antibody-mediated enhanced binding of dengue virus to human platelets is not Fc receptor dependent.

Figure 12.

Antibody-enhanced binding of dengue to human platelets using murine mAbs specific for dengue E protein. A: Culture fluid (T) from ^{35}S-methionine-labeled dengue-infected Vero cells was mixed with dilutions of mAbs and let stand 1h at 4°C. Mixtures were added to platelets and let stand a further 1h. Platelets were washed, solubilized and analyzed by SDS-PAGE (10-22% gradient) fluorography. Abbreviations as in legend to Fig. 11. B: relative binding of dengue virus to platelets in the absence (Con; binding normalized to 1) or presence of varying dilutions of mAbs or of human dengue-convalescent serum 7199. Binding was determined by densitometric scanning of E protein from fluorograms (as in panel A) by means of an Apple Color One Scanner linked to an Apple Macintosh Centris 610 computer using Scan Analysis software (Release 2.20; Biosoft, Cambridge, U.K.). Reciprocal dilutions of serum employed are noted below the x-axis. Serum dilutions shown are the ones found to give maximal virus-platelet binding for each serum tested.

Figure 12.

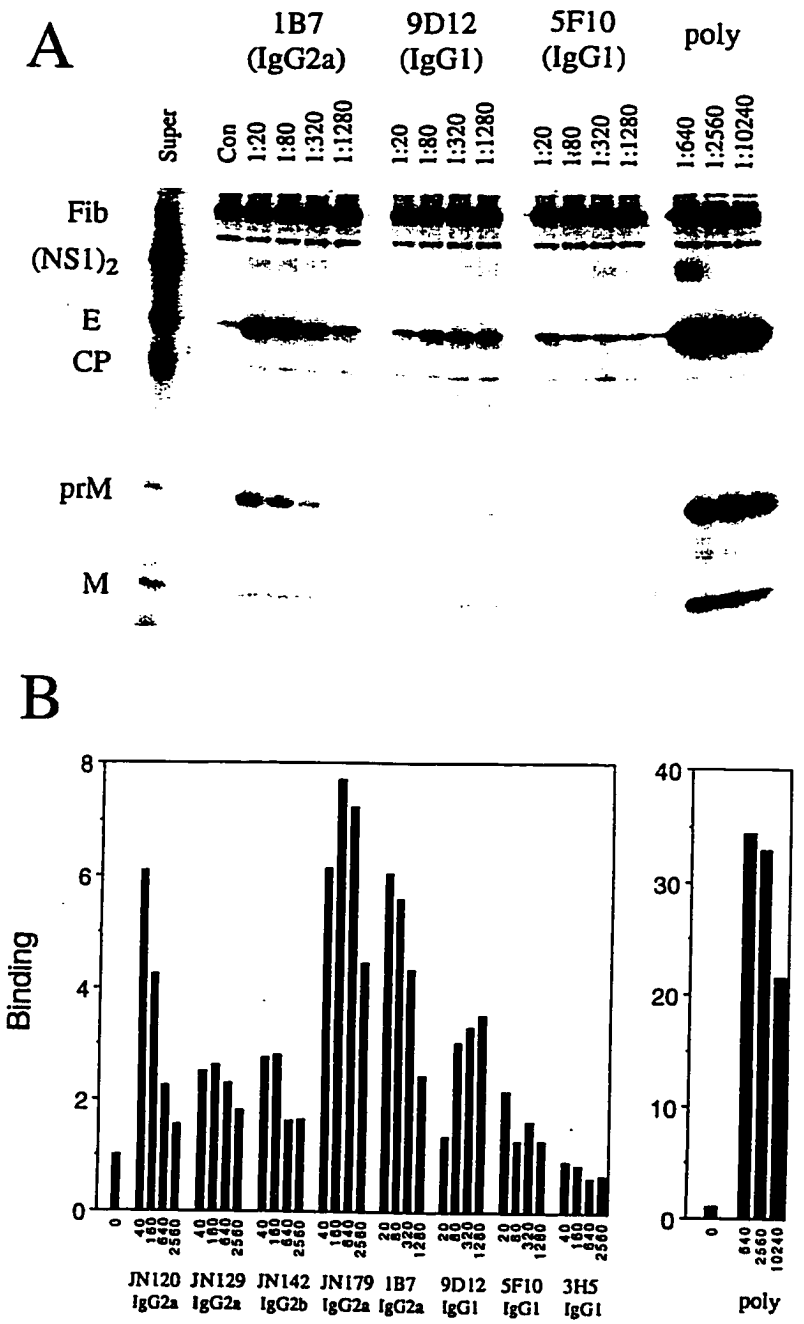
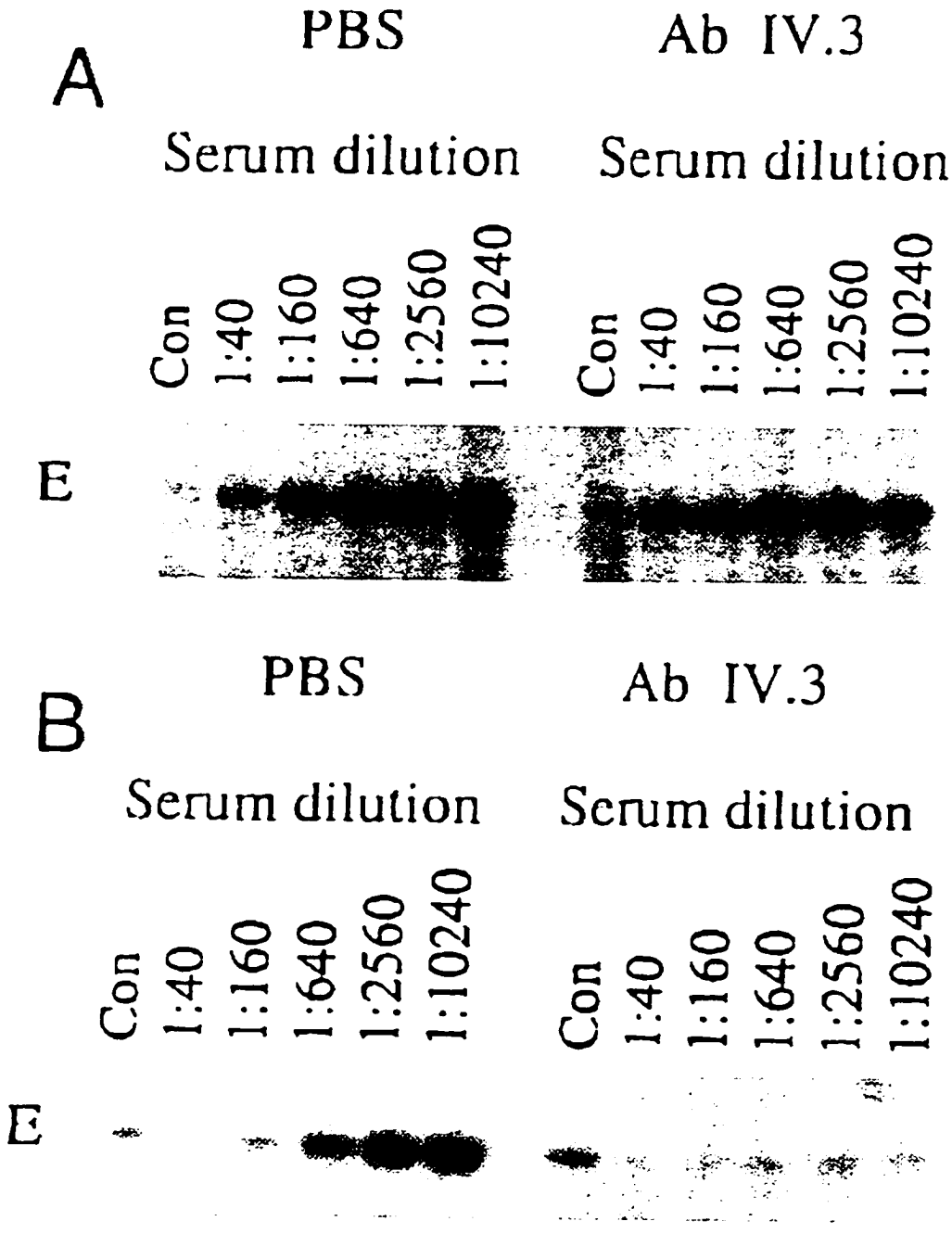

A
1B7 (IgG2a)
9D12 (IgG1)
5F10 (IgG1)
poly
Super
Con
1:20
1:80
1:320
1:1280
1:20
1:80
1:320
1:1280
1:20
1:80
1:320
1:1280
1:640
1:2560
1:10240
Fib
(NS1)2
E
CP
prM
M
B
Binding
JN120 IgG2a
JN129 IgG2a
JN142 IgG2b
JN179 IgG2a
1B7 IgG2a
9D12 IgG1
5F10 IgG1
3H5 IgG1
poly

Figure 13.

Lack of blocking of antibody-enhanced virus-platelet binding by pretreatment with mAb IV.3. Culture fluid from ^{35}S-methionine-labeled dengue-infected Vero cells was mixed with various dilutions of convalescent antiserum 7199 (1h at 4°C) and added to platelets (A) or K562 cells (B) which had been pretreated with either PBS or mAb IV.3 (100 mg/ml). After 1h at 4°C, cells or platelets were microcentrifuged, washed and solubilized for analysis by SDS-PAGE (10-22% gradient) fluorography. Excerpts of the gel showing the region containing the viral E protein are shown.

Figure 13.


A
PBS
Ab IV.3
Serum dilution
Serum dilution
Con
1:40
1:160
1:640
1:2560
1:10240
Con
1:40
1:160
1:640
1:2560
1:10240
E
B
PBS
Ab IV.3
Serum dilution
Serum dilution
Con
1:40
1:160
1:640
1:2560
1:10240
Con
1:40
1:160
1:640
1:2560
1:10240
E

3.5. Anti-platelet antibody can further enhance the binding of virus to platelets

We prepared mouse anti-human-platelet antisera to investigate if such antisera could block binding of virus-antibody complexes to platelets. Platelets were treated with two anti-platelet antisera, two anti-Vero cell antisera and one non-immune antiserum; a platelet sample without antiserum treatment was used as a mock control. They were then used in a binding assay with ^{35}S-methionine and radio-labeled dengue/antibody mixture (with a human anti-dengue antiserum). Surprisingly, treatment of platelets with anti-platelet antisera actually enhanced, rather than blocked, binding of the virus-antibody complex to platelets. No enhanced binding was observed with platelets treated with anti-Vero and non-immune antisera (Fig. 14A). Thus anti-platelet antibody can further enhance binding of virus-antibody complex to platelets. However, this enhancement activity is dependent on anti-dengue antisera. As shown in Fig. 14B, radio-labeled dengue virus without treatment with anti-dengue antiserum could not bind to platelets which were treated with anti-platelet antisera.

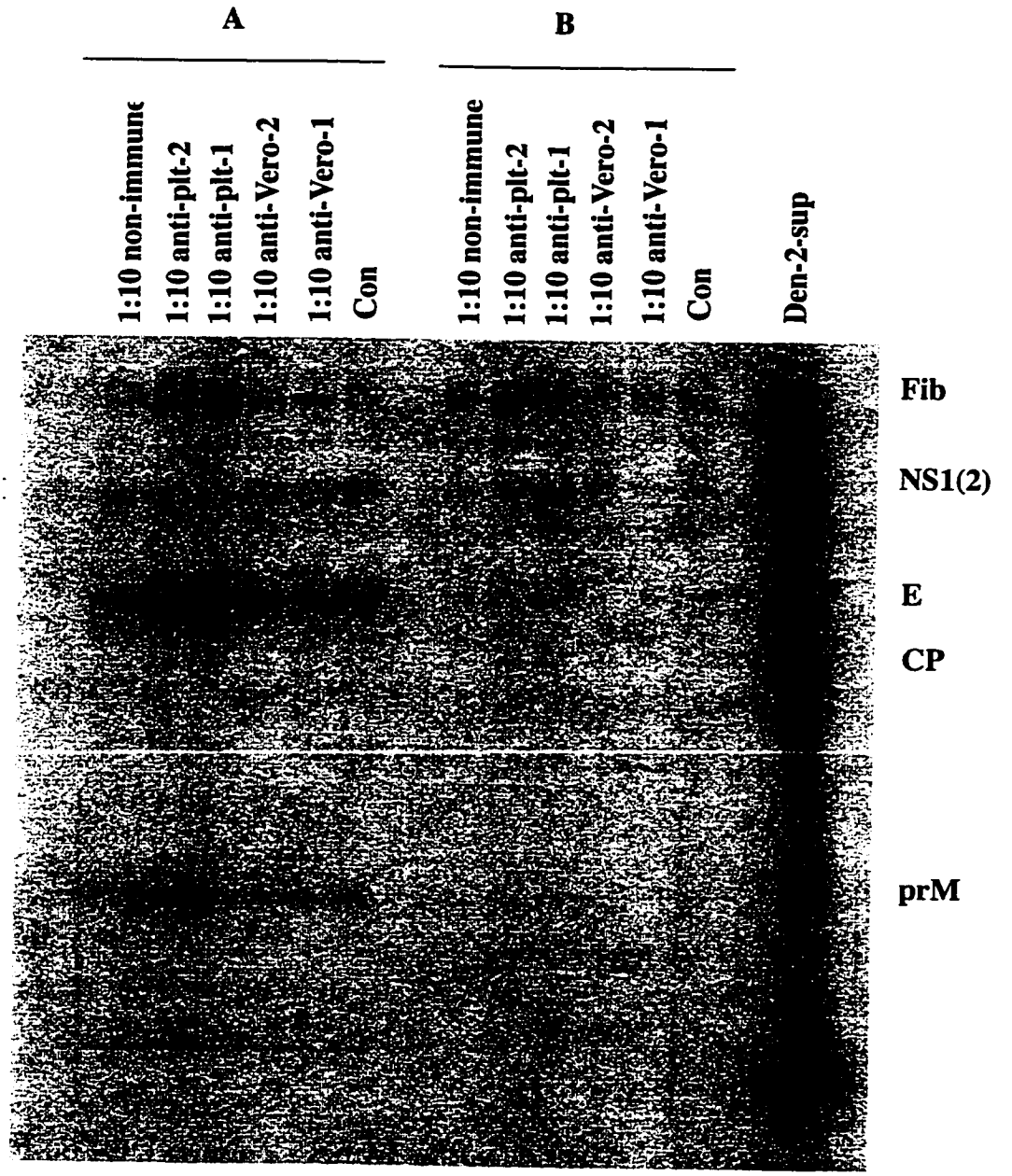
3.6. Anti- platelet and Vero antibodies do not enhance virus binding to Vero cells

The finding that anti-platelet antibody could further enhance virus-antibody binding to platelets indicated that it is possible that the affinity or the specificity of the virus-antibody-binding molecule(s) on the surface of platelets may be changed. We investigated whether a similar process may occur with Vero cells which bind dengue virus and therefore have virus receptors on the cell surface. Vero cells were pretreated with mouse anti-Vero antisera, anti-platelet antisera, non-immune serum and PBS. As a control, platelets were pretreated with anti-platelet antibodies. Radiolabeled dengue virus was mixed with dengue specific antisera and added to the pretreated Vero cells

Figure 14.

Anti-platelet antisera-enhanced binding of dengue-2 virus to human platelets. Convalescent antiserum 7202 was mixed with culture fluid from ^{35}S-methionine-labeled dengue virus-infected Vero cells at a final dilution of 1:1280 and incubated for 1 h at 4°C. Another aliquot of the labeled culture fluid without antibody treatment was also incubated for 1h at 4°C. In the meantime, platelets were treated with two anti-platelet antisera, two anti-Vero antisera and one non-immune antisera at a final dilution of 1:10 and incubated for 1h at 4°C. Samples of labeled culture fluid treated with (A) and without anti-dengue antisera (B) were then added to platelets and incubated a further 1 h at 4°C. Platelets were washed, solubilized (as in legend to Fig. 1) and analyzed by SDS-PAGE (10-22% gradient polyacrylamide) fluorography. Abbreviations as in legend to Fig.10. Anti-plt-2= anti-platelet antibody-2

Figure 14.


A
B
1:10 non-immune
1:10 anti-plt-2
1:10 anti-plt-1
1:10 anti-Vero-2
1:10 anti-Vero-1
Con
1:10 non-immune
1:10 anti-plt-2
1:10 anti-plt-1
1:10 anti-Vero-2
1:10 anti-Vero-1
Con
Den-2-sup
Fib
NS1(2)
E
CP
prM

and platelets. As shown in Fig. 15, compared with the enhanced virus binding to platelets, there was no apparent enhancement of virus-cell binding observed in Vero cells. Anti-platelet antisera had a slightly blocking effect on virus binding, while anti-Vero antisera had no enhancing or blocking activities. Thus, it is clear that the binding of dengue virus to Vero cells is quite different from that observed to platelets, and that the virus-binding molecules are probably different.

3.7. Dengue virus does not bind to human red blood cells

To further confirm that the enhanced binding to platelets is specific, we used red blood cells as another control. It is known that some viruses can bind to red blood cells (RBC) and cause hemagglutination. For example, measles virus (Shibahara et al., 1994;), influenza virus (Gunther et al., 1993) Sendai virus (Rapaport et al., 1995) have been shown to be able to bind to the surface of red blood cells. Radiolabeled dengue virus was mixed with serial dilutions of convalescent patient dengue-immune antiserum. The virus-antibody mixtures were used to assay binding to human red blood cells and human platelets. As shown in Fig. 16, dengue virus did not bind to red blood cells either in the presence or absence of virus-specific antisera. The control group with platelets showed strongly enhanced binding in the presence of virus-specific antisera (Fig. 16). As another control, we also treated red blood cells with anti-red blood cell antiserum to determine whether the virus-antibody complex binds to antibody-decorated red blood cells (eg to the Fc portion of IgG bound to the red blood cell). As shown in Fig. 16, there was no binding of dengue virus to the antibody-treated red blood cells. These results indicated that, in contrast to Vero cells or platelets, red blood cells do not have receptors for binding of dengue virus or of virus-antibody complexes. Furthermore, these results rule out the possibility that virus binding to anti-platelet antibody-treated platelets involves the Fc portion of the IgG molecule.

Figure 15.

Dengue virus binding to Vero cells and platelets in the absence and presence of anti- platelet and anti-Vero antibodies. Radiolabeled dengue virus was mixed with a convalescent patient dengue-immune serum (#7199). The virus-antibody mixture was added to the pretreated Vero cells and platelets. Samples were washed and dissolved in dissociation buffer. Cell extracts were resolved on SDS-PAGE and visualized by fluorography. Enhanced virus binding was only observed in platelet samples (Con= non-treated). For abbreviations, see Fig. 10.

Figure 15.

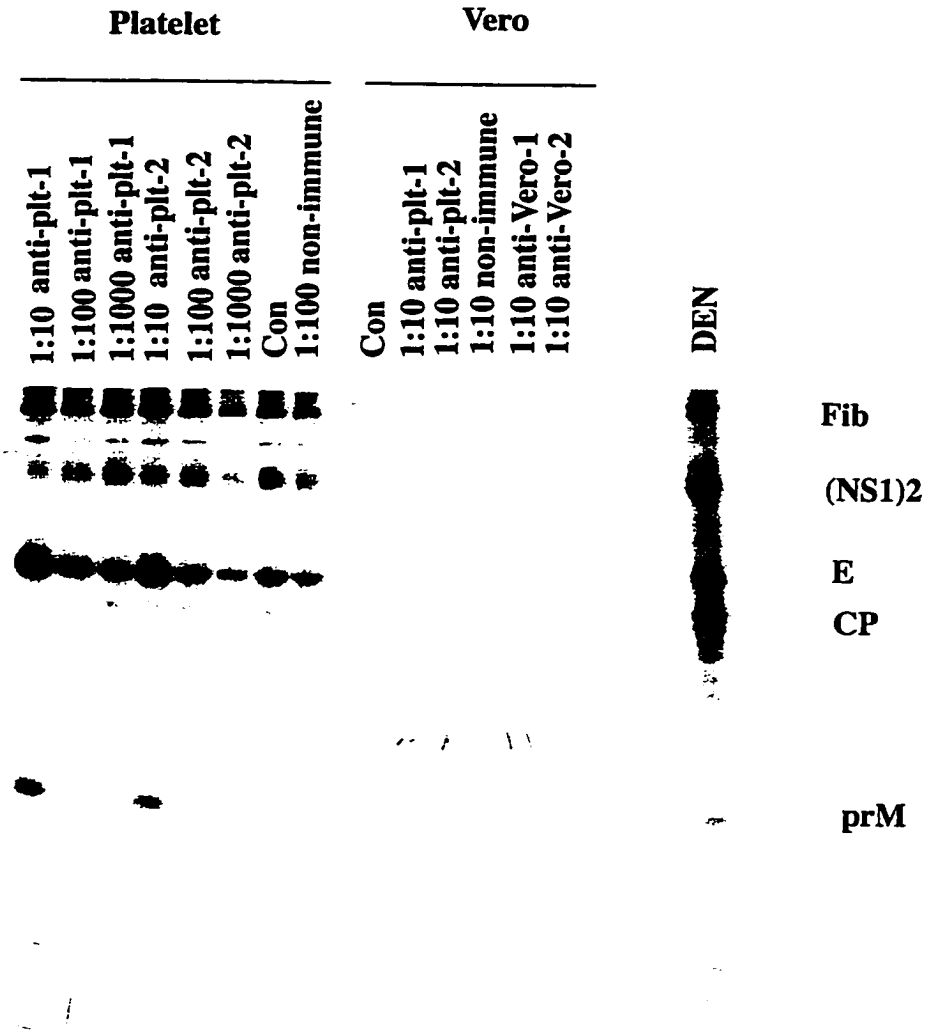
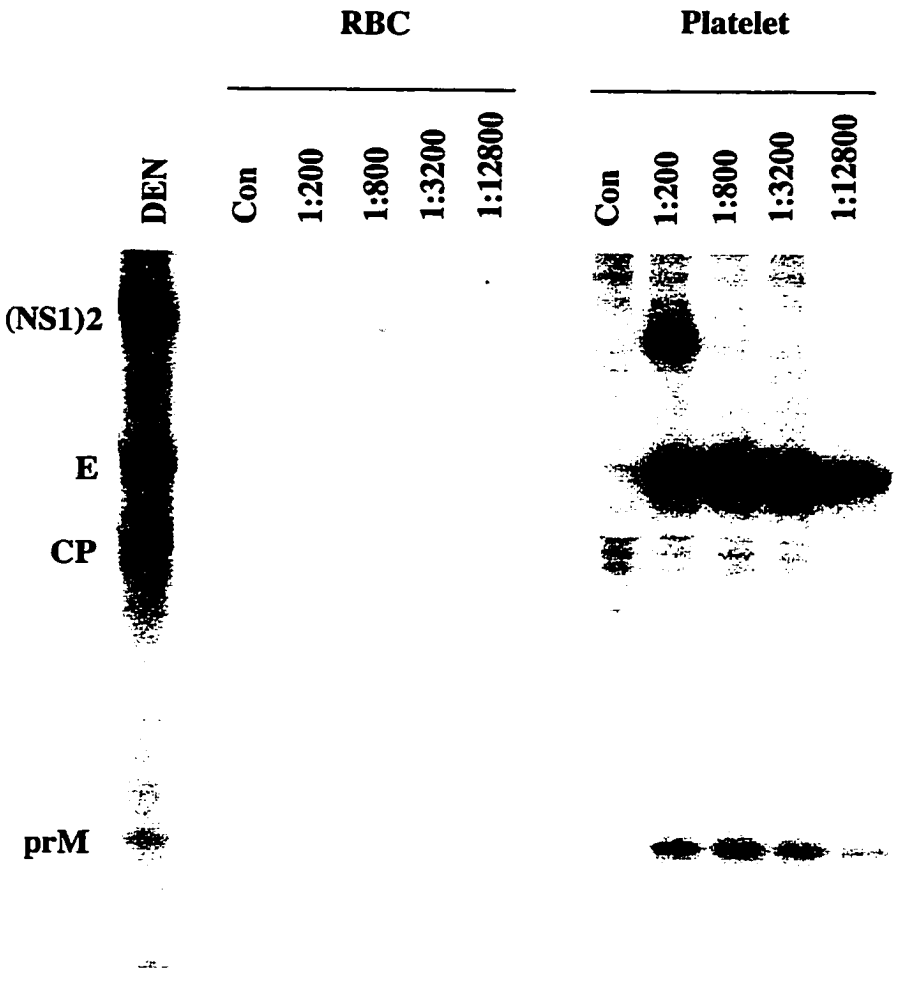

Platelet
Vero
1:10 anti-plt-1
1:100 anti-plt-1
1:1000 anti-plt-1
1:10 anti-plt-2
1:100 anti-plt-2
1:1000 anti-plt-2
Con
1:100 non-immune
Con
1:10 anti-plt-1
1:10 anti-plt-2
1:10 non-immune
1:10 anti-Vero-1
1:10 anti-Vero-2
DEN
Fib
(NS1)2
E
CP
prM

Figure 16.

Lack of binding of dengue virus to red blood cells. Radio-labeled dengue virus was mixed with dilutions of a convalescent dengue-immune serum. The virus antibody mixtures were added to RBCs and platelets. Cells were then washed, resolved by SDS-PAGE and radiolabeled proteins were visualized by fluorography. No virus binding to RBCs was observed. In the presence of dengue-immune serum, dengue virus binds to platelets (Con = virus alone). For abbreviations, see Fig. 10.

Figure 16.


RBC
Platelet
DEN
Con
1:200
1:800
1:3200
1:12800
Con
1:200
1:800
1:3200
1:12800
(NS1)2
E
CP
prM

4. DISCUSSION

To our knowledge, this is the first report showing the antibody-dependent enhanced binding of dengue virus to human platelets. Surprisingly the results suggest that molecule(s) other than Fc receptors may mediate antibody-enhanced virus binding to platelets. It is likely that this molecule(s) is platelet-specific, since it is not obvious on Vero cells or red blood cells. Alternatively, the same molecule may be shared by both platelets and Vero cells, but the conformation is different.

One of the best examples of non-Fc-receptor binding of antibody-antigen complexes comes from studies with drug-induced thrombocytopenias. It is known that given the appropriate accessory ligand (i.e. drug), IgG can bind to platelets through either the Fc receptor or other surface proteins. For example, heparin-induced thrombocytopenia is characterized by heparin-dependent IgG binding to the platelet Fc receptor (Kelton et al., 1988). On the other hand, it is known that quinine/quinidine-dependent thrombocytopenia involves IgG binding to a complex composed of drug and either GPIIb/IIIa or GPIb/IX (Christie et al., 1981; Kelton et al., 1988; Berndt et al., 1985). It will be important to establish whether similar mechanisms might operate in the antibody-enhanced binding of dengue virus to platelets and in dengue-associated thrombocytopenia.

It is possible that our study has identified a novel dengue virus receptor on platelets. Virus receptors are one of the most determinant factors of virus host range and tissue tropism. Virus receptors can be protein (such as CD4, CD46), carbohydrates (such as neuraminic acid) or lipids (such as gangliosides). Some examples of identified virus receptors are: CD4 (Dalgleish et al., 1984) and chemokine receptors, CCR5 for HIV (Choe et al., 1996; Rucker et al., 1996) and CXCR4 (Endres et al., 1996; Cocchi et al., 1996), integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ for

adenovirus type 2 (Wichham et al., 1993; Wickham et al., 1995), CD46 for measles virus (Naniche et al., 1993; Seya et al., 1997), gangliosides for influenza types A and B (Haywood et al., 1974) and N-acetylneuraminic acid (for type C) (Herrler et al., 1985), CD21 for Epstein-Barr virus (McClure, 1992), and ICAM-1 for rhinovirus (Greve et al., 1989; Staunton et al., 1989).

Despite the importance of virus receptors, the identities of many virus receptors and the mechanisms of virus-receptor interactions remain unknown (Norkin, 1995). As for many other viruses, the receptor(s) for dengue virus is not yet characterized. The question as to whether there is one receptor or several receptors in different host cells for dengue virus also remains to be answered. From our study, it is possible that there is more than one receptor (or different conformational states of the same receptor) on Vero cells and platelets. The receptor on Vero cells can mediate virus infection while the receptor on platelets binds to virus-antibody complexes which may play a role in platelet depletion in severe dengue disease. Having more than one virus receptor is not just found with dengue virus. Some other viruses also have multiple receptors. For example, HIV requires the CD4 molecule as well as one or more chemokine receptors for infection of T lymphocytes or macrophages. Blocking these chemokine receptors with antibodies or antagonists can abolish the virus infection (Choe et al., 1996; Rucker et al., 1996; Wu et al., 1996). For example, it has been shown that blocking the HIV chemokine receptor CCR-5 with chemokine ligands, or antibodies against the V3 loop of gp120 inhibits virus-cell fusion and infection (Wu et al., 1996). Other viruses, such as herpes simplex virus, bind heparan sulfate (via the viral glycoprotein C) (WuDunn and Spear, 1989), while glycoprotein D binds to another protein on the cell surface (Johnson et al., 1990). Furthermore, adenovirus type 2 binds (via its fiber protein) to cellular integrins $\alpha_v\beta_3$

and $\alpha_v\beta_5$ (Wickham et al., 1993, 1995; Roelvink et al., 1996; Mayr and Freimuth, 1997).

The conformational change of viral glycoproteins is very important for virus binding to host cells. A virus receptor is not only involved in initial virus binding, but is also involved in the subsequent steps including conformational changes of viral and cellular components, which modulate affinities of the binding and facilitate virus penetration (Haywood et al., 1994). For example, most viruses bind at 0-4°C, but penetrate cells only at physiological temperatures. Binding can occur in two or more stages. Primary or initial binding may be of lower affinity and may loosely attach the virus to the cell receptor. Subsequent or secondary binding is often of higher affinity and may lead to the process of penetration either by fusion or by endocytosis. In the case of Sendai virus which can utilize gangliosides as receptors, binding occurs with different affinities depending on the temperature. The virus binds to gangliosides weakly at low temperatures but binds tightly at higher temperatures, suggesting that conformational changes of the virus glycoproteins and cellular protein occur during the temperature transition (Haywood et al., 1982; Tsao et al., 1986). Studies have shown that heparin can reverse the initial binding of Herpes viruses to cells. However, the binding that follows the initial binding is heparin-resistant (Compton et al., 1993). Two proteins are thought to be involved in herpesvirus binding: glycoprotein C is involved in the early binding, while glycoprotein D is necessary for the heparin-resistant step and penetration (Compton et al., 1993).

Our studies suggest at least two kinds of receptors for dengue virus, eg. an antibody-independent virus receptor on Vero cells (Chapter 2) and an antibody-dependent virus receptor on platelets (this chapter). In the case of the latter, it is possible that anti-dengue antibodies might be able to change the conformation of the E

protein so that it allows the virus to bind to human platelets. It is known that the flaviviral E protein is subject to conformational changes induced by factors such as low pH or binding with prM (Roehrig et al., 1990; Heinz et al., 1994; Rey et al., 1995). It is also noteworthy that not all E-protein antibodies enhance binding of virus to platelets. For example, mAB 3H5 had no effect on virus binding to platelets. This suggests that certain epitopes on the dengue E protein may be required, either for direct receptor interaction or for reconforming the E protein into a shape required for receptor binding.

It is interesting that mouse-anti-platelet antisera further enhanced binding of virus-antibody complexes to platelets, suggesting that the conformational changes of both E protein and cellular factor(s) may play a role in binding virus to the platelet surface. This enhancement is also platelet-specific, as no enhancement was detected with anti-Vero cell or anti-red-blood-cell antibodies to red blood cells. Thus it may be that the virus-antibody binding molecule on the surface of platelets is very sensitive to conformational changes. An intriguing candidate may be a member of the integrin family. Integrins are abundant on the surface of platelets, are prone to conformational changes, and are able to bind RGD or RGD-like sequences, which are also contained in the E proteins of several mosquito-borne flaviviruses (Becker et al., 1990; Rey et al., 1995).

The family of integrin receptors is composed of series of heterodimers, formed from one α and one β chain. The different combinations of chains determine the specificities of receptors. Each heterodimer binds to one or a number of extracellular matrix proteins. Usually several integrin receptors are expressed by a given cell, allowing the cell to interact with proteins in the basement membrane or interstitial matrix (Schnaper, 1996). Integrins generally bind to a peptide or protein

which contains the amino acids arginine-glycine-aspartic acid, or RGD, giving rise to signal transductions in the cell. Some integrins bind to the RGD or RGD-like sequence of a single adhesion protein only, while others recognize groups of RGD or RGD-like sequences. The conformation of the RGD sequence in the individual proteins may be critical to its recognition specificity (Ruoslahti and Pierschbacher, 1987; Schnaper, 1996). Integrins can also recognize proteins containing Ig-like structures, such as platelet/endothelial cell-adhesion molecule-1 (Roos, 1991; Newman, 1994; Jackson et al., 1997) and intracellular adhesion molecule-1 (Rothlein et al., 1986; van-de-Stolpe et al., 1996). ICAM-1 has been shown to react with integrin molecules LFA and Mac-1 (Mazerolles et al., 1988; Duperray et al., 1997), while PECAM-1 has been shown to bind to the integrin $\alpha_5\beta_3$ (Buckley et al., 1996).

Many mosquito-borne flavirviruses contain an RGD sequence in domain III of the E protein (Stuart and Gouet, 1995; Rey et al., 1995). The RGD sequence is conserved in Japanese encephalitis virus, yellow fever virus and Murray Valley virus. However, dengue virus does not seem to have an obvious RGD containing sequence. However, this might not be required for binding of dengue E protein to integrin molecules because there is an Ig-like structure in domain III of the flavivirus E protein, which is proposed to be important for E protein to be recognized by integrins (Rey et al., 1995). Other than features in domain III, the dengue virus E protein contains an RGD-like sequence (RGWG) from residue 101 to 115 in domain I (Becker et al., 1990; Markoff et al., 1991) The question as to whether this RGD-like sequence can be recognized by integrins remains to be answered. We are in the process of doing binding assays using RGD containing peptides and integrin-binding molecules (fibronectin, fibrinogen and vitronectin) that contain RGD sequences to compete against binding of virus-antibody complexes to platelets.

Like integrin molecules which are expressed in a variety of cells and are able to mediate binding and infection of viruses, complement receptors are expressed in different kinds of human cells. It has been reported that complement proteins are involved in complement-mediated antibody-dependent virus binding to complement receptor expressing cells. For example, HIV can bind to CD4+ T lymphocytes via complement receptors (June et al., 1991, Montefiori et al., 1992). It has been shown that complement-mediated binding to T lymphocytes results in an accelerated cytopathic effect in T lymphocytes (Robinson et al., 1989). Also, it was found that two complement receptors CR1 (CD35) and (CD21) are involved in the antibody-dependent, complement mediated enhancement of HIV infection (Delibrias et al., 1993).

Can complement receptor(s) mediate antibody-dependent enhancement of dengue virus binding to platelets? To our knowledge, the C1q receptor is the only complement receptor that has been found on human platelets. This receptor has also been found on the surface of endothelial cells, neutrophils, macrophages and eosinophils (Andrews et al., 1981; Ghebrehiwet, 1989; Tenner, 1993; Peerschke et al., 1996). Data from our lab suggested that the C1q receptor does not mediate antibody-dependent binding of dengue virus to K562 cells, since anti-Fc receptor antibody IV.3 abrogated the enhanced binding of virus-antibody complexes to K562 cells (Fig. 13). Also, the binding and infection by virus-antibody complexes of endothelial cells, which express C1q receptor (Andrews et al., 1981), was not enhanced in the presence of patient antisera or monoclonal antibodies (Anderson et al., 1997). Based on these considerations, it is unlikely that C1q is involved in the enhanced binding of virus-antibody complexes to platelets.

Dengue virus has been reported to bind to platelets in the absence of antibody (Funahara et al., 1987). However it is evident from the present study that the levels of antibody-independent bound virus are very low compared to the levels of virus bound in the presence of dengue-specific antibodies. Our in vitro studies may have in vivo significance as indicated by the demonstration of dengue immune complexes on platelets from DHF patients (Boonpucknavig et al., 1979). Cohen and Halstead (1966) originally proposed the possibility that dengue virus interactions with platelets might be involved in the thromobocytopenia observed in severe dengue disease. Our finding that dengue virus binding to platelets is dependent on virus-specific antibody is consistent with epidemiologic and experimental data linking preexisting host antibodies to an increased risk of DHF/DSS.

CHAPTER 4

CHARACTERIZATION OF PrM- AND CELL-BINDING ACTIVITIES OF THE DENGUE E PROTEIN

1. INTRODUCTION

The flavivirus E protein is a multifunctional protein which is involved in cell receptor binding and virus entry, via fusion with a host cell membrane (Rice, 1996). Studies with some flaviviruses have shown that at low pH, the E protein undergoes conformational changes which expose the hydrophobic elements and cause virus-plasma membrane fusion (Gollins and Porterfield, 1985; Randolph et al., 1990; Guirakhoo et al., 1993; Heinz et al., 1994). In addition, certain monoclonal antibodies directed against the envelope protein have been shown to be able to block fusion (Guirakhoo et al., 1991; Randolph et al., 1990; Summers et al. 1989).

In addition to the flavivirus E protein, the prM protein is also thought to be involved in virus-membrane fusion. In TBE virus, prM is associated with the E protein in immature intracellular virions and is cleaved by a cellular protease before virus release (Chambers et al., 1990a). The cleavage of prM can be blocked in infected cells by using ammonium chloride or bafilomycin A1. This treatment allows the release of immature virions containing prM, which have reduced infectivity and do not induce cell-cell fusion at acidic pH (Randolph et al., 1990; Guirakhoo et al., 1991; Guirakhoo et al., 1992; Heinz et al., 1994). In the case of TBE, the association of prM with E stabilizes the pH-sensitive epitopes on the E protein and prevents the comformational changes which normally occur at acidic pH (Heinz et al., 1994). The prM-E association is commonly believed to be important to protect

against premature fusion and virus activation during virus transport through acidic intracellular vesicles (Allison et al., 1995).

Although prM-E association has been reported in some flaviviruses, much remains to be studied. In particular, the binding sites involved in prM-E interaction are not clear. Wengler et al (1989), reported that domain II of the E protein contained the prM binding site, while Heinz et al (1994) reported that prM interacts with both the ectodomain and anchor domain. In the following experiments we show that dengue prM and E form higher-order molecular structures and that the anchoring domain of the E protein rather than domain II is important for prM-E association. In contrast, the membrane anchoring domain was found not to be required for cell-binding of the E protein.

2. MATERIALS AND METHODS

2.1. Pulse chase ^{35}S-methionine labeling of virus-infected Vero cells

Dengue virus infected Vero cells were pulse-labeled for 30 min with ^{35}S-methionine (40 μCi/ml) at 48 h post infection. Cells were washed and incubated for various times in 0.5 ml chase medium with excess methione and cysteine. Cells were harvested after washing in tris-buffered saline (TBS) and then solubilized in 100 μl digitonin (1mg/ml). Extracts were microfuged for 15 min at 4°C and the supernatants used for immunoprecipitation using E-specific mAbs 3H5 and 1B7. Immunoprecipitates were analysed on SDS-PAGE and autoradiographed.

2.2. Sucrose gradient fractionation of pulse-chase labeled samples

Extracts from ^{35}S-methionine pulse-chase-labeled infected cells were overlaid on 10-50% (w/w) sucrose gradients in PBS and centrifuged for 16 h at 45k rpm in a Beckman SW60 rotor. Gradient tubes were pierced from the bottom and drained by gravity into eight microfuge tubes. Aliquots were analyzed by SDS-PAGE and fluorography.

2.3. Trypsin cleavage of dengue virus E protein

Dengue virus-infected Vero cells were labeled with ^{35}S-methionine for 12 h from 36-48 h post infection. Trypsin (1 mg/ml, 200 μg/ml and 40 μg/ml) was added to the harvested supernatant from the radiolabeled cells and kept on ice for 1h. The reaction was stopped by adding "stop" buffer containing bovine serum albumin (30 mg/ml) and 5 mM N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK). Samples were immunoprecipitated with mAbs 3H5 and 1B7

2.4. Sucrose gradient fraction of trypsin treated virus

Trypsin treated dengue virus was overlaid onto 10-50% (w/w) sucrose gradients in PBS and centrifuged for 16 h at 45k rpm in a Beckman SW60 rotor. Samples were collected in 7 fractions and immunoprecipated with E-specific mAb 1B7. Aliquots of each sample were analyzed by SDS-PAGE and fluorography.

2.5. Trypsin cleaved E protein binding to Vero cells

Trypsin treated radiolabeled virus was used in a virus-cell binding assay with Vero cells (see chapter 2). An untrypsinized virus sample was used as an control. Samples were solubilized with dissociation buffer and analysed by SDS-PAGE and fluorography.

3. RESULTS

3.1. Association of dengue virus E and prM protein

As mentioned the association of dengue virus prM and E protein is unknown. To investigate this question, we performed a pulse-chase assay with radiolabled dengue virus infected Vero cells followed by immunoprecipitation with E-specific mAb 3H5. As shown in Fig. 17 dengue virus prM becomes rapidly associated with E protein (i.e. within 30 min; Fig. 17 lane 1). The prM-E complex persists in the infected cell for about 2 h of chase before declining around 4 h likely because of export in progeny virions to the cell exterior.

3.2. Multimerization of prM/E complex

To investigate the formation of E/prM heteromers, we analyzed the pulse-chase radiolabeled cell extracts on sucrose gradients (Fig. 18). In the pulse-labeled sample, the prM/E complex was found to sediment near the top of the gradient (fraction 6) consistent with the complex being a simple heterodimer. In contrast, by 6 h of chase, the prM/E complex was also found in higher density fractions (fractions 4 and 5), indicating the formation of higher molecular structures.

3.3. Dengue E protein can be cleaved by trypsin

To investigate domains that contain the prM-E binding site(s) on the dengue virus E protein, we considered proteases that might separate the functional domains from the anchoring domain. Studies with the TBE E protein showed that treatment of virions with trypsin generates a soluble fragment consisting of most of the ectodomain (Rey et al., 1995). Trypsin is a protease that cleaves after basic amino acids such as lysine and arginine (Colletti et al., 1977; Hedstrom et al., 1992). The

Figure 17.

Association of dengue virus E and prM protein. Dengue virus infected Vero cells were pulse-labeled for 30 min with ^{35}S-methionine at 48 h post infection. Cells were washed and incubated for various times in 0.5 ml chase medium. Cells were then harvested after washing in tris-buffered saline (TBS) and solubilized in digitonin. Extracts were microfuged for 15 min at 4°C and the supernatants used for immunoprecipitation using E-specific mAb 1B7. Immunoprecipitates were analysed on SDS-PAGE and autoradiographed. For abbreviations, see Figure 10.

Figure 17.

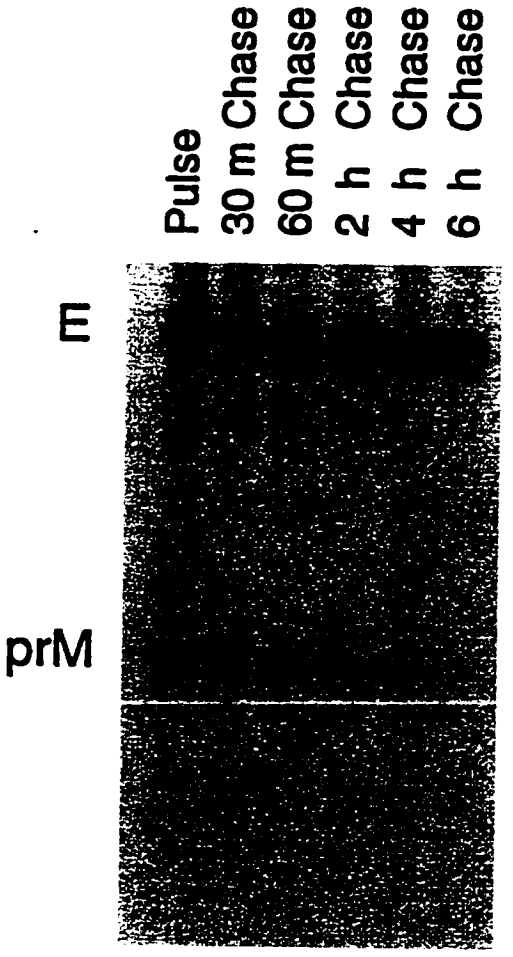
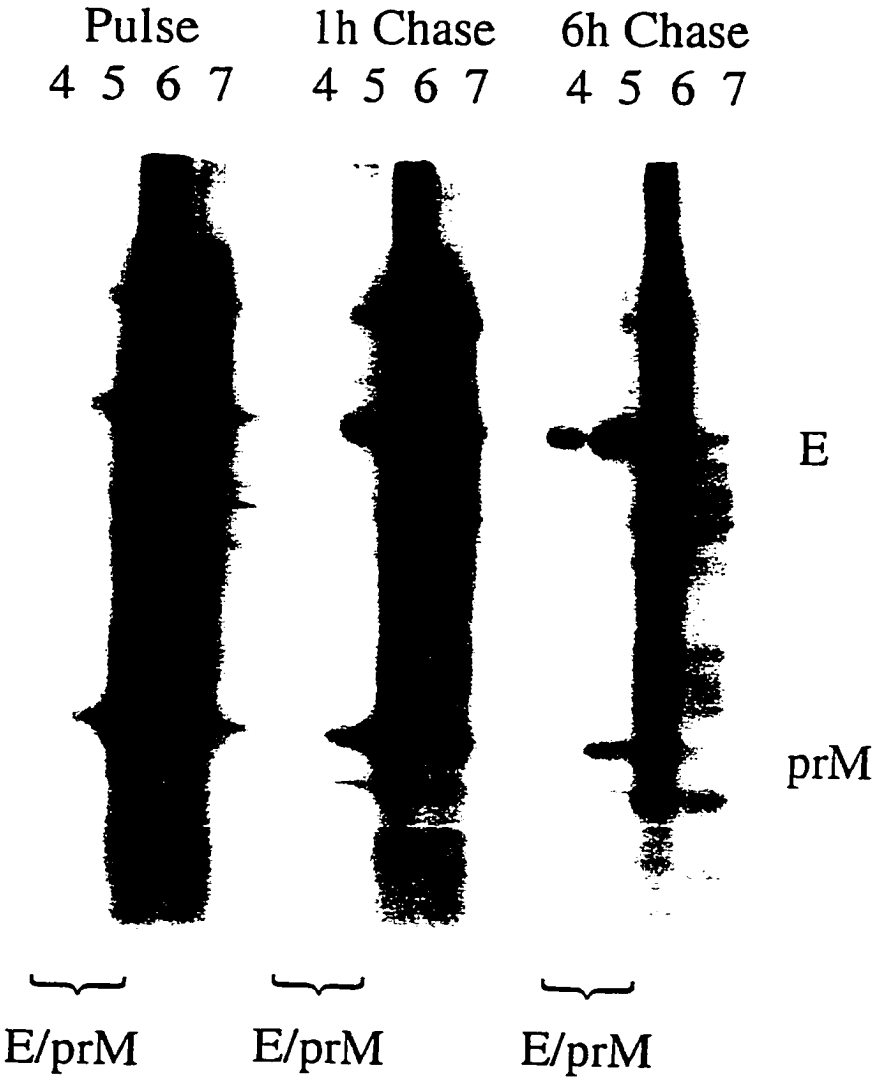


Figure 18.

Multimerization of prM/E complexes. Extracts from ^{35}S-methionine pulse-chase-labeled infected cells were overlaid on 10-50% (w/w) sucrose gradients and centrifuged for 16 h at 45k rpm. Gradient tubes were pierced from the bottom and drained by gravity into eight microfuge tubes. Aliquots were analyzed by SDS-PAGE and fluorography. Multimeric complexes of E/prM are found in fractions 4-5 (see 1 h and 6 h chase samples) while simple heterodimers are found at the top of the gradient in fractions 6-7. For abbreviations, see fig.10.

Figure 18



postulated cleavage sites on the TBE E protein are two basic sequences, Lys (395) and Lys-Lys (407-408). Similar but not identical sequences were also found in the dengue E protein (see Discussion). Based upon the above information, we chose to study whether trypsin would cleave the dengue virus E protein.

Dengue virus radiolabeled with ^{35}S-methionine was treated with trypsin and immunoprecipitated with mAbs 3H5 and 1B7. As shown in Fig. 19 both antibodies were able to immunoprecipitate a protein fragment of about 45 kD, indicating that the E protein could be cleaved by trypsin. In contrast, the apparent size of the prM was not affected by trypsin treatment.

3.4. The trypsin-released ectodomain of E protein does not bind to prM

To investigate the binding sites for prM/E interaction in dengue E protein, we used trypsin-cleaved E protein to investigate domains that are involved in prM-E binding. Dengue virus was digested with trypsin and the digested samples were run on a 15-50% (w/w) sucrose gradient. Fractions of the sucrose gradients were collected and immunoprecipitated with anti-dengue E protein mAb 1B7. Seven fractions were collected from the sucrose gradient, and immunoprecipitatied. Immunoprecipitates were run on SDS-PAGE. As shown in Fig. 20 the uncleaved virus sedimented in the bottom fractions 1-3. The trypsin-cleaved E protein with molecular weight about 45 kD, was mainly found in fraction 6. There was no detectable prM in this fraction, suggesting that the major prM binding sites are located in the anchoring sequence of the E protein.

Figure 19.

Dengue virus E protein can be cleaved by trypsin. Dengue virus-infected Vero cells were labeled with ^{35}S-methionine. Trypsin (1 mg/ml, 200 ug/ml and 40 μg/ml) was added to the harvested supernatant from the radiolabeled cells. The reaction was stopped by adding "stop" buffer containing bovine serum albumin and N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK). Samples were immunoprecipitated with mAbs 3H5 and 1B7. Both mAbs were able to immunoprecipitate the trypsin-cleaved E protein (ΔE). For other abbreviations, see Fig.10.

Figure 19.

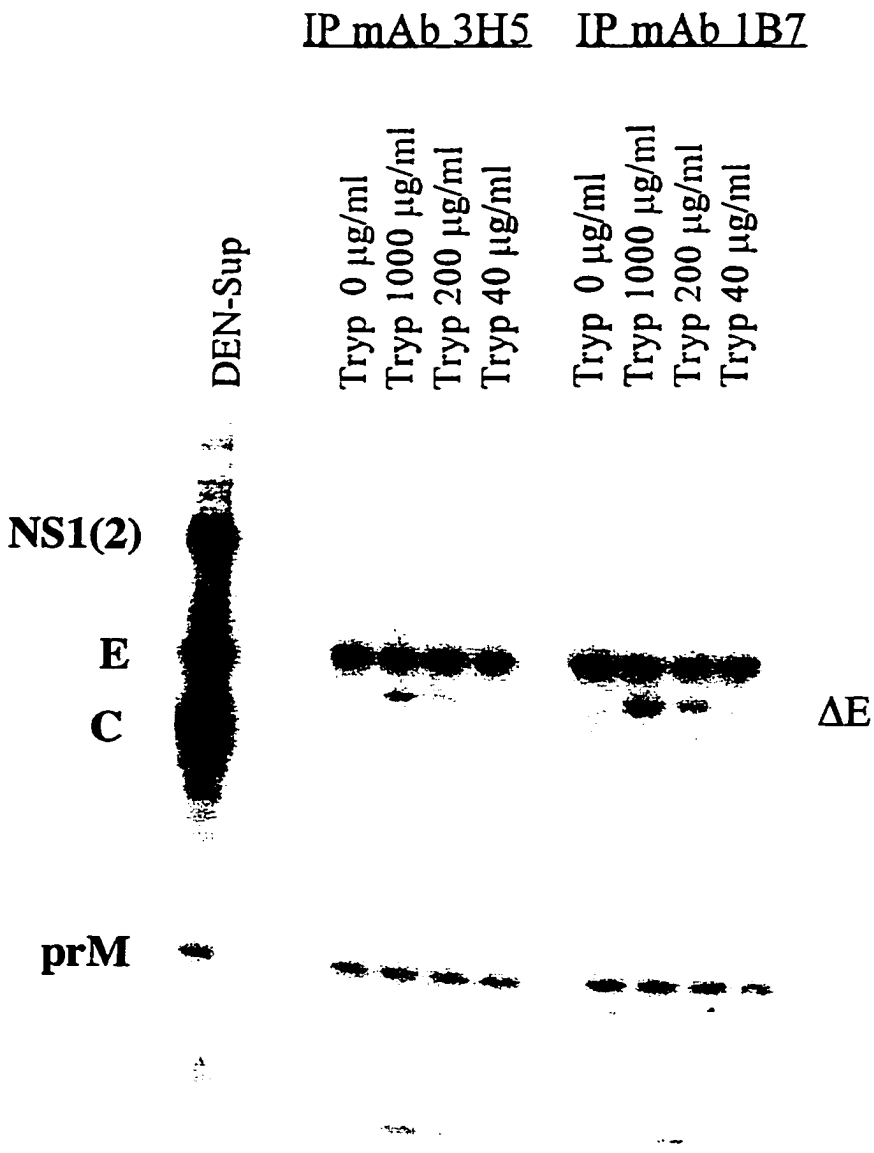
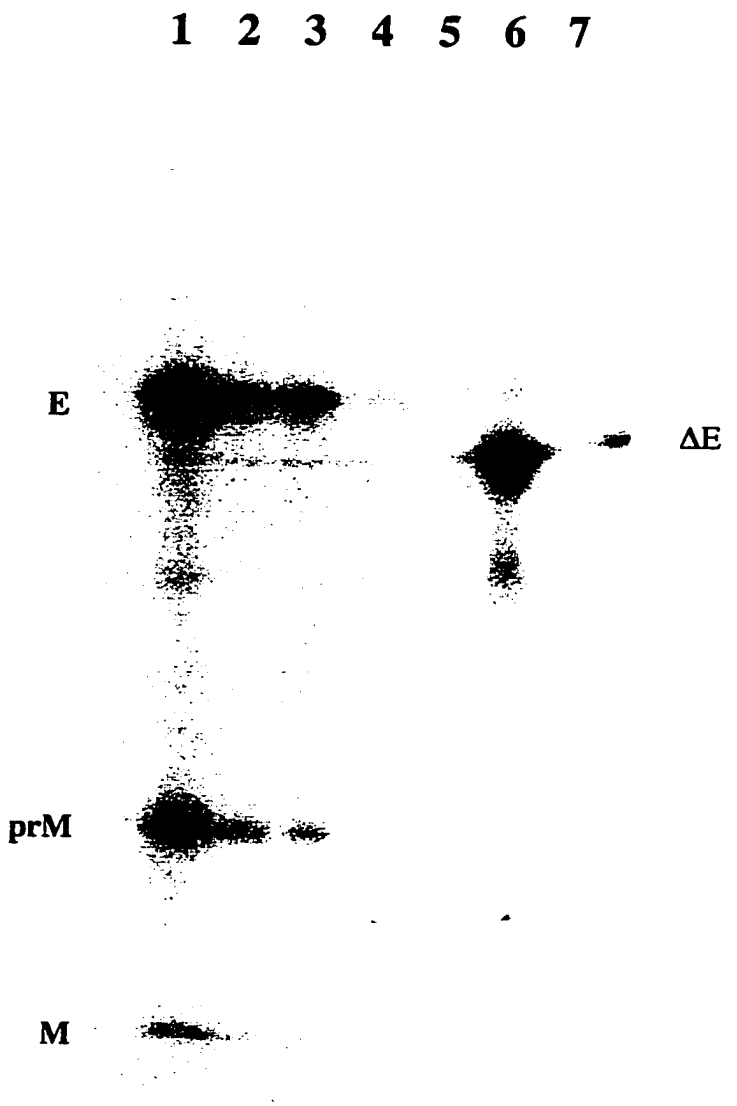

IP mAb 3H5
IP mAb 1B7
DEN-Sup
Tryp 0 µg/ml
Tryp 1000 µg/ml
Tryp 200 µg/ml
Tryp 40 µg/ml
Tryp 0 µg/ml
Tryp 1000 µg/ml
Tryp 200 µg/ml
Tryp 40 µg/ml
NS1(2)
E
C
prM
ΔE

Figure 20.

Release of trypsin cleaved E protein from dengue virions. Trypsin cleaved dengue virus was subjected onto 10-50% (w/w) sucrose gradients in PBS and centrifuged for 16 h at 45k rpm in a Beckman SW60 rotor. Samples were collected in 7 (from bottom to top) fractions and immunoprecipitated with E-specific mAb 1B7. Aliquots of each sample were analyzed by SDS-PAGE and fluorography.

Figure 20



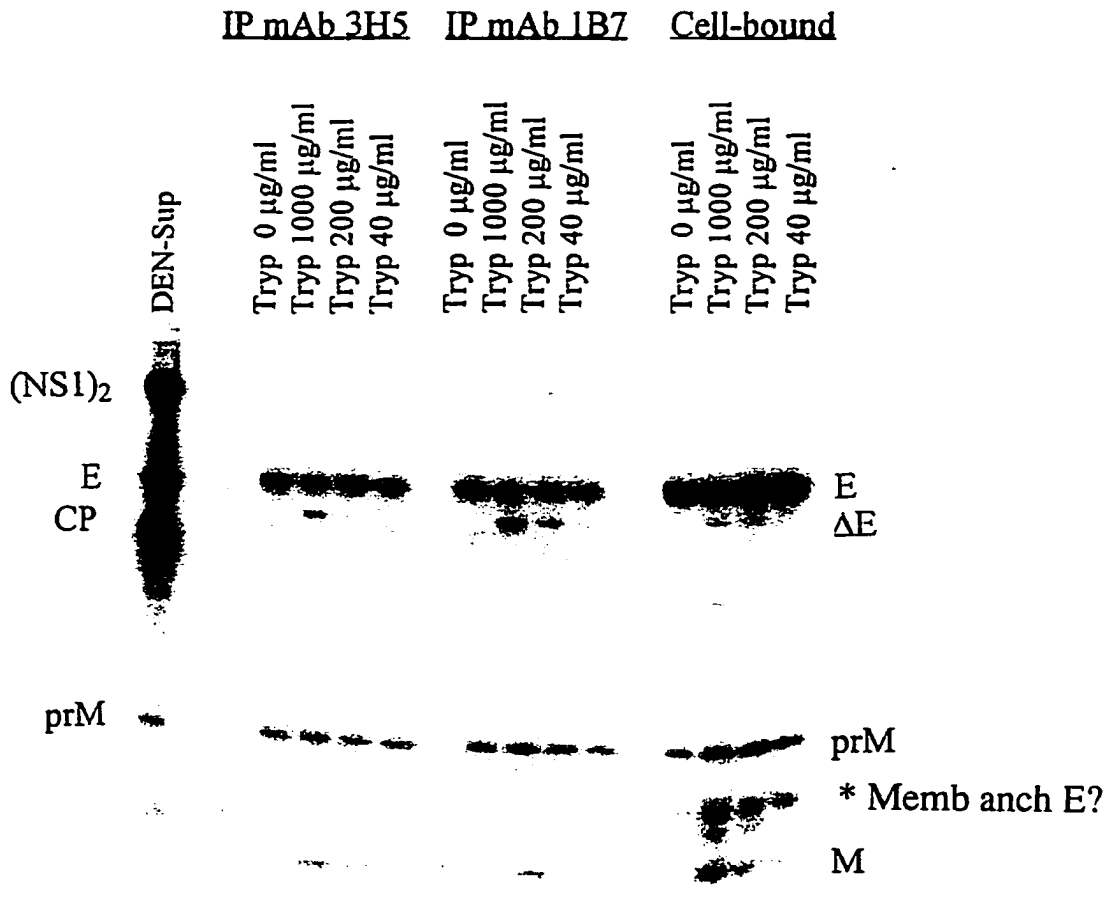
3.5. The trypsin-released ectodomain of E retains cell-binding activity

Even though the receptor-binding site on the dengue virus E protein has not been characterized, studies on several flaviviruses suggested that the distal face of domain III of E protein may contain important sites for E-receptor interactions (Rey et al., 1995; Hiramatsu et al., 1996). We used the trypsin-cleaved soluble E fragment to perform a virus-cell binding assay to determine whether this fragment contained the receptor-binding. As shown in Fig. 21 the cleaved dengue virus E protein retained partial receptor-binding activity compared with intact virus containing uncleaved E protein. This result supports the hypothesis that the receptor binding site is in the N-terminal 80% of the E protein. Stated differently, the cell receptor-binding portion of E can be released in functional form by trypsin cleavage of dengue virions.

Figure 21.

Trypsin-released E retains cell-binding activity. Radiolabeled virus was treated with serial dilutions of trypsin (1000 μg/ml, 200 μg/ml, 40 μg/ml and 0 μg/ml). Trypsin treated samples were used in a virus-cell binding assay with Vero cells. Samples were solubilized with dissociation buffer and analysed by SDS-PAGE. Some of the trypsin-released E (ΔE) was observed to bind to Vero cells.

Figure 21.


IP mAb 3H5
IP mAb 1B7
Cell-bound
DEN-Sup
Tryp 0 µg/ml
Tryp 1000 µg/ml
Tryp 200 µg/ml
Tryp 40 µg/ml
Tryp 0 µg/ml
Tryp 1000 µg/ml
Tryp 200 µg/ml
Tryp 40 µg/ml
Tryp 0 µg/ml
Tryp 1000 µg/ml
Tryp 200 µg/ml
Tryp 40 µg/ml
(NS1)2
E
CP
prM
E
ΔE
prM
* Memb anch E?
M

4. DISCUSSION

This study showed that, in dengue virus infected cells, the E protein rapidly associates with prM, initially as a simple heteromer, then as higher molecular structures. We also showed that the dengue E protein could be cleaved by trypsin into membrane-anchor and ectodomains, in which the membrane anchor of E binds to prM, while the ectodomain has cell-binding activity.

Studies with the TBE E protein have shown that at low pH, the E protein undergoes a conformational change and switches from a homodimer to a homotrimer structure, which can fuse with the cell membrane. The fusion is necessary for the virus to be released from the endosomal vesicle during endocytosis. However, during virus release, virus fusion with cell membranes can block virus transport through intracellular vesicles. The prM-E association has been shown to block this conformational change of the flavivirus E protein and prevents premature virus fusion during virus maturation and release (Heinz et al., 1994).

Similar to the TBE E protein, we showed that dengue virus prM and E form stable complexes. In addition, we also provided evidence that prM-E complexes form higher order heteromers rather than just simple heterodimers. This result supports the model of TBE virus prM/E association proposed by Heinz et al (1995). It was suggested that at neutral pH E protein consists of a lattice of closely packed E protein dimers which can associate together to form higher molecular structures. The prM may associate with the E protein in the lattice to prevent low-pH-induced dimer-trimer transition and premature activation of fusion with cell membranes during virus release.

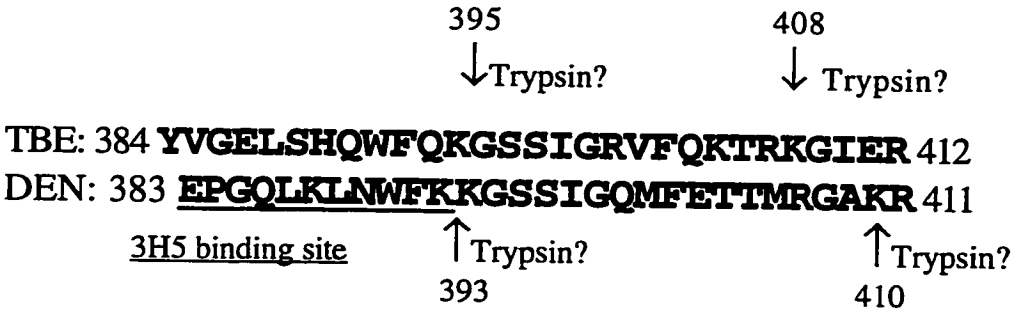
The prM-E binding site in E protein of flavivirus is still controversial. The prM-E binding sites on both proteins have not been well characterized. Heinz et al (1994) used mAbs to study the sites that are involved in the prM/E interaction. They found that the reactivity of an mAb against domain II of E protein was completely blocked when prM was associated with E in immature virions. In the same study, binding of two other monoclonal antibodies against domains I and III was shown not to be affected by prM binding, indicating that one of the prM/E binding sites is in domain II of the E protein (Heinz et al., 1994, 1995). However, this result was not definitive as antibody-binding to the E protein might cause conformational changes rather than precisely block the prM protein binding site. Another study with mammalian cell-expressed TBE E protein suggested that the stability of prM and truncated E association was much lower than that of the full-length E, indicating that the membrane anchoring sequence of the E protein was involved in the prM-E association (Heinz et al., 1995). Our own studies suggest that the involvement of the ectodomain in binding prM is likely very minor, since the trypsin-released ectodomain of E did not bind to prM.

The carboxy-terminal membrane-anchoring sequence may be important for conformational changes of the dengue virus E protein. Other than the fact that it is involved in prM-E binding, other crystallographic studies with the TBE E protein showed that the anchor-free E protein did not rearrange in the presence of low pH, suggesting that the carboxy terminus itself is important for the low pH dependent conformational change of the E protein (unpublished observations by FX Heinz, cited in Stuart and Gouet, 1995)

The trypsin cleavage site of the flavivirus E protein has not been completely characterized. Studies with TBE showed that treatment of virions with trypsin generated a dimeric soluble fragment of E. The cleavage site was reported at residue

408 (Heinz et al., 1991), or, at residue 395 claimed in a later paper by the same group (Rey et al., 1995). The carboxy-most amino acid discernible by X-ray crystallography of trypsin-cleaved TBE protein was lys 395 (Rey et al., 1995). However, it is likely that the fragment may contain a few more residues that could not be resolved by structure analysis. Using anion-exchange columns to elute the trypsin cleaved E protein, these authors found several peaks, which indicated that cleavage occurred at various sites from 395 to 408 (there are several potential trypsin cleavage sites in this region). Since the fragment with the highest number of positive charges, which is around residue 408 with a double lysine sequence, is expected to elute first from the anion-exchange column, It was proposed that the trypsin cleavage site of the TBE E protein is at residue 408 (Heinz, personal communication, 1997)

To investigate if the dengue E protein contains similar cleavage sites, we performed a sequence alignment of the dengue virus E protein with the TBE E protein around the putative TBE E protein trypsin cleavage site:



Similar to the TBE E protein, which contains basic amino acids at 395 and 407-408, the dengue E protein contains one Lys-Lys site at 393-394 and a Lys-Arg site at 411-412. These two sites are the likely trypsin cleavage sites of the dengue virus E protein.

Monoclonal antibody binding sites are useful to further characterize the trypsin cleavage site of the E protein. Both mAbs 3H5 and 1B7 were able to immunoprecipitate trypsin cleaved dengue virus E protein, indentifying a product with apparent molecular weight of 45 kD. Defining the binding sites of these two monoclonal antibodies helps to characterize the trypsin cleavage site. As mentioned before, 3H5 can bind to an incompletely characterized sequence probably encompassing residues 383-393 (Trirawatanapong et al., 1992; Megret et al., 1992; Hiramatsu et al., 1996; chapter 5 in this thesis), indicating that the trypsin cleavage site is downstream of the mAb 3H5 binding site. Further studies, such as C-terminal sequencing, will further help to define the trypsin cleavage site of the dengue E protein.

We also found in this study that prM, which was co-immunoprecipitated with E protein, remains intact after trypsin treatment. This result agrees with a report that the prM of another flavivirus Murray Valley encephalitis virus is resistant to trypsin cleavage (Stocks and Lobigs 1995).

Finally, the trypsin-released ectodomain of E showed binding activity with Vero cells, suggesting that the receptor binding domain resides in the N-terminal 80% of the protein. Even though the precise receptor-binding site on dengue virus E protein has not been defined, studies with several flaviviruses have shown that the distal face of domain III of E protein may contain important sites for E-receptor interactions (Rey et al., 1995. Hiramatsu et al., 1996). Results from our and other labs suggest that the mAb 3H5 epitope on the dengue E protein may be close to the receptor-binding site. We have shown that mAb 3H5 blocks dengue-2 virus binding to Vero cells (see chapter 2). The RGD sequence contained in domain III of other

flaviviruses is thought to be an important host-range determinant. It was shown with Murray Valley encephalitis virus that altering the RGD sequence changed the virus tropism and virus virulence (Lobigs et al., 1990). The dengue virus E protein does not have an obvious RGD sequence in domain III; however, there might be RGD-like sequences which may be important for virus tropism (Ruoslahti and Pierschbacher, 1987). Domain III has an immunoglobulin-like structure, which is similar to structures of many adhesion molecules (Rey et al., 1995). All these factors suggest that domain III, especially residues around the mAb 3H5 recognition site, may contain important epitope(s) for E-receptor interaction.

CHAPTER 5

CLONING AND EXPRESSION OF THE DENGUE VIRUS ENVELOPE PROTEIN

1. INTRODUCTION

The flavivirus E protein is the major structural protein exposed on the surface of the virus (Rice, 1996). It mediates virus infection via attachment to cell surface receptors and the subsequent fusion step with the endosomal membrane, which allows the virus to be released into the cytosol (Helenius and Marsh, 1982). It has been shown that dengue virus binding to and infection of Vero cells can be blocked with patient antisera and monoclonal antibodies against E (see Chapter 2). It also mediates antibody-dependent enhancement of infection of macrophage-like cells (Peiris and Porterfield, 1979; Halstead et al., 1984) and induces neutralizing antibodies and protective immunity (Putnak et al., 1991; Feighny et al., 1994).

Because of the importance of the dengue virus E protein, many studies have been conducted using expressed E protein products to investigate immunogenecity as well as structure/function relationships Both prokaryotic and eukaryotic systems have been used to express dengue E protein, including *E. coli* (Mason et al., 1990; Trirawatanapong et al., 1992; Srivastava et al., 1995), Salmonella (Cohen et al., 1990), baculovirus (Deubel et al., 1991; Putnak et al., 1991; Feighny et al., 1994 Staropoli et al., 1996) and vaccinia virus (Bray et al., 1989; Hahn et al., 1990). The expression systems vary according to yield, conformation and functional activity of the expressed E proteins. Generally speaking, E proteins expressed from eukaryotic systems are more appropriate for vaccine studies and functional analyses because the conformation of the protein is similar to the authentic E protein (Feighny et al.,

1994). To date E protein recombinants generated from prokaryotic systems have been commonly used in epitope mapping. However, this is probably because of simplicity rather than superiority of prokaryotic over eukaryotic expression systems.

Several studies have attempted to use expressed E proteins as recombinant vaccines. The expressed E protein has been shown to induce neutralizing antibodies against virus infection (Putnak et al., 1991, Feighny et al., 1994; Srivastava et al., 1995). Many functional epitopes have been mapped with expressed dengue E proteins (and fragments) (Trirawatanapong et al., 1992; Hiramatsu et al., 1996). Also, it has been shown that a eukaryotically expressed envelope protein inhibited infection by dengue virus of Vero cells, suggesting that the expressed E protein was able to block virus binding to its cellular receptor (Chen et al., 1996).

There are some limitations of the expression systems mentioned above, such as: 1) cost; even though E protein recombinants expressed from baculovirus and vaccinia virus have been shown to generate protective immunity, they are relatively expensive. In the case of another virus, rabies, for example, baculovirus-expressed G protein was shown to be able to induce neutralizing antibodies. However, since it was difficult and expensive to produce large quantities of the G protein, other expression systems had to be considered (Prehaud et al., 1989). 2) immunogenicity; prokaryotic expression systems can generate recombinant proteins at low cost, however, the immunogenicity of the products is usually much lower than that obtained from eukaryotic systems because of conformational and post-translational differences (Lenstra et al., 1990; Srivastava et al., 1995), 3) risk of contamination with viral or oncogenic DNA, it is known that polio vaccines which were used in the 1950's were contaminated with SV40, which was reported to increase the incidence of neural neoplasms in children (Gerber, 1967; Farwell et al., 1979; Geissler, 1985).

Also it was speculated that the AIDS pandemic may have originated with a contaminated polio vaccine (Elswood and Stricker, 1994).

Yeast offers expression systems which can produce eukaryotic proteins at low cost and authentic conformation. The yeast, *Saccharomyces cerevisiae* , has been particularly utilized as an expression system, since it has several advantages: 1) safety; yeast is a food organism. As such it is safe for human consumption and should be acceptable for the production of vaccines. This is in contrast to, for example, E. coli, which contains toxic components such as the cell wall pyrogen, and mammalian cells which can contain oncogenes or viral DNAs (Jansen-Durr, 1996). 2) cost; yeast can be grown rapidly on simple media and to high cell density (Bathurst et al., 1994). 3. High level expression; yeast is probably the best eukaryotic cell for high level expression of proteins, particularly post-translationally modified proteins, including glycoproteins (Sudbery, 1996; Sadhukhan and Sen 1996).

Yeast has been demonstrated to be an excellent host for the expression of recombinant, mammalian-derived proteins (Bathurst, 1994), including interleukin-2 (Bujdoso et al., 1995), gamma interferon (Levitz and North; 1996), HIV gp120 (Barr et al., 1987. Franzusoff et al., 1995), hepatitis B virus core antigen and surface antigen (HBsAg) (Hitzeman et al., 1983, Miyanohara et al , 1986, Itoh and Fujisawa, 1986). The expressed surface antigen of hepatitis B has been used in humans as a recombinant vaccine (Shouval et al., 1994. Chiaramonte et al., 1996). Yeast recombinant DNA technology has been successfully applied in diagnostics, therapeutics and vaccine production. If the dengue virus E protein can be expressed in yeast, it can be used not only as a recombinant vaccine candidate but also as a source of E protein to map the monoclonal antibody binding epitopes and to characterize other functional domains of E protein.

2. MATERIALS AND METHODS

2.1. Yeast growth media

YPD medium contained 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. YMI rich medium contained 1% Bacto-peptone, 0.5% Bacto-yeast extract, 1% succinic acid, 0.6% sodium hydroxide, and 0.67% bacto-yeast nitrogen base without amino acids (pH=5.8). The YNB synthetic minimal medium contained 1% succinic acid, 0.6% sodium hydroxide, and 0.67% bacto-yeast nitrogen base, with amino acids (40 μg/ml), 0.1% ammonium sulfate and selected purine and pyrimidine bases added to satisfy the selection requirements. The selection media were YNB supplemented with amino acids: adenine, arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, threonine, tyrosine, uracil and valine. For example, pEMBLyex4 is a *URA3*-marked plasmid. The selection medium for this plasmid was prepared with all the essential supplements except uracil. Unless specified, glucose was used in all non-induction yeast media as the carbon source. To prepare solid media, 1.5% agar was added to the above liquid media.

2.2. *E. coli* growth media

LB contained: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl. The pH was adjusted to 7.0 with 5 N NaOH. Double-strength YT contains: 1.6% Bacto-tryptone, 1% Bacto-yeast extract, 0.5% NaCl. pH was adjusted to 7.0 with 5 N NaCl. To prepare solid media, 1.5% agar was added to the above liquid media. For clone selection, ampicillin stock solution was made at 50mg/ml, and added to the growth media at a final concentration of 30-50 μg/ml.

Both 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropylthio-β-D-galactoside (IPTG) were used to screen positive clones of Bluescript. The stock solution of X-gal was made by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution. The stock solution of IPTG was made by dissolving 2g of IPTG in 8 ml of distilled H_2O, and sterilized by filtration through a 0.22 micron disposable filter. All the solutions were dispensed into 1 ml aliquots and stored at -20°C. For screening, 40 µl of X-gal and 4 µl of IPTG were added to an ampicillin containing LB plate before transformation.

2.3. Plasmids

2.3.1. Yeast plasmid pEMBLyex4

The pEMBLyex4 plasmid is a yeast expression vector. It has a polylinker for the insertion of the gene of interest. Upstream of the gene is a strong galactose inducible promoter; the GAL10-CYC promoter. Downstream of the gene is a termination codon. The vector has yeast auxotrophic makers URA3 and leu2-d. The vector is normally maintained in *E. coli* by selecting for the ampicillin resistance marker. Translation starts at the first AUG encountered on the encoded mRNA (Adams, 1972; Giniger et al., 1985).

2.3.2. E. coli plasmid pBluescript

pBluescript SK^+, obtained originally from Stratagene, was used as the expression vector in *E. coli*.. The vector is commonly used in cloning (Gal-On et al., 1990; Shankar et al., 1997) and expression (Ladror et al., 1991; Fleming et al., 1994; Burke et al., 1997). Gene expression in pBluescript is under the control of the *lac* promoter, which is inducible with IPTG. The expressed protein is a fusion protein with β-galactosidase (Voth and Lee, 1989).

The vector contains an ampicillin resistance marker and is usually maintained in *E. coli* . Other than specified, we maintained all our plasmids and clones in *E. coli* strain DHFα. For plasmid amplification, the vector-containing E. coli was grown up at 37°C overnight and used for plasmid extraction as mentioned bellow.

2.3.3. Plasmid extraction and transformation

E. coli plasmid DNA was prepared either by the alkaline-lysis method (Birnboim and Doly, 1979) with modifications or using Genclean kit from BioCan. *E. coli* cells were grown overnight in LB medium plus ampicillin. OD_{600} was around 0.6-0.8 before harvesting.

For the alkaline-lysis method, 1.5 ml of E. coli cells were microfuged at 12,000g in a microfuge tube for 30 seconds. All steps were done at room temperature unless specified. The pellet was resuspended in 200 µl of Solution I (25 mM Tris pH 8, 10 mM EDTA, and 50 mM glucose), and vortexed vigorously. Cells were further lysed in 200 µl of freshly prepared Solution II (0.2M NaOH and 1% SDS). Tubes were gently inverted for 10 times. 200 µl of Solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O) was then added to the mixture. The cell lysate was then microfuged for 5 min and the supernatant was transferred to another tube containing an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1). The mixture was microfuged for 2 min and the aqueous phase was transferred to another tube containing 2 volumes of 70% ethanol and let stand for 5 min at -70°C. DNA was pelleted by microcentrifugation for 15 min and the DNA pellet was dissolved in 30 µl of ddH_2O and stored at -20°C.

For the GenClean method, cells were lysed as mentioned in the alkaline-lysis procedure. After microfuging the cell lysate, 0.6 ml supernatant was transferred to another tube containing 0.45 ml isopropanol. Samples were put into -70°C freezer for 5 min and then microfuged for 15 min. Supernatants were poured off and 100 µl of NaI and 3-5 µl of glassmilk were added to the pelleted DNA. Samples were microfuged for 5 sec., and the glassmilk pellets were washed twice with 1 ml of New Wash (supplemented in the kit). After washing the pellets, 30 µl of H_2O was added to each sample and microfuged for 1 min Supernatants containing purified DNA were stored at -20°C.

2.3.4. Plasmid extraction from yeast

Plasmid extraction from yeast was performed as described by Lorincz Lorincz and Reed, (1984). Plasmid bearing yeast cells were grown in selection medium until OD_{600}=0.8-1. An aliquot of 1.5 ml of the culture was transferred to a microfuge tube and microfuged for 30 sec. An aliquot of 400 µl Breaking buffer (100 mM NaCl, 10 mM Tris, pH 8, 1 mMEDTA, and 0.1% SDS) was added to the pellet. Cells were vortexed with buffer, mixed with 200 µl of acid-washed glassbeads and vigorously vortexed for 2 min. Tris-buffered phenol (200 µl) was then added to the sample with another vigorous vortex for 2 min. The sample was then microfuged and the aqueous phase was extracted with phenol/chloroform/isoamyl alcohol (24:24:1). The supernatant was mixed with 2 volumes of 95% ethanol and placed at -70°C for 5 min. DNA was microfuged at 12,000 g for 15 min. The pellet was washed twice with ice cold 70% ethanol, air-dried for 1h. and then dissolved in 30 µl of ddH_2O.

2.4. Transformation

2.4.1. Transformation of *E. coli* cells

Most transformations were done by electroporation. Competent cells were prepared as follows: a single colony of *E. coli* was selected from an LB plate, and inoculated into a culture tube containing 5 ml of LB liquid medium. Cells were grown at 37°C in shaking incubator overnight. The over-night culture was then transferred to a flask containing 2 L of LB and continued to incubate at 37°C for 5-6 h. The OD was checked every half hour until it reached 0.5-0.6. Cells were then chilled on ice for 30 min and then washed twice with chilled ddH_20, once with 10% glycerol and spun at 6,000 rpm for 15 min in between the washings. The washed cells were resuspended in 10% glycerol and stored in 80 µl aliquots at -70°C.

Electroporation was done with 0.1 cm or 0.2 cm cuvettes (BioRad). Competent cells were thawed on ice and mixed with 1-3 µg of DNA, then transferred to a chilled cuvette and electroporated with a Gene Pulser (BioRad) following the manufacturer's instructions. A time constant between 4.0-4.6 was considered indicative of successful electroporation. Double-strength YT (1 ml) was added to the cuvettes, the mixture was then transferred to selection plate (usually amplicillin) and incubated overnight in a 37°C incubator.

2.4.2. Transformation of yeast cells

Yeast competent cells were prepared as follows: a single colony from a YPD or YMI plate was selected and inoculated into a 5 ml culture of YPD or YMI media. Cells were grown at 30°C overnight and then transferred to 250 ml of YPD or YMI media. Cells were grown at 30°C until an OD_{600} of 1.8-2.0 and then cooled on ice for 30 min. The ice-cooled cells were washed twice with 40 ml chilled ddH_2O,

and once with 40 ml chilled 1 M sorbitol. The washed cells were resuspended in 0.5 ml of 1 M sorbitol and used right away for electroporation.

Electroporation was conducted with 0.1 or 0.2 cm cuvettes and the Gene Pulser as mentioned above. After the transformation, 1 ml of 1 M sorbitol was added to the cuvette and transferred to a yeast selection plate. Cells were grown at 30°C for 3-6 days until colonies were seen on the plate.

In some cases, transformation was performed using a lithium-acetate method. Briefly yeast cells were grown up in 5 ml YPD or YMI until early stationary phase. About 0.5 ml of cells were harvested and microfuged. An aliquot of 10 µl salmon sperm DNA (10 µg/µl) plus 1 µg transforming DNA were added to the cells and vortexed vigorously. After vortexing, 0.5 ml Plate Mix (90 ml sterile 45% PEG400, 10 ml 1 M Li acetate, 1 ml 1 M Tris-HCl pH 7.5, 0.2 ml 0.5 M EDTA pH 7.5) were added and vortexed. The mixture was incubated overnight at room temperature and then microfuged for 10 sec to aspirate off the Plate Mix. Finally, 200 µl 0.1 M Tris-Cl pH 7.5 was added to the transformed cells, which were subsequently grown on a selection plate at 30°C.

2.5. DNA MANIPULATIONS

2.5.1. Restriction enzyme digestions

Restriction endonuclease digestion was used to screen positive clones, generate restriction maps or to insert a gene fragment into cloning/expression vectors. A 10-µl reaction mixture containing 1 µl of appropriate restriction enzyme buffer (provided by the manufacturer), 0.5-1 µg of DNA, and 5 units (usually 0.5 µl) of restriction enzyme and ddH2O was used to make up the final volume to 10 µl . The

reaction was usually carried out at 37°C, unless otherwise recommended by the manufacturer.

2.5.2. Polymerase chain reaction (PCR)

Primers used for these studies were the following:

Primer 1, full-length E 5': gtgagctc atgcgttgcataggaatg

Primer 2, full-length E 3': gtgagctcggcctgcaccatgactccca

Primer 3, carboxy-truncated E 5': gtgagctctcattagcgacctaagacatgtctt

Amino-truncated E 5': gtgacgagctccaaaatgtggggaaatggatgtggac

PCR was used to amplify DNA or add restriction sites onto the ends of DNA fragments for cloning. PCR reaction mixtures contained: 10 µl 10 X PCR buffer (10 mM KCl, 20 mM Tris-HCl (pH8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 9.1% Triton X-100) supplemented with 3 µl $MgSO_4$ (according to the manufacturer's instructions), 0.2 mM each of dATP, dTTP, dCTP, dGTP (pre-mixed as dNTP), 100 ng of DNA template, and 2 units of Vent polymerase (New England Biolab). Samples were denatured at 94°C for 5 min, followed by 30-40 cycles of denaturation (94°C), annealing (58°C) and extension (72°C). Times for annealing and extension varied depending on the sequence of the primer and DNA template. The product(s) of PCR reaction was purified as described below.

2.5.3. Agarose gel electrophoresis of DNA

DNA samples or digests (in loading buffer: 50% glycerol, 0.3% xylene cyanol, and 0.3% bromophenol blue) were analyzed in agarose gels (usually 1% in TAE buffer: 40 mM Tris-acetate, 1 mM EDTA pH 8.0) containing ethidium bromide (0.2 µg/ml).

2.5.4. Gel purification of DNA

Using either regular or low melting point agarose gels, DNA fragments were excised and purified using either the GenClean kit or QiaGen's Gel Purification kit.

2.5.5. Reactions in the agarose gel

Some reactions, such as restriction enzyme digestion and ligation, were performed inside the agarose gel. This procedure was especially useful for small DNA fragments which are difficult to recover from gel purification, or for a DNA fragment that cannot be PCR amplified. DNA samples were run at low voltage or at 4°C in a 0.8-1% low melting gel in TAE buffer. Desired fragments were excised and incubated at 50°C till gels were completely melted. Samples were transferred to a 37°C heating block where reagents were added.

2.5.6. Ligation and plasmid constructions

Concentrated T4 ligase from New England Biolabs was used for all the ligations. First, vector DNA and insert DNA were digested with restriction enzymes, and purified with DNA cleaning kits. Cohesive end ligations were carried out at room temperature for 1h. Cohesive-blunt end ligations and blunt-blunt end ligations were carried out at 16°C for over 4 h. For blunt-blunt end ligation, the vector was treated with alkaline phosphatase after restriction enzyme digestion to minimize the re-ligation of the vector. All ligation products were purified with DNA cleaning kits before doing further reaction or transformation.

2.6. RNA EXTRACTION

2.6.1. RNA extraction from yeast

For RNA preparation, all glassware and tips were baked for over 4 h in a 120°C oven. Solutions were made either in baked glassware or in disposable plasticware

Yeast cells were grown to 1 x 10^7 cells/ml and harvested by centrifugation at 3,000 to 3,500 rpm for 3 min. Cell samples were directly frozen at -70°C. Samples were thawed on ice, SDS was added to a final concentration of 1%, and yeast cells were broken by vigorous vortexing with acid-washed glass beads in the presence of water-saturated phenol/chloroform (pH 5). Samples were vortexed for 6 x 20 sec, cooled on ice in between vortexing and centrifuged at 12,000 rpm for 2 min. The supernatant was transferred to a fresh tube and extracted again with phenol/chloroform. The supernatant was then precipitated with 95% ethanol at -70°C for over 4 h and RNA was pelleted by microcentrifugation for 30 min at 4°C. Pellets were washed with 70% ethanol, air-dried and dissolved in ddH_2O. RNA concentration was measured by absorbance at 260nm. The OD260/OD280 should be between 1.9-2.2.

2.6.2. Total RNA extraction from mammalian cells

Vero cells were infected with dengue virus in several wells of a 24-well plate. Three days post-infection, cells were washed twice with PBS and harvested in 0.5 ml solution B (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and 50 µl of 2 M sodium acetate pH 4. Cells were aspirated thoroughly and transferred to a microfuge tube containing 0.5 ml water washed phenol pH 5, and 100 µl chloroform. The mixture was cooled on ice

for 15 min. and microfuged at 4°C for 15 min. The supernatant was transferred to a new microfuge tube, mixed with 0.5 ml of isopropanol and stored at -20°C for at least 2h. RNA was pelleted by microcentrifuging the sample at 4°C for 15 min. The supernatant was discarded; 200 µl diethyl pyrocarbonate (DEPC) treated water, 1.5 µl sodium acetate pH 5.2 and 450 µl of 95% ethanol were added to the pellet. The sample was stored at -70°C for 1 h and then microfuged for 15 min. DEPC-treated ddH_2O (10 µl) was added to the RNA pellet and stored at -20°C.

2.7. Northern blot analysis

Northern blots were performed together with Kendra Gillis and Dr. Chris Barnes. The gel apparatus chamber, gel tray and comb were all soaked in 1% SDS for 2h, and rinsed with ddH_2O to minimize potential RNase contamination. Each RNA sample contained 20 µg of total yeast RNA in a 1- 5 µl volume; 10 µl formamide; 3.5 µl formaldehyde and 1 X MOPS. The RNA mixture was denatured for 15 min at 65°C, and chilled on ice for 5 min RNA was then resolved by electrophoresis through a formaldehyde-containing agarose gel. The gel (in 200 ml volume) contained 2.6g of agarose, 20 ml of 10 x MOPS buffer (0.4 M 3-[N-morpholino] propane-sulfonic acid, 0.1 M sodium acetate, 0.01 M EDTA, pH 5.2), 37 ml of formaldehyde, and 7.5 µl of 10 mg/ml ethidium bromide. The gel was prerun for 5 minutes at 5V/cm and then run with samples in 1 x MOPS running buffer at 2V/cm. Ribosomal RNA (18S, 2366 bases and 28S, 6333 bases) in the total RNA was used as markers. The gel was then photographed on a UV transluminator to visualize the RNA. The gel was rinsed with 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) and then used to transfer RNA to a nylon membrane. The RNA transfer was carried out in 20x SSC buffer for 16-18 h. After the transfer, the membrane was dried at room temperature for 30 min and then baked at 80°C for 2h.

To eliminate non-specific binding, the membrane was incubated for 4 h in prehybridization buffer, which contains: 0.05 M sodium phosphate, pH6.5, 5 x SSC, 50% formamide, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA, 1% SDS and denatured salmon sperm DNA (100 μg/ml).

Blots were probed using a ^{32}P-labeled DNA probe. The probe was prepared by mixing aliquots of 30-100 ng of template DNA, pEMBL-E(1-495), and 2 μl random primers (Boehringer-Mannheim) in a 0.5 ml microfuge tube and boiling for 5 min. After cooling briefly on ice, the mixture was supplemented with 2.5 μl 0.5 mM 3dNTPs, 2.5 μl 10 x Klenow buffer (0.5 M Tris pH 7.5, 0.1 M $MgCl_2$, 10 mM DTT), 1 μl α^{32}P-dCTP , 1 μl Klenow (6 units/μl). The reaction was incubated at room temperature for 30 min, then heated 5-10 min to 100°C, cooled on ice for 5 min and then added to the nylon blot.

Hybridization was carried out in a sealed bag containing prehybridization buffer in a 42°C incubator for at least 12 h. After the hybridization, the membrane was washed twice for 10 min in 2 x SSC and 1% SDS at room temperature and then washed twice with 0.1 x SSC and 0.1% SDS at 60°C for 20 min The membrane was then sealed and exposed to X-ray film.

2.8. SCREENING POSITIVE CLONES

Screening positive clones were performed in *E. coli* by using restriction enzyme digestion and confirmed by sequencing. Briefly, transformed cells were randomly selected as single colonies from culture plates and inoculated into culture tubes containing LB medium and ampicillin. The selected colonies were grown up overnight at 37°C. After extraction and DNA digestion, samples were run on an

agarose gel and visualized under UV. Clones that showed the correct restriction digest patterns were selected as positive clones and stored at -70°C in LB containing 20% glycerol.

2.9. EXPRESSION OF CLONED GENE FROM *E. COLI*

Bluescript was used as an expression vector in *E. coli*. For expression, cells were grown overnight in LB containing ampicillin at 37°C. Aliquots of 100 µl of overnight cultures were inoculated into new tubes containing LB and ampicillin and further incubated for 4 h at 37°C. To induce expression, IPTG (final concentration 10 mM) was added. Samples were collected (1.5 ml) every 60 min. Cells were pelleted and resuspended in 200 µl of 1 x SDS gel-loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). If degradation of the expressed protein was a concern, samples were dissolved in preboiled 2 x dissociation buffer containing 8 M urea (0.3 M Tris Cl pH 6.8, 20% glycerol, 10% mercaptoethanol, 2.5% SDS, 0.1% bromophenol blue, 8M urea) and boiled for 5 min. Samples were stored at -70°C.

2.10. PROTEIN EXPRESSION FROM YEAST

Several yeast clones containing the desired gene were selected from a YNB selection plate, and inoculated into 5 ml YNB selection culture medium containing 2% glucose. Cells were grown up at room temperature or 30°C until OD_{600}=1-1.5 before harvesting. For induction, the glucose containing medium was replaced with galactose containing medium after $OD_{600} \cong 1$. Cells were further incubated for 12 to 18 h. Yeast extracts were then harvested either with glass bead treatment, or with dissociation buffer.

For glass bead treatment, cells were microfuged, washed with ddH2O and resuspended in breaking buffer (20 mM Tris, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 100 mM NaCl). The buffer-treated cells were frozen immediately in liquid nitrogen and thawed on ice. Acid-treated glass beads were added into the mixture. The following protease inhibitors were also added at concentration of 2 μg/ml: aprotinin, antipain, pepstatin and leupeptin. PMSF (1 mM) was added to the samples right before vortexing. The samples were then vortexed vigorously for 6 x 30 sec, and 1x 2 min. Samples were then microfuged; supernatants were boiled for 5 min immediately after harvesting. Aliquots of 2x dissociation buffer were added to an equal volume of supernatants and stored at -70°C. If the expressed protein was not soluble, the cell pellets were treated directly with 2 x DB containing 8 M urea. Samples were boiled for 5 min and then stored at -70°C. The samples were probed by Western blot.

2.11. WESTERN BLOT ANALYSIS

Western blot analysis was used to probe expressed proteins with anti-dengue E protein antibodies. After proteins were resolved by SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane as follows: The protein gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 15 min; the PVDF membrane was cut to the gel size and soaked in pure methanol briefly and then placed in transfer buffer and equilibrated for 2 min. Protein transfer was then carried out in a semi-dry trans-blot apparatus (BioRad). The transferred blot was then probed with dengue E protein-specific antibodies. The blot was first immersed in blocking buffer (5% skim milk, 0.5 casein in TBST: 10 mM Tris, pH 7.6, 0.88% NaCl, 0.1% Tween 20) at room temperature overnight to eliminate non-

specific binding, and then the blot was washed 3 x 5 min with TBST (0.1% Tween 20, 20 mM Tris base, 137 mM sodium chloride, pH 7.6). The primary antibody at 1:1,000 in TBST was then added to the blot and incubated at room temperature for 1 h. The blot was washed for 3 x 5 min with TBST and probed with the second antibody conjugated with horseradish peroxidase at 1:20,000 in TBST. After a further washing step, the blot was then incubated with enhanced chemiluminesent (ECL) reagent (Amersham) for 30 sec and exposed to X-ray film.

3. RESULTS

3.1. Selecting antibodies that can be used to probe expressed E protein

In order to identify E protein produced by *E. coli* or yeast, it was necessary to first identify mAbs which react with the E protein by immunoblotting. A panel of eight monoclonal antibodies was used to perform an immunoblot using lysates of dengue-infected Vero cells and uninfected Vero cells as a control. As shown in Fig. 22, two (3H5, 9D12) out of eight mAbs reacted with the E protein. Surprisingly, mAb 5F10 which was reported to be able to probe dengue E protein in immunoblots (Kaufman et al., 1987) showed no reactivity in our hands. MAbs 3H5 and 9D12 were therefore chosen to probe the E protein expression in *E. coli* and yeast.

3.2. Amplification of dengue virus E protein gene by RT-PCR

Total cell RNA was isolated from dengue infected Vero cells and used with random primers and reverse transcriptase to generate cDNA. The resultant cDNA was amplified by PCR with a pair of primers which amplify the E gene from residue 1 to 485. The two primers were also flanked with Sst I sites. The 3' primer also contained a termination codon TAA. Fig. 23 showed a single product about 1.5 kb (estimated yield = 100 μg).

Figure 22.

Selection of antibodies that can be used to probe expressed E protein. Extracts from dengue infected Vero cells (V) and molecular weight markers (M) were resolved by SDS-PAGE. Proteins were transferred to PVDF membrane and immunoblotted using eight dengue E-specific mAbs. MAbs 3H5 and 9D12 were able to recognize dengue E protein.

Figure 22.

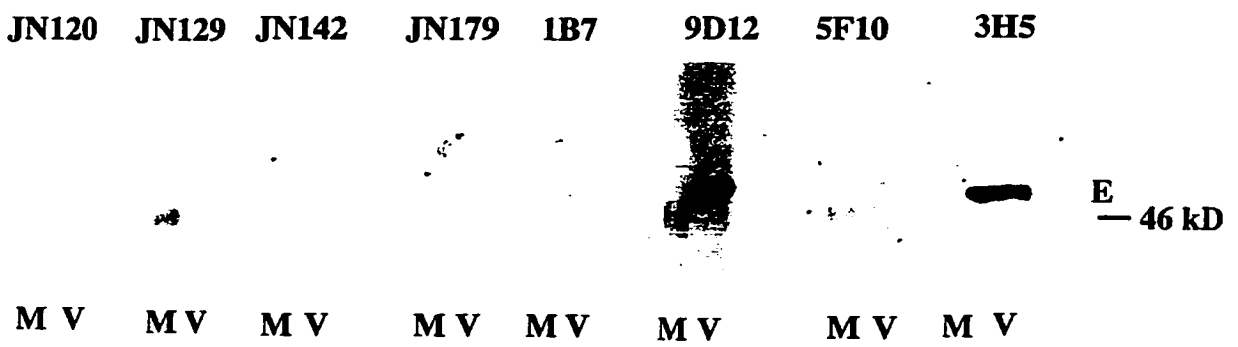
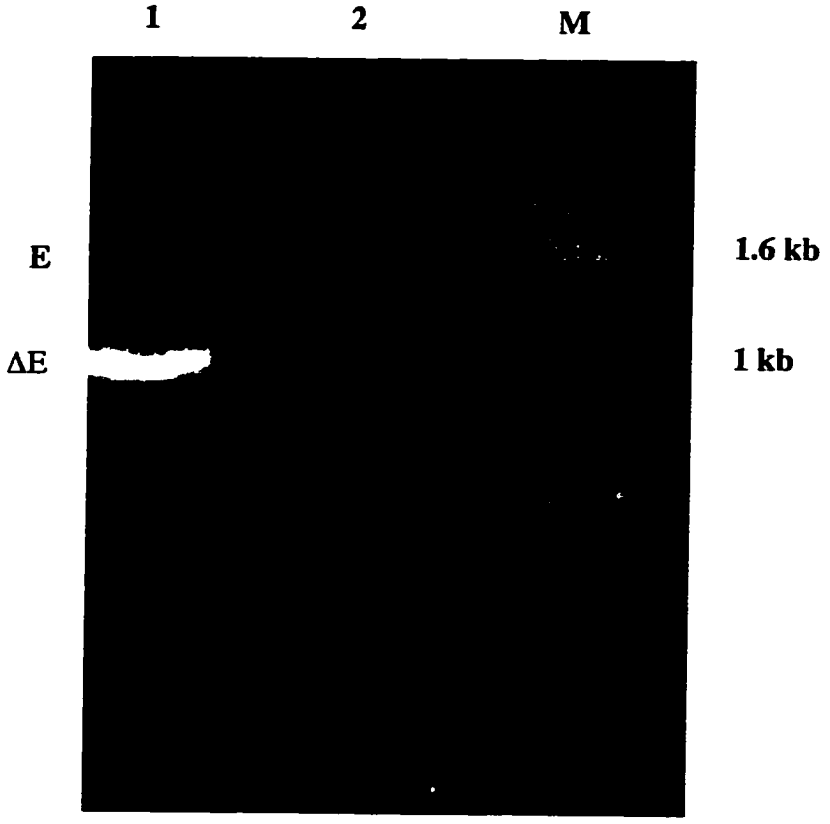

JN120
JN129
JN142
JN179
1B7
9D12
5F10
3H5
E
46 kD
M V
M V
M V
M V
M V
M V
M V
M V

Figure 23.

RT-PCR amplification of full-length and carboxy truncated dengue E protein. Total cell RNA was isolated from dengue infected Vero cells and used to generate cDNA which was then amplified by PCR with primers for full length and truncated E protein. PCR products were analysed on a 1% agarose gel and visualized under UV. The full-length E (lane 2, 1485 bp) and the truncated E (lane 1, 1179 bp) are seen on the gel.

Figure 23



3.3. Full length dengue E protein is toxic in pBluescript

The PCR-amplified full-length dengue E protein DNA and pBluescript were digested with Sst I, ligated and used to transform *E. coli*.. A total of forty insert-containing clones showed a 594 bp fragment after Pst I digestion, indicating the presence of the reversely oriented inserts. (analysis of the sequence by DNA Strider (Marck, 1988; Douglas, 1995) indicated that digestion with Pst I generates a 894 bp fragment from correctly oriented inserts and a 594 bp fragment from reverse inserts). Shown in Fig. 24 is a representative screen of 10 clones, four of which contained inserts, all in the reverse orientation. One of these four clones was designated pBlue-RFE. Thus, the results suggest that the correctly oriented full length dengue E protein may be toxic in *E. coli* .

3.4. Truncated dengue E protein can be cloned into pBluescript.

It has been reported that the full length E protein is toxic in *Salmonella* and could not be expressed, while the carboxy-truncated E (24% truncation at carboxy terminus) could be expressed (Cohen et al., 1990). We reasoned that toxicity might be due to a relatively high degree of hydrophobicity in the carboxy terminal portion of the E protein. By computer analysis (using SeqVu 1.0.1) there are four hydrophobic clusters at the carboxy terminus: residues 425-430, 441-449, 456-465, and 476-494. We speculated that these hydrophobic sequences might be related to the suspected toxicity, since hydrophobic sequences in other proteins have also proven difficult to clone and express in prokaryotic systems (see Discussion).

To investigate our hypothesis, we designed two PCR primers that amplify a truncated E protein from residue 1-1179, which excludes the four hydrophobic sequences. The two primers are flanked with Sst I (5') and Sal I (3') site, which were

Figure 24.

Full-length E protein is toxic in *E. coli* . Full-length E protein sequence was ligated into pBluescript and transformed into *E. coli* . Colonies were randomly selected and grown up in LB medium in the presence of ampicillin. Plasmids were isolated as mentioned in the Materials and Methods and digested with Pst I for 1 h at 37°C. Samples were analyzed on a 1% TAE agarose gel and visualized under UV. A 594 bp product was observed in clones containing the full-length E insert (lane 3, 4 and 5), indicating that the insert was in the reversed orientation.

Figure 24

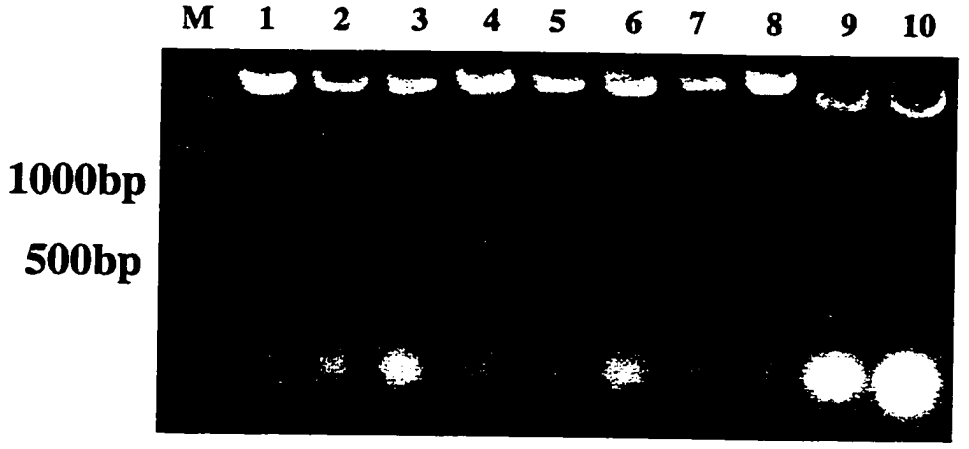
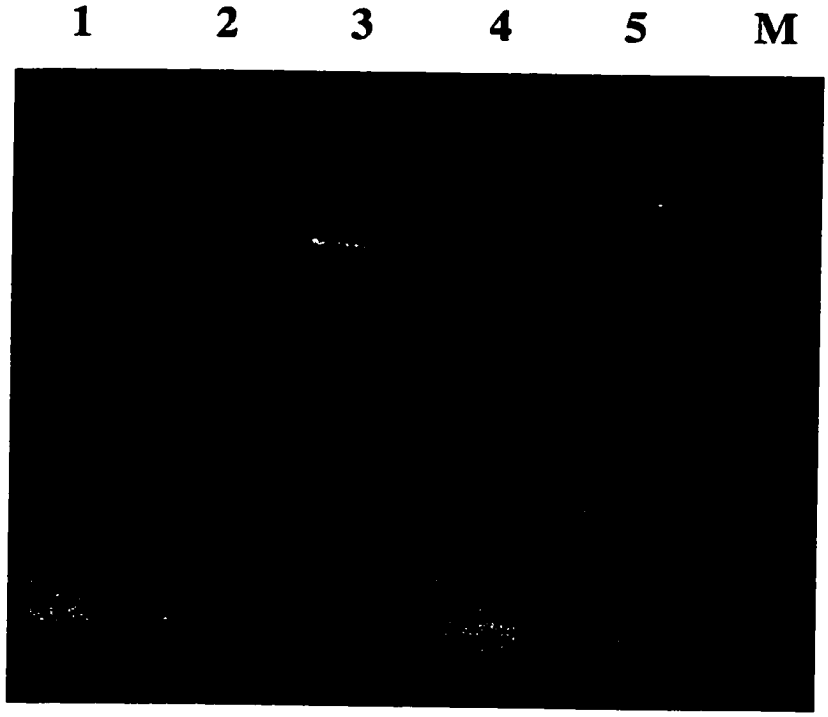


Figure 25.

Cloning of carboxy truncated E into pBluescript. The PCR amplified carboxy truncated E was ligated into pBluescript (in 5' Sst I and 3' Sal I site) and transformed into *E. coli* . Randomly selected colonies were grown up in LB medium in the presence of ampicillin. Plasmids were isolated and digested with Sst I and Sal I for 1 h at 37°C respectively. Samples were analyzed on a 1% agarose gel and visualized under UV. A 1179 bp product was observed in clones containing the truncated E insert (lanes 1, 2 and 5).

Figure 25.



designed to clone the truncated E protein in frame in pBluescript. The amplified fragment consists of approximately 80% of the full length E protein. The pBlue-RFE (see above) was used as the template for PCR amplification. As shown in Fig. 23 the size of obtained PCR product is around the expected 1200 bp. The PCR product was subsequently digested with Sst I and Sal I, and ligated into pBluescript. The ligation product was transformed into *E. coli*.. As shown in Fig 25, three out of ten clones contained the insert and were designated pBlue-E(1-1179). This result showed that 80% of the full length E can be cloned into pBluescript and grown in *E. coli* without lethal toxicity.

3.5. Truncated dengue E protein can be expressed in *E. coli*

To perform expression, fresh cultures of pBlue-E(1-1179)-transformed *E. coli* were induced (or not) with 10 mM IPTG. Cultures were harvested at various times and prepared for SDS-PAGE and immunoblotting using mAbs 3H5 (Fig 26) and 9D12 (Fig. 26). As shown in Fig. 26 the dengue E sequence 1-1179 was successfully expressed in *E. coli*. It also confirmed that the toxicity of the E protein resides in the carboxy terminus.

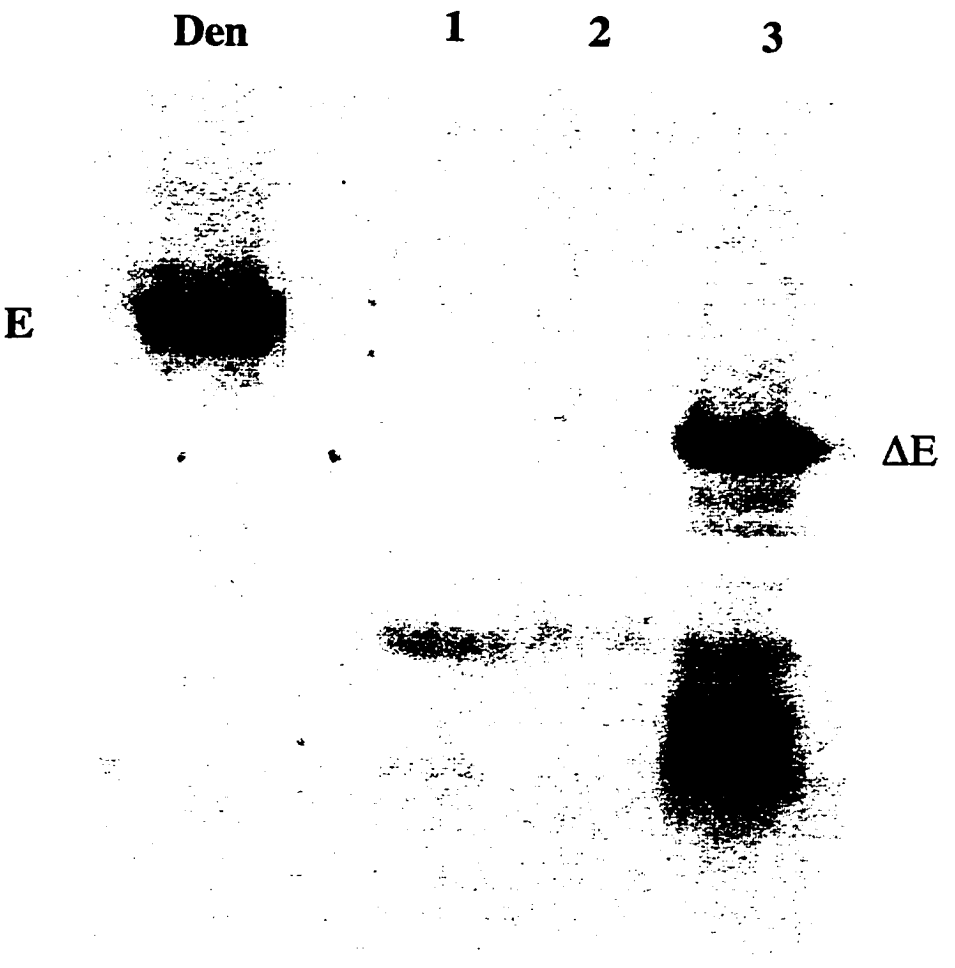
3.6. Both full-length E and carboxy-truncated E proteins can be cloned into the yeast vector pEMBLyex4

To explore the possibility of expressing full-length dengue E protein in yeast, we used the yeast expression vector pEMBLyex4. This vector contains a galactose-inducible promoter (GAL10-CYC) which is an advantage for the expression of potentially toxic proteins (galactose induction in yeast can stimulate transcription of both GAL1 and GAL10 up to 5000 fold; Giniger et al., 1985).

Figure 26.

Carboxy-truncated E protein expression in *E. coli* . A pBluescript-transformed *E. coli* clone (lane 3) containing the carboxy-truncated E was grown up in LB medium and induced with IPTG. The left hand lane is a cell extract from dengue infected Vero cells as positive control. A non-induced *E. coli* clone (lane 2) and an IPTG-induced, empty pBluescript-transformed *E. coli* clone (lane 1) were used as controls. Boiled cell extracts were resolved by 10% SDS-PAGE, transferred to PVDF membrane and probed with mAb 3H5. The expressed E band was visualized in lane 3.

Figure 26.



We amplified both full-length and carboxy-truncated (amino acids 1-393) E protein gene and ligated them into pEMBLyex4. The full-length E was cloned into the Sst I site, and the carboxy-truncated E was cloned into the Sst I (5') and Sal I (3') sites. Ligation products were transformed into *E. coli* for amplification and screening. Restriction enzyme digestion was used to select the positive clones. For full-length E screening, we used Hind III which generates a 878 bp fragment from correctly oriented clones and 610 fragment from reversed clones. Out of 12 selected transformants there were three clones with correct insert orientation and one with reversed orientation (Fig. 27A). For screening of the carboxy-truncated E, we used Sst I and Sal I, which generate 1179 bp fragment for correctly oriented clones. As shown in Fig. 27B, four out of five clones contained the truncated E sequence in the correct orientation. This result also showed that full-length and truncated dengue E protein gene can be cloned into yeast expression vectors.

3.7. Expression of the carboxy-truncated E, but not the full-length E in yeast

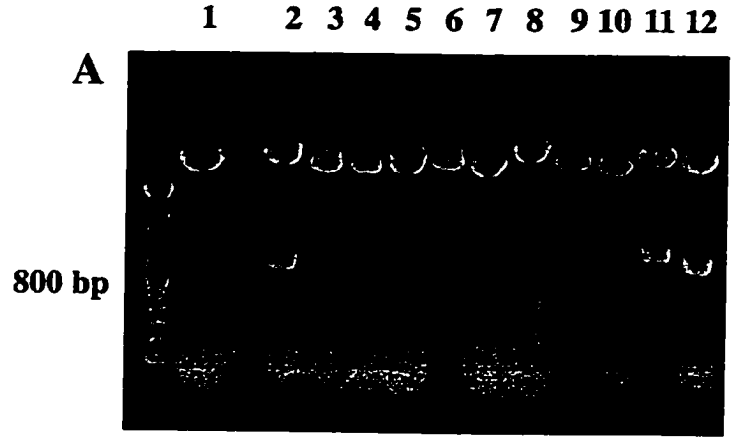
The selected yeast clones were grown in YNB uracil-deficient medium containing 2% glucose at 23°C for about 48 h until the OD_{600} was approximately 1 and then changed to galactose-containing medium as described in the Materials and Methods. Cultures were harvested and analyzed on 12% SDS-PAGE and Western blotting.

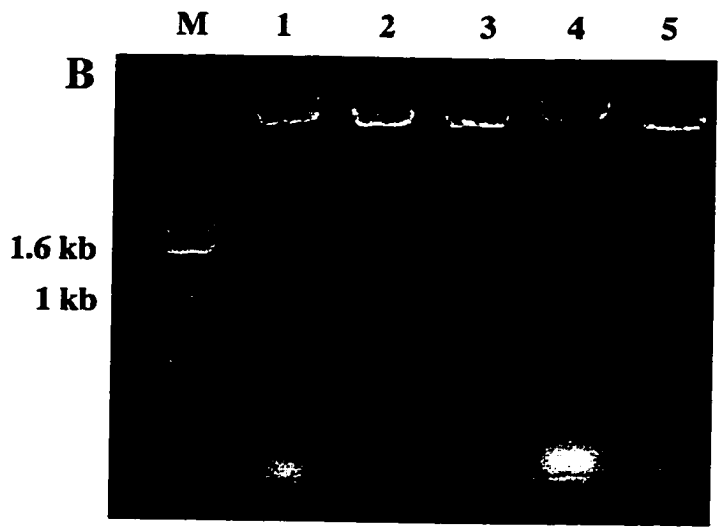
As shown in Fig. 28 no expression of full-length E protein was detected by either mAb 3H5 or 9D12, although both antibodies could detect authentic E protein from dengue-infected Vero cells. The mAb 3H5 did react with a 45kD protein in all

Figure 27.

Cloning of full-length and carboxy truncated E protein into pEMBL. PCR amplified full-length and carboxy-truncated E sequence were ligated into pEMBL and transformed into *E. coli*. Clones were randomly selected and grown up in LB medium containing ampicillin. Plasmids were isolated and digested with Hind III (A) or Sst I and Sal I (B) to screen full-length E and truncated E clones. In A, three clones (clones 2, 11 and 12) contain a 900 bp fragment (correct orientation); one clone (clone 8) contains a 690 bp fragment (reverse orientation). In B, four clones (clones 2, 3, 4 and 5) contain a 1200 bp fragment.

Figure 27.





galactose-induced samples including control samples, indicating that this protein was a yeast protein (i.e. not the E protein) which was inducible by galactose.

In contrast, the carboxy-truncated E was detected in yeast. Both mAbs 3H5 and 9D12 reacted with a 45 kD protein only in galactose-induced, pEMBL-E(1-393) transformed yeast. They did not react with any other proteins in galactose-induced, control samples.

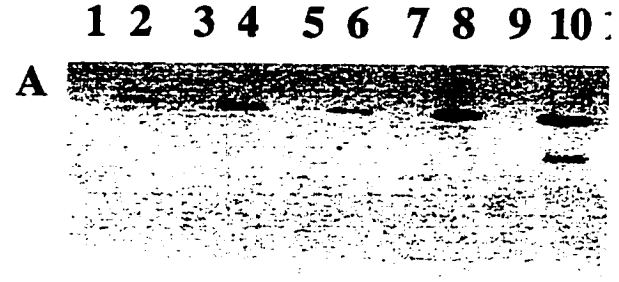
3.8. Yeast mRNAs for full-length E and carboxy-truncated E

To investigate whether the yeast clones transformed with pEMBL-E(1-495), pEMBL-E(1-393) and pEMBL-E(100-393), were transcribing E-specific mRNA, we performed a Northern blot analysis using total yeast RNA, probed with a ^{32}P-labeled E cDNA. As shown in Fig. 29 mRNAs corresponding to full-length E pEMBL-E(100-393), were observed, demonstrating transcription of both mRNAs in yeast. In the case of pEMBL-E(1-393) a much weaker signal for RNA was detected; however, this was likely due to the reduced level of RNA sample loaded on the gel (compare with ethidium bromide-stained gel in Fig. 29).

Figure 28.

Detecting full-length and truncated E protein expressed from yeast. Yeast alone (lane 1 and 2), and yeast transformed with pEMBL (lane 3 and 4), full-length-E, and truncated E (lane 5, 6, 7, 8, 9 and 10) were grown up and induced (odd numbers are non-induced, even numbers are induced). MAb 3H5 (A) and 9D12 were used to probe the expression. Mab 3H5 recognized a 46 kD protein in all induced samples, while mAb 9D12 only recognized proteins in induced truncated E protein transformed clones.

Figure 28.



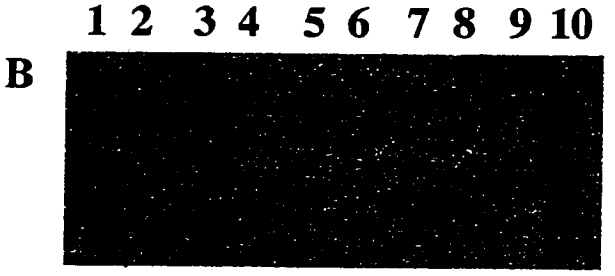
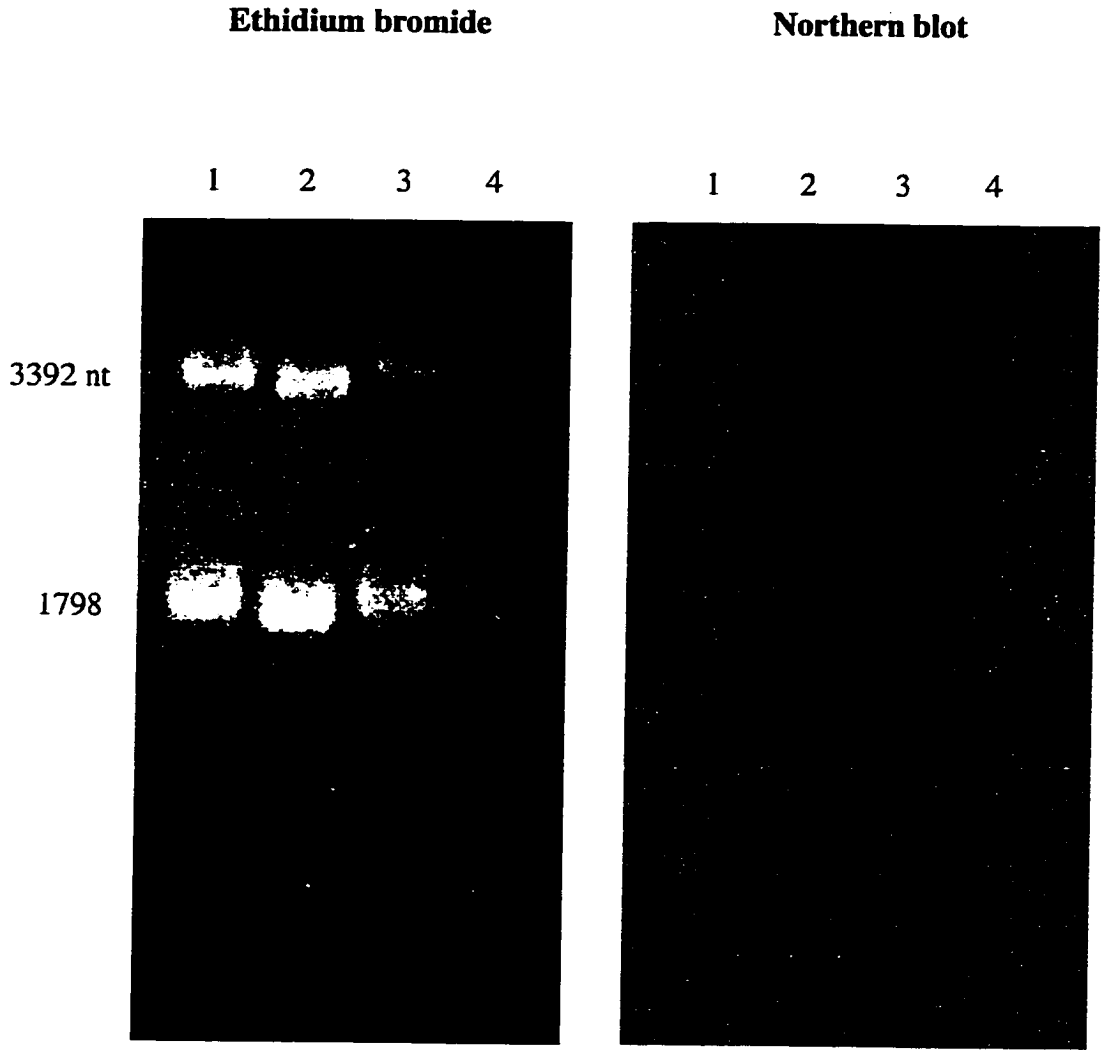


Figure 29

Northern blot for yeast full-length E (lane 2) and truncated E (100-393) (lane 3) and truncated E (1-393) (lane 4). Yeast total RNA was extracted, resolved by agarose gel electrophoresis, transferred to nylon membrane, and probed with E-specific ^{32}P-cDNA. E-specific mRNA was detected in both full-length and truncated E clones, but not in the control (lane 1) sample containing only pEMBL vector.

Figure 29



4. DISCUSSION

In this Chapter, we describe the expression of truncated dengue virus E proteins in both *E. coli* and the yeast, *S. cerevisiae*. These expressed proteins were used to investigate the binding sites of two mAbs, 3H5 and 9D12, and should be useful in the future for the further structure/function characterization of the E protein and for the production of potential vaccine candidates

In our studies, the full-length E gene was shown to be lethal in *E. coli* . This might be due to basal levels of expression, which can occur with genes under the control of the *lacZ* promoter (Tabor and Richardson, 1985). Basal expression of certain viral proteins may be enough to kill the bacterial host cells in which they are expressed (Brown and Campbell, 1993). Extreme toxicity of expressed viral proteins has been reported with some other viruses, such as the rotavirus VP7 protein (Williams et al., 1995) and the vesicular stomatitis virus G protein (Yoshida et al., 1997). Also, as has been mentioned previously, the full length dengue E protein was found to be toxic in *Salmonella*, while the carboxy-truncated E (24% truncation at carboxy terminus) could be expressed (Cohen et al., 1990).

The most common cause of toxicity in the expression of viral proteins in bacterial systems is the presence of hydrophobic sequences at either the carboxy or amino termini (Sheu and Lo, 1995, Sisk et al., 1992, Wan et al., 1995). Some hydrophobic viral protein sequences have been found to adversely affect or even abolish expression. For example, it has been reported that deletion or alteration of hydrophobic amino acids in transmembrane domains of hepatitis B surface antigen decreased toxicity and enhanced protein expression in *Escherichia coli.* (Sheu and Lo, 1995). It has also been shown that deletion of hydrophobic domains of HIV glycoproteins increases the level of their production in *E. coli* (Sisk et al., 1992). In

addition, the hydrophobic sequence in the HIV protease was shown to be toxic (Wan et al., 1995). Our results are consistent with the above reports and showed that deletion of hydrophobic sequences at the carboxy end of E protein decreased the toxicity and allowed the truncated E protein to be expressed in *E. coli*.

It has been shown that the toxicity of heterologous genes in cloning or expression can also be avoided by using different cloning systems (Chen, 1994). For example, we cloned the full length dengue virus gene in the yeast/*E. coli* shuttle vector pEMBLyex4 and found that the gene-containing vector could be amplified in *E. coli*, suggesting that the yeast promoter in *E. coli* may be silent. This result is consistent with the fact that yeast and *E. coli* have different promoter-polymerase recognition sequences, so that yeast promoters are often not active in *E. coli* and vice versa (Loison and Jund, 1981; Kwak et al., 1987).

However, we were not able to express the full-length dengue E protein in yeast, suggesting that the production of the protein may be altered at steps of transcription, translation or posttranslational processes. Most common factors that affect heterologous gene expression in yeast are:

a. initiation of transcription: Gene expression is more regulated at the level of transcription than at any other step (Ramanos et al., 1992). The level of mRNA is determined both by the rate of initiation and the rate of turnover (Mellor et al., 1987). It was suggested that reduced levels of expression are due to shorter half lives of the foreign transcripts (Chen et al., 1984. Guzowski et al., 1994). However, it is unlikely that this is the cause of lack of expression of the full length E because the level of mRNA of the full-length E is about the same as that of the truncated E.

b. RNA elongation: It has been shown that A-T rich sequences cause termination of transcriptional elongation. For example, HIV-env mRNA transcription was prematurely terminated because of the AT-rich sequence in the env gene. The fortuitous polyadenylation sequence resembles the polyadenylation consensus sequence in yeast and causes the premature termination (Ramanos et al., 1992 Roitsch and Lehle, 1989). In our studies, Northern blot analysis was performed to determine whether RNA transcription was prematurely terminated. The results indicated that the RNA transcript was the expected size. Therefore premature transcription did not appear to be a problem in yeast expression of the full-length dengue E sequence.

c. Toxicities of foreign proteins: Since factors that alter the level of full-length E expression do not seem to be acting at the transcriptional level, it is possible that the toxicity of the protein itself may play a role in reducing full-length E expression. For host cells, gene expression of a foreign protein is often a significant burden which causes reluctions in growth rate and the level of gene expression (Romanos et al., 1992).

Common mechanisms of protein toxicity of foreign proteins are: 1) Rare codons, which can deplete cognate tRNAs and inhibit the translation of host mRNAs containing rare codons (Purvis et al., 1987; Hennigan et al., 1996). 2) Anchoring sequences, which may be toxic by non-specific insertion and disruption of yeast intracellular membranes. For example, polyoma virus middle T antigen, influenza virus haemagglutinin are all toxic when expressed in yeast due to this reason (Belsham et al., 1986. Jabbar et al., 1985). 3) Acute toxicity: some heterologous proteins many interfere with host cell metabolism and cause toxicity right after the

foreign protein is expressed. This has been noted, for example, with poliovirus protease 3C pro and human interferon-β (Barco et al., 1995. Demolder et al., 1994).

As for the toxicity of full-length dengue virus E protein, it is likely that the toxicity is from the carboxy-terminal anchoring sequence because the carboxy-truncated E was expressed in yeast. We searched codon usage of the protein and did not find rare codon clusters (such as : AGG/AGA, CUA, AUA, CGA or CCC) (Makoff et al., 1989; Herrick et al., 1990; Kane, 1995; Zahn, 1996) in the sequence. Therefore we speculate that it is likely that the toxicity arises from the non-specific insertion of the carboxy-terminal sequence into intracellular membranes of yeast cells, or because of the interference of cell metabolism by the anchoring sequence.

We were able to utilize expressed E protein fragment to gain information on the location of epitopes recognized by the 3H5 and 9D12 mAbs. It was first reported that mAb 3H5 is dengue-2 specific and is able to recognize a sequence from a linear sequence from residue 385-397 in the E protein (Trirawatanapong et al., 1992). However, recent studies found that residues from 394-397 are highly conserved in all dengue serotypes, suggesting that residue 393 may be the last residue of the 3H5 recognition site (Hiramatsu et al., 1996). Furthermore, residues 383-385 (EPG or Glu-Pro-Gly) were found to be completely conserved among all dengue-2 viruses. Single amino acid replacement in this region affects 3H5 binding, suggesting that mAb 3H5 binding site is upstream of the previously reported binding site. However, in the same study, it was shown that oligopeptide 382-393 did not react with 3H5. The authors speculated that this might be because mAb 3H5 recognizes a conformational rather than a linear sequence (Hiramatsu et al., 1996). Our results showed that the carboxy-truncated E protein (residues 1-393) can be recognized by

mAb 3H5. This result shows that the mAb 3H5 recognition site is five bases further upstream than the previously reported. Since the Western blot was performed under denaturing conditions, we believe that the mAb 3H5 recognizes a linear sequence.

The binding site of mAb 9D12 is less characterized. However, studies have shown that there is an overlap between the 3H5 and 9D12 binding site (Megret et al., 1992). We observed that mAb 9D12 recognizes two forms of the truncated the truncated E protein (residues 1-393 and residues 100-393), thus localizing the 9D12 epitope to the middle one-half (residues 1-393) of the E protein.

In summary, we were able to express the known the truncated E protein in *E. coli.* as well as in yeast. To our knowledge this is the first successful cloning of any dengue virus protein in yeast and suggests the possibility of utilizing yeast for further studies in structure/function of dengue viral proteins as well as for the development of subunit vaccines.

REFERENCES

Adams BG, 1972, Induction of galactokinase in Saccharomyces cerevisiae: kinetics of induction and glucose effects, J Bacteriol, 111: 308-15

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

Amberg SM, Nestorowicz A, McCourt DW, Rice CM, 1994, NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: in vitro and in vivo studies, J Virol, 68: 3794-802

Anderson R, King AD, Innis BL, 1992, Correlation of E protein binding with cell susceptibility to dengue 4 virus infection, J Gen Virol, 73: 2155-9

Anderson R, Wang S, Osiowy C, Issekutz AC, 1997, Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes, J Virol, 71: 4226-3

Andrews BS, Shadforth M, Cunningham P, Davis JS 4th, 1981, Demonstration of a C1q receptor on the surface of human endothelial cells, J Immunol, 127: 1075-80

Arenzana Seisdedos F, Virelizier JL, Rousset D, Clark Lewis I, Loetscher P, Moser B, Arias CF, Preugschat F, Strauss JH, 1993, Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain, Virology, 193: 888-99

Armstrong SJ, Outlaw MC, Dimmock NJ, 1990, Morphological studies of the neutralization of influenza virus by IgM, J Gen Virol, 71: 2313-9

Armstrong SJ, Dimmock NJ, 1996, Varying temperature dependence of post attachment neutralization of human immunodeficiency virus type 1 by monoclonal antibodies to gp 120: identification of a very early fusion-independent event as a neutralization target, J Gen Virol, 77: 1397-402

Armstrong SJ, McInerney TL, McLain L, Wahren B, Hinkula J, Levi M, Dimmock NJ, 1996, Two neutralizing anti-V3 monoclonal antibodies act by affecting different functions of human immunodeficiency virus type 1, J Gen Virol, 77: 2931-41

Aung Khin M, Ma Ma K, Thant Zin, 1975, Changes in the tissues of the immune system in dengue haemorrhagic fever, J Trop Med Hyg, 78: 256-61

Back NK, Thiriart C, Delers A, Ramautarsing C, Bruck C, Goudsmit J, 1990, Association of antibodies blocking HIV-1 gp 160-sCD4 attachment with virus neutralizing activity in human sera, J Med Virol, 31: 200-8

Baim SB, Sherman F, 1988, mRNA structures influencing translation in the yeast Saccharomyces cerevisiae, Mol Cell Biol, 8: 1591-601

Baines NJ, Baird JB, Elmore DT, 1964, The kinetics of hydrolysis of derivatives of arginine, homoarginine and ornithine by trypsin, Biochem J, 90: 470-6

Barnes WJ, Rosen L, 1974, Fatal hemorrhagic disease and shock associated with primary dengue infection on a Pacific island, Am J Trop Med Hyg, 23: 495-506

Barr PJ, Steimer KS, Sabin EA, Parkes D, George Nascimento C, Stephans JC, Powers MA, Gyenes A, Van-Nest GA, Miller ET, et al, 1987, Antigenicity and immunogenicity of domains of the human immunodeficiency virus (HIV) envelope polypeptide expressed in the yeast Saccharomyces cerevisiae, Vaccine, 5: 90-101

Basaca Sevilla V, Halstead SB, 1966, Recent virological studies on haemorrhagic fever and other arthropod-borne virus infections in the Philippines, J Trop Med Hyg, 69: 203-8

Bathurst IC, 1994, Protein expression in yeast as an approach to production of recombinant malaria antigens, Am J Trop Med Hyg, 50: 20-6

Becker Y, 1990, Computer analysis of antigenic domains and RGD like sequences (RGWG) in the E glycoprotein of flaviviruses: an approach to vaccine development, Virus Genes, 4: 267-82

Bhamarapravati N, 1989, Hemostatic defects in dengue hemorrhagic fever, Rev Infect Dis, Suppl 4: S826-9

Bhamarapravati N, Tuchinda P, Boonyapaknavik V, 1967, Pathology of Thailand haemorrhagic fever: a study of 100 autopsy cases, Ann Trop Med Parasitol, 61: 500-10

Birnboim HC, Doly J, 1979, A rapid alkaline extraction procedure for screening recombinant plasmid DNA, Nucleic Acids Res, 7: 1513-23

Bokisch VA, Muller Eberhard HJ, Dixon FJ, 1973, The role of complement in hemorrhagic shock syndrome (dengue), Trans Assoc Am Physicians, 86: 102-10,

Bokisch VA, Top FH Jr, Russell PK, Dixon FJ, Muller Eberhard HJ, 1973, The potential pathogenic role of complement in dengue hemorrhagic shock syndrome, N Engl J Med, 289: 996-1000

Boltz Nitulescu G, Bazin H, Spiegelberg HL, 1981, Specificity of fc receptors for IgG2a, IgG1/IgG2b, and IgE on rat macrophages, J Exp Med, 154:374-84

Boonpucknavig S, Vuttiviroj O, Bunnag C, Bhamarapravati N, Nimmanitya S, 1979, Demonstration of dengue antibody complexes on the surface of platelets from patients with dengue hemorrhagic fever, Am J Trop Med Hyg, 28: 881-4

Bork P, Holm L, Sander C, 1994, The immunoglobulin fold, Structural classification, sequence patterns and common core, J Mol Biol, 242: 309-20

Brandt WE, McCown JM, Top FH Jr, Bancroft WH, Russell PK, 1979, Effect of passage history on dengue-2 virus replication in subpopulations of human leukocytes, Infect Immun, 26: 534-41

Brandt WE, McCown JM, Gentry MK, Russell PK, 1982, Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants, Infect Immun, 36: 1036-41

Bravo JR, Guzman MG, Kouri GP, 1987, Why dengue haemorrhagic fever in Cuba? 1, Individual risk factors for dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS), Trans R Soc Trop Med Hyg, 81: 816-20

Bray M, Lai CJ, 1990, Dengue virus premembrane and membrane proteins elicit a protective immune response, Virology, 185: 505-8

Bray M, Zhao BT, Markoff L, Eckels KH, Chanock RM, Lai CJ, 1989, Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against fatal dengue virus encephalitis, J Virol, 63: 2853-6

Brinton MA, Davis J, Schaefer D, 1985, Characterization of west nile virus persistent infections in genetically resistant and susceptible mouse cells, II, Generation of temperature-sensitive mutants, Virology, 140: 152-8

Brioen P, Dekegel D, Boeye A, 1983, Neutralization of poliovirus by antibody-mediated polymerization, Virology, 127: 463-8

Brown WC, Campbell JL, 1993, A new cloning vector and expression strategy for genes encoding proteins toxic to Escherichia coli, Gene, 127: 99-103

Brown WM, Axelrad AA, 1974, Effect of Friend leukemia virus on megakaryocytes and platelets in mice, Int J Cancer, 18: 764-73

Buckley CD, Doyonnas R, Newton JP, Blystone SD, Brown EJ, Watt SM, Simmons DL, 1996, Identification of alpha v beta 3 as a heterotypic ligand for CD31/PECAM-1, J Cell Sci, 109: 437-45,

Bujdoso R, Williamson M, Roy D, Hunt P, Blacklaws B, Sargan D, McConnell I, 1995, Molecular cloning and expression of DNA encoding ovine interleukin 2, Cytokine, 7: 223-31

Burioni R, Williamson RA, Sanna PP, Bloom FE, Burton DR, 1994, Recombinant human Fab to glycoprotein D neutralizes infectivity and prevents cell-to-cell transmission of herpes simplex viruses 1 and 2 in vitro, Proc Natl Acad Sci U S A, 91: 355-9

Burke RR, Heard DD, Nieuwkoop AJ, 1997, Cloning and expression in Pseudomonas putida of two of the histidine utilization genes from Rhizobium fredii, Curr Microbiol, 34: 55-60

Cane PA, Gould EA, 1988, Reduction of yellow fever virus mouse neurovirulence by immunization with a bacterially synthesized non-structural protein (NS1) fragment, J Gen Virol, 69: 1241-6

Cardiff RD, Lund JK, 1976, Distribution of dengue-2 antigens by electron immunocytochemistry, Infect Immun, 13: 1699-709

Castle E, Nowak T, Leidner U, Wengler G, 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: 227-36

Chamat S, Nara P, Berquist L, Whalley A, Morrow WJ, Kohler H, Kang CY, 1992, Two major groups of neutralizing anti-gp120 antibodies exist in HIV-infected individuals, Evidence for epitope diversity around the CD4 attachment site, J Immunol, 149: 649-54

Chambers TJ, Grakoui A, Rice CM, 1991, Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites, J Virol, 65: 6042-50

Chambers TJ, Hahn CS, Galler R, Rice CM,1990a, Flavivirus genome organization, expression, and replication, Annu Rev Microbiol, 44: 649-88

Chambers TJ, Weir RC, Grakoui A, McCourt DW, Bazan JF, Fletterick RJ, 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 U S A, 87: 8898-902

Chen CJ, Kuo MD, Chien LJ, Hsu SL, Wang YM, Lin JH, 1997, RNA-protein interactions: involvement of NS3, NS5, and 3' noncoding regions of Japanese encephalitis virus genomic RNA, J Virol, 71:3466-73

Chen CY, Oppermann H, Hitzeman RA, 1984, Homologous versus heterologous gene expression in the yeast, Saccharomyces cerevisiae, Nucleic Acids Res, 12: 8951-70

Chen E, 1994, Host strain selection for bacterial expression of toxic proteins, Methods Enzymol, 241: 29-46

Chen RL, Lin KH, Lin DT, Su IJ, Huang LM, Lee PI, Hseih KH, Lin KS, Lee CY, 1995, Immunomodulation treatment for childhood virus-associated haemophagocytic lymphohistiocytosis, Br J Haematol, 89: 282-90

Chen Y, Maguire T, Marks RM, 1996, Demonstration of binding of dengue virus envelope protein to target cells, J Virol, 70: 8765-72

Chiaramonte M, Majori S, Ngatchu T, Moschen ME, Baldo V, Renzulli G, Simoncello I, Rocco S, Bertin T, Naccarato R, Trivello R, 1996, Two different dosages of yeast derived recombinant hepatitis B vaccines: a comparison of immunogenicity, Vaccine, 14: 135-7

Chiewsilp P, Scott RM, Bhamarapravati N, 1981, Histocompatibility antigens and dengue hemorrhagic fever, Am J Trop Med Hyg, 30: 1100-5

Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH, 1969, Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population, Am J Epidemiol, 89: 449-63

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J, 1996, The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates, Cell, 85: 1135-48

Christensen ND, Cladel NM, Reed CA, 1995, Postattachment neutralization of papillomaviruses by monoclonal and polyclonal antibodies, Virology, 207: 136-42

Churdboonchart V, Bhamarapravati N, 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: 569-76

Churdboonchart V, Bhamarapravati N, Peampramprecha S, Sirinavin S, 1991, Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever, Am J Trop Med Hyg, 44: 481-93

Cigan AM, Feng L, Donahue TF, 1984, tRNAi(met) functions in directing the scanning ribosome to the start site of translation, Science, 242: 93-7

Cinque P, Landonio G, Lazzarin A, Nosari AM, Ruggieri A, Coen M, Meraviglia P, Gringeri A, Gallo L, Quirino T, et al, 1993, Long-term treatment with zidovudine in patients with human immunodeficiency virus (HIV)-associated thrombocytopenia: modes of response and correlation with markers of HIV replication, Eur J Haematol, 50: 17-21

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

Cocchi F, DeVico AL, Garzino Demo A, Cara A, Gallo RC, Lusso P, 1996, The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection, Nat Med, 2: 1244-7

Cohen S, Powell CJ, Dubois DR, Hartman A, Summers PL, Eckels KH, 1990, Expression of the envelope antigen of dengue virus in vaccine strains of Salmonella, Res Microbiol, 141: 855-8

Cohen SN, Halstead SB, 1966, Shock associated with dengue infection, I, Clinical and physiologic manifestations of dengue hemorrhagic fever in Thailand, 1964, J Pediatr, 68: 448-56

Coleman JC, 1972, Dengue viruses in mosquitoes, J Med Microbiol, 5: Pxv

Coleman JC, McLean DM, 1973, Dengue virus transmission by Aedes aegypti mosquitoes following intrathoracic inoculation, Am J Trop Med Hyg, 22: 124-9

Coletti Previero MA, Axelrud Cavadore C, 1977, Changes of substrate configuration during trypsin hydrolysis of arginine and lysine 2-phenyl-thiazol-5-ones, Biochem Biophys Res Commun, 62: 844-8

Compton T, Nowlin DM, Cooper NR, 1993, Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate, Virology, 193: 834-41

Connolly M, Junker AK, Chan KW, Farrell K, 1994, Cranial neuropathy, polyneuropathy and thrombocytopenia with Epstein-Barr virus infection, 36: 1010-5

Coyle TE, 1997, Hematologic complications of human immunodeficiency virus infection and the acquired immunodeficiency syndrome, Med Clin North Am, 81: 449-70

Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA, 1984, The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus, Nature, 312: 763-7

Daughaday CC, Brandt WE, McCown JM, 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, Infect Immun, 32: 469-73

Davis AC, Shulman MJ, 1989, IgM--molecular requirements for its assembly and function, Immunol Today, 10: 118-22, 127-8

Davis BR, Zauli G, 1995, Effect of human immunodeficiency virus infection on haematopoiesis, Baillieres Clin Haematol, 8: 113-30

Delenda C, Frenkiel MP, Deubel V, 1994, Protective efficacy in mice of a secreted form of recombinant dengue-2 virus envelope protein produced in baculovirus infected insect cells, Arch Virol, 139: 197-207

Delibrias CC, Kazatchkine MD, Fischer E, 1993, Evidence for the role of CR1 (CD35), in addition to CR2 (CD21), in facilitating infection of human T cells with opsonized HIV, Scand J Immunol, 38: 183-9

Della Porta AJ, Westaway EG, 1977, Immune response in rabbits to virion and nonvirion antigens of the Flavivirus kunjin, Infect Immun, 15: 874-82

Derrick EH, Bicks VA, 1958, The limiting temperature to the transmission of dengue. Aust Ann Med 7: 102-7

Deubel V, Digoutte JP, Mattei X, Pandare D, 1981, Morphogenesis of yellow fever virus in Aedes aegypti cultured cells, II, An ultrastructural study, Am J Trop Med Hyg, 30: 1071-7

Deubel V, Kinney RM, Esposito JJ, Cropp CB, Vorndam AV, Monath TP, Trent DW, 1988, Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue, J Gen Virol, 69: 1921-9

Deubel V, Laille M, Hugnot JP, Chungue E, Guesdon JL, Drouet MT, Bassot S, Chevrier D, 1990, Identification of dengue sequences by genomic amplification: rapid diagnosis of dengue virus serotypes in peripheral blood, J Virol Methods, 30: 41-54

Dietzschold B, Tollis M, Lafon M, Wunner WH, Koprowski H, 1987, Mechanisms of rabies virus neutralization by glycoprotein-specific monoclonal antibodies, Virology, 161: 29-36

Dimmock NJ, 1984, Mechanisms of neutralization of animal viruses, J Gen Virol, 65: 1015-22

Dimmock NJ, 1993, Neutralization of animal viruses, Curr Top Microbiol Immunol, 183: 1-149

Dittmar D, Cleary TJ, Castro A, 1979, Immunoglobulin G- and M-specific enzyme-linked immunosorbent assay for detection of dengue antibodies, J Clin Microbiol, 9: 498-502

Douglas SE, 1995, DNA Strider. An inexpensive sequence analysis package for the Macintosh, Mol Biotechnol, 3: 37-45

Duperray A, Languino LR, Plescia J, McDowall A, Hogg N, Craig AG, Berendt AR, Altieri DC, 1997, Molecular identification of a novel fibrinogen binding site on the first domain of ICAM-1 regulating leukocyte-endothelium bridging, J Biol Chem, 272: 435-41

Duperval R, Frost EH, Artsob H, 1993. Dengue fever with hemorrhagic manifestations in travellers returning to Quebec from Asia. Can J Infect Dis 4: 220-222

Eckels KH, Dubois DR, Summers PL, Schlesinger JJ, Shelly M, Cohen S, Zhang YM, Lai CJ, Kurane I, Rothman A, et al, 1994, Immunization of monkeys with baculovirus-dengue type-4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection, Am J Trop Med Hyg, 50: 472-8

Edelman R, Nisalak A, Pariyanonda A, Udomsakdi S, Johnsen DO, 1973, Immunoglo-bulin response and viremia in dengue-vaccinated gibbons repeatedly challenged with Japanese encephalitis virus, Am-J-Epidemiol, 97: 208-18

Ehrengut W, Sarateanu DE, Alswede U, Habib A, Tetzlaff G, 1975, Vaccinia virus variants as presumable cause of vaccinial complications, Arch Virol, 48: 229-38

Ellis RW, 1996, The new generation of recombinant viral subunit vaccines, Curr Opin Biotechnol, 7: 646-52

Elswood BF, Stricker RB, 1994, Polio vaccines and the origin of AIDS, Med Hypotheses, 42: 347-54

Endres MJ, Clapham PR, Marsh M, Ahuja M, Turner JD, McKnight A, Thomas JF, Stoebenau Haggarty B, Choe S, Vance PJ, Wells TN, Power CA, Sutterwala SS, Doms RW, Landau NR, Hoxie JA, 1996, CD4-independent infection by HIV-2 is mediated by fusin/CXCR4, Cell, 87: 745-56

Esposito JJ, Knight JC, Shaddock JH, Novembre FJ, Baer GM, 1988, Successful oral rabies vaccination of raccoons with raccoon poxvirus recombinants expressing rabies virus glycoprotein, Virology, 165: 313-6

Falconar AK, Young PR, 1990, Immunoaffinity purification of native dimer forms of the flavivirus non-structural glycoprotein, NS1, J Virol Methods, 30: 323-32

Falgout B, Pethel M, Zhang YM, Lai CJ, 1991, Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins, J Virol, 65: 2467-75

Falgout B, Miller RH, Lai CJ, 1993, Deletion analysis of dengue virus type 4 nonstructural protein NS2B: identification of a domain required for NS2B-NS3 protease activity, J Virol, 67: 2034-42

Fan WF, Mason PW, 1990, Membrane association and secretion of the Japanese encephalitis virus NS1 protein from cells expressing NS1 cDNA, Virology, 177: 470-6

Farwell JR, Dohrmann GJ, Marrett LD, Meigs JW, 1979, Effect of SV40 virus-contaminated polio vaccine on the incidence and type of CNS neoplasms in children: a population-based study, Trans Am Neurol Assoc, 104: 261-4

Fasel N, Begdadi Rais C, Bernard M, Bron C, Corradin G, Reymond CD, 1992, Dictyostelium discoideum as an expression host for the circumsporozoite protein of Plasmodium falciparum, Gene, 111: 157-63

Feighny R, Burrous J, McCown J, Hoke C, Putnak R, 1992, Purification of native dengue-2 viral proteins and the ability of purified proteins to protect mice, Am J Trop Med Hyg, 47: 405-12

Feighny R, Burrous J, Putnak R, 1994, Dengue type-2 virus envelope protein made using recombinant baculovirus protects mice against virus challenge, Am J Trop Med Hyg, 50: 322-8

Figueiredo LT, Simoes MC, Cavalcante SM, 1989, Enzyme immunoassay for the detection of dengue IgG and IgM antibodies using infected mosquito cells as antigen, Trans R Soc Trop Med Hyg, 83: 702-7

Finbloom DS, Hoover DL, Meltzer MS, 1991, Binding of recombinant HIV coat protein gp120 to human monocytes, J Immunol, 146: 1316-21

Fleming MG, Howe SF, Graf LH Jr, 1994, Expression of insulin-like growth factor I (IGF-I) in nevi and melanomas, Am J Dermatopathol, 16: 383-91

Franzusoff A, Volpe AM, Josse D, Pichuantes S, Wolf JR, 1995, Biochemical and genetic definition of the cellular protease required for HIV-1 gp160 processing, J Biol Chem, 270: 3154-9

Funahara Y, Sumarmo, Shirahata A, Setiabudy Dharma R, 1987, DHF characterized by acute type DIC with increased vascular permeability, Southeast Asian J Trop Med Public Health, 18: 346-50

Fust G, Toth FD, Kiss J, Ujhelyi E, Nagy I, Banhegyi D, 1994, Neutralizing and enhancing antibodies measured in complement-restored serum samples from HIV-1-infected individuals correlate with immunosuppression and disease, AIDS, 8: 603-9

Gaines PJ, Olson KE, Higgs S, Powers AM, Beaty BJ, Blair CD, 1996, Pathogen-derived resistance to dengue type 2 virus in mosquito cells by expression of the premembrane coding region of the viral genome, J Virol, 70: 2132-7

Gal-On A, Antignus Y, Rosner A, Raccah B, 1990, Nucleotide sequence of the zucchini yellow mosaic virus capsid-encoding gene and its expression in Escherichia coli, Gene, 87: 273-7

Geissler E, Konzer P, Scherneck S, Zimmermann W, 1985, Sera collected before introduction of contaminated polio vaccine contain antibodies against SV40, Acta-Virol, 29: 420-3

Gentry MK, Henchal EA, McCown JM, Brandt WE, Dalrymple JM, 1982, Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies, Am J Trop Med Hyg, 31: 548-55

Gerber P, 1967, Patterns of antibodies to SV40 in children following the last booster with inactivated poliomyelitis vaccines, Proc Soc Exp Biol Med, 125: 1284-7

Ghebrehiwet B, 1989, Functions associated with the C1q receptor, Behring Inst Mitt,: 204-15

Giniger E, Varnum SM, Ptashne M, 1985, Specific DNA binding of GAL4, a positive regulatory protein of yeast, Cell, 40: 767-74

Glatt AE, Anand A, 1995, Thrombocytopenia in patients infected with human immunodeficiency virus: treatment update, Clin Infect Dis, 21: 415-23

Gollins SW, Porterfield JS, 1985, Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry, J Gen Virol, 66: 1969-82

Gollins SW, Porterfield JS, 1986, A new mechanism for the neutralization of enveloped viruses by antiviral antibody, Nature, 321: 244-6

Gollins SW, Porterfield JS, 1986, pH-dependent fusion between the flavivirus West Nile and liposomal model membranes, J Gen Virol, 67: 157-66

Green EM, Armstrong SJ, Dimmock NJ, 1993, Mechanisms of neutralization of a nairovirus (Dugbe virus) by polyclonal IgG and IgM, J Gen Virol, 73: 1995-2001

Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, Kamarck ME, McClelland A, 1989, The major human rhinovirus receptor is ICAM-1, Cell, 56: 839-47

Gubler DJ, 1987, Dengue and dengue hemorrhagic fever in the Americas, P R Health Sci J, 6: 107-11

Gubler DJ, 1989, Aedes aegypti and Aedes aegypti-borne disease control in the 1990s, Am J Trop Med Hyg, 40: 571-8

Guirakhoo F, Heinz FX, Mandl CW, Holzmann H, Kunz C, 1991, Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions, J Gen Virol, 72: 1323-9

Guirakhoo F, Hunt AR, Lewis JG, Roehrig JT, 1993, Selection and partial characterization of dengue 2 virus mutants that induce fusion at elevated pH, Virology, 194: 219-23

Gunasegaran K, Lim TW, Ahmed A, Aaskov JG, Lam SK, Pang T, 1986, Hemadsorption immunosorbent technique for the detection of dengue immunoglobulin M antibody, J Clin Microbiol, 23: 170-4

Gunther I, Glatthaar B, Doller G, Garten W, 1993, A H1 hemagglutinin of a human influenza A virus with a carbohydrate-modulated receptor binding site and an unusual cleavage site, Virus Res, 27: 147-60

Guzman MG, Kouri G, Martinez E, Bravo J, Riveron R, Soler M, Vazquez S, Morier L, 1987, Clinical and serologic study of Cuban children with dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), 21: 270-9

Guzman MG, Kouri G, Morier L, Soler M, Fernandez A, 1984, A study of fatal hemorrhagic dengue cases in Cuba, 1981, Bull Pan Am Health Organ, 18: 213-20

Guzman MG, Kouri GP, Bravo J, Soler M, Vazquez S, Morier L, 1990, Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study, Am J Trop Med Hyg 42: 179-84

Guzowski JF, Singh J, Wagner EK, 1994, Transcriptional activation of the herpes simplex virus type 1 UL38 promoter conferred by the cis-acting downstream activation sequence is mediated by a cellular transcription factor, J Virol, 68: 7774-89

Hahn YS, Lenches EM, Galler R, Rice CM, Dalrymple J, Strauss JH, 1990, Expression of the structural proteins of dengue 2 virus and yellow fever virus by recombinant vaccinia viruses, Arch Virol, 115: 251-65

Hall RA, Brand TN, Lobigs M, Sangster MY, Howard MJ, Mackenzie JS, 1996, Protective immune responses to the E and NS1 proteins of Murray Valley encephalitis virus in hybrids of flavivirus-resistant mice, J Gen Virol, 77: 1287-94

Halstead SB, 1965, Dengue and hemorrhagic fevers of Southeast Asia, Yale J Biol Med, 37: 434-54

Halstead SB, 1982, Dengue: hematologic aspects, Semin Hematol, 19: 116-31,

Halstead SB, 1982, Immune enhancement of viral infection, Prog Allergy, 31: 301-64

Halstead SB, 1988, Pathogenesis of dengue: challenges to molecular biology, Science, 239: 476-81

Halstead SB, 1989, Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade, Rev Infect Dis, 11 Suppl 4: S830-9

Halstead SB, 1990, Dengue, p675-685, in K S Warren and AAF Mahnoudleds Tropical and Geographical Medicine McGraw Hill, New York, NY

Halstead SB, Diwan AR, Marchette NJ, Palumbo NE, Srisukonth L, 1984, Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells, I, Attributes of uncloned virus at different passage levels, Am J Trop Med Hyg, 33: 654-65

Halstead SB, Marchette NJ, Sung Chow JS, Lolekha S, 1976, Dengue virus replication enhancement in peripheral blood leukocytes from immune human beings, Proc Soc Exp Biol Med, 151: 136-9

Halstead SB, Shotwell H, Casals J, 1973, Studies on the pathogenesis of dengue infection in monkeys, I, Clinical laboratory responses to primary infection, J Infect Dis, 128: 7-14

Halstead SB, Simasthien P, 1970, Observations related to the pathogenesis of dengue hemorrhagic fever, II, Antigenic and biologic properties of dengue viruses and their association with disease response in the host, Yale J Biol Med, 42: 276-92,

Halstead SB, Udomsakdi S, Scanlon JE, Rohitayodhin S, 1969, Dengue and chikungunya virus infection in man in Thailand, 1962-1964, V, Epidemiologic observations outside Bangkok, Am J Trop Med Hyg, 18: 1022-33

Halstead SB, Venkateshan CN, Gentry MK, Larsen LK, 1984, Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies, J Immunol, 132: 1529-32

Hammon WM, 1969, Observations on dengue fever, benign protector and killer: a Dr. Jekyll and Mr. Hyde, Am J Trop Med Hyg, 18: 159-65

Hariharan K, Nara PL, Caralli VM, Norton FL, Haigwood N, Kang CY, 1993, Analysis of the cross-reactive anti-gp120 antibody population in human immunodeficiency virus-infected asymptomatic individuals, J Virol, 67: 953-60

Hase T, Summers PL, Cohen WH, 1989, A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis viruses, Arch Virol, 108: 101-14

Hase T, Summers PL, Eckels KH, Baze WB, 1987, An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito cells: maturation events, Arch-Virol, 92: 273-91

Hawkes RA, Lafferty KJ, 1967, The enchancement of virus infectivity by antibody, Virology, 33: 250-61

Haywood AM, 1974, Characteristics of Sendai virus receptors in a model membrane, J Mol Biol, 83: 427-36

He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D, 1997, CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia, Nature, 385: 645-9

He RT, Innis BL, Nisalak A, Usawattanakul W, Wang S, Kalayanarooj S, Anderson R, 1995, Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2, J Med Virol, 45: 451-61

Hedstrom L, Szilagyi L, Rutter WJ, 1992, Converting trypsin to chymotrypsin: the role of surface loops, Science, 255: 1249-53

Hegde UM, 1989, Platelet antibodies in immune thrombocytopenia, Blood Rev, 6: 34-42

Heinz FX, Stiasny K, Puschner Auer G, Holzmann H, Allison SL, Mandl CW, Kunz C, 1994, Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM, Virology, 198: 109-17

Helenius A, Marsh M, 1982, Endocytosis of enveloped animal viruses, Ciba Found Symp, 59-76

Henchal EA, McCown JM, Burke DS, Seguin MC, Brandt WE, 1985, Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies, Am J Trop Med Hyg, 34: 162-9

Hernandez LD, Hoffman LR, Wolfsberg TG, White JM, 1996, Virus-cell and cell-cell fusion, Ann Rev Cell Dev Biol, 12: 627-61

Herrler G, Rott R, Klenk HD, 1985, Neuraminic acid is involved in the binding of influenza C virus to erythrocytes, Virology, 141: 144-7

Hiramatsu K, Tadano M, Men R, Lai CJ, 1996, Mutational analysis of a neutralization epitope on the dengue type 2 virus (DEN2) envelope protein: monoclonal antibody resistant DEN2/DEN4 chimeras exhibit reduced mouse neurovirulence, Virology, 224: 437-45

Hitzeman RA, Chen CY, Hagie FE, Patzer EJ, Liu CC, Estell DA, Miller JV, Yaffe A, Kleid DG, Levinson AD, Oppermann H, 1983, Expression of hepatitis B virus surface antigen in yeast, Nucleic Acids Res, 11: 2745-63

Holzmann H, Kundi M, Stiasny K, Clement J, McKenna P, Kunz C, Heinz FX, 1996, Correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis, J Med Virol, 48: 102-7

Homsy J, Meyer M, Levy JA, 1990, Serum enhancement of human immunodeficiency virus (HIV) infection correlates with disease in HIV-infected individuals, J Virol, 64: 1437-40

Hospedales CJ, 1990, An update on Dengue fever in the Caribbean, West Indian Med J, 39: 131

Hugo H, Linde A, Abom PE, 1989, Epstein-Barr virus induced thrombocytopenia treated with intravenous acyclovir and immunoglobulin, Scand J Infect Dis, 21: 103-5

Ibrahim NM, Cheong I, 1995, Adult dengue haemorrhagic fever at Kuala Lumpur Hospital: retrospective study of 102 cases, Br J Clin Pract, 49: 189-91

Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, Puttisri P, Hoke CH, 1989, An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate, Am J Trop Med Hyg, 40: 418-27

Iorio RM, Bratt MA, 1983, Monoclonal antibodies to newcastle disease virus: delineation of four epitopes on the HN glycoprotein, J Virol, 48: 440-50

Iorio RM, Glickman RL, Riel AM, Sheehan JP, Bratt MA, 1989, Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment, Virus Res, 13: 245-61

Iorio RM, Syddall RJ, Sheehan JP, Bratt MA, Glickman RL, Riel AM, 1991, Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition, J Virol, 65: 4999-5006

Ishak R, Tovey DG, Howard CR, 1988, Morphogenesis of yellow fever virus 17D in infected cell cultures, J Gen Virol, 69: 325-35

Ishido S, Muramatsu S, Fujita T, Iwanaga Y, Tong WY, Katayama Y, Itoh M, Hotta H, 1997, Wild-type, but not mutant-type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus, Biochem Biophys Res Commun, 230: 431-6

Itoh Y, Fujisawa Y, 1986, Synthesis in yeast of hepatitis B virus surface antigen modified P31 particles by gene modification, Biochem Biophys Res Commun, 141: 942-8

Jackson DE, Ward CM, Wang R, Newman PJ, 1997, The protein-tyrosine phosphatase SHP-2 binds platelet/endothelial cell adhesion molecule-1 (PECAM-1) and forms a distinct signaling complex during platelet aggregation, Evidence for a mechanistic link between PECAM-1- and integrin-mediated cellular signaling, J Biol Chem, 272: 6986-93,

Jan LR, Yang CS, Henchal LS, Sumiyoshi H, Summers PL, Dubois DR, Lai CJ, 1993, Increased immunogenicity and protective efficacy in outbred and inbred mice by strategic carboxyl-terminal truncation of Japanese encephalitis virus envelope glycoprotein, J Gen Virol 48: 412-23

Jan LR, Yang CS, Trent DW, Falgout B, Lai CJ, 1995, Processing of Japanese encephalitis virus non-structural proteins: NS2B-NS3 complex and heterologous proteases, J Gen Virol, 76: 573-80

Jansen-Durr P, 1996, Viral oncogenesis and cell cycle control, Virus Res, 42: 187-91

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

Johnson DC, Burke RL, Gregory T, 1990, Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells, J Virol, 64: 2569-76

Johnson KM, Halstead SB, Cohen SN, 1967, Hemorrhagic fevers of Southeast Asia and South America: a comparative appraisal, Prog Med Virol, 9: 105-58

June RA, Schade SZ, Bankowski MJ, Kuhns M, McNamara A, Lint TF, Landay AL, Spear GT, 1991, Complement and antibody mediate enhancement of HIV infection by increasing virus binding and provirus formation, AIDS, 5: 269-74

Kabra SK, Verma IC, Arora NK, Jain Y, Kalra V, 1992, Dengue haemorrhagic fever in children in Delhi, Bull World Health Organ, 70: 105-8

Kamer G, Argos P, 1984, Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses, Nucleic Acids Res, 12: 7269-82

Kang CY, Nara P, Chamat S, Caralli V, Ryskamp T, Haigwood N, Newman R, Kohler H, 1991, Evidence for non-V3-specific neutralizing antibodies that interfere with gp120/CD4 binding in human immunodeficiency virus 1-infected humans, Proc Natl Acad Sci U S A, 88: 6171-5

Karpatkin S, 1989, Autoimmune thrombocytopenia and AIDS-related thrombocytopenia, Curr Opin Immunol, 2: 625-32

Karpatkin S, Nardi M, Lennette ET, Byrne B, Poiesz B, 1988, Anti-human immunodeficiency virus type 1 antibody complexes on platelets of seropositive thrombocytopenic homosexuals and narcotic addicts, Proc Natl Acad Sci U S A, 85: 9763-7

Kaufman BM, Summers PL, Dubois DR, Cohen WH, Gentry MK, Timchak RL, Burke DS, Eckels KH, 1989, Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection, Am J Trop Med Hyg, 41: 576-80

Kaufman BM, Summers PL, Dubois DR, Eckels KH, 1987, Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection, Am J Trop Med Hyg, 36: 427-34

Khanna M, Chaturvedi UC, Sharma MC, Pandey VC, Mathur A, 1990, Increased capillary permeability mediated by a dengue virus-induced lymphokine, Immunology, 69: 449-53

Kielian M, Jungerwirth S, 1990, Mechanisms of enveloped virus entry into cells, Mol Biol Med, 7: 17-31

Kimura T, Ohyama A, 1988, Association between the pH-dependent conformational change of West Nile flavivirus E protein and virus-mediated membrane fusion, J Gen Virol, 69: 1247-54

Kliks SC, Nimmanitya S, Nisalak A, 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: 411-9

Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke DS, 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: 444-51

Knudsen AB, Romi R, Majori G, 1996, Occurrence and spread in Italy of Aedes albopictus, with implications for its introduction into other parts of Europe, J Am Mosq Control Assoc, 12: 177-83

Konishi E, Pincus S, Paoletti E, Shope RE, Burrage T, Mason PW, 1992, Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection, Virology, 188: 714-20

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: 733-40

Kouri GP, Guzman MG, Bravo JR, 1987, Why dengue haemorrhagic fever in Cuba? 2, An integral analysis, Trans R Soc Trop Med Hyg, 81: 821-3

Kozak M, 1989, The scanning model for translation: an update, J Cell Biol, 108: 229-41

Kreil TR, Eibl MM, 1997, Pre- and postexposure protection by passive immunoglobulin but no enhancement of infection with a flavivirus in a mouse model, J Virol, 71: 2921-7

Kuno G, 1994, Review of the factors modulating dengue transmission, Epidemiol Rev, 17: 321-35

Kuno G, Gubler DJ, Santiago de-Weil NS, 1985, Antigen capture ELISA for the identification of dengue viruses, J Virol Methods, 12: 93-103

Kurane I, Innis BL, Nimmannitya S, Nisalak A, Rothman AL, Livingston PG, Janus J, Ennis FA, 1990, Human immune responses to dengue viruses, Southeast Asian J Trop Med Public Health, 21: 658-62

Kurane I, Innis BL, Nimmannitya S, Nisalak A, Meager A, Janus J, 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: 1473-80

Kurane I, Ennis FE, 1992, Immunity and immunopathology in dengue virus infections, Semin Immunol, 4: 121-7

Kurane I, Dai LC, Livingston PG, Reed E, Ennis FA, 1993, Definition of an HLA-DPw2-restricted epitope on NS3, recognized by a dengue virus serotype-cross-reactive human CD4+ CD8- cytotoxic T-cell clone, J Virol, 67: 6285-8

Kurane I, Rothman AL, Livingston PG, Green S, Gagnon SJ, Janus J, Innis BL, Nimmannitya S, Nisalak A, Ennis FA, 1994, Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome, Arch Virol Suppl, 9: 59-64

Kurtzberg J, Stockman JA 3rd, 1994, Idiopathic autoimmune thrombocytopenic purpura, Adv Pediatr, 41: 111-34,

Kwak JW, Kim J, Yoo OJ, Han MH, 1987, A method to isolate DNA sequences that are promoter-active in Escherichia coli and in yeast, Biochem Biophys Res Commun, 149: 846-51

La-Russa VF, Innis BL, 1995, Mechanisms of dengue virus-induced bone marrow suppression, Baillieres Clin Haematol, 8: 249-70

Ladror US, Gollapudi L, Tripathi RL, Latshaw SP, Kemp RG, 1991, Cloning, sequencing, and expression of pyrophosphate-dependent phosphofructokinase from Propionibacterium freudenreichii, J Biol Chem, 266: 16550-5

Laemmli UK, 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227: 680-5

Lafferty KJ, Oertelis S, 1963, The interaction between virus and antibody, III, Examination of virus-antibody complexes with the electron microscope, Virology, 21: 91-99

Lai CJ, Zhao BT, Hori H, Bray M, 1991, Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus, Proc Natl Acad Sci U S A, 88: 5139-43

Lai CL, Wong BC, Yeoh EK, Lim WL, Chang WK, Lin HJ, 1993, Five-year follow-up of a prospective randomized trial of hepatitis B recombinant DNA yeast vaccine vs, plasma-derived vaccine in children: immunogenicity and anamnestic responses, Hepatology, 18: 763-7

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV, 1992, Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction, J Clin Microbiol, 30: 545-51

Leblois H, Young PR, 1995, Maturation of the dengue-2 virus NS1 protein in insect cells: effects of downstream NS2A sequences on baculovirus-expressed gene constructs, J Gen Virol, 76: 979-84

Lee E, Fernon C, Simpson R, Weir RC, Rice CM, Dalgarno L, 1993, 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

Lenstra JA, Kusters JG, van-der-Zeijst BA, 1990, Mapping of viral epitopes with prokaryotic expression products, Arch Virol, 110: 1-24

Levitz SM, North EA, ,1996, gamma Interferon gene expression and release in human lymphocytes directly activated by Cryptococcus neoformans and Candida albicans, Infect Immun, 64: 1595-9

Lifson AR , 1996, Mosquitoes, models, and dengue, Lancet, 347: 1201-2

Lin B, Parrish CR, Murray JM, Wright PJ, 1994, Localization of a neutralizing epitope on the envelope protein of dengue virus type 2, Virology, 202: 885-90

Lin C, Amberg SM, Chambers TJ, Rice CM, 1993, Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site, J Virol, 67: 2327-35

Linsley PS, Ledbetter JA, Kinney Thomas E, Hu SL, 1988, Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the env protein of human immunodeficiency virus type 1, J Virol, 62: 3695-702

Littaua R, Kurane I, Ennis FA, 1990, Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection, J Immunol, 144: 3183-6

Livingston PG, Kurane I, Dai LC, Okamoto Y, Lai CJ, Men R, Karaki S, Takiguchi M, Ennis FA, 1995, Dengue virus-specific, HLA-B35-restricted, human CD8+ cytotoxic T lymphocyte (CTL) clones, Recognition of NS3 amino acids 500 to 508 by CTL clones of two different serotype specificities, J Immunol, 154: 1287-95

Lobigs M, Usha R, Nestorowicz A, Marshall ID, Weir RC, Dalgarno L, 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: 587-95

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: 2305-12

Lobigs M, Arthur CE, Mullbacher A, Blanden RV, 1994, The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants, Virology, 202: 195-201

Loison G, Jund R, 1981, Expression of a cloned Saccharomyces cerevisiae gene (URA1) is controlled by a bacterial promoter in E, coli and by a yeast promoter in S, cerevisiae, Gene, 15: 127-37

Lopez-Correa RH, Cline BL, Ramirez-Ronda C, Bermudez R, Sather GE, Kuno G, 1978, Dengue fever with hemorrhagic manifestations: a report of three cases from Puerto Rico, Am J Trop Med Hyg, 27: 1216-24

Lorincz AT, Reed SI, 1984, Primary structure homology between the product of yeast cell division control gene CDC28 and vertebrate oncogenes, Nature, 307: 183-5

Luckow VA, Summers MD, 1988, Signals important for high-level expression of foreign genes in Autographa californica nuclear polyhedrosis virus expression vectors, Virology, 167: 56-71

Malasit P, 1987, Complement and dengue haemorrhagic fever/shock syndrome, Southeast Asian J Trop Med Public Health, 18: 316-20

Mandl CW, Guirakhoo F, Holzmann H, Heinz FX, 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: 564-71

Mandl CW, Heinz FX, Kunz C, 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, Stockl E, 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

Marchette NJ, Halstead SB, Falkler WA Jr, Stenhouse A, Nash D, 1973, Studies on the pathogenesis of dengue infection in monkeys, 3, Sequential distribution of virus in primary and heterologous infections, J Infect Dis, 128: 23-30

Marchette NJ, Sung-Chow JS, Halstead SB, Lolekha S, Pongpanich B, 1975, Dengue virus replication in cultures of peripheral blood leukocytes during the course of dengue haemorrhagic fever, Southeast Asian J Trop Med Public Health, 6: 316-21

Marchette NJ, Halstead SB, Chow JS, 1976, Replication of dengue viruses in cultures of peripheral blood leukocytes from dengue-immune rhesus monkeys, J Infect Dis, 133: 274-82

Marck C, 1988, 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers, Nucleic Acids Res, 16: 1829-36

Mares-Guia M, Shaw E, Cohen W, 1967, Studies on the active center of trypsin, Further characterization of the hydrophobic binding site, J Biol Chem, 242: 5777-81

Markoff L, 1989, In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein, J Virol, 63: 3345-52

Markoff L, Chang A, Falgout B, 1994, Processing of flavivirus structural glycoproteins: stable membrane insertion of premembrane requires the envelope signal peptide, Virology, 204: 526-40

Markoff LJ, Innis BL, Houghten R, Henchal LS, 1991, Development of cross-reactive antibodies to plasminogen during the immune response to dengue virus infection, J Infect Dis, 164: 294-301

Marsh M, Helenius A, 1989, Virus entry into animal cells, Adv Virus Res, 36: 107-51

Mason PW, 1989, Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells, Virology, 169: 354-64

Mason PW, Zugel MU, Semproni AR, Fournier MJ, Mason TL, 1990, The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in Escherichia coli, J Gen Virol, 71: 2107-14

Mathew A, Kurane I, Rothman AL, Zeng LL, Brinton MA, Ennis FA, 1996, Dominant recognition by human CD8+ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1,2a, J Clin Invest, 98: 1684-91

Matsumura T, Shiraki K, Sashikata T, Hotta S, 1977, Morphogenesis of dengue-1 virus in cultures of a human leukemic leukocyte line (J-111), Microbiol Immunol, 21: 329-34

Mayr GA, Freimuth P, 1997, A single locus on human chromosome 21 directs the expression of a receptor for adenovirus type 2 in mouse A9 cells, J Virol, 71: 412-8

Mazerolles F, Lumbroso C, Lecomte O, Le-Deist F, Fischer A, 1988, The role of lymphocyte function-associated antigen 1 (LFA-1) in the adherence of T lymphocytes to B lymphocytes, Eur J Immunol, 18: 1229-34

McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR, 1985, Human hepatitis B vaccine from recombinant yeast, Nature, 307: 178-80

McClure JE, 1992, Cellular receptor for Epstein-Barr virus, Prog Med Virol, 39: 116-38

McKnight A, Wilkinson D, Simmons G, Talbot S, Picard L, Ahuja M, Marsh M, Hoxie JA, Clapham PR, 1997, Inhibition of human immunodeficiency virus fusion by a monoclonal antibody to a coreceptor (CXCR4) is both cell type and virus strain dependent, J-Virol, 71: 1692-6

McLean DM, Clarke AM, Coleman JC, Montalbetti CA, Skidmore AG, Walters TE, Wise R, 1974, Vector capability of Aedes aegypti mosquitoes for California encephalitis and dengue viruses at various temperatures, Can J Microbiol, 20: 255-62

Megret F, Hugnot JP, Falconar A, Gentry MK, Morens DM, Murray JM, Schlesinger JJ, Wright PJ, Young P, Van-Regenmortel MH, et al, 1992, Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein, Virology, 187: 480-91

Mellor J, Dobson MJ, Kingsman AJ, Kingsman SM, 1987, A transcriptional activator is located in the coding region of the yeast PGK gene, Nucleic Acids Res, 15: 6243-59

Men RH, Bray M, Lai CJ, 1991, Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice, J Virol, 65: 1400-7

Milne A, Moyes CD, Allwood GK, Pearce NE, Krugman S, 1988, Antibody responses to recombinant, yeast-derived hepatitis B vaccine in teenage New Zealand children, N Z Med J, 101: 67-9

Miyanohara A, Imamura T, Araki M, Sugawara K, Ohtomo N, Matsubara K, 1986, Expression of hepatitis B virus core antigen gene in Saccharomyces cerevisiae: synthesis of two polypeptides translated from different initiation codons, J Virol, 59: 176-80

MMWR Morb Mortal Wkly Rep, 1996, Imported dengue--United States, 1995, 45: 988-91

Monath TP, 1994, Dengue: the risk to developed and developing countries, Proc Natl Acad Sci U S A, 91: 2395-400

Monath TP, Heinz FX, Flaviviruses, In *Fields Virology*, eds. Fields BN, Knipe DM, Howley PM, Lippincott-Raven, Philadelphia-New York (1996) Volume 1, pp. 961-1034

Montefiori DC, Zhou J, Shaff DI, 1992, CD4-independent binding of HIV-1 to the B lymphocyte receptor CR2 (CD21) in the presence of complement and antibody, Clin Exp Immunol, 90: 383-9,

Morens DM, 1994, Antibody-dependent enhancement of infection and the pathogenesis of viral disease, Clin Infect Dis, 19: 500-12

Morens DM, Halstead SB, 1990, Measurement of antibody-dependent infection enhancement of four dengue virus serotypes by monoclonal and polyclonal antibodies, J Gen Virol, 71: 2909-14

Mori M, Kurane I, Janus J, Ennis FA, 1997, Cytokine production by dengue virus antigen-responsive human T lymphocytes in vitro examined using a double immunocytochemical technique, J Leukoc Biol, 61: 338-45

Murray JM, Aaskov JG, Wright PJ, 1993, Processing of the dengue virus type 2 proteins prM and C-prM, J Gen Virol, 74: 175-82

Mussgay M, Enzmann PJ, Horzinek MC, Weiland E, 1975, Growth cycle of arboviruses in vertebrate and arthropod cells, Prog Med Virol, 19: 257-323

Muylaert IR, Chambers TJ, Galler R, Rice CM, 1996, Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: effects on virus replication and mouse neurovirulence, Virology, 222: 159-68

Muylaert IR, Galler R, 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: 291-8

Naniche D, Varior-Krishnan G, Cervoni F, Wild TF, Rossi B, Rabourdin-Combe C, Gerlier D, 1993, Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus, J Virol, 67: 6025-32,

Neff JM, Morris L, Gonzalez-Alcover R, Coleman PH, Lyss SB, Negron H, 1967, Dengue fever in a Puerto Rican community, Am J Epidemiol, 86: 162-84

Nestorowicz A, Chambers TJ, Rice CM, 1994, Mutagenesis of the yellow fever virus NS2A/2B cleavage site: effects on proteolytic processing, viral replication, and evidence for alternative processing of the NS2A protein, Virology, 199: 114-23

Newman PJ, 1994, The role of PECAM-1 in vascular cell biology, Ann N Y Acad Sci, 714: 165-74

Ng ML, Lau LC, 1988, Possible involvement of receptors in the entry of Kunjin virus into Vero cells, Arch Virol, 100: 199-211

Niklasson B, Vene S, 1996, Vector-borne viral diseases in Sweden--a short review, Arch Virol Suppl, 11: 49-55

Nishioka K, 1974, Serum complement level in dengue hemorrhagic fever, Allerg Immunol Leipz, 20-21: 385-92

Norkin LC, 1995, Virus receptors: implications for pathogenesis and the design of antiviral agents, Clin Microbiol Rev, 8: 293-315

Nowak T, Farber PM, Wengler G, 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: 365-76

Nowak T, Wengler G, 1987, Analysis of disulfides present in the membrane proteins of the West Nile flavivirus, Virology, 156: 127-37

Ohyama A, Ito T, Tanimura E, Huang SC, Hsue J, 1977, Electron microscopic observation of the budding maturation of group B arboviruses, Microbiol Immunol, 21: 535-8

Olson KE, Higgs S, Gaines PJ, Powers AM, Davis BS, Kamrud KI, Carlson JO, Blair CD, Beaty BJ, 1996, Genetically engineered resistance to dengue-2 virus transmission in mosquitoes, Science, 272: 884-6

Olson NH, Kolatkar PR, Oliveira MA, Cheng RH, Greve JM, McClelland A, Baker TS, Rossmann MG, 1993, Structure of a human rhinovirus complexed with its receptor molecule, Proc Natl Acad Sci U S A, 90: 507-11

Osiowy C, Anderson R, 1995, Neutralization of respiratory syncytial virus after cell attachment, J Virol, 69: 1271-4

Outlaw MC, Dimmock NJ, 1991, Insights into neutralization of animal viruses gained from study of influenza virus, Epidemiol Infect, 106: 205-20

Pajot-Augy E, Couture L, Bozon V, Remy JJ, Biache G, Severini M, Huet JC, Pernollet JC, Salesse R, 1995, High-level expression of recombinant porcine LH receptor in baculovirus-infected insect cells or caterpillars, J Mol Endocrinol, 14: 51-66

Palladino G, Mozdzanowska K, Washko G, Gerhard W, 1995, Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice, J Virol, 69: 2075-81

Paradoa-Perez ML, Trujillo Y, Basanta P, 1987, Association of dengue hemorrhagic fever with the HLA system, Haematologia-Budap 20: 83-7

Patz JA, Epstein PR, Burke TA, Balbus JM, 1996, Global climate change and emerging infectious diseases, JAMA, 275: 217-23

Peerschke EI, Smyth SS, Teng EI, Dalzell M, Ghebrehiwet B, 1996, Human umbilical vein endothelial cells possess binding sites for the globular domain of C1q, J Immunol, 157: 4154-8

Peiris JS, Porterfield JS, 1979, Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines, Nature, 282: 509-11

Peiris JS, Porterfield JS, Roehrig JT, 1982, Monoclonal antibodies against the flavivirus West Nile, J Gen Virol, 58: 283-9

Pierschbacher MD, Ruoslahti E, 1987, Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion, J Biol Chem, 262: 17294-8

Pincus S, Mason PW, Konishi E, Fonseca BA, Shope RE, Rice CM, Paoletti E, 1992, Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis, Virology, 187: 290-7

Plow EF; Ginsberg MH, 1981, Specific and saturable binding of plasma fibronectin to thrombin-stimulated human platelets. J Biol Chem. 256: 9477-82

Poignard P, Klasse PJ, Sattentau QJ, 1996, Antibody neutralization of HIV-1, Immunol Today, 17: 239-46

Possee RD, Schild GC, Dimmock NJ, 1982, Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-

haemagglutinin) inactivates influenza virus in vivo by inhibiting virion transcriptase activity, J Gen Virol, 58: 373-86

Post PR, Carvalho R, Galler R, 1991, Glycosylation and secretion of yellow fever virus nonstructural protein NS1, Virus Res, 18: 291-302

Pothipunya S, Patarapotikul J, Rojanasuphot S, Tharavanij S, 1993, Mapping of functional epitopes of Japanese encephalitis virus using monoclonal antibodies, Southeast Asian J Trop Med Public Health, 24: 277-83

Prehaud C, Takehara K, Flamand A, Bishop DH, 1989, Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors, Virology, 173: 390-9

Pryor MJ, Wright PJ, 1993, The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus, Virology, 194: 769-80

Putintseva E, Vega G, Fernandez L, 1986, Alterations in thrombopoiesis in patients with thrombocytopenia produced by dengue hemorrhagic fever, Nouv Rev Fr Hematol, 28: 269-73

Putnak R, Cassidy K, Conforti N, Lee R, Sollazzo D, Truong T, Ing E, Dubois D, Sparkuhl J, Gastle W, Hoke C, 1996, Immunogenic and protective response in mice immunized with a purified, inactivated, Dengue-2 virus vaccine prototype made in fetal rhesus lung cells, Am J Trop Med Hyg, 55: 504-10

Putnak R, Feighny R, Burrous J, Cochran M, Hackett C, Smith G, Hoke C, 1991, Dengue-1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicits virus-neutralizing antibody in mice and protects them from virus challenge, Am J Trop Med Hyg, 45: 159-67

Ramasamy R, Richardson NE, Feinstein A, 1976, The specificity of the Fc receptor on murine lymphocytes for immunoglobulins of the IgG and IgM classes, Immunology, 30:851-8

Ramirez-Ronda CH, Garcia CD, 1994, Dengue in the Western Hemisphere, Infect Dis Clin North Am, 8: 107-28

Randolph VB, Winkler G, Stollar V, 1990, Acidotropic amines inhibit proteolytic processing of flavivirus prM protein, Virology, 174: 450-8

Rapaport D, Ovadia M, Shai Y, 1995, A synthetic peptide corresponding to a conserved heptad repeat domain is a potent inhibitor of Sendai virus-cell fusion: an emerging similarity with functional domains of other viruses, EMBO J, 14: 5524-31

Re MC, Furlini G, Zauli G, La-Placa M, 1994, Human immunodeficiency virus type 1 (HIV-1) and human hematopoietic progenitor cells, Arch Virol, 137: 1-23,

Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC, 1995, The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution, Nature, 375: 291-8

Rice CM, Flaviviridae: the viruses and their replication, In *Fields Virology*, eds. Fields BN, Knipe DM, Howley PM, Lippincott-Raven, Philadelphia-New York (1996) Volume 1, pp. 931-959

Rigg RJ, Carver AS, Dimmock NJ, 1989, IgG-neutralized influenza virus undergoes primary, but not secondary uncoating in vivo, J Gen Virol, 70: 2097-109

Roehrig JT, Hunt AR, Kinney RM, Mathews JH, 1988, In vitro mechanisms of monoclonal antibody neutralization of alphaviruses, Virology, 165: 66-73

Roehrig JT, Johnson AJ, Hunt AR, Bolin RA, Chu MC, 1990, Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation, Virology, 177: 668-75

Roelvink PW, Kovesdi I, Wickham TJ, 1996, Comparative analysis of adenovirus fiber-cell interaction: adenovirus type 2 (Ad2) and Ad9 utilize the same cellular fiber receptor but use different binding strategies for attachment, J Virol, 70: 7614-21,

Roitsch T, Lehle L, 1989, Requirements for efficient in vitro transcription and translation: a study using yeast invertase as a probe, Biochim Biophys Acta, 1009: 19-26

Roos E, 1991, Adhesion molecules in lymphoma metastasis, Cancer Metastasis Rev, 10: 33-48

Rosen L, Shroyer DA, Tesh RB, Freier JE, Lien JC, 1983, Transovarial transmission of dengue viruses by mosquitoes: Aedes albopictus and Aedes aegypti, Am J Trop Med Hyg, 32: 1108-19

Rosen L, 1986, Dengue in Greece in 1927 and 1928 and the pathogenesis of dengue hemorrhagic fever: new data and a different conclusion, Am J Trop Med Hyg, 35: 642-53

Rosen L, Khin MM, 1989, Recovery of virus from the liver of children with fatal dengue: reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever, Res Virol, 140: 351-60

Rothlein R, Dustin ML, Marlin SD, Springer TA, 1986, A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1, J Immunol, 137: 1270-4

Rothwell SW, Putnak R, La-Russa VF, 1996, Dengue-2 virus infection of human bone marrow: characterization of dengue-2 antigen-positive stromal cells, Am J Trop Med Hyg, 54: 503-10

Rucker J, Samson M, Doranz BJ, Libert F, Berson JF, Yi Y, Smyth RJ, Collman RG, Broder CC, Vassart G, Doms RW, Parmentier M, 1996, Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity, Cell, 87: 437-46

Ruiz-Linares A, Cahour A, Despres P, Girard M, Bouloy M, 1989, Processing of yellow fever virus polyprotein: role of cellular proteases in maturation of the structural proteins, J Virol, 63: 4199-209

Ruoslahti E, Pierschbacher MD, 1987, New perspectives in cell adhesion: RGD and integrins, Science, 238: 491-7

Sadhukhan R, Sen I, 1996, Different glycosylation requirements for the synthesis of enzymatically active angiotensin-converting enzyme in mammalian cells and yeast, J Biol Chem, 271: 6429-34

Sanchez IJ, Ruiz BH, 1996, A single nucleotide change in the E protein gene of dengue virus 2 Mexican strain affects neurovirulence in mice, J Gen Virol, 77: 2541-5

Schlesinger JJ, Foltzer M, Chapman S, 1993, The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice, Virology, 192: 132-41

Schnaper HW, 1996, Integrin receptors, the cytoskeleton, and glomerular cell function, Pediatr Nephrol, 10: 523-8

Scott RM, Russell PK, 1972, Complement fixation blocking activity of anti-dengue IgM antibody, J Immunol, 109: 875-7

Scott RM, Nimmannitya S, Bancroft WH, Mansuwan P, 1976, Shock syndrome in primary dengue infections, Am J Trop Med Hyg, 25: 866-74

Seya T, Kurita M, Iwata K, Yanagi Y, Tanaka K, Shida K, Hatanaka M, Matsumoto M, Jun S, Hirano A, Ueda S, Nagasawa S, 1997, The CD46 transmembrane domain is required for efficient formation of measles-virus-mediated syncytium, Biochem J, 322: 135-44

Shankar V, Pichan P, Eddy RL Jr, Tonk V, Nowak N, Sait SN, Shows TB, Schultz RE, Gotway G, Elkins RC, Gilmore MS, Sachdev GP, 1997, Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxy terminus, Am J Respir Cell Mol Biol, 16: 232-41

Sheu SY, Lo SJ, 1995, Deletion or alteration of hydrophobic amino acids at the first and the third transmembrane domains of hepatitis B surface antigen enhances its production in Escherichia coli, Gene, 160: 179-84

Shibahara K, Hotta H, Katayama Y, Homma M, 1994, Increased binding activity of measles virus to monkey red blood cells after long-term passage in Vero cell cultures, J Gen Virol, 75: 3511-6

Shouval D, Ilan Y, Adler R, Deepen R, Panet A, Even Chen Z, Gorecki M, Gerlich WH, 1994, Improved immunogenicity in mice of a mammalian cell-derived recombinant hepatitis B vaccine containing pre-S1 and pre-S2 antigens as compared with conventional yeast-derived vaccines, Vaccine, 12: 1453-9

Sisk WP, Bradley JD, Kingsley D, Patterson TA, 1992, Deletion of hydrophobic domains of viral glycoproteins increases the level of their production in Escherichia coli, Gene, 112: 157-62

Sittisombut N, Maneekarn N, Kanjanahaluethai A, Kasinrerk W, Viputtikul K, Supawadee J, 1995, Lack of augmenting effect of interferon-gamma on dengue virus multiplication in human peripheral blood monocytes, J Med Virol, 45: 43-9

Smith TJ, Olson NH, Cheng RH, Liu H, Chase ES, Lee WM, Leippe DM, Mosser AG, Rueckert RR, Baker TS, 1993, Structure of human rhinovirus complexed with Fab fragments from a neutralizing antibody, J Virol, 67: 1148-58

Smucny JJ, Kelly EP, Macarthy PO, King AD, 1995, Murine immunoglobulin G subclass responses following immunization with live dengue virus or a recombinant dengue envelope protein, Am J Trop Med Hyg, 53: 432-7

Songco RS, Hayes CG, Leus CD, Manaloto CO, 1987, Dengue fever/dengue haemorrhagic fever in Filipino children: clinical experience during the 1983-1984 epidemic, Southeast Asian J Trop Med Public Health, 18: 284-90

Sonnenberg A, 1993, Integrins and their ligands, Curr Top Microbiol Immunol, 184: 7-35

Sriurairatna S, Bhamarapravati N, Phalavadhtana O, 1973, Dengue virus infection of mice: morphology and morphogenesis of dengue type-2 virus in suckling mouse neurones, Infect Immun, 8: 1017-28

Sriurairatna S, Bhamarapravati N, 1977, Replication of dengue-2 virus in Aedes albopictus mosquitoes, An electron microscopic study, Am J Trop Med Hyg, 26: 1199-205

Srivastava AK, Putnak JR, Warren RL, Hoke CH Jr, 1995, Mice immunized with a dengue type 2 virus E and NS1 fusion protein made in Escherichia coli are protected against lethal dengue virus infection, Vaccine, 13: 1251-8

Staropoli I, Clement JM, Frenkiel MP, Hofnung M, Deubel V, 1996, Dengue virus envelope glycoprotein can be secreted from insect cells as a fusion with the maltose-binding protein, J Virol Methods, 56: 179-89

Staunton DE, Merluzzi VJ, Rothlein R, Barton R, Marlin SD, Springer TA, 1989, A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses, Cell, 56: 849-53

Stocks CE, Lobigs M, 1995, Posttranslational signal peptidase cleavage at the flavivirus C-prM junction in vitro, J Virol, 69: 8123-6

Stuart D, Gouet P, 1995, Viral envelope glycoproteins swing into action, Structure, 3: 645-8

Sudbery PE, 1996, The expression of recombinant proteins in yeasts, Curr Opin Biotechnol, 7: 517-24

Sullivan JL, Woda BA, Herrod HG, Koh G, Rivara FP, Mulder C, 1985, Epstein-Barr virus-associated hemophagocytic syndrome: virological and immunopathological studies, Blood, 65: 1097-104

Sullivan PS, McDonald TP, 1995, Evaluation of murine leukemia virus infection as a model for thrombocytopenia of HIV/AIDS: mechanism of thrombocytopenia and modulation of thrombocytopenia by thrombopoietin, AIDS Res Hum Retroviruses, 11: 837-42

Summers PL, Cohen WH, Ruiz MM, Hase T, Eckels KH, 1989, Flaviviruses can mediate fusion from without in Aedes albopictus mosquito cell cultures, Virus Res, 12: 383-92

Tabor S, Richardson CC, 1985, A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes, Proc Natl Acad Sci U S A, 82: 1074-8

Takeda A, Sweet RW, Ennis FA, 1990, Two receptors are required for antibody-dependent enhancement of human immunodeficiency virus type 1 infection: CD4 and Fc gamma R, J Virol, 64: 5605-10

Tan BH, Fu J, Sugrue RJ, Yap EH, Chan YC, Tan YH, 1996, Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity, Virology, 216: 317-25

Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, Davis SW, van-der-Hoeven J, Meignier B, Riviere M, et al, 1992, NYVAC: a highly attenuated strain of vaccinia virus, Virology, 188: 217-32

Taylor HP, Dimmock NJ, 1985, Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG, J Exp Med, 161: 198-209

Tenner AJ, 1993, Functional aspects of the C1q receptors, Behring Inst Mitt,: 241-53

Thant KZ, Morita K, Igarashi A, 1996, Detection of the disease severity-related molecular differences among new Thai dengue-2 isolates in 1993, based on their structural proteins and major non-structural protein NS1 sequences, Microbiol Immunol, 40: 205-16

Tignor GH, Kende M, Hanham CA, 1992, Chemotherapeutic prevention of complications caused by vaccinia virus-vectored immunogen, Ann N Y Acad Sci, 653: 334-43

Trent DW, Monath TP, Bowen GS, Vorndam AV, Cropp CB, Kemp GE, 1980, Variation among strains of St, Louis encephalitis virus: basis for a genetic, pathogenetic, and epidemiologic classification, Ann N Y Acad Sci, 354: 219-37

Trirawatanapong T, Chandran B, Putnak R, Padmanabhan R, 1992, Mapping of a region of dengue virus type-2 glycoprotein required for binding by a neutralizing monoclonal antibody, Gene, 116: 139-50

Trpis M, 1978, Genetics of hematophagy and autogeny in the Aedes scutellaris complex (Diptera: Culicidae), J Med Entomol, 15: 73-80

Trpis M, Hausermann W, 1986, Dispersal and other population parameters of Aedes aegypti in an African village and their possible significance in epidemiology of vector-borne diseases, Am J Trop Med Hyg, 35: 1263-79

Tuchinda M, Dhorranintra B, Tuchinda P, 1977, Histamine content in 24-hour urine in patients with dengue haemorrhagic fever, Southeast Asian J Trop Med Public Health, 8: 80-3

Vancura S, Steiner M, 1987, Identification of Fc and F(ab')2 IgG receptors on human platelets, Proc Natl Acad Sci, 84:3575-9

Van-de-Stolpe A, Jacobs N, Hage WJ, Tertoolen L, van-Kooyk Y, Novakova IR, de-Witte T, 1996, Fibrinogen binding to ICAM-1 on EA,hy 926 endothelial cells is dependent on an intact cytoskeleton, Thromb Haemost, 75: 182-9

Van-der-Lelie J, Lange JM, Vos JJ, van-Dalen CM, Danner SA, von-dem-Borne AE, 1987, Autoimmunity against blood cells in human immunodeficiency-virus (HIV) infection, Br J Haematol, 67: 109-14

Varma MG, Pudney M, Leake CJ, Wallace C, 1974, Proceedings: Cytopathic effect of dengue-2 virus on an established mosquito cell-line, Trans R Soc Trop Med Hyg, 68: 12

Vaughn DW, Hoke CH Jr, Yoksan S, LaChance R, Innis BL, Rice RM, Bhamarapravati N, 1996, Testing of a dengue 2 live-attenuated vaccine (strain 16681 PDK 53) in ten American volunteers, Vaccine, 14: 329-36

Venugopal K, Gould EA, 1994, Towards a new generation of flavivirus vaccines, Vaccine, 12: 966-75

Vishnuvardhan D, Kakiuchi N, Urvil PT, Shimotohno K, Kumar PK, Nishikawa S, 1997, Expression of highly active recombinant NS3 protease domain of hepatitis C virus in E, coli, Expression of highly active recombinant NS3 protease domain of hepatitis C virus in E, coli, FEBS Lett, 402: 209-12

Voth WP, Lee CS, 1989, Improved yield of helper P-element by switching its vector from pBR322 to pBluescript, Nucleic Acids Res, 17 : 6424

Wan M, Takagi M, Loh BN, Imanaka T, 1995, Comparison of HIV-1 protease expression in different fusion forms, Biochem Mol Biol Int, 36: 411-9

Wang S, He R, Patarapotikul J, Innis BL, Anderson R, 1995, Antibody-enhanced binding of dengue-2 virus to human platelets, Virology, 213: 254-7

Ward MA, Burgess NR, 1993, Aedes albopictus--a new disease vector for Europe? J R Army Med Corps, 139: 109-11

Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A, 1987, Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus, Am J Trop Med Hyg, 36: 143-52

Wengler G, Wengler G, 1993, The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity, Virology, 197: 265-73

Wengler G, Gros C, Wengler G, 1996, Analyses of the role of structural changes in the regulation of uncoating and assembly of alphavirus cores, Virology, 222: 123-32

Westaway EG, Brinton MA, Gaidamovich SYa, Horzinek MC, Igarashi A, Kaariainen L, Lvov DK, Porterfield JS, Russell PK, Trent DW, 1985, Flaviviridae, Intervirology, 24: 183-92

Whitton JL and Oldstone MB, 1996, Immune Response to Viruses, Fields Virology, Vol, I, 345-374

Wickham TJ, Mathias P, Cheresh DA, Nemerow GR, 1993, Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment, Cell, 73: 309-19

Wickham TJ, Carrion ME, Kovesdi I, 1995, Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs, Gene Ther, 2: 750-6

Williams KL, Emslie KR, Slade MB, ,1995, Recombinant glycoprotein production in the slime mould Dictyostelium discoideum, Curr Opin Biotechnol, 6: 538-42

Winkler G, Heinz FX, Kunz C, 1987, Studies on the glycosylation of flavivirus E proteins and the role of carbohydrate in antigenic structure, Virology, 159: 237-43

Wittesjo B, Eitrem R, Niklasson B, 1993, Dengue fever among Swedish tourists, Scand J Infect Dis, 25: 699-704

Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C, Sodroski J, 1996, CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5, Nature, 384: 179-83

WuDunn D, Spear PG, 1989, Initial interaction of herpes simplex virus with cells is binding to heparan sulfate, J Virol, 63: 52-8

Yoshida Y, Emi N, Hamada H, 1997, VSV-G-pseudotyped retroviral packaging through adenovirus-mediated inducible gene expression, Biochem Biophys Res Commun, 232: 379-82

Zhang YM, Hayes EP, McCarty TC, Dubois DR, Summers PL, Eckels KH, Chanock RM, Lai CJ, 1988, Immunization of mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis, J Virol, 62: 3027-31

Zhao BT, Prince G, Horswood R, Eckels K, Summers P, Chanock R, Lai CJ, 1987, Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus, J Virol, 61: 4019-22

Zin K, Morita K, Igarashi A, 1995, Sequences of E/NS1 gene junction from four dengue-2 viruses of northeastern Thailand and their evolutionary relationships with other dengue-2 viruses, Microbiol Immunol, 39: 581-90

Zon LI, Arkin C, Groopman JE, 1987, Haematologic manifestations of the human immune deficiency virus (HIV), Br J Haematol, 66: 251-6

Zucker Franklin D, 1994, The effect of viral infections on platelets and megakaryocytes, Semin Hematol, 31: 329-37

Zucker-Franklin D, Termin CS, Cooper MC, 1989, tructural changes in the megakaryocytes of patients infected with the human immune deficiency virus (HIV-1), Am J Pathol, 134: 1295-303

Zulkarnain E, Hotta S, Takegami T, 1994, Molecular comparison of dengue type 1 Mochizuki strain virus and other selected viruses concerning nucleotide and amino acid sequences of genomic RNA: a consideration of viral epidemiology and variation, Microbiol Immunol, 38: 581-5

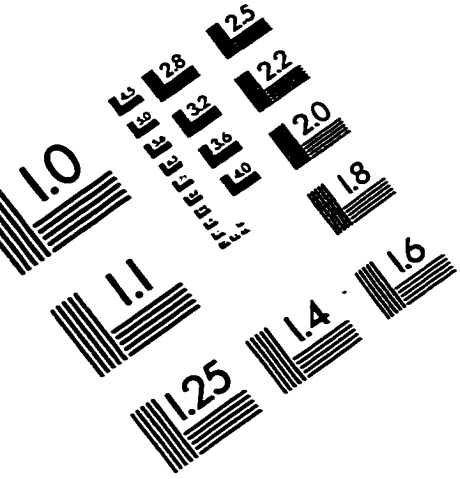
APPENDIX 1.

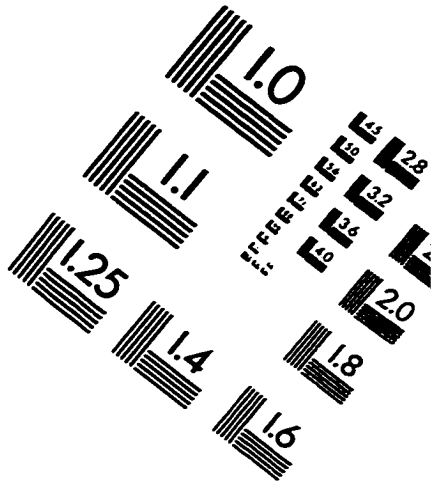
Characteristics of patient sera

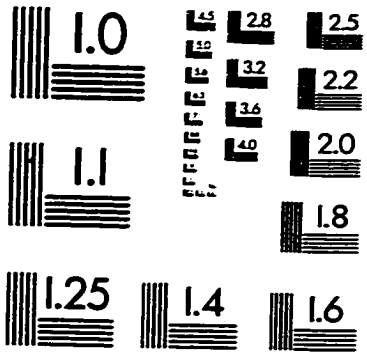
Patient	Age	Status	Anti-dengue EIA		Anti-dengue-2 activities	
			IgM	IgG	Neutralization	Block virus-cell attachment
0647	9m	0	10	5	8.6 ± 3.6	10.1 ± 2.6
0180	4m	0	10	15	12.6 ± 4.3	8.7 ± 8.7
0356	2m	0	27	9	6.7 ± 3.3	16.1 ± 2.7
0171	7m	0	39	0	13.4 ± 6.4	5.7 ± 5.7
0571	6m	0	40	0	16.8 ± 6.8	17.8 ± 8.0
0897	5m	0	46	0	27.5 ± 9.3	11.8 ± 0.4
0160	5m	0	46	4	17.9 ± 5.1	6.4 ± 6.4
0072	7m	0	47	0	12.8 ± 5.3	7.6 ± 5.0
0847	7m	0	91	48	38.5 ± 10.7	36.2 ± 5.6
1138	9m	0	124	0	25.4 ± 13.0	12.0 ± 6.1
1055	11m	0	150	6	20.4 ± 9.5	15.2 ± 9.9
1005	7m	0	174	7	11.5 ± 4.9	3.5 ± 3.5
0006	3	1	3	1	12.8 ± 7.4	5.3 ± 5.3
0546	8	1	32	5	11.6 ± 7.4	9.0 ± 0.4
1133	7	2	0	5	22.3 ± 16.3	5.3 ± 4.9
0083	12	2	0	15	38.0 ± 18.8	39.2 ± 4.4
0984	6	2	0	111	38.9 ± 7.6	40.6 ± 16.0
0510	11	2	0	142	89.7 ± 6.5	75.5 ± 6.4
1056	7	2	1	90	78.3 ± 8.8	75.2 ± 8.5
0939	2	2	2	26	20.3 ± 14.8	15.9 ± 0.9
1060	9	2	4	14	5.8 ± 4.1	16.6 ± 2.0
0059	3	2	4	91	87.7 ± 20.3	71.7 ± 12.3
0994	10	2	6	16	19.9 ± 8.3	6.6 ± 2.7
0857	4	2	6	29	43.2 ± 16.5	34.9 ± 9.0
0915	12	2	7	24	13.6 ± 12.7	20.4 ± 8.1
1136	11	2	8	18	27.6 ± 13.5	36.1 ± 3.6
0959	6	2	8	20	65.5 ± 19.0	74.6 ± 8.0
0063	10	2	8	120	45.7 ± 10.0	26.8 ± 1.6
1161	14	2	9	27	44.7 ± 8.0	55.8 ± 5.2
0127	8	2	9	34	36.4 ± 13.6	23.0 ± 2.1
0121	7	2	10	13	18.3 ± 5.6	12.2 ± 1.0
1054	10	2	11	204	81.5 ± 18.7	54.5 ± 11.8
1129	4	2	12	11	24.1 ± 11.6	8.7 ± 2.5
0980	6	2	12	233	61.3 ± 16.8	52.1 ± 3.7
1070	12	2	13	11	13.2 ± 5.5	9.7 ± 3.5
1192	7	2	14	38	18.6 ± 8.4	21.0 ± 17.6
0956	3	2	14	53	76.9 ± 30.7	69.1 ± 2.0
0855	13	2	15	68	55.1 ± 13.1	62.8 ± 4.5
1073	12	2	15	131	33.6 ± 11.8	38.2 ± 2.1
0066	10	2	16	13	8.5 ± 5.7	5.7 ± 2.0
1164	4	2	18	45	24.6 ± 10.9	30.0 ± 1.4
1083	13	2	19	9	17.4 ± 6.7	22.8 ± 2.3
1026	9	2	19	127	29.3 ± 14.1	23.2 ± 3.2
1102	6	2	20	24	39.7 ± 16.6	52.6 ± 15.2
1191	10	2	20	42	43.2 ± 8.7	36.1 ± 0.8
1068	13	2	23	126.	38.4 ± 17.4	26.0 ± 8.8
0118	7	2	26	18	28.5 ± 7.7	21.3 ± 12.1
1086	10	2	26	130	39.8 ± 13.5	23.4 ± 4.8
1062	10	2	28	209	67.8 ± 28.6	43.6 ± 4.1
1148	1	2	29	68	35.4 ± 16.2	45.6 ± 3.7
1132	4	2	31	53	33.3 ± 8.9	45.7 ± 9.4
1082	10	2	35	204	91.6 ± 28.8	76.3 ± 14.5
1113	8	2	37	128	68.3 ± 17.7	63.3 ± 12.7
1099	5	2	45	136	57.4 ± 15.8	73.4 ± 14.2
0109	10	2	46	58	38.8 ± 12.7	31.7 ± 10.1
0867	7	2	47	174	51.3 ± 13.8	44.4 ± 9.4
1075	11	2	57	34	34.3 ± 8.4	38.5 ± 8.3
1051	8	2	64	79	32.4 ± 14.8	25.2 ± 11.0
0105	9	2	69	175	62.8 ± 24.4	51.8 ± 4.1
0933	1	2	147	112	42.6 ± 17.2	56.0 ± 7.6
0999	4	2	169	165	69.0 ± 11.1	73.2 ± 6.1

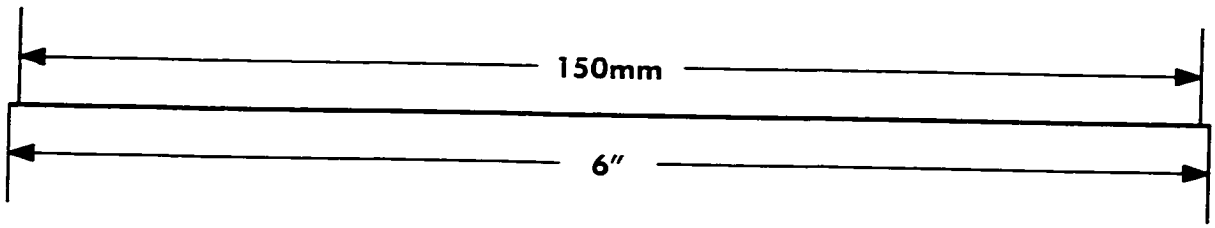
Status designations: 0, infants not previously infected with dengue virus and presumably bearing maternal antibodies; 1, older children in which infection elicited a primary antibody response to dengue antigens; 2, older children in which infection elicited a secondary antibody response to dengue antigens. Children's ages are recorded in years unless otherwise indicated (m=months). IgM and IgG are reported as EIA absorbance units (Innis et al, 1989) in sera diluted 1:100. Virus neutralizations and blocking of virus-cell attachment performed on sera diluted 1:100. Neutralization and virus-cell attachment blocking scores (mean ± standard error) are reported on a linear basis, ranging from 0 to 100% of maximum activity

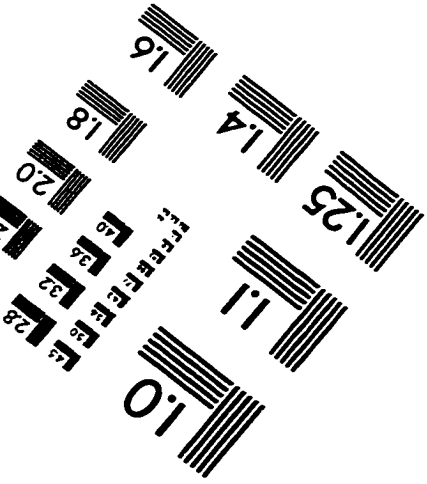
IMAGE EVALUATION TEST TARGET (QA-3)

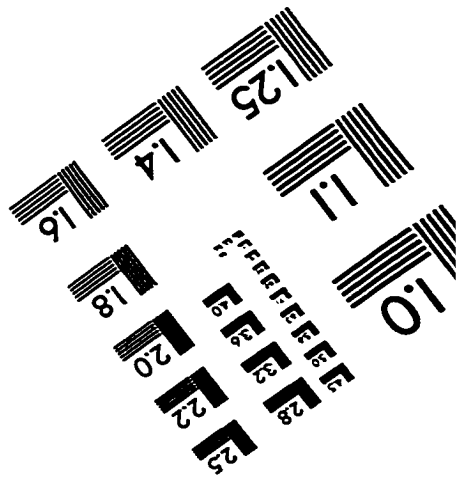












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989