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Progress Report No 1

Studies for Dengue Viral Antigens and Antibodies in sera
of Dengue Hemorrhagic Fever (DHF) Patients

A research project

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Submitted by

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Introduction

Dengue infection is caused by four distinct types of dengue viruses. Clinical manifestations of infection range from inapparent infection and classical dengue fever to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Classical dengue fever is a mild form of the disease and characterized by sudden onset of fever, headache, lymphadenopathy, myalgias and petichial hemorrhage or maculopapular rash. Hemorrhagic fever or shock are severe-disease manifestations that may be fatal, even when treatment is given (1). These severe forms of disease start with high fever but progress to nausea, vomiting, abdominal pain, hepatosplenomegaly, epistaxis, hematemesis, melena, and petichial hemorrhage. Shock usually occurs from day 5 to day 7 after the onset of fever.

At least 2 hypotheses for the pathogenesis of DHF/DSS have been proposed. The first hypothesis (dengue virus virulence) states that different strains of dengue viruses of all four serotypes possess varying degrees of virulence and vary in their pathogenic potential (2). The second hypothesis states that the severity of shock occurring in this disease is due to an immunopathologic mechanism caused by a second, heterotypic type, dengue infection. Timing of the second infection is also proposed to be critical in the causation of this immunological consequence (3). This later hypothesis is called the

second infection hypothesis.

The possibility that immunopathological processes are the factors determining the severity of clinical manifestations is suggested by the finding that most DHF/DSS are secondary, heterologous dengue virus infection (3,4,5). Observation of the anamnestic antibody responses with high concentrations of IgG antibody in sera of patients during their acute phase support this hypothesis (6). Because there is evidence that infectious virus and antibodies are present at the same time, antigen-antibody complexes have been postulated to exist in dengue hemorrhagic fever (5,7). Patients with DSS have low levels of complement C₃, C₄ and factor B, suggesting that complement consumption occurs in vivo via both classical and alternative pathways (7,8,9,10). The levels of depression of complement correlate with severity of disease and the depletion of complement is not due to extravasation (8). The complement consumption parallels signs which occurs in dengue hemorrhagic fever but not in dengue fever. These signs are an increase of vascular permeability, an decrease in plasma volume, hypotension, thrombocytopenia, and a hemorrhagic diathesis. These symptoms have rapid onset, and are shortlasting, and thus must be a consequence of rapidly reversible pathophysiologic processes. Histopathological vascular lesions are not found which suggested that the role of active mediators is important for generation of increased

vascular permeability (11,12). These circumstances lead to a hypothesis that an activation of the complement system yielding the formation of anaphylatoxins, C_{3a} and C_{5a} enhance the shock syndrome (8). Evidence of disseminated intravascular coagulation (DIC); low platelet counts, reduced fibrinogen levels, presence of fibrin degradation products (FDP) and reduced Hagemann factor, have been observed (8). These phenomena appear in the mild forms and are increasingly present in severe forms, suggesting their primary role in the shock syndrome. The changes in both complement and clotting systems are well correlated with the severity in cases of DHF/DSS (8). Since complement could initiate blood coagulation through platelets, Observed DIC may be a consequence of intravascular complement activations. Plasma enzyme inhibitors may be depleted due to the activation of proteolytic enzymes from the complement, coagulation, and possibly the kinin system. An imbalance between activated enzymes and inhibitors may produce an increase of vascular permeability and lead to development of a shock state.

The role of the immune response (at sub-neutralizing level) in enhancing dengue virus infection is suggested to be a major determinant of the development of hemorrhage and shock (13). In vitro, antibodies act through an Fc-mediated mechanism to enhance infection and virus yield from human monocyte culture. In vivo, passive antibody at subneutralizing level causes higher levels of viremia in rhesus monkeys. Shock in infants is postulated to be due

to maternal antibody in the same manner. Even though there are data that dengue viruses can cause primary hemorrhagic fever, there is more evidence supporting the importance of secondary antibody-mediated enhancement. These antibodies come from infection with other dengue serotypes or from passively transferred antibodies. e.g. maternal antibody. Thereby a host-virus interaction dependent on the humoral immune response seems to be very much tied to the severity of this disease.

Objectives

To investigate our general hypothesis that more severe forms of dengue infection have an immunopathogenesis, we propose to:

1. Completely describe the pattern of antigen reactivity of acute and convalescent sera from patients with all clinical degrees of dengue infection, i.e. asymptomatic (subclinical), dengue fever, dengue hemorrhagic fever and dengue shock syndrome. Additionally, the humoral response to attenuated dengue virus infection (vaccine volunteers) will be similarly described.

2. Completely describe, using collections of serial sera or plasma from patients with well-characterized clinical dengue syndromes, the type, the quantity and kinetics of circulating dengue antigens.

3. On the basis of these data, identify a viral antigen or anti-viral peptide antibody whose presence or absence is highly associated with the occurrence of severe symptoms.

4. Purify dengue viral polypeptide (s) using preparative gel electrophoresis and/or Fast protein liquid chromatography (FPLC) system and further reduce the peptide chain to fragments by either enzymes or chemicals.

5. Determine biologic properties of the purified proteins, i.e. immunogenic property, protective inducing and complement activation ability.

6. Develop a rapid, simple but sensitive laboratory test based on the conclusion above (#3) to prospectively select from the entire set of febrile children those likely to progress to a severe form of dengue infection. Such a test, in all likelihood, will require the preparative purification of a biologically active dengue peptide or peptide fragment for use either as a test antigen or to produce, in animals, a diagnostic antibody reagent.

Materials and Methods

A. Preparation of dengue viral antigen

A.1. Conventional systems for dengue viral antigen preparation

A.1.a. infected suckling mouse brain suspension

A.1.b. infected cell cultures such as infected mosquito cell (C6/36) or Rhesus monkey kidney cell (LLC-MK2)

A.2. New approaches

A.2.a. preparation of dengue viral polypeptides and peptide fragments.

A.2.a.1. Preparative gel electrophoresis to isolate viral proteins.

A.2.a.2. Western Blotting.

A.2.a.3. Proteolytic digestion and chemical cleavage to identify immunogenic peptides from interesting proteins e.g. NV3 etc.

B. Characterization of prepared dengue antigen

B.1. by polyacrylamide gel electrophoresis

B.2. Viral antigen strips/Enzymeimmuno assay (VAS/EIA)

B.3. by Crossed immunoelectrophoresis

C. Preparation of anti-dengue sera

C.1. antibody raised in rabbits using different conventional sources for antigen preparation as mentioned in A.1. as immunogens

- C.2. trial using each dengue viral polypeptide or its fragments from A.2 as an immunogen
- C.3. human hyperimmune sera collected from dengue hemorrhagic fever (DHF) patients during acute and convalescent phases
- C.4. monoclonal antibody (Mab)
- D. Characterization of prepared anti-dengue sera
 - D.1. by Viral antigen strips/Enzymeimmunoassay (VAS/EIA)
 - D.2. by Crossed immunoelectrophoresis
- E. Collection and Confirmation of DHF and NON-DHF cases by available diagnostic tests
 - E.1. Hemagglutination Inhibition test
 - E.2. Viral isolation
 - E.3. Crossed Immunoelectrophoresis
 - E.4. VAS/EIA test
- F. Detection for circulating dengue viral antigen (s), antibodies and complement split products
 - F.1. patients
 - Classical dengue fever patients
 - DHF/DSS patients
 - patients of other infectious diseases
 - F.2. healthy individual
 - adults (series of tests if possible)
 - children (in series if possible)
 - cord blood for maternal antibody survey
 - Vaccine trial volunteers.

G. Determine biologic interaction of polypeptides and polypeptide fragments

G.1. immunogenic property and protective ability

G.1.a. Immunize rabbit to elicit antibody

G.1.b. In Vivo test for protective ability using simple mouse model or rabbit model.

G.1.c. In Vitro test using monocyte culture with immunocytochemistry test to determine rate of infectivity by counting infected cells.

G.2. Complement activation capability

G.2.a. In vitro test using crossed immunoelectrophoresis test for determining complement split products.

H. Data collection and analysis

Results

Our first report covers the period between the month of July and December of 1987. All Laboratory instruments were ordered and now some already arrived such as the Fast protein liquid chromatography system (FPLC), the Enzymeimmuno assay reader (EIA reader), and the electrophoretic apparatus.

According to our activity plan (Table I, Page 14), all activities listed were already began except the item G. Preparation of dengue viral antigen with conventional techniques (A.1.a and A.1.b) and the characterization by methods listed (B.1, B.2, and B.3) were also started. With crossed immunoelectrophoresis, we reported the use of this technique to detect the third component of complement split products (C3sp) in DHF patients (13,14), and we are now applying this technique to quantitate and qualitate dengue antigen (s) and antibodies from different source. This work is now in progress in our laboratory.

In order to quantitate dengue antibodies at the molecular level (antibodies to dengue viral polypeptides), we proposed to employ 3 methods together. These are the polyacrylamide gel electrophoresis (PAGE) to separate viral proteins into single polypeptide chains, western blot technique to transfer the separated polypeptides onto the surface of nitrocellulose membrane, and enzymeimmunoassay (EIA) to detect dengue polypeptides and

the specific antibodies toward these separated/polypeptides. We managed to employ this technique and called the process as "the viral antigen strips/enzymeimmunoassay" or VAS/EIA. Sensitivity and specificity tests were done and reported by our team (15). With VAS/EIA, we can detect specific dengue antibodies toward polypeptides at following molecular weight; 130,000-110,000, 98,000-91,000, 85,000-77,000, 71,000-68,000, 66,000-64,000, 60,000-54,000, 55,000-50,000, 50,000-48,000, 46,000-44,000, 30,000-28,000, 26,000-24,000, 21,000-19,000 and 14,000 daltons. After publication, we received the monoclonal antibody (Mab) with specific activity toward envelope protein (E) from AFRIMS. This Mab confirmed that the antibody to polypeptides between molecular weight of 60,000-54,000 daltons is the antibody to E-glycoprotein. The molecular weight of E-protein were varies among 4 different dengue strains. Dengue type 4 demonstrated the smallest size envelope glycoprotein by moving faster than the other dengue types. Envelope protein of dengue type 3 move slower than dengue type 4 but faster than dengue type 1 and type 2. Dengue type 1 and type 2 move closest in an electric field. However dengue type 1 showed the shortest moving distance which indicated that its envelope polypeptide chain probably is larger than the others. By comparison with antibody to E-glycoprotein, the rest of antibodies to other polypeptides can be estimated and we are now preparing this manuscript for publication.

Two approaches were planned to separate and purify dengue viral proteins. The first one using FPLC system as a tool and the other will employ the preparative gel electrophoresis system. Proteins obtained from these two techniques will be in minute amount and will demand a very sensitive technique to qualitate and quantitate. We proposed the use of Enzyme-linked immunosorbant assay or ELISA and we already established this test in our laboratory to detect both minute amount of proteins and antibodies as well.

Source of dengue viral antigens is now from the infected suckling mouse brain. Tissue culture room was set up eventhough the laminar flow and the inverted microscope are not arrived yet. With the use of existing equipment, we are now infesting dengue viruses in mosquito cell line (C6/36) and monkey kidney cell line (LLC-MK2). This source of antigen will be compared with the mouse brain source after we can prepare and titrate them properly.

Another approach is to collect patients sera, diagnose by available serology tests, detect antibodies to dengue polypeptides, detect dengue antigen, and detect complement split products. This period we can collect over 500 sera from 150 clinically suspected dengue hemorrhagic fever cases. Sera from healthy population were also obtained from cord blood, children infected with other diseases and adults.

Remarks

We can manage to develop all experiments faster than we've planned (see attached activity plan on page-14). This leads us to face the shortage of funds to purchase chemicals and reagents for further application with clinical specimens. According to our plan, this fund was requested again in July 1988 (6 months from now). Due to outbreak of dengue viral infection in Bangkok in 1987, we can collect good series of sera from 150 patients and collect at least 200 specimens from children infected with other diseases. Again this is another unexpected and leads to shortage of proper storage place which should be the low temperature freezer (-85 °C) and the fund will be available in July-Sept period this year.

During this period we published one paper and enclosed herewith in picture's section (Page 25).

Activity Plan
 USAID grant No. 936-5542-G-00-7029-00
 "Studies for Dengue Viral Antigens and Antibodies in Sera of Dengue Hemorrhagic Fever (DHF) Patients"

Activity	1987		1988		1989		1990			
	July-Sept	Oct-Dec	Jan-Mar	Apr-June	July-Sept	Oct-Dec	Jan-Mar	Apr-June	July-Sept	Oct-Dec
A. Preparation of Dengue viral antigen										
A.1. Conventional systems										
A.1.a. Infected suckling mouse brain suspension										
A.1.b. Infected cell cultures										
-CS/36										
-LLC/ME2										
A.2. New approaches										
A.2.a. Preparation of dengue viral polypeptides and peptide fragments										
A.2.a.1. Preparative gel electrophoresis										
A.2.a.2. Western blot										
A.2.a.3. Proteolytic digestion and chemical cleavage										
B. Characterization of prepared dengue antigen										
B.1. Polyacrylamide gel electrophoresis										
B.2. VAS/EIA										
B.3. Crossed immunoelectrophoresis										
C. Preparation of anti-dengue sera										
C.1. Antisera raised in rabbit										
C.2. Dengue viral polypeptide or its fragments as an immunogen										
C.3. Human hyperimmune sera collected from DHF/DSS patients										
C.4. Monoclonal antibody										
D. Characterization of anti-dengue sera										
D.1. By VAS/EIA										
D.2. By Crossed immunoelectrophoresis										
E. Collection and Confirmation of DHF and NON-DHF cases										
E.1. Hemagglutination inhibition test (HI test)										
E.2. Viral isolation										
E.3. Crossed immunoelectrophoresis										
E.4. VAS/EIA										
F. Detection for circulating dengue viral antigen(s), antibodies, and complement split products										
F.1. Patients										
F.2. Healthy individual										
G. Determination for biological interaction of polypeptides and peptide fragments										
G.1. Test for immunogenic and protective ability										
G.2. Test for complement activation capability										
H. Data collection analysis and reports										

_____ : project activity plan
 _____ : work done
 - - - - - : work plan

Workplan for the next period

According to our activity plan (page 14), detection for circulating dengue viral antigen (s), antibodies, and complement split products (item F) should begin this period, but these works already started last period. We shall continue as planned (work plan on page 14) and hope that we can start making certain analysis of the obtained results. Since all techniques were already established and the clinical specimens are available, we shall test them as quick as possible, and hope that we may get some clue to understand more about immunopathologic mechanism of this disease. We are convincing that we can cope with all works as planned.

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Figures

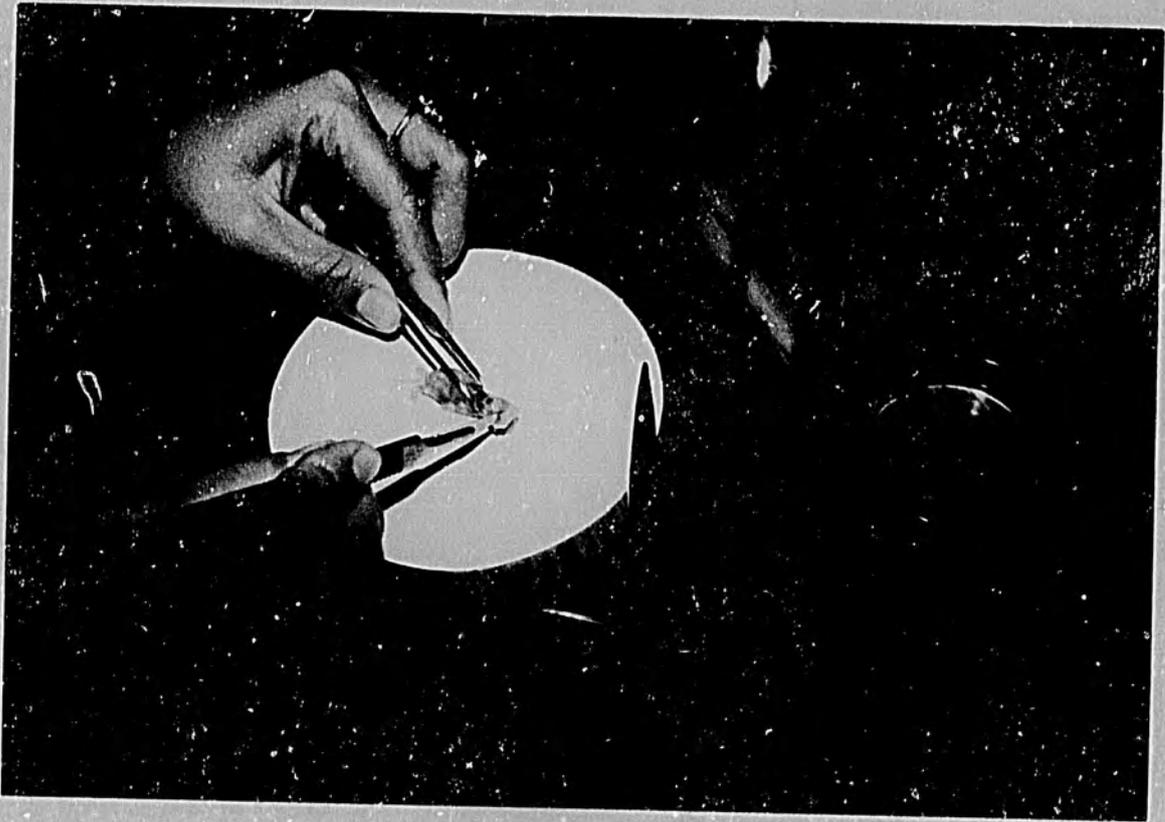


Fig 1. Preparation of dengue viral antigen using suckling mouse



Fig 2. Preparation of dengue viral antigen using mosquito cell culture



Fig 3. Detection of dengue virus infected cells by peroxidase-anti-peroxidase (PAP) test

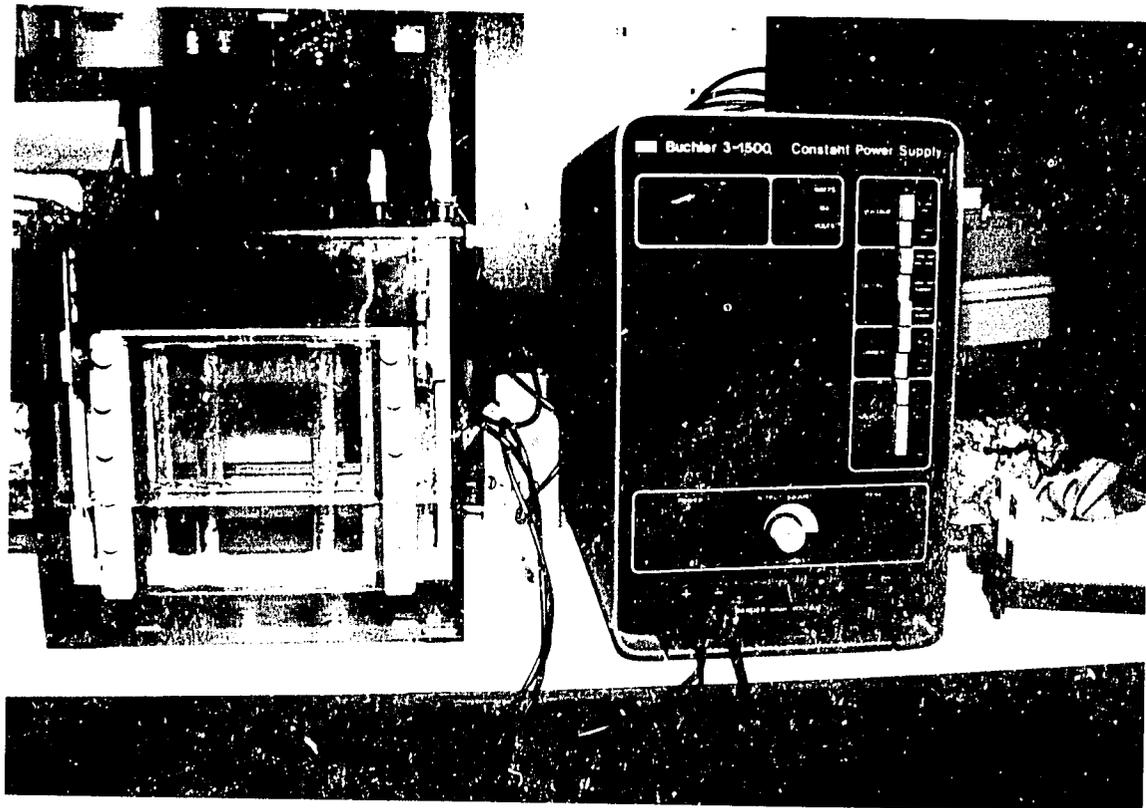


Fig 4. Polyacrylamide gel electrophoresis (PAGE) for separation and detection of dengue viral antigen

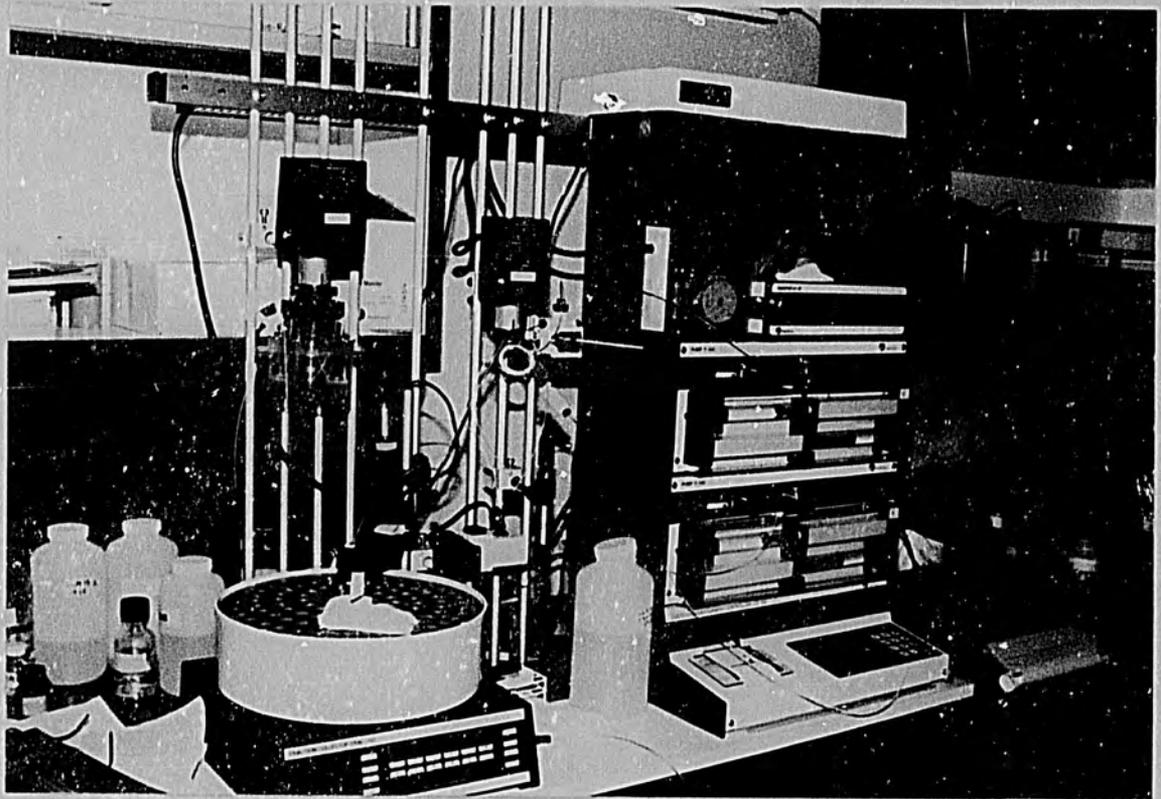


Fig 5. Fast protein liquid chromatography system (FPLC) for purification of dengue viral polypeptides

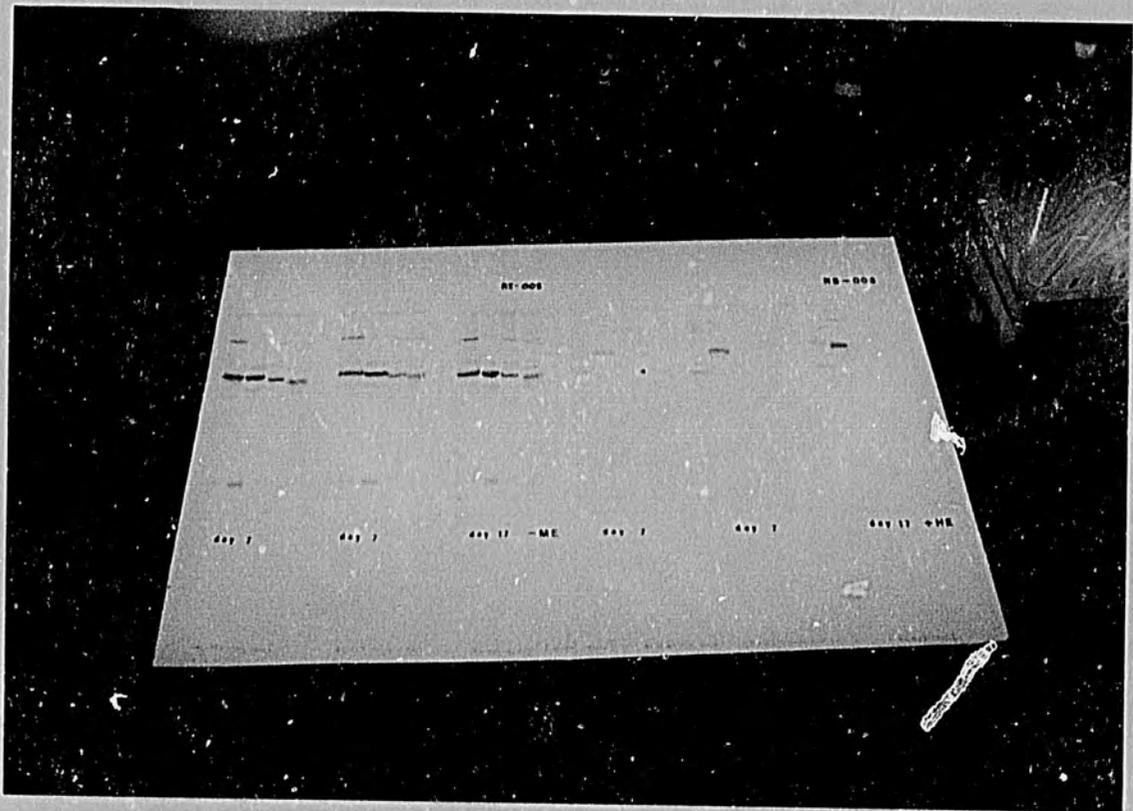


Fig 6. VAS/EIA test reveals specific antibodies to dengue polypeptides.

ANTIBODIES TO DENGUE VIRAL POLYPEPTIDES I. SENSITIVITY AND SPECIFICITY OF THE VIRAL-ANTIGEN-STRIPS/ENZYMEIMMUNO ASSAY (VAS/EIA)

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INTRODUCTION

Many tests are employed for titration of antibodies to Flaviviruses, and the most frequently employed techniques are the hemagglutination inhibition (HI) test (Clarke and Casal, 1958), the plaque reduction neutralization test (PRNT) (Russell *et al.*, 1967) and the antibody capture enzyme-linked immunoabsorbent assay (Burke and Nisalak, 1982). None of these tests can describe the antibodies specifically react toward individual dengue viral proteins. Seventeen polypeptides were found during *in vitro* dengue infection using different kinds of target cells (Westaway and Shew, 1977; Westaway *et al.*, 1980; Heinz *et al.*, 1984). Three polypeptides are the structural or virion proteins including the E or major envelope glycoprotein, C or capsid protein, and M or membrane-like protein. Envelope glycoprotein (E, or VSP-3, or GP59) has molecular weight (M.W.) of 51,000-60,000 daltons, while the capsid proteins, C or VSP-2, has approximately M.W. of 13,500 daltons and the membrane-like proteins, M or VSP-1, has M.W. of 7,700 daltons. The other 13 proteins were detected in dengue-2 infected Vero cells namely P98, p82, P67, gp54, GP46, p30, p28, gp22, GP20, p18, gp16, p15 and p14. In dengue-2 infected *Aedes albopictus* C6/36 cells, gp54 and GP13 were absent (Smith and Wright, 1985); while in dengue-2 infected BHK21 cells, p130, p83, p55 and p21 were demonstrated with suggestive role of non

stable precursor proteins (Ozden and Porter, 1985).

The role of the immune response (at sub-neutralizing level) in enhancing dengue virus infection was suggested to have a major role for the development of haemorrhagic and shock syndromes (Halstead, 1981). Titrations for the humoral immune responses were either based on the ability of antibodies to neutralize or to inhibit agglutination reaction, or to attach specifically to any portion of the viral antigen. Information concerning antibody response toward individual proteins is lacking and this information may lead to a better understanding for the immunopathologic mechanism of this disease. Three techniques; SDS-PAGE, western blot, and enzymeimmuno assay were employed and modified. The whole process was called "the Viral-Antigen-Strips/Enzymeimmunoassay" (VAS/EIA). We have used the classical SDS-PAGE which was described by Laemmli with western blot and enzymeimmunoassay and found that the antibody to dengue viral envelope protein (E or GP59) was almost absent in both human hyperimmune sera and in healthy population (Churdboonchart *et al.*, 1986). With this study, certain chemical and physical treatment were investigated for their effects to dengue viral polypeptides which reflect the binding of human antibodies. The VAS/EIA results were scored from 0 to 4 (Schupback *et al.*, 1985). These results can be obtained in 48 hours. The

specificity, sensitivity and reproducibility are discussed.

MATERIALS AND METHODS

Serum samples : Dengue haemorrhagic fever (DHF) patients' sera and negative control serum were provided by Dr. B.L. Innis and Dr A. Nisalak of the Virology Department, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok 10400, Thailand.

Viral antigens : Suckling mouse brain seed viruses of dengue-1(d1); Hawaii M199 passage # 17; dengue-2 (d2), New Guinea C, passage # 33; dengue-3 (d3), H-87, passage # 27, dengue-4 (d4), passage # 32, and Japanese encephalitis (JE), Nakayama, passage # 17, were obtained from the Virology Department, AFRIMS, Bangkok 10400, Thailand. Dengue viral antigens were raised in suckling mouse brain. Sucrose acetone extraction for these antigen was performed as previously described (Clarke and Casals, 1958). They were titrated by hemagglutination (HA) test and confirmed to have following HA titers : d1 (1:1280), d2 (1:1280), d3 (1:1280), and d4 (1:1280).

Gel electrophoresis and blotting : SDS polyacrylamide gel electrophoresis (8% separating gel with 2.5% stacking gel) was performed as described by Laemmli (Laemmli, 1970). The reducing agent, 2-mercaptoethanol (2ME), was added with varying concentrations in order to evaluate its effects on dengue viral polypeptides.

The separated proteins in gels were blotted onto the surface of 0.1 μ m nitrocellulose membrane (NC) (Schleicher and Schuell; PH 79, Germany), according to the method described by Towbin *et al.*, (1979). Transferring was made for 3, 6, 12 and 18 hours at 3 v/cm.

For this experiment; dengue viruses, type 1 to 4, and normal mouse brain (NMB) as a

negative control were loaded in sequence. After electrophoresis, the gels were cut so that one gel strip will contain the separated proteins of dengue viruses type 1, 2, 3, 4 and NMB. The NC membranes were also cut to the same size as the gel. The NC strip after blotting will contain the separated dengue viral polypeptides and was called as the viral antigen strip or VAS.

Enzymeimmunoassay for the detection of proteins on NC : Bovine serum albumin, BSA, (Sigma, St. Louise, MO 63178, USA) and non-fat dry milk, NF, (Slim, Iverson Dairy Group, Wisconsin, USA) were used as blocking agents to prevent nonspecific background after immunochemical reaction. VAS were incubated with diluted test serum which was diluted with 10 mM Tris-hydrochloric acid (pH 7.4) with 0.9% NaCl (TBS) and BSA (5%) or NF (10%), 3 hr or overnight, at 37°C. They were washed extensively with TBS containing 0.5% (v/v) Tween 20 before incubation with peroxidase labelled antibody, rabbit-anti-human-IgG-HRP (214, Dakoimmunoglobulin, Denmark). After another washing cycle (4x, 5 min each), the strips were soaked with freshly made solution of 3-amino-9-ethylcarbazole (AEC) and H₂O₂ for 15 min (Broe and Ingild, 1983). Then they were rinsed with distilled H₂O and blot dried. The color reaction developed with different level of intensity allowing a possibility to score them as follows (Schupback *et al.*, 1985) : 0 or (-) was for negative (no color), 1 was for barely detected or faint pink color, 2 was for clearly positive (sharp bands with pink color), 3 was for strong positive (red), and 4 was for very strong positive reaction or dark red color.

RESULTS

Dengue viral antigen from dengue viruses type 1 to 4 were separated by SDS-PAGE

(8%), and transferred onto the surface of NC membrane. The NC containing viral antigens (VAS) were immersed in diluted test serum allowing specific antibody towards these separated proteins to bind specifically. Bounded antibodies were located by immunoenzymatic reaction using enzyme labelled antibody and substrate reaction. Different protocols were set up by varying concentration of reducing agent (2-mercaptoethanol) with or without heat treatment, varying volume and titer of viral antigens, and varying dilutions of test serum. The results were impressive and are shown in Figs. 1 to 8. Since 2-mercaptoethanol (a reducing agent) used by Laemmli was suspected to cause the lost of antigenicity for certain proteins, this reagent was excluded in the first two following experiments.

Fig. 1 shows the optimum condition using sucrose acetone extracted dengue-2 antigen

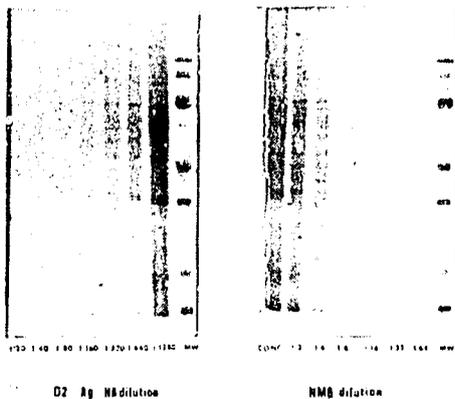


Fig. 1—SDS-PAGE results for serially diluted sucrose acetone extracted dengue type-2 antigen (HA 1:1,280 to HA 1:20) and normal mouse brain treated with the same protocol as negative control. Note the higher number of stained bands in serially diluted SAE-d2 in comparison with serially diluted mouse brain proteins. Molecular Weight markers were placed on the far left side of each gel strip.

with SDS-PAGE systems. 10 μ l of viral antigen and the sample buffer (1:1 ratio) were loaded into each well. The right side gel strip shows the separated dengue-2 viral

proteins when serially diluted (from HA 1:1280 to 1:20), while the left side gel shows the serially diluted proteins from normal mouse brain as a negative control. The separated bands from dengue-2 with HA titer of 1:1280 were seen sharply and well separated. With larger volume of viral antigen, 20 μ l and 30 μ l; these bands were broader but not as sharp. At this stage, proteins from mouse brain or viral proteins can not be differentiated.

In order to determine the specificity, serially diluted dengue-2 viral antigen (form HA titers of 1:1280 to 1:20) were loaded in sequence. After blotting to NC membrane, serum with anti-dengue-2 activity proven by HI test (HI titer to dengue type 2 was 1:2560) and negative control serum, proven by both HI test and the neutralization test, were separately incubated with 2 identical twin NC strips. No specific enzyme-immunological reaction with the negative serum was found, but at least 3 positive bands with dengue-2 antigen at 1:1280 can be observed (Fig. 2). These 3 bands were compared with molecular weight markers and their molecular weights were estimated as 71,000, 66,000, and 59,000

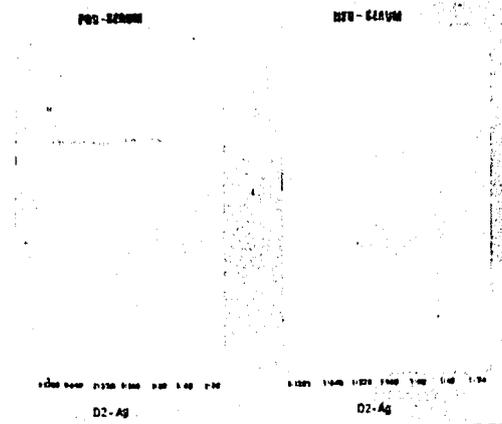


Fig. 2—Specificity test for antibodies detected by VAS/EIA. Positive serum from dengue-2 infected patients bound to 3 polypeptides while negative serum demonstrated an absence of bound antibody.

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daltons. The best blocking agent for this test was the non-fat dry milk, 10% (W/V).

In order to find out the effect of 2ME dengue-2 antigen (HA 1:1280) and non-dilute sucrose acetone extracted normal mouse brain were treated with different amount of 2ME (Fig.3). The final concentration of 2ME

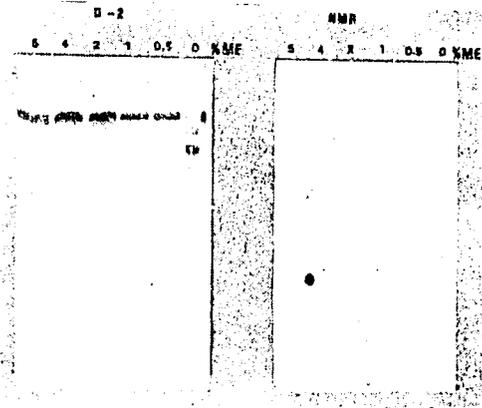


Fig. 3—Effect of 2-mercaptoethanol demonstrated by varying final concentration of this reducing agent. Normal mouse brain showed negative result while SAE-d2 (HA:1280) was sensitive to this treatment. Note the disappearance of bands at 71,000-68,000, 60,000-54,000, 50,000-48,000, 21,000-19,000, and an appearance of strongly positive band at 85,000-77,000. Also note for the higher color intensity at 85,000-77,000 and at 45,000.

were 5, 4, 2, 1 and 0.5, leaving the last well for control (without 2ME or 0%). This serum had anti-dengue-2 HI titer of 1:2560 and demonstrated at least 8 antibodies when 2ME was not included. These polypeptides are as following : between M.W. of 130,000-110,000 (p130-110), 71,000-68,000 (p71-68), 66,000-64,000 (p66-64), 60,000-54,000 (GP 60-54), 55,000-50,000 (p55-50), 50,000-48,000 (p50-48), 46,000-44,000 (GP46-44), and 21,000-19,000 (GP21-19) daltons. When 2ME was included, some of the positive bands disappeared such as the polypeptides with M.W. of 71,000-68,000, 60,000-54,000, 55,000-50,000, and 21,000-19,000 daltons. At the sametime, this treatment brought about an increasing of intensity to certain

Table 1

Antibodies to dengue 2 polypeptides by VAS/EIA in an absence and in presence of 2-mercaptoethanol.

	Intensity scores for antibodies to dengue 2 polypeptides					
	% 2ME					
	0	0.5	1	2	4	5
p130-110	1	1	1	1	1	1
p 85-77	-	3	3	4	4	4
p 71-68	2	-	-	-	-	-
p 66-64	2	1	1	1	1	1
GP 60-54	3	-	-	-	-	-
p 55-50	2	1	1	1	1	1
p 50-48	1	-	-	-	-	-
GP 46-44	1	1	2	2	2	2
GP 21-19	2	-	-	-	-	-

polypeptides especially the one with M.W. of 85,000-77,000 and 45,000 daltons. These results are shown in Table I. NF is still the choice for blocking agent.

Another factor that can cause the lost of antigenicity is the heat treatment, 5 min at 100°C. This process is needed for 2ME to reduce di-sulfide bonds and normally all proteins were treated to reach a condition of being a straight single polypeptide chain for estimation of their molecular weight. A patient serum (C0909), collected 15 days after hospital admission, was used to compare the effect of 2ME with and without heat treatment, and without the 2ME. HI titers of this serum are as follows : d1 (1:5,120), d2 (1:2,560), d3(>1:10,240) and d4(>1:10,240). The results are demonstrated in Fig 4 and Table 2. With 2% of 2ME and heat, antibodies to p85-77 and GP45 can be observed. When 2ME was included, volume of all dengue viral proteins and negative control must be raised up to 20 µl, otherwise the

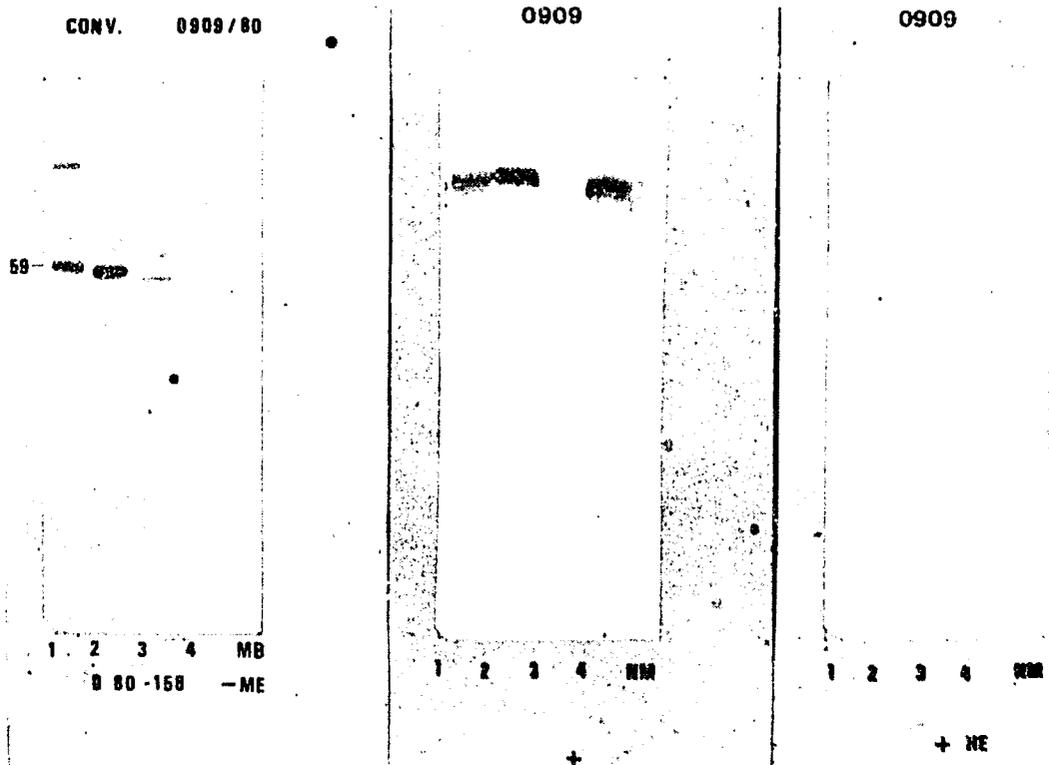


Fig. 4 -Comparison of the results obtained from 3 protocols: A-strip was from VAS EIA without 2ME, B-strip from VAS EIA with 2% 2ME, and C-strip from 2% 2ME with heat treatment at 100 C for 3 min. Strip B and C were tested at the same time, so the bands can be compared while A-strip was run separately using different M.W. markers to determine approx M.W.. Note lower intensity of color development at 85,000-77,000 and higher intensity at 45,000 after heat treatment.

immunoenzyme reaction will be poor and can not be compared with the results obtained when 2ME was excluded.

In order to compare the rising of antibodies in DHF patients : dengue viral antigens, from type 1 to 4, and NMB, were tested with sera collected on day 1 (0192/80) and day 15(0317/80) after hospital admission. On day 1, HI titers were as follows: d1(1:5,120), d2 (1:2,560), d3(1:5,120) and d4(1:2,560). Dengue type 2 was isolated on day 1 indicated that the patient was secondary infected with dengue virus type 2. Antigens used in this experiment were not treated with 2ME. From Fig. 5., antibodies were found against all 4 dengue

serotypes. Comparison between 2 strips was made and the scored for color-intensity were assigned to each individual bands. These scores are shown in Table 3. Ten antibodies to dengue-2 polypeptides were noted in sera collected during convalescent phase.

With another pair sera, 2% of 2ME was used to treat the antigens but heat treatment was exempted. The results are shown in Fig. 6 and Table 4. Eventhough the antibodies were found to react with lesser number of polypeptides, the seroconversion can be clearly seen by visualization.

Normal adult sera were subjected to VAS/ EIA with exemption of 2ME. Examples of

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Table 2

Antibodies in human hyperimmune serum (C0909) to dengue viral polypeptides detected under different conditions.

Antibodies to dengue viral polypeptides	Highest VAS/EIA score to each polypeptide		
	Non-reducing condition	Reducing condition (+ 2% 2ME) without heat treatment	Reducing condition (+ 2% 2ME) with 3 min heat treatment
p130-110	3 (d1)	2 (d2)	-
p 85-77	-	4 (d1,2,4)	3 (d2)
p 71-68	2 (d2)	2 (d2,3)	-
p 66-64	2 (d2)	-	-
GP 60-54	4 (d1,2)	2 (d1)	2 (d1)
p 55-50	2 (d2)	-	-
p 50-48	2 (d1)	-	-
GP 45	-	1 (d all)	2 (d1,2,3)
p 30-28	-	-	-
p 26-24	-	-	-
GP 21-19	-	-	-
p 14	-	-	-

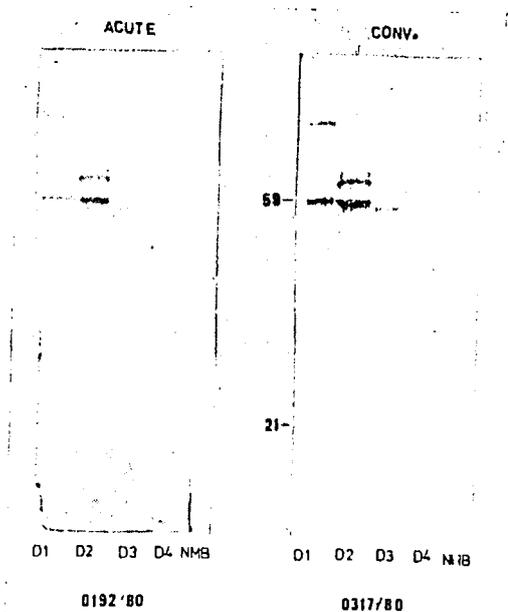


Fig. 5—Sero-conversion to all types of dengue viruses (d1, d2, d3 and d4) as seen by VAS-EIA without 2ME. 0192 is the serum collected on day 1 after hospital admission and 0317 is the serum collected on day15. Note the higher intensity of color development and number of positive bands in 0317 (Convalescent serum).

Table 3

Antibodies to dengue viral polypeptides	Antibodies to dengue viral polypeptides in patients (SDS-PAGE without 2ME)	
	Serum 0192	Serum Number 0314
p130-110	2 (d1)	3 (d1)
p71-68	2 (d2)	2 (d2)
p66-64	3 (d2)	3 (d2)
GP60-54	3 (d2)	4 (d1, 2)
VAS/EIA results	p55-50 1 (d1,2)	1 (d1, 2)
	p50-48 1 (d1, 2, 3)	3 (d1)
	GP46-44 1 (d1, 2, 3)	2 (d2)
	p30-28 1 (d1)	1 (d1)
	p26-24 1 (d1)	2 (d2)
	GP21-19 1 (d2)	3 (d2)

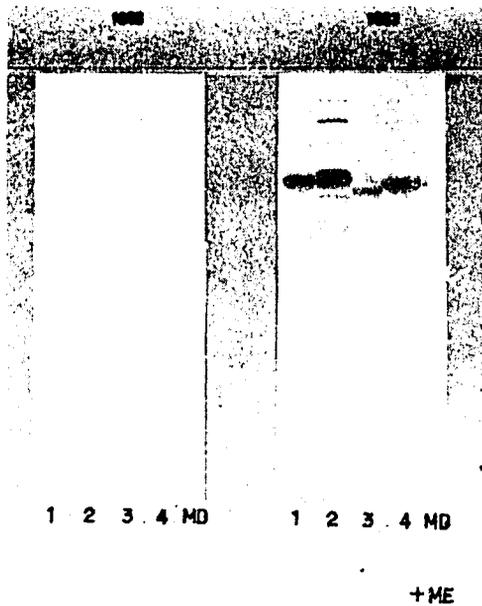


Fig. 6 -Sero-conversion to all types of dengue viruses as seen by VAS/EIA with 2% 2ME without heat treatment. Note the sero-conversion especially at d2 and at M.W. of 45,000 daltons.

Table 4

Sero-conversion between sera collected during acute and convalescent phase in presence of 2% 2-mercaptoethanol.

Antibodies to dengue viral polypeptides	Serum Number	
	1099/80	1267/80
p130-110	1 (d1,2)	2 (d2)
p85-77	1 (d all)	4 (d2)
VAS/EIA results		
p71-68	-	1 (d2)
GP 59	1 (d2)	1 (d2)
p52-50	1 (d1, 2)	1 (d1,2)
GP45	1 (d1)	2 (d1)
d1	1:40	1:2560
HI titers		
d2	1:40	1:2560
d3	1:160	1:10240
d4	1:40	1:5120

Table 5

Antibodies to dengue viral polypeptides in healthy adult male by VAS/EIA without 2 ME.

Antibodies to dengue viral polypeptides	Serum Number		
	NHS 19	NHS 20	NHS 21
p 130-110	2 (d1,3)	3 (d1)	3 (d1)
p 71-68	2 (d2)	3 (d2)	4 (d2)
p 66-64	3 (d2)	3 (d2)	4 (d2)
GP 60-54	3 (d1, 2, 3)	4 (d 1, 2, 3)	4 (d 1, 2, 3)
VAS/EIA results			
p 55-50	-	-	-
p 50-48	1 (d1)	2 (d1)	1 (d1)
GP 46-44	-	-	-
p 30-28	-	-	-
p 26-24	-	-	-
GP 21-19	-	2 (d1, 2)	2 (d1, 2)
HI titers			
d1	< 1 : 20	1 : 80	1 : 160
d2	1 : 20	1 : 40	1 : 80
d3	1 : 80	1 : 80	1 : 160
d4	1 : 20	1 : 40	1 : 160

D1 D2 D3 D4 NMB D1 D2 D3 D4 NMB D1 D2 D3 D4 NMB
NHS 19 NHS 20 NHS 21

Fig 7. Antibodies to all types of dengue viruses in normal adult male sera by VAS/EIA without 2ME. Note appearance of antibodies to all dengue viruses and different level of color intensities among the sera.

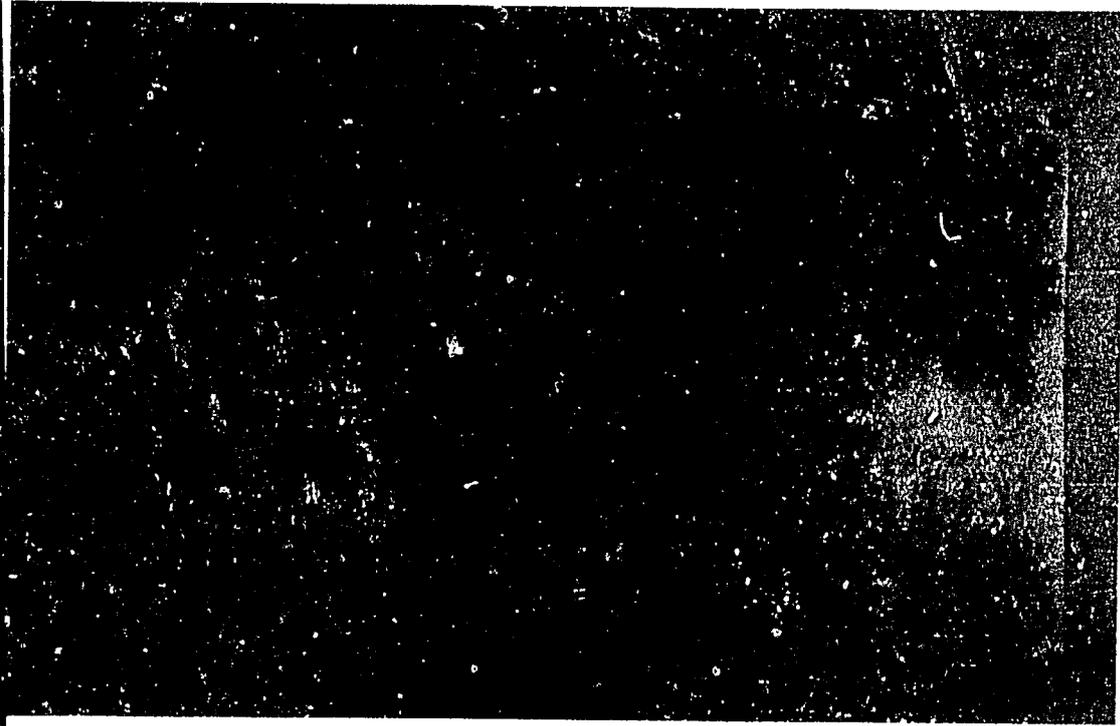


Fig 8. Sero-conversion to all type of dengue viruses in a patient's sera serially collected from day 1, day 15, day 30, to day 60. VAS/EIA was performed in non-reducing condition. Note for the appearance of antibody to polypeptide with approximate m.w. around 14,000 daltons on day 15.

this result are demonstrated in Fig 7. VAS/EIA scores and HI titers are shown in Table 5. VAS/EIA demonstrated higher sensitivity than HI test i.e. when HI titer was lesser than 1:20 for d1 in NHS 19.

Antibody to protein with M.W. around 14,000 was found in a certain case. This antibody appeared on day 15 after hospital admission, and cannot be detected on day 30 and 60 in which the samples volume were increased to 20 μ l (Fig. 8).

DISCUSSION

The VAS/EIA used in this study was proven useful to study dengue viral polypeptides and human humoral immune responses specifically against these individual proteins. With classical SDS-PAGE protocol (Laemmli, 1970), 2-ME was included in the sample buffer and this mixture must be heated at 100 C for 5 min. After electrophoresis, the separated proteins can be stained by Coomassie brilliant blue and observed as shown in Fig 1. After blotting, these proteins on surface of NC membrane can be traced by Indian ink. We have selected 15 hr to transfer these proteins since we found no Coomassie stain in gel and many strong Indian ink bands on NC strips.

When serially diluted sucrose acetone extracted dengue type 2 antigens were tested with positive and negative control serum (proven by HI and PRNT tests), we found that strong antigen with HA titers of 1:1,280 gave the best result while the required antigen volume is only 5 μ l. After mixing this antigen with equal volume of sample buffer, the final volume loaded into each sample well is only 10 μ l.)

Hyperimmune sera, diluted from 1:5, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000, were incubated with viral antigens strip and normal mouse brain strip. Then the bound antibodies

were traced by second labelled antisera, rabbit anti-human IgG. The optimum result was seen with 1:50 dilution. Therefore, all test sera were diluted to 1:50 dilution by buffer A with addition of 5% BSA or 10% NF for non-specific blocking.

When we applied the test sera to the viral antigen strips, few pinkish-red color bands developed indicating antibodies against these polypeptides. With classical Laemmli SDS-PAGE method, where 5% 2-mercaptoethanol was used as a reducing agent and heat treatment at 100 C for 5 min was required, we observed antibodies towards the polypeptides with estimated M.W. between 130,000-110,000, 85,000-77,000, 71,000-68,000 and 45,000. With BSA as non-specific blocking agents, the antibodies to smaller polypeptides (30,000-23,000, 26,000-24,000 and 21,000-19,000-daltons), were often found. One drawback of BSA is that the non-specific band can be seen with normal mouse brain strip causing difficulty to interpret the results.

Replacing BSA with NF, all non-specific background disappeared, however the intensity of positive bands were also decreased, and certain bands diminished. This problem was solved by increasing sample size from 10 μ l to 20 μ l. Each band was broader, but eventually brought up color intensity. However, the separation of protein bands was not as good as when the sample size was 10 μ l.

To study the effect of 2ME, various concentrations of this reducing agent were mixed with the sample buffer from 0, 0.5, 1.0, 2.0, 4.0 to 5.0 percent. The samples were not heated when 2ME was not included. As seen in Fig. 3, without 2ME and without heat treatment, more antibodies can be demonstrated especially to the polypeptides at M.W. of 130,000-110,000 (P130-110), 71,000-68,000 (p71), 66,000-64,000 (p66), 60,000-54,000 (E or GP59), 66,000-64,000 (p66), 55,000-

50,000 (p55) 50,000-48,000 (p50), 46,000-44,000 (GP45) and 21,000-19,000 (GP21 or prM). At 1-2% 2ME, antibodies at p130-110, p66, p55, and GP45 can be seen with an extra band at M.W. of 85,000-77,000 daltons (p85-77). Serum collected during convalescent phase (15 days after hospital admission) was used to compare 3 different following protocols.

- (1) excluding 2ME and heat treatment;
- (2) including 2ME at 2% and excluded heat treatment;
- (3) including 2ME at 2% and treat sample at 100 C for 3 min.

The results indicated clearly that GP 59 is very sensitive to 2ME and also the antibodies to protein p130-110, p71-68 p66-64, p55-50, and p50-48. With 2ME and heat treatment only few antibodies can be seen with lower scores. However, the antibodies to p85-77 GP45 can be best observed.

With the same scoring system that was applied to quantitate HIV antibodies in AIDS significant seroconversion can be observed by VAS/EIA such as seen in Fig. 5. Higher color intensities were recorded and also there were antibodies towards more polypeptides. This changing pattern is interesting and allowing the analysis of antibody responses at the molecular or polypeptide level. Realizing the effect of 2ME and heat treatment investigation for interesting antibody or monoclonal antibody may be undertaken by careful selection of a proper protocol.

Sensitivity of VAS-EIA when applied with other virus such as the avian leukosis viruses (Esenman *et al.* 1980), it was calculated that a specific viral protein (p27) as little as 1-2 ng can be detected by hyperimmune rabbit serum at 1:50 dilution. In our experiment (Table 5), when antibody to dengue type 1 was found lower than 1:20, antibodies to dengue 1 polypeptides still can be recorded as follows :

2 for polypeptides with M.W. at p130-110, 3 for antibody to GP59, and 1 for antibody to p50. Therefore, the sensitivity can be claimed to be higher than that of HI test.

The new nomenclature for flavivirus proteins were designated according to their gene expression after the entire RNA genome of the yellow fever was described (Rice *et al.*, 1985). The first structural protein is the Capsid (C) protein with M.W. between 13,000-16,000 daltons and the antibody toward this protein can be seen with the non-reducing condition in certain hyperimmune sera (Fig. 8). The next structural protein synthesized in sequence is the virion M protein (M.W. of 8,000-8,500 daltons). This protein has not been found in infected cells and was postulated to be derived from a precursor glycoprotein (prM) which was also called as GP23, GP19, NV2, or NV2 1/2 and has estimated M.W. of 19,000-23,000 daltons. With VAS/EIA, antibodies to M was so far absent but the antibodies to protein at prM position can be detected. Envelope (E) protein follows M with M.W. between 51,000 to 60,000 daltons. Under non-reducing condition, human hyperimmune sera reacted with at least 3 polypeptides at the approximate M.W. of 60,000-54,000, 55,000-50,000 and 50,000-48,000 daltons. We can say that the antibodies to structural proteins can be detected under non-reducing condition and they are quite sensitive to 2ME and heat treatment.

The antibodies to the non-structural-proteins, NS1 (NV3 or GP45 with M.W. of 44,000-49,000 daltons) and NS3 (NV4, or p77) with M.W. of 67,000-76,000 daltons) can be seen best with the classical SDS-PAGE when the concentration of 2ME was decreased to 1-2 percent and the heat treatment period was also reduced to only 2-3 min. The ns 2a was found to have similar molecular weight to prM, therefore the antibody to this polypeptide or prM must be differentiated by the

use of their specific antisera. This situation must also be applied to ns2b because its molecular weight is about 12,000 to 15,000 daltons and cannot be differentiated from antibody to C protein until the specific antisera is employed. With ns4a (NVX or NV2 1/2 with M.W. between 24,000-32,000 daltons, we observed at least 2 bands (at M.W. of 30,000-28,000 and 26,000-24,000 daltons). The antibodies to ns4b (NV1 with M.W. of 10,000-11,000) was absent, while antibody to NS5 (NV5 with M.W. of 91,000-98,000 daltons) were observed with reducing condition in certain patients' sera.

Besides the proteins mentioned, VAS/EIA revealed antibodies toward at least 2 more proteins : the proteins with M.W. between 130,000-110,000 and the other with M.W. of 85,000-77,000 daltons. These antibodies are probably toward the p130 and p83 respectively.

In this study, VAS/EIA was proven as a powerful tool in monitoring antibodies to dengue viral polypeptides. It can be used to study humoral immune responses in vaccinees, patients, and in population as well. The results may lead to a better understanding of the immunopathogenesis and may as well lead to further application as rapid diagnostic method. This study is now in progress in our laboratory.

SUMMARY

The authors applied polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), western blot, and enzyme immunoassay (EIA) for the detection of antibodies toward individual dengue viral proteins or polypeptides. SDS-PAGE procedure as described by Laemmli *et al.*, was applied and modified. The results can be observed by visualization. The scores, from 0 to 4, can be assigned by comparing the intensity of the color development. Besides

being sensitive and rapid, this technique yields information of the polypeptides or molecular level. An increasing of intensities of positive reactions indicated rising in antibodies titers and all serotypes of dengue viruses (from type 1 to type 4) can be tested together allowing reliable comparison among serotypes. With hyperimmune human sera, at least 13 polypeptides reacted with sera while negative non-immune subject showed no reaction. It is highly possible to use this technique as a rapid quantitative and for qualitative analysis of antibodies to individual viral proteins as well.

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