PN-ABA-569

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 8/10/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose VO

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 7, 269
PR #2 dtd 7/1/88

Attachment

PN-AEH 567

Progress Report No 2

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

A research project

Grant no 936-5542-G-00-7029-00

Submitted by

Dr. Vina Churdboonchart

Project Leader

Dept of Pathobiology

Faculty of Science, Mahidol University

Rama VI Rd. Bangkok 10400

Thailand

July 1, 1988

Project Profile

Country	:	Thailand
Grant number	:	936-5542-G-00-7029-00
Program	:	Program on Science and Technology Cooperation
Project Title	:	Studies for Dengue Viral Antigens and Antibodies in Sera of Dengue Hemorrhagic Fever (DHF) Patients
Project Leader	:	Vina Churdboonchart, Ph.D., Associate Professor
Organization	:	Department of Pathobiology, Faculty of Science, Mahidol University Rama VI Rd.,Bangkok 10400, THAILAND
Co-investigators	:	1. Dr. Suporn Peampramprecha , M.Sc., D.V.M. 2. Miss Amporn Chaiyotha, B.Sc. 3. Miss Kanchana Sirisidhi, B.Sc. 4. Dr. Ananda Nisalak, M.D. 5. Dr. Suchitra Nimmanitya, M.D., 6. Dr. Sutee Yoksan, M.D. 7. Dr. Kingkarn Laohatai, M.D., Ph.D. 8. Dr. Sakol Panyim, Ph.D.
Technical Assistants	:	1. Miss Rusama Nangnuan, B.Sc. 2. Miss Kanitha Kenpitak, B.Sc.

3. Mrs. Maliwan Swasti-Xuto

4. Miss Tasanee Chaiyalirk

5. Miss Aranya Wongmool

6. Mr. Artakorn Vichaiboon

Project Consultants : 1. Prof. Natth Bramarapravati (Mahidol University; Rector)

2. Dr. Bruce Innis (Dept of Virology, AFRIMS, Bangkok 10400)

Authorized Officer : Dr. Pairoj Prampree, Dean of Faculty of Science

Total project budget : 3,806,640.00 baht (US$ 149,280)

Project Duration : July 1, 1987 - Dec 31, 1990

Reporting Period : Jan 1, 1988 - June 30, 1988

Budget Allocation for this Period : 590,398.95 Baht

Contents

	Page
1. Introductions:.....	1-4
2. Objectives:.....	5-6
3. Materials and Methods:.....	7-9
4. Results/Discussion/Tables:.....	10-12
5. Remarks:.....	13-14
6. Workplan for the Next Period:.....	15
7. References:.....	16-18
8. Annex/Pictures:.....	19-25

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 in given (1). These severe forms of disease start with high fever but progress to nausea, vomitting, 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 hypotheses (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 occuring 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, heterologus 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 infections 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 C_3, C_4 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 ncrease 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 (10, 11).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 (12). In <u>In Vitro</u> experiment, antibodies act through the Fc-mediated mechanism to enhance infection and virus yield from human monocyte culture. Experiment in animal, passive antibody at sutneutralizing level causes

higher levels of viremia in rhesus monkeys. Shock in infants is postulated to be due to maternal antibody in the similar 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. Develope 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 perparation 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. Hemaglutination 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 infections 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 elecit 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

Progress since last report

A. preparation of dengue viral antigen

A.1. Preparation for dengue viral antigen by existing methods were done and the antigen were characterized. With the listed methods for characterization (Section B/Materials and Methods), we selected the first protocol using suckling mice to prepare our antigen (sucrose acetone extracted antigen, SAE) dengue viral antigen(s) after blotting onto surface of nitrocellulose membrane is still retaining the immunogenic reactivity and reacted with the specific dengue antisera including the monoclonal antibody, rabbit sera, and hyperimmune human sera (13). SAE antigen was also used as the starting material to purify each structural and non-structural proteins by either the chromatographic or electrophoretic system.

A.2. New approaches to purify dengue viral polypeptides and polypeptide fragments

Three approaches are being made for this purpose. The preparative gel electrophoretic system is not functioned as expected. The first reason may be due to the fact that most dengue viral polypeptides are sensitive to 2-mercaptoethanol (2 ME) and this reagent is neccessary. Second reason due to the flow rate which can not be adjusted to allow enough protein separation. We observed irregular patterns of protein migration and a non-reproducible results.

With crossed immunoelectrophoresis, the envelope protein (E) is the best protein to work with this method. Few other unidentified proteins can be obtained as well (Fig 1), and they are now being studied in our unit.

The column chromatography offers the most promissing results. Gel filtration system, using superose 6 HR 10/30 column with MW range 5,000-500,000 daltons from Pharmacia (Sweden), revealed 6 interesting peaks (Fig 2). These peaks were collected for further purification.

B. Characterization of prepared dengue antigen

All proposed methods were established and being used. In addition, we are now establishing the enzyme-linked immunonassorbent assay (ELISA) by collaborateion with Dr. Bruce Innis and Dr. Ananda Nisalak from AFRIMS, (Bangkok). The test itself are now functioned, and the AFRIMS's authorities will assist for standardization and interpretation part since this test for dengue virus system was established by them.

C. Preparation of anti-dengue sera

C.1. Rabbit anti-dengue sera

Rabbits were immunized with dengue type 1 to type 4, Japanese B encephalitis (JEV) and Chikungunya. Specific antisera to Japanese B encephalitis and Chikungunya viruses are the first two that can be obtained. As shown in Fig 3, rabbit antisera to JEV reacted with at least 5 polypeptides of Japanese B

encephalitis and anti-chikungunya react with one chikungunya polypeptide. Dengue type 1 to type 1 were demonstrated in Fig 4. These polyclonal antibodies reacted well with their homologous antigen. Cross reaction among dengue serotypes must be absorbed. Dengue type 2 antisera has very little crossed reactivity and we hope to have the specific reaction from dengue type 2 antisera in a very near future.

C.2. Dengue viral polypeptide or its fragments are still not available to use as immunogen

C.3. Human hyperimmune sera were collected and retrospectively investigated.

C.4. Monoclonal antibody (Mab)

The results using monoclonal antibody with VAS/EIA test for both characterization and identification were shown in Fig 5 . With our modified system, we can demonstrate monoclonal antibody specificity toward dengue viral polypeptides. The antibody to envelope protein can be clearly seen. We hope to receive antisera to NS1 or to other non-structural proteins to test for their specificities as well. This can be done by collaboration with Dr. Joel M. Dalrymple of the US Army Medical Research Institute of Infectious Diseases whom has done many works for preparation of monoclonal antisera.

D. Characterization of prepared anti-dengue sera

Both methods, VAS/EIA and Crossed immumoelectrophoresis were established. The characterization are

being done together with other techniques such as the hemagglutination inhibition (HI) test, and ELISA test.

E. and E. Studies with patients' specimens

From July 1987 to December 1987 we were able to collect at least 150 cases of clinically suggested Dengue Hemorrhagic Fever (DHF) and Dengue Fever (DF) cases. Out of this, 21 cases were confirmed by HI and ELISA tests to be the primary cases whom has never been infected before with dengue viruses. Studies for complement system by our reported protocol (14, 15) and the platelet system in primary cases indicated that in severely ill cases, grade III and IV, both systems were activated regardless of the age of the patients (Fig 6). Both HI and ELISA tests revealed negative anti-dengue activity indicated that the shock syndrome can occur in absence of the dengue antibody. The report for this finding is under preparation.

Further study to separate human immunoglobulin G and M (IgG and IgM) was done using our own protocol. Preliminary results are demonstrated in Fig 7 using PAGE and Western blot, we can identify IgG and IgM peaks which was eluted from Mono -Q column (anion exchange chromato graphy). The IgG and IgM fragtions were sent to AFRIMS again to test for their dengue specific antibodies. The protocol for separation will be reported in a separate manuscript.

Remarks

We are now preparing 4 manuscripts. The first report describes how to produce rabbit anti-dengue sera from immunoprecipitin bands. Second report will be the protocal for separation of human immunoglobulin G and immunoglobulin M by chromatography and its application with the Western blot analysis. The third report will cover the findings in primary infected cases emphasizing the complement system and antibodies. The fourth one involves the identification of dengue polypeptides by monoclonal antibody and how to use VAS/EIA to characterize specific antibody activity towards dengue viral polypeptides.

Workplan for the next period

Collection of specimens from clinically suspected patients will be continued because during this period, from July to September, we often see more cases of Dengue Hemorrhagic Fever.

Sensitive and reliable method will be searched for to detect the circulating dengue viral antigen besides the viral isolation and countercurrent immunoelectrophoresis.

Besides continuation of laboratory works, manuscripts will be prepared. Collaboration with other loboratory in order to obtain monoclonal with specific activity to single polypeptide shall be made. A trip to Washington D.C to visit Dr. Joel Dalrymple is expected for this venture.

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	Jan-Mar	Apr-June	July-Sep	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														
-[illegible]														
-[illegible]														
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														

References

1. Chow, C.Y., Gratz, N.G., Halstead, S.B., Hammon, W.M., Lim, K.A. Marshall, I.D., Montoya, J.A., Saenz, A.C. and P. Tuchinda. Mosquito born haemorrhagic fever of South-East Asia and the Western Pacific. Bull WHO, 35 : 17-30, (1966).
2. Rosen, L. The emperor's new clothes, revisited or reflections on the pathogenesis of dengue hemorrhagic fever. Amer. J. Trop. Med. Hyg., 26 : 337-343 (1977).
3. Halstead, S.E., Nimmanitya, S., Yamarat, C., and P. K. Russell. Hemorrhagic fever in Thailand, newer knowledge regarding etiology. Jap. J. Med. Biol., 20 (Suppl.) : 96-103, (1972).
4. Winter, P.E., Yuill, T.M., Udomsakdi, S., Gould, D.I. Nantapanich, S., and P.K. Russell. An insular outbreak of dengue hemorrhagic fever. I. Epidermiologic observations. Amer. J. Trop. Med. Hyg., 17 : 590-599, (1968).
5. Russell, P.K., Yuill, T.M., Nisalak, A., Udomsakdi, S., Gould D.J., and P.E. Winter. An insular outbreak of dengue hemorrhagic fever. II. Virologic and serologic studies. Amer. J. Trop. Med. Hyg., 18 : 159-165, (1969).

6. Scott, R.M., McCown, J.M., and P.K. Russell. Human immunoglobulins specificity after group B arbovirus infections. Infect Immun., 6 : 277-281, (1972).

7. Jayavasu, J., Jayavasu. C., Phengsrithong. C., Anavil, A., and P. Tuchinda. Immunoelectrophoretic study of B_1c globulin in the serum in dengue hemorrhagic fever. Biken. J., 10 : 253-255, (1967).

8. Report of an international collaborative study. Pathogenetic mechanism in dengue hemorrhagic fever. Bull. WHO., 48: 117-133, (1973).

9. Kuberski, T., Rosen. L., Reed, D., and J. Mataika. Clinical and laboratory observations on patients with primary and secondary dengue type I infections with hemorrhagic manifestation in Fiji. Am. J. Trop. Med. Hyg., 26 : 775-783, (1977).

10. Bhamarapravati, N., Boonpacknavig V., and P. Nimsomburana. Pathology of Thai Hemorrhagic Fever. WHO. Bulletin 35 : 47-48, (1966).

11. Bhamarapravati, N., Tuchinda, P., and V. Boonpucknavig. Pathology of Thai Hemorrhagic fever. An autopsy study. Ann Trop. Med & Parasit, 61 : 55, (1967).

12. S.B. Halstead. The pathogenesis of dengue : Molecular epidemiology in infectious disease. Am J. Epidemiol. 114 : 632-648, (1981).

13. Churdboonchart, V., Bhamarapravati, N., Peampramprecha, S., Pinthong, C., Sirisidhi, K., and A. Chaiyotha. Antibodies to dengue viral polypeptides. I. Sensitivity and specificity of the viral-antigen-strips/Enzymeimmuno assay (VAS/EIA). Southeast Asian J. Trop.Med. Pub.Hlth. 18,362-372, (1987).

14. Churdboonchart, V., Bhamarapravati, N., and P Futrakul. Crossed Immunoelectrophoresis for the detection of the third complement in Dengue Hemorrhagic Fever. I. Observations in patients' plasma. Am. J. Trop. Med. and Hyg. 32, 569-576, (1983).

15. Churdboonchart, V., Bhamarapravati, N., and S. Yoksan. Crossed Immunoelectrophoresis for the detection of split products of the third complement component in Dengue Hemorrhagic Fever. II. In Vitro activation by dengue viral antigen. Am. J. Trop. Med. and Hyg, 33 : 1239-1247, (1984).

Figures

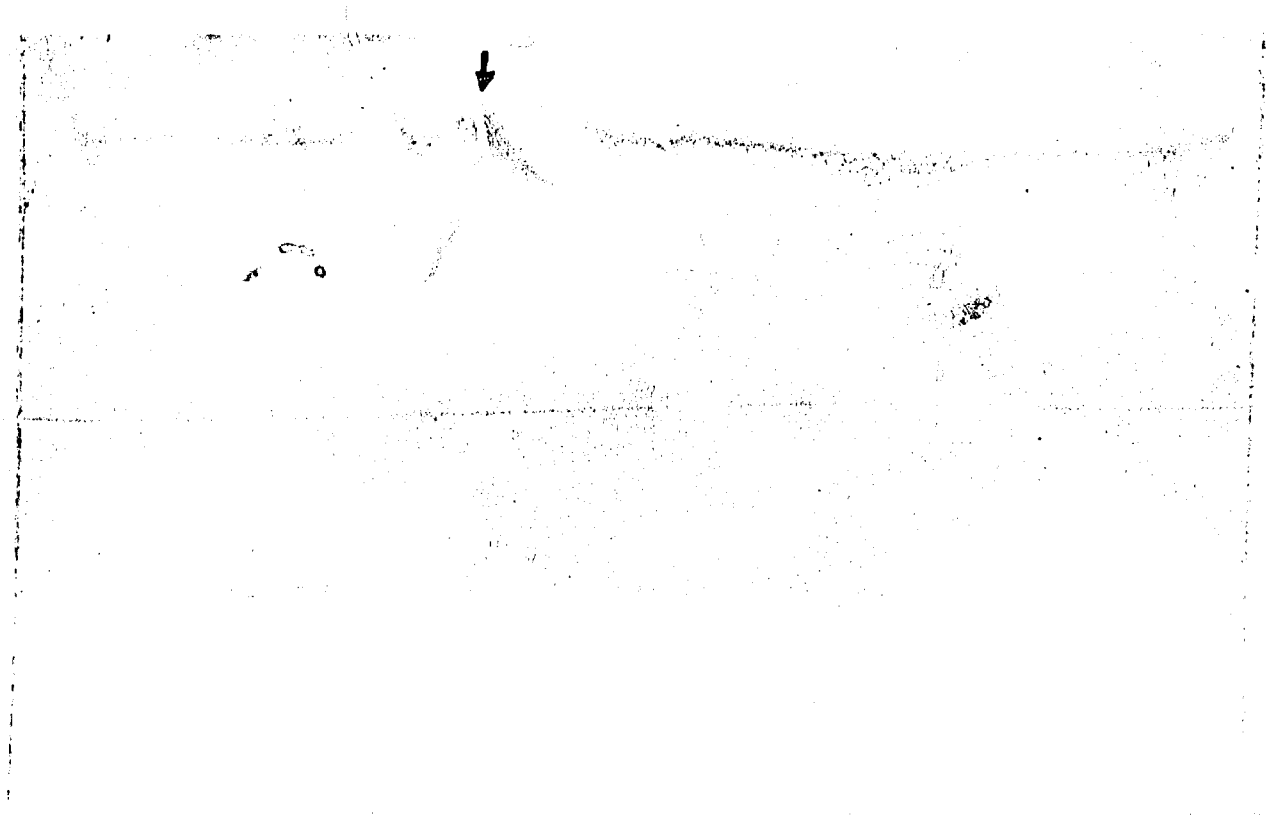


Fig 1. Crossed immunoelectrophoresis of PAGE separated dengue type-2 viral antigen with agarose containing human hyperimmune sera. The second peak from left side is probably the precipitin complexes between envelope (E) protein and its specific antibody.

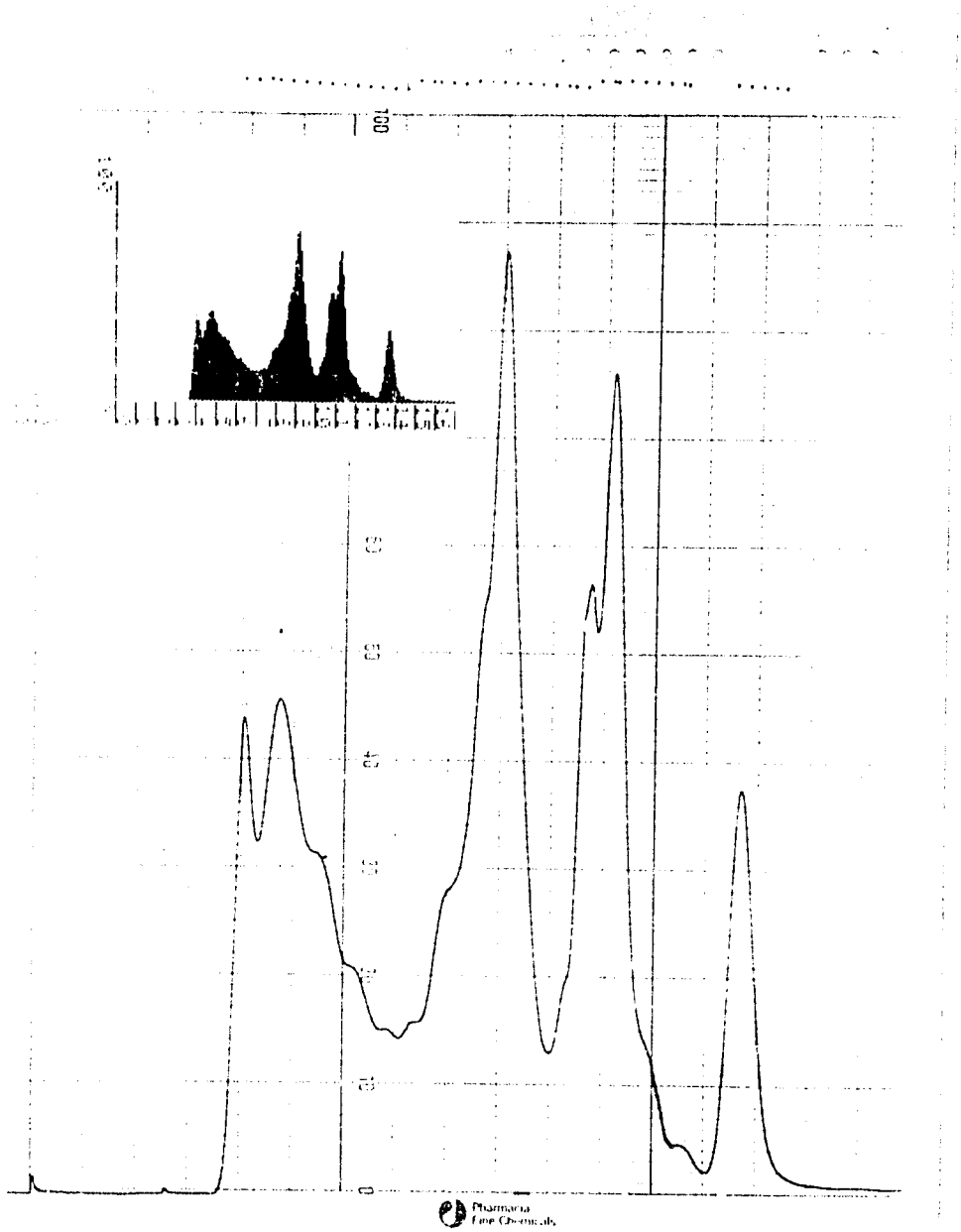
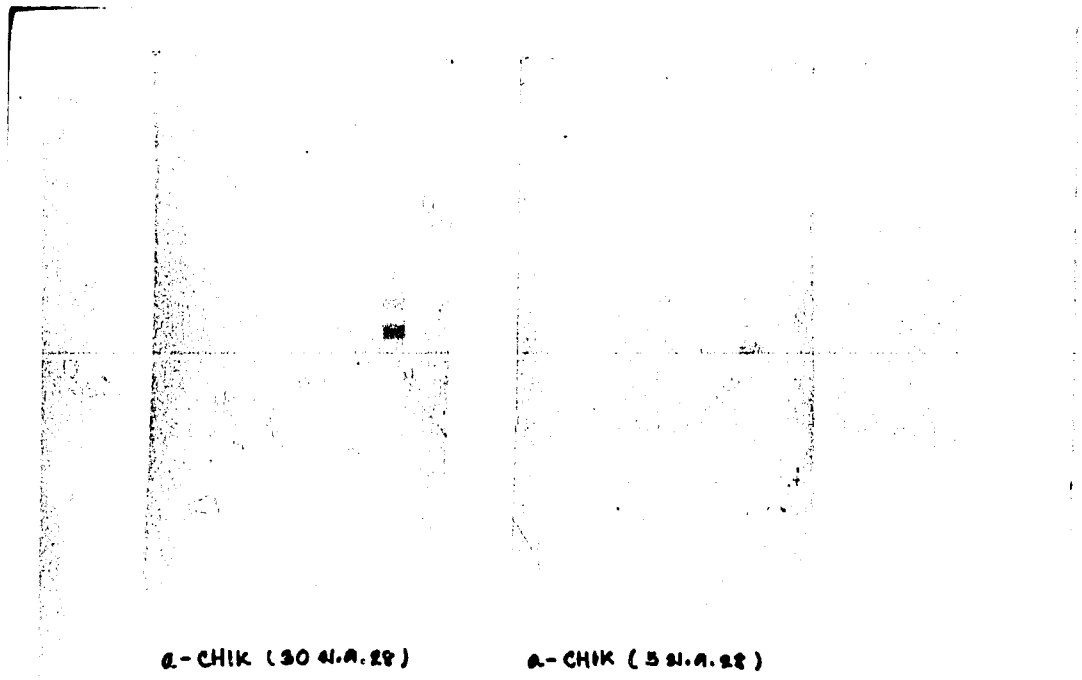


Fig 2. Result from chromatographic separation of dengue type 2 viral antigen using 6 HR 10/30 column with FPLC (Fast protein liquid chromatography) system. At least 6 protein peaks can be collected in separate container allowing further separation by other type of chromatography system.

A.



B.

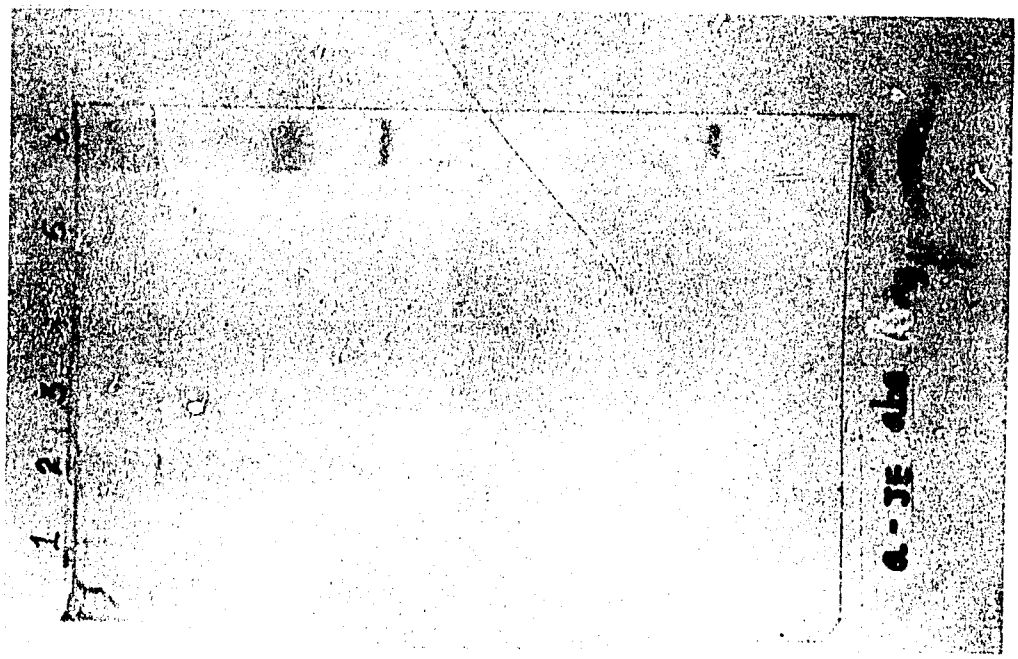
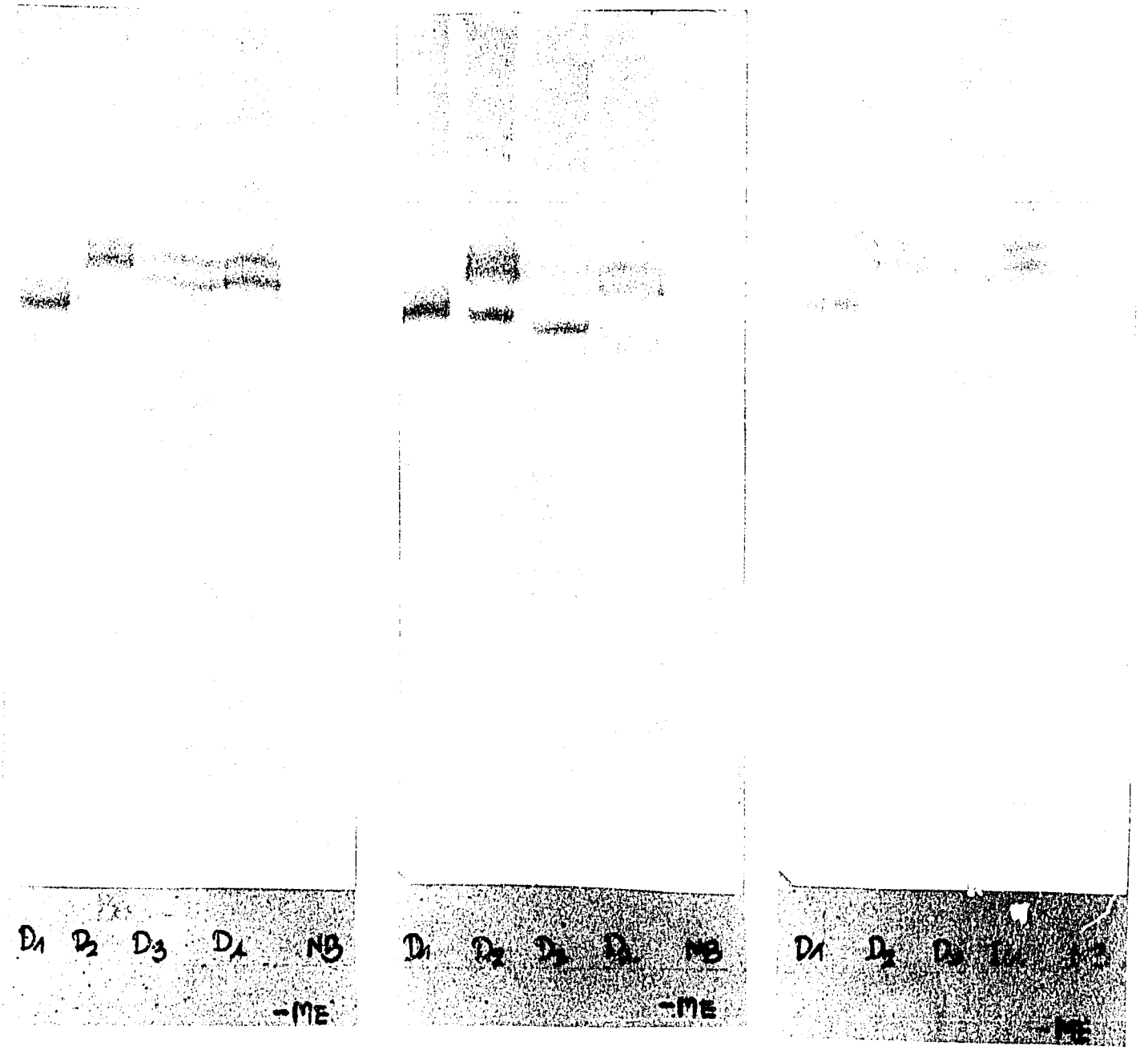


Fig 3. A. VAS/EIA result showing specific antigen-antibody reaction between chikungunya and rabbit anti-chikungunya sera. B. Japanese B encephalitis antigen and rabbit anti-JEV sera specifically bound to each other on the last lane leaving separated proteins from dengue type 1 to type 4, (Lane 1-4) normal mouse brain, (Lane 5) and JEV antigen free yielding negative VAS/EIA results



A B C

Fig 4. These are the results confirming anti-dengue viral antigen in rabbit sera. Crossed reaction among types can be seen. For example in section 4.A. rabbit as immunized with dengue virus type 1 (D1) showing antibody to PAGE separated protein of dengue type 2 (D2), type 3 (D3) and type 4 (D4)

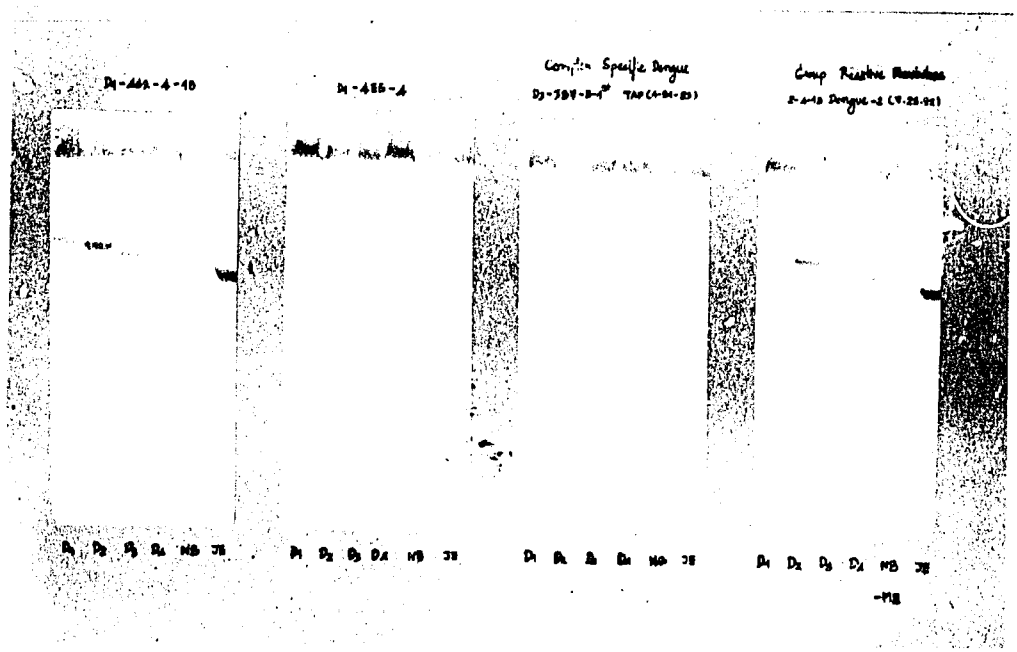


Fig 5. Monoclonal antibody demonstrate antibody specifically reacted with envelope protein (E, 59,000 daltons). Some of them, D1-4G2-4-15 (5.A.) and the antibody classified as group reactive Flavivirus (5.D.) also showed antibody to a higher molecular weight protein (of M.W. approx. >120.000 daltons)

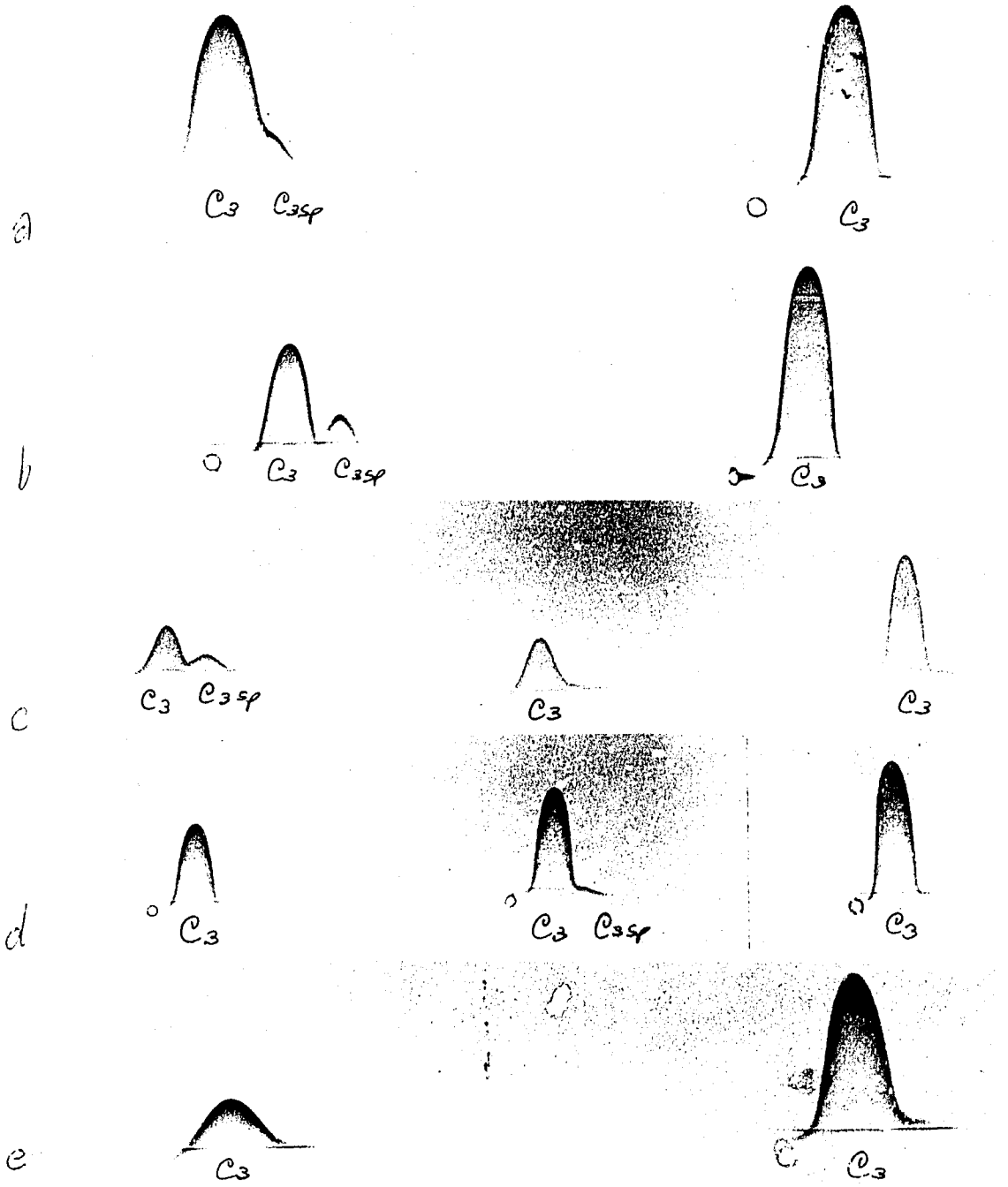


Fig 6. Complement (C3) and Complement split products (C3sp) in cases of primary dengue hemorrhapic fever (P-DHF). Profile of C3/C3sp in sera collected during acute and convalencent phase were compared case by case as they demonstrated here in each row (a, b, c, d and e). a, b and c are from infant cases (6, 8 and 10 months old respectively) and d and e are from 2 children of 6 and 7 years old.

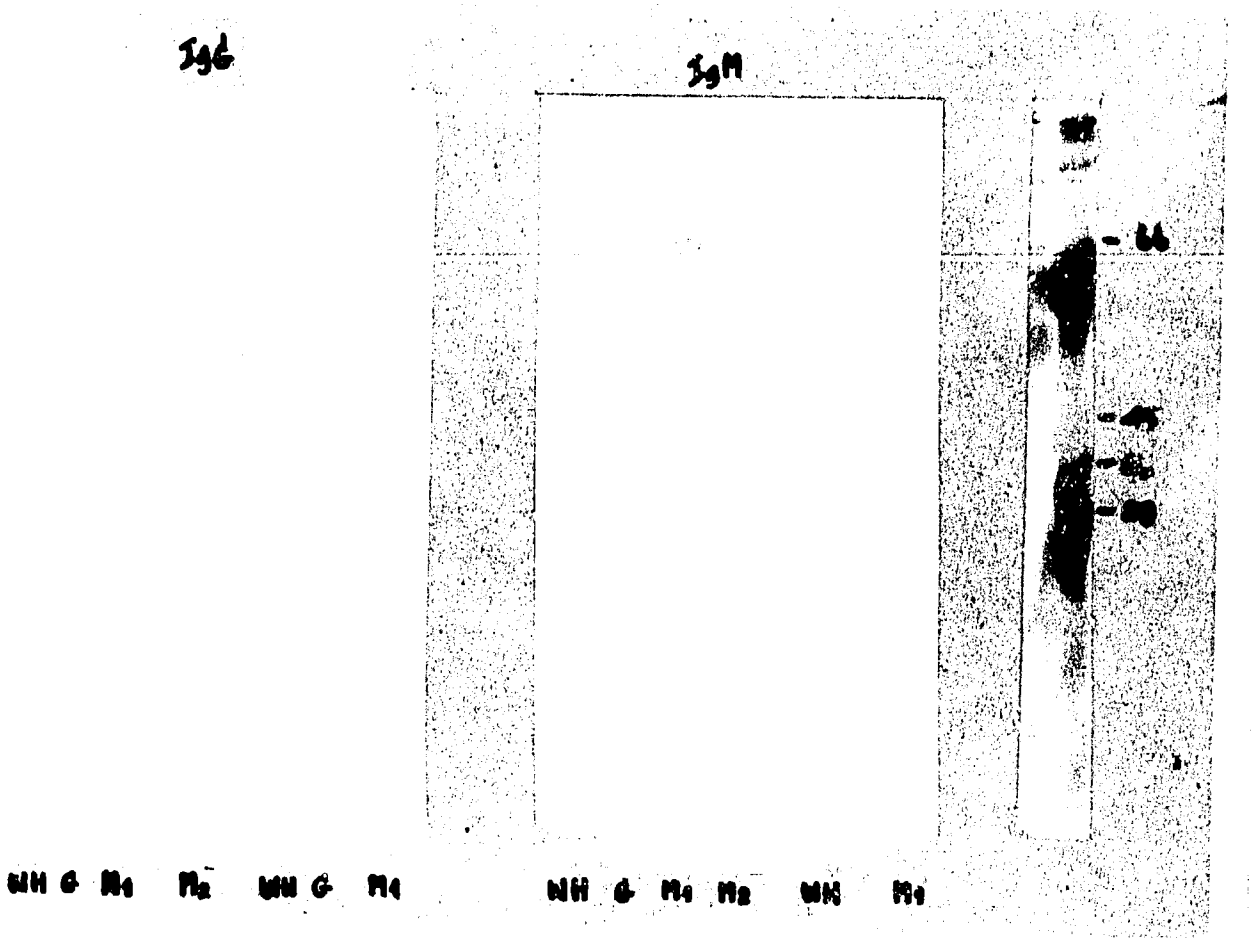


Fig 7. Three fractions, G, M1 and M2, obtained by ionexchange chromatography were characterized by VAS/EIA test. When Rabbit anti-human-IgG labelled with peroxidase was used (left sheet), G showed the strong positive band at M.W. around 51,000 dalton while M1 and M2 showed faint positive band at the same area. With rabbit anti-human-IgM, G fraction demonstrated negative result while M1 and M2 showed strong positive band at M.W. around 66,000 daltons. These result suggested that fraction M1 and M2 contain only IgM while fraction G contains mainly IgG with low amount of IgM