PROGRESS REPORT NO. 3

PROJECT TITLE

IMMUNOPATHOGENESIS OF DENQUE
HEMORRHAGIC FEVER/DENGUE SHOCK SYNDROME

A RESEARCH PROJECT

GRANT NO. USAID/PSTC PROGRAM
936-5512-G-06-0025-00

SUBMITTED BY

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PROJECT LEADER

DEPARTMENT OF PEDIATRICS

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1 JUNE 1987 - 30 NOVEMBER 1987
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**Total Project Budget** | $149,700 (3,892,200 Bahts)

**Project Duration** | 3 years

(7 May 1986 - 31 December 1989)

**Reporting Period** | 1 June 1987 - 30 November 1987

**Budget Allocation**

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<td>$16,750</td>
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PROJECT PROFILE

Country: Thailand
Grant No.: 936-5542-G-00-6025-00
Program: Program on Science and Technology Cooperation
Project Title: Immunopathogenesis of Dengue hemorrhagic fever/Dengue shock syndrome
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1. Background/Introduction:

Despite many active researches in dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in all aspects of the disease during the past decade, DHF/DSS still remains one of the major health problems, not only in Southeast Asia but also in the Pacifics and lately in the Caribbean areas. DHF/DSS is a severe febrile disease of children and adolescents characterized by sudden onset of fever, nausea, vomiting, abdominal pain, hepatomegaly, petechial hemorrhage, epistaxis, hematemesis, melena and shock on the fifth to seventh day of illness with significant mortality. The major pathophysiologic hallmarks in DHF/DSS are shock and excessive hemorrhage which are the leading causes of death. Previous studies suggested that immunological processes are involved in the pathogenesis of this disease, however, the exact mechanisms are largely unknown. Further investigations are needed to understand the whole immunopathophysiologic processes especially factors leading to the development of shock which will lead to better treatment and ultimate prevention of this disease by vaccination.

There are two main areas in the study of DHF/DSS that this proposal tries to emphasize. The first deals mainly with the questions of how cellular immunity manifests in the face of secondary heterotypic dengue infection. The identification and enumeration of subsets of the peripheral blood leukocytes in different phases of the disease will give a better information on what population of cells are mostly affected and indirectly will give a general picture of the cellular circuit operated in this condition. The identification of sites in which the virus replicated and the derangement of the architecture of the lymphoid tissue and
reticulo-endothelial system, together with the data of changes of T-lymphocyte subsets, will give a better insight into which population of cells are involved in cell-mediated immunity as the results of infection and which population of cells are responsible for killing or immune elimination of virus-infected cells.

The second area deals mainly with the role of complement and immune complexes. The possibility of the anaphylatoxins resulted from massive complement activation, to generate "shock" in severe cases will be thoroughly explored. Other possible mechanisms leading to active complement activation will be investigated including the capability of the different serotypic viral antigens and/or the infected cells to activate the complement system. The results will render weight to the notion that complement activation perse can lead to the syndrome of shock and if so, what and how many possible mechanisms are involved. These data will be very essential for the successful employment of dengue vaccine in the future.
2. Objectives:

The overall aim is to explore the immunopathological mechanisms of Dengue hemorrhagic fever (DHF)/Dengue shock (DSS) with the view of obtaining a better guide to the therapeutic intervention and the development of dengue vaccine which is being developed in Bangkok, Thailand. The specific Objectives are:

I. To determine sites of dengue virus replication and virus-cell interactions with mononuclear phagocytes.

II. To examine in detail the derangement of architecture and subpopulation of cells of the lymphoid organs.

III. To study the kinetics of peripheral blood leukocytes in different stages of disease, including subpopulation of T-lymphocytes and natural killer (NK) cells in relation to the derangement of immune response and immune regulation during the course of DHF/DSS.

IV. The complement system will be studied in details with the following emphasis,
   a. detail analysis of complement components, both antigenically and functionally, in various stages of the disease.
   b. correlation between the presence of anaphylatoxins mainly C3a, C5a and clinical activity.
   c. The capacity to activate the complement system of isolated viral antigens from different sources with and without the presence of enhancing antibodies
   d. activation of complements by virus infected cells and susceptibilility to cell lysis by the complement system.
   e. haplotype of complement C1 and factor B in patients with and without shock.
V. Detection of circulating antigen-antibody complexes using various assays with the emphasis on the identification of the subclasses and class of the antibodies, the type of viral antigens and other complement components. Isolation of the complexes in large amounts and detail identification of the antigens by poly and monoclonal antibodies to specific serotypes of the virus.

3. Materials and Methods:

3.1 Patient population to be studied

Children diagnosed as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) who were admitted at Department of Pediatrics, Siriraj Hospital and Children's Hospital, Bangkok, Thailand were included in this study. Approximately 100 serologic proven cases of either primary or secondary dengue hemorrhagic fever will be studied in details during the course of illness. The criteria for the clinical diagnosis of DHF/DSS and grading of disease severity were used as described previously. (1)

3.2 Kinetics of peripheral blood leukocytes in different stages of disease

The peripheral blood leukocytes were obtained every day beginning from the first day of admission to approximately 5-7 days of hospitalization, from 50 confirmed cases of DHF/DSS. The pattern and nature of various kinds of leukocytes were determined sequentially by Wright's stain using light microscope, with particular attention on the accurate enumeration of monocytes, mature lymphocytes and atypical or transformed lymphocytes. The absolute number of these peripheral white blood cells was then calculated from the total white cell count.
At the same time, 10 ml heparinized venous blood samples were obtained and peripheral blood mononuclear cells (PBM) were isolated on Picoll-Hypaque density gradient centrifugation method. The cells were washed 3 times with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 50 μg/ml gentamicin. Subpopulations of PBM cells were then determined by monoclonal antibodies specific for surface antigens on these cells. The unconjugated antibodies of OKT series, T₃ (total T cells), T₄ (inducer/helper T cells), T₈ (suppressor/cytotoxic T cells), B₁ (total B cells) and HNK-1 (natural killer cells) were used to identify lymphocyte subpopulation. The secondary antibodies included FITC-conjugated goat anti-mouse Ig M for T₃, T₄, T₈, Mo₂, B₁ and HNK-1 were used for indirect immunofluorescence assays. In addition, the natural killer (NK) cell function were determined sequentially by ⁵¹Cr-specific release assay using myeloid cell line K 562 as target cells. The age and sex matched normal individual controls were included in each study.

3.3 Complement assays

Assay for complement components, mainly C₃, C₄, factor B, C₇ and C₉ were performed using radial immunodiffusion techniques with monospecific antisera. Functional assays for the classical and the alternative pathways were determined by methods previously described. The complement activations in vivo and in vitro were evaluated by various assays to detect different degree of complement activation as followed:

a. Assay for the presence of C₃a des-arg and C₅a des-arg by radioimmunoassays.

b. Detection of C₃d by rocket immunodiffusion assay.

c. Detection of the soluble terminal complement complex (SC 5b-9) by a modified ELISA method.
3.4 Immune complexes

Assays for the presence of immune complexes in EDTA plasma were employed by 2 different methods:

a. CIq binding test.\(^{(9)}\)

b. Solid phase conglutinin binding.\(^{(10)}\)

Heat aggregated human IgG was used as standard control. Further isolation of circulating immune complexes were performed by a column of polymethylmetacrylate beads coated with either bovine conglutinin or purified human CIq.\(^{(11)}\) The isolated complexes were then labelled with \(^{125}\)I by IODOGEN methods.\(^{(12)}\) The subclasses and classes of immunoglobulins in the complexes could be identified by co-precipitation of the \(^{125}\)I complexes with monospecific antisera. The serotypic viral antigens in the complexes were also identified by co-precipitation, using serotypic - specific polyclonal or monoclonal antibodies (provided by the Department of Virology, AFRIMS).

4. Results/Discussion/Tables:

The results of the first part of the research project during the first half of the study project are consisted with the study of the kinetics of peripheral blood leukocytes and the determination of complement activation and immune complexes during the course of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS).

4.1 Kinetics of peripheral blood leukocytes in different stages of disease

In the previous progress report (No.2) we have studied the kinetics of the peripheral blood leukocytes in 52 serological proven secondary dengue infection, aged 9 months to 12 years. It was found that atypical lymphocytes could be found in significant number 1-2 days before
the onset of shock or subsidence of fever and reach the peak in the shock stage of the disease and then were gradually decreased to normal value at 4 days after shock or defervescence (Figure 1). Whereas the monocytes were increased in the early days of disease and then declined to minimum at 3 days before shock or subsidence of fever. Further kinetic study of lymphocyte subpopulations and NK cell cytotoxic activity were performed in 61 DHF/DSS patients aged 3 months to 12 years and 59 age-matched normal controls. There were 36 patients in grade 2 and 25 patients in grade 3 of the disease severity and none received steroid therapy. The subpopulation of T-lymphocytes, B-lymphocytes and NK-cells were determined serially during the course of the disease using monoclonal antibodies on the febrile stage, shock stage or 1st and 2nd day of subsidence of fever, convalescent stage or 3rd and 4th day of subsidence of fever and once on the recovery stage (approximately 11-15 days after subsidence of fever).

The study revealed that all lymphocyte subpopulations including total T-lymphocyte (T3+), T-helper/inducer (T1+), T-suppressor/cytotoxic (T8+) and natural killer (HNK-1+) cells were decreased on febrile stage and their lowest values were noted on the 1st day of shock or subsidence of fever, while B cells were within the normal range (Figure 2). Thereafter, all lymphocyte subpopulations were increased predominately T3+, T8+ and B lymphocytes reaching peak on the third day after shock and return to normal values in the convalescent stage. Similarly, the absolute number of NK cells (HNK-1+) was decreased during the shock phase and then gradually increased to attain the normal value in convalescent stage (Figure 3). However, the NK cell cytotoxic activity was increased on the first day of shock in spite of decreasing absolute number of NK cells and then declined to normal value in the convalescent stage (Figure 4).
Thus, the lymphopenia on the 1st day of shock was due to the decrement of T cells (both T4+ and T8+ cells) and HNK-1+ cells. The lymphocytosis thereafter was mainly due to the increment of T8+ cells and B cells. However, the NK cell cytotoxic activity was increased on the 1st day of shock inspite of decreasing absolute number of HNK-1+ cells.

1.2 Complement studies

On examining for anaphylatoxin C5a in the plasma obtained from Dengue patients collected in the latter half of 1986 and early 1987, a significant sharp increase of plasma C5a coincided with the period of shock and leakage was found (figure 5). This is the first time that C5a has been demonstrated to be raised in vivo. The finding is very significant since C5a is the most potent complement anaphylatoxin with strong spasmogenic and chemotactic activity. In the presence of polymorphs and prostaglandins especially PGE2 (Jose PJ et al, J Immunol 127, 2376, 1981), C5a produces severe and prolonged vascular leakage. Attempts will be made to investigate the possibility of finding appropriate inhibitors to the anaphylatoxins with the view of being able to influence the course of the disease in some severe cases of DHF in future.

Additional analysis of our data confirms the role of C3a in the pathogenesis of shock. C3a was found to be significantly raised within 24 hours before or after the period of subsidence of fever or the appearance of shock in severe cases. The levels of C3a was extremely high in Grade 3 and 1 patients during the period of shock (figure 6).
Additional information on the role of complement activation in the pathogenesis of shock in DHF was obtained from the study of SC5-9 complex in plasma collected from patients during the same period. The complex is the result of binding of protein S to the newly formed C5b7. Definite increase of the SC5-9 complex at the peak of the disease was obtained (figure 7). Excellent correlation with clinical severity was also demonstrated (figure 8).

All the above finding demonstrated an active role of complement in the pathogenesis of shock and leakage in DHF.

5. Conclusion/Remarks:

5.1 The kinetic study of the peripheral blood leukocytes and lymphocyte subpopulations in DHF/DSS revealed that atypical lymphocytes were significantly increased and reached the peak in the shock stage of the disease and then were gradually declined to normal value at 1 days after shock or defervescence. During shock phase there were increased B lymphocyte while T-lymphocytes (both T-helper/inducer and T-suppressor/cytotoxic) and natural killer (NK) cells. The lymphocytosis thereafter was mainly due to the increment of T-suppressor/cytotoxic (Th1+) and B cells. However, the NK cell cytotoxic activity was increased on the 1st day of shock inspite of decreasing absolute number of HNK-1+ cells.
5.2 The complement system is actively involved in DHF/DSS.

The peak of complement activation and the presence of complement fragments especially C3a and C5a coincides with the onset of shock and leakage. The raised levels of C3a and C5a in patients without shock suggested that C3a and C5a act synergistically producing severe leakage. The role of the circulating immune complexes and the virus infected cells in the activation of complement needs to be reinvestigated.
Figure 1  Serial determination of atypical lymphocyte, monocyte and eosinophil in different stages of DHF/DSS.
LYMPHOCYTE SUBPOPULATIONS IN DHF (62)

**Figure 2** Serial determination of total T-lymphocytes (T3⁺), and subpopulations of T-lymphocytes including T-helper/inducer (T₄⁺), T-suppressor/cytotoxic (T₈⁺) and B-lymphocytes (B₁⁺) in different stages of DHF/DSS.
Serial determination of natural killer cells (HNK 1+) in different stage of DHF/DSS
Figure 4 Serial determination of NK cell cytotoxic activity in different stage of DHF/DSS
Fig 5
Plasma C5a in patients with different grades and at different period of the disease. Highest peaks belong to grade 4 patients. The levels returned to normal after 18 hours.

Fig 6
Plasma C3a levels in different severity grades taken at 24 hours prior to and after the drop of temperature or the appearance of shock. Grade 4 patients had highest levels and were significantly different from all other grades.
Fig. 7
Plasma levels of SC5-9 complex as measured at different time periods. At least two samples from each patient taken at acute and convalescent phase of the disease were taken. Patients with grade 4 showed the highest plasma levels during the period of shock.

Fig. 8
Plasma levels of SC5-9 complex in different grades of patients taken during 24 hours prior to and after the subsidence of fever or the appearance of shock. Patients with grade 4 had the highest levels and was significantly different from all other grades (p < 0.001).
6. **Workplan for the next period**

Approximately fifty more patients with DHF/DSS will be studied for the kinetics of peripheral blood leukocytes and complement profiles including immune complexes. The lymphocyte subpopulations will be also studied, including the derangement of lymphoid tissues in autopsy cases.

7. **References**


