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PROGRESS REPORT No. 6

PROJECT TITLE

IMMUNOPATHOGENESIS OF DENGUE HEMORRHAGIC FEVER/DENGUE SHOCK SYNDROME

A RESEARCH PROJECT

GRANT No. USAID/PSTC PROGRAM

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PROJECT PROFILE

Country	:	Thailand
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Program	:	Program on Science and Technology Cooperation
Project Title	:	Immunopathogenesis of Dengue hemorrhagic fever/ Dengue shock syndrome
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CONTENTS

1. Background/Introductions :

Despite many active researches in dengue hemorrhagic fever/dengue shock syndrome (D H F / D S S) 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 Carribean 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 immunopathogenetic 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 emphasise. The first dealts 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 dealts 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 capabability 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 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 syndrome (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 susceptibillity to cell lysis by the complement system.

c. haplotype of complement C4 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 Ficoll-Hypaque density gradient centrifugation method.[2] The cells were washed 3 times with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 50 μgm/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, T_3 (total T cells), T_4 (inducer/helper T cells), T_8 (suppressor/cytotoxic T cells), B_1 (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 T_3, T_4, T_8, Mo_2, B_1 and HNK-1. In addition, the natural killer (NK) cell function were determined sequentially by ^{51}Cr-specific release assay using myeloid cell line K 562 as target cells.[3] The age and sex matched normal individual controls were included in each study.

3.3 Complement assays

Assay for complement components, mainly C_3, C_4, factor B, C_7 and C_9 were performed using radial immunodiffusion techniques with monospecific antisera. Functional assays for the classical and the alternative pathways were determined by methods previously described.[4,5] 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.[6]

b. Detection of C3d by rocket immunodiffusion assay.[7]

c. Detection of the soluble terminal complement complex (SC 5b - 9) by a modified ELISA method.[8]

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 contrdl. Further isolation of circulating immune complexes were performed by a column of polymethylmetacylate beads coated with either bovine conglutinin of 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 two years 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) which have been described in the previous progress reports (No. 2-4). The results of the study on natural killer (NK) cell numbers and NK cell activity during the course of DHF/DSS were described in the progress report No.5. In This progress report a further study of the plasma terminal complement complex in dengue hemorrhagic fever/ dengue shock syndrome (DHF/DSS) is described.

Our previous data had indicated active roles of the complement system in the pathogenesis of shock and leakage in severe patients with DHF/DSS. The complement profile in DHF/DSS is unique; with moderate reduction of C3, C4 during the acute phase but relatively larger amount of complement anaphylatoxins, mainly C3a and C5a are generated (please see previous reports). The amount of C3a generated is comparable to the levels (upto 9000 ng/ml) found in patients with shock associated with hypersensitivity to dialysis membranes[13], and patients with serum sickness from anti-thymocyte globulin treatment.[14] In severe cases of DHF/DSS (Grade IV) the levels reach 40,000 ng/ml.

In order to complete the total picture of the complement activation, we also measured the plasma levels of the fluid phase terminal complement complex (SC5b-9complex) in the same group of patients. The measurement of SC5b-9 complex was performed by an ELISA method as developed by Professor S. Bhakdi, one of our collaborators in the project.[15] One of the prime reasons for this part of investigation is based on our finding just published in the Journal of Immunology and also presented at the XII th International Complement Workshop at Chamonix, France in 1987,[16] that the factors responsible for the activation of C5 and terminal complement sequence depend on the density of activation sites of the activator molecules and the system will be inefficient if the initiators are small soluble immune complexes. In case activation happens on the surface of cells or large particles, the C3 : C5 and C5 : SC5b-9 ratios reached 30 : 1 and 1 : 1, instead of 100 : 1 and 10 : 1 in small complexes respectively. In other words, complement activation will be more efficient when the activity occurs on relatively larger surfaces. The study of the levels of SC5b-9 complex in relationship

to the levels of C3, C5a in DHF/DSS would indirectly indicate the nature of the activator. The results of the SC5b-9 study were summarized in the attached figures. Figure 1 showed the levels of plasma SC5b-9 complex at different period of the disease in patients with different severity. A sharp rise of SC5b-9 complex was observed coincided with the appearance of shock or subsidence of fever (day 0), with grade IV patients showing highest levels. In the convalescent period (day 12-14), the SC5b-9 complex decreased to the normal levels. Figure 2 shows the mean±standard errors of the levels of SC5b-9 complex in DHF/DSS patients with different grades of severity and compares them with the data obtained from other immune complex diseases (severe systemic lupus erythematosus with active glomerulonephritis and acute phase of post streptococcal glomerulonephritis) in which active complement activation has been demonstrated. Our findings that relatively large amount of SC5b-9 complex was generated in the face of moderate C3 consumption would indicate a very efficient complement activation in DHF/DSS, leading not only the generation of potent anaphylatoxins but also activating the terminal complement sequence. The levels of SC5b-9 complex are significantly higher in DHF/DSS and low in SLE and normal population. This indicates that complement activation in DHF/DSS is much more efficient, reaching the terminal sequence; where as in SLE, a disease in which circulating antigen-antibody complexes activate the complement system, the complement consumption merely reach the levels of C3 or C5. It is reasonable to speculate that the complement activation in DHF/DSS is unlikely to be due to circulating antigen-antibody complexes (CIC) as substantiated by our previous data of not being able to detect considerable amount of CIC by the two sensitive radio-immunoassay

methodes (please see our previous reports), but by direct activation by the virus itself or possibly by the surface of the virus infected cells. In the latter case, the consequences of the activation would have far reaching effects, either leading to cell lysis, releasing many phlogistic mediators or the activation of the arachinodic acid cascade, leading to shock or circulatory leakage. Further investigation into the interaction between the virus infected cell and the complement is therefore indicated. We are currently investigating this problem.

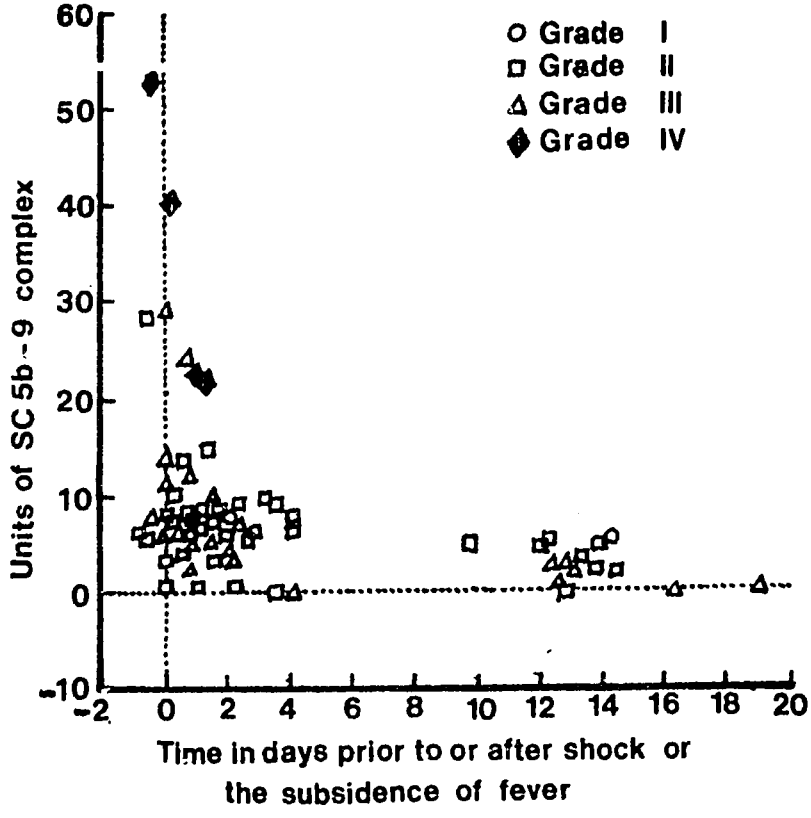

Figure 1 Plasma levels of SC5b-9 complex in 34 patients with different grades of severity at acute and convalescent phase of the disease.

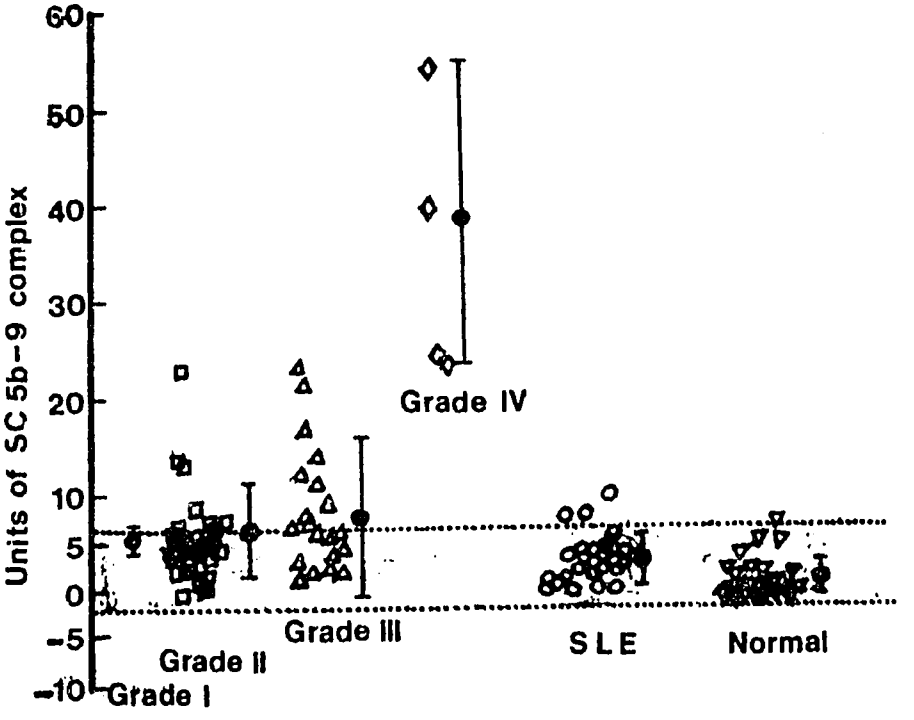

Figure 2 Plasma levels of SC5b-9 complex (mean and standard error) in DHF/DSS with different grades of severity, compared with the levels found in patients with active SLE and in normal individuals.

6. Workplan for the next period

The plan of the research study in the next period will be done including detailed study of the derangement of lymphoid tissues in autopsy cases, sites of dengue virus replication and virus-cell interaction with mononuclear phagocytes and megakaryocytes in the bone marrow. The capacity of dengue virus infected cell to activate the complement system as well as isolated viral antigens from different sources with and without enhancing antibodies to participate in the complement activation will be also investigated. Furthermore, the nature of atypical or transformed lymphocytes in the peripheral blood of DHF/DSS patients will be investigated in details using monoclonal antibody and FACS scan machine.

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