PN-ABM-704

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: December 27, 1988

MEMORANDUM

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

FROM: AID/SCI, Victoria Ose

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

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 4.528 PR# 5

"Immunopathogenesis of Dengue Hemorrhagic Fever/Dengue Shock Syndrome"

Attachment

PN-ABM-704
79838

DUPLICATE

PROGRESS REPORT No. 5

PROJECT TITLE

IMMUNOPATHOGENESIS OF DENGUE HEMORRHAGIC FEVER/DENGUE SHOCK SYNDROME

A RESEARCH PROJECT

GRANT No. USAID/PSTC PROGRAM

936-5542-G-00-6025-00

SUBMITTED BY

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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
Project Leader	:	Dr. Vinai Suvatte,M.D., Ph.D.
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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 1988 - 30 November 1988
Budget Allocation- for this period	:	$ 20,300 (527,800 Bahts)

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 precesses 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 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 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 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.

e. 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 were used for indirect immunofluorescence assays. 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 control. 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). In this report, the results of the study on natural killer (NK) cell numbers and NK cell activity during the course of DHF/DSS will be described in details.

Natural killer (NK) cell activity against K-562 target cells and HNK-1$^+$ cell levels were serially determined in peripheral blood of 62 Thai children with dengue hemorrhagic fever/dengue shock syndrome aged 4-12 years and 59 age-matched normal controls. The studies were performed on febrile stage (F), first (S1)and second (S3) day of subsidence of fever (shock stage), third (C1) and fourth (C2) day of subsidence of fever (early convalescent stage) and once again on the late convalescent stage(C3, approximately 14-18 days after subsidence of fever)

4.1 HNK-1$^+$ cell levels and NK cell activities of normal controls

The HNK-1$^+$ cell levels and NK cell activities of 59 normal controls were shown in Table 1. These values between male and female showed no significant gender difference. Hence, we used a mean value obtained from these 59 children as a normal value for studied patients.

4.2 HNK-1$^+$ cells levels in patients with DHF/DSS

When compared with the normal controls, the patients demonstrated significant decreased absolute absolute number of HNK-1$^+$ cell levels in febrile (F) and shock stage (S1 and S3) but showed no significant difference in the early (C1 and C2), and late convalescent stage (C3) (Figure 1 and Table 3). When the patients were subdivided according to disease severity into grade II and grade III, the patients with grade III of severity showed lower HNK-1$^+$ cell levels in febrile stage when compared with the patients with grade II (Table 2)

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4.3 NK cell functional activities in patients with DHF/DSS.

The NK cell activity against K-562 target cells was assessed in parallel with HNK-1$^+$ cell enumeration. As shown in Figure 2, the level of NK cell activity was higher in the early disease stages (F,S1 and S3) than that in the late disease stages (C1, C2 and C3) and the normal controls. However, the differences were not statistically significant. These results, however, were in contrast with those of the HNK-1$^+$ cell level (Figure 1). Thus, when the NK cell activity on a per-cell basis was considered, it was found that the activity on a per-cell basis was significantly increased in the early disease stages with a peak in the day of shock (Figure 3 and Table 3). The patients with grade 2 and grade 3 of disease severity were found to exhibit comparable NK cell activity in all but the febrile stage which is higher in grade 3. When the NK cell activity per-cell was considered, the patients with grade 3 showed more NK cell activity significantly on febrile stage and the first day of shock (Table 2).

4.4 Discussion

Most studies have shown that NK cells which lyse virus-infected cells have the same phenotypic and morphologic characteristics of those active against K-562 cells.[13,14] However, heterogeneity of NK cells has also been reported in human system.[15,16] The present study has demonstrated that there was significant increase in the activity of each HNK-1$^+$ cells, previously shown to exhibit almost all NK and killer (K) cell functional activities[17], in febrile and shock stages of the patients with DHF/DSS. This study also revealed

that NK cell activity per one HNK-1$^+$ cell was higher on febrile stage and the first day of shock in grade 3 patients when compared with those in grade 2, suggests that NK cell function might also determine the severity of disease. It has been reported that NK cell activity is raised during infection with several viruses in experimental animals and man.[18,19] Based on the pathogenic studies in the Rhesus monkey, it was found that following subcutaneous inoculation of dengue virus, in the presence of enhancing dengue antibody, the virus is replicated in histiocytes, macrophage in regional lymph node, spleen, lungs and in Kuffer cells in the liver.[20] It was hypothesized that those dengue-infected cells will be killed by the process of immune elimination of the hostresulting in the release of various mediators leading to dengue shock syndrome.[21] It is possible that the NK cells may be responsible for the destruction of dengue-infected macrophage. Thus, the results of this study suggest the possibility that NK cells play role in the pathogenesis of DHF/DSS by killing the dengue-infected macrophages which cause the release of such vasoactive mediators.

5. Conclusions/Remarks.

The study of natural killer (NK) cell activity and HNK-1$^+$ cell levels during the course of DHF/DSS revealed that the NK cell activity was not change significantly from the normal controls. In contrast, the level of HNK-1$^+$ cells, which exhibited almost all NK and killer cell functional activities, was significantly decreased

in the febrile and the shock stages and were normal in the early and late convalescent stages. The NK cell activity, on the per-cell basis, was significantly increased in the early disease stage when compared to that of the later period of the disease and of the normal controls. The study also revealed that patients with grade 3 of disease severity exhibited significantly more NK cell functional activities per cell than grade 2 on febrile stage and the first day of shock. These results suggest that natural killer cells were active in defense against dengue viral infection and might play some role in the pathogenesis of DHF/DSS.

This is the first report to demonstrate the NK cell functional activity and levels at various stages of dengue viral infection. Though a definite conclusion as to the role of NK cells in DHF/DSS can not be made, it is hoped that future research will use the findings in this study as a guideline for further improving our understanding of the role of NK cells in defense against dengue viral infection in general and of the involvement, if any, of NK cells in the pathogenesis of DHF/DSS.

Table 1 : HNK-1⁺ cell levels and NK cell activity in peripheral blood of healthy male and female children.

	Males+Females	Males	Females	P-value[b]
Number	59	33	26	-
Age range(mean)	3-13 (8.6)	4-12 (8.1)	3-13 (9.3)	-
% HNK-1⁺ cell	14.3 ± 4.7[a]	14.9 ± 5.3	13.5 ± 3.9	NS[c]
Absolute HNK-1⁺ (cells/mm³)	526.4±231.3	549.8±226.8	496.6±242.3	NS
% Cytotoxicity	56.69±11.42	57.71±10.82	55.39±12.44	NS

[a] : Mean ± standard deviation

[b] : Mann-Whitney U test was used to test the two independent samples (males and females)

[c] : NS = Not significant ($P > 0.05$)

Table 2 : The HNK-1⁺ cell levels, % NK cell cytotoxicity and % cytotoxicity per 1 HNK-1⁺ cell at various stages of illness in DHF/DSS patients with grade II and grade III of disease severity.

Disease stages	% NK-1⁺cells			Absolute HNK-1⁺cells (cells/mm³)			% Cytotoxicity			% Cytotoxicity per 1 HNK-1⁺ cell (x 10^{-4})		
	Grade II	Grade III	P-value[c]	Grade II	Grade III	P-value	Grade II	Grade III	P-value	Grade II	Grade III	P-value
F	9.7±3.8[a] (n = 13)[b]	10.4±5.1 (n = 2)	NS[d]	207.4±123.4	87.6±13.3	P<0.05	59.21±11.69	76.76±0.4	P<0.05	17.8±7.2	23.9±11.5	P<0.05
S1	9.5±5.6 (n = 18)	7.2±3.6 (n = 18)	NS	252.2±226.8	209.9±183.9	NS	65.70±10.7[illegible]	59.51±12.64	NS	20.9±7.9	24.0±9.1	P<0.05
S3	8.9±2.7 (n = 20)	8.6±4.0 (n = 12)	NS	330.6±169.7	244.0±219.[illegible]	NS	58.72±13.73	57.59±12.73	NS	17.7±5.1	19.8±7.9	NS
C1	9.5±2.4 (n = 17)	10.4±4.3 (n = 17)	NS	467.5±210.4	459.2±274.1	NS	53.82±11.79	56.60±11.39	NS	14.7±3.6	14.6±3.5	NS
C2	10.7±3.1 (n = 23)	11.3±4.2 (n = 13)	NS	491.1±233.6	397.1±310.7	NS	57.36±12.97	54.18±12.88	NS	14.2±4.5	13.1±4.3	NS
C3	14.3±4.3 (n = 36)	13.7±3.3 (n = 26)	NS	509.2±270.5	454.4±226.0	NS	53.09±14.41	53.91±15.33	NS	9.8±3.3	10.1±3.2	NS

a : Mean ± standard deviation
b : Number of cases
c : Mann-Whitney U test was used to test the two independent samples (grade II and grade III values)
d : NS = Not significant (P > 0.05)

Table 3 : The HNK-1⁺ cell levels, % NK cell cytotoxicity and % cytotoxicity per 1 HNK-1⁺ cell at various stages of illness in 62 DHF/DSS patients and 59 normal controls.

Disease stages	%HNK-1⁺ cells	P-value[c]	Absolute HNK-1⁺cells (cells/mm³)	P-value	%Cytotoxicity	P-value	%Cytotoxicity per 1HNK-1⁺cell ($\times 10^{-4}$)[b]	P-value
F	9.8±4.0[a]	P<0.05	191.5±122.0	P<0.05	61.6±12.4	NS	18.6±8.2	P<0.05
S1	8.4±4.8	P<0.05	231.1±207.5	P<0.05	62.6±12.1	NS	22.5±8.6	P<0.05
S3	8.8±3.3	P<0.05	298.1±194.3	P<0.05	58.3±13.4	NS	18.5±6.4	P<0.05
C1	10.0±3.5	P<0.05	463.3±244.4	NS	55.2±11.7	NS	14.6±3.6	NS
C2	10.9±3.5	P<0.05	477.0±268.8	NS	56.2±13.0	NS	13.8±4.5	NS
C3	14.0±4.0	NS[d]	486.2±254.2	NS	53.5±14.9	NS	10.0±3.5	NS
Normal controls	14.3±4.7	-	526.4±231.3	-	56.7±11.4	-	10.2±2.3	-

[a] : Mean ± standard deviation

[b] : Average of calculated values for individual samples

[c] : Mann-Whitney U test was used to test the values at each disease stage with the normal controls

[d] : NS = Not significant (P > 0.05)

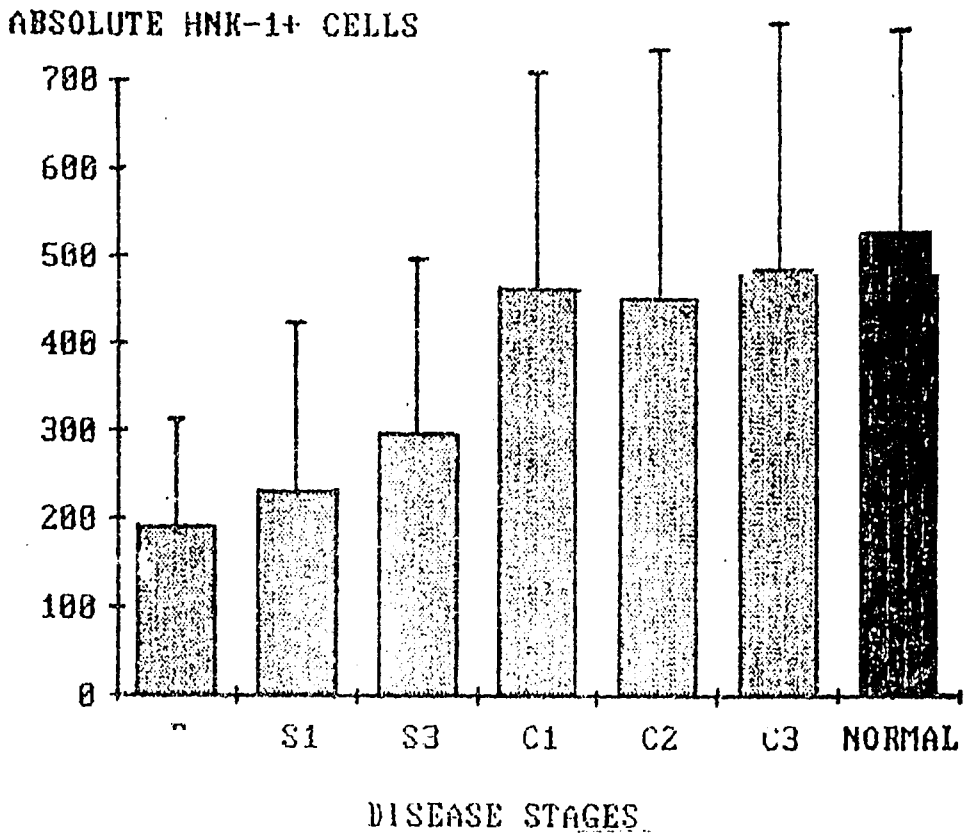

Fig. 1 Absolute numbers of HNK-1+ cells per mm³ at various stages of illness in 62 patients with DHF/DSS compared with the normal controls.

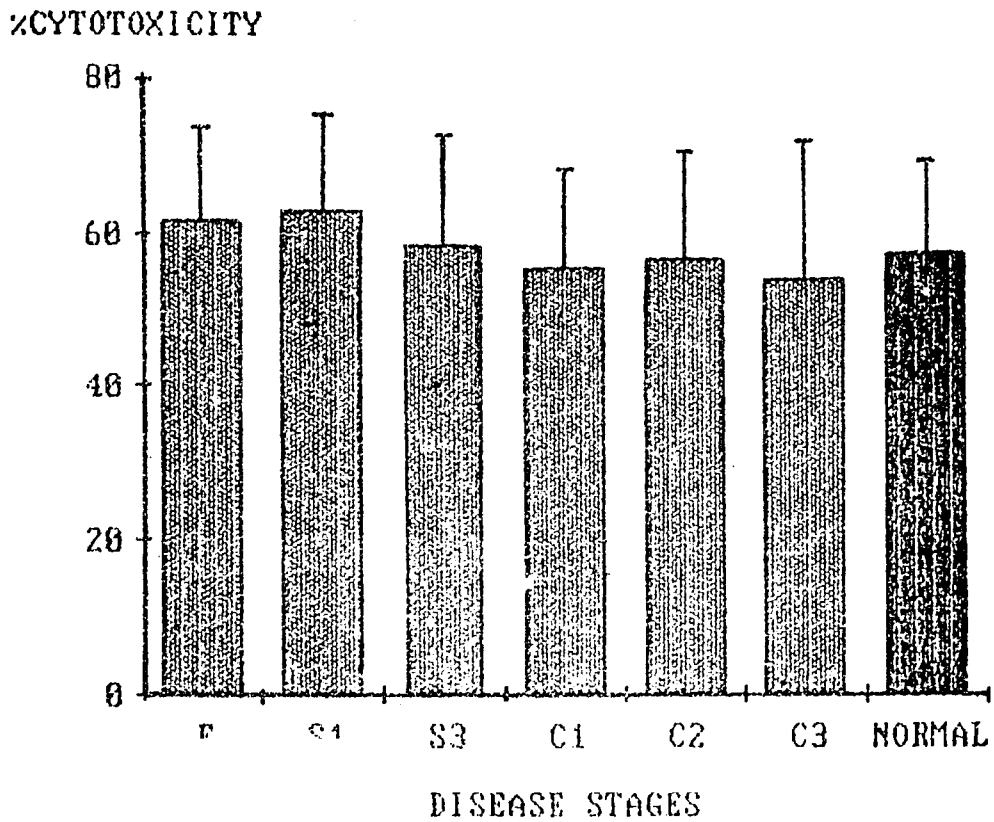

Fig. 2 The percentage of NK cell cytotoxic activity (at E:T= 40:1) at various stages of illness in 62 patients with DHF/DSS compared with the normal controls.

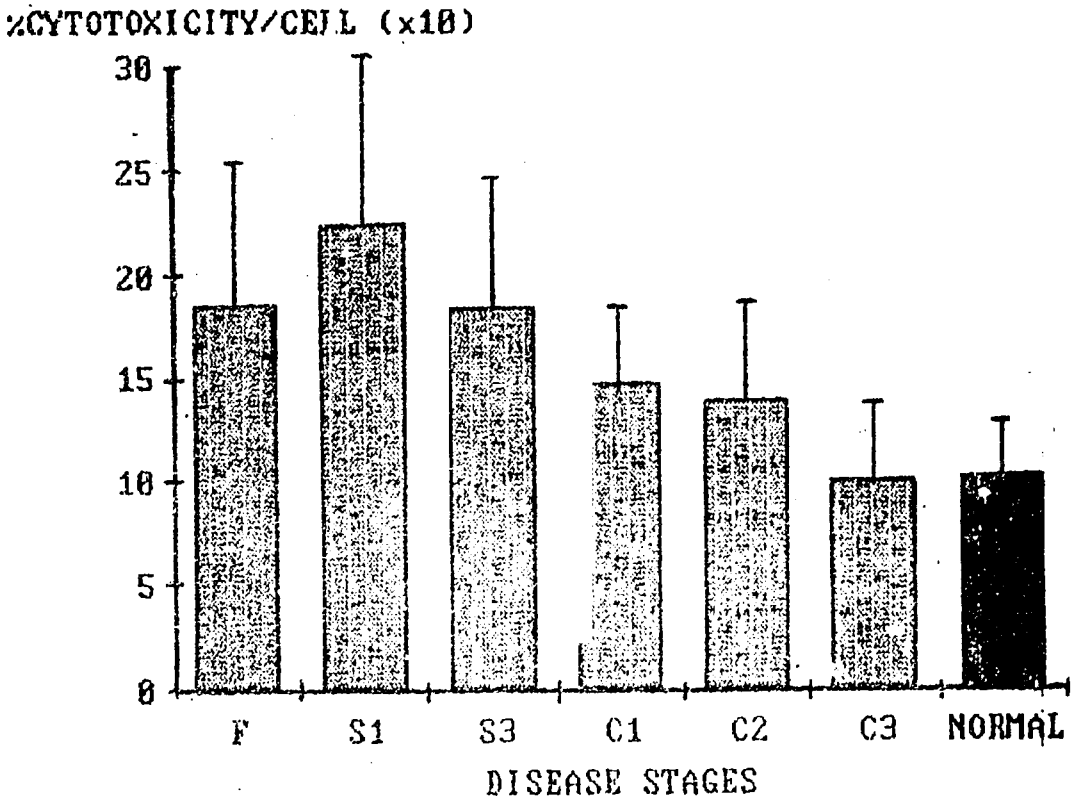

Fig. 3 The percentage of NK cell cytotoxic activity on a per-cell basis at various stages of illness in 62 patients with DHF/DSS compared with the normal controls.

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 interactions with mononuclear phagocytes and megakaryocytes in the bone morrow. The capacity to activate the complement system of dengue virus infected cells as well as isolated viral antigens from different source with and without enhancing antibodies will be also investigated.

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