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**HUMAN T CELL RESPONSES TO
DENGUE VIRUS INFECTIONS:
CD8+CTL AND ACUTE
IMMUNOSUPPRESSION**

A Dissertation Presented By

Anuja Mathew

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
Worcester
in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY AND VIROLOGY
January 1999

HUMAN T CELL RESPONSES TO DENGUE VIRUS INFECTIONS: CD8+CTL AND
ACUTE IMMUNOSUPPRESSION

A Dissertation

By

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Parts of this dissertation have been presented in the following publications

Mathew, A., Kurane, I., Rothman, A.L., Zeng, L.L., Brinton, M.A., and Ennis, F.A. (1996). Dominant Recognition by Human CD8+ Cytotoxic T Lymphocytes of Dengue Virus Nonstructural Proteins NS3 and NS1.2a. *J.Clin.Invest.* 98 (7): 1684-1692.

Mathew, A., Kurane, I., Green, S., Stephens, H.A.F., Vaughn, D.W., Kalayanarooj, S., Suntyakorn, S., Chandanayingyong, D., Ennis, F.A., and Rothman, A.L. (1998). Predominance of HLA-Restricted Cytotoxic T-Lymphocyte Responses to Serotype-Cross-Reactive Epitopes on Nonstructural Proteins following Natural Secondary Dengue Infection. *J.Virol* 72(5): 3999-4004.

Mathew, A., Kurane, I., Green, S, Vaughn, D.W., Kalayanarooj, S., Suntyakorn, S., Ennis, F.A., and Rothman, A.L. (1998). Impaired T cell proliferation during acute dengue infection. Submitted for publication.

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ABSTRACT

There are four serotypes of dengue virus designated dengue 1, 2, 3 and 4 (D1, D2, D3 and D4) and epidemiological studies indicate that a severe complication of dengue virus infection - dengue hemorrhagic fever (DHF) is more likely to occur following a secondary infection. DHF is hypothesized to be immunologically mediated and may be triggered by virus-specific T cells. It is also likely that dengue virus-specific cytotoxic T lymphocytes (CTLs) are important for recovery from dengue virus infections. An analysis of the immune response during acute illness and when the patient has recovered from the infection (immune state) is therefore important as it will provide insights into the immunopathological nature of the disease. This thesis initially examines the CD8⁺ CTL responses in volunteers who have received live attenuated dengue vaccines and then investigates acute and immune T cell responses in children following natural infection with dengue.

When this project was initiated, there was little available information on the human CD8⁺ T cell responses to dengue viruses. PBMC from one donor had generated memory CD8⁺CTL to the nonstructural protein NS3 of dengue virus. Memory CD8⁺CTL responses were therefore analyzed to determine the diversity of the T cell response to dengue virus and to identify immunodominant proteins using PBMC from eight healthy adult American volunteers who had received monovalent live-attenuated

candidate vaccines of the 4 dengue serotypes. All the donors had specific T cell proliferation to dengue viruses and to other flaviviruses that we tested. CTLs were generated from the stimulated PBMC of all donors and in the seven donors tested, dengue virus-specific CD8+CTL activity was demonstrated. The nonstructural proteins NS3 and NS1.2a and the structural protein E were recognized by CD8+CTLs from six, five and three donors respectively. All donors recognized either NS3 or NS1.2a. In a donor who received a dengue 4 vaccine, CTL killing was seen in bulk culture against the premembrane protein (prM). This is the first demonstration of a CTL response against the prM protein. The CTL responses using PBMC of two donors were serotype-specific whereas all other donors had serotype-cross reactive responses. For one donor, CTLs specific for E, NS1.2a and NS3 proteins were all HLA-B44 restricted. For the three other donors tested the potential restricting alleles for recognition of NS3 were HLA-B38, A24 and/or B62 and B35. These results indicate that the CD8+CTL responses of humans after immunization with a single serotype of dengue virus are diverse and directed against a variety of proteins. The nonstructural proteins NS3 and NS1.2a appear to be immunodominant and should be considered when designing subunit vaccines for dengue.

Previously T cell responses had not been examined in people who have had natural infections with dengue. The HLA diversity between North American Caucasians and populations where dengue is a serious health problem, calls for the analysis of immune responses in people who have been infected with natural circulating strains of the virus. We examined the memory cytotoxic T lymphocytic (CTL) responses of

peripheral blood mononuclear cells (PBMC) obtained from patients in Thailand 12 months after natural symptomatic secondary dengue infections. In all four patients analyzed, CTLs were detected in bulk culture PBMC against nonstructural dengue proteins. Numerous CD4⁺ and CD8⁺ CTL lines were generated from the bulk cultures of two patients, KPP94-037 and KPP94-024, which were specific for the NS1.2a and NS3 proteins respectively. All CTL lines derived from both patients were crossreactive with other serotypes of dengue virus. The CD8⁺ NS1.2a specific lines from patient KPP94-037 were HLA-B57 restricted and the CD8⁺ NS3 specific lines from patient KPP94-024 were HLA-B7 restricted. The CD4⁺ CTL lines from patient KPP94-037 were HLA-DR7 restricted. A majority of the CD8⁺ CTLs isolated from patient KPP94-024 were found to recognize a.a. 221-232 on NS3. These results demonstrate that after symptomatic secondary natural dengue infections in Thai patients, CTLs are mainly directed against nonstructural proteins and are broadly crossreactive. The data correlate with our observations that nonstructural proteins are immunodominant proteins in volunteers who received dengue vaccines.

We were interested in examining CTL responses in children during their acute illness and comparing them to memory CTLs obtained from the same children a year or more after the infection. A detailed analysis on samples from nine patients during their acute illness failed to generate any dengue virus-specific CTL responses. We therefore decided to determine if cell mediated responses are altered during acute dengue infection. Decreased proliferative responses to mitogens and recall antigens have been observed in

PBMC obtained during several acute human viral infections. All responses of PBMC during acute illness were compared to the same patients PBMC obtained at least 6 months after their infection. Proliferative responses to PHA, anti-CD3, tetanus toxoid and dengue antigens were significantly decreased in PBMC obtained during the acute infection. The proliferative responses to PHA were restored by the addition of gamma-irradiated autologous immune or allogeneic PBMC. Cell contact with the irradiated PBMC was necessary to restore proliferation. Non-T cells from the acute PBMC of dengue patients did not support proliferation of T cells from control donors in response to PHA, but T cells from the PBMC of patients with acute dengue proliferated if accessory cells from a control donor were present. Addition of anti-CD28 antibodies restored anti-CD3-induced proliferation of the PBMC of some patients. The percentage of monocytes was reduced in the acute sample of PBMC of the dengue patients. Addition of IL-2 or IL-7, but not IL-4 or IL-12 also restored proliferation of acute PBMC stimulated with anti-CD3. The results demonstrate that both quantitative and qualitative defects in the accessory cell population during acute dengue illness result in a depression of in vitro T cell proliferation.

The data generated from this project shed light on the nature of the immune responses during acute natural dengue infections. It strengthens the existing data on the human memory CD8+CTL responses to dengue viruses and validates the observations by examining memory CTL responses after natural dengue infection in patients from Thailand. In addition, we demonstrate a profound defect in lymphoproliferative responses

during dengue illness.

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ABBREVIATIONS

ADE	antibody-dependent enhancement
ALT	alanine aminotransferase
APC	antigen-presenting cell
BLCL	B-lymphoblastoid cell line
C	capsid protein
cAMP	cyclic adenosine monophosphate
Cpm	counts per minute
CTL	cytotoxic T lymphocyte
CMV	cytomegalovirus
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
DV	dengue virus
E	envelope protein
E/T	effector:target ratio
FBS	fetal bovine serum

GAG	glycosaminoglycan
HCMV	human cytomegalovirus
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
HTLV-1	human T lymphotropic virus type 1
IL	interleukin
JE	japanese encephalitis
M	membrane protein
MHC	major histocompatibility complex
NS	nonstructural protein
OFI	other febrile illness
PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E2
PFU	plaque forming units
PHA	phytohemagglutinin
TBE	tick borne encephalitis
TCR	T cell receptor
TNF	tumor necrosis factor

Vac

vaccinia

WNV

West Nile virus

YFV

Yellow fever virus

CHAPTER I

INTRODUCTION

There are estimated to be up to 100 million cases of dengue infection each year (Halstead, 1988). Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) affect people in South America, South and South-East Asia, the Caribbean and Africa and are caused by a group of antigenically related viruses called dengue viruses (Halstead, 1988). There are four serotypes of dengue virus designated dengue 1, 2, 3 and 4 (D1, D2, D3 and D4) (Sabin, 1952). All four serotypes of virus can cause the milder form of illness (DF) and the more severe form of disease (DHF/DSS).

Dengue virus is transmitted to humans by mosquitoes belonging to the genus *Aedes*. The species *Aedes aegypti* is the main vector although other *Aedes* species have been known to transmit disease (Rodhain and Rosen, 1997). This species bites during the daytime and lives near humans, which accounts for higher infection rates among adult women and children compared to adult men. *Aedes aegypti* is indigenous to the Southern part of the United States, and in 1995 there were 7 cases of DV infection reported in Texas in individuals who did not travel outside the country (CDC, 1996). The emergence of the severe form of disease, DHF/DSS, as a public health problem is thought to be due to the circulation of multiple serotypes of virus in areas with large populations and mosquito vectors. Dengue fever occurs endemically or as epidemics in populations that

are predominantly non immune. These outbreaks occur typically in the rainy season.

A. VIRUS STRUCTURE AND COMPOSITION

Dengue viruses (DV) belong to the family Flaviviridae, which includes Yellow fever virus (YFV), Japanese encephalitis virus (JE) and West Nile virus (WNV); the majority of these viruses are arthropod borne (Monath and Heinz, 1996). DV contain a single stranded positive sense RNA genome. The genome is about 11Kb long and genome length RNAs appear to be the only virus-specific messenger RNA molecules in infected cells. The genome encodes for 3 structural and 7 nonstructural proteins and the primary translation product is synthesized as a single polypeptide that gets cleaved cotranslationally or posttranslationally by viral and host proteases to produce the different proteins (Chambers, et al., 1990) (Figure I-1).

The structural proteins include the nucleocapsid (C), the membrane (prM) and the envelope (E) proteins, and the genes that encode them are located at the 5' end of the genome (2325 of the 10173 nucleotides). The C protein is the first protein to be synthesized during viral translation. It is a highly basic protein and forms a structural component of the nucleocapsid (Henchal and Putnak, 1990). The prM protein is synthesized as a precursor that is cleaved to a mature M and a nonM portion that is secreted into the extracellular medium. The formation of the mature M is crucial for the morphogenesis of the virion and results in an increase in viral infectivity (Randolph, et al., 1990). The envelope protein (E) is thought to be important in several different

processes including virion assembly, receptor binding and membrane fusion (Kaufman, et al., 1987). It is the target for neutralizing antibodies, and mutations on E seriously affect viral pathogenesis in experimental animals. Neutralizing antibodies to E at subneutralizing concentrations and nonneutralizing antibodies have been shown to mediate antibody dependent enhancement (ADE) of infection (Kurane, et al., 1991c). ADE is a serological phenomenon in which productive viral infection of susceptible cells (cells that bear Fc receptors for antibodies like monocytes and macrophages) is modified by the addition of virus-reactive antibody at certain concentrations.

The xray structure of the envelope protein of tick borne encephalitis (TBE), a related flavivirus, has been solved (Rey, et al., 1995). The complex is a head to tail oligomer anchored to the membrane by its distal ends. The architecture of the E dimer indicates that it is flat and extends in a direction parallel to the surface of the virion. This is in contrast to the spike-like projection of the influenza hemagglutinin protein, which has analogous functions.

Chen et al recently showed that a recombinant dengue virus envelope protein expressed in Cos cells bound to heparan sulfate (HS) molecules on Vero cells, and they speculated that HS is the target cell receptor for DV (Chen, et al., 1997). They showed that highly sulfated HS inhibited binding and infectivity of DV to a greater extent than any low sulfate HS. Treatment of Vero cells with glycosaminoglycan (GAG) lyases, which degraded heparin, prevented envelope protein binding; treatment of DV with

suramin, a polyionic sulfate, also prevented infection. Others have hypothesized that HS is a primary receptor, which serves to localize the virus on the surface of the cell and enhance the uptake of virus by an unidentified high affinity second receptor in close proximity.

The genes encoding the 7 nonstructural proteins, NS1-NS5 are found on the C terminal two-thirds of the genome. The NS1 glycoprotein, which is the soluble complement fixing antigen, exists in cell-associated, cell-surface or extracellular nonvirion forms (Lee, et al., 1989, Westaway, 1987). The NS2a protein is important for proteolytic processing of the NS1 protein and the NS2b protein is involved in proteolysis along with NS3 (Falgout, et al., 1989, Falgout, et al., 1993). The NS3 protein is the second largest dengue viral protein (618-623 a.a.) and is highly conserved among flaviviruses (Mandl, et al., 1989). At its N terminus it has sequence homology to trypsin-related serine proteases (Bazan and Fletterick, 1989). The C terminus shares homology with a family of RNA helicases and there is some evidence that this region is involved in the capping and methylation of viral RNA (Gorbalenya, et al., 1989). The NS4a and 4b proteins are hydrophobic with no known function as yet. NS5, the largest protein (900-905 a.a.) is also highly conserved (Mandl, et al., 1989). It is an RNA dependent RNA polymerase and may be involved in RNA capping (Grun and Brinton, 1987, Sumiyoshi, et al., 1987).

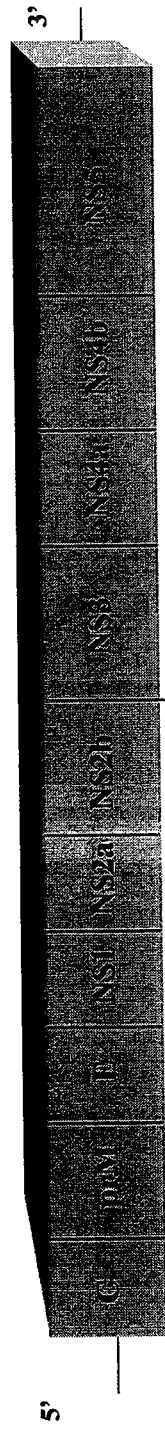


Figure I-1. Schematic of the dengue virus genome. The dengue virus genome is approximately 11,000 base pairs in length and codes for three structural and seven nonstructural proteins, and is flanked by 5' and 3' non-coding regions. Note: not to scale.

B. CLINICAL SPECTRUM OF ILLNESS AND DIAGNOSIS

Dengue virus infections are often asymptomatic, but infection with any serotype of dengue virus can cause a wide spectrum of illness ranging from a viral fever (DF) to a fatal hemorrhagic disease (DHF/DSS) (Burke, et al., 1988, Halstead, 1980). If a person gets infected with any one serotype of dengue virus (primary infection) he has life long immunity to that serotype, but no cross-protective immunity to the other three serotypes (Sabin, 1952).

DF lasts about 5-7 days after an incubation period of a few days and is a self-limited illness. The fever usually does not exceed 40.5°C , and may return to normal during the middle of illness. Some patients do not show this typical 'saddleback fever' curve. DF is characterized by extreme malaise, muscleache, headache, backache, nausea and vomiting. Development of a macular or maculopapular rash on days 2 to 6 of illness is common. In 5-30% of cases, DF is associated with hemorrhage. The symptoms are usually followed by defervescence and patients start to recover. Petechiae are seen at the time of defervescence and last for a few days. Classical dengue fever is associated with neutropenia, leukopenia, presence of atypical lymphocytes and thrombocytopenia (in 30-50% of confirmed cases).

DHF occurs in a smaller number of patients but the early clinical features of DF are indistinguishable from DHF. The characteristic features of DHF appear at the time of defervescence when patients experience increased capillary permeability, resulting in

leakage of plasma into interstitial spaces. There is marked hemoconcentration, thrombocytopenia and a prolonged bleeding time. The World Health Organization (WHO) clinical definition of dengue hemorrhagic fever includes continuous fever, a positive tourniquet test, hepatomegaly, thrombocytopenia ($< 100,000/\text{mm}^3$) and hemoconcentration (hematocrit increased by $\geq 20\%$ above baseline value). The WHO established a grading system for the severity of DHF (WHO, 1986), from less severe (grade 1) to severe (grade 4). Grades 3 and 4 are also known as dengue shock syndrome (DSS) in which plasma leakage is excessive resulting in hypovolemia and circulatory shock. Without treatment, death will occur in up to 50% of patients with DSS (Monath and Heinz, 1996). It is common to detect pleural effusion during plasma leakage in DHF cases. Most patients recover from these severe symptoms with fluid replacement therapy with no longlasting organ dysfunction.

Liver function tests (serum ALT and AST) are increased in patients with DF and DHF (Kuo, et al., 1992, Nimmannitya, 1987). Kalayanarooj et al found that these elevations are more common in the early stages of dengue than in other non-dengue febrile illnesses (Kalayanarooj, et al., 1997). Severe gastrointestinal bleeding, which is more common in DHF/DSS patients, may complicate the course of illness and can be the direct cause of death. Hepatomegaly with hepatic failure may occur in severe cases.

C. PATHOGENESIS OF INFECTION

1. Virulence of Virus Strains

Studies in some populations have suggested that increased virulence of certain strains of virus cause DHF (Burke, et al., 1988, Sangkawibha, et al., 1984). It is known that dengue viruses differ in genotype and phenotype and these differences influence in vitro experimental parameters such as plaque size. Propagation of dengue virus in suckling mouse brains have yielded strains of viruses that have attenuated virulence in humans (Sabin, 1952). However, variations in location of natural strains of dengue viruses make any differences in the virulence among strains difficult to assess.

Outbreaks of severe disease in the Americas have been associated with the introduction of a dengue 2 genotype of Asian origin that has been associated with DHF in that region (Gubler and Clark, 1995, Rico-Hesse, 1990). These observations are difficult to interpret because during this time period there has also been increased circulation of multiple serotypes of dengue virus. The data are not clear and definitive studies have not yet been performed to indicate that certain strains of dengue virus cause DHF. The lack of a suitable animal model has also made identifying markers of virulence difficult.

Burke et al and Sangkawibha et al suggested that infections with dengue virus serotype 2 were associated with an increased risk for the development of DHF/DSS

(Burke, et al., 1988, Sangkawibha, et al., 1984). Strains of D2 virus associated with DHF have been reported to replicate to higher levels in monocytes than DF associated strains (Kliks, 1990, Morens, et al., 1991). Other serotypes of virus were not examined in these studies. Since DHF/DSS has been shown to occur after infection with each of the four serotypes of virus (Gubler and Trent, 1995), the concept that dengue 2 viruses cause severe disease needs to be reexamined. Changes in the envelope sequence of the genome have not been linked to disease severity amongst different strains of virus (Blok, et al., 1989, Chungue, et al., 1993, Lee, et al., 1993). Research has been performed mostly on the envelope protein and therefore this does not exclude the possibility that other regions of the genome may be associated with severity of illness. Significant differences in viremia titres have not been detected between patients with DF or DHF; however recent studies suggest that the degree of capillary leakage in DHF patients infected with dengue 2 correlated with levels of viremia (Vaughn et al unpublished data).

2. Immunopathology

a) Epidemiologic and pathologic evidence for immunopathogenesis of DHF

There is considerable evidence that host immune factors play an important role in determining if a person develops DHF. Most of the evidence is epidemiological but these strongly implicate prior exposure to the virus as a critical determinant of pathogenesis.

The overwhelming percentage of cases (80-90%) of DHF occur during secondary

infections, that is infection with a serotype of DV other than that which caused their first infection (Burke, et al., 1988, Guzman, et al., 1987, Halstead, 1980). There are a minority of cases of DHF which occur during primary infections. DHF during primary infections also occurs in infants 6-12 months of age who have anti-dengue IgG enhancing antibodies from their mothers in their circulation (Kliks, et al., 1988).

DHF commonly occurs in areas where multiple serotypes of dengue virus are circulating. In these areas people that are visiting for the first time experience classical DF whereas those that live in the area appear to develop DHF from the same strain of virus (Herrera-Basto, et al., 1992, Phillips, et al., 1992). Most of the cases of DHF are confirmed to have secondary infections by serologic testing. Cuba had an outbreak of dengue 2 virus in 1981. Four years earlier, Cuba had experienced an outbreak with dengue 1 after many years during which the island had been free of dengue infections. Children between the ages of 1-3 in 1981 who had no prior exposure to any other dengue serotype, developed classical dengue fever. The majority of DHF cases occurred in the age group above 4 years who had prior dengue infection in 1976 and in some infants under 1 whose mothers were dengue antibody positive (Bravo, et al., 1987, Guzman, et al., 1987).

Pathological studies of patients who died from DHF reveal no major organ pathology, no sequelae post infection, vascular changes are non-specific and virus antigen has been located only in cells of the reticuloendothelial cell compartment. The vascular

injuries associated with severe disease may result from the direct destruction of endothelial cells or by mediators that are secreted which alter the function of the cells indirectly. Replication of dengue viruses in endothelial cells has not been demonstrated in vivo although the 4 serotypes of virus can infect these cells in vitro (Butthep, et al., 1993). Dengue virus antigens have not been detected in vascular endothelial cells of patients who succumbed to DHF. Electron microscopy has shown loss of integrity of tight endothelial cell junctions (Sahaphong, et al., 1980). Most observations do not support the direct destruction of cells by the virus but rather a functional alteration of endothelial cells through the release of soluble mediators.

b) Antibody dependent enhancement of infection

Antibodies to certain viruses, including flaviviruses, have been shown to enhance virus infection by a process known as antibody dependent enhancement (ADE) of infection (Halstead, 1977a, Kurane, et al., 1991c). These antibodies are generally of the IgG fraction, nonneutralizing and mediate their effect only at certain dilutions. ADE requires binding of the antibody to the Fc γ R on the monocytes because monoclonal antibodies to the Fc γ R have been shown to inhibit ADE (Peiris, et al., 1981) and enzymatic removal of the immunoglobulin Fc region blocks ADE but not neutralization. The antibodies that mediate ADE in dengue are directed against the envelope and the prM protein (Henchal, et al., 1985). The consequences of ADE in vivo could be to increase the

viral burden and/or altered tissue tropism (Halstead, 1977a, Halstead and O'Rourke, 1977b) which could contribute to the pathogenesis of DHF. Eighty-five to ninety percent of DHF cases are seen in children over 1 year of age with secondary infections who could theoretically have enhancing antibodies from their primary infection.

Kliks et al examined preinfection sera of 40 children who subsequently developed either DHF/DSS or had asymptomatic dengue infections. They showed that undiluted sera from children (5/7) who developed severe disease (DHF/DSS) enhanced dengue-2 virus infection of human monocytes while sera of children who had asymptomatic secondary infections did not (Kliks, et al., 1989). Thus during a secondary DV infection, a patient is likely to have circulating antibodies generated during primary infection that are crossreactive to the presently infecting serotype, but cannot neutralize it. These data were generated using a prototype strain of dengue 2 virus, not the virus strain that infected the children. A lack of a suitable animal model of dengue makes confirmation of these observations in vivo difficult.

c) Complement activation

Patients with DHF have been shown to have abnormal levels of different complement components. Serum levels of C3, C4 and C5 are decreased and C3a levels are increased (Malasit, 1987). C3a and C5a stimulate tissue mast cells to secrete histamine and also help neutrophils move across the endothelium. Histamine is known to

cause formation of gaps between the endothelial cells. Immune complexes and complement activation have also been shown to be present in patients with DF (Shaio, et al., 1992). The exact role that complement plays in the pathogenesis of infection remains unclear. It has been proposed that the high levels of antibody present in patients with DHF may form immune complexes, which activate complement and cause immune complex disease in the blood vessels. The alteration of serum levels of complement occur during the capillary leakage phase of DHF; therefore they may be a response to another stimulus that triggered the capillary leakage. To date no direct evidence exists to confirm this hypothesis.

d) Role of T cells in Immunopathogenesis

The hypothesis of our laboratory is that T cells play a crucial role in the immunopathogenesis of dengue disease. Several observations have been made by analyzing patients samples which suggest that T cells play an important role in mediating disease. In vivo evidence of T cell activation has been shown by Kurane et al in the sera of patients with dengue infections in Thailand (Kurane, et al., 1991a). Levels of sIL-2R, sCD4 and sCD8 were more elevated in patients with DHF compared to patients with DF, which in turn were higher than normal controls. Atypical lymphocytes, which probably include activated T lymphocytes, are seen in peripheral blood smears of patients with acute disease (Kalayanarooj, et al., 1997, Thisyakorn, et al., 1984). Although these data

do not conclusively prove that these T cells are the mediators of immunopathogenesis, they suggest a potential for harm that these activated T cells may cause.

D. CYTOTOXIC T CELLS AND THEIR POTENTIAL ROLE IN DENGUE VIRUS INFECTIONS

1. CTLs in clearance of virus infections

Cytotoxic T cells have been shown to play an important role in clearing and recovery from viral infections. Virus-specific CTLs may exert their effects by directly lysing virus-infected antigen presenting cells and/or releasing cytokines (including IFN- γ and TNF- α) and other factors with direct or indirect antiviral activity. Neutralizing antibodies can prevent viral infections but only virus-specific CTLs can eliminate established infections.

CD8+CTLs have been traditionally shown to be the major cytotoxic component of the T cell subsets. Many studies in mice have demonstrated their role in the elimination of virus-infected cells in vivo. CD4+ T cells were initially thought to only provide help for CD8+CTLs to become activated and lyse foreign targets or help antibody producing B cells. Many reports have since demonstrated virus specific CD4+CTLs in both humans and mice. Although several reports have examined the role of CD4+CTLs in vivo (Muller, et al., 1992, Neal and Splitter, 1995, Wijburg, et al., 1996), CD8+CTLs are

thought to be the main protective immune mechanism against several virus infections

Lukacher et al showed that influenza virus-specific CD8+CTLs of two specificities protected recipient Balb/c mice from a lethal influenza infection by reducing pulmonary virus titers (Lukacher, et al., 1984). A subtype-specific clone protected mice that were subsequently infected with a lethal dose of influenza virus in a subtype-specific manner whereas a crossreactive clone protected mice infected with different subtypes of virus. McIntyre et al demonstrated the specificity of LCMV immune class I restricted T cells because they reduced LCMV spleen titres in mice infected with LCMV but they had no effect on reducing titres of Pichinde virus, a related arenavirus, in Pichinde virus-infected mice (McIntyre, et al., 1985). These results indicate the specificity of the T cell clones, and suggest that direct contact between the killer and target cell is necessary for elimination of virus-infected cells, arguing against nonspecific mechanisms that work indirectly.

The main mechanism of direct lysis by CTLs is via the perforin/granzyme pathway . Perforin is a pore-forming protein that is released by the killer cell and causes loss of plasma membrane integrity of the target cells leading to its eventual lysis and elimination. Another mechanism by which infected cells are eliminated is the interaction of Fas ligand on T cells with Fas, a molecule which can be constitutively expressed on the APC's. This interaction has been shown to induce apoptotic death of target cells.

2. Memory cytotoxic T lymphocytes in dengue infection

Our laboratory has focused on characterizing and understanding T lymphocyte responses to dengue infection. Most of these studies were performed on PBMC obtained from young American volunteers who had received live attenuated monovalent dengue vaccines as a primary infection. Kurane et al have demonstrated dengue virus-specific cytotoxic T cells from these human volunteers (Kurane, et al., 1991b). Some of these T cells were crossreactive with other serotypes of dengue virus indicating the potential for them to be reactivated during a secondary infection. Rothman et al have shown dengue-specific T cells in the mouse which were crossreactive with other serotypes of virus (Rothman, et al., 1996, Rothman, et al., 1993). Although CD4+ and CD8+ CTL responses were detected, previously most studies were performed on CD4+CTLs.

Seven dengue virus-specific CD4+CTL clones were initially generated from one donor who received a live dengue 3 vaccine (Kurane, et al., 1991b, Kurane, et al., 1989b). These T cell clones were analyzed in detail and were found to be directed against the nonstructural protein NS3. Numerous virus-specificities including virus subcomplex specific (crossreactive with some but not all serotypes), virus serotype crossreactive and also flavivirus crossreactive, were detected in these clones. At the bulk culture level, the dengue virus-specific proliferative responses were also serotype crossreactive, though the highest response was to the infecting serotype. Since then, CD4+CTL responses have been analyzed in 5 dengue immune donors and many clones with varying patterns of

virus specificity have been characterized. Epitopes were mapped to C, E, NS1/NS2a and NS3 proteins of dengue virus (Gagnon, et al., 1996, Green, et al., 1993, Green, et al., 1997, Kurane and Ennis, 1994, Livingston, et al., 1994). CD4+ and CD8+ CTLs with multiple specificities and against many different proteins (prM, E, NS1/NS2a and NS3) were also detected in mice that had been infected with dengue-2 virus (Rothman, et al., 1996, Rothman, et al., 1993).

When this project was initiated, PBMC from one D4 immune donor had been examined for CD8+CTLs in bulk culture. Bukowski detected memory crossreactive T cells directed against target cells expressing the structural protein (E) or expressing several nonstructural proteins (NS1-NS4) (Bukowski, et al., 1989). Following the demonstration of CD8+CTLs in bulk culture, CTL clones were isolated from this donor. The T cell clones generated recognized the nonstructural protein NS3 and were all restricted by HLA-B35 (Livingston, et al., 1995). Three patterns of specificities were detected with these clones 1) serotype-specific 2) dengue subcomplex-specific (D2/4 crossreactive) 3) serotype-crossreactive (D1/2/3/4 crossreactive). Epitopes were mapped on NS3 using synthetic peptides and 3 of the clones recognized a.a. 500-508 on D4NS3. The serotype specific clone did not lyse targets expressing the corresponding peptide of D2NS3, whereas the crossreactive clones lysed D2NS3 expressing targets.

Many of the CD4+CTLs obtained in our laboratory secrete IFN- γ and IL-2 after stimulation with dengue antigens and thus have a Th1 cytokine profile. The T cell clones

established from immune individuals could thus contribute to the cytokine milieu proposed to be involved in DHF (Kurane, et al., 1989a, Kurane, et al., 1989b). T cells may be activated by the antigen presenting cells and the activated T cells through the release of soluble mediators such as cytokines can result in the abnormalities seen in DHF. The fact that capillary leakage occurs quickly and there is no direct endothelial cell damage, supports our hypothesis that locally acting cytokines may induce the plasma leakage seen in DHF. Dengue viruses are known to infect cells of the reticuloendothelial system including Kupffer cells in the liver, alveolar macrophages, and cells of the spleen and thymus in vivo (Boonpucknavig, et al., 1979, Yoson and Bhamarapravati, 1982). It is possible that infection of these monocyte/macrophage cells lead to activation of T cells, release of chemical mediators and activation of complement, all of which contribute to disease severity. T cells may therefore have opposing roles, one in eliminating virus infected cells which occurs in all dengue infections, but in some infections, the T cell and other responses appear to trigger the immunopathogenesis of disease.

3. Cytotoxic T cells during acute viral infections

Although the main function of CTLs is to help in control viral infection they have also been shown to play a role in the immunopathogenesis of disease (Rouse, 1996). Cannon et al have shown that intravenous transfer of an RSV specific T cell line into RSV-infected mice cleared the virus infection but also caused acute respiratory disease

(Cannon, et al., 1988). Mice injected with 10^6 or more RSV-specific CTLs showed more severe pathology on bronchoalveolar lavage. Mice injected with lower numbers of CTLs had less severe disease but still cleared the infection. In murine LCMV infection, viral clearance is mediated by CTLs but this is also associated with increased pathology (Buchmeier, et al., 1980, Kagi, et al., 1996). Athymic mice that have been infected with LCMV show no brain lesions and instead proceed to a persistent viral infection . Therefore there is a fine balance between protective effects of CTLs and the deleterious consequences that they may induce.

In humans, the function of CTLs during acute infections cannot be studied in as great detail. It is assumed that human T cells may also have dual effects in that they can clear virus in certain cases but may contribute to the immunopathology seen after some viral infections. A number of studies have been done in patients infected with Epstein Barr Virus, which causes acute infectious mononucleosis. Strang et al isolated T cell lines that could lyse autologous B lymphoblastoid cell lines (BLCL's) from PBMC of patients with infectious mononucleosis after expansion in vitro with IL-2 and stimulator cells (Strang and Rickinson, 1987). These CTLs had a complex pattern of recognition because they also lysed to varying degrees allogeneic targets with no shared HLA antigens. Schendel et al showed that CTLs with dual specificity could be isolated from four unrelated donors which lysed both HLA-C restricted EBV targets and allotargets (Schendel, et al., 1992). Tomkinson et al analyzed the alloreactive cytotoxic responses in patients with infectious mononucleosis and concluded that MHC class I expression was

sufficient for recognition because class I specific antibodies strongly inhibited lysis of allogeneic targets (Tomkinson, et al., 1987). EBV antigenic expression was not required on the allogeneic targets as the CTLs could lyse cell lines (HSB2, HTLV-1 and K562) devoid of the EBV genome. Therefore, in acute EBV infection, CTLs which lyse both virus-specific T cells that may contribute to the recovery from infection and alloreactive T cells that can lyse MHC incompatible target cells may be generated.

Kreth et al isolated mumps virus-specific CTLs that were MHC restricted from the peripheral blood and cerebrospinal fluid of patients with acute mumps infection. In a direct CTL assay the T cells lysed mumps virus-infected autologous PHA blasts but not histoincompatible target cells (Kreth, et al., 1982). Demonstration of lysis by T cells without in vitro stimulation indicated that the CTL were sensitized in vivo. These CTL were virus-specific and showed no recognition of allogeneic target cells, unlike the observation in acute EBV. HLA-restricted CTL have also been isolated in acute measles and CMV infection in a small number of patients (Kreth, et al., 1979). Directly activated CTLs have also been isolated in patients with persistent virus infections such as HIV-1 and HTLV-1.

The above data and our interests in isolating dengue virus-specific CTLs in acute disease initially warranted the examination of acute PBMC samples for in vivo activated dengue-specific CTLs. The results from these studies prompted us to further explore T cell responses in the acute samples obtained from the Thai children. They led us to

analyze the in vitro unresponsiveness of PBMC from samples obtained during acute illness.

E. IMMUNOSUPPRESSION IN ACUTE VIRAL INFECTIONS

Several viral infections have been known to cause a generalized suppression of host immune responses. As early as 1905, physicians observed that the morbidity of viral infections was exacerbated by secondary bacterial infections, such as *Haemophilus influenzae* and *Streptococcus pneumoniae* (Mills, 1984). Von Pirquet in 1908 noticed that DTH responses to tuberculin were transiently depressed in PPD positive individuals during acute measles (Von Pirquet, 1908, Wainberg and Mills, 1985). Tamashiro et al showed that children with complicated measles illness remained skin test negative longer (mean = 4 weeks after the appearance of rash) than children with uncomplicated illness (mean = 2.3 weeks) (Tamashiro, et al., 1987). Measles kills nearly one million children per year and a significant proportion of these deaths is thought to be due to secondary infections. Susceptibility to these secondary infections is thought to be the result of immunosuppression seen following the acute measles virus infection.

Although viruses mediate their effects in multiple ways, they can alter the function of the immune system in two general ways. Regulation of the immune system may be altered due to the direct replication of virus in immunocompetent cells. Viruses such as HTLV-I, HIV-1 and certain parvoviruses are lymphotropic and infection can result in profound lymphopenia (Bloom, 1984, Dent, 1972, Markham, et al., 1985). Viruses may directly infect lymphocytes and lyse or functionally impair them (Measles,

EBV, certain parvoviruses) or they can infect and damage the antigen presenting cell compartment namely the monocyte/macrophage compartment which is a critical component in triggering a good immune response (Herpesviruses, influenza, canine distemper virus). The other major mechanism involved is indirect, and could involve the release of antigens from the virus (feline leukemia virus) (Rojko and Olson, 1984), soluble factors like lymphokines from host cells, and inhibitors of lymphokine production (Rodgers, et al., 1985). This latter mechanism could be an advantage for the virus because relatively few infected cells could cause a wide ranging disruption in the entire immune system by the release of immunoregulatory factors.

Viruses have variable cytopathic effects on host cells. Productive infection, leading to the replication of infectious virus, could result in a loss of vital cell functions, loss of membrane integrity, and eventual cell lysis (Figure I-2). Other viruses, like LCMV, HCMV and Measles, have restricted viral replication in immune cells (Doyle and Oldstone, 1978, Joseph, et al., 1975, Schrier, et al., 1985). The cell morphology remains normal and there is no disturbance of housekeeping functions, but specialized functions of the cell, synthesis of products necessary for their immune function, are altered (Borrow and Oldstone, 1995).

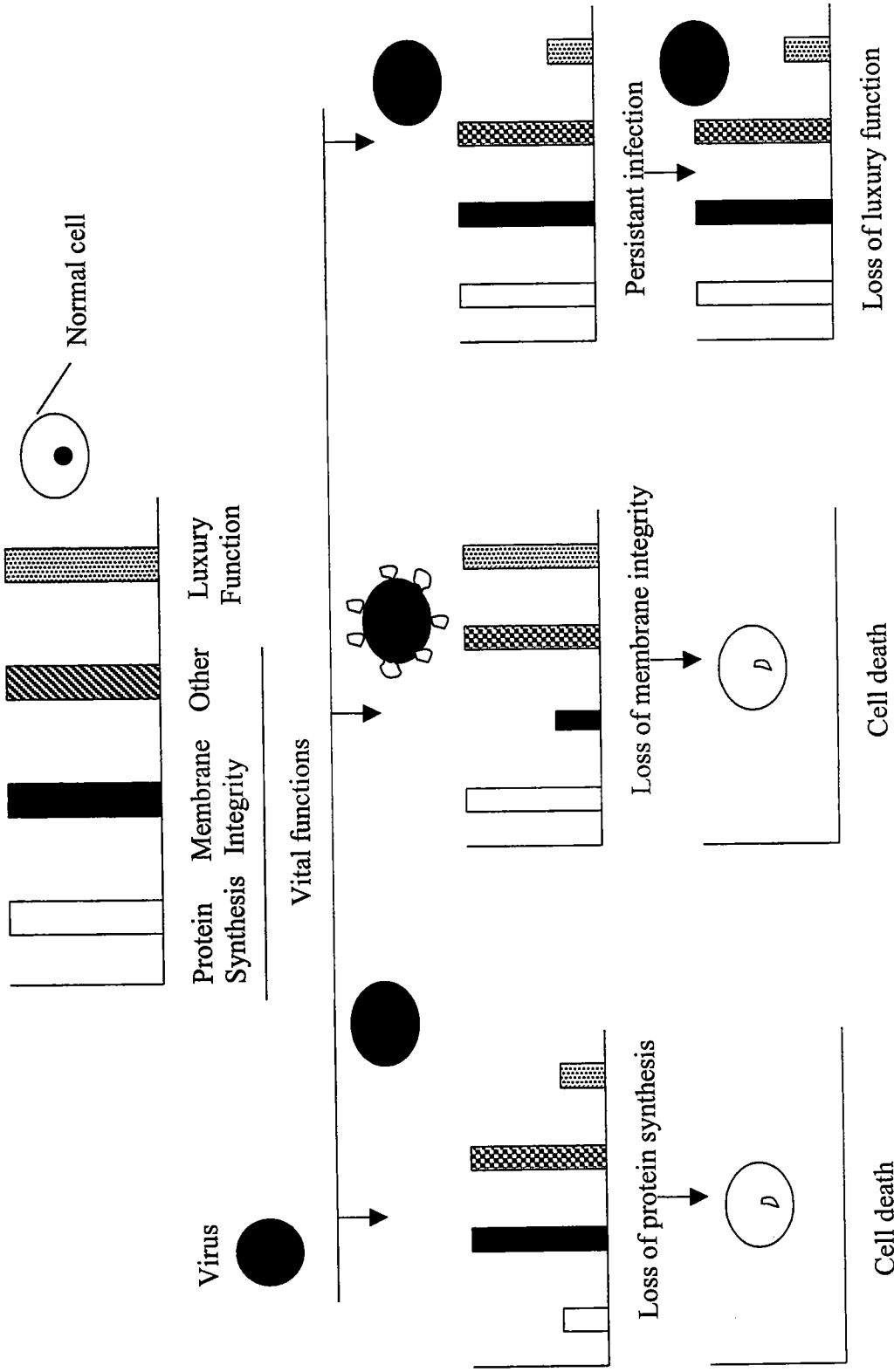


Figure I-2. Mechanisms by which viruses can cause disease (From McChesney and Oldstone, Ann. Rev.immunol. 1987. 5:279-304)

Although there is in vivo evidence, of virus-induced immunosuppression, many of the initial observations have been extended to in vitro systems. Smithwick and Berkovich demonstrated that the proliferative response to tuberculin, of immune lymphocytes isolated during the course of acute measles infection, is depressed (Smithwick and Berkovich, 1969). Several other investigators have examined the in vitro responses of PBMC obtained during acute viral infections to specific antigens and mitogens. Numerous cell surface molecules, and cytokines released from lymphocytes play an important role in shaping the nature of the proliferative response.

1. Factors and cell surface interactions that may influence lymphocyte proliferation

a). Interleukin-1 (IL-1).

IL-1 α and IL-1 β are proteolytically cleaved to the mature proteins (Paul, 1993). In vitro many cell types secrete IL-1 including monocytes/macrophages, T cells and endothelial cells, but macrophages and keratinocytes are thought to be the main producers of this cytokine in vivo. IL-1 is generally considered a mediator of inflammation; it can costimulate T cell proliferation, increase IL-2 secretion and make dendritic cells more efficient APC's. However, IL-1 also induces production of corticosteroids and prostaglandins such as PGE2 from T cells, which are known powerful inhibitors of T cell proliferation. Macrophages secrete IL-1, IL-6, IL-8, TNF, prostaglandins, and plasminogen activators in response to IL-1. Therefore, even though IL-1 can promote

inflammatory responses, it can also have considerable negative effects on T cell proliferation.

b). Interleukin-4 (IL-4)

IL-4 is secreted by T cells, provides help for B cells and stimulates growth and differentiation of Th2 cells (Paul, 1993). It down regulates Th1 type cells and can also affect macrophages. It decreases the expression of IL-2, IL-12 and IFN- γ specifically and attenuates the antiviral immune response. It can also alter IL-2 dependent in vitro lymphoproliferative responses to various antigens. DTH responses are classically mediated by Th1 cells and secretion of IL-4 can suppress cell mediated immunity and affect in vivo tuberculin responses.

c). Interleukin-10 (IL-10)

Produced by T and B lymphocytes and monocytes, IL-10 is known for its anti-inflammatory and immunosuppressive properties (Moore, et al., 1993). By affecting the antigen presenting capacity of monocytes it can prevent antigen-induced proliferation of PBMC and T cell clones. It can downregulate costimulatory molecules, such as B7-1, B7-2 and ICAM-1 that are critical for the activation of T cells. It can induce long lasting anergy in human CD4+ T cells. However IL-10 has also been shown to rescue human T cells starved of IL-2 from apoptotic death.

d). Interleukin-12 (IL-12)

IL-12 is produced by phagocytic cells. It induces cytokine production, mainly IFN- γ , by

NK and T cells, favors Th1 cell differentiation and promotes proliferation of differentiated cells in response to specific antigens (Trinchieri, 1995). It enhances proliferation of preactivated T cells to mitogens, alloantigens and anti-CD3 stimulation. It has an obligatory role in antigen-induced Th1 differentiation in vivo. A decrease in the production of IL-12 could therefore lead to a suppression of cell mediated immunity. Known inhibitors of IL-12 include IL-10, TGF- β , IL-4, IL-13 and PGE2.

e). Tumor Necrosis Factor (TNF)

Monocytes and macrophages are the main producers of TNF (Paul, 1993). TNF is also an inflammatory mediator and can increase the expression of cell adhesion molecules such as ICAM-1 on the surface of cells. It can activate phospholipase A2 (PLA2) an enzyme that cleaves arachidonic acid (AA) from phospholipids. AA has been reported to inhibit IL-2 production in PBMC and T cell lines and have antiproliferative effects on T and B cells. Therefore, increased production of these products directly or via secretion of TNF may affect T cell proliferative responses in viral infections.

f). Prostaglandins

Prostaglandins, also termed eicosanoids, are involved in development and regulation of immunological and inflammatory responses. PGE2 is known to inhibit secretion of lymphokines and also affects T and B cell proliferation. PGE2 mediates its antiproliferative effects by inhibiting IL-2 production (Santoli, et al., 1990), inhibiting IL-2 receptor expression, influencing cAMP levels, and modulating the protein kinase C

pathway. It is mainly thought to be released by monocytes. PGE₂ appears to have a direct inhibitory effect on T cells rather than acting through the APC's or generating other suppressor populations.

g). B7-CD28 interactions

CD28 is expressed on 80% of human T cells and levels are increased upon T cell activation (Lenschow, et al., 1996). B7-1 is expressed on activated APC's and T cells and maximal levels of expression occur 48-72 hours after stimulation. B7-2 is expressed on resting monocytes to a low level and its expression is upregulated within 6 hours of stimulation. The CD28-B7 signaling pathway is one of the most important costimulatory pathways studied to date and is critical for the triggering of T cell activation. Blocking this pathway using CTLA-4 Ig leads to inhibition of T cell proliferation and in some cases induces T cell anergy or antigen-specific hyporesponsiveness (Yi-qun, et al., 1997). Antibodies to B7-1, B7-2 and CTLA-4 induced anergy of memory T cells to specific antigens and this could be reversed by the addition of IL-2 and autologous monocytes. Cytokines differentially regulate expression of these molecules on APC's and this may result in distinct effects during an immune response.

h). CD2-LFA-3 and LFA-1-ICAM-1

CD2 is expressed on all T cells and its interaction with its ligand LFA-3 initiates strong antigen independent adhesion, induction of IFN- γ production, and expansion of naïve T helper cells. The LFA-1-ICAM-1 interaction also promotes cell adhesion and plays a role

in stimulating the proliferation of memory T cells. One or more of these molecules may play an important role in affecting T cell responses during acute viral infections (Wingren, et al., 1995).

Viruses may affect both T cell and antigen presenting cell populations and mediate their effects. Many studies have examined *in vitro* proliferative responses by focussing on one cell population and concluded that the primary defect was due to either abnormal T cells or abnormal APC's.

2. Effects of acute virus infection on the APC compartment

a) Monocyte/Macrophage involvement

1.) In vitro responses of acute PBMC obtained following viral infection in vivo

CMV is a herpesvirus that causes an infectious mononucleosis-like syndrome. PBMC of patients with acute CMV infection have diminished responses to mitogens, specific antigens and low production of IFN- γ *in vitro* (Carney and Hirsch, 1981, Kapasi and Rice, 1988). Monocytes of patients with CMV infection may play an important role in mediating suppression of immune responses, as the adherent cell population of PBMC could suppress the response of cultured lymphocytes *in vitro*. Patients at an earlier clinical stage of illness (group 1, approximately two weeks after the onset of symptoms) had markedly depressed Con A responses; depletion of autologous monocytes or addition

of increasing doses of fresh autologous monocytes had no effect on proliferation of PBMC. Depletion of monocytes from samples of patients at a later clinical stage, (group 2, three weeks after onset of symptoms) decreased Con A responses by 80% and addition of fresh autologous monocytes improved proliferation. This indicated that monocytes helped improve proliferation of T cells.

Mononuclear cells from 4 patients, who had CMV isolated from monocytes in the PBMC were cultured for 7 days without Con A. On day 7, the lymphocytes were collected and compared with unseparated mononuclear cells that had also been cultured without Con A for 7 days. The responsiveness of cultured lymphocytes from group 2 patients to Con A was reduced in the presence of autologous CMV-infected monocytes by 72% whereas uninfected monocytes from control PBMC under similar culture conditions showed no suppressive effect. This indicated that CMV-infected monocytes could suppress reactivity of cultured lymphocytes to Con A. Therefore, depending on the culture conditions, monocytes could either help or suppress lymphocyte proliferation.

PBMC obtained from patients with measles 1-9 days after rash, from children with other infectious diseases, and normal laboratory controls were evaluated for suppressor activity (Hirsch, et al., 1984). Cells from these patients were cultured in PBS or Con A for 4 days and then mixed with an equal number of normal donor cells treated with mitomycin C. The percentage of suppression was calculated relative to the Con A response of normal donor cells mixed with an equal number of fresh normal donor cells

treated with mitomycin. There were no differences between the control group and measles patients in the ability of their cells, when precultured with Con A, to suppress the response of fresh cells to Con A. However, only cells from measles patients when precultured with PBS, suppressed Con A responses of normal donor cells. Infectious virus was not detected in the culture supernatants. The results suggested that a soluble factor was responsible for suppressing the allogeneic Con A responses.

2.) In vitro responses following in vitro infection with a virus

Most of the research performed to date has examined in vitro responses following in vitro infection with different viruses. Human monocytes were infected with the Edmonston strain of measles virus and stimulated with LPS, *Staphylococcus aureus* Cowan strain 1 (SAC) or preincubated with IFN- γ . Compared to uninfected control monocytes, the production of both the p40 and p70 units of IL-12 was downregulated in infected cells (Karp, et al., 1996). Secretion of TNF- α , IL-6 and MIP-1 β was not altered by this stimulation. Soluble inhibitors of IL-12 production, IL-10, TGF- β , IL-4 and IL-13, were not detected in culture supernatants, which implied that a direct interaction of the virus with monocytes may be important. Crosslinking of the measles virus receptor CD46 also markedly decreased IL-12 production and the authors speculated that reduced production of IL-12 from monocytes and macrophages leads to the suppression of cell mediated immunity observed following acute measles infection. However, the authors did

not demonstrate that T cell proliferation to a variety of stimuli depended on IL-12 secretion from monocytes. Other in vitro studies have shown increased IL-1 β and decreased TNF- α production by measles virus-infected peripheral blood monocytes and reduced capacity of the infected cells to present antigen (Griffin, et al., 1994, Ward, et al., 1991).

Nokta et al showed that supernatants from monocytes infected in vitro with CMV (Strains C-87 and AD-169) inhibited T cell proliferation by > 95% (Nokta, et al., 1996). This was associated with increased levels of cAMP, arachidonic acid and PGE₂, and increased TNF- α release. A monoclonal antibody to TNF and an inhibitor of PGE₂, indomethacin, abolished the inhibitory effects of the supernatants. The authors concluded that increased TNF- α -dependent release of PGE₂ induced antigen unresponsiveness of T cells in acute HCMV disease. Elevated levels of TNF- α have not been reported in patients with acute CMV.

Gern et al showed that human rhinovirus (HRV) binding to ICAM-1 on monocytes inhibited T cell proliferation to tetanus toxoid, candida antigens or varicella zoster virus (VZV) but did not affect the response to mitogens and IL-2 (Gern, et al., 1996). HRV was not directly toxic to T lymphocytes but rather mediated its effects by affecting the antigen presenting cells.

b) Dendritic cell (DC) involvement

Dendritic cells are professional antigen presenting cells that are potent stimulators of naive T cells to initiate T cell-dependent immune responses. High levels of expression of MHC Class II, adhesion and costimulatory molecules make DC's very efficient APC's. Fugier-Vivier et al studied the effect of measles virus infection on CD40-activated DC's (Fugier-Vivier, et al., 1997). They found that measles virus replicated to much higher levels in activated DC's than in monocytes and induced syncytia formation. Infection of DC's resulted in decreased production of IL-2 by T cells and rendered the T cells unable to proliferate in coculture experiments. Both DC's and T cells were induced to undergo apoptosis. They suggest that measles virus-infected DC's in the PBMC are the primary cell type that induce suppression of T cell proliferation. This study, therefore, has examined the role of DC's following in vitro infection with measles virus; whether DC's are infected in vivo during acute measles infection is unknown.

Borrow et al analyzed the effect of an LCMV variant clone 13 on suppression of immune responses (Borrow, et al., 1995). This variant leads to persistent infection in BALB/c ByJ mice. They found a CD8-dependent loss of interdigitating DC's in the spleen that resulted in an inability of APC's from infected mice to stimulate a primary MLR. However, PHA-induced T cell proliferation was not affected in these mice. They conclude that CD8+CTL lysis of DC's may be a mechanism of virus-induced immunosuppression. Although DC's may play a critical role in influencing the immune

response, there have been no reports that address the role of DC's in mediating virus-induced immunosuppression in PBMC from humans that have been infected in vivo with a virus.

3. Effects of acute virus infection on the T cell compartment

a) Immunosuppressive T cell activity

In the early 1980's several researchers noted an increase in CD8+ T cells which they thought mediated suppressive activity (T_s), or a decrease in the CD4+ T cell (T_h) population and concluded that an abnormal ratio of T_s / T_h cells mediated the immunosuppression seen following acute viral and bacterial infections. With herpesviruses, such as EBV and CMV, an increase in the number of CD8+ cells following virus infection was noted (Carney, et al., 1981, Reinherz, et al., 1980). Diminished mitogen responses in vitro and increased susceptibility to secondary infections were associated with an abnormal CD4 to CD8 ratio (associated with an increased number of CD8+ T cells). These CD8+ T cells were thought to directly kill helper T cells or secrete cytokines and other soluble factors which were antiproliferative in nature.

In several experimental models of infectious diseases in mice, including HSV, Influenza and Reovirus infection, virus-specific suppressor cells were generated (Liew

and Russell, 1980, Rubin, et al., 1981, Whittum, et al., 1984). However, most of the studies did not show any conclusive proof that the T_s cells were directly responsible for the effects observed. There was also no unified concept of how T_s cells worked in spite of the demonstration of suppressor T cells in several different viral and bacterial systems (Bloom, et al., 1992, Vijayakumar, 1990). This area of research has not been active in recent years.

b) Direct lympholysis

Some viruses can infect all lymphocytes, but typically most viruses infect a certain subtype of cells, e.g. T or B cells, resulting in a more selective defect in the immune system. Canine distemper virus and some parvoviruses directly infect lymphocytes resulting in an inability to generate *in vitro* and *in vivo* immune responses (Bloom, 1984, Krakowka, et al., 1975). This immunosuppression is thought to be partly responsible for the secondary bacterial infections and neurologic involvement that affects the animals. HIV-1 infects CD4⁺ T cells. Patients with AIDS show decreased DTH responses to many recall antigens and also impaired *in vitro* cell mediated responses (Shannon, et al., 1985).

Some viruses have a direct effect on lymphocytes but the actual mechanism of suppression may not be the result of direct lympholysis. EBV infects B cells primarily, immortalizes them, induces polyclonal activation of B cells and the production of

antibodies to EBV by specific cells. These infected B cells are thought to stimulate atypical T cells, which may remove B cells from the circulation and may also account for the anergy seen in cases of infectious mononucleosis (Tosato, et al., 1979). Measles virus can infect both T cells and monocytes in vitro and undergo restricted replication in unstimulated cells. In vivo the main cell population that is infected are the monocytes. It is possible that productively infected T and B cells are removed early from the circulation which accounts for the inability to detect measles virus in these populations.

c) Apoptosis of T cells

Over the last several years, studies have examined whether mature T lymphocytes in acute viral infections undergo apoptotic death upon antigenic stimulation. Viruses may sensitize T cells to undergo activation-induced cell death and this could be a general mechanism of virus-induced immunodeficiency. T lymphocytes from patients with EBV, VZV and HIV have been shown to undergo apoptosis in vitro. CD4⁺ and CD8⁺ T lymphocytes from patients with acute infectious mononucleosis, which are CD45RO⁺, were shown to undergo spontaneous in vitro apoptotic death (Tamaru, et al., 1993, Uehara, et al., 1992). These activated cells could be rescued by the addition of IL-2 to the cultures, which resulted in an increase in bcl-2 expression of these cells. These T cells were shown to have less expression of the bcl-2 protooncogene compared to T cells from normal healthy controls. Bcl-2 is an antiapoptotic gene known to prolong cell survival in

activated, mature B and T cells. The results suggest that differential expression of bcl-2 in activated T cells may play a crucial role in determining the balance between T cell death and survival following viral infections.

In acute LCMV infection, T cells die in vitro by activation-induced apoptosis upon stimulation of the TCR-CD3 complex with anti-CD3 antibodies (Razvi and Welsh, 1993). Addition of recombinant IL-2 improved proliferation. However, in contrast to EBV infection, IL-2 sensitized the T cells to undergo apoptosis upon TcR triggering. Some memory cells against unrelated antigens bear the high affinity IL-2 receptor, and release of IL-2 during an acute viral infection may cause these cells to actively cycle and sensitize them to undergo activation-induced cell death. This would account for impaired responses to recall antigens, such as tetanus toxoid, following an acute viral infection. Therefore, depending on the culture conditions and the state of the T cells, IL-2 can either rescue cells or accelerate their apoptosis upon subsequent stimulation.

4. Summary of Virus Infection-Induced Immunosuppression

To date there has not been any conclusive proof that one cell type or factor is responsible for the decreased T cell proliferative responses that have been seen during acute virus infection. A great deal of controversy exists in several virus systems because initial data suggested the predominant defect to be due to either T cells or APC's. The observations are difficult to interpret, and very few studies have been done to analyze patients' samples to understand which population of cells are responsible for decreased in

vitro proliferative responses.

Upon closer examination, researchers have found several defects in different cell populations. The mechanisms that are involved in mediating the immunosuppression induced by virus infections are probably multifactorial. Many viral infections are associated with immunologic abnormalities. A single virus could theoretically affect several arms of the immune system. They could be directly cytopathic for the host cells (HIV, EBV), destroy virus infected target cells by antiviral immune effector mechanisms (LCMV, HIV), impair the function of the infected cells (HCMV, Influenza), encode for homologues of the immune system (EBV) that suppress immune function, trigger an imbalance in the immune system by generating suppressor T cells (McChesney and Oldstone, 1987, Rouse and Horohov, 1986) or elicit autoimmune responses. These strategies may enable viruses to evade host defense mechanisms and persist longer in the host.

In acute measles infection, defects have been found in the monocyte/macrophage, B cell, dendritic cell and T cell compartments (Fugier-Vivier, et al., 1997, Fujinami, et al., 1998, Griffin, et al., 1986, Karp, et al., 1996). The mechanisms underlying the immunosuppression following acute viral infections are complex and difficult to dissect and interpret especially in humans. It is likely the decreased proliferative responses are the result of triggering of a complex network of cytokines and cell-surface interactions involving both T cells and antigen presenting cells. There does not appear to be one

single general mechanism for the immunosuppression observed during and following an acute virus infection but rather a series of events triggered by a virus affecting several components of the immune system directly or indirectly. Different viruses may use different strategies to downregulate the immune system. This could result in common defects in the lymphocyte/monocyte pool that are manifested as similar defective responses in vitro to the different stimuli tested.

F. QUESTIONS TO BE ADDRESSED AND AIMS OF THIS THESIS

F1. Human CD8+CTL responses to dengue vaccines

Only a limited analysis had been previously done on the CD8+ memory responses to dengue virus. Therefore, a more detailed characterization of these responses in several dengue-immune volunteers was performed in order to better understand the nature of the CD8 responses to dengue virus. We wished to identify immunodominant proteins or epitopes encoded by the dengue genome that were recognized by CD8+CTLs. These results would complement the available data on dengue-specific CD4+CTLs and determine whether different proteins are recognized by these two distinct populations of CTLs. These results would also be important when considering new approaches for vaccine development and contribute to the basic understanding of the immune response to this virus.

F2. Human CD8+CTL responses following natural secondary dengue infection

Dengue infections occur mainly in South and South East Asia, South America and Africa; therefore, analyzing CTL responses from patients who are naturally infected with DV is very important. Most of the research performed on human CTL responses to dengue viruses had been in Caucasian volunteers who had no prior exposure to dengue

and therefore received the vaccine as a primary infection. The viruses used for the vaccines were laboratory passaged in cell lines and were thought to be attenuated strains. On the other hand, the complications seen in DV infection (DHF and DSS) are more common in patients who have preexisting immunity to one serotype of virus during infection with another serotype (secondary dengue infection). Our laboratory was therefore interested in analyzing CTL responses from patients after natural secondary infections as it could provide insights into the mechanisms of T-cell mediated immunopathology.

F3. Immunosuppression during acute dengue

Since a main interest of the laboratory is to better understand the role that T cells play in the pathogenesis of severe dengue illness, we wanted to characterize the in vivo activated T cells that were dengue-specific in the acute PBMC samples obtained from children when they were admitted into the hospital. A second sample of PBMC from the same patients several months after infection to examine which population of cells would become memory T cells. Results observed during these experiments led me to investigate the decreased in vitro T cell responses of PBMC obtained during acute dengue infection.

My specific aims were:

1. Analysis of bulk culture memory CD8+CTL responses in 8 volunteers who received live attenuated candidate monovalent dengue vaccines.
2. Analysis of memory CTL responses in Thai children following natural dengue infection.
3. Analysis of T cell responsiveness in Thai children during and after natural dengue infection.

CHAPTER II

MATERIALS AND METHODS

A. VIRUSES

Dengue virus type 1 (Hawaii strain) and dengue virus type 2 (New Guinea C strain) were provided by Walter E Brandt, Walter Reed Army Institute of Research. Dengue virus type 3 (CH53489 strain) was provided by Bruce L. Innis, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Dengue virus type 4 (814669 strain) was provided by Jack McCown, Walter Reed Army Institute of Research. Yellow fever virus (YFV) (17D strain) was provided by Jacob J Schlesinger, University of Rochester School of Medicine and Dentistry. West Nile virus (WNV) (E101 strain) was provided by Margo Brinton, Georgia State University. Viruses were propagated in C6/36 mosquito cells as previously described and frozen at -70°C until use (Kurane, et al., 1984). Viral titres were 10^7 to 10^8 PFU/ml by plaque assays on CV-1 monolayers.

Recombinant vaccinia viruses that contain the genes coding for dengue viral proteins were produced as previously described (Falgout, et al., 1989, Zeng, et al., 1996, Zhao, et al., 1989). Vaccinia viruses expressing dengue 1 proteins were kindly provided

Table II-1 Recombinant vaccinia viruses expressing different portions of the dengue genome

C	prM.	E	NS1	NS2a	NS2b	NS3	NS4a	NS4b	NS5
---	------	---	-----	------	------	-----	------	------	-----

Vac. D1 C.prM.E	[]								
Vac. D1 E.NS1.2a			[]						
Vac. D1 E.NS1.2a.2b			[]						
Vac. D2 C.prM.E	[]								
Vac. D2 NS1.2a			[]						
Vac. D2NS1			[]						
Vac. D2NS3						[]			
Vac. D3 NS3						[]			
Vac. D4 prM.	[]								
Vac. D4 NS1.2a			[]						
Vac. D4 NS3						[]			

The range of proteins expressed is listed based on the order of proteins in the flavivirus polyprotein, C-prM-E-NS1-NS2a-NS3-NS4a-NS4b-NS5 (not drawn to scale), where prM is the precursor to the membrane. The dengue proteins expressed by these recombinant vaccinia viruses are designated based on the dengue serotype that served as the source for cloning and the genome segment included.

by Dr. Enzo Paoletti, Virogenetics Co. ; those expressing dengue 4 proteins, dengue 2 C.prM.E (D2 C.prM.E) and dengue 2 NS1.2a (D2NS1.2a) were kindly provided by Dr. C.J. Lai, National Institutes of Health (N.I.H.). The other dengue 2 and dengue 3 vaccinia recombinants were generously donated by Dr. Margo A. Brinton, Georgia State University. Most of the recombinant vaccinia viruses used in this study are shown in Table II-1. The amino acids expressed by the truncations of the D3 and D4 nonstructural genes are indicated in parenthesis in the tables and figures.

B. PREPARATION OF A RECOMBINANT VACCINIA VIRUS EXPRESSING THE DENGUE 2 VIRUS NS1 PROTEIN

RNA was extracted from the New Guinea C strain of dengue 2 virus using Ultraspec reagent. cDNA synthesis was performed using a downstream primer specific for the dengue 3' noncoding region ALD-2 (5' -TCT CTC CCA GCG TCA ATA -3'). The NS1 region was amplified by PCR using a specific 5' primer CTG TAT TTG GGA GTT ATG GTG, which had an ATG in it for initiation of translation, and a 3' primer AGT GAA AAG TTG TCA ATC TGC. The PCR product, which was 1112 bp in length, was cloned into the vector pCR™ 2.1. The pCR™ 2.1 vector containing the D2NS1 cDNA, was digested with Spe I and Xho I, and the D2NS1 cDNA insert was cloned into the vaccinia virus recombination vector pSC11. The pSC11 vector was digested with XbaI and Hind III to confirm the insertion of D2NS1 cDNA, and from 1 of 10 clones fragments of the

right size were generated. CV-1 cells were then transfected with the D2NS1 cDNA insert and infected with a wildtype vaccinia virus. As a result of homologous recombination, the vaccinia virus thymidine kinase (tk) gene was replaced by the D2NS1 cDNA insert rendering them tk negative. After 3 rounds of plaque purification a recombinant vaccinia virus was selected. The expression of the D2NS1 protein in the vaccinia recombinant was tested by immunofluorescence using rabbit anti NS1 serum followed by FITC horse anti rabbit serum. The expression was also confirmed by infecting BLCL's with the vaccinia recombinant virus and using them as target cells in a CTL assay.

B. PREPARATION OF DENGUE ANTIGENS

Vero cell monolayers were infected with dengue viruses at a multiplicity of infection of approximately 1 PFU per cell and incubated at 37°C in minimal essential medium (MEM)/2% FBS until approximately 50% of the cells displayed cytopathic effects (Kurane, et al., 1989a). Cells were then harvested by scraping, washed, fixed in 0.025% glutaraldehyde in phosphate buffered saline (PBS) for 15 min on ice, washed again and resuspended at 3×10^8 cells/ml in RPMI 1640. The suspension of fixed cells was then sonicated on ice in a sonic dismembrator (Fisher Chemical Co.) and centrifuged at 1500xg for 10 min at 4°C. The supernatant was collected, aliquoted and frozen at -70°C as viral antigen. Control antigen was prepared similarly from uninfected vero cell monolayers.

D. EXPERIMENTAL VACCINES AND PBMC

All donors included in the studies had no detectable hemagglutinating-inhibiting serum antibody to all four serotypes of dengue virus. They were immunized subcutaneously with undiluted vaccine viruses of the different serotypes as indicated in Table II-2. They were proven to be infected with dengue virus by antibody responses and virus isolation. Most of the volunteers had few symptoms (Bhamarapravati, et al., 1987, Green, et al., 1993, Hoke, et al., 1990) and two had symptoms compatible with dengue fever (Gagnon, et al., 1996, Innis, et al., 1988). PBMC were isolated at 4 months or later after vaccination, resuspended at 1×10^7 / ml in RPMI 1640 with 20% FBS (Sigma) and 10% dimethyl sulfoxide and cryopreserved until use. HLA typing was performed in the Tissue Typing Laboratory at the University of Massachusetts Medical Center - Worcester.

E. STUDY DEFINITION AND SAMPLES OBTAINED FROM PATIENTS IN THAILAND

Blood samples were obtained from children enrolled in a prospective study of dengue infections at the Queen Sirikit National Institute for Child Health (Bangkok Children's Hospital), Bangkok, Thailand, and the Kamphaeng Phet Provincial Hospital, Kamphaeng Phet, Thailand, in 1994 (Kalayanarooj, et al., 1997, Vaughn, et al., 1997).

TABLE II-2 Summary of Vaccination and HLA Class I Types of Donors who received live attenuated experimental dengue vaccines

DONOR #	SEROTYPE OF VACCINE RECEIVED*	MONTHS AFTER VACCINATION P BMC OBTAINED	CLASS I HLA TYPE		
			A	B	Cw
1	D1	4	2, 11	27, 60	1, 3
2	D2	12	2, 29	44	5
3	D2	12	1	8, 38	
4	D2	12	2, 3	7, 44	5, 7
5	D3	36	2, 24	7, 62	3, 7
6	D4	22	26, 28	27, 39	1
7	D4	6	2, 23	35, 44	4
8	D4	5	2, 28	51, 57	6

Virus strains administered were: dengue-1 strain 45AZ5 (McKee, et al., 1987), dengue-2 strain 16881 (Dharakul, et al., 1994), dengue-3 strain CH53489 (Innis, et al., 1988) and dengue-4 strain 341750 (Gagnon, et al., 1996).

We thank Sharone Green, David Vaughn and Ananda Nisalak, who supplied us with these valuable specimens. We thank the nurses and pediatricians at the Queen Sirikit National Institute of Child Health and the Kamphaeng Phet Provincial Hospital, Thailand for volunteer enrollment and patient management. We thank the research nurses, technicians, technologists, and administrative personnel of the Department of Virology, AFRIMS for specimen collection, specimen processing, and data management. We also thank the patients and their families for their participation in this study.

Previously healthy children who presented to either hospital with fever of 72 h or less in duration and facial flushing without an obvious cause were eligible to participate in this study. Children who had an identifiable cause of fever (e.g. those with measles, sinusitis, gastroenteritis) were excluded from the study. A diagnosis of acute dengue infection was based on serologic tests (antibody capture EIA and hemagglutination-inhibition HAI) and isolation of dengue virus (in *Toxorhynchites splendens* mosquitoes). Clinical diagnoses of DF and DHF were assigned according to WHO criteria (Anonymous, 1997). Standard serological criteria were used to identify an acute primary or secondary dengue infection. A diagnosis of secondary dengue infection was made based on the following criteria: a) a dengue IgM-to-IgG ratio of $< 1.8:1$, b) a 2-fold increase in IgG to dengue between acute and immune samples, with an absolute value of $\geq 100U$ in the absence of IgM to dengue of $\geq 40U$ c) A HAI titer of $>1:1280$, one week after the onset of illness.

For all the analyses done in Chapter IV, PBMC samples obtained from 4 patients 12 months after infection were used as indicated in Table II-3. HLA-A, B and C class I typing was performed using a standard microlymphocytotoxicity assay, and HLA class II typing was performed by PCR-based amplification and hybridization with HLA-DRB1, DRB3, DRB5, DQA1, DQB1 and DPB1 sequence-specific oligonucleotide probes as previously described (Chandanayingyong, et al., 1997). Four subjects with documented secondary dengue-2 or dengue-4 virus infections were selected for this study.

Study day 1 was defined as the calendar day the subject was enrolled in the study. For the studies performed in Chapter V, a sample was considered to be an acute sample up to study day 11, although the subjects were not febrile at that time. Immune samples were obtained from the same patients 6 months or later after their acute infection. All sample numbers in Chapter V are denoted by the study days on which the samples were obtained. Fever day 0 was defined as the calendar day during which the temperature fell and stayed $< 38^{\circ}\text{C}$, and days before and after this point were numbered consecutively (fever days -1, -2 etc. occurred before defervescence and fever days +1, +2 etc. occurred after defervescence). Patient information and the diagnoses are given in Table II-4. Sample numbers indicate acute (A) or immune (I) PBMC and the study day on which they were obtained. PBMC obtained from patients during and after their illness (acute and immune samples) were separated and cryopreserved in liquid nitrogen. Frozen PBMC samples were shipped to the University of Massachusetts Medical Center for testing.

Table.11-3. Clinical, viral and immunogenetic profiles of the study subjects from Thailand involved in the immune CTL studies

PATIENT	DISEASE	VIRAL SEROTYPE	HLA CLASS I ALLELES			HLA CLASS II ALLELES		
			A	B	C	DR	DQ	DP
KPP94-037	DF	D2	1,11.1	46,57	1,6	DRB1 *0701 DRB1 *0901 DRB4 *0101	DQA1 *0201 DQA1 *03011 DQB1 *03032	DPB1 *0501 DPB1 *1301
KPP94-024	DHF Grade 3	D2	2, 24	7,46	1,7	DRB1 *0901 DRB1 *1201 DRB3 *0301 DRB4 *0101	DQA1 *03011 DQA1 *0102 DQB1 *03032 DQB1 *0502	DPB1 *0501 DPB1 *1301
CHD94-134	DHF Grade 3	D2	11.1.1,28	27,57	3,6	DRB1 *0701 DRB1 *1202 DRB3 *0301	DQA1 *0201 DQA1 *0601 DQB1 *0301 DQB1 *03032	DPB1 *0401 DPB1 *1301
CHD94-020	DHF Grade 2	D4	2,11.1	7,46	1,7	DRB1 *0901 DRB1 *1110 DRB3 *0202 DRB4 *0101	DQA1 *03011 DQA1 *05011 DQB1 *0301 DQB1 *03032	DPB1 *0501 DPB1 *1301

Table II-4 Patient information and clinical diagnosis of study subjects from Thailand involved in the acute T cell studies

PATIENT	DIAG-NOSIS ^a	SERO-LOGY ^b	SERO-TYPE	ACUTE/IMMUNE BLEED ^c	STUDY DAY	FEVER DAY ^d
1(CHD94-090)	DF	1 ⁰	D1	A I	4, 11 374	-1, +6
2(CHD94-067)	DF	1 ⁰	D2	A I	1, 11 368	-2, +8
3(CHD94-139)	DF	2 ⁰	D1	A I	2, 3 354	0, +1
4(KPP94-017)	DF	2 ⁰	D2	A I	1 366	-1
5(CHD94-115)	DF	2 ⁰	D3	A I	9 725	+8
6(CHD94-089)	DF	2 ⁰	D3	A I	2, 10 725	-1, +7
7(CHD94-118)	DF	2 ⁰	D4	A I	2, 3 367	0, +1
8(KPP94-013)	DF	2 ⁰	D4	A I	1 364	-1
9(CHD94-094)	DHF Gr 1	2 ⁰	D4	A I	1 336, 725	-1
10(CHD94-073)	DHF Gr 2	2 ⁰	D4	A I	9 368	+5
11(CHD94-020)	DHF Gr 2	2 ⁰	D4	A I	2, 3 367, 724	0, +1
12(CHD94-138)	DHF Gr 2	2 ⁰	D2	A I	2 354	-2
13(CHD94-095)	DHF Gr 2	2 ⁰	D2	A I	11 196	+7
14(CHD94-134)	DHF Gr 3	2 ⁰	D2	A I	4 372	1
15(KPP94-041)	DHF Gr 3	2 ⁰	D1	A I	2, 5, 8 366	-4, -1, +2
16(KPP94-035)	DHF Gr 3	2 ⁰	D2	A	9	+7
17(CHD94-081)	DHF Gr 3	2 ⁰	D3	A	3	-1

^a DF = dengue fever, DHF = dengue hemorrhagic fever. ^b 1⁰ = primary infection, 2⁰ = secondary infection. ^c A = acute PBMC samples, I = immune samples

^d Fever day 0 = calendar day when the temperature fell and stayed < 38°C

Written consent was obtained from each subject's parent or guardian. The study protocol was approved by the Institutional Review Boards established by the Ministry of Health, Thailand, the Surgeon General's Office of the Department of the Army, and the University of Massachusetts Medical Center.

F. PROLIFERATIVE RESPONSES OF PBMC FROM DENGUE VACCINE RECIPIENTS AND THAILAND DONORS

Proliferation assays were performed as previously described (Kurane, et al., 1989a). PBMC (2×10^5) from vaccine recipients were cultured with the different flaviviruses at various dilutions in 0.2 ml of AIM-V medium (GIBCO BRL Life Technologies, Gaithersburg, MD) containing 10% heat inactivated human AB (Hu ABS; Advanced Biotechnologies Inc, Columbia, MD) serum (AIM/10%) in duplicate or triplicate wells of 96-well round-bottom microtiter plates (Costar, Cambridge, MA) at 37°C for 7 days. PBMC from patients in Thailand (both the acute and immune phase of infection at 10^5 /well) were thawed, resuspended in AIM/10% medium and added to a v-bottom 96 well plate in the presence of either PHA (1:1000), anti-CD3 (12F6; provided by Johnson Wong, Massachusetts General Hospital) (0.1 µg/ml), or with the indicated concentrations of other antigens. When cytokines were added to the wells, 10U/ml of recombinant IL-2 (Collaborative Biochemical Products, Bedford, Mass.), 100U/ml of IL-4 (Genzyme), 100U/ml of IL-7 (Genzyme) or 25 ng/ml of IL-12 (Genetics Institute,

Cambridge, Mass.) were added to PHA-stimulated or anti-CD3 stimulated cells. 1×10^5 gamma-irradiated (3500 rads) autologous immune PBMC or allogeneic PBMC from healthy control donors were added to wells containing PBMC from acute dengue patients as indicated. The anti CD28 antibodies were purchased from Pharmingen and used at a concentration of 5 or 10 $\mu\text{g/ml}$ together with the anti-CD3 antibody 12F6 (0.1 $\mu\text{g/ml}$). The negative controls were cell suspensions in medium alone without any stimulation. Optimal concentrations of antigens were determined using PBMC of control donors. Cells were incubated for 5-7 days as this was shown to be optimal for proliferation using 10^5 cells/well. The cells were pulsed with 1.25 μCi of ^3H thymidine (^3H TdR) on day 4 after the PHA or anti CD3 stimulation or on day 6 for flavivirus antigens and tetanus toxoid for approximately 18 hours before harvest onto filters with a multiharvester (Titertek; Skatron Inc, Sterling VA). ^3H -TdR incorporation was counted in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland). Significant virus-specific proliferative responses after immunization were defined using the following criteria: (Dharakul, et al., 1994) the maximum stimulation index (SI) induced by each of the flaviviruses had to be two or more times greater than that induced by control antigen at the same dilution, the differences had to be statistically different and ^3H -TdR incorporation had to be greater than 1000 cpm.

G. BULK CULTURE OF PBMC.

PBMC from donors who received the live attenuated monovalent vaccines (data in Chapter III) or patients from Thailand (data in Chapter IV), were cultured at approximately $2-5 \times 10^6$ / well in AIM/10% medium with the homologous dengue virus at a final dilution of stock virus between 1:2-1:6 in 24 or 48 well cluster plates. Virus-stimulated cells from most of the vaccine donors were tested in bulk culture CTL assays between days 7-9.

In certain cases, cells were restimulated on day 7 with gamma irradiated (3500rad) autologous PBMC in 1 ml of fresh medium containing 10% HuABs, 10U/ml IL-2 and dengue virus (for vaccine donors). Restimulated cells were assayed 7 days later for cytolytic activity. For the patients in Thailand, cells were restimulated with 1×10^6 gamma-irradiated (3500 rad) allogeneic PBMC and anti-CD3 monoclonal antibody 12F6 (0.1 μ g/ml), kindly provided by Johnson Wong, Massachusetts General Hospital in 0.5 ml of fresh medium containing 10% Hu ABS and 25-50U/ml of recombinant IL2 (Collaborative Biochemical Products, Bedford MA) as indicated. Bulk cultures were restimulated every two weeks and cells were assayed 7-10d after the last restimulation for cytolytic activity in CTL assays.

H. CLONING OF PBMC.

Immune PBMC (Chapter IV) which had been stimulated in bulk culture for 7 days were collected, and plated at a concentration of 10 and 30 cells per well in 96-well round-bottom microtiter plates in 200 μ l of AIM/10% medium, 10⁵ allogeneic gamma-irradiated PBMC, anti-CD3 (0.1 μ g/ml) and 25U/ml IL2. Every 3-4 days cells were fed with fresh AIM-V medium containing 10%Hu ABS and 25U/ml IL2. Cells were restimulated with allogeneic PBMC and IL-2 every 2 weeks. The T cell lines were initially screened in cytotoxicity assays using target cells infected with vaccinia recombinants expressing dengue proteins. Growing cells that showed positive lytic activity against any dengue protein were expanded into 48-well plates (Costar) and restimulated with 10⁶ allogeneic PBMC and anti-CD3 in a final volume of 1 ml.

I. ESTABLISHMENT OF LYMPHOBLASTOID CELL LINES

Lymphoblastoid cell lines (BLCLs) were established by culturing PBMC (approximately 2x10⁶) with EBV from an infected marmoset cell line, B95-8 (American Type Culture Collection) in RPMI 1640 containing 20% FBS, penicillin, streptomycin, glutamine and HEPES. Cyclosporin A was added at a final concentration of 1 μ g/ml.

J. CELL SURFACE ANTIGEN ANALYSIS

Approximately $4-5 \times 10^5$ T cells were washed with cold PBS and stained with fluorescein isothiocyanate-conjugated (FITC) anti-CD4, anti-CD8 or control IgG antibodies for 30 minutes on ice. The cells were washed in PBS and fixed with 4% paraformaldehyde for 7 minutes. The lines were washed again and analyzed for CD4 or CD8 expression using a fluorescence microscope. FITC-conjugated monoclonal antibodies to CD14 and CD19 were purchased from Pharmingen (San Diego, CA). The FITC-conjugated CD3, CD4 and CD8 antibodies were purchased from Beckton Dickinson. FACS analysis was performed on unstimulated acute and immune PBMC (approximately $1-3 \times 10^6$ cells).

K. PEPTIDE SYNTHESIS

Peptides were synthesized with the RAMPS multiple peptide synthesis system (New England Nuclear Products, Boston, MA) or the Symphony peptide synthesizer (Rainin Instruments, Woburn, MA) at the University of Massachusetts Peptide Core Facility.

L. PREPARATION OF TARGET CELLS.

BLCLs (approximately 5×10^5) in RPMI/10% FBS were infected with vaccinia viruses for 1.5 - 2 hours at 37°C . The cells were then diluted in 2 mls of media and further incubated for 12 - 16 hours . Target cells were then washed and labeled with 0.25 mCi of ^{51}Cr (Dupont NEN, Boston, MA) for 60 mins at 37°C . After four washes to remove unincorporated ^{51}Cr , target cells were counted and diluted to 10^4 cells/ml for use in the cytotoxicity assay. The allogeneic target cells used in the assays were either produced in our laboratory in Massachusetts from unrelated donors or obtained from the National Institutes of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository or the American Society for Histocompatibility and Immunogenetics (ASHI) Cell Bank and Repository.

M. ^{51}Cr RELEASE CYTOTOXICITY ASSAYS.

Cytotoxicity assays were performed in 96 round-bottom plates as previously reported (Bukowski, et al., 1989). Effector cells were added to 1×10^3 ^{51}Cr -labeled target cells at an effector to target (E:T) ratio of approximately 100:1 in bulk culture CTL assays and at the indicated E:T ratios when T cell lines were tested. In CTL assays with synthetic peptides, peptides at the indicated concentrations were added to target cells and incubated at 37°C for 30 min, after which effector cells were added. Plates were

centrifuged at 200 x g for 5 mins and incubated for 4-5 hours at 37⁰C. Supernatant fluids were harvested using the supernatant collection system (Skatron) and ⁵¹Cr content was measured in a gamma counter. The percent specific ⁵¹Cr release was calculated with the following formula: (cpm experimental release - cpm spontaneous release)/(cpm maximum release - cpm spontaneous release) x 100. Maximum values were obtained by incubating target cells alone with a detergent and spontaneous values were obtained by incubating target cells alone with media. All assays were performed in triplicate, and the results were calculated from the average of the triplicate wells. The standard error of the mean was less than 10% in all of the experiments.

N. MONOCLONAL ANTIBODY DEPLETION ASSAYS .

Anti-OKT3 (CD3), anti-OKT4 (CD4), anti-OKT8 (CD8); (all from Ortho Diagnostic Systems, Inc., Raritan, N.J.) and anti-Leu11b (CD16; Becton Dickonson Co.) antibodies were used in antibody-complement depletion assays. Dengue virus-stimulated effector cells ($1-1.5 \times 10^6$) were resuspended in 0.5 ml of RPMI 1640 supplemented with 2% FBS with 50 μ l of the individual antibodies. Following 30 mins. of incubation at 4⁰C, the cells were washed twice in cold RPMI/2% FBS and then resuspended in 0.6 ml to which 0.2 ml of rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added. After 60 mins of incubation at 37⁰C, cells were washed three times,

resuspended in RPMI/10% FBS and then used as effector cells in cytotoxicity assays.

O. CELL SEPARATIONS

2×10^6 PBMC from patients with acute dengue or control healthy donors were used to isolate T cells using anti-CD4 and anti-CD8 coated magnetic beads (Dynal MPC). After the CD4 and CD8 coated beads adhered to the magnet, the remaining cells were collected and used as a source of non-T cells. The non-T cell population was irradiated (3500 rads). As controls, unfractionated PBMC from both acute dengue patients and control healthy PBMC were stimulated with PHA.

P. TRANSWELL EXPERIMENTS

PBMC from 4 patients with acute dengue infection and control allogeneic PBMC were resuspended at 1×10^6 /ml in AIM/10% medium. The transwells (Costar, Cambridge, MA), consist of a lower and upper compartment which are separated by a polycarbonate-treated membrane with pores of $0.4 \mu\text{m}$ in size. $100 \mu\text{l}$ of acute or control PBMC were added to the upper well of the transwell and $600 \mu\text{l}$ of media were added to the lower well with PHA at a final concentration of 1:1000. For coculture experiments, acute PBMC and gamma-irradiated allogeneic PBMC were cultured together in $100 \mu\text{l}$ in the upper well of the transwell as a positive control. To see if cell contact was essential to increase

proliferation, 100 μ l of the acute PBMC were transferred to the upper well and 600 μ l of gamma- irradiated, allogeneic PBMC were transferred to the lower well with PHA at 1:1000. The plate was incubated at 37°C. On day 4, 75 μ l of the cell suspensions from the upper and lower wells were transferred to a 96 well plate in a final volume of 200 μ l, pulsed with 1.25 μ Ci of 3 HTdR and harvested approximately 18 hours later.

CHAPTER III

ANALYSIS OF CD8+ T CELL RESPONSES OF PBMC FROM DENGUE VACCINE RECIPIENTS

A. LYMPHOCYTE PROLIFERATIVE RESPONSES TO FLAVIVIRUSES

We first examined the proliferative responses of the PBMC from all 8 donors who received monovalent live attenuated dengue vaccines to different flaviviruses including dengue viruses. Uninfected supernatants from C6/36 mosquito cells were used as a negative control in these assays. Proliferation was measured on day 7 by uptake of ^3H -TdR. Previous studies have shown that both dengue virus-specific CD4+ and CD8+ T cells can be stimulated in this assay (Bukowski, et al., 1989). All 8 donors had significant proliferative responses to one or more of the viruses tested (Table III-1). The PBMC of all the donors responded strongly to the serotype that they had been vaccinated with and the PBMC of most donors responded to a lesser degree to the other serotypes of dengue virus, West Nile virus or Yellow fever virus. These results suggest that dengue virus specific memory T lymphocytes from the eight donors after primary dengue virus infection are predominantly serotype-specific and that crossreactive cells are also present.

TABLE III-1 Proliferation of PBMC from dengue vaccine recipients to flaviviruses in bulk culture*

Donors (vaccine)	SI after stimulation with										Control [†]
	D1V 1:8 1:16	D2V 1:8 1:16	D3V 1:8 1:16	D4V 1:8 1:16	WNV 1:8 1:16	YFV 1:8 1:16	Control [†]				
Donor 1 (D1)	<u>5</u> 3	2 1	4 2	1 1	2 2	1 1	1 1	1 1	1 1	1 1	1 1
Donor 2 (D2)	<u>26</u> 21	<u>44</u> <u>55</u>	<u>15</u> 2	<u>15</u> 13	<u>15</u> 14	<u>21</u> 11	5 12	5 12	5 12	5 12	5 12
Donor 3 (D2)	7 5	<u>54</u> <u>40</u>	12 6	12 7	<u>35</u> <u>37</u>	5 5	15 11	15 11	15 11	15 11	15 11
Donor 4 (D2)	<u>5</u> 3	<u>10</u> <u>14</u>	<u>6</u> 7	5 4	<u>10</u> 4	<u>5</u> 3	2 1	2 1	2 1	2 1	2 1
Donor 5 (D3)	3 3	3 2	<u>6</u> <u>6</u>	2 1	3 3	2 2	3 1	3 1	3 1	3 1	3 1
Donor 6 (D4)	1 2	<u>12</u> <u>11</u>	5 8	<u>19</u> <u>10</u>	2 5	<u>8</u> 6	4 4	4 4	4 4	4 4	4 4
Donor 7 (D4)	1 1	2 2	1 1	2 2	1 2	1 1	1 1	1 1	1 1	1 1	1 1
Donor 8 (D4)	3 4	2 3	1 1	<u>10</u> 4	5 3	7 5	4 4	4 4	4 4	4 4	4 4

* PBMC (2×10^5 cells) were incubated for 6 days in the presence of the indicated dilutions of flaviviruses. Cells were pulsed with 1:25 μCi of ^3H -TdR for 12 hrs and ^3H -TdR incorporation was measured. SI is calculated as mean cpm of cultures with virus/mean cpm of cultures with medium. Data are stimulation index (SI) of immune PBMC of each subject (average of 3 or 2 wells). Underlined values indicate significantly elevated levels of proliferation as defined in the Methods section.

† Control, C6/36 mosquito cell supernatants.

B. PROTEIN SPECIFICITY OF CTLs GENERATED FROM PBMC OF DENGUE IMMUNE DONORS

We next analyzed the CTL responses to different dengue proteins in PBMC of these vaccine recipients. BLCLs were infected with vaccinia recombinants expressing portions of the dengue genome (Table II-1) and used as target cells in cytotoxicity assays. The effector cells generated from PBMC of donor 1, who was immunized with a monovalent D1 vaccine, lysed targets expressing the D1 structural proteins C.prM.E and also those expressing the E and NS1.2a proteins (Table III-2 exp 1). CTLs from donors 2 and 3 who received a D2 vaccine, recognized target cells expressing the three D2 structural proteins C.prM.E (Table III-3 exp 1) and were further shown to lyse target cells expressing only the envelope protein E (Table III-3 exp 3), indicating that specific killing was directed against the envelope protein. CTLs from both these donors also recognized recombinant vaccinia viruses expressing the nonstructural proteins NS1.2a and NS3. On the other hand, CTLs from donor 4, who received the same D2 vaccine demonstrated no lytic activity against any of the structural proteins but recognized the nonstructural proteins NS1.2a and NS3 (Table III-3 exp 1). Using target cells expressing D3NS3, CTLs from donor 5, who received a D3 vaccine, were found to recognize the nonstructural protein NS3 (Table III-3 exp 1).

Donor 6 had memory CTL responses against the prM (precursor of the membrane) and NS3 proteins. Target cells expressing the structural proteins

TABLE III-2 Recognition of dengue virus proteins by CTLs generated from PBMC of Dengue-1 immune donor 1 *

	Target cells infected with	% Specific ⁵¹ Cr release
Exp. 1 [†]	Vac. D1 (C.prM.E)	58
	Vac. D1 (E.NS1.NS2a)	51
	Vac. (control)	26
Exp. 2 [§]	Vac. D1 (C.prM.E)	22
	Vac. D1 (E.NS1.NS2a)	17
	Vac. D2 (E)	4
	Vac. D2 (C.prM)	5
	Vac. D2 (NS1.NS2a)	7
	Vac. D4 (E)	5
	Vac. (control)	4

*Autologous target cells (10^3) were infected with DV/VV recombinants and incubated with effector cells for 4-5 hours. The effector/target ratio was approximately 100:1.

[†] Donor 1 PBMC were stimulated twice in vitro with D1V.

[§] Donor 1 PBMC were stimulated once in vitro with D1V.

TABLE III-3 Recognition of dengue virus proteins by CTLs generated from PBMC of dengue-2 immune donors 2, 3, 4 and dengue-3 immune donor-5.

	Target cells infected with	% Specific ⁵¹ Cr release			
		Donor 2 (D2)	Donor 3 (D2)	Donor 4 (D2)	Donor 5 (D3)
Exp. 1	Vac.D2 (C.prM.E)	49	35	17	
	Vac.D2 (C.pr.M)		6	10	
	Vac.D2 (E)		25	16	
	Vac.D2 (NS1.2a)	54	41	92	
	Vac.D2 (NS3)	71	50	89	
	Vac.D3 (NS3)				82
	Vac. (Control)	26	7	19	32
Exp. 2	Vac.D2 (NS3)	51	43	52	70
	Vac.D3 (NS3)	5	45	17	82
	Vac.D4 (NS3)	16	0	11	54
	Vac. (Control)	4	0	10	32
Exp. 3 [†]	Vac.D2 (E)	32	16		
	Vac.D4 (E)	6	0		
	Vac.D2 (NS1.2a)	33	72		
	Vac.D1 (ENS1.2a.2b)		13		
	Vac.D4 (NS1.2a)	11	9		
	Vac. (Control)	10	0		

*PBMC from Donors 2-5 were stimulated with autologous virus once in vitro. The effector/target ratio was approximately 100:1.

[†]The effector target ratio was approximately 200:1 for donor 2.

TABLE III-4 Recognition of dengue virus proteins recognized by CTLs generated from PBMC of dengue-4 immune donors 6,7 and 8

	Target cells infected with	% Specific ⁵¹ Cr release		
		Donor 6 (D4)	Donor 7 (D4)	Donor 8 (D4)
Exp. 1	Vac. D4 (C.prM.E)	29	41	16
	Vac. D4 (pr.M)	26	27	
	Vac. D4 (E)	11	51	14
	Vac. D4 (NS1.2a)	7	27	32
	Vac. D4 (NS3)	30	58	8
	Vac. (Control)	0	21	0
Exp. 2	Vac. D2 (NS3)	-2	59	
	Vac. D3 (NS3)	16	42	
	Vac. D4 (NS3)	24	73	
	Vac. D2 (NS1.2a)			11
	Vac. D1 (E.NS1.2a)			46
	Vac. (Control)	1	26	-1

PBMC were stimulated with D4V twice for donor 6 and once for donors 7 and 8 as described in Materials and Methods. Effector/target ratio was approximately 100:1

(D4 C.prM.E), were lysed by donor 6 bulk cultures (Table III-4 exp 1). We then narrowed the response down to the prM protein using target cells expressing the individual structural proteins. This is the first time a T cell response has been detected against the prM protein. CTLs from donor 7 recognized the envelope protein (E) and nonstructural protein NS3 (Table III-4 exp 1). Our laboratory has previously detected CD8+ CTL clones from this donor that recognized the NS3 protein. CTLs from donor 8 mainly recognized target cells expressing D4 NS1.2a and the E protein to a lesser degree (Table III-4 exp 1).

The results from the above experiments indicate that CTLs were generated against at least one viral protein in all 8 donors. The dengue proteins predominantly recognized by CTLs were the nonstructural proteins NS1.2a and NS3 and the envelope protein E.

C. MAPPING OF THE prM EPITOPE RECOGNIZED BY PBMC OF DONOR 6

To define the on epitope the prM protein recognized by donor 6 PBMC, we used constructs that expressed the cleaved portion (nonM) and the mature portion (M) of the prM protein to infect targets for use in a CTL assay. We found that the nonM portion was recognized by Donor 6's CTLs in bulk culture (Table III-5 exp. 1). Using overlapping 15mer peptides that spanned the entire nonM protein, we found that a.a. 1-15 (peptide prM #1) on the nonM protein was lysed by bulk culture CTL (Table III-5 exp. 2). Further analysis using a dilution of the peptide indicated that prM peptide #1 was recognized at a

Table III-5 Recognition of amino acids 1-15 of the D4V pr.M protein by bulk culture CTLs generated from Donor 6

		% Specific ⁵¹ Cr release
	Target cells infected/pulsed with	
Exp. 1	Vac. D4 prM.	34
	Vac. D4 nonM.	33
	Vac. D4 M	18
	Vac. D2 nonM	13
	Vac. control	12
Exp. 2	Vac. D4 nonM	42
	No peptide	0
	#1 (a.a.1-15)	25
	#2 (a.a.8-22)	0
	#3 (a.a.15-29)	0
	#4 (a.a. 22-36)	2
	#5 (a.a. 29-43)	0
	#6 (a.a.36-50)	1
	#7 (a.a.43-57)	5
	#8 (a.a.50-64)	2
	#9 (a.a. 57-71)	1

Table III-5 continued

Exp. 3		
	Vac. control	12
	Vac. D4 nonM	54
	No peptide	12
	#1 (1-15) 25 $\mu\text{g/ml}$	48
	#1 (1-15) 2.5 $\mu\text{g/ml}$	52
	#1 (1-15) .25 $\mu\text{g/ml}$	53
	#1 (1-15) .025 $\mu\text{g/ml}$	30
	#10 (a.a. 64-78)	13
	#11 (a.a. 71-85)	10
	#12 (a.a. 78-92)	16
	#13 (a.a. 85-99)	6

PBMC were stimulated with D4V twice for donor 6 as described in Materials and Methods. Effector/target ratio was approximately 100:1. Targets were pulsed with 25 $\mu\text{g/ml}$ of the indicated peptide unless otherwise stated.

analysis using a dilution of the peptide indicated that prM peptide #1 was recognized at a concentration of .025 µg/ml (Table III-5 exp. 3). This is the first CTL epitope that has been detected on the nonM portion of the prM protein.

D. SEROTYPE SPECIFICITY OF CTLs GENERATED FROM PBMC OF DENGUE IMMUNE DONORS

To analyze the serotype specificity of the CTL responses in all donors we used target cells expressing dengue proteins of serotypes other than the one used to immunize the donor. For donor 1, the CTL response was serotype-specific, as target cells expressing dengue proteins from serotypes other than D1 were not recognized (Table III-2 exp. 2). For donor 2, the responses to E and NS1.2a proteins were serotype-specific (Table III-3 exp. 3) whereas recognition of NS3 was cross-reactive with D4NS3 but not D3NS3 (Table III-3 exp 2). CTLs from donor 3 had primarily serotype-specific responses to both E and NS1.2a but recognition of NS3 was cross-reactive with D3NS3 and not D4NS3 (Table III-3 exp 2). The responses were serotype-specific for CTLs generated against NS1.2a (data not shown) and NS3 (Table III-3 exp 2) from donor 4 PBMC. CTLs from donor 5 were cross-reactive with both D2 and D4 NS3 (Table III-3 exp 2).

For donor 6, the CTL response against the nonM protein was serotype-specific (Table III-5 exp 1). Using target cells expressing NS3 from serotypes other than D4 for donors 6 and 7, CTLs were shown to be cross-reactive with D3NS3 for donor 6 and with

both D2 and D3NS3 for donor 7 (Table III-4 exp 2). CTLs from donor 8 were also cross-reactive as target cells expressing D1E.NS1.2a were lysed (Table III-4 exp 2). These results suggest that the CTL responses to nonstructural proteins were predominantly cross-reactive and the responses to structural proteins were serotype-specific.

E. LOCALIZATION OF THE EPITOPE WITHIN THE NS3 PROTEIN RECOGNIZED BY CTLs FROM DONORS 3, 5, 6 and 7.

Donor 3 PBMC recognized D2 and D3NS3 (Table III-3 exp 2 and Table III-6 exp 1) to similar levels. We used recombinant vaccinia viruses expressing truncated D3NS3 proteins to localize the epitopes on NS3 recognized by this donor's CTLs. The epitope was localized to a region between a.a. 247 and 618, since a truncated vaccinia recombinant containing a.a.1-247 (Vac D3-8) of D3NS3 was not recognized (Table III-6 exp 1). From exp 2 the area was further localized to the region from a.a. 247- 412 since Vac D3-C2 (a.a. 412-618) was not recognized . Exp 3 narrows the epitope down to a region between a.a. 247 and 354. Similarly, for donor 5, it appears that the epitope lies between a.a. 214 and 247 (Table III-6 exp 2 and 3).

TABLE III-6 Localization of the epitopes on NS3 recognized by CTLs generated from PBMC of a dengue 2 immune donor 3 and a dengue 3-immune donor 5

	Target cells infected with	a.a. †	% Specific ⁵¹ Cr release	
			Donor 3 (D2)	Donor 5 (D3)
Exp. 1	Vac.D2 (NS3)	1-618	32	
	Vac.D3 (NS3)	1-618	26	
	Vac.D3-8	1-247	3	
	Vac.D3-3	1-214	4	
	Vac.D3-14	1-176	2	
	Vac. (Control)		0	
Exp. 2	Vac.D3 (NS3)	1-618	46	54
	Vac.D3-5	1-548	51	60
	Vac.D3-3	1-214		39
	Vac.D3-C1	447-618	8	42
	Vac.D3-C2	412-618	9	
	Vac. (Control)		7	34
Exp. 3	Vac.D3 (NS3)	1-618	76	78
	Vac.D3-15	1-354	54	87
	Vac.D3-8	1-247		71
	Vac. (Control)		3	29

* PBMC were stimulated with autologous virus once in vitro and used as effectors. Autologous target cells (10^3) were infected with DV/VV recombinants and incubated with effector cells for 4-5 hours. The effector/target ratio was approximately 100:1.

† The numbers indicate amino acid residues of NS3 expressed by the various recombinant VV's.

From the results in Table III-7 exp 1, we conclude that CTLs from donor 6 recognized an epitope between a.a. 1-183 of NS3. The CTLs of donor 7, on the other hand, recognized a region between a.a. 453 and 618 on NS3. Using synthetic peptides we confirmed that the recognition was directed against a peptide containing a.a. 500-508 of NS3 (Table III-7 exp 2). This is in agreement with previous work in our laboratory, which showed that CD8+ CTL clones isolated from this donor recognized a synthetic peptide containing a.a. 500-508 of NS3. CTLs in bulk culture were also shown to lyse synthetic peptides containing a.a. 500-508 of D2 and D3NS3, confirming the cross reactive nature of this response at the peptide level (Table III-7 exp 2).

F. PHENOTYPE OF DENGUE-SPECIFIC CTLs IN BULK CULTURES.

To identify the phenotypes of dengue virus-specific cytotoxic T cells in each of the donors, cell depletion studies with monoclonal antibodies and complement were carried out. The assays were done 7-9 days after stimulation with autologous virus for donors 2, 3, 4, 5, 7 and 8. For donors 1 and 6 effector cells were restimulated on day 7 and used on day 14. The effector cell populations were tested against target cells infected with vaccinia recombinants expressing portions of the dengue genome that had elicited significant lysis in previous experiments, a control vaccinia virus, and a natural killer (NK) cell-sensitive tumor cell line, K562. For donor 2, autologous BLCLs persistently infected with D2V were used as targets.

TABLE III-7 Localization of the epitope on NS3 recognized by PBMC of dengue 4-immune donors 6 and 7

	Target cells infected or pulsed with	a.a.*	% Specific ⁵¹ Cr release	
			Donor 6 (D4)	Donor 7 (D4)
Exp. 1	Vac.D4.NS3	1-618	23	58
	Vac.D4 (1-452)	1-452	18	29
	Vac. D4 (183-452)	183-452	4	
	Vac. D4 (453-618)	453-618	5	58
	Vac. (Control)		-4	21
Exp. 2	Vac.D4 (453-618)			54
	Vac. (Control)			24
	Pep.D2.500	500-508		46
	Pep.D3.500	500-508		41
	Pep.D4.500	500-508		62
	None			-3

* The number indicates amino acid regions on D4 NS3 expressed by the recombinant vaccinia viruses. Peptides were present at a final concentration of 25µg/ml for the duration of the assay.

Treatment with anti-CD3 antibody and complement depleted the specific killing activity (Table III-8). These results indicate that the killing was mediated by T cells in all donors. For donor 1, killing of target cells expressing the envelope and nonstructural proteins NS1.2a (infected with recombinant Vac D1E.NS1.2a) was mediated by CD4+ CTLs because depletion with anti-CD4 and complement decreased the killing substantially. Killing of donor 1 targets infected with a construct containing all three structural proteins (Vac D1C.prM.E) (Table III-8) was mediated by CD4+ and CD8+ CTLs since depletion with either anti-CD4 or anti-CD8 decreased killing.

For the other donors tested, killing was mediated almost exclusively by CD8+ CTLs as treatment with anti-CD8 and complement significantly decreased the lytic activity. No other antibody had a significant effect on the levels of lysis for these donors. Taken together the results suggest that stimulation of immune PBMC in vitro with infectious dengue virus activated mainly CD8+ memory CTLs, and the levels of CD8+ CTL activity varied between donors.

Table III-8 Characterization of Cytotoxic T cells generated in bulk culture

DONOR	TARGET CELLS INFECTED WITH	TREATMENTS					Phenotype of cells
		C'	Anti-CD3 + C'	Anti-CD4 + C'	Anti-CD8 + C'	Anti-CD16 + C'	
Donor 1 (D1)	Vac. D1 (E.NS1.2a)	33	<u>2</u>	<u>0</u>	21	16	CD4
	Vac. D1 (C.prM.E)	36	<u>6</u>	<u>9</u>	<u>15</u>	29	CD4 + CD8
	Vac. (control)	1					
	K562	29	24	12	30	12	
Donor 2 (D2)	D2V infected	18	<u>0</u>	19	<u>0</u>	21	CD8
	Uninfected	-1					
	K562	19	9	12	14	7	
Donor 3 (D2)	Vac. D2 (NS1.2a)	34	<u>3</u>	27	<u>2</u>	38	CD8
	Vac. D2 (NS3)	20	<u>-1</u>	15	<u>3</u>	18	CD8
	Vac. (control)	0					
	K562	10	5	8	10	2	
Donor 5 (D3)	Vac. D3 (NS3)	21	<u>8</u>	14	<u>9</u>	14	CD8
	Vac. (control)	10					
	K562	36	21	24	24	7	
Donor 6 (D4)	Vac. D4 (nonM)	26	<u>0</u>	15	<u>-1</u>	17	CD8
	Vac. (control)	0					
	K562	13	4	7	11	1	
Donor 7 (D4)	Vac. D4 (NS3) ₄₅₃₋₆₂₈	52	<u>9</u>	31	<u>-4</u>	23	CD8
	Vac. D4 (E)	22	<u>5</u>	<u>10</u>	<u>-1</u>	6	CD8 + CD4
	Vac. (control)	0					
	K562	11	11	7	5	0	
Donor 8 (D4)	Vac. D4 (NS1.2a)	27	<u>0</u>	33	<u>5</u>	26	CD8
	Vac. (control)	5					
	K562	12	4	12	6	0	

Values are % specific ⁵¹Cr. release from target cells. Bulk cultures from donors 1 and 6 were restimulated and tested at 14 days whereas all other donors' PBMC were tested at 7 days in CTL assays. Underlined values are those that were decreased by $\geq 50\%$ compared to controls with the indicated treatment. The E/T ratio was between 100 and 150:1 before treatments.

TABLE III-9 Determination of HLA Class I restriction in recognition of dengue proteins by CD8+ CTLs

	TARGET	HLA CLASS I TYPE			% SPECIFIC CYTOTOXICITY	POTENTIAL RESTRICTING ALLELE
		A	B	Cw		
DONOR 2 (Vac D2E)	Autologous	2,29	44	5	18	
	JK	2,24	7,62	3,7	9	
	CB	2,3	35,44	4	24	B44
	TomG	23,29	7,44	4	25	
	9038	2	18	7	-8	
DONOR 2 (Vac D2NS1.2a)	Autologous	2,29	44	5	28	
	JK	2,24	7,62	3,7	9	
	CB	2,23	35,44	4	14	B44
	VA17	2,25	18,44	5	19	
	9038	2	18	7	0	
DONOR 2 (Vac D2 NS3)	Autologous	2,29	44	5	71	
	CB	2,23	35,44	4	73	B44
	9038	2	18	7	-11	
	9049	33	65	8	5	
DONOR 3 (Vac D3NS3)	Autologous	1	8,38		73	
	VA 12	1,24	35	4	10	B38
	GM3105A	26,28	18,38		49	
	GM3162	2	8,35		12	

Table III-9 continued

DONOR	TARGET	HLA CLASS I TYPE			% SPECIFIC CYTOTOXICITY	POTENTIAL RESTRICTING ALLELE
		A	B	Cw		
DONOR 5 (Vac D3NS3)	Autologous	2,24	7,62	3,7	24	
	UM27	<u>24,30</u>	<u>13,62</u>	3,6	16	
	63390	3,24	<u>7,62</u>	<u>4,7</u>	17	
	Puzzro	2,3	9	<u>2,3</u>	3	
						A24 and/or B62
	Autologous	2,24	7,62	3,7	52	
	CB	<u>2,23</u>	<u>35,44</u>	4	12	
	CP	<u>2,28</u>	<u>51,57</u>	6	1	
	9022	1	8	7	16	
	VA16	<u>2,28</u>	<u>7,14</u>	5	9	
DONOR 7						
(Pulsed with	Autologous	2,23	35,44	4	52	
a.a. 500-508	VA03	<u>2,24</u>	<u>7,35</u>	3,7	32	B35
of D4NS3)	JC	3,24	<u>35</u>	4	45	

PBMC from Donor 2 were stimulated with D2V for 7 days and used as effectors. (10^5) allogeneic targets were infected with the indicated vaccinia virus recombinants or pulsed with the indicated peptide and incubated with effectors for 4-5 hours. Shared HLA antigens are underlined. For clarity, values are given as % specific cytotoxicity for the VV recombinant infected targets - % specific cytotoxicity for vaccinia control infected targets.

G. HLA RESTRICTION OF THE LYSIS OF TARGET CELLS BY CD8+ BULK
CULTURE CTLs

HLA restriction of the CD8+ bulk cultures was examined in four immune donors to identify the predominant restricting alleles in these cultures. Target cells were infected with the recombinant vaccinia viruses that had previously elicited significant lysis or with a control vaccinia virus. From Table III-9, we conclude that recognition of E, NS1.2a and NS3 by PBMC of donor 2 were all restricted by HLA-B44, as only allogeneic target cells having HLA-B44 in common with the autologous BLCLs showed significant lysis.

The nonstructural protein NS3 was recognized by CTL of a majority of our donors, therefore we tested the HLA restriction of three additional donors that had NS3-specific CTLs. The results indicate that recognition of NS3 by donor 3 CTL was B38 restricted. For donor 5, it appears that either A24 or B62 (or both) may restrict recognition of D3NS3. In bulk culture, CD8+ CTLs from donor 7 lysed allogeneic targets pulsed with a.a.500-508 that have only HLA B35 in common with autologous targets, confirming what we observed earlier with CD8+ CTL clones of this donor.

CHAPTER IV

ANALYSIS OF DENGUE VIRUS-SPECIFIC CTL RESPONSES AFTER NATURAL SECONDARY DENGUE INFECTIONS

A. PROTEIN SPECIFICITY OF CTLs IN BULK CULTURE

To detect dengue virus-specific CTL, we stimulated immune PBMC (12 months after infection) with a homologous dengue virus (i.e. the same serotype of virus as the patient's isolate) and tested for cytolytic activity in bulk culture against the homologous dengue virus proteins. We detected CTL activity in bulk culture PBMC from patient KPP94-037 against target cells infected with vaccinia recombinants expressing the nonstructural proteins D2NS1.2a primarily and to those infected with Vac. D2NS3 to a lesser degree (Table IV-1). For patients KPP94-024 and CHD94-020, the predominant CTL response was directed against the homologous NS3 protein (Table IV-1). Even though the level of lysis of wild type vaccinia virus-infected target cells was high for patient KPP94-024, there was substantial killing against targets expressing D2NS3. For patient CHD94-134 low level CTL activity was detected in bulk culture against all the dengue vaccinia recombinants tested. These results show that, in bulk culture experiments, PBMC from all four of the patients had detectable cytolytic activity against target cells expressing nonstructural proteins NS1.2a or NS3.

Table IV-1: Bulk Culture CTL of PBMC obtained from children 1 year after natural infection with dengue virus

Target cells infected with	Percent dengue specific ⁵¹ Cr. release			
	KPP94-037	KPP94-024	CHD94-020	CHD94-134
Vac. D2CprME	0	-11		15
Vac. D2NS1.2a	38	5		16
Vac. D2NS3	13	38		18
Vac. D4CprME			-12	
Vac. D4NS1.2a			-6	
Vac. D4NS3			29	

PBMC ($1-3 \times 10^6$) were stimulated in vitro with the homologous serotype of dengue virus (dengue-2 for KPP94-037, KPP94-024 and CHD94-134 and dengue 4 for CHD94-020). Cells were restimulated with anti CD3 and allogeneic feeders on day 7 and then every two weeks. The cells were used as effectors and tested in CTL assays on days 14 to 35 of culture. % specific lysis represents lysis of target cells infected with recombinant vac/dengue viruses - lysis of target cells infected with control vaccinia virus. The vaccinia control values for KPP94-037 was 6%, KPP94-024 - 51%, CHD94-020 - 29% and CHD94-134 - 33%. Effector to target ratios were 70:1 for KPP94-037 and 50:1 for other donors

B. SEROTYPE CROSSREACTIVITY OF T CELL LINES ESTABLISHED FROM PBMC OF PATIENTS KPP94-037 AND KPP94-024

T cell lines were established by limiting dilution from the bulk cultures obtained from patients KPP94-037 and KPP94-024. For patient KPP94-037, the lines were initially screened using Vac. control, Vac D2NS3 and Vac D2NS1.2a. All lines that had dengue-specific lytic activity were found to recognize the nonstructural proteins NS1.2a (data not shown). Among 12 T cell lines, 4 lines had the CD3+CD4+CD8- phenotype and 8 lines had the CD3+CD4-CD8+ phenotype (Table IV-2). These NS1.2a specific CTL lines were tested for crossreactivity against Vac.D1ENS1.2a and Vac.D4NS1.2a constructs. Recognition of NS1.2a by all the T cell lines was crossreactive against dengue 1, dengue 2 and dengue 4 (Table IV-2).

For patient KPP94-024, 12 NS3 specific T cell lines were isolated after initial screening against target cells infected with Vac.control and Vac.D2NS3 (data not shown). These T cell lines were then tested for crossreactivity to other dengue serotypes (Table IV-3). All of the CTL lines were crossreactive against dengue 3 and dengue 4. These results indicate that all the dengue virus-specific T cell lines established from patient KPP94-037 were serotype crossreactive and recognized the NS1.2a proteins, and that those established from PBMC of patient KPP94-024 were also serotype crossreactive but recognized the NS3 protein. The protein specificities of T cell lines isolated from donors.

Table IV-2: Serotype crossreactivity of T cell lines generated from PBMC of patient KPP94-037 1 year after secondary D2V infection.

Cell Line	Phenotype	Percent specific ⁵¹ Cr. release			
		Vac. control	Vac. D1ENS1.2a	Vac. D2NS1.2a	Vac. D4NS1.2a
2G3	CD4 ⁺	0	57	91	64
2E8	CD4 ⁺	5	59	81	67
2D11	CD4 ⁺	5	64	98	73
2B5	CD4 ⁺	9	59	93	73
3C11	CD8 ⁺	9	56	80	59
3E7	CD8 ⁺	8	57	97	100
3F3	CD8 ⁺	8	65	100	57
2C8	CD8 ⁺	9	63	89	78
2G7	CD8 ⁺	4	56	77	58
2D9	CD8 ⁺	5	58	96	65
3F11	CD8 ⁺	3	51	67	40
3G10	CD8 ⁺	4	66	80	59

T cell lines were established from bulk culture of PBMC from patient KPP94-037 by the limiting dilution method. The phenotype of the cells was determined by fluorescent antibody staining. Percent specific ⁵¹Cr. release of indicated target cells was measured at E/T ratios between 20:1 and 60:1 for all the lines used.

Table IV-3: Serotype crossreactivity of CTL lines generated from PBMC of patient KPP94-024 1 year after secondary D2V infection

Cell Line	Phenotype	Percent specific ⁵¹ Cr. release			
		Vac. control	Vac D2NS3	Vac D3NS3	Vac. D4NS3
2B2	CD4 ⁺	1	80	82	76
2D11	CD4 ⁺	0	53	40	29
2C9	CD4 ⁺	-1	81	75	72
3E5	CD4 ⁺	0	72	72	68
2B11	CD4 ⁺	0	68	65	63
2E3	CD8 ⁺	0	80	75	76
3C3	CD8 ⁺	-3	68	82	77
3B11	CD8 ⁺	0	76	73	89
2G10	CD8 ⁺	0	63	64	61
2C8	CD8 ⁺	2	84	88	84
3C2	CD8 ⁺	2	77	77	83
2F5	CD8 ⁺	-2	86	77	78

T cell lines were established from bulk culture of PBMC from patient KPP94-024 by the limiting dilution method. The phenotype of the cells was determined by fluorescent antibody staining. Percent specific ⁵¹Cr. release of indicated target cells was measured at E/T ratios between 20:1 and 60:1 for all the lines used.

KPP94-037 and KPP94-024 were consistent with those of the bulk culture CTLs from the same subjects, shown in table IV-1C.

HLA RESTRICTION OF THE LYSIS OF TARGET CELLS BY CD8+ AND CD4+
CTL LINES FROM PBMC OF PATIENTS KPP94-037 AND KPP94-024

For patient KPP94-037, allogeneic BLCLs which had HLA alleles in common with autologous cells were infected with Vac. D2NS1.2a and used as targets. Experiment 1 in Table IV-4 shows that three representative CD8⁺ lines were also HLA B57 restricted because only targets having B57 in common with the autologous BLCLs were lysed efficiently by CD8⁺ CTLs. Other CD8⁺ lines established from patient KPP94-037 were B57 restricted. Experiments 2 and 3, using allogeneic BLCLs that had Class II alleles in common with the autologous line, indicate that 2 of the CD4⁺ CTL lines from patient KPP94-037 were HLA DR7 restricted.

For patient KPP94-024, allogeneic BLCLs sharing Class I alleles with autologous BLCLs were infected with recombinant vaccinia expressing D2NS3. Four representative CD8⁺ lines generated from patient KPP94-024 were HLA B7 restricted (exp. 1 and 2, Table IV-5). Other CD8⁺ CTL lines established from patient KPP94-024 were also B7 restricted (data not shown).

Table IV-4: HLA restriction of recognition of dengue viral proteins by CTL lines from patient KPP94-037

					% specific ⁵¹ Cr. release		
					T cell lines		
Experiment 1		HLA CLASS I			3E7	2E4	2C8
	Autologous	A1,11.1	B46,57	C1,6	95	91	79
	HCV57	A2.1,3	B7,37	C6,7	11	-4	-4
	9052	A1,3	B27,57	C2,6	29	22	21
	GM3098A	A2,2	B 57,57		71	51	53
Experiment 2		HLA CLASS II			2D11	2G3	
	Autologous	DP5	DQ9	DR7,9	56	55	
	9077	DP5	DQ3	DR9,12	-3	-7	
	CP	DP4	DQ2,3	DR5,7	30	30	
Experiment 3	Autologous	DP5	DQ9	DR7,9	71	39	
	KPP94-024	DP5	DQ9	DR9,12	-3	1	
	CHD94-134	DP4	DQ9,7	DR7,12	78	34	
	MS		DQ1,3	DR1,4		-7	

BLCLs were infected with vaccinia recombinants expressing D2NS1.2a. Allogeneic targets that shared one or more HLA Class I alleles with the autologous line were used in Experiment 1 and those that had HLA Class II alleles in common with the autologous line were used in Experiments 2 and 3. The E/T ratios were between 16:1 and 40:1 for all the T cell lines used.

Table IV-5: HLA restriction of recognition of dengue viral proteins by CTL lines from patient KPP94-024

			% specific ⁵¹ Cr. release		
			T cell lines		
Experiment 1		HLA CLASS I	2F5	2C8	
	Autologous	A2,24 B7,46 C1,7	70	68	
	VA03	A2,24 B7,35 C3,7	32	23	
	9077	A2 B46 C11	-5	-5	
	CP	A2,28 B51,57 C6	-4	0	
Experiment 2		HLA CLASS I	2F5	3C2	3B11
	Autologous	A2,24 B7,46 C1,7	78	75	82
	Tom G	A23,29 B7,44 C4	28	56	51
	GM3161	A3,3 B7,7	41	44	46
	GM6825A	A23 B7,7	26	53	52

BLCLs were infected with vaccinia recombinants expressing D2NS3. Allogeneic targets that shared one or more HLA Class I alleles with the autologous line were used in Experiments 1 and 2. The E/T ratios were between 16:1 and 40:1 for all the T cell lines used

D. SEROTYPE CROSSREACTIVITY, HLA RESTRICTION AND EPITOPE
ANALYSIS OF BULK CULTURE CTL GENERATED FROM PBMC OF PATIENT
CHD94-020.

For patient CHD94-020, bulk culture CTLs lysed targets expressing the NS3 proteins of D2, D3 and D4 virus (Table IV-1 and IV-6). Using recombinant vaccinia viruses expressing truncated D3NS3, we were able to localize a CTL epitope to a.a 1-176 of NS3 (Table IV-6 exp. 2). Using allogeneic targets having HLA class I alleles in common with CHD94-020, bulk culture CTL activity was found to be HLA-A11.1 restricted, based on recognition of CHD94-134 BLCL target cells, which share only A11.1 in common with the autologous line (Table IV-6 exp 3). These results indicate that the NS3 specific CTLs detected in bulk culture from this subject are A11.1 restricted and are also serotype crossreactive. We tried to isolate CTL lines from this donor by the limiting dilution method but were not successful.

E. LOCALIZATION OF THE EPITOPE WITHIN THE NS3 PROTEIN RECOGNIZED
BY T CELL LINES FROM PBMC OF PATIENT KPP94-024.

CTL lines from patient KPP94-024 lysed target cells expressing NS3; therefore we infected target cells with vaccinia recombinants expressing truncations of the D3NS3 gene to localize the epitopes recognized by these CTL lines.

TABLE IV-6: Serotype crossreactivity, Epitope localization and HLA Restriction of Bulk Culture CTL obtained from PBMC of Patient CHD94-020.

	Target cells infected with	HLA CLASS I ALLELES			% specific ⁵¹ Cr release
Experiment 1					
	Vac. control				24
	Vac. D2C.prM.E				25
	Vac. D2NS1.2a				28
	Vac. D2NS3				45
Experiment 2					
	Vac. control				17
	Vac. D3NS3				54
	Vac D3 (1-176)				54
Experiment 3					
		A	B	C	
	Autologous	2,11.1	7,46	1,7	49
	KPP94-024	2, 24	7,46	1,7	17
	KPP94-037	1,11.1	46,57	1,6	56
	CHD94-134	11.1,28	27,57	3,6	45

PBMC from patient CHD94-020 were stimulated with D4 virus and 7 days later restimulated with anti-CD3 and allogeneic feeders. Cells were restimulated every two weeks and tested in CTL assays 7-10 days after the last restimulation. The E/T ratio was 50:1 for exp.'s 1 and 2 and 106:1 for exp. 3.

Table IV-7: Localization of the epitope on NS3 recognized by CTL lines of patient KPP94-024

Target cells infected or pulsed with	% specific ⁵¹ Cr. release							
	T cell lines							
	2G10	2F5	3C2	3C3	2E4	3E5	3B11	2C9
Experiment 1								
Vac. control	ND	ND	2	-7	-3	-5	-1	-1
Vac. D3NS3			70	63	64	37	62	55
Vac. D3NS3 (a.a 1-133)			-2	-5	-2	-4	-4	-6
Vac. D3NS3 (a.a 1-176)			-4	0	-2	-2	-2	-3
Vac. D3NS3 (a.a 1-216)			11	-3	2	-2	3	0
Vac. D3NS3 (a.a 1-247)			63	71	59	37	61	61
Experiment 2								
No peptide	2	ND	0	ND	1	ND	ND	ND
D4NS3 221 (a.a 221-235)	21		50		70			
D4NS3221a (a.a 224-235)	2		1		1			
D4NS3 221c (a.a 226-235)	-1		1		1			
D4NS3 221d (a.a 227-235)	-1		1		2			
D4NS3 221f (a.a 221-233)	12		35		50			
D4NS3 221g (a.a 221-232)	5		24		58			
Experiment 3								
No peptide		1		-5	0		1	3
D4NS3 221 (a.a 221-235)		61		53	48		39	33
D4NS3 221g (a.a 221-232)		47		-	34		52	22
D4NS3 222z (a.a 222-230)		30		33	18		11	9

Exp. 1 BLCLs were infected with vaccinia recombinants expressing different truncations of the NS3 protein as indicated and used as target cells. Exp 2 & 3. BLCLs were pulsed with 25µg/ml of the indicated peptide for 30 mins. after which the T cells were added and further incubated for 4-5 hours. The E/T ratios were between 10:1 and 60:1 for all of the T cell lines used in these assays.

We mapped the region on the NS3 protein recognized by all T cell lines tested from patient KPP94-024 to a.a 216-247 of NS3 (Table IV-7, exp 1). We then tested for CTL activity against overlapping peptides which spanned that region and found lytic activity only against peptide 221, which is a 15mer corresponding to a.a 221-235 (LAPTRVVAEMEEAL) (data not shown).

We used synthetic peptides with truncations of peptide 221 to detect the minimum epitope recognized by the T cell lines (Table IV-7, exp 2 and 3). Peptide targets pulsed with truncations 221g and 221f were lysed by most CTL whereas target cells pulsed with truncations 221a, 221c and 221d were not lysed. T cell lines 2F5 and 3C3 recognize a.a. 222-230 of NS3 and most of the other T cell lines recognize targets that were pulsed with a.a. 221-232 of NS3.

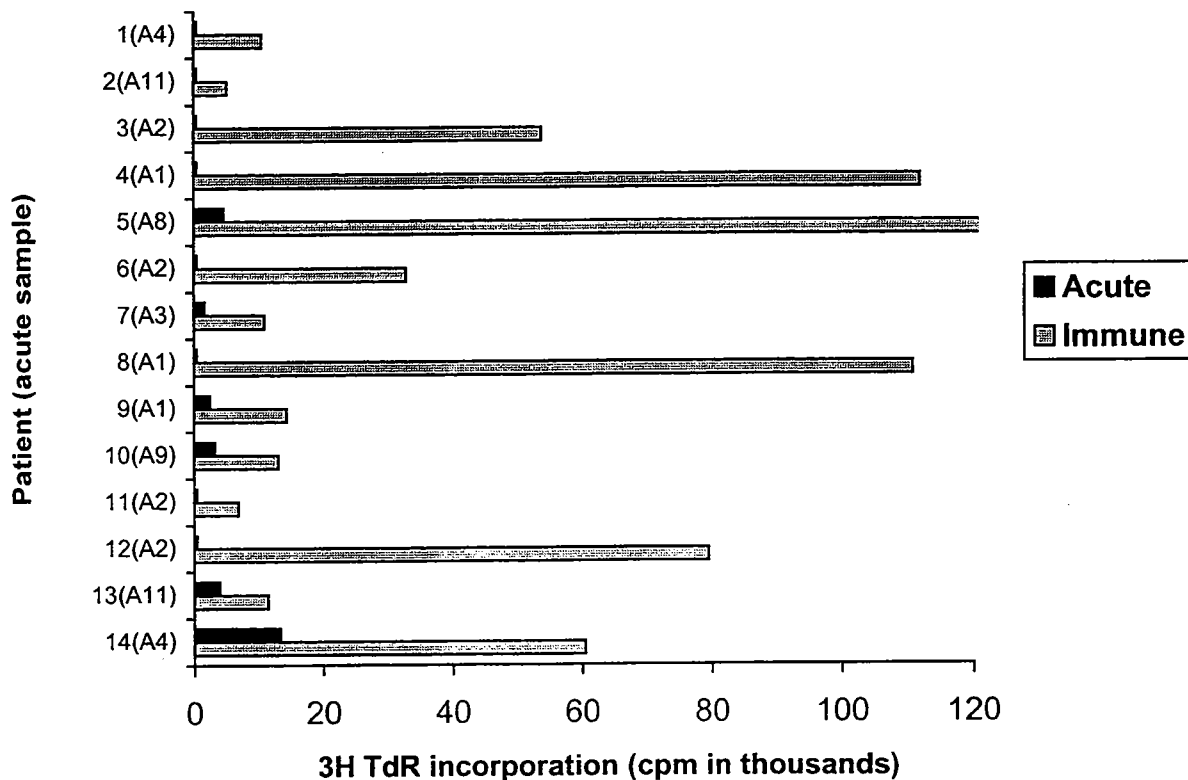
CHAPTER V

ANALYSIS OF IN VITRO PROLIFERATIVE RESPONSES OF ACUTE AND IMMUNE PBMC FROM THAILAND

A. PBMC FROM CHILDREN WITH ACUTE DENGUE INFECTION HAVE DECREASED LYMPHOPROLIFERATIVE RESPONSES TO PHA, DENGUE ANTIGENS AND RECALL ANTIGENS.

We analyzed the proliferative responses of acute and immune PBMC from patients with varying grades of severity of dengue illness to mitogens and other antigens. Acute PBMC samples obtained from patients at different time points after they were admitted into the study (study day 1 up to study day 11) were tested in this assay (Table II-4). The responses during acute infection were compared to the proliferation of the same patients' PBMC obtained 6-24 months after their acute infection. In all 14 patients there was a significant decrease in proliferation of acute PBMC in response to stimulation with PHA (Figure V-1). In the 4 patients tested there was a decrease in the response to dengue antigen and also to a recall antigen, tetanus toxoid, in the acute samples of PBMC (Table V-1). Background cpm of acute PBMC were consistently lower in all patients compared to their immune PBMC but the stimulation indices still indicated a substantial decrease in proliferation of acute PBMC compared to that of

Figure V-1 PBMC from patients with acute dengue infection do not respond in vitro to mitogen stimulation.



1×10^5 acute and immune PBMC from patients were stimulated with a 1:1000 dilution of PHA. Patient numbers are given along the vertical axis with acute sample numbers (A, study day) in parenthesis. All immune bleeds were obtained at one year or later after infection except for #13 which was obtained at 6 months. For patients #9 and #11, a two year bleed was used in this assay as the immune sample. Cells were pulsed with $^3\text{HTdR}$ for 18 hours and harvested 5 days after incubation at 37°C . The data presented are from the 9 different experiments which were performed.

Table V-1 PBMC obtained during acute dengue infection do not respond to dengue antigens and recall antigens in proliferation assays ^a

PATIENT NUMBER	STUDY DAY	Dengue antigen	Tetanus Toxoid	No antigen
9	(A1)	141	140	128
	(I336)	14166	45391	387
11	(A3)	80	58	49
	(I367)	2939	5090	227
14	(A4)	78	55	58
	(I372)	2094	1370	169
15	(A5)	83	80	75
	(I366)	2344	5376	856

^a 1×10^5 PBMC from acute (A) and immune (I) phases of infection from the same patient were stimulated with a 1:40 dilution of dengue antigen or a 1:5000 dilution of tetanus toxoid and incubated for 6 days in a 96 well plate at 37°C. Cells were pulsed with ³HTdR on day 6 for 18 hours and harvested. The results presented are from 4 different experiments performed. Data are mean cpm of 2 or 3 replicates

immune PBMC (Table V-1 and data not shown). The results indicate that in vitro proliferative responses of PBMC to mitogens and specific antigens are suppressed in all grades of acute dengue infection. Samples tested up to 11 days after the patient was admitted into the study showed decreased responses compared to the same individuals' immune samples.

B. RECOMBINANT IL-2 AND IRRADIATED PBMC RESTORE PROLIFERATION OF ACUTE PBMC

To characterize the immunological unresponsiveness of the acute PBMC, we treated the PHA-stimulated cells with 10U/ml of recombinant IL-2. Inadequate production of IL-2 by T cells is thought to be one of the factors that contribute to the immunosuppression seen during acute measles and CMV infection. In 8 of 9 patients tested, the in vitro proliferative responses to PHA were restored by the addition of exogenous IL-2 (Table V-2). PBMC samples tested from study day 1, when the patients were sick and had severe symptoms, also responded to IL-2. In one patient (#12), addition of up to 100U/ml of IL-2 did not restore proliferation (data not shown).

Table V-2 Decreased proliferative responses of acute PBMC are partially restored upon the addition of IL-2 or gamma-irradiated autologous or allogeneic PBMC ^a

	PHA	PHA + IL-2	PHA + irradiated PBMC	
			Autologous	Allogeneic
2(A1) ^b	308	62514	ND ^c	ND
3(A3)	899	23637	ND	23146
5(A9)	4600	73073	22886	ND
7(A3)	627	17593	59555	ND
9(A1)	1049	26314	ND	ND
10(A9)	3240	71969	ND	ND
12(A2)	102	128	ND	ND
13(A11)	3917	54815	130788	ND
16(A9)	1396	64821	ND	ND

^a10U/ml of recombinant IL-2 or 1×10^5 gamma irradiated autologous immune (for patients 5,7 and 13) or allogeneic healthy PBMC (for patient 3) were added to acute PBMC stimulated with a 1:1000 dilution of PHA and incubated for 5 days. Cells were pulsed overnight on day 4 with ³HTdR and harvested. The results presented are from 8 different experiments performed. Data are mean cpm of 2 or 3 replicates.

^b Acute sample numbers are indicated in parenthesis

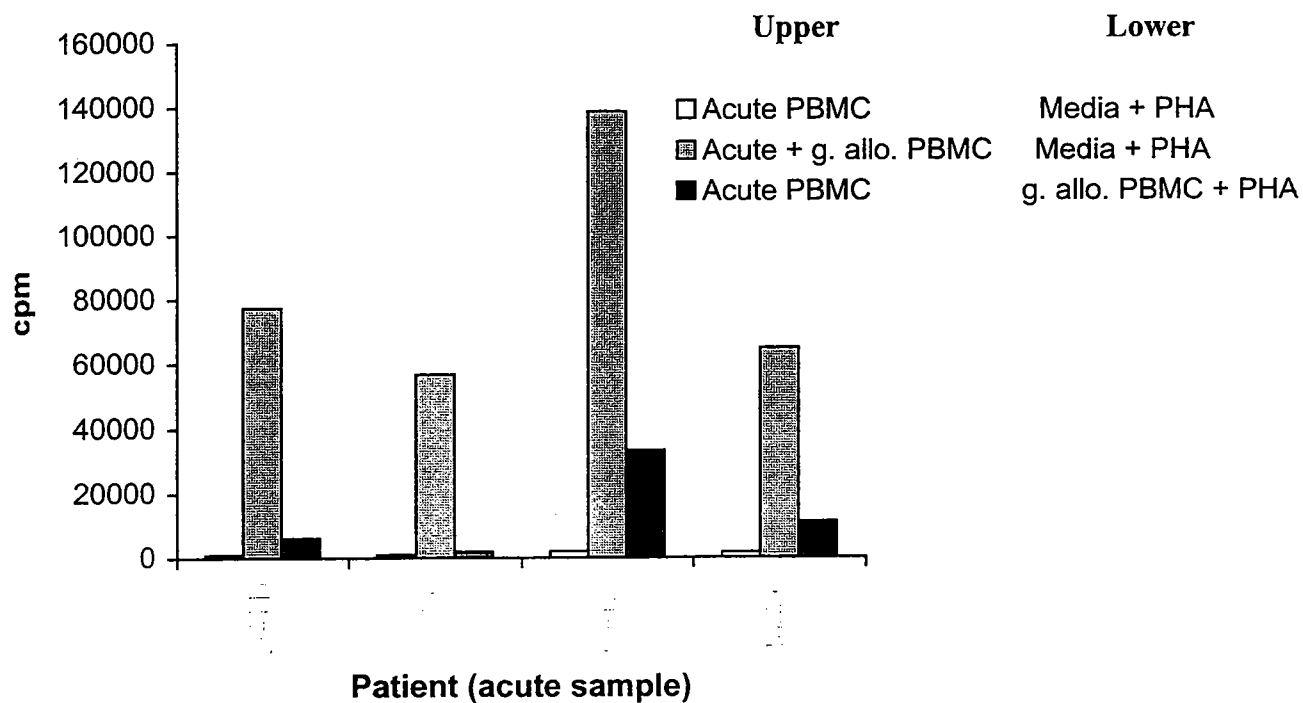
^c ND = not done.

Addition of 1×10^5 gamma-irradiated, autologous immune or allogeneic control PBMC also improved proliferative responses of acute PBMC (Table V-2). Our data show that the unresponsive acute PBMC can be induced to proliferate by the addition of the cytokine IL-2 or irradiated control PBMC. However, these results do not indicate if released soluble factors or cell contact with the irradiated PBMC are essential to restore the proliferation of the acute PBMC.

C. ACUTE PBMC REQUIRE CELL CONTACT WITH GAMMA-IRRADIATED CONTROL PBMC TO INCREASE PROLIFERATIVE RESPONSES

To determine if cell contact with the irradiated PBMC is needed to mediate proliferation, we used a transwell system in which acute cells were separated from allogeneic control PBMC by a polycarbonate membrane. When acute and gamma-irradiated allogeneic cells were incubated together in the upper well, there was a substantial increase in proliferation compared to acute T cells alone stimulated with PHA (Figure V-2). When gamma-irradiated PBMC were separated from the acute cells in the upper well by a polycarbonate membrane, proliferation was much lower in all 4 patients' PBMC compared to when the cells were incubated together. In patient 6, acute cells proliferated somewhat when they were separated from the gamma-irradiated control cells by the membrane (approximately 30,000 cpm compared to 135,000 cpm) suggesting that

Figure V-2 Acute PBMC from patients with dengue viral infection require contact with control PBMC to increase proliferative responses.



1×10^5 acute PBMC were added to the upper well of the transwell. 6×10^5 gamma irradiated allogeneic PBMC were added to the upper or lower well of the transwell as indicated. Cpm of the irradiated allogeneic PBMC incubated with PHA was less than 1148 cpm. Patient numbers are given along the X axis and sample numbers of the acute bleeds are given in parenthesis. The results presented are from two different experiments performed.

both soluble factors and cell contact were important in restoring proliferation. However, these results suggest that cell contact with gamma-irradiated allogeneic PBMC is predominantly required to restore proliferation of acute cells.

D. ACCESSORY CELLS IN PBMC OBTAINED DURING ACUTE DENGUE ILLNESS ARE UNABLE TO PROVIDE ADEQUATE STIMULI TO INDUCE PROLIFERATION OF T CELLS

To further demonstrate that there is a defect in the accessory cells in the acute PBMC samples, both T and non-T cells were isolated from patients with acute dengue and mixed in proliferation assays with T and non-T cells from a control donor. When T cells from a control donor were added to gamma-irradiated non-T cells from the acute PBMC, responses were substantially reduced, indicating that the non-T cells in the acute PBMC sample could not support the proliferation of T cells from a control donor (Table V-3). In contrast, when T cells from an acute dengue patient were mixed with non-T cells from a control donor, substantial proliferation was observed. The results indicate that the T cells in the acute PBMC samples from dengue patients are able to respond when optimal stimuli from the PBMC of a control donor are provided.

Table V-3 Non T cells from acute PBMC of patients with dengue cannot support the proliferation of healthy T cells

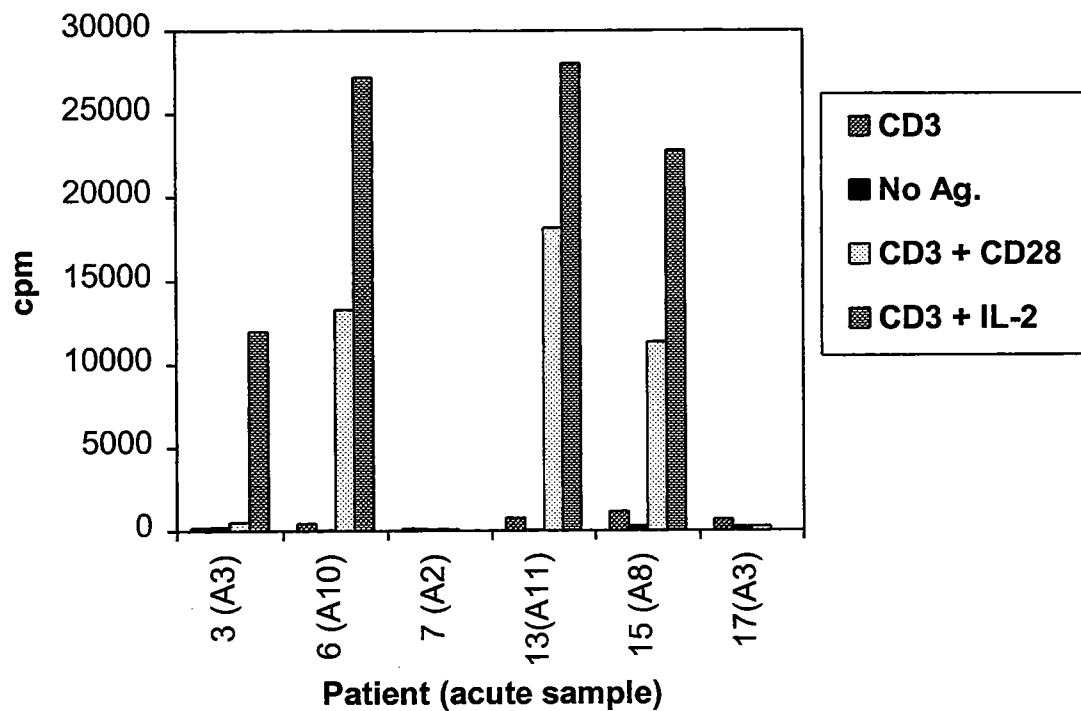
	Patient (acute sample)		
	10(A9)	13 (A11)	15 (A2)
T (acute PBMC) + γ nonT (acute PBMC) + PHA	308	67	199
T (acute PBMC) + γ nonT (healthy PBMC) + PHA	15302	16661	12693
T (healthy PBMC) + γ nonT (healthy PBMC) + PHA	21671	5028	7471
T (healthy PBMC) + γ nonT (acute PBMC) + PHA	1420	3534	736
γ nonT (healthy PBMC) + PHA	727	339	911
γ nonT (acute PBMC) + PHA	70	38	116
Acute PBMC + PHA	409	616	99
Healthy. PBMC + PHA	45830	45830	25671

T cells were isolated from acute PBMC of patients with dengue infection or healthy control PBMC and mixed with non T cells from either healthy or acute PBMC. For patient 10, 26000 T cells and 110,000 non T cells were added per well, for patient 13, 22000 T cells and 70,000 nonT cells were added per well and for patient 15, 12000 T cells and 33000 nonT cells were added per well. Cells were incubated at 37°C for 5 days, pulsed and then harvested. The results presented are from two different experiments performed.

E. CD28 ANTIBODIES INCREASE PROLIFERATION OF ACUTE PBMC IN CERTAIN PATIENTS WITH ACUTE DENGUE ILLNESS

To analyze whether costimulatory molecules may play a role in the cell-mediated suppression of acute PBMC samples, we treated the PBMC of 6 subjects with anti-CD28 and anti-CD3 antibodies. In three patients (#6, #13 and #15), addition of anti-CD28 restored proliferation of acute PBMC to levels comparable to stimulation with anti-CD3 + IL-2 (Figure V-3). The PBMC samples from these patients were obtained at least 8 days after enrollment, at which time the subjects were no longer febrile or viremic. PBMC from three other patients (#3, #7 and #17) did not respond to costimulation with either 5 or 10 μg of anti-CD28. The PBMC were obtained from these patients when they had the most severe symptoms (days 2 or 3 after they were enrolled into the study) and were acutely ill in the hospital. Study days 2 or 3 also represent days when the absolute monocyte counts drop to the lowest levels. The results suggest that anti-CD28 antibodies can restore proliferation of PBMC samples when the patients are recovering from illness but have no effect on PBMC obtained from the patients at the peak of their illness.

Figure V-3 Anti-CD28 antibodies increase proliferation of anti-CD3 stimulated acute PBMC obtained at later time points during acute infection



1×10^5 acute PBMC were stimulated with $0.1 \mu\text{g/ml}$ of anti-CD3 antibodies, anti-CD3 + anti-CD28 ($10 \mu\text{g/ml}$) or anti-CD3 + IL-2 (10U/ml) for 5 days. Cells were pulsed overnight and harvested. Patient numbers are given along the X axis with acute sample numbers in parenthesis. The results presented are from 4 different experiments performed. PBMC from patients 7 and 17 were not tested for stimulation with CD3+IL-2.

F. ACUTE PBMC SAMPLES HAVE DECREASED NUMBERS OF MONOCYTES BUT NORMAL PERCENTAGES OF T AND B CELLS

We found a decrease in the number and percentage of monocytes in the acute PBMC samples compared to the convalescent samples (Table V-4). However, there was no decrease in the percentage of either CD4 or CD8 T cells in the acute PBMC. Therefore, a reduction in the number of T cells cannot be the primary reason for inadequate proliferative responses. There was also no difference in the percentage of B cells during acute infection.

G. IL-2 AND IL-7, BUT NOT IL-4 OR IL-12, IMPROVE PROLIFERATION OF PBMC OBTAINED DURING ACUTE DENGUE INFECTION

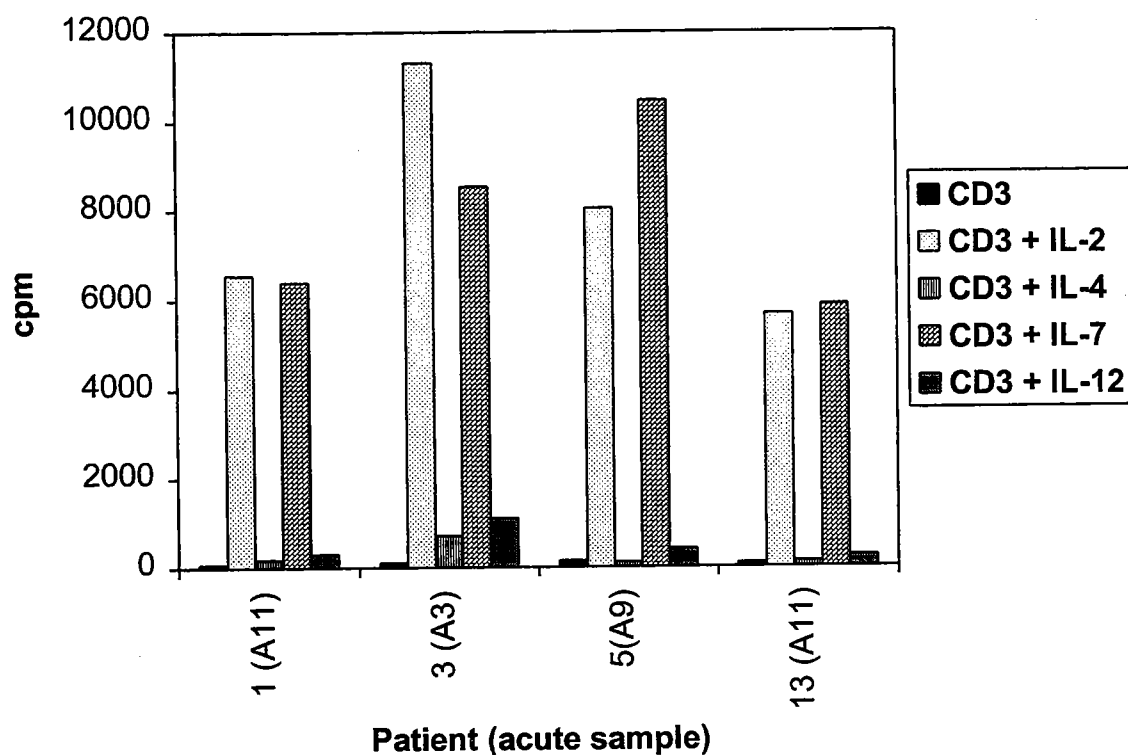
Since receptors for the cytokines IL-2, IL-4, IL-7 and IL-15 are known to share a common gamma chain, we examined whether cytokines other than IL-2 would also help overcome the unresponsiveness of the acute phase PBMC. We chose IL-12 as a cytokine that did not utilize the common gamma chain. In 4 of 4 patients tested, the acute PBMC samples responded to anti-CD3 in the presence of IL-2 or IL-7 but not in the presence of IL-4, even at a concentration of 500U/ml (Figure V-4 and data not shown). No increase in proliferative responses to anti-CD3 was detected in the presence of IL-12. The results indicate that several but not all, cytokines whose receptors share the common gamma chain are able to restore the proliferation of the PBMC obtained during acute infection.

Table V-4 Percentages of T cells, B cells and monocytes in acute and convalescent PBMC populations. ^a

PATIENT	SAMPLE NUMBER	% T CELLS		% MONOCYTES	% B CELLS
		CD4	CD8		
3	A8	27	21	6	28
	C354	25	12	26	25
5	A9	21	20	3	19
	C725	15	23	8	17
13	A11	ND	ND	7	23
	C196			13	22

^a Cryopreserved PBMC from acute and convalescent samples from each patient were thawed and stained with FITC conjugated antibodies to CD4, CD8, CD14 and CD19 and analyzed by flow cytometry.

Figure V-4 Acute PBMC respond to CD3 + IL-7 stimulation but not IL-4 or IL-12 stimulation



1×10^5 cells were stimulated with $0.1 \mu\text{g/ml}$ of anti CD3 antibodies and either 10U/ml IL-2, 100U/ml IL-4, 100U/ml IL-7 or 25 ng/ml IL-12. Cells were pulsed with $^3\text{H-TdR}$ on day 4 overnight and harvested on day 5. The results presented are from two different experiments performed. Patient numbers are given along the X axis with acute sample numbers in parenthesis.

CHAPTER VI

DISCUSSION

A. CD8+ CTL RESPONSES IN VACCINE RECIPIENTS

Our aim in this study was to analyze the diversity of bulk culture CD8+ CTL responses in volunteers who received candidate monovalent live attenuated dengue vaccines (D1, D2, D3 or D4).The data generated would complement existing information on CD4+ CTL responses to dengue infection.

I first examined the proliferative responses of PBMC from all donors to varying concentrations of live flaviviruses. Most of the studies that had been done prior to the outset of the project had examined dengue-specific T cell responses following stimulation with noninfectious dengue antigen. The PBMC of all subjects exhibited proliferative responses with the most significant proliferation to the serotype of virus contained in the vaccine that they had received and a variable level of cross reactive proliferation to other dengue serotypes or to other flaviviruses. The results demonstrated that dengue virus-specific memory T cells were generated after primary immunization and the patterns of specificities of these T cell responses varied.

We also detected dengue-specific CTL activity in all 8 donors, indicating that these live-attenuated vaccines elicited dengue-specific CTL responses. We found CTL

killing against a wide variety of proteins including the envelope (E), premembrane (prM) and nonstructural proteins (NS1.2a and NS3) of dengue viruses as summarized in Table VI-1. Seven of eight donors had CD8⁺ CTL responses directed against one or more of these proteins, although the levels of lysis varied between donors.

In initial studies of dengue virus-immunized inbred mice, bulk culture CD8⁺ CTLs recognized only a very limited number of epitopes on dengue virus proteins - as few as one to three on the entire genome, which encodes 3,386 amino acids (Rothman, et al., 1993). CTLs from H-2^k mice recognized an epitope on the NS3 protein. CTLs from H-2^b mice recognized an epitope on the NS4a or NS4b proteins. CD8⁺ CTLs generated from H-2^d mice recognized an epitope on a structural protein, the NS1 or NS2a and the NS3 proteins. In humans, CD8⁺ CTL clones were established from only one donor, Donor 7, which recognized a.a 500-508 on the nonstructural protein NS3 (Livingston, et al., 1995). This preliminary study suggested that there were a limited number of CD8⁺ CTL epitopes on dengue proteins in humans as well.

The results generated from the study done in Chapter III illustrate that there are multiple CD8⁺ CTL epitopes on both structural and nonstructural proteins, which is similar to our previous results demonstrating that multiple epitopes were recognized by dengue virus-specific CD4⁺ CTLs in both humans and mice (Gagnon, et al., 1996, Green, et al., 1993, Kurane, et al., 1991b, Kurane, et al., 1989b, Zeng, et al., 1996, Zivny, et al., 1993). T cell cloning experiments on the PBMC from different volunteers is likely to

generate numerous CD8+ CTL clones consistent with the bulk culture results.

1. Immunodominance of nonstructural proteins as CTL targets

All donors had CTLs that recognized at least one nonstructural protein (NS3 or NS1.2a) (Table VI-1). The predominant protein that was recognized by the PBMC of the 8 donors was the nonstructural protein NS3. NS3 is the second largest viral protein (containing 618 amino acids) and is one of the most highly conserved proteins among flaviviruses. Table VI-2 shows the serotype specificities of the bulk culture CTL responses to the different dengue proteins in these 8 donors. The results from Tables VI-1 and 2 indicate that CTLs recognizing NS3 are usually cross reactive whereas CTLs recognizing other proteins (E, prM and NS1.2a) are mainly serotype specific. The cross reactive nature of CTLs against NS3 may be due to the high levels of amino acid conservation among the NS3 proteins of the different serotypes.

NS3 has both a protease (N terminus) and a nucleotide triphosphatase/helicase (C terminus) activity. At the outset of this project, our laboratory had previously identified multiple sites on NS3 recognized by human CD4+ CTLs and one site recognized by CD8+ CTLs as shown in Figure VI-2. Here we show that there are at least four sites on NS3 recognized by CD8+ CTLs. Therefore, although there is a predominance of

TABLE VI-1 Summary of CTLs generated in bulk culture from PBMC of dengue immune donors 1-8

Donor	Serotype of Vaccine Received	Proliferation to infectious virus	Dengue Proteins Recognized by CTL	Serotype specificity or cross reactivity of CTL	Localization of Epitope	Responding Cell Type	Potential HLA Restricting Allele
1	D1	+	Cpr.M.E E.NS1.2a	Specific Specific	- -	CD4+ CD8 CD4	ND ND
2	D2	+	E NS1.2a NS3	Specific Specific Cross- Reactive	- - -	CD8 (virus infected targets)	B44 B44 B44
3	D2	+	E NS1.2a NS3	Specific Specific Cross-Reactive	- - 247-354	ND CD8 CD8	ND ND B38
4	D2	+	NS1.2a NS3	Specific Specific	- -	ND ND	ND ND
5	D3	+	NS3	Cross-Reactive	214-247	CD8	A24 and B62
6	D4	+	pr.M NS3	Specific Cross-Reactive	- 1-183	CD8 ND	- -
7	D4	+	E NS3	Specific Cross-Reactive	- 500-508	CD8+ CD4 CD8	- B35
8	D4	+	NS1.2a	Cross Reactive		CD8	ND

¹ determined by monoclonal antibody and complement depletion

ND = not determined.

Table VI-2 Summary of serotype specific and crossreactive CTL responses to dengue virus proteins in 8 vaccine recipients.

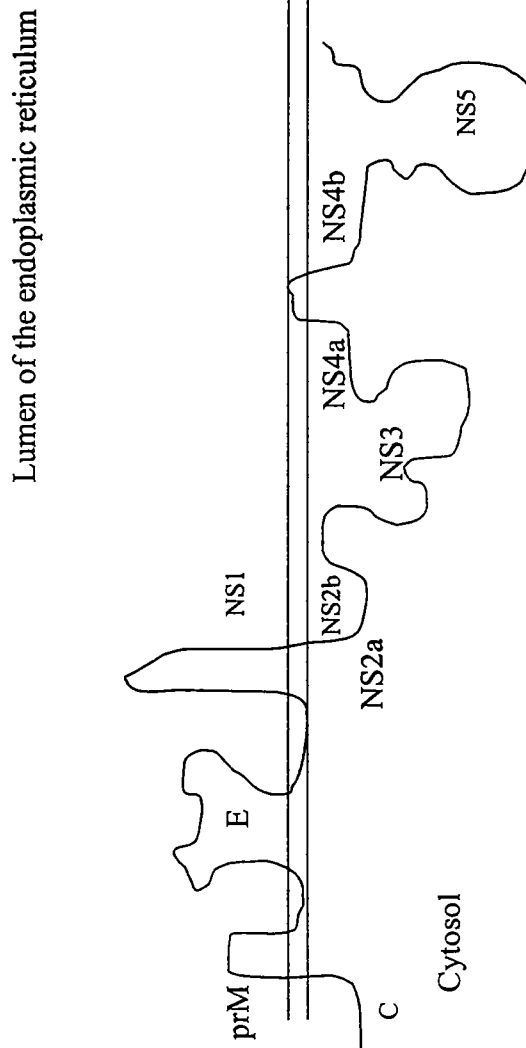
PROTEIN(S)	# of DONORS WITH CTL RESPONSE TO PROTEIN	# OF DONORS WITH SEROTYPE SPECIFIC RESPONSES	# OF DONORS WITH SEROTYPE CROSSREACTIVE RESPONSES
NS3	6	1	5
NS1.2a	5	4	1
E	3	3	0
prM	1	1	0
C.prM.E	1	1	0

recognition of determinants on the NS3 protein, there is no single immunodominant epitope.

In the murine system we have shown that the nonstructural protein NS3 is the major target for dengue virus-specific H-2^k CTL (Rothman, et al., 1993). Other laboratories have also found immunodominant murine CTL determinants on nonstructural proteins of other flaviviruses. The Murray Valley Encephalitis (MVE) NS3 protein has been shown to be a dominant source of H-2^k restricted antigenic determinants recognized by MVE and WN-immune CTLs (Lobigs, et al., 1994). Using Kunjun and WNV to identify determinants for CTLs in mice of five H-2 haplotypes, the nonstructural proteins were shown to have the strongest response (Hill, et al., 1992, Parrish, et al., 1991). For mice with the H-2^k haplotype, a 98 amino acid fragment spanning the junction between NS3 and NS4a of Kunjun virus was shown to be an immunodominant region. Nonstructural proteins from viruses belonging to other families such as the bluetongue virus (Reoviridae), MCMV and HSV have also been shown to be an immunodominant source of peptides for CTL recognition (Banks, et al., 1991, Del Val, et al., 1991, Jones, et al., 1996). ~~These data support the hypothesis that proteins with an intracellular location are a major source of T cell determinants.~~

The flavivirus genome encodes for a single polyprotein that gets co- and posttranslationally cleaved to the individual proteins. The structural proteins (C, prM,E),

Figure VI-1 Flavivirus nascent polypeptide chain



Schematic representation of a flavivirus polyprotein in the membrane of the endoplasmic reticulum
(From Coia et al, J. Gen. Virol 1988; 69: 1-21)

NS1 and the N terminus of NS2a are translocated into the lumen of the endoplasmic reticulum via a series of signal and stop-transfer sequences as shown in Figure VI-1 (Coia, et al., 1988, Rice, et al., 1986). On the other hand, the NS3 and NS5 proteins, which are the largest proteins encoded by the dengue genome, have a cytoplasmic localization. NS3 constitutes approximately 25% of the cytoplasmic region of the viral polyprotein. This may be one of the reasons why NS3 is an immunodominant protein. Since we have not tested vaccinia recombinants expressing NS5, it is possible that there are also T cell epitopes on NS5.

Viral polyprotein processing may not be a major factor influencing the immunodominance of NS3, since in most of the studies cited vaccinia virus recombinants expressing individual flaviviral proteins were used to infect target cells. Also, when a large polyprotein fragment that was not correctly folded was used in the MVE studies, it efficiently sensitized target cells for lysis (Lobigs, et al., 1994). Numerous other factors, such as the efficiency of transport and peptide stability are likely to influence why peptides from nonstructural proteins are preferentially presented on MHC molecules (Barber and Parham, 1994).

Since NS3 is recognized by a wide variety of CTLs from dengue-immune donors, and antibodies to NS3 are unable to mediate antibody-dependent enhancement of infection, this protein is an attractive candidate for inclusion in a subunit vaccine. However, it is also possible that NS3 specific CTL may contribute to the

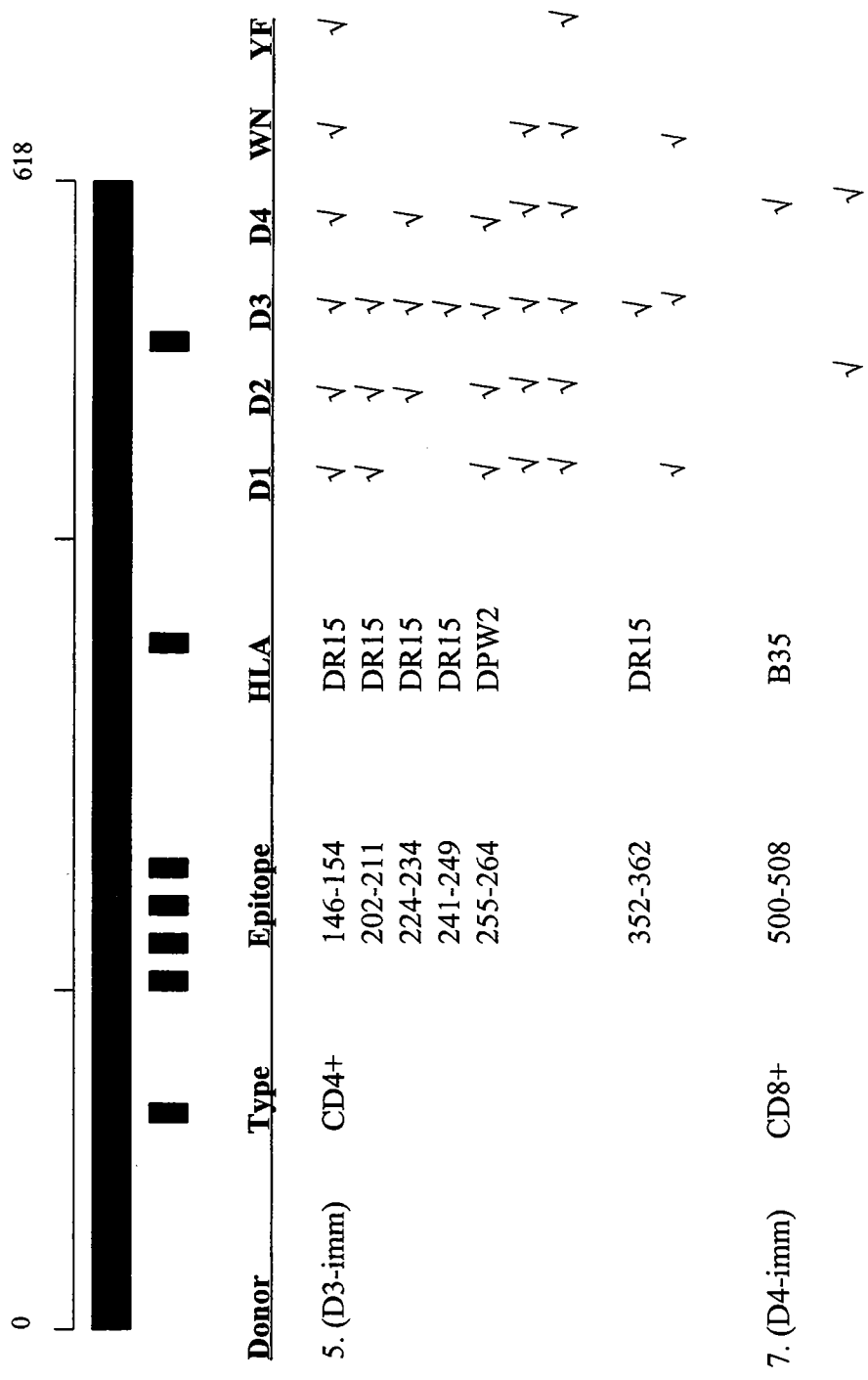
immunopathology of DHF, as they can be reactivated following exposure to a heterologous serotype of virus.

2. Serotype specificity of T cell clones and bulk cultures

The demonstration that dengue virus-specific memory CTLs are serotype cross-reactive after a primary dengue infection supports the possibility that these memory T cells can be activated in secondary infections with a heterologous serotype of virus. We have previously isolated human CD4+ CTL clones from different donors with varying patterns of virus specificities (Figure VI-2). These include: dengue virus type specific clones, dengue virus subcomplex specific clones, dengue virus serotype-crossreactive clones and flavivirus-crossreactive clones that recognize WNV and/or YFV. The CD8+ CTL clones isolated from Donor 7 had three patterns of serotype specificity, including serotype-specific clones, dengue virus subcomplex-specific and serotype crossreactive clones (Livingston, et al., 1995). These T cells may play important roles in recovery from infection by other flaviviruses or in immunopathology.

The results from this current study on 8 vaccine donors extend the observation that human CD8+bulk cultures also have multiple specificities. We detected only serotype-specific CTLs from 2 of 8 donors (donors 1 and 4, Table VI-1). In the other donors, we detected CTLs with various patterns of serotype-crossreactivity, including

Figure VI-2 Recognition of the Dengue NS3 Protein by CTLs



recognition of D2 and D4 NS3 (donor 2), D2 and D3NS3 (donor 3), D3 and D4NS3 (donor 6), D2, D3 and D4 (donors 5 and 7) and D1ENS1.2a and D2NS1.2a (donor 8). Data from these experiments further lend support to the possibility that both CD4+ and CD8+ CTLs are capable of being activated in secondary infections with a different serotype of virus and may contribute to the pathogenesis seen in DHF.

3. HLA restriction and dengue infection

Genetic factors may also play a critical role in accounting for the differences between individuals in susceptibility to severe dengue infection. Since NS3 was the immunodominant protein recognized by CTLs, we examined the MHC restriction of NS3-specific CTLs generated in bulk cultures of PBMC from 4 donors to identify class I alleles that might present different portions of the NS3 genome. For donors 2, 3, 5 and 7 the potential restricting alleles for NS3-specific CTL are B44, B38, A24 &/or B62, and B35, respectively.

It is interesting that for donor 2, three CD8+ T cell epitopes on dengue viral proteins (E, NS3 and NS1.2a) were recognized in the context of HLA-B44. A peptide binding motif has been identified for HLA-B44 with an acidic amino acid (glutamic acid) at P2 and a less rigid requirement for a basic or hydrophobic amino acid at P9 (DiBrino, et al., 1995). It is likely that if we were to identify nonamer peptides with the above motif

in the three proteins, one or more of them would be able to sensitize target cells for lysis. Using the HLA-B44 motif, three different antigenic peptides from both influenza NS1 and NP proteins were shown to be presented by HLA-B44 to antiinfluenza type A-specific CTLs from one donor (DiBrino, et al., 1995). In an earlier study of Thai children who had been hospitalized with severe dengue infection, HLA A2 and HLA B blank appeared to be statistically associated with severe disease (Chiewslip, et al., 1981).

The studies performed in Chapter III strengthen the information that exists on the human T cell responses to dengue infection and show that, in bulk culture, CD8+ CTL responses can be generated against several proteins encoded by the dengue genome.

B. CTLs FOLLOWING NATURAL SECONDARY INFECTION WITH DENGUE VIRUS

Most of the research on human cytotoxic T cell responses to dengue viruses has been performed using Caucasian volunteers who received experimental live monovalent dengue vaccines. These individuals had no known prior exposure to dengue viruses and therefore they received the dengue vaccine as a primary infection. However, the complications seen in dengue infection (DHF and DSS) are more common in patients who have preexisting immunity to one serotype of virus during infection with another serotype (secondary infection) of virus. Activation of dengue virus-specific memory T cells has been implicated in the pathogenesis of severe dengue infection (Kurane and

Ennis, 1992). Analysis of T cell responses in patients after natural secondary infections is therefore important because it may provide insights into the mechanisms of T cell mediated immunopathology.

1. Immunodominance of nonstructural proteins as CTL targets

In the study done in Chapter IV, memory CTL responses were detected against dengue proteins in the PBMC of all four patients examined. This is the first report of dengue-specific CTLs after natural secondary dengue infections. In our previous work on CD4+ CTLs and the work done in Chapter III on CD8+ CTLs, we have shown that nonstructural proteins, in particular NS3, are a predominant target for CTLs (Livingston, et al., 1995, Mathew, et al., 1996, Zeng, et al., 1996). In the present study, we also found that, all 4 donors had CTL responses to NS3, and two donors had CTLs that recognized the nonstructural proteins NS1.2a (Table VI-3). Therefore NS3 is an immunodominant protein recognized by CTLs even in patients who have had a natural infection with dengue virus.

2. Serotype-specificity of CTL responses

Our earlier work, analyzing CTL responses to primary dengue infection in PBMC of vaccine recipients, generated several T cell lines from dengue immune donors that had varying specificities (Kurane, et al., 1991b, Mathew, et al., 1996). These typically include

serotype-specific, dengue-subcomplex-specific and virus serotype-crossreactive T cell clones. We speculated that the serotype crossreactive memory T cells generated during the primary infection would be reactivated in a secondary infection and contribute to the immunopathology of DHF, which is observed much more frequently in secondary infections. This hypothesis predicted that most of the T cells isolated following a secondary infection would be serotype-crossreactive.

In the study done in Chapter IV we examined CTL responses after secondary infection in 4 Thai patients. All the NS1.2a specific CTL lines generated from patient KPP94-037 and the NS3 specific CTLs from patient KPP94-024 were crossreactive with the other serotypes of dengue virus. The bulk culture CTLs from patient CHD94-020, who was infected with dengue-4 virus, were also crossreactive with dengue 2 and dengue 3 NS3. We have not yet analyzed the CTL responses to primary dengue virus infection in Thai children; therefore, we cannot conclusively define the relationship between primary and secondary dengue infections. However, these results suggest that the CTL responses in secondary infections are predominantly due to reactivation of memory cross-reactive CTL from the primary infections, consistent with our hypothesis. The CTL responses of Thai patients to natural secondary dengue infection are directed against nonstructural proteins and are mainly serotype-crossreactive.

3. HLA restriction of dengue-specific CTLs from Thai children

Immune responsiveness is affected by the major histocompatibility complex and the pathogenesis of some diseases has been associated with specific HLA alleles. In areas where DHF is endemic, only a small percentage of individuals exhibit severe disease. This suggests that host genetic factors may play a role in susceptibility to severe disease. One study found a positive association between HLA-A2 and B blank and the development of DSS and a negative relationship for HLA-B13 (Chiewslip, et al., 1981).

Since it is known that there is diversity in both HLA and non-HLA gene loci between South East Asians and Caucasians (Chandanayingyong, et al., 1997), it was important to analyze the recognition of dengue viral proteins by T cells in the context of typical HLA molecules in Thai patients. We previously isolated NS3 specific CD4⁺ and CD8⁺ CTL clones from Caucasian volunteers which were restricted by the HLA alleles B35 (Livingston, et al., 1995), DR15 (Kurane, et al., 1991b) and DPw2 (Kurane, et al., 1993) (Figure VI-2).

The NS1.2a -specific CTLs from patient KPP94-037 in the present study were B57 restricted and DR7 restricted; the NS3 specific CTLs from patient KPP94-024 were B7 restricted and the NS3 specific CTLs from patient CHD94-020 were A11.1 restricted. These results indicate that despite differences in HLA alleles between Thais and Caucasians, peptides from dengue nonstructural proteins are also predominantly recognized by T cells from Thai children. These studies confirm our previous results of

the dominance of nonstructural proteins as CTL targets in Caucasian dengue vaccine recipients.

4. Epitope analysis of T cell lines from patient KPP94-024

All of the T cell lines isolated from patient KPP94-024 recognized autologous targets pulsed with a.a 221-235 of the NS3 protein. This 15mer, which is lysed by all the B7 restricted CD8+ CTLs from this patient, contains a proline at position 223 . These results are consistent with reports that antigenic peptides bearing a proline at position 2 and aromatic or hydrophobic residues at their C-terminus bind to HLA-B7 and related class I alleles sharing the B7-like supertype (Sidney, et al., 1996).

There are a wide variety of factors that may contribute to the development of DHF and DSS. Vaccine strategists therefore need to carefully weigh the options as they have to develop a vaccine that stimulates protective responses without increasing the risk of immunopathological consequences. Considerable work still needs to be done to elucidate the roles of CD4 and CD8 T cells in viral clearance and pathology. These studies identifying CTL responses in volunteers who have received candidate live dengue virus vaccines and in children following natural dengue infection will guide scientists in the development of a protective and effective vaccine to prevent dengue virus infections.

Table VI-3 Summary of CTLs generated from dengue-immune donors in Thailand

Patient	Disease/ Diagnosis (viral serotype)	Primary/ Secondary	Proteins recognized in bulk culture	Proteins recognized by T cell lines (CD4 and CD8)	Serotype Specificity
KPP94-037	DF (D2)	Secondary	NS1.2a NS3	NS1.2a	All crossreactive
KPP94-024	DHF grade 3 (D2)	Secondary	NS3	NS3	All crossreactive
CHD94-020	DHF grade 3 (D2)	Secondary	NS3	Not successful	Bulk culture crossreactive
CHD94-134	DHF Grade 2 (D2)	Secondary	C.prM.E NS1.2a NS3	ND	ND

ND = not determined.

C. ATTEMPTS TO ISOLATE DENGUE-SPECIFIC CTLs DURING ACUTE INFECTION

We were interested in characterizing dengue-specific T cells in patients with acute DF or DHF. We hypothesized that these T cells would be activated in vivo, play an important role in the pathogenesis of DHF, and also contribute to the recovery from infection. We wanted to isolate dengue-specific CTLs in samples obtained when the patients were sick and admitted into the hospital, and determine their protein recognition, serotype specificity and HLA restriction. Our eventual aim was to follow these in vivo activated T cells during the acute infection and determine if a subpopulation of acute cells would become memory cells using T cell receptor gene sequences as markers for analysis of 6 or 12 month follow-up bleeds from the same patients.

As shown in Table VI-4, we attempted several techniques to isolate and characterize acute phase T cells specific for dengue but were not successful. Since we speculated that some dengue-specific T cells would be activated in vivo during acute illness and have upregulated their IL-2 receptors, as one approach we initially cultured the PBMC with IL-2 to preferentially select for these activated cells. Both bulk cultures and T cell lines grew well in vitro when cells were cultured in IL-2, but neither bulk cultures or lines lysed dengue-infected target cells. Whenever IL-2 was not initially added to the cultures, cell growth was very poor and the cultures could not be maintained..

Table VI-4 Techniques used to isolate or detect dengue-specific CTLs in acute PBMC samples from Thailand

		Methods attempted	No. of patients tested	Results
1.	Bulk culture	Initial culture with 50-100U/ml of IL-2 for 7-10 days; subsequent stimulation with anti-CD3, IL-2 and allogeneic irradiated PBMC	6	No dengue-specific CTL activity
2.		Initial stimulation with dengue virus/ antigen for 7-10 days; restimulation with anti-CD3, IL-2 and allogeneic irradiated PBMC	1	"
3	No culture	Direct CTL at high E/T ratios	2	"
4.	Limiting dilution	Limiting dilution of 7-14 day bulk cultures established in 1 & 2 at 10, 30 and 100 cells/well; stimulation with anti-CD3, IL-2 and allogeneic irradiated PBMC	3	No dengue-specific CTL clones
5.		Limiting dilution of PBMC at 500 and 1000 cells/well at day 0 with IL-2 and stimulation with anti-CD3, IL-2 and allogeneic irradiated PBMC.	1	"

These observations prompted us to examine the acute PBMC to see if they proliferated in vitro to various stimuli

D. IMPAIRED CELL MEDIATED RESPONSES DURING ACUTE DENGUE INFECTION

The results in Chapter V demonstrate decreased in vitro proliferative responses of PBMC to mitogens and to several antigens during acute dengue infection. All the responses of PBMC from children with acute dengue infection were compared to the responses of PBMC obtained from the same patients 6-24 months after the infection. In all patients studied (Table II-4), in vitro proliferative responses were abrogated during acute dengue infection. Decreased proliferative responses to PHA and anti-CD3 were seen in patients with DF and in those with DHF, in patients with primary infections and those with secondary infections, and in those infected with any of the four dengue serotypes. Therefore, neither the severity of illness nor the serotype of virus that the person had been infected with appeared to influence the extent of unresponsiveness of PBMC to the different stimuli tested.

This study is the first report of impaired cell mediated responses of the PBMC of patients with acute dengue infection. Our results are consistent with observations in several other viral infections, such as measles, EBV and CMV (Carney and Hirsch, 1981, Hirsch, et al., 1984). Patients' PBMC samples obtained during acute illness from these

various other viral infections have shown similar unresponsiveness to stimulation with PHA, ConA and specific antigen.

A variety of mechanisms for virus-induced immunosuppression have been suggested including, 1) virus killing of infected lymphocytes, 2) production of suppressor T cells, 3) a shift towards a Th2 response by the release of Th2 cytokines such as IL-4 and a lack of a potent Th1 proinflammatory cytokine such as IL-12, 4) induction of apoptosis of T cells upon antigenic challenge in vitro, 5) impairment of monocytes by release of TNF and PGE₂, and, 6) impairment of dendritic cells leading to decreased IL-12 production (Fugier-Vivier, et al., 1997, Karp, et al., 1996, Nokta, et al., 1996, Rouse and Horohov, 1986). One or more of these factors could play a role in contributing to the immunosuppression seen.

1. Role of T cells and APC's in mediating unresponsiveness of PBMC

Changes in both T cells and antigen presenting cells have been implicated in mediating the suppression of in vitro proliferation. T lymphocytes from patients with HIV and EBV have been shown to undergo apoptosis in vitro upon stimulation of the TcR (Lauener, et al., 1995, Uehara, et al., 1992). In acute EBV infection, both CD4⁺ and CD8⁺ T cells were shown to die upon culture, and the dying cells were restricted to the activated (CD45RO⁺) population (Tamaru, et al., 1993). Death of the T cells could be prevented by the addition of recombinant IL-2. Similarly, in acute LCMV infection virus-

induced immunodeficiency was shown to be predominantly a result of T cells undergoing apoptosis (Razvi and Welsh, 1993). However, in this situation IL-2 appears to sensitize and accelerate apoptosis of memory T cells specific for nonviral antigens upon subsequent stimulation. These studies did not examine whether the accessory cell population contributed to the observed immunosuppression.

Since the background thymidine incorporation was lower in acute PBMC in all cases in our study, it could imply that the acute PBMC cultured in medium alone were more susceptible to apoptosis than normal PBMC. However, the addition of gamma-irradiated feeder cells, IL-2, IL-7 or antiCD28 antibodies could restore proliferation of the acute T cells, indicating that these cells could be rescued. Though we have not directly tested to see if acute T cells in dengue infection undergo apoptosis upon *in vitro* stimulation, our results suggest that the T cells can proliferate if provided with an adequate stimulus.

To test if one cell type predominantly accounts for the unresponsiveness of the PBMC, we separated T and non-T cells using magnetic beads and performed mixing experiments. The results demonstrate that the accessory cells in the acute PBMC sample are incapable of providing the signals to help the T cells proliferate, because both acute and control T cells are incapable of responding to PHA in the presence of irradiated acute non-T cells. However, the T cells in the acute PBMC samples are capable of responding to the appropriate signals, because proliferation increased substantially when irradiated

non-T cells from a control donor were added to the culture.

The defect in the accessory cell population in the acute PBMC is not exclusively an antigen processing defect because it was also observed with mitogens. The accessory cells in the acute PBMC sample either lack an adequate stimulus or are providing a negative signal that makes them incapable of triggering the activation of T cells from samples of acute or control PBMC. Incubation of acute PBMC with autologous immune PBMC did not decrease the proliferation of the immune PBMC (data not shown); therefore we believe that during acute dengue infection the accessory cells in the PBMC are unable to provide the necessary stimulus to activate the T cells. Although we cannot entirely exclude the possibility that a small proportion of the acute T cells are triggered into activation-induced cell death, our results indicate that the primary defect is in the antigen presenting cell population. Cell surface molecules on the accessory cells in the irradiated control non-T population may provide the necessary signals needed to trigger the activation of acute T cells. Accessory cells are necessary to help polyclonal activation of T cells with PHA by crosslinking the TcR and by providing second signals or soluble factors to induce activation.

Both cell contact dependent and soluble factors released from APC's have been shown to mediate unresponsiveness of T cells. Inhibition of proliferation of antigen specific T cells by human rhinoviruses was shown to be dependent on virion binding to ICAM-1 on monocytes (Gern, et al., 1996). Supernatants from CMV-infected monocyte

cultures were shown to inhibit mitogenic T cell proliferative responses through the TNF-dependent release of arachidonic acid and prostaglandin E2 (Nokta, et al., 1996). Recently, suppression of antigen specific proliferation by measles virus infection was shown to be mediated by a soluble factor which the authors speculate is an undescribed cytokine (Fujinami, et al., 1998, Sun, et al., 1998). However, Karp et al have shown that crosslinking of the measles virus receptor CD46 inhibited monocyte production of IL-12, which is known to be important for the generation of cell mediated responses (Karp, et al., 1996). Salonen et al suggested that early inhibition of proliferation is due to the release of IFN- α from monocytes and subsequent cell death of the mitogen stimulated cells (Salonen, et al., 1989). All of these studies analyzed the unresponsiveness of T cells by infecting monocytes or PBMC with the respective viruses in vitro, and then examining the effect of these infected monocytes or PBMC on T cell proliferation. How this relates to the observed suppression seen during or following in vivo infection with any of these viruses is still in question. Analysis of patients' PBMC samples obtained during an acute viral illness has not yet been done in as great detail as the described in vitro studies in those systems. Demonstration of altered plasma levels of IL-12, TNF, PGE2 in patients with acute measles or CMV compared to healthy or immune controls would complement the in vitro studies and provide more direct evidence that these cytokines are important mediators of immune suppression in infected individuals.

Our rationale for using the transwell system was to see if gamma irradiated autologous or allogeneic PBMC would restore proliferation of the T cells in the acute

samples of PBMC by the release of soluble factors. If this was the case, the acute cells should have been able to proliferate when separated by a membrane from the irradiated allogeneic PBMC. Since the patients' acute cells had significantly lower proliferation when separated by the polycarbonate membrane, our results suggest that the initial triggering between acute PBMC and gamma-irradiated control PBMC requires cell-cell contact. It is possible that soluble growth factors are produced by the acute T cells as a result of this initial cell contact dependent stimulation, which can then act to restore proliferation. This may explain why cytokines such as IL-2 or IL-7, which are produced downstream of signaling events, are able to increase the proliferative responses in the acute cells. The inability of the PBMC from one patient to respond to even high doses of IL-2 may reflect a more severe state of immunosuppression and this patient did exhibit more severe disease manifestations (DHF grade 2). Since we had a limited number of PBMC from this patient it was not possible to further characterize this defect.

The absolute monocyte and absolute neutrophil counts are transiently decreased in patients with dengue illness compared to children with other febrile illnesses (Kalayanarooj, et al., 1997). The *in vitro* T cell responses we have observed are significantly depressed for at least two weeks after the appearance of fever in these patients. The reduction in monocytes could result in fewer total costimulatory ligands, such as B7-1 and B7-2, and adhesion molecules available to activate the T cells in the acute samples. The results suggest that one of the reasons for the decrease in cell-

mediated responses in children with acute dengue infection may be a decrease in the expression of costimulatory molecules on the antigen presenting cells as a result of the decreased number of monocytes seen during acute dengue infection.

Depletion of monocytes from PBMC of control donors decreases the anti-CD3 response but not the PHA response, whereas depletion of B cells does not affect the proliferation of PBMC (data not shown). This suggests that decreases in the monocyte counts in the acute PBMC of the dengue patients do not solely account for the marked *in vitro* suppression observed. Infection of monocytes, which are the main target of dengue infection, may cause a series of abnormalities and result in the inability of T cells to respond to most stimuli *in vitro*.

Plasma levels of IL-10 are increased in patients with DF and DHF (S. Green et al manuscript submitted). IL-10 is a cytokine with known immunosuppressive properties (Schols and De Clercq, 1996, Taga and Tosato, 1992). It downregulates monocyte production of several costimulatory molecules, including B7-1 and B7-2, which are important for the activation of T cells. Groux et al showed that IL-10 suppresses proliferation and induces alloantigen-specific unresponsiveness of human CD8⁺ T cells (Groux, et al., 1998). This effect was due to the downregulation of costimulatory molecules on the monocytes. It is possible that increased levels of IL-10 in dengue patients *in vivo* alters the expression of costimulatory molecules on monocytes, making them incapable of activating T cells in the presence of mitogens.

Addition of anti-CD28 antibodies did not induce T cell proliferation of the acute samples of PBMC obtained on study days 2 or 3, when patients are typically viremic and have their most severe symptoms of disease. When the patients were recovering from their acute illness and the monocyte levels were slowly returning back to normal levels, the anti-CD28 antibodies improved proliferation of acute PBMC samples (Figure IV-4). It is possible that the addition of costimulatory molecules at the earlier stage of illness cannot trigger sufficient production of IL-2 for T cell proliferation and therefore had no effect on the proliferation in the acute PBMC samples. Previous studies of patients with measles and CMV have not examined the effect of antiCD28 antibodies on acute T cell proliferation. It is interesting to speculate that in acute dengue infection the lower density of surface B7 molecules might preferentially trigger the high affinity receptor CTLA-4 (instead of the low affinity receptor CD28), which has been shown to provide inhibitory signals and prevent proliferation. This would provide one explanation why in vitro proliferation is abrogated in acute dengue infection. Other interactions between T cells and APC's, like CD40/CD40L and LFA-3/ICAM-1, may also be important to generate a good proliferative response.

The receptors for several cytokines, e.g. IL-2, IL-4, IL-7, IL-13 and IL-15, have common motifs and share the γ_c chain, resulting in cytokine redundancy (Lin, et al., 1995). IL-2, IL-4, IL-7, IL-9 and IL-15 can all act as T cell growth factors and therefore might be able to help restore proliferation of the unresponsive acute T cells. Purified CD4 T cells that had been anergized by ligation of gp120 with CD4 were unable to respond to

anti TcR antibodies (Selliah and Finkel, 1998). Addition of IL-2, IL-4 or IL-7 restored proliferation of these cells whereas cytokines that bound to receptors without the γ_c chain (IL-6 and IL-12) could not. The cytokines that share a common γ_c activate the Jak family tyrosine kinases Jak1 and Jak3. IL-2 and IL-7 induce different Stat proteins (Stat 3 and Stat 5) compared to IL-4 and IL-13 in preactivated peripheral blood lymphocytes even though they all activate the same Jak kinases (Lin, et al., 1995). Our data indicate that IL-2 or IL-7, but not IL-4, can restore proliferation of PBMC obtained during acute dengue infection. Therefore, although these cytokines share common receptor motifs, there appears to be selectivity in the ability of the acute cells to respond to them.

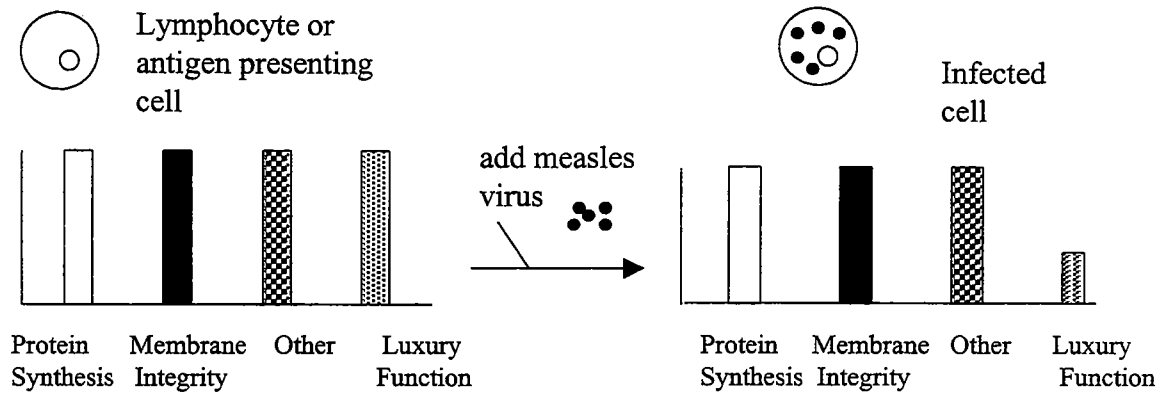
The analysis of the unresponsiveness of PBMC to in vitro stimuli during acute dengue infection in the present study poses several questions that might be addressed in the future. It will be interesting to examine which Stat proteins are induced in the PBMC following stimulation with CD3 and IL-2. We would like to see whether antiapoptotic genes, such as bcl_{XL} , are induced in the acute PBMC and if signaling through the Jak/Stat pathway is altered in these cells. We would also like to determine which population of antigen presenting cells is responsible for the alterations in T cell proliferation.

The suppression of in vitro proliferation seen in measles infection is associated with in vivo immune defects. The tuberculin skin test response is depressed during acute measles from prior to the appearance of the rash till 7-20 days afterwards (Tamashiro, et al., 1987). Children with measles are more susceptible to secondary infections, which

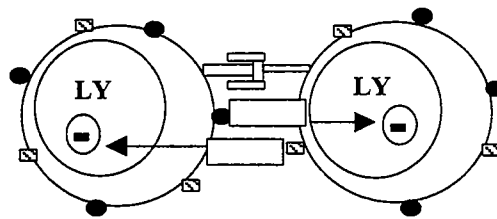
contributes to the nearly one million measles-related deaths worldwide each year (Borrow and Oldstone, 1995). There are no published studies on the susceptibility to secondary infections after acute dengue virus infections or on DTH responses in patients with acute dengue. Unlike measles, dengue virus infections do not involve mucosal surfaces. However, studies of the *in vivo* implications of these *in vitro* observations may be warranted. It would be of interest to perform *in vivo* testing of delayed type hypersensitivity of dengue patients to an antigen such as PPD.

There are theories concerning the mechanism of measles virus-induced immunosuppression (Figure VI-3) (Borrow and Oldstone, 1995, Oldstone, 1996). One idea put forth is that measles virus infection selectively interferes with the cells' ability to make product(s) necessary for their differentiated function i.e. their roles in immune responses (A), because there is no interference with protein synthesis and no direct cell lysis of measles infected lymphocytes. A second hypothetical mechanism by which immunosuppression may occur is via cell-cell interactions mediated by measles virus glycoproteins. The interaction of a lymphocyte (LY) with the measles virus hemagglutinin (H) and fusion (F) proteins on infected cells delivers a transmembrane signal (arrows) that arrests the proliferation of the responding cell. Adhesion molecules may facilitate suppression by enhancing cell-cell interactions. This suppressive activity can occur only between infected cells (B), or an uninfected lymphocyte could be affected by an infected APC (C). The infected APC could be a monocyte/macrophage, dendritic cell or B cell as indicated by several reports. This second hypothesis is attractive because

A)



B)



C)

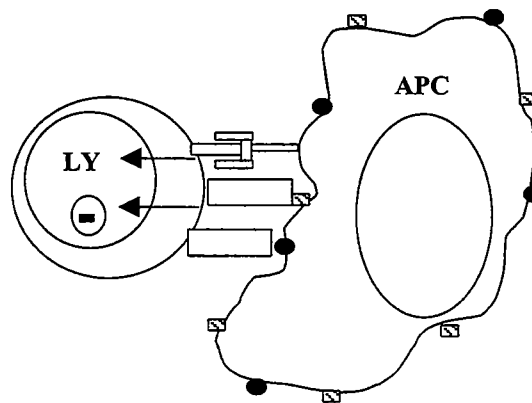


Figure VI-3. Hypothetical mechanisms by which measles virus-induced suppression occur (From Borrow et al *Curr.Top.Microbiol.Immunol.* 191:85-100)

● Virus hemagglutinin protein H, ◻ virus fusion F protein, — cell adhesion molecules
 APC = antigen presenting cell, LY = lymphocyte, ⊖ = negative signal

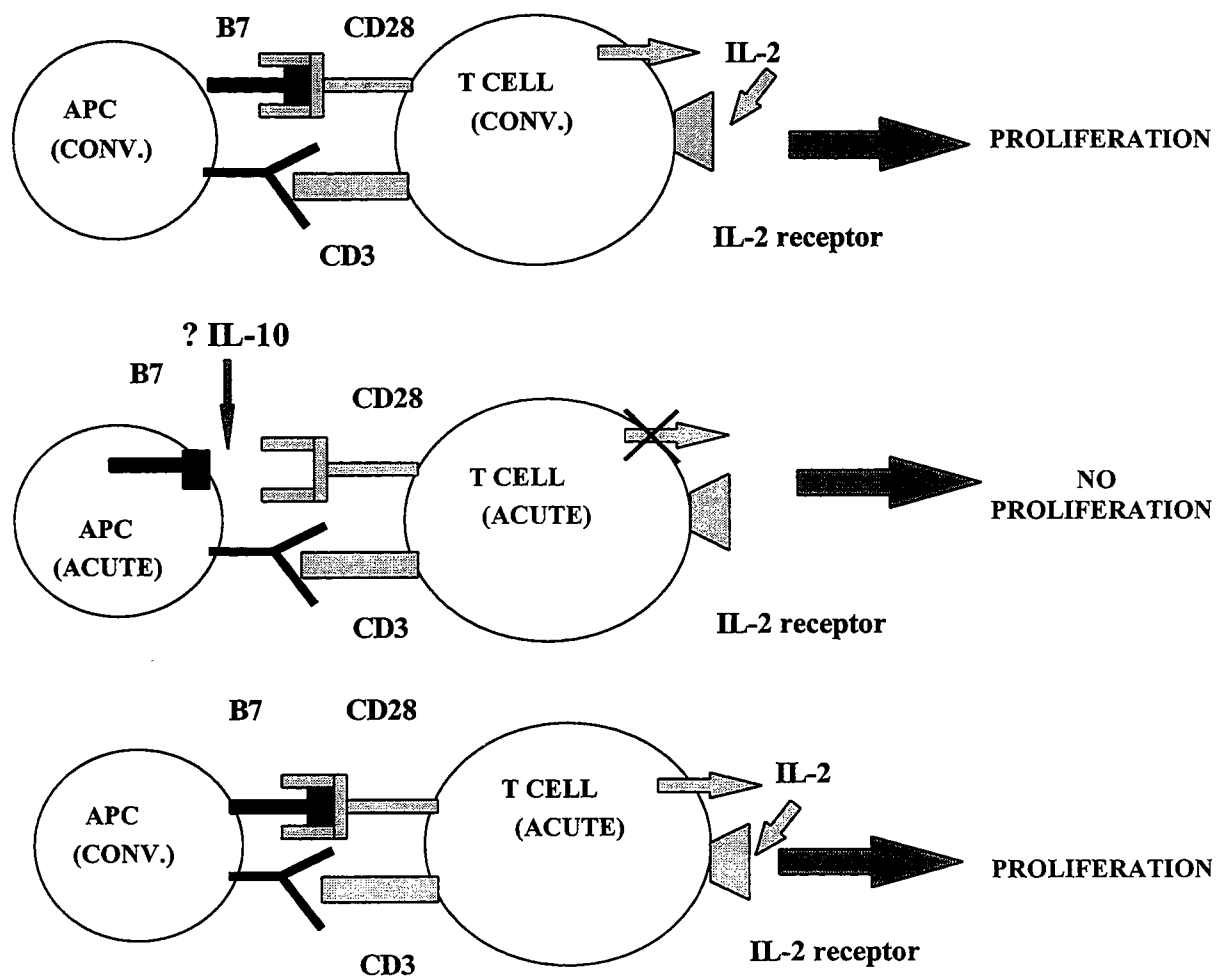
a relatively small number of cells infected by measles virus may have a far-reaching effect on the immune response mounted by a large population of uninfected cells.

In light of the data that exist in several other viral systems of acute viral immunosuppression, our results indicate that generation of immunosuppressive T cells or suppressor macrophages is not likely to be the mechanism by which T cell responses are abrogated in acute dengue infection. If this were the case, we would have expected reduced proliferative T cell responses of convalescent healthy PBMC when they were incubated with acute PBMC from patients with dengue infection. Instead, our data point to inadequate stimulation of the T cells by antigen presenting cells. This defect is global because it affects T cell responses to both specific antigens and mitogens. It is not primarily an antigen processing defect, since even responses to PHA were reduced in patients' PBMC samples. We have not established if a particular subset of accessory cells is responsible for this immunological unresponsiveness.

We hypothesize that the increased levels of IL-10 in patients with DF and DHF alter APC's by decreasing expression of costimulatory molecules and other adhesion molecules on their surface (Figure VI-4). Acute APC's therefore cannot provide adequate secondary stimulation to the T cells in order to induce proliferation. Although we have not specifically examined T cells in acute dengue infection to see if they are undergoing apoptosis, we believe that the majority of T cells are functional because when they are provided with the right signals (from gamma-irradiated autologous convalescent or

allogeneic PBMC, anti-CD28 antibodies, IL-2 and IL-7) they will proliferate. The results suggest that there is a primary defect in the APC population in acute dengue infections.

Figure VI-4 MODEL: Acute APC's in dengue infection are unable to provide adequate stimulus to trigger activation of T cells



The results presented in this thesis examine T cell responses during acute dengue illness and CD8+ memory CTL responses following dengue virus infection. The major conclusions of this thesis are:

1. CD8+ CTL responses can be detected in most volunteers who have received a monovalent dengue vaccine. Nonstructural proteins, in particular NS3, are dominant targets for recognition by CTLs. There is no immunodominant epitope on NS3.
2. Dengue-specific memory CTL responses are detected in Thai children following natural secondary dengue infection. Nonstructural proteins are immunodominant targets for CTLs, consistent with our data on healthy dengue vaccine recipients. The majority of T cell lines established from these patients are crossreactive with the other serotypes of dengue virus.
3. During acute dengue infection, in vitro T cell proliferative responses are abrogated. These responses can be restored by the addition of IL-2, IL-7, irradiated autologous convalescent or allogeneic PBMC, and, in some cases, by anti-CD28 antibodies. The primary defect appears to be in the antigen presenting cell population. This suppression of acute PBMC observed in vitro may explain our inability to isolate dengue-specific CTLs in the same samples.

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