QUANTITATION OF T LYMPHOCYTE SUBSETS HELPS TO DISTINGUISH DENGUE HEMORRHAGIC FEVER FROM CLASSIC DENGUE FEVER DURING THE ACUTE FEBRILE STAGE

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Abstract. Activation of immunoregulatory T lymphocyte subsets has been observed in dengue viral infection, being more evident in dengue hemorrhagic fever (DHF) than in classical dengue fever (DF). There are, however, as yet no well-defined host markers to determine which patients with dengue viral infection will develop severe complications during the acute febrile stage of the disease. A study was performed to compare the cellular immune status in DHF, DF and non-dengue viral infections (NDF) in order to determine the value of these parameters in distinguishing DHF from classic DF and other viral infections during the acute febrile stage of the disease. This study involved 109 febrile patients admitted because of suspected DHF. Fifty patients were serologically confirmed cases of dengue infection, of which 25 had grade 1 or 2 DHF.

There was a reduction in total T (CD3), CD4 and CD8 cells in DHF and demonstrated that a low level of CD3, CD4, CD8 and CD5 cells discriminated DHF from DF patients during the febrile stage of the illness. In contrast, B (CD19) cells and natural killer (NK) cells did not appear to be discriminatory in this study. Receiver operating characteristic (ROC) curve analysis showed that a combination of CD3 cell of ≤45% and CD5 cell of ≤55% was the best marker to identify DHF patients (sensitivity = 84% and specificity = 52% for CD3 cell of ≤45%; sensitivity = 92% and specificity = 71% for CD5 cell of ≤55%). CD4 cell of ≤25% and CD8 cell ≤30% were equally good in discriminating DHF from DF patients. On the other hand, the ROC curves indicated no clear difference between the immunoregulatory cell counts in DF from NDF. Lymphopenia, atypical lymphocytosis and thrombocytopenia were significantly more evident in dengue compared to non-dengue infection but did not appear to be discriminatory among DHF and DF patients. The reduction in CD3, CD4, CD8, CD5 cells correlated with the degree of thrombocytopenia in DHF ($p < 0.05$) which suggests that these cells probably participate in a common pathogenetic mechanism.

INTRODUCTION

Outbreaks of dengue infection pose a serious threat to more than 85 tropical and sub-tropical countries throughout the world (Lam, 1993). Annually 500,000 cases occur worldwide and about 1.8 billion people are estimated to be at risk of dengue infection (Leduc, 1994). Diagnosis of dengue infection in the early febrile phase may be difficult because the nonspecific constitutional symptoms mimic a wide spectrum of viral or bacterial infections and specific dengue serological tests may yield negative results. Dengue virus infection presents as two clinical syndromes: classic dengue fever (DF) and dengue hemorrhagic fever (DHF) (Halstead, 1980). Classic DF is a self-limited febrile disease and is the most common type of dengue illness. Some patients with dengue infection leak plasma into the interstitial space resulting in hypovolemia and sometimes circulatory collapse. This severe syndrome, which is always accompanied by thrombocytopenia and sometimes frank hemorrhage or shock, is termed DHF (Halstead, 1980). The case fatality rate of DHF remains significantly high, around 40-50%, without prompt institution of treatment to combat impending shock (Nimannitya, 1993). Time is a critical factor in the management of DHF as early effective intervention is crucial in reducing morbidity and mortality.

The pathogenesis of DHF differs from classic DF. Plasma leakage differentiates DHF from DF, and also determines the severity of DHF. Previous studies have demonstrated the role of T lymphocytes in the pathogenesis of DHF and recovery from dengue virus infection (Kurane *et al*, 1989). It has been observed that activation of T lymphocytes is more evident in DHF than in DF, and may contribute to the plasma leakage and severe hemorrhage seen in DHF (Kurane *et al*, 1991). There are, however, as yet no well-defined host markers to deter-

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mine which patients with dengue viral infection will develop severe complications in the acute febrile stage of the disease.

A study was performed to compare the cellular immune status of dengue infection from other viral infections, in order to determine the value of these parameters in distinguishing early cases of DHF from classic DF, and also to attempt to predict which patients will develop severe complications during the acute febrile stage of the disease.

MATERIALS AND METHODS

Study patients

This study was conducted in 1995 and 1996 at the medical faculty of Universiti Kebangsaan Malaysia in Kuala Lumpur. We examined serial serum specimens from 109 febrile patients, ages 13-63 years of either sex, who were hospitalized because of suspected DHF. The study groups consisted of patients who had symptoms and signs similar to dengue viral infection with fever for at least 5 days. Fifty patients were serologically confirmed cases of dengue of which 25 had grade 1 or 2 DHF. Diagnosis of DHF were assigned to patients when the level of thrombocytopenia and signs of hemorrhage and plasma leakage met established criteria (Technical Advisory Committee, 1980). Patients were excluded from the study if they were pregnant, if they were infected with human immunodefiency virus (HIV), if they had underlying autoimmune or hematological disorders, if they were immunocompromised or if they had just received blood or blood products. A careful history was taken including the onset of fever, previous dengue infection, bleeding tendency, drug intake, and cigarette smoking. A detailed physical examination was performed to look for hemorrhagic manifestations (positive tourniquet test for capillary fragility, skin hemorrhages, epistaxis, gingival, gastrointestinal, or urinary tract hemorrhage), signs of plasma leakage (pleural or pericardial effusion, ascites), signs or circulatory failure (cold extremities, cyanosis, hypotension, tachycardia), hepatomegaly and other major organ involvement. When clinically indicated, chest radiography, electrocardiography and abdomen ultrasonography were performed. Upon hospital discharge, the WHO grade of dengue illness was determined by a review of the clinical record. Without knowledge of the immune status parameters studied [T (CD3) cells, B (CD19) cells, T helper (CD4) cells, T suppressor (CD8 cells, CD4/CD8 ratio and natural killer NK cells] the authors reviewed every patient's record and confirmed the assignment of WHO grade. Hospitalized cases of dengue infection that did not meet diagnostic criteria for DHF were classified as DF. Dengue viral infection was confirmed by detection of antiviral IgM.

Collection of samples and flow cytometric analysis

Three milliliter samples of heparinized venous whole blood were obtained in a vacuitable tube containing EDTA for full blood picture and enumeration of lymphocyte subsets. The samples were taken between 8-11 am on day 4 or 5 of fever onset and processed within 6 hours after blood taking.

The immune status parameters studied were CD3, CDI9, CD4, CD8, NK and CD5 cells and CD4/CD8 ratios. The immunophenotypes of the lymphocytes were determined by flow cytometry. The method of the test performed was the lysed whole blood technique of Becton Dickinson (Anonymous, 1988). An appropriate quantity of murine monoclonal antibodies against the different cell surface markers was mixed and incubated in separate tubes with 100 µl of blood at room temperature. The erythrocytes were lysed when the above mixture was incubated with FACS lysing solution. The stained leukocytes were washed with phosphate buffered saline (PBS) and fixed in 1% paraformaldehyde. The cells were subsequently analyzed using the Simulset Analysis software in the FACScan within 24 hours of preparation.

The total white cell count was determined by using a coulter counter (model JR) and absolute lymphocyte count was estimated from manual differential count of 100 white cells on the Wright-stained blood films.

Statistical analysis

The data obtained from the FACScan computer were expressed as a percentage of the total lymphocytes. All results were expressed as mean ± SD. Analysis of variance (ANOVA) was used for comparison of the values of the lymphocyte subsets and peripheral blood count between the three study populations If the differences between the values examined were significant, the Duncan Multiple Range test was performed to determine whether the difference between two means was significant. The values of the lymphocyte subsets from patients with DHF, DF or NDF were compared with those of healthy Malaysian adults. The reference range for the lymphocyte subsets in the peripheral blood of

healthy Malaysian adults is based on a previous study by Choong *et al* (1995). The correlation between the platelet count and lymphocyte subsets was assessed by Pearson correlation coefficient. Differences yielding p-values of ≤ 0.05 were regarded as significant.

The ability of a laboratory parameter to discriminate between two groups is a consequence of the relative position of the two distributions characterized by the mean and standard deviation, whereas comparison between means is consequence of the relative position of the two mean distributions (characterized by mean and standard error of the mean). A small difference between means could be detected by comparisons of means, if the sample sizes are sufficiently large, but this small difference will not be correctly classify patients into one group or the other. For these reasons, the receiver operating characteristic curve (ROC) was used, as it directly related to the discriminant ability of a laboratory marker expressed in terms of sensitivity and specificity. When a laboratory parameter was identically distributed in diseased and non-diseased patients, the ROC curve was reduced to the first diagonal of the Unit Square. The more the ROC curve approaches a 90° angle, the more the discriminant is the corresponding parameter (Metz, 1978).

RESULTS

Table 1 shows the age, sex and ethnic distribution among the 3 study groups (DHF, DF, non-dengue infection or NDF). Even though it appears that more male patients had DHF, this was not statistically significant. A previous study showed that the lymphocyte subsets were not significantly different between the sexes (Choong *et al*, 1995).

Peripheral blood profile in patient with DHF, DF and NDF

The mean leukocyte, neutrophil, lymphocyte and monocyte counts were decreased in DHF and DF patients but the difference in the means was not statistically significant. Atypical lymphocytosis was more evident in dengue infection than NDF ($p < 0.05$) (Table 2). The mean platelet count was lowest in DHF patients but it was not significantly different from DF patients. However, the platelet count was significantly reduced in dengue cases compared to non-dengue cases (Table 2). Severe thrombocytopenia (a platelet count of $< 20 \times 10^9/l$) was noted in nearly half of the DHF cases, 4% of DF cases and none of the NDF cases. There was a significant correlation between the platelet count and CD3, CD4, CD8 and CD5 cell counts in DHF patients ($p < 0.05$).

Lymphocyte subset in patients with DHF, DF and NDF

T (CD3) cells: The mean percentage of D3 cells was significantly lower in DHF than in DF and NDF patients ($p < 0.05$) (Table 3). When compared with healthy adult populations, reduced CD3 was observed in 92% of DHF and 52% of DF patients. The ROC curve indicates that CD3 was able to distinguish DHF from DF patients with a sensitivity of 84% and specificity of 52% at a cut off value of 45% or less. A CD3 count of 50% was also able to discriminate DHF from NDF with a sensitivity of 88% and specificity of 78%.

B (CD19) cells: The mean percentage of CD19 cells was significantly lower in DHF than in DF and NDF ($p < 0.05$) (Table 3) . When compared with healthy adult populations, reduced %CD19 was observed in approximately half of the DHF patients, while ma-

Table 1
Age, sex and ethnic distribution of the study patients.

Study patients	No.	Average age ± SD (Range)	Sex		Ethic		
			Male	Female	Malay	Chinese	Indian
DHF	25	28 ± 9.1 (16-50)	18	7	14	9	2
DF	25	26 ± 9.3 (16-48)	13	12	16	7	2
NDF	59	27 ± 9.4 (13-63)	35	24	44	13	2

DF = Classic dengue fever, DHF = dengue hemorrhagic fever, NDF = non dengue fever

Table 2
Peripheral blood profile among the 3 study groups.

Parameters	DHF	DF	NDF
Leukocyte ($x10^9$/l)	3.88 ± 0.4	3.40 ± 0.3	6.97 ± 1.4
Neutrophil ($x10^9$/l)	2.16 ± 0.3	1.77 ± 0.3	2.77 ± 0.2
Lymphocyte ($x10^9$/l)	1.15 ± 0.1	0.99 ± 0.1	* 2.03 ± 0.15
Atypical lymphocyte ($x10^9$/l)	0.76 ± 0.1	0.98 ± 0.1	* 0.37 ± 0.07
Monocyte ($x10^9$/l)	3.78 ± 0.5	5.28 ± 0.6	5.68 ± 0.4
Platelet ($x10^9$/l)	41.52 ± 5.7	72.48 ± 5.5	* 129.47 ± 9.3

All values were expressed as means ± SD (standard deviation)
* Indicates significant difference ($p < 0.05$)

Table 3
Mean plasma level of lymphocyte subsets among DHF, DF, NDF patients and healthy Malaysian adults.

Lymphocyte subsets (%)	DHF	DF	NDF	Healthy Malaysian adults
CD3 cell	* 22.28 ± 4.2	46.10 ± 3.8	59.67 ± 1.9	67.5 ± 8.5
CD19 cell	* 9.0 ± 2.2	17.96 ± 1.69	16.88 ± 1.2	12.4 ± 4.5
CD4 cell	* 14.52 ± 2.6	26.96 ± 1.4	33.23 ± 1.3	35.5 ± 7.8
CD8 cell	* 16.12 ± 2.9	34.96 ± 3.0	42.05 ± 1.4	36.8 ± 8.5
CD4/CD8 ratio	0.96 ± 0.1	0.87 ± 0.2	0.88 ± 0.3	1.1 ± 0.6
CD5 cell	* 24.40 ± 3.9	53.20 ± 4.0	61.27 ± 2.2	ND
NK cell	195.18 ± 57.432	101.29 ± 15.5	365.09 ± 32.6	17.9 ± 8.1

All values were expressed as means ± SD (standard deviation)
* Indicates significant difference ($p < 0.05$)

The lymphocyte subsets in the peripheral blood of healthy Malaysian adults is obtained from a study by Choong *et al*, 1995

NK = natural killer cells, ND = not done

jority of the DF patients had normal %CD19 count. The ROC curves indicated no clear difference in %CD19 among the different study groups.

CD4 cells: The mean percentage of CD4 cells was significantly lower in DHF than in DF and NDF ($p < 0.05$) (Table 3) . Seventy-two percent of DHF and 8% of DF patients had low CD4 counts. The ROC curve indicated that CD4 of ≤ 20% or 25% were both discriminatory in terms of distinguishing DHF from DF, with a sensitivity of 72% and specificity of 68% if the CD4 was ≤ 20%, and a sensitivity of 80% and specificity of 52% if the CD4 was ≤ 25%. A CD4 of ≤ 25% could also discriminate between DHF and NDF patients with a sensitivity of 80% and specificity of 80%.

CD8 cells: The mean percentage of CD8 cells was significantly lower in DHF than in DF and NDF ($p < 0.05$) (Table 3). When compared with the normal range, 72% of DHF and 8% of DF patients had reduced CD8 counts. A CD8 count of ≤ 30% was discriminatory in terms of distinguishing DHF from DF patients, with a sensitivity of 84% and specificity of 52%. A CD8 of ≤ 35% could also distinguish DHF from NDF patients with a sensitivity of 92% and specificity of 75%.

CD4:CD8 ratio: There was no significant difference in the mean CD4:CD8 ratio among the study patients. Reduced CD4:CD8 ratio was observed in only 12% and 8% of DHF and DF patients, respectively.

CD5 cells: The mean percentage of CD5 cells was significantly lower in DHF than in DF and NDF ($p < 0.05$) (Table 3). A CD5 count of ≤ 55% could discriminate between DHF and NDF patients with a sensitivity of 92% and specificity of 52%. The same CD5 count could discriminate DHF from NDF patients (sensitivity of 92% and specificity of 71%).

Natural killer (NK) cells: Although the mean percentage NK cells in DHF patients was not signifi-

cantly different from that in DF patients, nearly 50% of DHF patients had reduced NK cells % when compared with healthy adult populations. The ROC curves indicate that NK cell counts were unable to discriminate between DHF, DF and NDF patients.

DISCUSSION

Dengue viral infection continues to be a major public health problem in Malaysia with several hundred thousand of cases per year. The diagnosis of grade 1 or 2 dengue hemorrhagic fever (DHF) in the acute phase of the disease may be difficult because of the non-specific nature of the symptoms, and specific dengue serological tests may yield negative results during the early stage of the illness. Thus, all patients with hemorrhagic manifestations or thrombocytopenia are hospitalized for observation. Most of these patients recover completely without going into the shock syndrome. Based on a previous study (Cheong, personal communication), unnecessary admissions for presumed DHF occur in up to 40-50% of cases; these cases eventually turned out to be either classic DF or non-dengue viral infections. On the other hand complicated cases of DHF might be missed in the absence of a definitive disease marker that can identify potentially fatal dengue cases in the early febrile phase of the disease.

The distribution of the immunoregulatory cells in the peripheral blood has now been established as an important diagnostic tool in the management of diseases that involve alterations in lymphocyte subsets. Activation of immunoregulatory T lymphocyte subsets has been observed in dengue viral infection, being more evident in DHF than in DF. Lymphokines (IL-2 and IFN-gamma) and soluble cell surface proteins (sIL-2, sCD4, and SCD8) released from activated T lymphocytes in patients with DHF have been shown to be associated with the hemorrhage and plasma leakage that are pathognomonic features of DHF (Kurane *et al*, 1991). Since the kinetics of lymphocyte subsets occur early during the course of the illness when plasma leakage is minimal and the specific dengue serology may still be negative, lymphocyte subset quantitation can be a useful tool to detect potentially complicated cases so that early, appropriate therapy can be administered in order to prevent morbidity and mortality from DHF. On the other hand, exclusion of benign dengue cases earlier in the course of the disease would minimize health care costs from unnecessary or prolonged hospitalization, repeated investigations and inappropriate therapy. Since a bench top flow cytometer can quantitate immunoregulatory cells in the peripheral blood within 2 hours after blood sampling, patients can be observed in a day care center rather than as inpatients while waiting for the results.

This study was conducted to determine the differences in the cellular immune response in DHF, DF and NDF. It was postulated that the immunoregulatory cell counts were of value in: (1) distinguishing dengue from non-dengue viral infections during the acute febrile stage of the disease when a confirmatory dengue serology test might not yet be available, (2) discriminating benign or classic DF from potentially complicated grade 1 or 2 DHF which usually share similar symptoms and signs at presentation, and (3) predicting the clinical severity and outcome among DHF cases earlier by determining its relationship with the degree of thrombocytopenia and plasma leakage.

This study showed that the immunoregulatory lymphocyte subsets were significantly different between the study patients during the acute febrile stage of the disease. The T (CD3), B (CD19), CD4, CD8 and CD5 cells were significantly reduced in DHF patients compared to the other groups of patients. In addition, DF patients had significantly lower CD3, CD19, CD4, CD8, CD5 and NK cells than NDF patients. These findings indicate that lymphocyte depletion occurs in dengue viral infection and the level of depletion is greater in DHF than DF patients, suggesting its major role in the pathogenesis of DHF in support of the immune enhancement hypothesis (Halstead *et al*, 1970). It is interesting that in this study more males had DHF ($p > 0.05$), as it may suggest that dengue infection tend to be more aggressive among male patients.

In order to define threshold values that discriminate between DHF, DF and NDF patients, ROC curves were established for the lymphocyte subsets for each of the 3 groups of patients. The ROC curves showed that CD3, CD4, CD8 and CD5 cell counts were able to discriminate DHF from both DF and NDF patients. Low levels of CD3, CD4, CD8 and CD5 cells discriminated DHF from DF at levels of 45%, 25%, 30% and 55% respectively. On the contrary, NK and CD19 cells were non-discriminatory.

In the present study, both CD4 and CD8 cells were significantly markedly reduced during the acute febrile stage in DHF patients. The peak decrement of these cells was obtained during the febrile stage in one study (Sarasombath *et al*, 1988). In the same study, the number of CD4 cells was greater than that of CD8 cells during the febrile stage, which

gave a CD4/CD8 ratio of greater than 1.0. The peak of the reversed ratio was on day 2 after the onset of shock, and it returned to normal thereafter. In contrast to this observation, the mean CD4/CD8 ratio in all the DHF patients in the present study was less than 1.0 as the number of CD4 cells was lower than CD8 cells. The depletion of CD4 cells is possibly due to their involvement in the immune elimination mechanism in response to dengue viral infection. Based on animal studies, the dengue virus induces a calcium-dependant lymphokine called cytotoxic factor (CF) with proteinase-like activity (Khanna *et al*, 1990). CF kills macrophages, CD4 cells and megakaryocytes and increases capillary permeability. Such a lymphokine may be responsible for the thrombocytopenia, CD4 cell depletion and plasma leakage in DHF/dengue shock syndrome (DSS). CD8 cells lyse dengue virus-infected monocytes and in the process, themselves become lysed. Proliferating CD4 and CD8 cells produce interferon-gamma that will augment this process (Kurane *et al*, 1988, 1989, 1992). The depletion in both CD4 and CD8 cells contributes to the decrement in total T cells seen in DHF. Fig 1 illustrates the possible pathogenetic mechanism of the T lymphocyte subset depletion and thrombocytopenia and their relationship in DHF based on findings from previous studies (Kurane *et al*, 1988, 1998, 1992; Khanna *et al*, 1990) and the present study.

In contrast to other studies (Sarasombath *et al*, 1988; Cornain *et al*, 1987), NK cells in DHF patients in the present study were not decreased. The differences in the observations between other studies and the present might be due to several factors, including genetic variation and differences in dietary patterns. Most studies done elsewhere concerned children. In this study, CD5 cells were significantly lower in DHF than DF or NDF patients. The low CD5 cells in DHF are expected as the CD5 marker is expressed on T cells. Therefore a reduction in total T cells will be accompanied by a similar reduction in CD5 cells. The possible participation of CD5 cells in the pathogenesis of DHF calls for establishment of a normal reference range in this country. Previous studies had not included these cells in analyzing the cellular immune response to dengue viral infection. The results of this study showed that the peripheral blood profile is a useful additional diagnostic tool in distinguishing dengue viral from non-dengue viral infection but of less value in distinguishing DHF from DF patients. The prominent leukopenia in dengue infection is mainly due to both the early lymphopenia and neutropenia, while in non-dengue infection the leukopenia is relatively milder because of the relative lymphocytosis which is commonly noted in many acute viral diseases (Carpenter and Sutton, 1995). In agreement with the observation made by Boonpucknavig *et al* (1979) and Thisyakorn *et al* (1984), the present study also noted that atypical lymphocytes were significantly increased in dengue infection. The mean monocyte counts were lower in DHF compared to DF or NDF. Monocytes have been identified as cells that can support dengue viral infection, and the number of infected monocytes has been shown to correlate with the severity of dengue infection (Kurane *et al*, 1991). The constant finding of a sudden drop in the platelet count preceding the onset of shock is unique to DHF. Severe thrombocytopenia (platelet count of less than $20x10^9/l$) was noted in almost half of our DHF patients, a minority of the DF patients and none of the NDF patients. Bleeding severity correlates with the grade of DHF (Mitrakul *et al*, 1987). In this study hemorrhagic manifestations, which were mostly mild were noted in 76% (19/25) of DHF patients and 84% (16/19) of them had platelet count of $\leq 50 \times 10^9/l$. There was a significant correlation between CD3, CD4, CD8 and CD5 cell counts

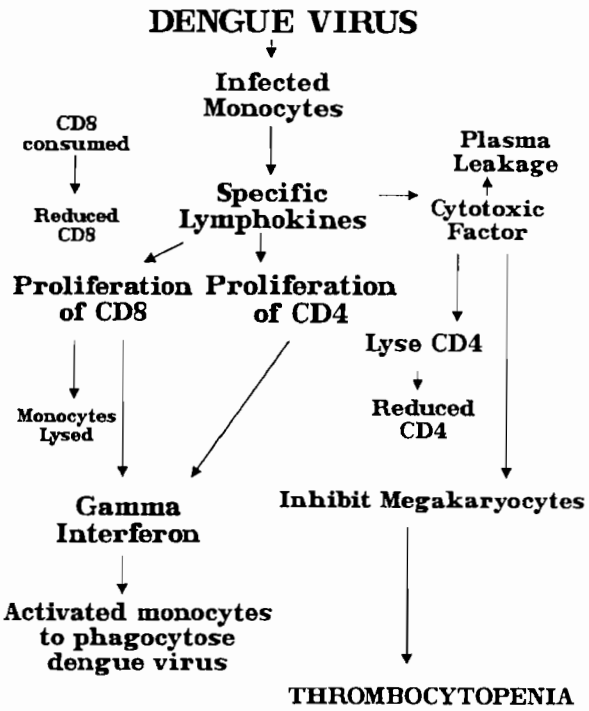

Fig 1–The possible pathogenetic mechanism of the CD4 and CD8 lymphocyte subsets depletion and thrombocytopenia in DHF.

and platelet counts in DHF. This finding suggest that T lymphocyte subsets might be involved in the mechanism of thrombocytopenia in DHF. Animal experiments have shown that dengue virus induces the release of CF (Khanna *et al*, 1990). As CF kills both CD4 cells and megakaryocytes, this could be one of the possible explanations for CD4 cell depletion and thrombocytopenia observed in the present study (Fig 1).

In this study, two of the DHF patients developed bilateral transudative pleural effusion while one patient had transudative ascites due to plasma leakage. The serum albumin and total protein were within normal limits in these patients. Early institution of treatment contributes to the low incidence of plasma leakage among our DHF patients. DHF patients with clinical evidence of plasma leakage had a lower CD3, CD4, CD8 and CD5 cell counts (data not shown), however we were unable to demonstrate the significance in the difference noted as the number of patients was too small. It would be interesting to compare the T lymphocyte subsets in DHF patients with plasma leakage to those without plasma leakage. It would also be desirable to serially evaluate the levels of the lymphocyte subsets early in the course of the disease until the recovery period in order to identify markers that could determine which patients with grade 1 or 2 DHF will progress to dengue shock syndrome.

In conclusion, our data suggest that CD3, CD4, CD8 and CD5 cells are markers of the aggressiveness of dengue viral infection and can be used to differentiate DHF from DF patients in the early stage of the disease. Of these immunoregulatory cells, both CD3 of $\leq 45\%$ and CD5 of $\leq 55\%$ were the markers that most clearly distinguished DHF from DF patients. Therefore quantitation of T lymphocyte subsets is useful in the management of dengue viral infection. This study has also enhanced our understanding in the pathogenesis of DHF and the possible mechanism that could lead to DSS. The admission and management policies could be revised so that only DHF cases are admitted and closer observation and prompt treatment would be given to potentially complicated cases.

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