

Characterization of Atypical Lymphocytes and Immunophenotypes of Lymphocytes in Patients with Dengue Virus Infection

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SUMMARY To characterize the immunophenotypes of lymphocytes in patients with dengue infection, we performed flow cytometric analysis of peripheral blood mononuclear cells collected from 49 dengue hemorrhagic fever (DHF), 25 dengue fever (DF), and 26 dengue-like syndrome (DLS) cases. The mean total atypical lymphocytes in DHF (916.1 ± 685.6 cells/ μ l) and DF (876.2 ± 801.9 cells/ μ l) were higher than those of DLS (310.5 ± 181.4 cells/ μ l). An atypical lymphocyte count of 10% or higher was a good indicator of dengue infection (sensitivity 50% and specificity 86%). Flow cytometric studies showed that the percentages of atypical lymphocytes correlated with those of CD19⁺ B lymphocytes and inversely correlated with the percentages of CD69⁺ lymphocytes. The mean absolute counts of atypical lymphocytes and CD19⁺ cells on the discharge day were significantly higher than those on the admission day. Low percentages of TdT⁺ cells were found in all groups of patients. We concluded that atypical lymphocyte and CD19⁺ cell counts may be a useful diagnostic tool for dengue infection and the recovery from the disease could be judged when numbers of both cell types are significantly elevated.

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are increasingly important public health problems in tropical and subtropical regions. It has been estimated that 2.5 billion people live in areas of risk to the infection and tens of millions of cases occur each year worldwide.^{1,2} Clinical manifestations of dengue virus infection range in severity from simple febrile illness (dengue fever; DF) to hemorrhagic fever that can be complicated with shock (dengue shock syndrome; DSS). The fatality rates vary from 0.5% to 3.5% in Asian countries. There is no specific treatment or vaccine currently available for this infection. The most challenging problem associated with the patient management is early and rapid diagnosis.¹⁻⁴ The value of the presence of atypical lymphocytes in flow cytometric analysis can be used as a presumptive diagnostic tool.

Clinical diagnosis is often difficult and can usually be confirmed only retrospectively on the basis of serology. The examination of the buffy coat preparation for the high percentages of atypical lymphocytes is an early and rapid diagnostic aid in the secondary infection.^{5,6} This examination can be done by using automated white cell differential counters.⁵ Changes in the atypical lymphocytes counts as the infection progresses could be useful markers of the disease activity. Descriptive study of the changes in the numbers of platelets, basophils and "large un-

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stained cells" (LUC) using an automated white blood cell counter and manual atypical lymphocyte counting was thus retrospectively carried out with serological secondary dengue fever confirmed cases. It has been found that the basophil, LUC and atypical lymphocyte counts rose in tandem with the drop in platelets and decreased when the platelets recovered.⁷

In a previous study, atypical lymphocytes in the peripheral blood of dengue cases were immunophenotyped by the alkaline phosphatase-anti-alkaline phosphatase method (APAAP).⁷ However, the origin of the atypical lymphocytes in dengue infection is still undefined. B cell ontogeny is suggested as the origin, contrary to the general expectation that T cells may be the source of atypical lymphocytes due to the presence of T cell activation and proliferation seen in most viral infections. The previous immunophenotyping methods used, however, may not have allowed restriction of phenotype identification to the atypical lymphocytes.⁸

Flow cytometry has been used to study immune activation in dengue hemorrhagic fever (DHF) by T cell surface activation marker detection in blood. Absolute CD4⁺ T cell, CD8⁺ T cell, natural killer (NK) cell, and $\gamma\delta$ T cell counts were decreased in children with DHF compared with those with dengue fever (DF) during early course of illness.^{8,9} These data demonstrate that cellular immune activation is present in early acute dengue infection and related to the disease severity.⁹ The percentages of CD8⁺ T cells and NK cells expressing CD69 were higher in children who developed DHF than those with DF.¹⁰ There was a reduction in total CD3⁺ cells and CD4⁺ and CD8⁺ T cells in DHF. The study demonstrated that a low level of CD3⁺, CD4⁺, CD8⁺ and CD5⁺ cells discriminated DHF from DF patients during the febrile stage contrary to B (CD19⁺) cells and NK cells.¹¹

In this study, monoclonal antibodies against CD69, CD87, and cytoplasmic TdT were used to examine their possible application in the early detection of dengue virus infection by flow cytometry. In order to distinguish early dengue hemorrhagic fever (DHF) from dengue-like syndrome (DLS) and to predict the severity of disease, the cellular immune status of dengue infection was evaluated. CD4, CD8,

CD19 and CD69, the markers of severity for dengue viral infection,^{10,11} were used to differentiate DHF from DF and DLS patients in the early stage of the disease.

MATERIALS AND METHODS

Blood sample collection

One hundred blood samples were collected from in-patients clinically suspected of dengue infection from the Hospital for Tropical Diseases, Ramathibodi Hospital, and B.M.A. Medical College and Vajira Hospital, Thailand, during November 2002 to October 2003. The WHO diagnostic criteria were used to classify the dengue-infected patients.¹² Blood samples were collected twice from each patient in tubes containing potassium ethylene diamine tetraacetic acid (K₃EDTA) on the days of hospital admission and discharge. All samples were tested by enzyme-linked immunosorbent assay (ELISA) for anti-dengue virus IgM and IgG antibodies. Complete blood count (CBC) and flow cytometry were performed within 24 hours after the blood was drawn.

The study protocol was approved by three Ethics Committees including the Faculty of Tropical Medicine and the Faculty of Medicine Ramathibodi Hospital, Mahidol University, and the Bangkok Metropolitan Administration.

ELISA

Plasma samples from each patient were tested for anti-dengue virus IgM and IgG antibodies by capture ELISA.¹³ The ELISA results were used for diagnosis and classification of dengue infection. A ratio of anti-dengue IgM to IgG > 1.8 (if IgM > 40 units) was the criterion for primary dengue infection and a ratio of IgM to IgG < 1.8 was considered as secondary infection.¹³

Complete blood count (CBC)

CBC was determined by automated white blood cell counter (Technicon H*3, Tarrytown, New York) and blood film stained with Giemsa was reviewed. Atypical lymphocytes were defined as large mononuclear cells having a fine homogeneous nu-

clear chromatin, some of which resemble blast cells, and a dark staining cytoplasm as seen in the Giemsa stain.

Flow cytometric studies

Whole blood (100 μ l) was dually stained with 5 μ l of R-phycoerythrin covalently linked to cyanin 5.1 (PECY5)-conjugated anti-human CD45, and 5 μ l of monoclonal antibody specific to other cell surface or intracellular markers. These include PE-conjugated anti-CD87 for mononuclear phagocyte and activated T cells staining, fluorescence isothiocyanate (FITC)-labeled anti-CD4 for T lymphocyte staining, FITC-labeled anti-CD8 for T and NK lymphocyte staining, FITC-labeled anti-CD19 for B lymphocyte stain, and FITC-labeled anti-TdT for mononuclear cell and blast population staining.

After incubation in the dark at 26°C for 15 minutes, 1 ml of diluted 1:10 FACS lysing solution (Becton Dickinson, San Jose, CA) was added and incubated in the dark at room temperature for 10 minutes. Samples were centrifuged at 500 \times g for 5 minutes, then the supernatant was discarded. The pellet was washed with 3 ml of 2% goat serum in PBS, pH 7.2. After centrifugation, the supernatant was removed. The cell pellet was resuspended with 1 ml of PBS and fixed with 100 μ l of 0.1% paraformaldehyde.

For intracellular staining, after the surface staining procedure, without resuspension in PBS and fixation in 1% paraformaldehyde, the cells were permeabilized with 500 μ l of FACS permeabilizing solution (Becton Dickinson, San Jose, CA) at 1:10 dilution, and incubated at 26°C in the dark for 10 minutes. The cell mixture was washed with 3 ml of PBS and centrifuged. After removal of the supernatant, 5 μ l of monoclonal antibody specific to TdT conjugated with FITC were added and incubated at 26°C in the dark for 10 minutes. The samples were centrifuged, the supernatant was discarded, and the pellet was resuspended in 1 ml of PBS and fixed in 100 μ l 1% paraformaldehyde.

Flow cytometry analysis

Samples were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) within 24 hours after

preparation. Cell Quest software, providing automatic acquisition, gating, and computation were used for this study. The lymphocyte population was identified based on forward and side light-scatter properties in combination with the expression of common leukocyte antigen (CD45). This gated population of lymphocyte cells was further analyzed for expression of various lymphocyte antigens.

Statistics

SPSS 10.0 for Windows was used for the statistical analysis. The correlation between the percentage of atypical lymphocyte count and lymphocyte subsets was assessed by Pearson correlation coefficient. *P* values lower than 0.05 were considered statistically significant. The data obtained from the FACScan computer were expressed as a percentage of total lymphocytes. All results were expressed as mean \pm SD. The receiver operating characteristic curve (ROC) was used to evaluate the ability of laboratory marker to discriminate distinct patient populations.

RESULTS

Subjects

The patients recruited in this study were 58 male and 42 female with age ranged between 2-28 years. According to the ELISA results, 26 negative cases were classified as dengue-like syndrome (DLS) and 74 positive cases were classified as dengue infection with 8 (10.81%) cases of primary infection and 66 (89.19%) cases of secondary infection. The 74 positive ELISA cases were clinically diagnosed as DHF (49 [66.22%]) and DF cases (25 [33.78%]), respectively. According to the WHO criteria, DHF cases were classified as DHF grades I, II, and III (25 [51.02%], 16 [32.65%] and 8 [16.33%], respectively) (Table 1). The average ages of the DHF, DF and DLS patients were 10, 9, and 10 years, respectively (Table 1). There were no statistically significant differences (*p* > 0.05) between patients with DHF, DF, and DLS related to age and duration of illness.

White blood cell and lymphocyte counting

The means of numbers of total WBC on admission day of DHF and DF (3,477.5 \pm 1,879.2 and 3,307.6 \pm 1,423.1 cells/ μ l) were significantly lower

Table 1 Demographic data of the studied groups

Variables	DHF (n = 49)	DF (n = 25)	DLS (n = 26)
Male/Female	28/21	19/6*	11/15
Age ^a	10 (3-19)	9 (2-15)	10 (3-28)
Days ill at study entry (Admission) ^b	4.6 ± 1.2	4.8 ± 1.4	4.8 ± 1.9
Days ill at study exit (Discharge) ^b	6.5 ± 1.4	7.1 ± 2.4	7.3 ± 3.1
Duration of admission ^b	4.2 ± 1.1	4.7 ± 1.6	5.1 ± 2.3
Serologic response			
Primary	1	7	-
Secondary	48	18	-
Grading severity			
Grade I	25	-	-
Grade II	16	-	-
Grade III	8	-	-

^aData shown as mean (range lower, upper limits)

^bData shown as mean ± S.D. (day[s])

*Significantly different from DLS at the 0.05 level, $p < 0.05$.

than on discharge day ($5,265.0 \pm 2,497.7$ and $5,179.0 \pm 2,766.7$ cells/ μ l, respectively; $p < 0.05$), but this was not observed in patients with DLS. The means of numbers of total WBC counts among these three groups were not significantly different, either on admission or discharge days. The means of total lymphocyte counts on admission day for DHF and DLS ($1,229.8 \pm 749.1$ and $1,083.7 \pm 640.1$ cells/ μ l) were significantly lower than that on discharge day ($2,558.6 \pm 1,701.4$ and $1,799.9 \pm 1,188.5$ cells/ μ l; respectively) ($p < 0.05$), but not significantly different between the three groups of the patients. On admission day, patients with dengue infection had lower absolute atypical lymphocyte counts (618.5 ± 748.2 and 420.8 ± 422.4 cells/ μ l) than on discharge day (916.1 ± 685.6 and 876.2 ± 801.9 cells/ μ l, respectively) ($p < 0.05$). The means of absolute counts of atypical lymphocytes on admission day and discharge day were higher in patients with DHF (618.5 ± 748.2 and 916.1 ± 685.6 cells/ μ l) than in those with DLS (236.2 ± 187.2 and 310.5 ± 181.4 cells/ μ l) ($p < 0.05$); patients with DF had higher atypical lymphocytes on discharge day (876.2 ± 801.9 cells/ μ l) than DLS (310.5 ± 181.4 cells/ μ l; $p < 0.05$), but there was no significant difference between those of DHF and DF. The mean percentages of atypical lymphocyte counts among total WBC on admission day and discharge day in DHF (13.3 ± 10.1 and 17.0

$\pm 8.3\%$) and DF (11.1 ± 9.3 and $14.9 \pm 9.3\%$) which were significantly higher than in DLS (5.9 ± 2.8 and $8.0 \pm 5.9\%$, respectively) ($p < 0.05$). The mean percentages of atypical lymphocyte counts among total lymphocytes on admission day and discharge day in DHF (36.4 ± 33.0 and $41.1 \pm 24.1\%$) and DF (30.5 ± 24.9 and $35.3 \pm 23.0\%$) were significantly higher than in DLS (16.2 ± 8.7 and $19.2 \pm 13.2\%$, respectively) ($p < 0.05$).

Correlation of white blood cell and lymphocyte counts in DHF, DF and DLS

At one day before defervescence (day -1), the means of absolute white blood cell counts in the DHF and DF were significantly higher than the mean of WBC counts in the DLS ($p < 0.05$) (Fig. 1). At the later time point, *i.e.* on one day after defervescence (day 1) the DLS had rising numbers of WBC counts and were significantly higher than the WBC counts in the DHF detected at the same time point. There was no difference in absolute lymphocyte counts between the DHF, DF and DLS patients on any day. At the day of defervescence (day 0), the mean absolute counts of atypical lymphocytes of the DHF and DF patients were significantly higher than those in the DLS patients. At the later time points, the atypical lymphocytes in the DHF on day 1 were

Table 2 Mean values of counts of total white blood cells (WBC), lymphocytes, atypical lymphocytes, and lymphocyte phenotypes expressed in percentages and absolute cell counts

Cell type	DHF (n = 49)		DF (n = 25)		DLS (n = 26)	
	Admission	Discharge	Admission	Discharge	Admission	Discharge
Total WBC (absolute count ^a)	3,477 ± 1,879	5,265 ± 2,497*	3,307 ± 1,423	5,179 ± 2,766*	3,925 ± 1,956	4,401 ± 1,759
Total lymphocytes						
% total WBC ^b	35.9 ± 13.6	48.9 ± 14.7	38.7 ± 13.9	43.8 ± 11.3	40.4 ± 16.8	42.3 ± 13.0
Absolute count ^a	1,229 ± 749	2,558 ± 1,701*	1,525 ± 1,049	2,358 ± 1,418	1,083 ± 640	1,799 ± 1,188*
Atypical lymphocytes						
% total WBC ^b	13.3 ± 10.1*	17.0 ± 8.3*	11.1 ± 9.3*	14.9 ± 9.3*	5.9 ± 2.8	8.0 ± 5.9
% total lymphocytes ^c	36.4 ± 33.0*	41.1 ± 24.1*	30.5 ± 24.9*	35.3 ± 23.0*	16.2 ± 8.7	19.2 ± 13.2
Absolute count ^a	618 ± 748*	916 ± 685***	420 ± 422	876 ± 801***	236 ± 187	310 ± 181
CD4⁺ T						
% total lymphocytes ^b	25.2 ± 13.3	20.9 ± 7.0*	25.1 ± 11.1	23.7 ± 8.7	27.7 ± 9.8	29.0 ± 13.5
Absolute count ^a	324 ± 293	523 ± 394**	309 ± 208	543 ± 365**	428 ± 320	538 ± 379
CD8⁺ T						
% total lymphocytes ^b	25.1 ± 8.4	29.8 ± 11.2	26.4 ± 13.0	32.7 ± 14.4	28.9 ± 9.2	27.1 ± 9.7
Absolute count ^a	349 ± 326	750 ± 549**	350 ± 302	847 ± 929**	438 ± 316	519 ± 330
CD19⁺ B						
% total lymphocytes ^b	22.3 ± 10.7*	23.9 ± 11.4*	18.9 ± 9.8*	20.3 ± 9.5*	14.2 ± 5.3	13.3 ± 7.0
Absolute count ^a	342 ± 390	615 ± 577***	251 ± 193	478 ± 369***	221 ± 190	246 ± 169
CD69⁺						
% total lymphocytes ^b	25.3 ± 12.6	18.5 ± 10.9	25.6 ± 14.7	23.4 ± 14.4	27.3 ± 14.9	16.7 ± 10.6
Absolute count ^a	272 ± 181	445 ± 363	292 ± 233	559 ± 573	363 ± 230	363 ± 338
CD87⁺						
% total lymphocytes ^b	1.0 ± 1.8	1.6 ± 3.1	1.3 ± 1.9	0.8 ± 2.0	1.8 ± 6.1	0.5 ± 1.4
Absolute count ^a	12 ± 20	46 ± 111	15 ± 23	48 ± 162	16 ± 38	12 ± 28
TdT⁺						
% total lymphocytes ^b	0.9 ± 1.6	0.5 ± 0.6	2.9 ± 11.4	0.4 ± 0.3	4.8 ± 19.4	3.8 ± 10.9
Absolute count ^a	10 ± 17	14 ± 22	50 ± 208	10 ± 11	36 ± 119	65 ± 175

^aResults expressed as mean of absolute counts (cells/ μ l) \pm SD

^bResults expressed as mean percentages of total WBC \pm SD.

^cResults expressed as mean percentages of total lymphocyte counts \pm SD.

*Significantly different from DLS, at 0.05 level, $p < 0.05$.

**Significantly different from Admission at 0.05 level, $p < 0.05$.

significantly higher than the atypical lymphocytes of the DF and DLS patients.

Lymphocyte immunophenotyping

The mean percentage of CD4⁺ cells on discharge day was significantly lower in DHF than in DLS patients ($p < 0.05$) (Table 2). The mean absolute counts on admission day in DHF and DF were significantly lower than on discharge day in DHF and DF ($p < 0.05$). The mean percentage of CD8⁺ cells was not different in DHF, DF and DLS. The mean absolute counts on admission day in DHF and DF were significantly lower than on discharge day ($p < 0.05$). The mean percentage of CD19⁺ cells on admission day and discharge day were significantly higher in DHF and DF than in DLS patients ($p < 0.05$). The mean absolute counts of CD19⁺ cells on

admission day in DHF and DF were significantly lower than on discharge day ($p < 0.05$). The mean absolute counts on discharge day in DHF and DF were significantly higher than in DLS ($p < 0.05$).

Correlation of lymphocyte immunophenotypes in DHF, DF and DLS

There was no significant difference in absolute CD4⁺ T cell counts between the DHF, DF and DLS at different time points ($p > 0.05$) (Fig. 2). At day -3, the mean absolute counts of CD8⁺ T cells in DHF were significantly lower than the CD8⁺ T cells in the DLS ($p < 0.05$). At day -3, the mean absolute counts of CD19⁺ B cells in DHF were significantly lower than the CD19⁺ B cells in the DLS ($p < 0.05$). At the later time points, *i.e.* on days 0 and day 1, the CD19⁺ B cells in DHF were increased and signifi-

cantly higher than the CD19⁺ B cells in DLS ($p < 0.05$) at the same time points.

Activated lymphocyte subsets immunophenotyping

A high percentage of CD69⁺ cells was expressed in DHF, DF, and DLS patients. The mean percentages and the absolute counts of CD69⁺ cells expressed on the CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, and CD19⁺ B lymphocytes, were not significantly different in DHF, DF, and DLS patients ($p > 0.05$) (Table 3). All patient groups had higher percentages of CD69⁺ cells expressed on CD8⁺ T lymphocytes than on CD4⁺ T lymphocytes and CD19⁺ B lymphocytes. A low percentage of lymphocytes (1.0 ± 1.8 , 1.3 ± 1.9 and 1.8 ± 6.1 on admission day and 1.6 ± 3.1 , 0.8 ± 2.0 and 0.5 ± 1.4 on discharge day) expressed CD87 in DHF, DF, and DLS patients. The mean percentages and the absolute counts of CD87⁺ cells expressed on the CD4⁺ T lymphocytes and CD8⁺ T lymphocytes were not significantly different in DHF, DF, and DLS patients ($p > 0.05$), but on the CD19⁺ B lymphocytes, they were significantly higher in DHF than in DLS ($p < 0.05$). Low percentages of TdT⁺ expressed lymphocytes were found in DHF, DF, and DLS patients. The mean percentages of TdT⁺ cells expressed on the CD69⁺ lymphocytes were not significantly different in DHF, DF, and DLS patients ($p > 0.05$). The absolute count of TdT⁺ expressed CD87⁺ cells in DHF patients was significantly increased compared with DF patients ($p < 0.05$), while those of DHF/DF patients had no significant differences with that of DLS patients ($p > 0.05$).

Correlation of immunophenotypes of activated lymphocyte subsets in DHF, DF and DLS

At two days before defervescence (day -2), the mean absolute counts of CD69⁺ cells in DHF were significantly lower than the CD69⁺ cells in DLS ($p < 0.05$). There was no significant difference in absolute CD87⁺ cell counts between DHF, DF, and DLS patients. There was no significant difference in absolute TdT⁺ cell counts between DHF, DF, and DLS patients.

Receiver operating characteristic analysis

In order to classify the results obtained from microscopic examination and flow cytometry (FCM)

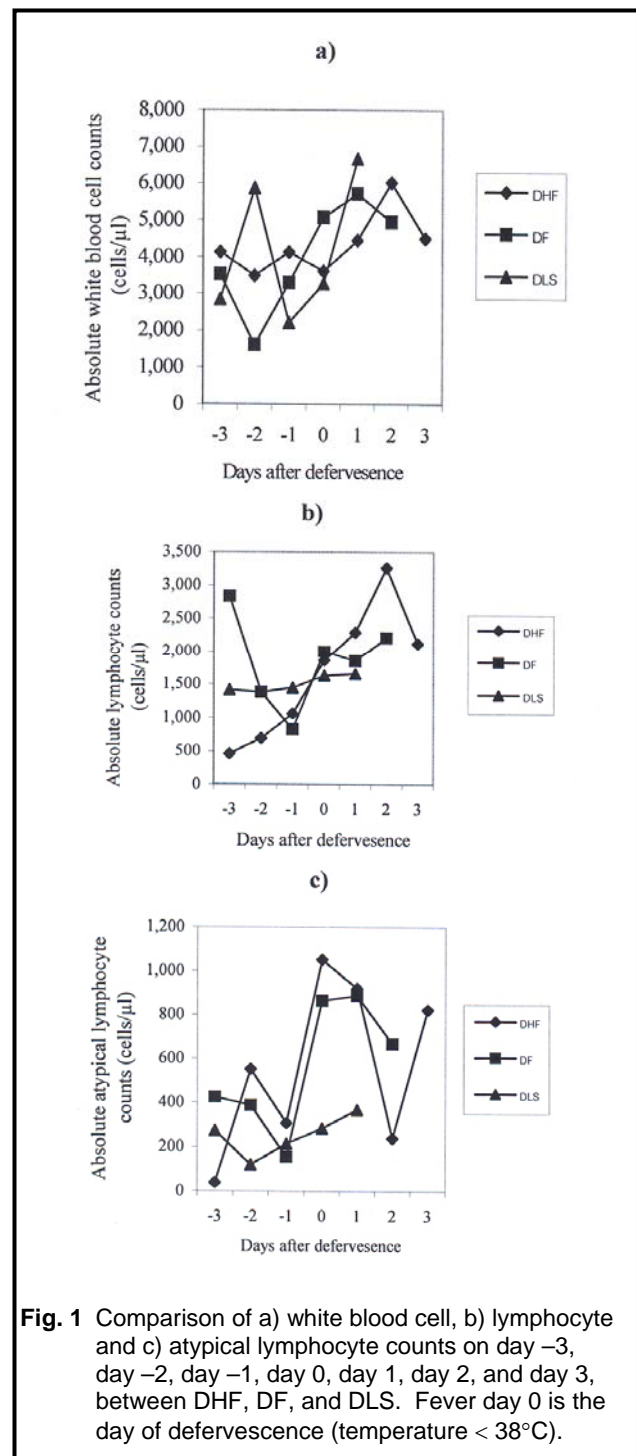


Fig. 1 Comparison of a) white blood cell, b) lymphocyte and c) atypical lymphocyte counts on day -3, day -2, day -1, day 0, day 1, day 2, and day 3, between DHF, DF, and DLS. Fever day 0 is the day of defervescence (temperature $< 38^{\circ}\text{C}$).

as positive or negative, cutoff point was determined using the Receiver Operating Characteristics (ROC) curve that was plotted from the results on admission day from microscopic examination and FCM, compared with ELISA results. A conventional ROC plot of the atypical lymphocyte is illustrated in Fig. 3. The curve displays the sensitivity and specificity of

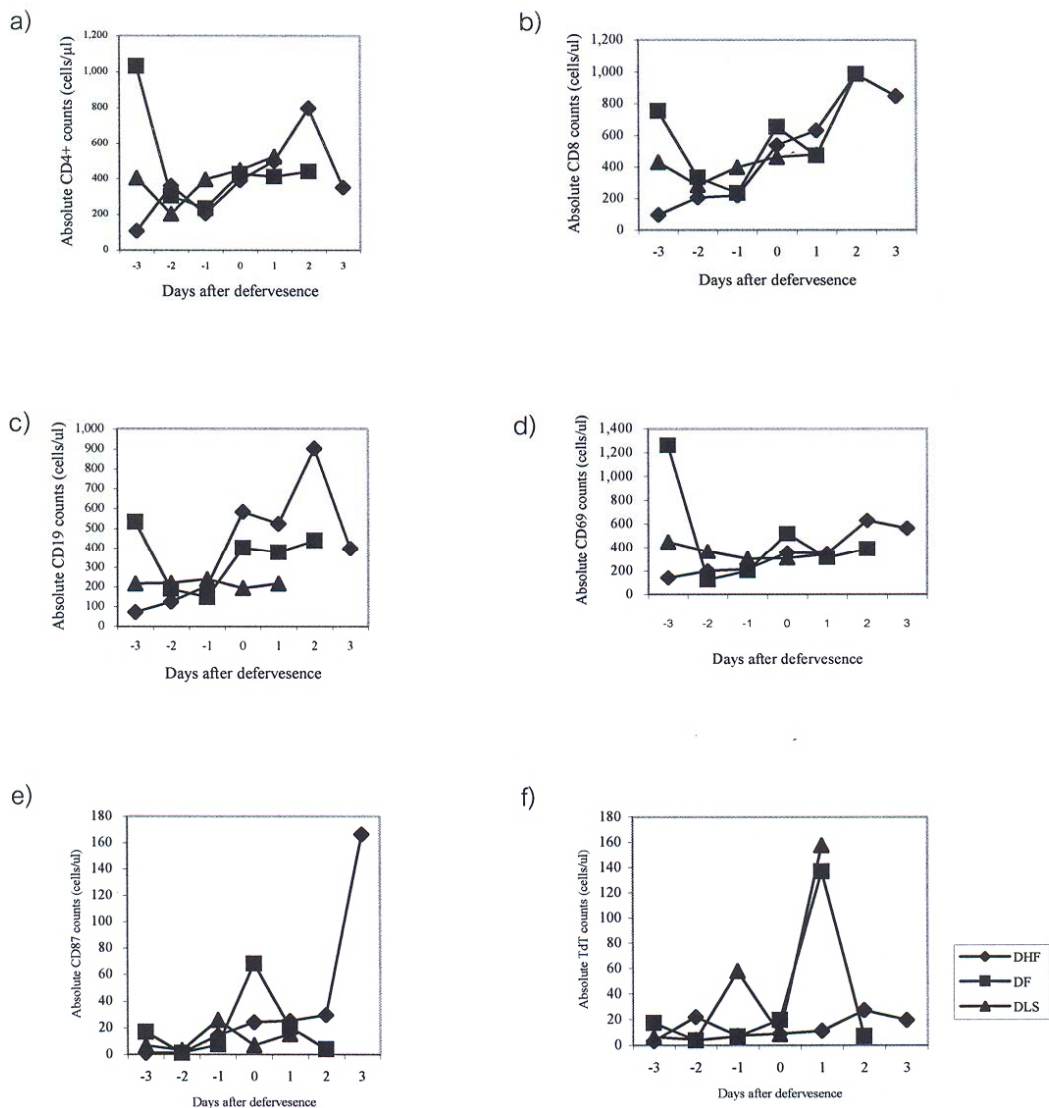


Fig. 2 Comparison of a) CD4⁺ T cell, b) CD8⁺ T cell, c) CD19⁺ B cell, d) CD69⁺ cell, e) CD87⁺ cells, and f) TdT⁺ cells on day -3, day -2, day -1, day 0, day 1, day 2, and day 3, between DHF, DF, and DLS. Fever day 0 is the day of defervescence (temperature < 38°C).

microscopic examination of the atypical lymphocytes at cutoff values ranging from 3 to 36.30. In this study, the selected cutoff value for discriminating patients with dengue viral infection (DF/DHF) from patients with non-dengue viral infection (DLS) was 10%, which would be the best sensitivity and specificity (50% and 86%) when compared with the ELISA. Therefore, the results obtained from microscopic examination $\geq 10\%$ were classified as positive results and the results $< 10\%$ were classified as negative results. The ROC curve of CD4⁺, CD8⁺, CD19⁺, CD69⁺, CD87⁺, and TdT⁺ on admission day obtained

from FCM were compared with the ELISA results. The ROC curve indicated that CD4⁺ and CD8⁺ counts were unable to distinguish DHF from DF and DLS patients. The ROC curve indicated no clear difference in the percentages of CD69⁺, CD87⁺, and TdT⁺ among the study groups. A cutoff point of CD19⁺ count at $\geq 15\%$ or 20% could be used for distinguishing DF/DHF from DLS patients, with the sensitivity of 69% and 49% and specificity of 50% and 85%, when CD19⁺ count was $\geq 15\%$ and $\geq 20\%$, respectively.

Table 3 The expression of activation markers on lymphocyte subsets in acute whole blood specimens from patients hospitalized with dengue hemorrhagic fever (DHF), dengue fever (DF), and dengue-like syndrome (DLS).

Cell type	Activation marker	DHF (n = 49)		DF (n = 25)		DLS (n = 26)	
		Admission	Discharge	Admission	Discharge	Admission	Discharge
CD4 ⁺ T	CD69						
	% total lymphocytes ^b	4.3 ± 9.1	3.2 ± 4.1	6.0 ± 6.9	5.3 ± 5.2	5.4 ± 4.4	3.8 ± 3.4
	Absolute count ^a	46 ± 115	85 ± 129	61 ± 65	105 ± 100	74 ± 71	83 ± 88
	CD87						
	% total lymphocytes ^b	2.4 ± 13.3	0.7 ± 2.7	1.1 ± 3.5	0.2 ± 0.3	0.4 ± 1.5	0.1 ± 0.2
	Absolute count ^a	30 ± 167	16 ± 54	18 ± 65	6 ± 11	4 ± 10	3 ± 7
CD8 ⁺ T	CD69						
	% total lymphocytes ^b	8.8 ± 5.0	8.5 ± 10.0	7.7 ± 5.3	12.0 ± 14.3	10.2 ± 7.4	6.9 ± 8.0
	Absolute count ^a	96 ± 69	225 ± 376	86 ± 87	412 ± 1,036	132 ± 99	150 ± 205
	CD87						
	% total lymphocytes ^b	1.5 ± 4.9	3.2 ± 10.4	1.0 ± 3.2	2.0 ± 7.7	1.9 ± 6.9	1.5 ± 5.2
	Absolute count ^a	14 ± 55	101 ± 378	8 ± 21	141 ± 605	16 ± 56	30 ± 86
CD19 ⁺ B	CD69						
	% total lymphocytes ^b	3.1 ± 4.1	2.9 ± 2.8	2.8 ± 1.9	3.2 ± 2.7	2.7 ± 2.1	2.1 ± 2.2
	Absolute count ^a	36 ± 51	70 ± 106	35 ± 33	91 ± 101	42 ± 43	51 ± 68
	CD87						
	% total lymphocytes ^b	0.2 ± 0.3	0.2 ± 0.7	0.4 ± 1.3	0.2 ± 0.4	0.0 ± 0.1	0.0 ± 0.0
	Absolute count ^a	3 ± 5*	8 ± 28	4 ± 10	4 ± 7	1 ± 1	0 ± 1
CD69 ⁺	TdT						
	% total lymphocytes ^b	0.5 ± 0.7	0.5 ± 1.8	1.8 ± 6.3	0.6 ± 0.7	4.2 ± 18.1	0.8 ± 2.4
	Absolute count ^a	5 ± 5	13 ± 37	30 ± 116	14 ± 21	29 ± 109	15 ± 39
CD87 ⁺	TdT						
	% total lymphocytes ^b	0.2 ± 0.6	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.1	3.3 ± 16.3	0.1 ± 0.3
	Absolute count ^a	2 ± 5	3 ± 6 [#]	2 ± 4	1 ± 1	21 ± 98	1 ± 5

^aResults expressed as mean of absolute counts (cells/ul) ± SD.

^bResults expressed as mean percentages of total lymphocyte counts ± SD.

*Significantly different from DLS at 0.05 level, $p < 0.05$.

[#]Significantly different from DF at 0.05 level, $p < 0.05$.

Correlation of immunophenotypes of atypical lymphocytes and lymphocytes

The relationships between the percentages of atypical lymphocytes and the percentages of cells with different cellular and activated markers were also analyzed. The percentages of atypical lymphocytes, CD19⁺ lymphocytes and CD69⁺ lymphocytes had significant correlation coefficients ($r = 0.403$ and -0.241), respectively. The atypical lymphocytes had a positive linear correlation with the CD19⁺ lymphocytes and was negatively correlated with the CD69⁺ lymphocytes at $\alpha = 0.01$.

DISCUSSION

In this study, we found a significant increase in the absolute count of atypical lymphocytes on day of defervescence and one day after defervescence during acute dengue viral infection, especially in DHF patients. This finding has been reported by

others and the atypical lymphocytes were proposed by Boonpucknavig *et al.*⁸ to represent B cells, since they found an increase in mouse erythrocyte rosette-forming cells (B cells) at the same time as increased atypical lymphocytes.^{6,8,15,16} The atypical lymphocytes may represent the anamnestic antibody response to dengue virus, since anti-dengue IgG antibody has been found to rise rapidly to high titers early in the course of secondary dengue infection.¹⁷ If these lymphocytes proliferate and differentiate into antibody-producing cells, they must be derived from B lymphocytes. In investigation of subpopulations of lymphocytes in DHF patients, it was found that the percentage of T lymphocytes decreased, whereas that of B lymphocytes increased.¹⁸ Thus, it was most likely that these atypical lymphocytes were antibody-producing cells. However, previous study of immunophenotyping using the APAAP technique demonstrated that atypical lymphocytes were predominantly of T lymphocyte origin, because most cells carrying markers for both CD2 and CD7.⁷ Therefore,

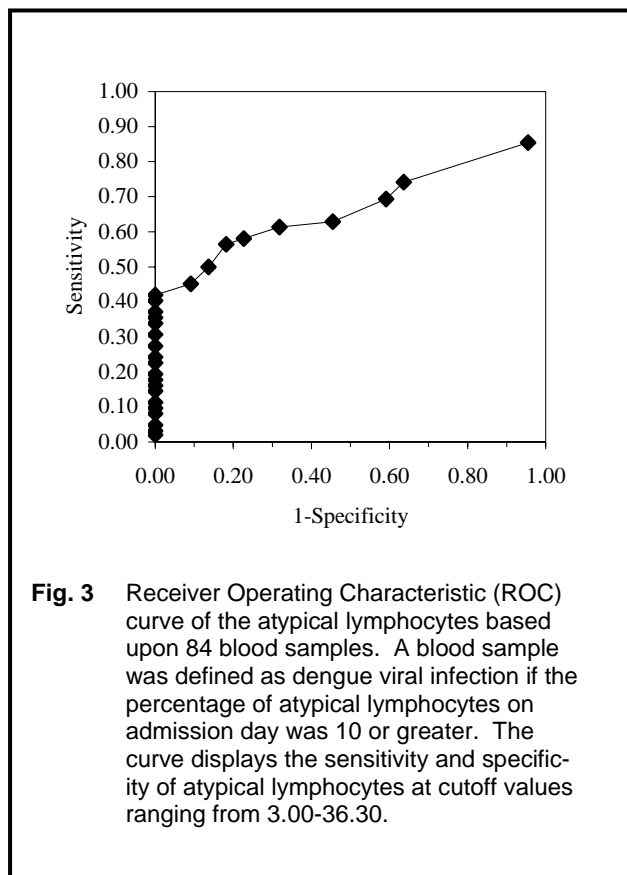


Fig. 3 Receiver Operating Characteristic (ROC) curve of the atypical lymphocytes based upon 84 blood samples. A blood sample was defined as dengue viral infection if the percentage of atypical lymphocytes on admission day was 10 or greater. The curve displays the sensitivity and specificity of atypical lymphocytes at cutoff values ranging from 3.00-36.30.

the possibility exists that atypical lymphocytes may also represent the cell-mediated immune response of the host to dengue virus.⁵

The CD4⁺ T cells on the day of discharge were significantly reduced in DHF patients compared with DLS patients. The CD8⁺ T cells were not significantly different among DHF, DF, and DLS patients, except three days before defervescence, when the CD8⁺ T cells in DHF were significantly lower than in DLS patients. We have confirmed reports by others that the absolute lymphocyte counts and CD4⁺ and CD8⁺ T cell counts, were significantly decreased during acute DHF.^{19,20} In contrast, DHF patients had significantly higher CD19⁺ B cells than DLS patients on the day of defervescence and one day after defervescence. This finding was consistent with the general observation that B lymphocytes increased in the acute phase of illness in secondary viral infection, as shown by Boonpucknavig *et al.*⁸ It may be hypothesized that B lymphocytes play a significant role in the elimination of circulating dengue-anti-dengue complex, and this may be the reason why there is an increase in their number.

Green *et al.*¹⁰ noted a significant increase in the percentage of cells expressing an early activation marker, CD69⁺ on CD8⁺ T cells and NK cells in children with DHF compared with those with DF during the febrile period of their illness. CD69 expression on CD4⁺ T cells and $\gamma\delta$ T cells was elevated at this same early time point in children with DHF and DF compared with controls, but there were no differences in expression between children with DHF and those with DF. This agrees with our result, which the mean absolute counts of CD69⁺CD8⁺ T lymphocytes tended to be greater in DHF than in DF, even though a significant difference was not demonstrated. We found high percentages of CD69⁺ but low percentages of CD87⁺ and TdT⁺ lymphocytes in DHF, DF, and DLS patients. We hypothesized that the atypical lymphocytes were not transformed into blast forms, because low percentages of TdT⁺ cells were found in DHF, DF, and DLS patients. TdT and CD87 may not be appropriate for use in differentiating DHF from DF and DLS patients, because of the very low numbers of cells expressing these markers. We found a significant increase in the absolute count of cells expressing an early activation marker, *i.e.* CD87 on CD19⁺ B cells in patients with DHF, compared with those with DLS, and the absolute counts of TdT cells that expressed on the CD87⁺ cells in DHF patients were significantly increased than DF patients during the febrile period of illness. To confirm our finding, further study should be conducted by increasing the counting events. The percent of cells expressing CD69 was increased on CD8⁺ T cells higher than CD4⁺ T cells and CD19⁺ B cells, respectively. But the absolute count of CD69 that expressed on all lymphocytes was higher than the absolute count of CD69 on total B and T lymphocytes. In other studies, immunophenotyping using the APAAP technique showed the composition of atypical lymphocytes to be predominantly of T lymphocyte origin, with most cells carrying markers of both CD2 and CD7. But CD2 and CD7 can be expressed also on NK cells.⁷

In conclusion, our data suggested that CD4, CD8, CD19 and CD69 were the markers of severity for dengue viral infection and could be used to differentiate DHF from DF and DLS patients in the early stage of disease. The depletion of CD4⁺ T cells is possibly due to their involvement in the immune elimination mechanism, in response to dengue viral

infection. We postulated that the atypical lymphocyte is an activated lymphocyte in response to stimulation and expressed activation markers on the cell. The atypical lymphocyte was probably not transformed into a blast form, as indicated by low percentage of TdT expressed cells. We concluded that atypical lymphocyte and CD19⁺ cell counts should be considered, besides platelets and white blood cell counts, as a useful diagnostic tool for dengue infection.

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