THE REFERENCE RANGE OF $CD4^+$ AND $CD8^+$ T-LYMPHOCYTES IN HEALTHY NON-INFECTED INFANTS BORN TO HIV-1 SEROPOSITIVE MOTHERS; A PRELIMINARY STUDY AT SIRIRAJ HOSPITAL

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Abstract. The results of $CD4^+$, $CD8^+$ T- lymphocyte values as percentage, number, and ratio were studied in infants aged 1 to 29 months. The 283 subsequent blood samples from 89 infants born to HIV-1 seropositive mothers were investigated. From 208 sequential samples of 70 healthy non-infected infants, the reference values of $CD4^+$ and $CD8^+$ T-lymphocytes have been established and compared to Caucasian reference values. The results were analysed in 4 difference age groups (1-5, 6-11, 12-17 and ≥ 18 months). At age 12 months, CD4 number and percentage declined significantly while CD8 percent increased. At age 6 months CD4/CD8 ratio decreased. Of 19 infected infants $CD4^+$ percentage and number as well as CD4/CD8 ratio declined at age 6 months and showed significant differences from uninfected infants. A significantly elevated CD8 percentage was demonstrated in infected infants at age of 12 months. In 9 infants who showed symptoms at age 6-18 months, the CD4 and CD8 values were different from the reference range and 6 of 9 patients showed lower CD4 percentage, CD4 number and reversed CD4/CD8 ratio before the symptoms appeared. In 10 infants who were asymptomatic at age 18 months, there was no evidence of immunosuppression at age 6 months or before. After age 6 months, lymphocyte subset values of some asymptomatic infected children were beyond the reference range. These preliminary findings should be very useful for monitoring children born to HIV infected mothers. The results of $CD4^+$ and $CD8^+$ T-lymphocytes in uninfected infants could be used as reference values for the Thai and other Southeast Asian pediatric populations.

INTRODUCTION

Early diagnosis of HIV in young infants has been difficult by the conventional antibody tests, due to persistence of placental transferred maternal IgG in infants' blood, often up to 15-18 months (Blanche *et al,* 1989; Rogers *et al,* 1989). Diagnostic tests of HIV infection in infants are the detection of HIV RNA or proviral DNA, p24 antigen with immune complex dissociation and anti-HIV testing. Evidence of immune suppression in HIV-infectd infants and adult is seen by the alteration of lymphocyte subsets *ie* CD4 and CD8 lymphocytes. Since 1991, more than 40 flow cytometers have been installed in hospitals and institutes throughout Thailand. (Pattanapanyasat, 1996). The enumeration of lymphocyte subsets is used for monitoring and management of HIV infected cases *ie* giving prophylactic treatment for opportunistic infections, starting antiretroviral therapy and use of other drugs. Progressive depletion of $CD4^+$ T-lymphocytes is associated with HIV disease progression. Many studies have shown that lymphocyte subsets in normal populations were significantly different by age and sex. (Babcock *et al,* 1987; Denny *et al,* 1992; Yanase *et al,* 1986). In the first year of life, the $CD4^+$ cell count was higher in infants than in adults (Yanase *et al,* 1986; Lee *et al,* 1996). In addition, there are many factors that affect lymphocyte immunophenotyping results such as ethnic group, and physiological condition (Nicholson and Landay, 1994; Webster *et al,* 1996). The CD^+ and $CD8^+$ lymphocyte subsets in Thai children who were born to HIV seropositive mothers were studied. The results of uninfected children provide a normal reference value in young children.

MATERIALS AND METHODS

Specimens

Blood samples were collected from 89 infants born to HIV seropositive mothers who attend the

special pediatric clinic at Siriraj Hospital, Mahidol University during April 1992 to April 1994. At first enrollment all were seropositive and asymptomatic, none of them had taken any antiretroviral drug and all received formula feeding. The samples were collected at age 1±2, 6±2, 12±3, and 18±4 months. A total of 283 subsequent blood samples were examined. Specimen analysis by age groups in presented in Table I. HIV infection in children was diagnosed by the presence of anti-HIV at age beyond 15 months and/or the positivity of HIV-1 p24 antigen after immune complex dissociation by ELISA and proviral DNA detection by PCR (Report on a consensus workshop 1992; Sutthent *et al,* 1996).

(about 2 ml) was used to diagnose HIV infected status by proviral DNA PCR, p24 Ag after immune complex dissociation and anti-HIV IgG and IgA (Report on a consensus workshop, 1992; Sutthent *et al,* 1996).

T-cell subsets analysis by flow cytometry

Each of whole blood sample was stained by using two Becton-Dickinson antibodies (a) Simultest control (b) Simultest CD4/CD8 and was analyzed by flow cytometry. Twenty microliters of each monoclonal antibody were placed into two 12x75 mm test tubes, and 100 µl of whole blood were

Table 1

The sequential collection of the 283 blood samples from 89 children born to HIV seropositive mothers by their age group.

Age group (month)	Number of samples collected from		
		HIV - Infected with	
	Uninfected (U) (age 1-25 mo) N = 70	No symptom (A) (age 1-26 mo) N = 10	Symptom (S) (age 1-29 mo) N = 9
1-5	68	11	9
6-11	50	7	8
12-17	66	9	12
≥18	24	12	7
Total	208	39	36

Sample preparation

Approximately 3 ml of venous blood was collected by scalp vein closed system into vacutainer tube containing EDTA (Becton-Dickinson, USA) during the morning session of the special pediatric clinic (9.00 - 12.00 am) The specimens were kept at room temperature and processed within 3 hours after collection. 0.5 ml whole blood was separated for $CD4^+$, $CD8^+$ T-lymphocyte enumeration (FACScan, Becton-Dickinson, USA) and total leukocyte count and percentage lymphocyte were determined by automated cell analyzer (Model JT3, Coulter, USA). The remaining part of specimens

added to each reagent tube, which was vortexed thoroughly at low speed for 3 seconds and incubated for 15-30 minutes at room temperature in the dark. Two ml of 1x FACS lysing solution was added to each tube and immediately mixed by vortex throughly at low speed for 3 seconds and incubated for 10 minutes at room temperature in the dark. After incubation, all tubes were centrifuged at 200g for 5 minutes at room temperature. The supernatants were aspirated and leaved 50 µl of residual fluid in the tube to avoid disturbing the pellet. The cell pellet was resuspended in residual fluid. Two ml of PBS with 0.1% sodium azide was added to each tube and mixed with cell suspension,

which was centrifuged at 200g for 5 minutes at room temperature. The supernatant was aspirated, and 0.5 ml paraformaldehyde was added into each tube for fixing the cells. Then, the sample tubes were passed through the flow cytometer and data were acquired in list mode file using consort 30 software's. All samples were analyzed with in 24 hours after collection.

Statistical analysis

Statistical analysis was performed using two types of software. First, Staview IV plus (McIntosh) was used for comparison of T-lymphocyte subsets in uninfected, asymptomatic and symptomatic HIV-1 infected children born to seropositive mothers. Second, SPSS/PC$^+$ (nonparametric Kruskal-Wallis) was used for further assessment of the difference by infection status, clinical symptoms and age group. The difference between each pair - group was determined by using Bonferroni pairwise comparison (Neter *et al,* 1985).

RESULTS

At the initial visit, the 89 children (56 girls and 33 boys) were 16 days to 10 months old (median = 1 month). In this study, they were followed up at 3 to 6 month intervals until 18 months old. The 70 children (43 girls and 27 boys) showed anti-HIV negative results in 2 sequential samples at the age 6 to 18 months and were classified as uninfected children. Of the 19 infected children (13 girls and 6 boys), 17 showed anti-HIV positive beyond 18 months old. Two children were lost follow up after age 6 months but their plasma gave positive results for p24 Ag (ICD). After followed-up, 9 cases developed clinical AIDS. (Centers for Disease Conrol, 1987). The other 10 cases were asymptomatic at that time. The PCR result in all 89 children confirmed the serological diagnosis (Report on a consensus workshop, 1992; Sutthent *et al,* 1996; Yourno and Conroy, 1992).

The lymphocytes and T-cell subsets (mean, SD, median) of 70 uninfected HIV children are shown by age group (Table 2), CD4 number and percentage, CD8 percentage and CD4/CD8 ratio were significantly different by age, ($p < 0.05$). A decline of CD4 number, percentage and CD4/CD8 ratio but increase of CD8 percentage were demonstrated. The CD4/CD8 ratio first declined significantly at age 6 months, ($p < 0.05$). The other parameters were significantly changed at age 12 months. The CD8 number did not show any significantly difference by age.

In HIV infected children, CD4 number, percentage, CD4/CD8 ratio and CD8 percentage also changed significantly by age similarly to the findings in uninfected children, except CD8 number in the symptomatic patients (Table 3). The CD4 number , CD4 percentage and CD4/CD8 ratio declined, but in CD8 the number and percentage increased.

The absolute lymphocyte counts in non-infected infants increased with peaked at age 60-11 months (Table 2). But no significant difference was observed at all groups in infected group (Table 3).

Comparison of T - lymphocyte subsets among uninfected and infected children (asymptomatic and symptomatic) are presented as boxplots in Fig 1. The pairs of lymphocyte subsets that showed significant differences are indicated at the top of each Fig 1A-1E. At age < 6 months, no significant difference was observed in the T - lymphocyte values among uninfected and infected groups. In the symptomatic children, the number and percentage of CD4$^+$ lymphocytes as well as CD4/CD8 ratio were significantly lower than those in uninfected children at age of 6 months while the number and percentage of CD8$^+$ lymphocytes significantly increased at age 12 months ($p = 0.0001$).

In the group of asymptomatic HIV-infected children, the CD4$^+$ lymphocyte count significantly decreased at age 6 months, while the other values were significantly different at age 12 months in comparison to the values from uninfected children ($p = 0.0001$).

The T-lymphocyte subsets of each infected individual were matched with the range of uninfected children (mean±SD) or control range. The results are shown in Figs 2-3. Of 9 symptomatic cases, 6 of the first blood samples (Fig 2B) and 4 of 10 samples from asymptomatic cases (Fig 3B) showed lower values of CD4 percentage than the control range. In the consequent samples from all symptomatic cases (9/9), the CD4 number and percentage (Fig 2A, 2B) were lower than the control range in all age groups, while only some cases of asymptomatic infected children showed a difference (Fig 3A, 3B).

Table 2

Lymphocytes and T-cell subset (mean, SD, median) in 70 uninfected HIV children.

	Age group (m)	Mean	SD	Median	(P25-P75)
Absolute lymphocyte ($x10^3$ /mm^3)					
	1-5	7.86	2.38	7.30	(6.3 - 9.1)
	6-11	9.26	2.83	8.8[a]	(7.3 - 11.1)
	12-17	7.87	2.08	7.10	(6.3 - 9.6)
	≥18	7.70	1.94	7.80	(6.4 - 9.6)
CD4+ lymphocyte ($x10^3$ /mm^3)					
	1-5	3.31	0.97	3.20	(2.6 - 4.0)
	6-11	3.49	1.07	3.20	(2.8 - 4.2)
	12-17	2.70	0.92	2.40[a]	(2.2 - 3.2)
	≥18	2.57	0.74	2.60[a]	(1.9 - 3.0)
% CD4					
	1-5	42.60	7.00	41.0	(37 - 48)
	6-11	38.30	6.70	38.0	(33 - 43)
	12-17	34.50	4.90	34.0[a]	(31 - 38)
	≥18	33.70	6.10	35.0[a]	(28 - 39)
CD8+ lymphocyte ($x10^3$ /mm^3)					
	1-5	1.79	0.95	1.70	(1.0 - 2.3)
	6-11	2.21	1.02	2.00	(1.4 - 2.8)
	12-17	2.02	0.69	1.80	(1.5 - 2.4)
	≥18	2.24	0.87	2.30	(1.7 - 2.8)
% CD8					
	1-5	22.0	7.40	21	(17 - 26)
	6-11	22.70	6.70	21	(20 - 26)
	12-17	25.60	5.30	26[a]	(21 - 29)
	≥18	28.80	7.30	28[a]	(23 - 32)
CD4 : CD8 ratio					
	1-5	2.18	0.86	2.0	(1.6 - 2.8)
	6-11	1.81	0.71	1.70[a]	(1.3 - 2.2)
	12-17	1.42	0.43	1.40[a]	(1.1 - 1.7)
	≥18	1.31	0.43	1.40[a]	(1.0 - 1.6)

[a] show significance difference by the pair age group (Bonferroni pairwise comparison) p-value <0.05
P25 = Percentile 25
P75 = Percentile 75

After 6 months old, the number and percentage of $CD8^+$ lymphocytes increased in the infected group, both symptomatic and asymptomatick proportionally until 15 months old and then the $CD8^+$ lymphocyte number declined while $CD8^+$ percentage was still higher than the control ranges (Figs 2C,2D,3C,3D).

Of 9 symptomaic cases, 6 children showed sequential decline of CD4/CD8 ratio to lower than the control range. In asymptomatic children, the CD4/CD8 ratio was higher in the first year of life and then declined to a value lower than the control range (Figs 2E, 3E).

DISCUSSION

The lymphocyte subset patterns in uninfected and infected infants born to HIV seropositive moth-

Table 3

Lymphocytes and T-cell subset (mean, SD, median) in HIV infected children.

	Age (months)	Lymphocyte subset [Median, (P 25-P 75)] in HIV - Infected with: No symptom (A) N=10		Symptom (S)* N=9	
Absolute lymphocyte ($x10^3$ /mm^3)					
	1-5	7.9	(7.1-10.4)	8.9	(7.7-10.7)
	6-11	10	(7.1-13)	7.5	(4.8-11.5)
	12-17	9.9	(9-11.1)	8.5	(7.4-10.2)
	≥18	6.0	(5.1-10.5)	4.1	(2.4-8.5)
$CD4^+$ lymphocyte ($x10^3$ /mm^3)					
	1-5	2.5	(2.3-3.0)	2.5	(2.2-3.3)
	6-11	2.9[b]	(2-3.3)	1.6[b]	(0.7-2.0)
	12-17	2.2	(2-2.6)	1.0[a,b]	(0.8-1.5)
	≥18	1.4[a,b]	(1-1.6)	0.6[a,b]	(0.07-1.28)
% CD4					
	1-5	37	(26-40)	28	(25-39)
	6-11	27	(26-30)	19[b]	(12-23)
	12-17	22[b]	(17-28)	12[a,b]	(11-14)
	≥18	22[a,b]	(14-27)	10[a,b]	(2-15)
$CD8^+$ lymphocyte ($x10^3$ /mm^3)					
	1-5	1.9	(1.5-3.3)	2.4	(1.7-3.0)
	6-11	3.3	(1.8-4.5)	2.3	(1.2-4.0)
	12-17	5.0[a,b]	(3.2-6.2)	4.8[b]	(2.4-5.7)
	≥18	2.8	(2.2-5.1)	1.9	(1.5-3.9)
% CD8					
	1-5	26	(19-37)	25	(22-30)
	6-11	34	(22-37)	31	(23-51)
	12-17	46[a,b]	(38-54)	50[a,b]	(40-56)
	≥18	47[a,b]	(37-57)	46[a,b]	(44-65)
CD4 : CD8 ratio					
	1-5	1.31	(0.7-2.3)	1.0	(0.9-1.8)
	6-11	0.81	(0.7-1.5)	0.58[b]	(0.27-1.07)
	12-17	0.44[a,b]	(0.34-0.67)	0.26[a,b]	(0.2-0.31)
	≥18	0.55[a,b]	(0.26-0.65)	0.15[a,b]	(0.04-0.34)

*Data from children who developed symptoms within two years old.

[a] show significant difference between pair age group p-value <0.05.

[b] show significant difference between uninfected and infected p-value ≤ 0.0001

ers were studied. Infants have a greater number of lymphocyte than the number in adults, and have also shown different distribution of the major lymphocyte subsets (Babcock *et al,* 1987; Denny *et al,* 1990; Mekinney and Wilfert, 1992). There are striking age dependent differences in absolute number of total lymphocyte, $CD4^+$ and $CD8^+$ lymphocytes (The European Collaborative Study, 1992; Vigano *et al,* 1993). Many factors affect the values of lymphocytes and their subpopulations *ie* age, sex, ethnic group and other conditions (The European Collaborative Study, 1992; Martino *et al,* 1991). The immunologic status of young infants must always be compared with age matched

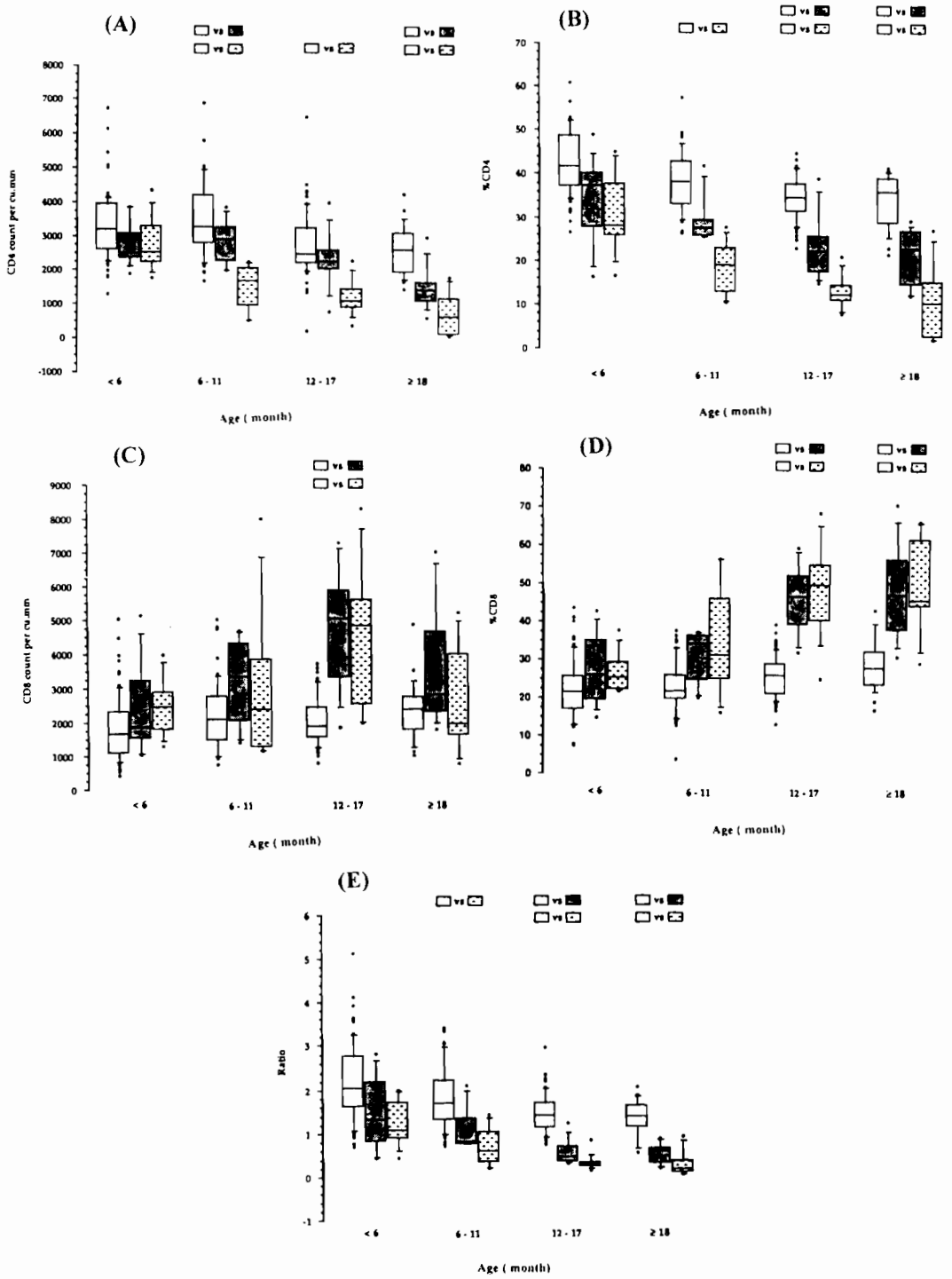

Fig 1–The CD4 lymhocyte count (A), % CD4 (B), CD8 lymphocyte count (C), % CD8 (D) and CD4/CD8 ratio (E), in uninfected (□) asymtomatic (□) and symtomatic (□) HIV infected children examined sequentially from first month of life to 18 months are shown as boxplot. Statistically significant difference are shown in some age groups. (p=0.000)

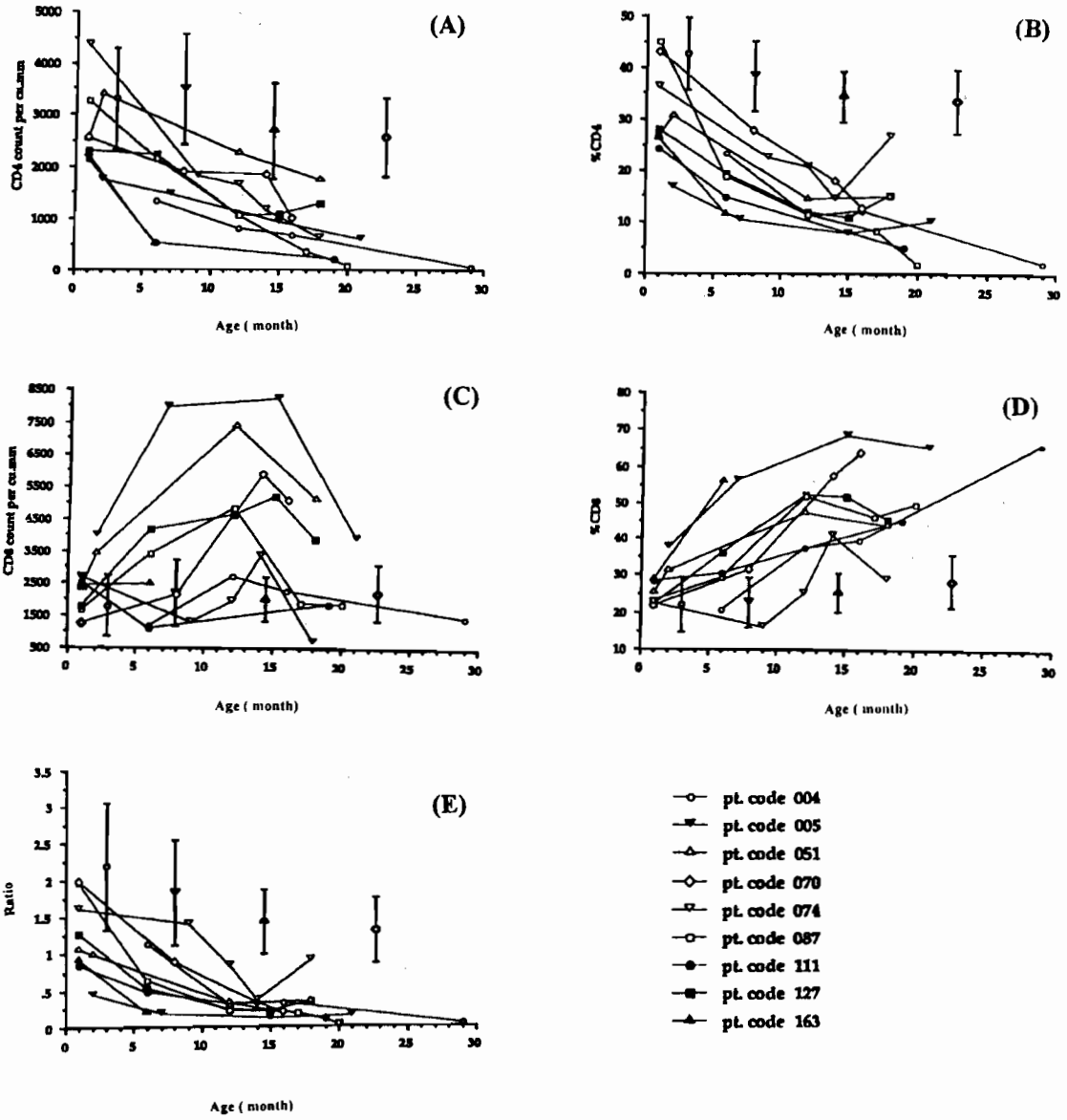

Fig 2–The CD4 lymphocyte count (A), %CD4 (B), CD8 lymphocyte count (C), % CD8 (D) and CD4/CD8 ratio (E), in seqential blood samples from individual HIV infected children who showed symptom after age of 6 months compare to reference value from uninfected children (mean ± ISD, the error bar;) in each age group.

controls in the same population. For monitoring or management of HIV infected children, the normal reference value of the lymhocyte immunophenotype should be established.

The lymphocyte subsets in Asian infants at age group 2 days 11 months has been reported (Lee *et al,* 1996). In our study, the results of T- lymphocyte subsets in healthy uninfected HIV children born to HIV-1 infected mothers was determined and served as controls for comparison purpose with infected children. The first blood samples of most subjects were collected at or after 1 month old except two

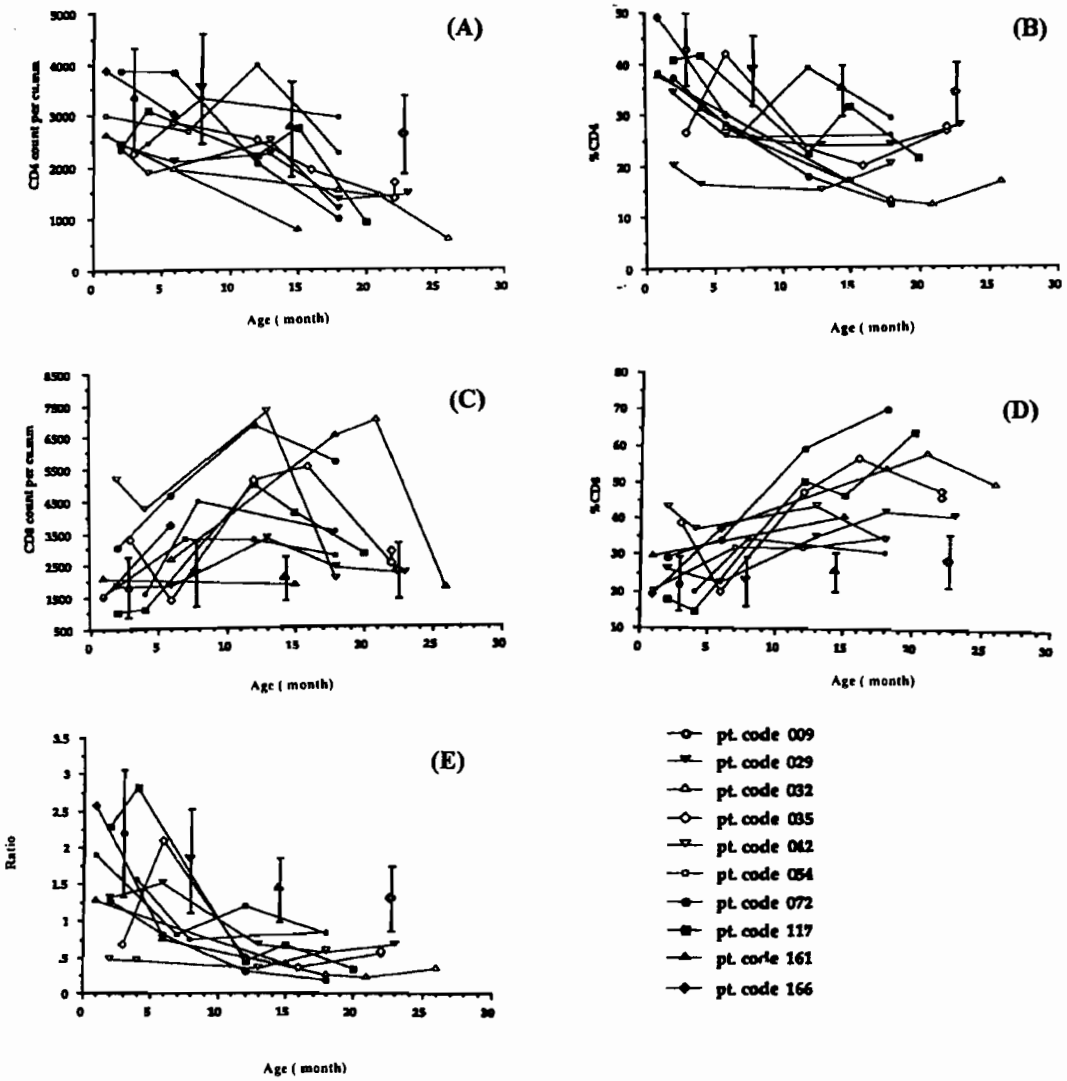

Fig 3–The CD4 lymphocyte count (A), %CD4 (B), CD8 lymphocyte count (C), %CD8 (8) and CD4/CD8 ratio (E), in seqential blood samples from individual HIV infected children who showed no symptom after age of 18 months compare to reference value from uninfected children (mean ± ISD, the error bar;) in each age group.

uninfected children at age 16 and 25 days. The results of girls and boys in the studied group were combined for analysis since the number of the children in this study was small and no significant differences by sex in infant groups was reported (The European Collaborative Study, 1992); we analysed all infants by age. The absolute lymphocyte count in uninfected children (Table 2) was approximately three times higher than in adult, CD4 percent was higher at age < 6 months and declined toward adult level at age group > 6 months, CD8 percent in age group < 6 months were lower than adult and increases to adult value at age > 18 months. In infants age below 6 months, the DC4/CD8 ratio was high (nearly 2) and then declined to the adult ratio (1.3) at age> 18 months. However

decline in total lymphocytes and CD4+lymphocytes and lowered CD4/CD8 ratios in infants born to IV drug using mothers have been reported (Webster *et al,* 1996). In this series, one uninfected child was born to an injecting drug user mother but his T-lymphocyte subsets were in the control range.

The European Collaborative Study Group showed that absolute lymphocytes, $CD4^+$ T-lymphocytes, $CD8^+$ T-lymphocytes, were high at birth, peaked in number at 6 to 9 months and then declined toward adult values. The CD4 percentage and CD4/CD8 ratio declined steadily from birth onwards. (The European Collaborative Study, 1992). Reports from Singapore (The European Collaborative Study, 1992) and our results showed similar trends. The absolute number of lymphocytes in our study showed a peak number at age 6-11 months, higher than in Caucasians (8.8×10^3 *vs* 7.2×10^3). The CD4 percentage in Thai infants was lower than that of Caucasians (38% *vs* 44%). However, the calculated CD4 lymphocyte number in our studied infants was still higher. In adult studies, it was also demonstrated the CD4 percentage in Asian was lower than in Caucasians (Lee *et al,* 1996; The European Collaborative Study, 1992). The CD8 percentage were slightly increased by age whereas $CD8^+$ cell counts were not significantly different between age groups (Table 2). In comparison of T-cell subsets between our study and the results from Caucasian children, we found that the absolute lymphocyte $CD4^+$ cell count and $CD8^+$ number in Asian children were higher, while the CD4 percentage or CD8 percentage was lower.

In infected children, all T-cell subsets were significantly different by age as well as reported in most studies (Table 3) (Yanase *et al,* 1986; The European Collaborative Study, 1992; Vigano *et al,* 1993; Martino *et al,* 1991; Plaeger-Marshall *et al,* 1993). In our studies no significant difference in the absolute lymphocyte number was seen. At the age 6 months all infected infants were asymptomatic and the T-cell subsets did not change. After 6 months, the CD4 number and percentage, as well as CD4/CD8 ratio decreased and showed significant difference between infected and uninfected infants. The CD8 percentage increased at age of 12 months in infected infants. The decline of CD4 number and percentage, CD4/CD8 ratio were correlated well with clinical progression. The children infected with HIV-1 were reported to show more rapid progression and fulminant disease than adults (Martino *et al,* 1991; Oleske *et al,* 1983). The persistence of CD8 level and the CD4 depletion leads to a progressive reversal of CD4/CD8 ratio (Levey, 1988; Salazer-Gonzalexz *et al,* 1985). Besides the CD4 count, the other subsets such as CD8 number and percent could be differentiated between infected and uninfected children. Such observations have already been reported (Vigano *et al,* 1993; Martino *et al,* 1991). The CD8 count and percentage were markedly high at age 12 months in the infected cases.

In the indetermined group of infants born to HIV infected mothers, the CD4 number, CD4 percentage, and CD4/CD8 ratio were powerful indicators for prognosis of disease progression after the age of 6 months (Fig 1A, 1B, 1E). CD4 count was the most sensitive indicator since the significant difference was observed between the asymptomatic or symptomatic infected cases and uninfected infants especially at age of 6 months or after (Fig 1A). However, in our study, CD4 percentage, and CD4/CD8 ratio in asymptomatic cases were not significantly different from uninfected infant at age of 6 months, as shown in the previous reports (Vigano *et al,* 1993; Martino *et al,* 1991).

In this study, the range (mean ± 1SD) of T-cell subsets from 70 uninfected infants was set up and compared to the T-cell subsets in symtomatic and asymptomatic infected cases. Of 9 infants who showed symptoms at 6 months or within 18 months, the CD4 percentage, CD4 cell count and CD4/CD8 ratio were lower than the normal range at the age < 6 months, before the symptoms appeared. This immunosuppressive evidence could be used for early treatment (Fig 2 A-E). Of 10 HIV infected infants who did not show symptom at age 18 months, the sequential T-cell subset values were scattered. In the first 6 months the values of T-cell subsets were in normal range but after 6 months, some of them showed evidence of immunosuppression before symptoms appeared.

The sample size of infected infants in this study was small for statistical calculation of the value (as a function of mean) of disease progression. Our results from 70 uninfected children at different ages could be used as normal reference values in children under 18 months old. $CD4^+$ T-lymphocyte depletion is a major consequence of HIV infection and is responsible for many of the severe manifestations of HIV infection in adults. By CDC classification for severity of HIV-infected pediatric cases, three immunologic categories were established for

severity. (Centers for Disease Control, 1994). When this classification was used as the reference, we found that in uninfected cases, all had CD4 percentage > 25%, whereas the 66% (6/9) symptomatic HIV infected cases had CD4 percent < 25% at 6 months of age and 89% (8/9) showed CD4 percent < 25% at age > 15 months. This reference is difficult with the CD4 count, since only 33% (3/9) had CD4 counts < 1,500 cell µl. So the normal value of CD4 percent and counts should be further investigated.

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