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Flow Cytometric Detection of Intracellular Cytokines in Peripheral Blood of HIV-1 infected Thai Children

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SUMMARY **The objective of this study was to determine changes in Th1/Th2 cytokine production at the cellular level which occur during the progression of HIV-1 subtype E infection in Thai children born to HIV–1 subtype E infected mothers. Mitogen stimulated whole blood cultures from 12 uninfected and 27 HIV-1 subtype E infected Thai children were stained intracellularly with fluorescein labelled monoclonal antibodies against interleukin (IL)-2 and IFN- γ (Th1 cytokines) and IL-4 (Th2 cytokine). Additionally, co-staining of $CD4^+$ and $CD8^+$ T cells was performed. Results were analyzed by two and three color flow cytometry. The percentage of IFN-γ expressing cells in $CD4^+$ T cells was increased in HIV-1 subtype E infected Thai children with mild and moderate immunosuppression (Immunological categories 1 + 2, Centers for Diseases Control and Prevention (CDC) staging system, 1994). The percentages of IFN-γ expression was continuously enhanced accompanied by remaining preserved in the proportion of IL-2 producing T cells in HIV-1 subtype E infected Thai children with severe immunosuppression (Immunological category 3, CDC staging system, 1994). The percentages of IFN-γ expression was continuously augmented whereas the proportion of IL-2 producing T cells remained unchanged in HIV-1 subtype E infected Thai Children with severe immunosuppression (immunological category 3, CDC staging system, 1994). The percentage of Th2 cytokine producing cells within the $CD4^+$ ad $CD8^+$ T cells increased in HIV-1 subtype E infected individuals and showed a significant difference in HIV-1 subtype E infected Thai children with AIDS compared with uninfected infants. These results suggest that in vertically acquired HIV-1 infection with severe immunosuppression, the percentages of IL-2 producing $CD4^+$ T cell was consistent but the percentages of IL-4 and IFN-γ producing cell were increased. Similar results were found for $CD8^+$ T cells in which IL-4 producing cells were increased in conjunction with a remaining in the number of IL-2 producing cells in HIV–1 subtype E infected Thai children. Thus, changes in the Th1 and Th2 cytokine pattern during HIV-1 infection may contribute to the prognosis of HIV disease in children.**

$CD4^+$ T cells are the preferred targets of HIV replication *in vivo* and *in vitro*, resulting in a reduction of this subset of cells during progression to AIDS and loss of T helper cell function. These cells represent a functionally heterogeneous population with specific profiles of cytokine production.[1,2] The major T helper derived cytokines are broadly categorized as type 1 cytokines (from Th1 cells) and type 2 cytokines (from Th2), which are important in cell mediated and humoral immunity, respectively. Th1 cells produce IL-2 and interferon gamma (IFN-γ) whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13.[3,4,5,6] Cytokines are also secreted by $CD8^+$ T cells and non-T cells.[7]

Methods for analyzing cytokine production measure the amount of secreted cytokine present in serum or supernatant using enzyme-linked immunosorbent assay (ELISA), or mRNA production in cells using polymerase chain reaction (PCR). However, these techniques require long activation times and cannot determine the frequencies and the phenotypes of the cytokine-producing cells at the single cell level.[8,9,10,11] The multiparameter capability of flow cytometry allows the measurement of two cytokines simultaneously at the single cell level and has the advan-

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tage of rapidly determining intracellular cytokine production after incubation with mitogen or antigen using a large number of cells. In this technique, intracellular accumulation of cytokines is induced by incubation with monensin or enhanced the sensitivity of cytokine detection by incubation with Brefeldin A.[8,9,10,12]

Several research groups have studied cytokine abnormalities in patients with HIV-1 infection and stimulus-induced cytokine responses in circulating peripheral blood cells or lymph nodes. Enhanced production of IL-4, IL-6 and IL-10 with decreased IL-2, IL-12 and IFN-γ secretion has been reported in HIV-1 infected children[2,4] and adults.[13,14] Although most of the cytokine profiles have been studied in HIV-1 infected adult, however, little information is available in HIV-1 infected children. To investigate stimulus induced type 1 and type 2 cytokine responses in HIV-1 infected children, we measured cytokine expression in $CD4^+$ and $CD8^+$ T cells using whole blood. These studies may lead to an understanding of the role of cytokines in HIV disease progression in HIV-1 infected pediatric subjects.

MATERIALS AND METHODS

Subjects

Peripheral blood was obtained from 39 children born to HIV seropositive mothers at Phramongkutklao Hospital, Bangkok, Thailand. T lymphocyte subsets were determined by FACS analysis using the simulset standard protocol from Becton Dickinson.[6] The diagnosis for HIV-1 infection was based on detection of HIV antibody by Vironostika HIV Uni-form II plus O ELISA (Organon Teknika) and confirmed positive by nested PCR using primers in the gp41 coding region as described.[15] We found that all HIV-1 infected children are HIV-1 subtype E infected. These children were then divided into 2 groups: Group 1 included HIV-1 infected children whose disease stages were classified using 1994 revised Centers for Diseases Control and Prevention (CDC) staging system, based on age, percentage and absolute number of $CD4^+$ T cells; these are referred to as immunological categories 1, 2 and 3.[16] Category 1 = no evidence of suppression; category 2 = moderate suppression and category 3 = severe suppression. It was found that 11 were in category 1, 9 in category 2 and 7 in category 3. In this study, however, category 1 and category 2 were combined. Group 2 included controls which were uninfected children born to HIV-1 infected mothers. These children (twelve subjects) were confirmed HIV negative by ELISA and PCR testing.

Whole blood from infected and uninfected children was collected in sodium heparin and EDTA tubes to measure cytokines and to lymphocyte subsets determination, respectively. The activation assay was modified from Jung *et al.*[8] and Suni *et al.*[11] Whole blood was diluted in RPMI-1640 (Gibco, Gaithersburg, MD) and stimulated with 25 ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St Louis, MO) and 1 μg/ml Ionomycin (I; Sigma) in the presence of 10 μg/ml Brefeldin-A (BFA; Sigma). Unstimulated blood (cultured in the absence of PMA and Ionomycin) was used as a baseline control. The culture tubes were incubated upright in a humidified 37°C, 5% CO_2 incubator for a period of 4 hours.

Surface staining and intracellular analysis of cytokine production

Following incubation, 100 μl of 20 mM EDTA was added directly to whole blood cultures, mixed and incubated for 15 minutes at room temperature. Red blood cells were lysed with FACS lysing solution (Becton Dickinson, San Jose, CA) for 10 minutes and washed. The cells were then permeabilized for 10 minutes using FACS permeabilizing solution (Becton Dickinson).

The anti-human monoclonal antibodies (mAbs) used were: CD45 (Hle-1) Per CP, CD3 (Leu-4) Per CP, CD4 (Leu-3a) Per CP, CD8 (Leu-2a) Per CP, CD4 FITC, CD8 FITC, anti-human IL-2 FITC, anti-human IL-2 PE, anti-human IL-4 PE, anti-human interferon-γ FITC, anti-human interferon-γ and FAST-IMMUNE™ interferon-γ FITC/IL-4 PE. All of these antibodies were obtained from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA)

After staining in the dark for 30 minutes, samples were washed and fixed in 1% paraformaldehyde and stored at 4°C until analysis using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, USA). Two and three-color flow cytometric analysis were performed and data were acquired and analyzed by using CELLQuest software (BDIS).

Statistical analyses

The mean and median cytokine production for each group of children was compared for various cell types. Statistical analysis was performed using the Wilcoxon's rank sum and Mann-Whitney tests to compare control and HIV-infected children in different immunologic categories. Statistical

significance was established at a cut-off value of $p < 0.05$ when comparing uninfected with HIV-1 infected children from each immunological category and between HIV-1 infected children categories 1 + 2 with category 3.

RESULTS

Demographic and CDC classification of characterization of children born from HIV seropositive mothers

Of 39 children born to HIV-1 infected mothers; 12 were uninfected and 27 were HIV-1 infected, 11 HIV-1 infected children were classified as category 1, nine were considered to have CDC category 2 and seven were considered to have CDC category 3. The percentage and absolute numbers of $CD4^+$ T cells were significantly reduced in both category of HIV-1 infected children (categories 1 + 2 and category 3) compared with uninfected children. Similarly, significantly decrease in $CD4^+$ and $CD8^+$ ratio was also found in HIV-1 infected children. Finally, there were enhanced significantly of the percentage of $CD8^+$ T cells in HIV-1 infected children (classified by CDC as categories 1 + 2 and category 3) compared with uninfected children. Whereas no difference were detected in the absolute number of $CD8^+$ T cells among both category of HIV-1 infected children compared with uninfected children (Table 1).

Intracellular type 1 cytokine production

Analysis of the Th1 type cytokines IL-2 and IFN-γ expression in $CD4^+$ T cells was enhanced in HIV-1 infected children classified as CDC categories 1 + 2 (Fig. 1). Whereas the frequency of IL-2 was reduced among $CD4^+$ T cells for children with AIDS (category 3). In contrast, the percentage of IFN-γ production from $CD4^+$ T cells increased from 2.97 % in HIV negative children to 17.84 % in HIV-1 infected children classified implying as categories 1+2 and to 22.89 % in HIV-children classified as category 3 (Table 2), implying there was no such difference between the two groups of HIV-1 infected children. The proportion of $CD4^+$ T cells expressing IFN-γ was significantly ($p < 0.05$) higher in cells from HIV-1 infected children from both categories.

In Table 2 and Fig. 1, the median amount of IL-2 expressing $CD8^+$ T cells from HIV-1 infected children was decreased. The percentage of IL-2 expressing $CD8^+$ T cells from both categories of HIV-1 infected children did not differ significantly with uninfected subject controls or in comparison with each individual category. In contrast, the majority of IFN-γ expressing $CD8^+$ T cell was 15.37 % in healthy controls, increasing to 62.88% from HIV-1 infected individuals classified as categories 1 + 2 and reduced to 42.17 % in HIV-1 infected children with AIDS (Table 2). The frequency of IFN-γ expression was increased significantly ($p < 0.01$) among $CD8^+$ T cells for HIV-1 infected without AIDS whereas significant reduced in children with AIDS. The IFN-γ expressing $CD8^+$ T cells was maximal in early stage of the disease and more than three times compared with uninfected control subject.

Table 1 Demographic and laboratory characteristics of study population

Characteristic	Uninfected	CDC Immunologic category	
		Categories 1+2	Category 3
Age (years)	1.2 ± 1.1[a]	3.2 ± 2.4	5.2 ± 2.6
Gender ratio (M/F)	4/8	10/10	2/5
% CD4	41 (20 -48)[b]	28 (14-53)*	9 (2-14)*
Absolute CD4 count (cell/mm^3)	2402 (1,680-6,882)	1043 (329-2442)*	200 (35 -444)*
%CD8	24 (16-35)[b]	40 (21-54)*	51 (23-56)*
Absolute CD8 Count (cell/mm^3)	1790 (727-3,514)[b]	1676 (684-2915)	1018 (380-2179)
CD4/CD8	1.9 (0.6-2.5)[b]	0.7 (0.3-2.3)*	0.2 (0.1-0.4)*

a = mean ± S.D.
b = median (range)
*$p < 0.05$ comparing uninfected with HIV-infected individuals of Centers for Disease Control and Prevention (CDC) 1994 criteria, categories 1+2 and category 3.

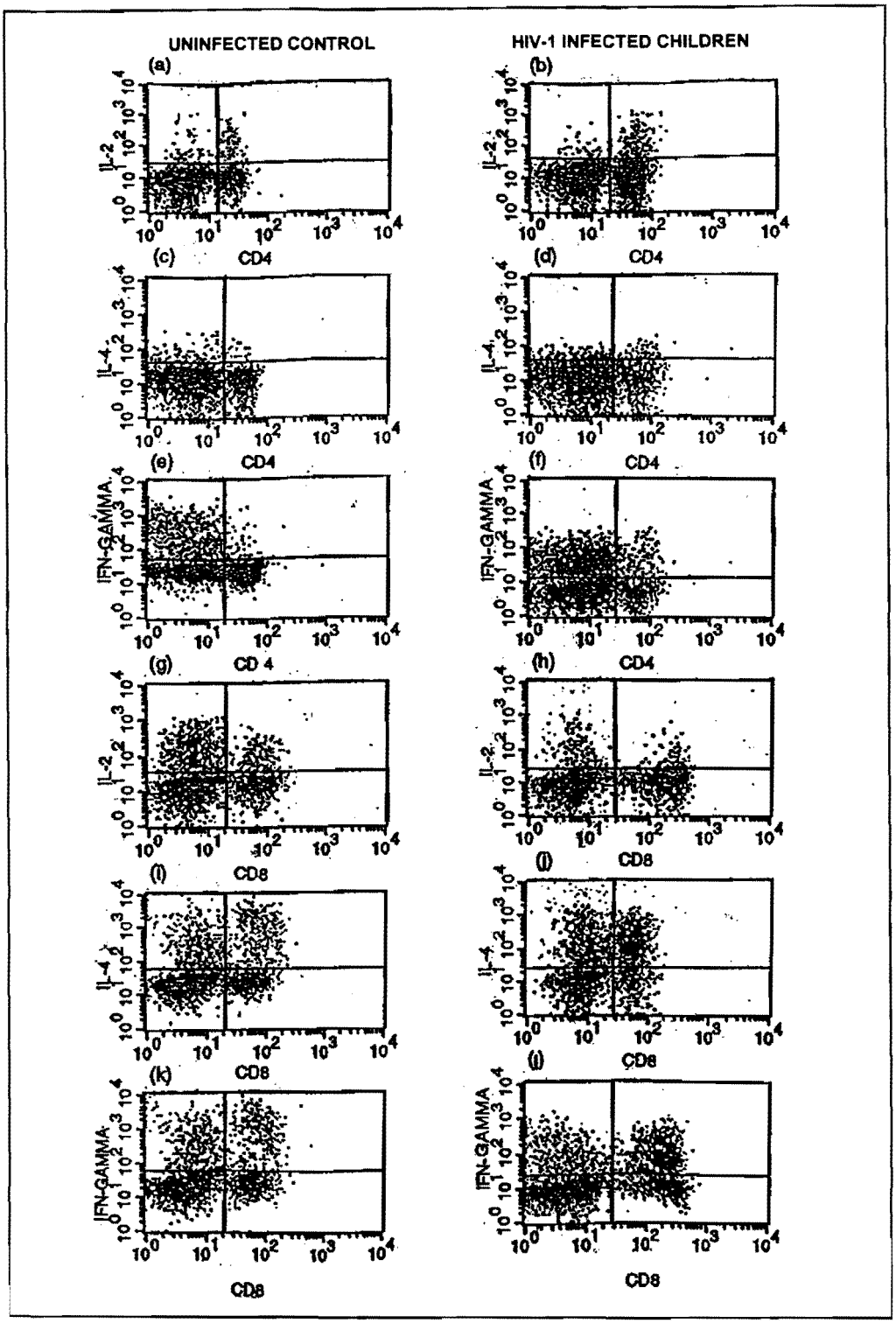

Fig. 1 Fluorescence-stimulated whole blood lymphocyte cell dot plot of PMA/Ionomycin/Brefeldin-A in the lymphocyte gate of uninfected control (left panels) and of HIV-1 infected children (right panels). Activated cells were stained with the combination of antibody as follow: (a) and (b), CD4 and IL-2; (c) and (d), CD4 and IL-4; (e) and (f) CD4 and IFN-gamma; (g) and (h) CD8 and L-2; (i) and (j) CD8 and IL-4; (k) and (l) CD8 and IFN-gamma.

Table 2 Percentage of IL-2, IFN-gamma and IL-4 expressing cells among $CD4^+$ or $CD8^+$ T cells from study children

		$CD4^+$ T cells			$CD8^+$ T cells		
		IL-2	IFN-gamma	IL-4	IL-2	IFN-gamma	IL-4
Uninfected (n = 12)	**Median** (range)	7.80 (4.2-39.45)	2.97 (2.16-7.16)	2.19 (0.68-2.34)	4.95 (1.74-18.94)	15.37 (3.07-52.78)	3.5 (0.4-30.65)
Categories 1+2 (n = 20)	**Median** (range)	17.58 (3.02-36.95)	17.84 * (1.42-70.92)	4.30 * (1.0-13.6)	3.94 (1.21-24.54)	62.88 * (4.49-81.09)	5.43 (1.93-67.61)
Category 3 (n = 7)	**Median** (range)	11.46 (1.57-52.52)	22.89 * (6.9-61.36)	10.32 * (4.05-22.81)	3.61 (2.03-13.45)	42.17* (6.25-74.52)	7.12 (0.71-31.13)

*$p < 0.05$ comparing uninfected with HIV-infected individuals CDC classified as categories 1 + 2 and category 3

Intracellular type 2 cytokine production

IL-4 production among $CD4^+$ T cell increased from 2.19% in uninfected control to 4.30% and 10.32% in HIV-1 infected children without AIDS and children with AIDS, respectively (Table 2 and Fig. 1). The frequency of $CD4^+$ T cell from HIV-1 infected children categories 1 + 2 expressing IL-4 was significantly ($p < 0.05$) higher compared that in with uninfected children. As shown in Table 2 and Fig. 1, the percentage of IL-4 secreting $CD4^+$ T cells in HIV-1 infected children categories 1 + 2 was significant higher than children in category 3 ($p < 0.05$). Similarly, the number of $CD8^+$ T cells which synthesize IL-4 showed increasing trend in HIV-1 infected children from categories 1 + 2 and category 3 in comparison with uninfected controls. Additionally, there was no significant IL-4 production by $CD8^+$ T cells obtained from HIV-1 infected children classified as CDC categories 1 + 2 and category 3 as compared to uninfected children.

DISCUSSION

This study is the first report of the intracellular cytokine detection in HIV-1 subtype E infected Thai children. In this study we investigated the correlation of cytokine pattern to immune status with disease progression in HIV-1 infected children. The method of examination the intracellular cytokine we use PMA and Ionomycin (mitogens) to stimulate intracellular cytokine in blood lymphocyte culture for 4-6 hrs and analyze by flow cytometry as described.[12,17] It is now well known that type 1 and type 2 cytokines are produced not only from $CD4^+$ T cells but also from $CD8^+$ T cells and non T cell such as macrophage.[5] Data of $CD4^+$ T cells and $CD8^+$ T cells were focused because $CD4^+$ T cells are the major targets of immune on AIDS progression and $CD8^+$ T cells may be of critical importance in resistance to HIV infection. For cell activation with PMA and Ionomycin has been reported to cause a great and more sustained decrease in $CD4^+$ expression and also a decrease in $CD8^+$ expression at different stage of disease in human peripheral T lymphocyte.[23] In additional of this effect was occurred from cell immunophenotype activation, the choice of mitogen and anticytokine antibodies and the time of cell harvest after activation.[23]

Our result showed that subtype E, IFN-γ expressing cell among $CD4^+$ T cells in mild and moderate stage of HIV-1 infection were increased compared with uninfected controls. In contrast, the other studies of perinatally HIV-1 infected children (non subtype E infection), the IFN-γ expressing cells were decreased which detect cytokine production by using ELISA[4,5] and RT PCR (reverse transcribed polymerase chain reaction).[5] It is also possible that the level of increasing IFN-γ contribute to the process of program cell death that has been reported in HIV-1 infection.[25] Otherwise IFN-γ production may be impaired at the $CD4^+$ T cells level.[3] However, there is controversy result may be partly due to the different experimental system used. The type 1 cytokine profile in the severely immunosuppression children (category 3) showed the reduction of IL-2 production among $CD4^+$ T cells implying that IL-2 expressing cell was decreased correlated with the declining of $CD4^+$ T cells.[5,19] It may be a critical step towards to development of AIDS. Whereas no differences in production of IL-2 was detected among different immunological categories of HIV-1 infected children compare with uninfected control subjects.[2]

Some studies have shown that IL-4 production among $CD4^+$ T cell by all categories of HIV-1 infected children was enhanced.[4,6,20] It may be due to T cells from HIV-1 infected children have impaired response to polyclonal T cell activation signals *in vitro*. Moreover, the average IL-4 production per $CD8^+$ T cells was increased in early stage (categories 1+2) and gradually increasing in late stage of HIV infection in category 3, confirming earlier reports[2,3,19] but not identity with recent data.[13,20,22] Although the cause and significance of this phenomenon still have to be identified.

As demonstration in Table 2 and Fig. 1, significant increase for IFN-γ production was greater than other cytokines in early stage (categories 1+2), especially in $CD8^+$ T cell. However, there was a trend toward a further increase IFN-γ expressing cells among $CD4^+$ T cells in more advance disease (category 3). These result confirm similar earlier reported data.[18,19,22] This may be reflect an enhanced activation of cytotoxic T cell and supports the hypothesis of an increasing population of anti-HIV specific $CD8^+$ T cells in course of infection. Another possibility reason that natural killer cell might be other source of IFN-γ expression can not be exclude from the study. The progression HIV specific Th1 cytokine response can generated from T cell proliferation[21] and HLA class 1-restricted cytotoxic T lymphocyte (CTLs).[1,24] It is therefore possible that exposure to low infection dose of HIV-1 would be optimal for generating as strong type 1 response.

Furthermore, the loss of immunoregulatory of Th 1 and Th 2 cytokine expression had been established as a mechanism of T cell depression via induction of apoptosis, antigen epitope, HLA genotype, antigen presenting cell and antiretroviral treatment.[19,24]

In summary, our finding suggest that there was an imbalance in type 1 and type 2 cytokine production in HIV-1 infected children. Thus, the disease progression of HIV-1 infected is associated with an increase in IL-4 production, we proposed that strong type 2 cytokine production pattern would be associated with the progression of HIV-1 infection. Whereas type 1 cytokine, IL-2 production was remained stable but the production of IFN-γ was enhanced. Further attention on data of single cell cytokine production with virus production, CTL and apoptosis is likely to resolve some of paradoxes in cytokine production of HIV infection. Thus, studies of type 1 and type 2 cytokine production have been useful in establishing the nature of protective and pathogenic immune response to pathogens.

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