Cytokine Profiles in HIV-1 Subtype CRF01_AE Infected Individuals with Different Rates of Diseases Progression: A Multiplex Immunoassay

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Objective: *Cytokines play an important role in controlling the homeostasis of the immune system and contribute to the pathogenesis of HIV infection. The measurement soluble cytokines in plasma of HIV-1 infected individuals with different rates of disease progression may provide additional information to complement prognostic markers and understand disease process. The aim of the present study was to determine the cytokine profiles in plasma of Thai HIV-1 CRF01_AE infected individuals with different rates of disease progression by using a multiplex system for simultaneous detection of 7 cytokines.*
Material and Method: *The authors used a multiplex immunoassay method to measure 7 cytokines (IL-2, IL-4, IL-6, IL-7, IL-10, IL-15 and IFN-gamma) in plasma of 23 progressors (PRs; symptomatic or AIDS within 5 years and CD4+ < 200/mm^3), 23 slower progressors (SPs; asymptomatic more than 5 years and CD4+ > 350/mm^3) and 23 normal healthy individuals.*
Results: *Both PRs and SPs demonstrated significantly higher levels of IL-7, IL-10 and IFN-gamma than healthy controls ($p < 0.05$). No significant difference in IL-6 between SPs and healthy controls but significant difference between RPs and controls were found. Furthermore, PRs showed significantly higher levels of plasma IL-6 ($p = 0.001$), IL-7 ($p = 0.016$), IL-10 ($p < 0.001$) and IFN-gamma ($p = 0.026$) than SPs. No significant difference in IL-2, IL-4 and IL-15 was found among 3 groups (PRs, SPs and healthy control).*
Conclusion: *These results suggested that a Th1 to Th2 cytokine switch did not occur. However, the measurements of plasma levels of cytokines could be used for predicting disease progression.*

Keywords: *Cytokine profiles, HIV-1 subtype CRF01_AE, Disease progression, Multiplex immunoassay*

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Cytokines are essential signal polypeptides within the immune system and play a important roles in controlling the homeostasis of the immune system[1]. They contribute to pathogenesis and disease progression of HIV-1 infection[2,3]. Cytokines have a complex effect on HIV-1 replication. Moreover, direct effect of HIV-1 itself may contribute to cytokine production in HIV-1 infected individuals. HIV-1 infection resulted immunodeficiency in humans was related to CD4+ T cell depletion and altered T helper (Th) cell function[4]. Th1 type cells produce Interleukin-2 (IL-2), and Interferon-gamma (IFN-gamma and they are the principal effectors of antiviral cell-mediated immunity[5], whereas Th2 type produce IL-4, IL-5, IL-6, IL-10 and IL-13[6] and also promote antibody production[4] and are related with immuno suppression and progression of HIV-1 infection[6,7]. An imbalance between the level

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of Th1 and Th2 cytokines was suggested to be involved in HIV disease progression. Several studies reported a Th1 to Th2 cytokine shift in HIV-1 infection[6,8,9]. However, controversial results were demonstrated[10-12]. In addition, other interleukins, such as IL-7 and IL-15, have gained considerable attention recently as homeostatic factors that may play a vital role in the survival, proliferation, differentiation and function of T lymphocytes[13,14]. Many studies reported increased IL-7 plasma levels in HIV-1 infected patients and associated with disease progression[15-17]. However, it was demonstrated reduced levels of IL-15 in sera from HIV-1 infected/AIDS patients[18]. On the other hand, higher IL-15 serum levels in HIV-1 infected individuals were reported[19].

HIV infection remains one of the critical public health problems in Thailand. Currently, the major subtype of HIV-1 circulating in Thailand is CRF01_AE. A study reported HIV-1 CRF01_AE was found in 97.3% and a predominance of subtype CRF01_AE was found in all geographic regions[20]. In addition, there are reports that the pathogenesis of different HIV-1 subtypes may differ[21,22]. However, there have been a few studies in cytokine profiles in HIV-1 CRF01_AE infected persons related to disease progression.

The aim of the present study was to investigate the plasma cytokines levels of Thai HIV-1 CRF01_AE infected individuals with different rates of disease progression and to analyze how these cytokines provide additional information to complement prognostic markers and understand disease process.

Material and Method

Study subjects

Thai HIV-1 CRF01_AE infected individuals were selected from patients enrolled in the hospitals in the North of Thailand by nested PCR method[23]. The patients were separated into progressors and slower progressors according to differences in CD4+ T cell counts and duration to show the symptom. Twenty-three subjects are progressors (PRs; CD4+ cells < 200/mm^3 and symptomatic or AIDS within 5 years) and 23 subjects are slower progressors (SPs; asymptomatic more than 5 years and CD4+ cells > 350/mm^3). Twenty-three normal healthy adults (HIV-1 seronegative) who were routine blood donors to the Army Institute of Pathology, Bangkok, Thailand voluntarily recruited, were included as controls. Blood from the patients and controls were collected into EDTA anticoagulant tubes. The study protocol was approved by the institutional review boards of Royal Thai Army Medical Department. The informed consent was obtained from all patients and controls.

Multiplex plasma cytokine assay (Luminex)

The following 7 cytokines were measured: IL-2, IL-4, IL-6, IL-7, IL-10, IL-15 and IFN-gamma in plasma of 23 PRs, 23 SPs and compared with 23 normal healthy individuals. Measurements using 25 microlitres of plasma were made by Luminex-based bead array method using the LINCOplex simultaneous multianalyte detection system (Linco Research, Inc) following the manufacturer's instructions. In brief, the principle of this assay is conventional sandwich assay technology. The antibody specific to each cytokine is covalently coupled to Luminex microspheres, with each antibody coupled to a different microsphere uniquely labeled with a fluorescent dye. The authors incubated the microspheres with standards, controls and samples (25 microlitres) in a 96-well microtiter fiber plate for 1 hour at room temperature. After incubation, the plate was washed to remove excess reagents and detection antibody, in the form of a mixture containing each of the seven antibodies, was added. After incubation 30-minutes at room temperature, streptavidin-phycoerythrin was added for an additional incubate 30 minutes. After the final wash step, the beads were resuspended in buffer and read on the Luminex 100 instrument to determine the concentration of the cytokines. All cytokines were simultaneously measured from a single specimen. Standard curves for each cytokine were generated by using the reference cytokine concentrations supplied by the manufacturer.

Statistical analysis

All data were presented as mean and standard deviation (SD). Mann-Whitney U test was used to compare cytokine mean values between groups. A p-value of < 0.05 was considered statistically significant.

Results

A total of 46 HIV-1 subtype CRF01_AE infected patients were enrolled in the present study. Twenty-three patients were PRs and 23 were SPs. The mean $\pm$ SD of CD4+ cell counts of PRs and SPs were 92 $\pm$ 61 and 624 $\pm$ 219 cells/mm^3, respectively. All patients were naive to anti-retroviral therapy. The authors evaluated plasma Th1 cytokines (IL-2 and IFN-gamma), Th2 cytokines (IL-4, IL-6, IL-10), IL-7 and IL-15 levels in Thai HIV-1 CRF01_AE infected patients with different rate of disease progression (both PRs and SPs) and 23

healthy controls in cross-sectional study by multiplex immunoassay.

Comparison of Th1 cytokines (IL-2, IFN-gamma) in PRs, SPs and healthy controls

The plasma levels of Th1 cytokines (IL-2, IFN-gamma), Th2 cytokines (IL-4, IL-6 and IL-10), IL-7 and IL-15 was simultaneously measured from a single specimen. The mean and SD values of the plasma levels of cytokine profiles of all groups and statistical results are shown in Table 1. No significant difference in plasma IL-2 level was found among PRs (mean = 8.74 pg/ml), SPs (mean = 4.13 pg/ml) and healthy controls (mean = 4.71 pg/ml). In contrast, the mean levels of IFN-gamma in PRs (mean = 42.24 pg/ml) was significantly higher than SPs (mean = 7.71 pg/ml, $p = 0.027$) and healthy controls (mean = 1.04 pg/ml, $p < 0.001$).

Comparison of Th2 cytokines (IL-4, IL-6 and IL-10) in PRs, SPs and healthy controls

As shown in Table 1, the authors found that there was no significant difference in IL-4 levels among PRs (mean = 98.85 pg/ml), SPs (mean = 30.90 pg/ml) and healthy controls (mean = 28.69 pg/ml). Notably, the mean IL-4 in PRs was higher when compared with SPs and healthy controls but this difference was not significant. For the levels of IL-6, PRs showed significantly higher than SPs (mean = 14.11 versus 1.71 pg/ml, $p = 0.002$) and healthy controls (mean = 14.11 versus 1.38 pg/ml, $p = 0.009$) but IL-6 levels in SPs was not significantly different from healthy controls (mean = 1.71 versus 1.38 pg/ml).

Both PRs and SPs demonstrated significantly higher levels of IL-10 than healthy controls [mean = 472.91 pg/ml (PRs), 35.12 pg/ml (SPs) versus 9.04 pg/ml (controls), $p < 0.001$]. Moreover, PRs also showed IL-10 levels was significantly higher than SPs (mean = 472.91 versus 35.12 pg/ml, $p < 0.001$).

Comparison of IL-7 and IL-15 in PRs, SPs and healthy controls

The mean levels of IL-7 in PRs was significantly higher than SPs (PRs = 10.99 pg/ml, SP = 0.94 pg/ml, $p = 0.041$). Both IL-7 levels of PRs and SPs showed significantly higher than healthy controls ($p < 0.001$, $p = 0.019$, respectively). Notably, IL-7 was undetectable in Thai healthy controls.

There was no significant difference in the mean IL-15 levels among the 3 groups. The mean levels of IL-15 in PRs, SPs and healthy controls were 10.78 pg/ml, 1.99 pg/ml and 2.86 pg/ml respectively.

Discussion

In the current study, for Th1 cytokines (IL-2 and IFN-gamma), the authors have demonstrated plasma IL-2 levels of HIV-1 CRF01_AE infected patients (both PRs and SPs) was not significantly different from healthy controls. Many studies reported the decreased IL-2 in HIV-1 infected patients with low CD4+ T cells[10,24,25]. However, the present study of Granzioai et al consistent with the present finding was shown[11]. They reported on the cytokine expression in unfractionated and sorted T cell populations isolated from peripheral blood and lymph nodes of HIV infected patients at different stages of the disease. It was found that IL-2 and IL-4 expression was scarcely detectable at any stage of disease. Interestingly, although IL-2 and IFN-gamma are Th1 cytokines, they demonstrated different changes in HIV-1 disease progression in the present study. Unlike IL-2, IFN-gamma levels in PRs were greater than SPs and healthy controls. It suggested that IFN-gamma is elevated progressively with more advanced disease. The present results concur with the findings that elevated plasma levels of IFN-gamma[26,27] and IFN-gamma expression[10] have been described in patients with HIV infection. The increase in IFN-gamma should be produced by cells other than CD4+ T cells such as CD8+ T cells and NK cells[28]. As HIV disease progression, the CD8+ T cells are a large proportion of T cells. A continuous decrease in IFN-gamma in the course of HIV-1 infection were controversial in some studies[6,8].

Th2 cytokines studied in the present study are IL-4, IL-6 and IL-10. Like IL-2, the authors found no significant difference in IL-4 among 3 groups. Although the increasing in plasma IL-4 levels were observed in some studies[6,25], the present study supported that IL-4, a regulatory cytokine, were not elevated in HIV-1 infected patients plasma[11,29]. In fact, the levels of plasma cytokine do not only reflect the production of cytokines but also are modified by cell-bound receptors removal, neutralization by soluble receptors, metabolism, and excretion. Highest levels of IL-6 and IL-10 in PRs were well demonstrated in the present study. The present data suggested that increasing of IL-6 and IL-10 levels in HIV-infected patients was associated with the progression of AIDS. Elevated levels of IL-6, IL-10 in serum or plasma have been described previously in the advanced stage of HIV-1 infected patients[25,26,30]. Poli et al demonstrated that IL-6 induced HIV replication by transcriptional and post-transcriptional mechanisms, affecting the late steps of HIV biological cycle[31]. However, Marfaing-

Table 1. Levels of cytokines in plasma of HIV-1subtype CRF01_AE Thai individuals with different rates of disease progression and normal controls

Variable	Mean (SD), Median (range)			p-value*		
	PRs n = 23	SPs n = 23	Controls n = 23	PRs vs. SPs	PRs vs. Controls	SPs vs. Controls
CD4+ (cells/mm^3)	92 (61) 100 (6-188)	624 (219) 545 (392-1,232)		< 0.001		
IL-2 (pg/ml)	8.79 (10.41) 4.71 (0.00-30.76)	4.13 (5.42) 1.79 (0.00-23.16)	4.71 (9.44) 1.96 (0.45-45.69)	NS	NS	NS
IL-4 (pg/ml)	98.85 (223.96) 0.00 (0.00-994.87)	30.90 (94.38) 0.00 (0.00-349.04)	28.69 (66.53) 0.00 (0.00-250.82)	NS	NS	NS
IL-6 (pg/ml)	14.11 (24.20) 3.80 (0.00-95.06)	1.71 (5.97) 0.00 (0.00-27.83)	1.38 (3.69) 0.00 (0.00-16.47)	0.002	0.009	NS
IL-7 (pg/ml)	10.99 (18.29) 0.00 (0.00-59.75)	0.94 (2.99) 0.00 (0.00-13.85)	0.00 (0.00) 0.00 (0.00-0.00)	0.041	< 0.001	0.019
IL-10 (pg/ml)	472.91 (516.84) 327.08 (0.00-993.91)	35.12 (46.12) 26.82 (0.00-215.20)	9.04 (10.62) 6.56 (0.00-34.74)	< 0.001	< 0.001	0.006
IL-15 (pg/ml)	10.78 (21.90) 0.00 (0.00-86.17)	1.99 (5.88) 0.00 (0.00-26.15)	2.86 (8.40) 0.00 (0.00-37.12)	NS	NS	NS
IFN-g (pg/ml)	42.24 (56.29) 16.63 (0.00-187.60)	7.71 (14.77) 0.00 (0.00-60.06)	1.04 (3.13) 0.00 (0.00-14.50)	0.027	< 0.001	0.040

* Mann-Whitney U test, NS = not significant

Koka et al showed increased IL-6 production in HIV-infected patients at a late stage of the infection may not stimulate HIV replication in vivo, but it may represent a key mechanism contributing to the metabolic and immunological dysbalance of the disease[32]. Compared with nonprogressors, the patients with disease progression had increasing IL-10 levels in serum, in contrast to non-progressing patients where levels were stable[33]. In addition, Elrefei et al reported that the presence of the IL-10 positive CD8+ T cells was associated positively with plasma HIV viral load. They suggested that these IL-10 positive CD8+ T cells may have an important regulatory role in the immune dysfunction observed in HIV infection[34].

IL-7 is a important cytokine regulating T-lymphocyte development. Elevated levels of IL-7 in PRs were demonstrated in the present study and they also supported several studies that increased IL-7 plasma levels indicated HIV-1 induced T cell depletion and disease progression[15-17,35]. Some studies demonstrated IL-7 enhanced HIV-1 infection, replication and cytopathic *in vivo*[15,17]. Increased plasma IL-7 in PRs suggests that IL-7 may acte as a regulator of T cell homeostasis by increased endogenous production to stimulate lymphocytes development and expansion in response to CD4+ T cell depletion. Notably, plasma levels of IL-7 was undetectable in Thai normal controls. However, the lack of circulating levels of IL-7 was found in both controls and patients with good immunologic response to treatment[35].

IL-15 plays an important role in maturation and differentiation of T cells, B cells and NK cells. The present study demonstrated no significant changes in plasma levels of IL-15 in HIV-1 CRF01_AE infected patients compared with healthy controls. The authors observed here that there was a trend of increasing in IL-15 levels in PRs, which indicated IL-15 levels might be associated with disease progression. Kacani et al reported serum levels of IL-15 were significantly elevated in HIV-1 infected individuals and correlated with immunoglobulin serum levels, indicating that IL-15 may contribute to the pathogenesis of HIV-1 associated hypergammaglobulinaemia even in the late stages of infection[19]. In contrast, Ahmad et al showed reduced levels of IL-15 in sea from HIV-infected/AIDS patients[18]. They explained that IL-15 was known to enhance both innate and adaptive immune responses *in vitro* and *in vivo*, its reduced concentrations in the sera of AIDS patients was found in these patients who were immunodeficiency. The reasons of discordant results can not be clearly explained. There might be some difference in the present study cohorts of patients and also the criteria of PRs and SPs that the authors used in the present study. It was noteworthy that the authors did not find the difference between IL-2, IL-4 and IL-15 among 3 groups. One possibility was the present results showed wide variabilities among individuals in the cytokine-production pattern.

Both Th1 and Th2 cytokines have been involved in the pathogenesis of HIV infection. Increased production of cytokines may affect the equilibrium between the virus and host-derived immune response in HIV-1 disease progression. Furthermore, progression of HIV disease is associated with increasing dysregulation of Th1 lymphocytes and cytokines, with diverse changes in Th2 cytokines.

In conclusion, The authors demonstrated that Th1 to Th2 cytokine switch did not found in the present study. Elevation of plasma cytokines levels of IL-6, IL-7, IL-10 and IFN-gamma are associated with more advanced HIV infection, whether it is a cause or effect or may be both is not known. However, they could be used as prognostic markers of disease progression.

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Potential conflicts of interest

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การศึกษาไซโตไคน์ในคนไทยผู้ติดเชื้อเอชไอวีสับทัยป์ อี ที่มีอัตราการลุกลามของโรคแตกต่างกัน โดยวิธี Multiplex Immunoassay

ทิพย์วรรณ ชื่นจิตร, พิธภัณฑ์ ชายทวีป, สุจิตรา สุขวิทย์, ศักดิ์ชัย เดชตรัยรัตน์, สุชชนา แทบประสิทธิ์, สุรางค์รัตน์ ศรีสุรภานนท์, สุรพล เกาะเรียนอุดม, ดลนภัทร กุวานนท์, รวงผึ้ง สุทเธนทร์, ณรงค์ฤทธิ์ ศิริโสภณา, สรชัย นิตยพันธ์

วัตถุประสงค์: *ไซโตไคน์มีบทบาทสำคัญในการควบคุมระบบภูมิคุ้มกัน และมีส่วนร่วมในการเกิดพยาธิสภาพของการติดเชื้อ เอชไอวี การตรวจวัดไซโตไคน์ในน้ำเหลืองของผู้ติดเชื้อเอชไอวีที่มีการลุกลามโรคแตกต่างกัน อาจนำมาใช้เป็นเครื่องบ่งชี้การลุกลามโรคเร็วขึ้น วัตถุประสงค์ของการศึกษานี้ เพื่อหาไซโตไคน์ในน้ำเหลืองของคนไทยผู้ติดเชื้อเอชไอวีสับทัยป์อี ที่มีอัตราการลุกลามโรคที่แตกต่างกัน*

วัสดุและวิธีการ: *การศึกษานี้ใช้ระบบ multiplex เพื่อตรวจหาไซโตไซน์พร้อมกันถึง 7 ชนิด คือ IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-15 และ IFN-g ในน้ำเหลืองของกลุ่มดำเนินโรคเร็ว (มีอาการภายใน 5 ปี หรือเข้าสู่ระยะเอดส์และมี CD4+ < 200/ลบ.มม.) จำนวน 23 ราย และกลุ่มดำเนินโรคช้า (ไม่มีอาการภายใน 5 ปี หรือเข้าสู่ระยะเอดส์และมี CD4+ < 200/ลบ.มม.) จำนวน 23 ราย เปรียบเทียบกับกลุ่มควบคุม (คนปกติที่มีสุขภาพดี) จำนวน 23 ราย*

ผลการศึกษา: *ทั้งกลุ่มดำเนินโรคเร็ว และกลุ่มดำเนินโรคช้า มีระดับของ IL-10, IL-15 และ IFN-g สูงกว่าคนปกติอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ไม่พบความแตกต่างของ IL- 6 ระหว่าง กลุ่มดำเนินโรคช้าและกลุ่มควบคุม แต่พบว่ามีความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มดำเนินโรคเร็วและกลุ่มควบคุม ยิ่งไปกว่านั้นเราพบว่า IL-6 ($p = 0.001$), IL-7 ($p = 0.016$), IL-10 ($p < 0.001$) และ IFN-g ($p = 0.026$) ในกลุ่มดำเนินโรคเร็ว สูงกว่าในกลุ่มดำเนินโรคช้าอย่างมีนัยสำคัญทางสถิติ แต่ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของ IL-2, IL-4 และ IL-15 ในทั้งสามกลุ่ม*

สรุป: *ผลการศึกษาดังกล่าวแสดงให้เห็นว่า ไม่มีการเปลี่ยนแปลงของไซโตไคน์ชนิด Th1 เป็น ไซโตไคน์ชนิด Th2 อย่างไรก็ตามการวัดระดับของไซโตไคน์ในน้ำเหลืองสามารถใช้ในการทำนายการลุกลามโรคได้*