Virological and immunological characteristics of HIV-infected individuals at the earliest stage of infection

Jintanat Ananworanich1,2,3,*, Carlo P. Sacdalan1, Suteeraporn Pinyakorn1,2,3, Nicolas Chomont4, Mark de Souza1, Tassanee Luekasemsuk1, Alexandra Schuetz2,3,5, Shelly J Krebs2,3, Robin Dewar6, Linda Jagodzinski2,3, Sasiwimol Ubolyam7, Rapee Trichavaroj5, Sodsai Tovanabutra2,3, Serena Spudich9, Victor Valcour9, Irini Sereti10, Nelson Michael3, Merlin Robb2,3, Praphan Phanuphak1,7, Jerome H. Kim3,11, and Nittaya Phanuphak1 on behalf of the RV254/SEARCH010 Study Group

1SEARCH, The Thai Red Cross AIDS Research Centre, Bangkok, Thailand 2The Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, MD, USA 3United States Military HIV Research Program; Walter Reed Army Institute of Research, Silver Spring, MD, USA 4Department of Microbiology, Infectiology, and Immunology, Université de Montréal, Faculty of Medicine, and Centre de Recherche du CHUM, Montreal, Quebec, Canada 5Armed Forces Research Institute of Medical Sciences, US Component, Bangkok, Thailand 6Leidos Biomedical Research Inc, Virus Isolation and Serology Laboratory, Frederick, MD, USA 7HIV-NAT, The Thai Red Cross AIDS Research Centre, Bangkok, Thailand 8Department of Neurology, Yale University, New Haven, CT, USA 9Department of Neurology, University of California, San Francisco, CA, USA 10National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA

Abstract

**Background**—The challenges of identifying acute HIV infection (AHI) have resulted in a lack of critical information on early AHI that constrains the development of therapeutics that are designed to eradicate HIV from the infected host.

**Methods**—AHI participants were recruited from the Thai Red Cross Anonymous Clinic in Bangkok, Thailand into the RV254/SEARCH010 protocol and categorised according to Fiebig stages as follows: Fiebig I (HIV-RNA+, p24 Ag−, HIV IgM−) and Fiebig II–IV (HIV-RNA+, p24 Ag + or −, HIV IgM− or +, Western blot- or indeterminate). Proviral and viral burden and immune activation levels were compared between Fiebig stage groups at the time of AHI. CD4 and CD4/CD8 ratio were also compared between groups before and up to 96 weeks of ART.
Results—Median age was 27 years and 96% were male. Fiebig I individuals had lower median HIV-DNA in mononuclear cells from blood (3 vs. 190 copies/10^6 cells) and gut (0 vs. 898 copies/10^6 cells), and lower HIV-RNA in blood (4.2 vs. 6.2 log_{10} copies/mL), gut (1.7 vs. 3.1 log_{10} copies/mg) and cerebrospinal fluid (2.0 vs. 3.8 log_{10} copies/mL), when compared to Fiebig II–IV individuals (all \( P < 0.01 \)). Median plasma sCD14 level was lower (1.1 vs. 1.6 μg/mL) in Fiebig I individuals as was the frequency of CD8+HLADR+CD38+ T cells in blood (7.6 vs. 14.9%, both \( P < 0.05 \)). The median plasma interleukin 6 levels were similar between stages (0.6 in Fiebig I vs. 0.5 pg/mL in Fiebig II–IV, \( P > 0.05 \)). The frequencies of CD4+HLA-DR+CD38+ T cells were also similar between these stages (2.1 vs. 2.6%, \( P > 0.05 \)). Median CD4 count and CD4/CD8 ratio were higher in Fiebig I: 508 vs. 340 cells/mm^3 and 1.1 vs. 0.7, respectively (both \( P < 0.001 \)). After ART, CD4 cell count normalised by week 24 in Fiebig I and week 48 in Fiebig II–IV. However, CD4/CD8 ratio was lower in both groups after 96 weeks of ART compared to healthy Thais (\( P = 0.02 \)).

Conclusions—Compared to later AHI stages, Fiebig I was associated with lower HIV burden in blood and tissue compartments, lower immune activation and higher CD4 and CD4/CD8 ratio. ART in Fiebig I–IV resulted in normalisation of CD4 cell count within the first year, supporting the benefit of early ART. However, the CD4/CD8 ratio was not normalised after 2 years of ART in all AHI stages, suggesting some degree of persistent immunological dysfunction even when ART was instituted as early as Fiebig I.

Keywords
acute HIV infection; Fiebig I; reservoir; immune activation; CD4; CD4/CD8 ratio

Background

Information is limited on the immunological and virological events that occur during the earliest stages of acute HIV infection (AHI) when HIV serology is still non-reactive, due mainly to difficulties in identifying such individuals [1]. The AHI period usually spans the first month of infection and is categorised into four stages by Fiebig and Busch et al. [2]. During the first few hours to days after HIV infection, the virus replicates in tissue but is not yet detected in blood. This so-called ‘eclipse phase’ is immediately followed by the Fiebig I stage in which HIV-RNA in plasma rises to a level of at least 100 copies/mL but the standard HIV diagnostic tests for p24 antigen and HIV antibody are still negative. Documenting immunological and virological parameters during Fiebig I is important to the understanding of HIV pathogenesis [3]. The timing and extent of HIV seeding in blood and tissue cells, CD4 depletion and immune activation are not well understood at the earliest stages of HIV infection [4,5]. Such knowledge could inform therapeutic strategies to mitigate the impact of HIV on the host.

Here we describe a large cohort of individuals with AHI in Thailand and compare immunological and virological data from two Fiebig groups: Fiebig I versus Fiebig II–IV (after the initial detection of p24 antigen and/or HIV antibody). The objective is to identify unique characteristics of Fiebig I individuals with regards to HIV proviral and viral burden, CD4 depletion and immune activation. We also report the CD4 and the CD4/CD8 responses to antiretroviral therapy (ART) in these two groups, since CD4 and CD4/CD8 ratio before
and after ART are predictors of long-term morbidity and mortality in HIV [6,7]. Existing
data demonstrate that these may not normalise in individuals who start treatment late [8].
However, data comparing these responses in individuals who initiated ART very early have
been limited [9,10].

Methods

The current analysis encompassed data collected between April 2009 and July 2015 from
participants enrolled in the RV254/SEARCH010 study (clinicaltrials.gov identification
NCT00796146), which is an ongoing prospective study of AHI in Thailand. Briefly, clients
of the Thai Red Cross Anonymous HIV testing Clinic in Bangkok were screened in real time
for AHI by pooled nucleic acid testing (NAT) or sequential immunoassay as previously
described [11]. Participants with a positive NAT and a non-reactive HIV IgG were invited to
join the study.

Further testing was performed to categorise them into Fiebig stages as follows: Fiebig I
(HIV-RNA+, p24 antigen-, HIV IgM−), Fiebig II (HIV-RNA+, p24 antigen+, HIV IgM−),
Fiebig III (HIV IgM+, Western blot-) and Fiebig IV (HIV IgM+, Western blot
indeterminate). The corresponding mean cumulative durations from onset of HIV viraemia
according to Fiebig et al. are 5 (Fiebig I), 10.3 (Fiebig II), 13.5 (Fiebig III) and 19.1 (Fiebig
IV) days [2]. For this study, we reported estimated infection duration from history of HIV
exposure within the last 30 days. Flexible sigmoidoscopy and biopsy, and cerebrospinal
fluid (CSF) collection were optional procedures. ART was also optional and offered as part
of an accompanying protocol (clinicaltrials.gov identification NCT00796263). Treatment
included standard doses of a three-drug regimen (tenofovir, lamivudine or emtricitabine, and
efavirenz) with some participants receiving a five-drug regimen with the addition of
raltegravir and maraviroc during the first 24 weeks. The Thai Chulalongkorn University and
relevant US and Canadian institutional review boards approved these studies. All
participants provided informed consent.

Laboratory methods were based on assays previously described by our group [12,13]. CD4
cell count was measured by dual-platform flow cytometry (Becton-Dickinson, USA). HIV-
RNA in plasma and CSF was performed using the COBAS AMPLICOR HIV-1 Monitor
Test v1.5 or Cobas Taqman v2.0 (Roche Molecular Systems, USA). The Siemens
Quantiplex HIV-1 3.0 assay was used to measure HIV-RNA (copy/mg of gut tissue). Total
HIV-DNA in peripheral blood mononuclear cells (PBMCs) and sigmoid colon were
quantified using a modified nested PCR assay for CRF01_AE and B [14].
Immunophenotyping was performed on PBMCs for activated CD4 (CD4+HLA-DR
+CD38+) and CD8 (CD8+HLA-DR+CD38+) T cells as previously described [5]. Plasma
soluble CD14 (sCD14) and interleukin 6 (IL-6) were measured by ELISA (R&D Systems,
Minneapolis, Minnesota, USA). HIV subtyping was performed using the multi-region
hybridisation real-time PCR assay for subtypes B, C and CRF01_AE [15].

Statistical analysis

Median (IQR) values were described for each variable. Comparison between Fiebig I and
Fiebig II–IV groups was carried out using Mann–Whitney U test or Student t-test for
continuous variables; Chi-squared or Fisher’s exact test were used for categorical variables. Data from HIV-uninfected Thais were used for comparison when available, and these were either from published data [16] or our concurrent RV304/SEARCH 013 study (clinicaltrials.gov identification NCT01397669). Generalised Estimating Equation model (GEE) was used to estimate mean changes in CD4/CD8 ratio, adjusted by baseline values. Logistic regression model was used to determine predictors for achieving CD4/CD8 ratio ≥ 1 after 96 weeks of treatment. Statistical tests were two-sided and $P$ values <0.05 were considered statistically significant. Analyses were performed using StataCorp 2013 (StataCorp LP, College Station, TX, USA). Figures were generated using Prism version 6.02 for Windows (GraphPad Software, La Jolla, California, USA).

Results

The RV254/SEARCH010 study screened 147,563 samples to identify 353 acutely infected individuals. Of these, 292 were enrolled in the study and the first 268 cases were included in this analysis because they were in Fiebig I to IV at time of ART initiation. Twenty-four were excluded either because they progressed to Fiebig V/VI at enrollment ($n=21$) or they did not initiate ART at enrolment ($n=3$). The majority of enrollees were young men who have sex with men. The most common HIV clade was CRF01_AE. Fiebig I individuals constituted 16% of participants. The estimated infection duration by history was shorter in Fiebig I (Table 1).

Figure 1a features the viral and proviral burden in different compartments. The HIV-RNA in blood, gut and CSF, and the total HIV-DNA in blood and gut were all significantly lower in Fiebig I versus Fiebig II–IV, $P<0.05$. The frequency of activated CD8 T cells and sCD14 level were lower in individuals captured in Fiebig I, whereas the frequency of activated CD4 T cells and IL-6 level were not different between Fiebig groups (Figure 1b). Compared to HIV-uninfected Thais, we found that both Fiebig groups had similar IL-6 levels, but higher sCD14 levels ($P=0.02$ for Fiebig I, $P<0.0001$ for Fiebig II–IV) and higher frequencies of activated CD8 T cells ($P=0.008$ for Fiebig I, $P<0.0001$ for Fiebig II–IV). The median (interquartile ranges, IQR) values from uninfected Thais were 0.5 (0.3–0.8) pg/mL for IL-6 ($n=29$), 0.8 (0.8–1.0) μg/mL for sCD14 ($n=10$), and 3.0% (2.8–3.5) for activated CD8 T cells ($n=9$). The frequency of activated CD4 T cells was similar to nine uninfected Thais (median 1.5%, IQR 1.2–2.1) for Fiebig I ($P=0.18$), but not for Fiebig II–IV ($P=0.03$) individuals.

The absolute CD4 T cell count (Figure 2a) and CD4/CD8 ratio (Figure 2b) were compared from baseline and post-ART between the two groups. Before ART, the CD4 cell counts were higher in Fiebig I versus Fiebig II–IV ($P<0.001$), but both were significantly lower than in 59 controls (mean 730, SD 190), $P<0.001$ for all. Fiebig I individuals also had higher CD4/CD8 ratio than the Fiebig II–IV individuals (1.1 vs. 0.7, $P<0.001$), but again both were lower than in uninfected Thai controls ($P=0.01$ for Fiebig I, $P<0.001$ for Fiebig II–IV). The mean CD4/CD8 ratio from 216 HIV-uninfected Thais was 1.35 (SD 0.48). The proportions with CD4/CD8 ratio ≥1 were 61% in Fiebig I versus 28% in Fiebig II–IV, $P<0.001$. 

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ART was initiated at a median (IQR) of 0 (0–1) days from study enrollment in both groups. The majority received the three-drug ART regimen (n=187, 70%) and 81 (30%) received the five-drug regimen. After ART, the proportions of participants with plasma HIV-RNA <50 copies/mL were 92% at week 24, 98% at week 48 and 99% at week 96. The CD4 T cell counts and CD4/CD8 ratio were not statistically different between the two Fiebig groups following ART. The CD4 cell count normalised by week 24 in Fiebig I and by week 48 in Fiebig II–IV participants. The CD4/CD8 ratio after treatment did not change from baseline in the Fiebig I group, but in the Fiebig II–IV group, the mean (95% CI) changes by GEE analysis, adjusted for baseline values were 0.27 (0.22–0.32) at week 24, 0.31 (0.26–0.36) at week 48 and 0.36 (0.30–0.42) at week 96 (P<0.001 for all). However, the CD4/CD8 ratio remained persistently low in both groups up to week 96 compared to uninfected controls, P=0.02. By univariate logistic regression analysis, baseline predictors for achieving CD4/CD8 ratio ≥1 after 96 weeks of treatment were CD4>350 cells/mm$^3$ [odds ratio (95% confidence interval, CI) 3.7 (1.7–8.1), P=0.001] and CD4/CD8 ratio higher than the median value of 0.8 [odds ratio (95% CI): 13.0 (5.0–33.5), P<0.001]. Types of ART regimens did not affect HIV-RNA, CD4 and CD4/CD8 ratio.

**Discussion**

The earliest stage of acute infection, Fiebig I, was associated with significantly lower viral and proviral burden, less immune activation and a better CD4 cell count and CD4/CD8 ratio compared to the later stages of Fiebig II–IV; suggesting that intervening with ART in Fiebig I may be more beneficial in mitigating HIV persistence and immune activation, and protecting immune function.

Based on self-reported history of exposure, the duration of HIV infection was shorter in Fiebig I by a median of 6 days with marked lower plasma HIV-RNA by 2 log$_{10}$ and total HIV-DNA in PBMCs by 1.8 log$_{10}$ in Fiebig I compared to the other group. These differences may impact long-term outcomes. Plasma HIV-RNA predicts CD4 depletion and subsequent progression to AIDS and death in untreated HIV [17], and both virological failure and poor CD4 recovery are associated with these outcomes in treated HIV [18]. HIV-DNA in PBMCs before ART is correlated with post-treatment HIV-DNA levels, residual viraemia and immune activation [19–22]. Importantly, in the SPARTAC trial, pre-treatment total PBMC HIV-DNA predicted time to viral rebound when ART was removed [23]. Indeed our group has shown that ART initiated at the Fiebig I stage is associated with extremely low frequencies of latently infected cells in PBMCs and in all CD4 subsets [24].

The viral and proviral burden in sigmoid biopsies were also significantly lower in the Fiebig I participants. The gut represents a major HIV reservoir site due partly to the high frequencies of target cells including CCR5+CD4+ and Th17 cells [25,26]. In the Fiebig I participants, the HIV-RNA was 1.4 log$_{10}$ lower and the total HIV-DNA was 2.7 log$_{10}$ lower in the gut compared to the Fiebig II–IV group. The central nervous system (CNS) is another important HIV reservoir site where brain microglial cells and astrocytes can harbour HIV, and infected peripheral blood cells traffic to the CNS [27]. CSF HIV-RNA is used as an indirect marker of brain infection, and we show that the CSF HIV-RNA is much lower if infection is identified in Fiebig I.
There was a higher frequency of activated CD8 T cells and a higher level of sCD14 in Fiebig II–IV. In contrast, the frequency of activated CD4 T cells and plasma IL-6 levels were similar in both groups. This is in line with previous reports showing that CD8 T cells are activated early to control viral load set-point in untreated acutely infected persons [28,29]. IL-6 slowly rises following infection; therefore, it was not yet elevated in our participants [30]. sCD14 is a marker of monocyte activation that could be elevated in response to gut endothelial damage and microbial translocation. We have previously shown that gut CD4 depletion occurs after Fiebig I [5], which correlates with the higher sCD14 level observed in later stages of acute infection. Persistent elevation of activated CD8 T cells, sCD14 and IL-6 are associated with AIDS and non-AIDS deaths in chronic HIV infection [31–33]. These data suggest that if HIV is diagnosed early enough, there is an opportunity to intervene and prevent or reverse immune activation. Early ART was associated with normalisation of cellular immune activation, and some, but not all, soluble inflammatory biomarkers [5,34].

Our data showed that the CD4 T cell count and CD4/CD8 ratio were better in Fiebig I participants. Over half of Fiebig I but only one-third of Fiebig II–IV individuals had CD4/CD8 ratio ≥1, a ‘normal’ threshold used in several studies [6,7,35]. Both groups, however, had CD4 cell count and CD4/CD8 ratio that were significantly lower than those in uninfected Thai controls [16]. This illustrates the rapidity of immune damage caused by HIV. Low CD4 and CD4/CD8 ratio correlate with poorer HIV disease outcomes [6,7,36]. The CD4/CD8 ratio has been proposed as a better marker for immune dysfunction in HIV than CD4 cell count because it reflects both the CD4 depletion and the activation and proliferation of CD8 T cells in HIV infection [35].

Studies in chronically HIV-infected individuals showed that CD4 and CD4/CD8 ratio recovery were slow even after years of suppressive ART [8,9]. In a large study of adults with chronic HIV infection with a median CD4/CD8 ratio of 0.39, the probability of achieving CD4/CD8 ratio ≥1 was 11.5% at 2 years after ART [8]. Whereas, adults treated within the first 6 months of infection had superior CD4 and CD4/CD8 ratio recovery [9,10]. Here we show that if ART is initiated in Fiebig I–IV AHI, CD4 cell counts recover to normal levels within the first year, with a faster recovery in the Fiebig I individuals. Intriguingly, the CD4/CD8 ratio in Fiebig I remained stable post-ART, whereas in the Fiebig II–IV group, there was a rapid rise in CD4/CD8 ratio within the first 24 weeks of ART. After 96 weeks of ART, both groups had a median CD4/CD8 ratio above 1, but the ratio was lower than in uninfected Thais, and suggests that some persistent immune dysfunction exists even when ART is initiated as early as Fiebig I.

Our rationale for combining the Fiebig II–IV as a group is as follows. First, our intention here is to focus on the Fiebig I group, for whom published data are most lacking. Second, the virological and immunological profiles are largely similar amongst the Fiebig II–IV individuals, particularly for proviral DNA in blood and tissue, and immune activation markers (data not shown). Our data has limitations. The available data from healthy Thais varied, and for some markers, there were as few as nine controls (e.g. activated T cells). This limits the interpretation of the data from our acutely infected participants. Our analysis lacked longitudinal comparisons for most markers for which studies are ongoing. Finally,
identifying Fiebig I individuals is not easy. Here we screened large numbers of samples from clients who sought HIV testing at a single centre to enrol 44 Fiebig I participants. Diagnosing AHI requires increased awareness for early testing by the at-risk persons and the healthcare providers. Nucleic acid testing is needed to diagnose Fiebig I acute infection. However, new developments using sample-to-cut-off ratio from standard fourth-generation antigen–antibody combo assays are being validated for early acute infection diagnosis, which could significantly improve the identification of Fiebig I and later stages of AHI [37].

In summary, our study contributes data on events during the very early AHI period. We demonstrate stark differences in the virological and immunological profiles between Fiebig I and Fiebig II–IV acute infection, highlighting the dynamic processes during AHI. The Fiebig I participants had limited viral and proviral burden, immune activation and CD4 depletion. Initiating ART in Fiebig I may afford the opportunity to markedly mitigate further HIV reservoir seeding and immune damage. The rapid recovery of CD4 cell counts during ART supports initiation of therapy in acute infection. However, the low CD4/CD8 ratio even after very early ART in Fiebig I suggests persistent immune dysfunction and raises the potential need for additional immune therapies that could enhance immune recovery and dampen immune activation.

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Figure 1.

**Figure 1a.** Viral and proviral burden in Fiebig I vs. Fiebig II–IV acute HIV infection. The number of participants included for each value is as follows: plasma HIV-RNA (Fiebig I, n=44, Fiebig II–IV, n=224); gut (sigmoid colon) HIV-RNA (Fiebig I, n=9, Fiebig II–IV, n=35); cerebrospinal fluid (CSF) HIV-RNA (Fiebig I, n=7, Fiebig II–IV, n=35); total HIV-DNA in peripheral blood mononuclear cells (PBMCs) (Fiebig I, n=11, Fiebig II–IV, n=60) and total HIV-DNA in gut (sigmoid colon mononuclear cells) (Fiebig I, n=5, Fiebig II–IV, n=21)
**Figure 1b.** Immune activation markers in Fiebig I vs. Fiebig II–IV acute HIV infection. The number of participants included for each value is as follows: CD4+/HLA-DR/CD38+ T cells (Fiebig I, n=8, Fiebig II–IV, n=33); CD8+/HLA-DR/CD38+ T cells (Fiebig I, n=8, Fiebig II–IV, n=33); interleukin-6 (IL6) (Fiebig I, n=12, Fiebig II–IV, n=66); soluble CD14 (sCD14) (Fiebig I, n=11, Fiebig II–IV, n=61)
Figure 2.

**Figure 2a.** CD4+ T cell counts in Fiebig I vs. Fiebig II–IV acute HIV infection before and after antiretroviral therapy. The dotted line represents the mean value in HIV-uninfected Thais. The number of participants included for each time-point is as follows: Week 0 (Fiebig I, n=44, Fiebig II–IV, n=224); Week 24 (Fiebig I, n=41, Fiebig II–IV, n=198); Week 48 (Fiebig I, n=34, Fiebig II–IV, n=162); Week 96 (Fiebig I, n=24, Fiebig II–IV, n=98).

**Figure 2b.** CD4/CD8 ratio in Fiebig I vs. Fiebig II–IV acute HIV infection before and after antiretroviral therapy. The dotted line represents the mean value in HIV-uninfected Thais. The number of participants included for each time-point is as follows: Week 0 (Fiebig I, n=44, Fiebig II–IV, n=224); Week 24 (Fiebig I, n=41, Fiebig II–IV, n=197); Week 48 (Fiebig I, n=34, Fiebig II–IV, n=159); Week 96 (Fiebig I, n=24, Fiebig II–IV, n=97).
## Table 1

Characteristics of Fiebig I vs. Fiebig II–IV acute HIV-infected participants

| Characteristics                      | All (n=268) | Fiebig I (n=44) | Fiebig II–IV (n=224) | P value |
|--------------------------------------|-------------|-----------------|----------------------|---------|
| Age (years)                          | 27 (23–32)  | 26 (23–31)      | 27 (23–32)           | 0.97    |
| Gender male:female, n                | 257:11      | 41:3            | 216:8                | 0.40    |
| Infection duration (days), median (IQR) | 19 (14–25)  | 14 (12–21)      | 20 (15–25)           | 0.001   |
| Risk behaviour, n (%)                |             |                 |                      |         |
| MSM                                  | 249 (93)    | 39 (89)         | 210 (94)             | 0.36    |
| Heterosexual female                  | 11 (4)      | 3 (7)           | 8 (4)                |         |
| Heterosexual male                    | 8 (3)       | 2 (4)           | 6 (2)                |         |
| Fiebig stage, n (%)                  |             |                 |                      |         |
| I (RNA+, p24 antigen−, HIV IgM−)     | 44 (16)     | 44 (100)        | –                    | –       |
| II (RNA+, p24 antigen+, HIV IgM−)    | 80 (30)     | –               | 80 (36)              |         |
| III (HIV IgM+/WB−)                   | 113 (42)    | –               | 113 (50)             |         |
| IV (HIV IgM+/WB indeterminate)       | 31 (12)     | –               | 31 (14)              |         |

WB: Western blot

** * P<0.001, P<0.05