Stability of Plasma Levels of Cytokines and Soluble Activation Markers in Patients with Human Immunodeficiency Virus Infection

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Cytokine and immune activation marker levels in plasma are valuable measurements of immune status and treatment effects in human immunodeficiency virus (HIV) infection and AIDS. Five populations representing various stages of disease were studied: controls, 2 AIDS groups with <50 mm$^3$ CD4 cells, and 2 groups of HIV-positive subjects—1 with stable CD4 T cells (median, 545/mm$^3$) and 1 with >100 mm$^3$ CD4 cell decline in 1 year. Relatively stable levels of tumor necrosis factor (TNF)-α, soluble TNF receptor (R)II, soluble interleukin-2R, neopterin, and β$_2$-microglobulin (β$_2$M) were documented over 5–8 weeks in patients with AIDS and for 1–4 years in the other groups. β$_2$M was generally the most stable marker. Interferon-γ levels, however, fluctuated substantially. Individuals, whether normal or HIV-positive, maintained characteristic plasma levels of cytokines and immune activation markers. Thus, documented changes, in excess of the variability observed in this study, are likely to be significant indicators of change in disease status or effects of therapy.

Evaluation of the effects of therapy or adverse events, such as coinfection, in patients with human immunodeficiency virus (HIV) disease is best made by serial testing of individual patients. Plasma levels of cytokines and soluble markers (CSMs) of immune activation are important parameters of HIV disease [1–4]. Thus, changes in these markers are important disease indicators [5–7]. However, change can occur for biologic or technical reasons [8–13]. Thus, knowledge of the variability in plasma levels of CSMs can be valuable in assessing the effects of therapy or untoward events on immune activation and dysregulation that are essential elements of the immunopathology of HIV infection.

The response to clinician requests for data on the intrapatient variability in CSMs is framed here in several contexts. One relates to time (e.g., days, weeks, months, and years). Another relates to disease stage as minimal, moderate, and advanced as reflected in CD4 T cell numbers. Still another context relates to rate of HIV progression to AIDS. A fourth context is more complex, relating to different patterns of change over time (e.g., steady loss or inflection to adverse course) in CD3 T cells, CD4 T cells, and CSMs [14, 15].

Several patterns of cytokine and immune activation marker abnormalities in the course of HIV infection have been demonstrated in recent studies at the University of California at Los Angeles (UCLA). In one context, a persistent, relatively stable level of CSMs within the normal range or slightly or moderately elevated without other evidence of disease progression is associated with maintained CD4 cell level for years without further fall or progression to AIDS [14, 15]. In contrast, HIV-positive persons who progressed to AIDS with accompanying CD4 cell reduction showed two patterns of CSM changes: either a steady increase in CSM parameters prior to AIDS or an inflection point where the steady level shifted to a new, progressively increasing pattern, which often presaged the fall in CD4 cells and occurred 1–4 years before AIDS was diagnosed [15]. Of interest, those who had the inflection point did not have the clinical diagnosis of AIDS until much more abnormal levels of CSM or CD4 T cells were reached than in the group with the steady progression [15].

Studies were undertaken to evaluate HIV-positive subjects with nonprogressing or with progressing disease and in 2 groups with AIDS and <50 CD4 cells/mm$^3$. Changes in plasma levels and intrasubject variability in levels of two cytokines (tumor necrosis factor [TNF]-α and interferon [IFN]-γ) and of four major markers of immune activation (soluble TNF receptor II [sTNF-RII], soluble interleukin-2 receptor α [sIL-2Rα], neopterin, and β$_2$-microglobulin [β$_2$M]) were measured in several groups, and IL-6 in 1 group.
Materials and Methods

Study subjects. Five populations were studied in detail (table 1). Study group A (controls) comprised a multiracial group of 10 healthy medical center employees (aged 19–42 years; 40% men, 60% women; Asian, Hispanic, African American, and white). Each subject had 5 consecutive daily visits in 1 week. Two HIV-positive populations (study groups B and C) were homosexual men participating in the Los Angeles cohort of the Multicenter AIDS Cohort Study (MACS). These men have been followed since 1984, and details on the recruitment and characteristics of the cohort have been published [16]. The participants have been recruited and followed at regular intervals. Study group B included 10 seropositive asymptomatic subjects who did not develop AIDS and had 5 visits at 3-month intervals for 1 year. These subjects had approximately equal CD4 cells at baseline (median, 310/mm³) that declined ≥100 cells/mm³ in the following year. The CD4 cells drop averaged 198 ± 42 cells/mm³/year (range, 141–262). Study group C (table 1) included 10 selected HIV-seropositive and asymptomatic persons who had seroconverted >1 year earlier, did not develop AIDS in 2 years after the follow-up period, had stable CD4 cells during the follow-up, and had 8 or 9 visits at 6-month intervals during 3.5–4 years of follow-up [14]. Group C had a median of 545 CD4 cells/mm³ at baseline. Study group D comprised participants in AIDS Clinical Trials Group (ACTG)-160, a study that evaluated pentoxifylline in AIDS patients [17]. The mean CD4 T cell count at baseline was 31/mm³ (range, 0–232). Plasma samples were obtained at baseline and at weeks 2, 4, and 8. From this study group, 16 subjects were tested for IFN-γ and TNF-α, and 36 subjects were tested for β,M and neopterin. TNF, neopterin, and β,M levels did not change appreciably in these subjects [17] and thus were used in this study of intrasubject variability. Study group E was drawn from ACTG-251, a study that evaluated pentoxifylline in AIDS patients [17]. The mean CD4 T cell level was 150/mm³ (range of median 5% would be considered very small and a CV >25% would be considered large. The within-person variation divided by the total variation also measures the consistency of the assay. Smaller numbers of this ratio are an indication of a more useful assay. The total variation is the between-person plus the within-person variation. These are calculated by fitting a repeated measures random effects model to the log transformed data.

Results

Plasma levels of 3 cytokines (TNF-α, IFN-γ, and IL-6) and 4 soluble immune activation markers (sTNF-RII, sIL-2Rα, neopterin, and β,M) were evaluated in serial studies of 5 populations: 1 reference population and 4 groups with HIV infection—representing different CD4 T cell levels and different states of CD4 cell depletion and disease progression. The fre-

Table 1. Characteristics of the study groups.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Population source (no.)</th>
<th>Frequency and no. of tests</th>
<th>Median CD4 T cells/mm³</th>
<th>CD4 T cells/mm³ (range of median levels, all subjects in group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal subjects (9)</td>
<td>Daily, 5 days</td>
<td>772</td>
<td>495–1059</td>
</tr>
<tr>
<td>B</td>
<td>MACS HIV-positive subjects (8)</td>
<td>Every 3 months/5 tests in 1 year</td>
<td>310</td>
<td>271–469</td>
</tr>
<tr>
<td>C</td>
<td>MACS HIV-positive subjects (10)</td>
<td>Every 6 months/9 tests in 4 years</td>
<td>545</td>
<td>326–674</td>
</tr>
<tr>
<td>D</td>
<td>ACTG-160 subjects (16–36)</td>
<td>4 tests in 8 weeks</td>
<td>31</td>
<td>0–232*</td>
</tr>
<tr>
<td>E</td>
<td>ACTG-251 subjects (16)</td>
<td>4 tests in 6 weeks</td>
<td>28</td>
<td>0–241*</td>
</tr>
</tbody>
</table>

NOTE. MACS, Multicenter AIDS Cohort Study; ACTG, AIDS Clinical Trials Group.

* Mean range of CD4 T cells at baseline.
quency of testing, the number of tests per subject, and the median CD4 T cell level for each group are summarized in table 1. For each marker, the mean plasma concentration of the serial measurement in each subject was calculated. Then the median (and range) for entire group was identified (table 2). The CVs of the markers were calculated for each person in the group. Table 3 shows the median (range) of the CV data for all subjects.

Cytokines. Plasma TNF-α levels were elevated in HIV infection (table 3). The median CV ranged from 11% to 26%, with a mean of 20% for TNF-α (table 3). Variations in collection procedures may have contributed to the dispersion of data.

IFN-γ plasma levels were elevated in HIV infection (table 2). Wide variability in the CV was observed (median, 69%; range, 46%–145%), due largely to the fact that IFN-γ levels are near or below the limits of detection by currently methods. IFN-γ levels could not be measured (i.e., were below the limits of detection) in 60% of the normal reference population samples.

Plasma IL-6 levels were evaluated at Case Western Reserve University in a study of 16 HIV patients with mean 150/mm³ CD4 cells. The median IL-6 level was 1.57 pg/mL. The median IL-6 level was 15.4 pg/mL.

CSMs. Plasma sTNF-R-II levels were elevated in HIV infection (table 2). The median CV was 12% (range, 8%–19%; table 3). Plasma sIL-2Ra was elevated in HIV infection (table 2). The median CV in the 3 available studies was 19% (range, 5%–22%; table 3). Neopterin was increased in HIV infection to different degrees in the 4 HIV-positive populations (table 2). The median CV was 16% (table 3).

The normal reference population, with samples collected on 5 sequential days, showed low variability in the measurements of the 4 CSMs of immune activation (median CV, 5%–11%). In contrast, the cytokines showed moderate or marked variability: TNF-α and IFN-γ had median CVs of 25% and 65%, respectively.

An HIV-positive population with stable CD4 T cell levels for 4 years (group C) had a median of 545 CD4 T cells/mm³ at baseline. These samples were collected before the advent of highly active antiretroviral regimens. However, zidovudine and related compounds were used at various times by some of these subjects. The plasma activation marker levels were on average slightly elevated (mean neopterin level, 9.9 nmol/L; β₂M, 1.86 mg/L). The variability in this population was moderate, with a mean CV of 19% for the plasma activation markers. The CVs for cytokines were substantially greater.

In group B, an HIV-positive population sampled at 3-month intervals for 1 year, CD4 T cells fell ≥100 cells/mm³ (from a starting mean of 442 cells/mm³). This group had high levels of immune activation, with mean plasma levels of 15.3 nmol/L. The CVs for cytokines were substantially greater.

### Table 2. Mean levels and ranges of plasma cytokines and immune activation markers in 5 study groups.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Normal subjects</th>
<th>HIV-positive CD4 cell fall</th>
<th>HIV-positive CD4 cells stable</th>
<th>ACTG-160</th>
<th>ACTG-251</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td>15.4 (11.3–25.8)</td>
<td>40.4 (24.4–46.4)</td>
<td>19.8 (10.5–33.6)</td>
<td>31.8</td>
<td>57.1</td>
</tr>
<tr>
<td>IFN-γ (U/L)</td>
<td>21 (0–36)</td>
<td>130 (21–251)</td>
<td>60 (7–203)</td>
<td>28 (0–373)</td>
<td>3.75</td>
</tr>
<tr>
<td>sTNF-R-II (ng/mL)</td>
<td>1.97 (1.58–2.44)</td>
<td>4.58 (3.38–5.32)</td>
<td>2.92 (1.92–5.57)</td>
<td>3.75</td>
<td>1.64</td>
</tr>
<tr>
<td>sIL-2R (U/mL)</td>
<td>366 (198–769)</td>
<td>1246 (710–1759)</td>
<td>841 (544–1144)</td>
<td>20.7</td>
<td>6.40</td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>3.7 (3.1–6.0)</td>
<td>15.3 (11.4–20.1)</td>
<td>9.9 (6.2–15.2)</td>
<td>15.8 (9.2–40.8)</td>
<td>20.7 (6.5–77.4)</td>
</tr>
<tr>
<td>β₂-microglobulin (mg/L)</td>
<td>0.95 (0.73–1.14)</td>
<td>2.97 (2.21–3.95)</td>
<td>1.86 (1.53–3.33)</td>
<td>3.13 (1.47–6.01)</td>
<td>2.41 (1.40–6.21)</td>
</tr>
</tbody>
</table>

### Table 3. Coefficient of variation (CV) for plasma levels of cytokines and immune activation marker in 5 study groups.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Normal subjects</th>
<th>HIV-positive CD4 cell fall</th>
<th>HIV-positive CD4 cells stable</th>
<th>AIDS</th>
<th>AIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td>25% (9%–30%)</td>
<td>19% (8%–42%)</td>
<td>26% (15%–40%)</td>
<td>11%</td>
<td>17%</td>
</tr>
<tr>
<td>IFN-γ (U/L)</td>
<td>65% (33%–119%)</td>
<td>69% (31%–167%)</td>
<td>145% (68%–283%)</td>
<td>68%</td>
<td>70%</td>
</tr>
<tr>
<td>sTNF-R-II (ng/mL)</td>
<td>9% (5%–12%)</td>
<td>8% (5%–17%)</td>
<td>19% (10%–32%)</td>
<td>15%</td>
<td>15%</td>
</tr>
<tr>
<td>sIL-2R (U/mL)</td>
<td>5% (3%–14%)</td>
<td>19% (9%–37%)</td>
<td>22% (15%–45%)</td>
<td>16%</td>
<td>21%</td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>11% (3%–14%)</td>
<td>13% (7%–31%)</td>
<td>23% (13%–28%)</td>
<td>16%</td>
<td>21%</td>
</tr>
<tr>
<td>β₂-microglobulin (mg/L)</td>
<td>7% (3%–12%)</td>
<td>8% (5%–14%)</td>
<td>11% (6%–22%)</td>
<td>8%</td>
<td>9%</td>
</tr>
</tbody>
</table>

NOTE. Data are median and range of coefficient of variation for all subjects in this group (range). ACTG, AIDS Clinical Trials Group; IFN, interferon; IL, interleukin; R, receptor; s, soluble; TNF, tumor necrosis factor.
for neopterin and 2.97 mg/L for $\beta_2$M. The median CV for plasma activation markers was 11%; the cytokines had somewhat greater variability.

Two groups of patients with AIDS and low CD4 T cells (medians of 28 and 31 cells/mm$^3$) had high plasma activation marker levels (means of 20.7 and 15.8 nmol/L for neopterin and 2.4 and 3.1 mg/L for $\beta_2$M, respectively). Four measurements were made with samples obtained over 6–8 weeks. Variability was not great for the immune activation marker levels or for plasma TNF-$\alpha$; Median CVs were 11% and 17% in the two studies.

**Assay consistency.** The CVs for the markers in each group are presented in table 3. The general similarity of the markers in each group is evident, except for IFN-$\gamma$, which had extreme variability. The consistency of an assay can be measured by the ratio of the within-person variation to the total variance for each of the 5 study groups, as shown in table 4. Numbers greater than ~0.30 are indicative of less precise assays. In this study, IFN-$\gamma$ was the weakest and $\beta_2$M performed the best.

**Characteristic CSM levels for individuals.** Individuals had characteristic CSM levels over time. This phenomena was valid in a variety of populations, including uninfected controls and HIV-positive subjects at different stages of infection, and for various time frames (days, weeks, months, and years). Figure 1 shows representative data: sIL-2R in normal persons (figure 1A), characteristic levels of TNF-$\alpha$ for persons with AIDS (figure 1B), sTNF-RII levels for HIV-positive subjects whose CD4 T cells declined $\geq 100$ cells/mm$^3$ in 1 year of follow-up (figure 1C), and stable levels of $\beta_2$M in HIV-positive subjects with only moderately reduced CD4 T cells during 4 years of follow-up (figure 1D). This stability is also reflected in the data for the median CVs in table 3 and the within-person variations noted in table 4.

### Discussion

Cytokines and immune activation markers are produced by cells that mediate host responses to infection or inflammatory stimuli [1, 21–24]. It is well established that cytokine and immune activation marker levels correlate with the severity of disease and/or clinical outcome in HIV infection and inflammatory disorders [5–7, 25–30]. In addition, cytokines can up-regulate directly or indirectly the replication of HIV [2]. Many factors may influence the plasma levels and the measurements of these immune cell factors [31]. Thus, it was important to determine the variability of plasma levels of CSMs of immune activation in HIV-positive persons. In these studies, we used the CV as the measure of variability.

The variation observed could be due to biologic factors, technical issues, coinfections, or changes in HIV disease activity. To reduce technical differences, all samples of individual patients were tested in the same batch. Intratest variability in our laboratory is <10% for most of these parameters [20]. The tests differed themselves in variability. The $\beta_2$M assay, which had the least variability, is partially automated, which may contribute to the low CV of this test.

Some of the assays, notably IFN-$\gamma$, are conducted near the limit of sensitivity. In fact, several control and HIV-positive subjects had levels below the limits of detection. Thus, the low plasma levels of several cytokines may contribute to high CV in those samples (e.g., modest absolute variability may appear as substantial percentage changes because the baseline values are so low and the test precision is weak). The greatest ratio of within-person variability to the total variance was seen with IFN-$\gamma$ (table 4), which confirms the poor consistency and thus the questionable usefulness of this plasma assay. This conclusion is based on the results from the assay kits now available, and appears to be an inherent problem of these kits. If more sensitive kits become available, they may overcome the problems of high CV and the low limit of detection we have observed.

Biologic factors that can contribute to changing CSM levels within the time frames of this study include diurnal variation and undetected infections [32–36]. Disease factors that may induce changes include intercurrent infections such as tuberculosis and autoimmune diseases. In these studies, samples were obtained at the same general time of day at regularly scheduled clinic hours to reduce diurnal factors. At each visit, each subject had temperature taken, current health history obtained, and relevant physical examinations as well as white blood cell counts and differential tests done to reduce the likelihood of undetected infections and other relevant diseases. Antiretroviral therapy may have had some effects, but changes were not seen in the short-term studies. However, therapies may have contributed to variability in the longer term studies, especially the 4-year study. A recent study of HIV-positive patients with $<100$ CD4 cells noted that serum levels of TNF-$\alpha$ tended to rise as disease advanced [37].

Good stability was generally evident in the measurements of the soluble markers of immune activation ($\beta_2$M, neopterin, sIL-2R, and sTNF-RII) and with TNF-$\alpha$. Improvements are needed, however, for many cytokine measurements. Present methodologies for many cytokines do not allow their accurate detection in normal plasma or in many persons with HIV-infection.

#### Table 4. Ratio of within-person variation to the total variation in the group for the 5 studies.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>A Normal subjects</th>
<th>B HIV-positive CD4 cell fall</th>
<th>C HIV-positive CD4 cells stable</th>
<th>D AIDS</th>
<th>E AIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-$\alpha$</td>
<td>0.47</td>
<td>0.79</td>
<td>0.49</td>
<td>0.12</td>
<td>0.41</td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>0.59</td>
<td>0.63</td>
<td>0.84</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>sTNF-RII</td>
<td>0.30</td>
<td>0.41</td>
<td>0.33</td>
<td>0.33</td>
<td>0.14</td>
</tr>
<tr>
<td>sIL-2R</td>
<td>0.04</td>
<td>0.31</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neopterin</td>
<td>0.22</td>
<td>0.64</td>
<td>0.52</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>$\beta_2$-microglobulin</td>
<td>0.22</td>
<td>0.22</td>
<td>0.28</td>
<td>0.13</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**NOTE.** IFN, interferon; R, receptor; s, soluble; TNF, tumor necrosis factor.
Figure 1.  Serial levels of cytokines and soluble immune activation markers.  A, Soluble interleukin-2 receptor (sIL-2R) measured on 5 consecutive days in 8 HIV-uninfected controls.  B, Tumor necrosis factor (TNF-α) determined before baseline (screen), baseline, and at weeks 2 and 4 in 15 AIDS patients.  C, sTNF-RII quantified at 3-month intervals for 1 year in 8 HIV-positive subjects with declining CD4 T cells.  D, β2-microglobulin (β2M) measured at 6-month intervals for 4 years in 10 HIV-positive persons with stable CD4 T cells.

Infection. This problem is illustrated with the IFN-γ measurements in this report.

The level of immune activation was not a factor in contributing to the CV as the patients with high levels of activation markers showed similar CV even though CD4 cells were 300–400/mm³ for 1 group and <50/mm³ for 2 others. Overall the tests for TNF-α and for the CSMs did not have great variability. Thus, the description of upward inflection patterns for plasma cytokine and CSMs prior to AIDS [14, 15] are not likely to be due to measurement artifacts, but do reflect major adverse changes in disease activity.

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References