Elevated Levels of Serum-Soluble CD14 in Human Immunodeficiency Virus Type 1 (HIV-1) Infection: Correlation to Disease Progression and Clinical Events

By Egil Lien, Pål Aukrust, Anders Sundan, Fredrik Müller, Stig S. Frøland, and Terje Espevik

Soluble (s) CD14, a marker for monocyte/macrophage activation and a mediator of bacterial lipopolysaccharide (LPS) action, was elevated in serum from human immunodeficiency virus type 1 (HIV-1)-infected individuals (n = 92) compared with seronegative controls. The highest levels were found in patients with advanced clinical and immunological disease. Patients with ongoing clinical events had significantly higher sCD14 levels than symptomatic HIV-1-infected individuals without clinical events, with especially elevated levels in patients infected with Mycobacterium avium complex (MAC). On longitudinal testing of patients (n = 26) with less than 100 × 10^6 CD4 lymphocytes/L at baseline, we found that increasing sCD14 serum concentrations per time unit were associated with death, whereas no differences in CD4 cell number decrease were found between survivors and nonsurvivors. In vitro studies showed that HIV-1 glycoprotein 120 and purified protein derivative (PPD) from M. avium (MAC-PPD) stimulated normal monocytes to release sCD14. Furthermore, MAC-PPD induced tumor necrosis factor (TNF) release from monocytes through interactions with CD14 and, importantly, the addition of sCD14 enhanced this MAC-PPD stimulatory effect. Our findings suggest that the CD14 molecule may be involved in the immunopathogenesis of HIV-1 infection, and it is conceivable that serial determination of sCD14 may give useful predictive information concerning disease progression and survival in HIV-1-infected patients.

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PATIENTS AND METHODS

Patients

From September 1991 through December 1994, serum samples were obtained from consecutively recruited HIV-1-infected patients from The Section of Clinical Immunology and Infectious Diseases, Medical Department A, the National Hospital (Oslo, Norway). The patients were clinically classified according to Center for Disease Control and Prevention (CDC)-1993 revision in groups of asymptomatic HIV-1 infection (CDC group A, n = 29; median, 30 years; range, 19 to 34 years), symptomatic non-acquired immunodeficiency syndrome (AIDS) (CDC group B, n = 22; median, 35 years; range, 25 to 56 years), and AIDS (CDC group C, n = 41; median, 38 years; range, 16 to 54 years). Twenty-one healthy, HIV-1-seronegative blood donors served as controls (median, 36 years; range, 22 to 60 years). Of the HIV-1-infected patients, 42 received zidovudine in combination with Pneumocystis carinii prophylaxis, and 7 of those received didanosine in addition. None of the patients had initiated therapy or changed dosage regimen during the last 6 months. For the longitudinal study, patients (n = 26)
followed twice a year were included in the study the first time their blood CD4 lymphocytes were less than 100 × 106/L. After inclusion, three to five blood samples were taken, with median intervals of 160 days. All serum samples were prepared as earlier described82 and were stored at −70°C until assaying for sCD14. Informed consent for blood sampling was obtained from each patient.

**Enzyme-Linked Immunosorbent Assay (ELISA) for sCD14**

Measurements of sCD14 were performed using a sandwich ELISA with CD14-specific monoclonal antibodies (MoAbs) 3C10 and 5C5. Immunoplates (Maxisorp, Nunc, Denmark) were coated for 12 hours with 10 µg/mL MoAb 3C10 (hybridoma obtained from American Type Culture Collection, Manassas, VA; ATCC TIB-228) diluted in phosphate-buffered saline (PBS). Nonspecific binding was blocked with 0.5% bovine serum albumin (BSA; RIA grade, Sigma, St Louis, MO) in PBS for 1 hour at room temperature (RT). After washing the plates three times with PBS/0.05% Tween 20 (Merck, Darmstadt, Germany), the plates were incubated for 1 hour at RT with 50 µL of samples or standard (recombinant soluble CD14; a kind gift from Drs H. Lichtenstein and M. Zukowski, Amgen Inc, Boulder, CO) diluted in 0.1% BSA, 0.05% Tween 20 in PBS. The MoAb 5C5 (non-LPS neutralizing CD14 MoAb from a hybridoma developed in our laboratory after immunization of Balb/c mice with sCD14) was labeled with digoxigenin (DIG antibody labeling kit; Boehringer Mannheim, Mannheim, Germany), diluted to 1 µg/mL in the BSA/Tween 20/PBS buffer, and added to the immunoplates after washing. Furthermore, after 1 hour of incubation at RT and subsequent washing, peroxidase-conjugated antidigoxigenin Fab fragments (Boehringer Mannheim) were used for detection with ortho-phenylene diamine (OPD; Dako, Glostrup, Denmark) as peroxidase substrate. The detection limit of the assay was 0.8 ng/mL, and LPS, LPS-binding protein, HIV-1 glycoprotein 120 (gp120), and purified protein derivative (PPD) from *M avium* had no effect on sCD14 quantification in the assay. Interassay and intra-assay variations were less than 10%. Serum samples from 1 patient were analyzed on the same plate to minimize run-to-run variations.

**Analysis of Blood CD4 Lymphocytes**

The number of CD4 lymphocytes in blood were determined by immunomagnetic quantification as described.83

**Measurement of HIV-1 RNA Copies in Plasma**

HIV-1 RNA copy numbers in plasma were measured by quantitative reverse transcriptase polymerase chain reaction (PCR; Amplicor HIV monitor; Roche Diagnostic Systems, Branchburg, NJ). The detection limit of the assay was 200 copies/mL.

**Stimulation of Cells**

Cells. Monolayer cultures of adherent human monocytes from healthy blood donors were prepared using gradient centrifugation of peripheral blood mononuclear cell fraction (2 × 10^6 cells/well) was incubated in 24-well cell culture plates (Costar, Cambridge, MA) for 1.5 hours in culture medium (see below) containing 40 µg/mL gentamicin and 1% glutamin. Nonadherent cells were removed by washing three times with Hank’s Balanced Salt Solution (GIBCO, Paisley, UK). This method gave typically more than 92% CD14+ cells as determined by flow cytometry (results not shown). Cell isolations yielded 4 to 8 × 10^5 monocytes/well.

Stimulations with HIV-1 gp120. Recombinant HIV-1 IIIB gp120 derived from baculovirus-infected insect cells (obtained through MRC AIDS Reagent Project, Potters Bar, UK) was added to monocytes in RPMI 1640 medium (GIBCO) containing 10% fetal calf serum (FCS; GIBCO) for 72 hours. For immunodepletion of gp120, 100 µL goat antimouse sepharose gel (Zymed, San Francisco, CA) was incubated in the presence or absence (for control) of 50 µg gp120 MoAb (obtained through MRC AIDS Reagent Project, UK) with end-over-end rotation for 4 hours. The gel was washed, and nonspecific binding was blocked with 0.1% BSA in PBS and incubated with a 10 µg/mL gp120 solution for 14 hours with end-over-end rotation. The gp120 content in solution after depletion were analyzed in an ELISA with recombinant sCD4 (Intracel, London, UK) coating, sample incubation, and detection with polyclonal rabbit anti-gp120 antibodies (IgG fraction; Intracel) and peroxidase-conjugated goat anti-rabbit antibodies/OPD substrate (Dako). The ELISA demonstrated removal of more than 99.6% of gp120 in the immunodepletion. Equal amounts of liquid from the gp120-immunodepleted solution and control (equivalent to 0.4 and 0.2 µg/mL gp120 according to the ELISA) were then added to monocytes and incubated for 72 hours. By use of Limulus amoebocyte lysate assay (Chromogenix, Mölndal, Sweden), endotoxin contamination was measured to 8 pg/µg for gp120. Cell supernatants were analyzed in the sCD14 ELISA as described above.

**Stimulations with purified protein derivative from M avium (MAC-PPD) and LPS.** Adherent monocytes were stimulated with PPD from *M avium* (MAC-PPD) or LPS from Escherichia coli O26:B6 (catalogue no. L8274; Sigma) in AIM V serum-free medium (GIBCO). MAC-PPD (generously provided by Reidar Bjørlo, The National Veterinary Institute, Oslo, Norway) was produced from *M avium* strain D4ER (provided by Central Veterinary Laboratory, Weybridge, UK) after main principles, as described.28 Briefly, the bacteria were grown on Sutong-medium, and PPD was prepared from filtrate after precipitation with 40% trichloroacetic acid and subsequent washings with 2% trichloroacetic acid, aceton, and ether. For study of sCD14-release, cells were stimulated for 72 hours. In separate experiments, monocytes were stimulated for 8 hours to investigate the release of TNF. To modulate the stimulatory response mediated by MAC-PPD, the following reagents were used: LPS-neutralizing MoAb 3C10 against CD14, control MoAb 6H8 against a widely distributed myeloid cell surface protein (B. Naume and T. Espesvik, unpublished results), and recombinant sCD14 (Amgen). Supernatants were analyzed for bioactive TNF in the WEHI 164 clone 13 bioassay, as described.39 The endotoxin content of MAC-PPD was 0.7 pg/µg as measured by the Limulus assay (Chromogenix).

**Statistics**

For statistical analysis, the Wilcoxon rank-sum (Mann-Whitney U) test was used for comparing two groups. The Kruskal-Wallis test was used for comparing more than two groups, and correlation analysis was performed using Spearman’s rank correlation coefficients. Results are given as medians with the corresponding 25 to 75 percentiles, unless otherwise specified. For calculations of change in parameters over time, linear regression was used. The influence of different parameters on survival was studied using Kaplan-Meyer analysis and the log rank test. *P* values less than .05 were considered significant. Data were analyzed with the SPSS for Windows statistical software (SPSS Inc, Chicago, IL).

**RESULTS**

**Increasing Levels of sCD14 Throughout HIV-1 Infection**

We found a significant increase of serum sCD14 levels in all clinical stages of HIV-1 infection compared with controls (Fig 1). In fact, the increase in sCD14 was more pronounced in patients with AIDS (CDC group C) than in individuals from the asymptomatic group (CDC group A) or the symptomatic non-AIDS group (CDC group B). The differences between sCD14 levels in AIDS (CDC group C) and non-AIDS (CDC groups A and B, respectively) patients were highly significant. Our earlier studies have indicated elevated activation markers

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Correlation of sCD14 Concentrations to Viral Load

To correlate amounts of sCD14 in serum to the viral load in patients, we measured HIV-1 RNA copy numbers by PCR in plasma samples from 49 patients in the study (median, 50,954 copies/mL; 25 to 75 percentile, 6,790 to 311,751 copies/mL). Interestingly, we found a highly significant correlation ($r = .69$, $P < .001$) between sCD14 and the amount of plasma HIV-1 RNA, as shown in Fig 3. In addition to the relationship between sCD14 and different immunological parameters, this further demonstrates the close correlation between disease activity and serum sCD14 concentrations.

Longitudinal Testing

For HIV-1-infected patients with markedly reduced numbers of CD4 lymphocytes, the CD4 cell number is not a good prognostic marker. To investigate if levels of sCD14 might give predictive information concerning survival, some of the HIV-1-infected patients were tested longitudinally. Twenty-six patients were included in the study when their blood CD4 cell numbers decreased to less than 100 $\times$ 10^3/L. Sera from the HIV-1-infected individuals were collected at different time points, and the patients were divided into two groups: one group of the patients who died during the study period (up to 6 months after the last serum collection; $n = 14$) and a second group of the patients who survived the same period ($n = 12$). As shown in Fig 3, the patients who died had a significant increase in sCD14 per time unit compared with the patients who survived.
Patients in both groups had similar values for decrease in CD4 lymphocyte number per time unit (Fig 4). At the time of inclusion, survivors and nonsurvivors did not differ significantly in serum sCD14 concentrations (nonsurvivors, 4.01 µg/mL [3.55 to 4.76 µg/mL]; survivors, 3.95 µg/mL [3.31 to 4.92 µg/mL]). They also did not differ significantly in CD4 lymphocyte numbers (nonsurvivors, 43 × 10^6 cells/L [19 to 63 × 10^6 cells/L]; survivors, 52 × 10^6 cells/L [43 to 68 × 10^6 cells/L]). However, at the last blood sampling, nonsurvivors had significantly higher sCD14 values (6.07 µg/mL [4.58 to 9.67 µg/mL]) compared with the inclusion sample (P < .001), and the number of blood CD4 lymphocytes was also significantly lower (15 × 10^6 cells/L [9 to 31 × 10^6 cells/L]) than at the first sampling time point for these patients (P = .003). In contrast, the surviving patients did not have a significant difference in sCD14 concentrations at the last time point (4.25 µg/mL [3.74 to 5.24 µg/mL]) compared with the time of inclusion, whereas the CD4 cell numbers also in these patients were significantly depressed at the last sampling time point (19 × 10^6 cells/L [11 to 24 × 10^6 cells/L]) compared with the first (P = .01). Additionally, at the last sampling time point, the nonsurviving patients had significantly higher serum sCD14 concentrations than the patients who survived the study period (P = .008), whereas there were no difference between the two groups in the number of blood CD4 cells.

To study the importance of different parameters on survival time, we divided the patients into groups according to the median values for all 26 patients participating in the longitudinal study, and the data were analyzed by Kaplan-Meyer survival analysis. Kaplan-Meyer curves for sCD14 versus survival are shown in Fig 5, in which patients are divided into two groups with either higher or lower sCD14 increase compared with median. The log rank test showed that sCD14 increase (median, 0.24 µg/mL per 100 days) was predictive for death (P < .001), as was the sCD14 concentration at the last sampling time.
AIDS patients with MAC infection were found to have markedly elevated serum levels of sCD14 compared with patients with other clinical events (Fig 2). Therefore, we tested the ability of MAC-PPD to induce sCD14 release from monocytes. As can be seen from Fig 7B, MAC-PPD stimulated monocytes to increased release of sCD14 in a dose-dependent manner, but, however, with less potency than LPS. This demonstrates that mycobacterial components may contribute to the raised levels of serum sCD14 found in AIDS patients with MAC infection.

Components From M avium Stimulate Monocytes Via the CD14 Pathway

Mycobacterial components such as LAM have the ability to stimulate monocytes through interactions with mCD14. MAC-PPD induced release of TNF from adherent monocytes in a dose-dependent manner after 8 hours of stimulation (Fig 8A); to study if this stimulation is mediated via the CD14 pathway, adherent monocytes were stimulated with MAC-PPD in the context of mCD14 expression.
absence or presence of the LPS neutralizing CD14 MoAb 3C10. When added 15 minutes before MAC-PPD, 3C10 clearly inhibited MAC-PPD-induced release of TNF from the monocytes (Fig 8B), indicating that mCD14 may be directly involved in the signaling mediated by cell components from MAC. To assess the role of sCD14 in this system, we added recombinant sCD14 together with MAC-PPD and found a significant increase in TNF release in these monocyte cell cultures (Fig 8B). Thus, both mCD14 and sCD14 seem to play important roles in the stimulation of monocytes by components from MAC and may also thereby have clinical significance during MAC infection.

DISCUSSION

In the present study, we show that there is a marked increase in serum concentrations of sCD14 in all clinical stages of HIV-1 infection, with the highest levels in the AIDS group. Particularly high levels were found in patients with ongoing disseminated MAC infection. Furthermore, increased levels of sCD14 were significantly correlated with the degree of immunodeficiency and HIV-1 replication, as measured by number of blood CD4+ lymphocytes and plasma HIV-1 copy numbers, respectively. Also, we found a strong correlation between sCD14 and sTNFR, earlier shown to be important immunological prognostic markers both in HIV-1 infection\(^2\) as well as other diseases.\(^6,47\) The fact that the number of HIV-1 RNA copies in plasma also showed a highly significant correlation to sCD14 serum concentrations (Fig 8B) further highlights that sCD14 serum concentrations reflect disease activity and viral load.

In addition to virus-bound HIV-1 gp120, free gp120 can be detected in circulation.\(^48\) Therefore, in vitro cell responses to this envelope glycoprotein may be relevant to the situation found in vivo during HIV-1 infection. Recombinant or natural glycoproteins are shown to stimulate the release of inflammatory cytokines from monocytes\(^43,44\); in our experiments, treatment of monocytes with recombinant gp120 resulted in increased release of sCD14. These experiments show that monocytes constantly exposed to virus-bound or circulating free viral antigens may release elevated levels of sCD14 and that these viral glycoproteins may be partly responsible for the increased serum sCD14 levels found in HIV-1-infected individuals.

Infection with the opportunistic pathogen MAC results in a poor prognosis for HIV-1-infected patients. The MAC infection in AIDS is characterized by high morbidity, weight loss, fever, and short survival times.\(^49\) HIV-1-infected patients with MAC infection showed significant higher serum sCD14 concentrations.

<table>
<thead>
<tr>
<th>gp120 (µg/mL)</th>
<th>gp120 MoAb in Immunodepletion</th>
<th>sCD14 (ng/mL)</th>
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<tbody>
<tr>
<td>0.4</td>
<td>–</td>
<td>76</td>
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<tr>
<td>0.4</td>
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<td>46</td>
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<td>0.2</td>
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A separate experiment showed that immunodepletion of gp120 with a gp120-specific MoAb removed the stimulating effect on sCD14 release from monocytes. Adherent monocytes from healthy donors were treated for 72 hours with gp120-immunodepleted solution and compared with equal amounts of liquid from the control gp120 solution (without gp120 MoAb in immunodepletion procedure) in sCD14-releasing ability. Numbers are the mean of duplicates. The concentration of sCD14 in unstimulated culture was 49 ng/mL.
tions than other patients, including symptomatic HIV-1-infected patients with clinical events other than MAC. These results may, at least in part, be explained by directly stimulated release of sCD14 from monocytes by mycobacterial antigens, as shown in the present study. Serum levels of inflammatory cytokines and cytokine receptors, as TNF/sTNFR, are also elevated in HIV-1-infected patients with MAC infection, and mononuclear cells from these patients release more TNF, IL-6, and IL-1 when challenged with LPS. Our findings demonstrating enhancement of MAC-PPD-induced TNF-release from monocytes by sCD14 suggest that sCD14 may directly contribute to immunological disturbances in MAC patients.

Monocytes from HIV-1-infected patients show reduced ability to take up and kill MAC. This may be a result of direct interaction between monocytes and HIV-1 or virus components, such as gp120. The monocyte response to LAM or MAC-PPD (Fig 8B) is increased in the presence of sCD14; in addition, mCD14 may play a role in cell activation by components from MAC, because CD14 MoAbs inhibited MAC-PPD stimulatory effects. A consequence of LPS or MAC stimulation of cells in HIV-1-infected patients in vivo may therefore be enhanced replication of virus, because CD14 is involved in the LPS-induced upregulation of HIV-1 replication. Activation of monocytes/macrophages can lead to the release of cytokines such as TNF, which in turn may further activate these cells with both increased HIV-1 replication and subsequent effector molecule release. The involvement of sCD14 and mCD14 in cell stimulation may indicate that these molecules may be parts of a vicious circle leading to more advanced disease in HIV-1-infected individuals. Interestingly, a recent study stressed the important role of cells of the macrophage lineage in HIV-1 infection as high-producers of virus. Furthermore, infection with common opportunistic pathogens such as Pneumocystis carinii and MAC dramatically increased the virus production by macrophages. Monitoring of monocyte/macrophage activity may therefore be of high clinical relevance in later stages of HIV-1 infection, in which monocytes/macrophages may be important reservoirs of HIV-1.

A recent report shows that mice with a disrupted and dysfunctional CD14 gene have increased resistance to dissemination of bacteria and lethality caused by gram-negative infection. CD14 is involved in phagocytosis of bacteria into monocytes, and high concentrations of sCD14 can block uptake of Mycobacterium tuberculosis, a common pathogen in HIV-1-infected patients, by microglia. In addition, the modulation of cell response to bacteria may be dependent on the concentration of LPS-binding protein. These facts show the complex pattern of reactions involving CD14 and indicate a possible important role of CD14 both in dissemination and control of certain secondary infections during HIV-1 disease.

In the longitudinal study, the prognostic value of serially measured sCD14 was emphasized. Increased serum sCD14 concentrations over time were associated with death in the patient group with blood CD4 lymphocytes less than 100 × 10⁶/L, whereas no differences in the decrease of CD4 cells were observed between surviving and nonsurviving patients. In chronic diseases such as systemic lupus erythematosus and rheumatoid arthritis, sCD14 levels have been associated with changes in immunological and clinical status. Our findings suggest that the CD14 molecule may be involved in the immunopathogenesis of HIV-1 infection. Therefore, it is interesting that, in the present study, we have demonstrated that an increase in serum levels of sCD14 over time was associated with death in the patients. Thus, it is conceivable that serial
determination of sCD14 might give useful predictive information concerning disease progression and survival in HIV-1-infected patients.

Lines of evidence demonstrate increased levels of activation parameters during HIV-1 infection, and, as this study shows, the soluble monocyte/macrophage activation marker sCD14 is found in increasing concentrations in serum during disease progression. Monocytes/macrophages are the most important producers of sCD14, and these cells also harbor HIV-1. Therefore, activation of monocytes/macrophages may result in both enhanced virus replication and in release of sCD14, which may further aggravate the disease. The direct role of sCD14 in HIV-1 infection and secondary clinical events remains unclear, but with the ability of sCD14 to modulate interactions between cells and bacterial components, this molecule may have an important role in regulating immune functions.

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