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Plasma Levels of Viro-Immunological Markers in HIV-Infected and Noninfected Ethiopians: Correlation with Cell Surface Activation Markers

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Cross-sectional studies were conducted to measure soluble viral and immunological markers in plasma in order to determine the prognostic value of these markers for HIV disease progression in Ethiopians and to see their association with cell surface markers in HIV-1-infected and noninfected Ethiopians. Whole blood samples were collected from 52 HIV-1-negative Ethiopians, 32 HIV-1-positive Ethiopians with absolute $CD4^+$ T-cell count >200/μl whole blood and no AIDS defining conditions, and 39 HIV-positive Ethiopians with $CD4^+$ T-cell count <200/μl and/or AIDS defining conditions. Plasma levels of b_2-microglobulin (b_2m), soluble CD27 (sCD27), soluble tumor necrosis factor alpha receptor type II (sTNFR-II), IgG, IgA, IGE, and IL12 were elevated in HIV-1-infected individuals. The plasma levels of sTNFR-II, sCD27, b_2m, IL12, and IgG were inversely correlated with numbers of $CD4^+$ T-cells, the proportion of naïve ($CD45RA^+CD27^+$) $CD8^+$ T-cells, and the proportion of $CD8^+$ T-cells expressing CD28 ($CD8^+CD28^+$) were positively correlated with the proportions of activated ($HLA\text{-}DR^+CD38^+$) $CD4^+$ T-cells, as well as activated ($HLA\text{-}DR^+CD38^+$) $CD8^+$ T-cells. A strong positive correlation was also observed when soluble immune markers were compared to each other. Multivariate regression analyses of soluble markers with numbers of $CD4^+$ T-cells showed that sCD27 is the best independent marker for $CD4^+$ T-cell decline in the HIV-1-infected Ethiopians. Our results indicate that measurement of soluble immune markers, which is relatively easy to perform, could be a good alternative to the quantification of T-cell subsets for monitoring HIV-1 disease progression in places where there is no facility for flow cytometric measurements.

Key Words: **HIV-1; Ethiopians; sCD27; sTNFR-II; β_2m; IL12; IgG; IgE; IgA.**

INTRODUCTION

There is a wide variation in the course of disease among HIV-1-infected persons and thus considerable efforts have been put into the identification of factors that have a predictive value for the clinical stages of the disease, so-called surrogate markers. For example, the levels of HIV-1 replication (as expressed by viral loads) and the type of immune response during primary HIV-1 infection have a major impact on the ultimate course of HIV-1 disease (1–3). In addition, recent studies have indicated the association of levels of markers of immune activation and inflammatory cytokines with HIV-1 disease progression (4, 5).

HIV-1 disease progression is reportedly faster and the time of survival after the onset of AIDS is shorter in African patients (6, 7). Virologic and immune correlates of this altered disease course need to be identified by comparing HIV-1-infected and noninfected Africans, as well as through prospective studies in cohorts of HIV-1-infected Africans in order to understand the course of HIV-1 disease and design therapeutic trials for this setting. However, there are still relatively few data available detailing surrogate markers and their use in monitoring HIV-1 disease progression in Africans.

We previously have compared several $CD4^+$ and $CD8^+$ T-cell subsets in HIV-1-infected and noninfected Ethiopians, to assess their potential use as predictors for HIV-1 disease progression (8). In summary, we observed a gradual increase of activated ($CD38^+HLA\text{-}DR^+$) $CD4^+$ and $CD8^+$ T-cells, a decrease of $CD8^+$ T-cells expressing CD28, and a decrease of effector ($CD45RA^+CD27^-$) $CD8^+$ T-cells when comparing HIV^- subjects to HIV^+ subjects and to subjects with AIDS. Also, a decrease of naïve ($CD45RA^+CD27^+$) and an increase of memory ($CD45RA^+CD27^-$) $CD8^+$ T-cells were observed in Ethiopians with AIDS.

Flow cytometry is not a widely available facility in Africa. On top of that, few laboratories in Africa have settings for manipulation of peripheral blood mononuclear cells (PBMC). Therefore, it was considered important to evaluate surrogate markers which can be mea-

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sured using relatively cheap and easy to perform tests on plasma samples. In addition, surrogate markers reflect different biologic processes and their evaluation in African patients may contribute to the understanding of HIV disease pathogenesis.

In the present study we measured plasma viral load and several soluble immune markers and assessed their association with different stages of HIV-1 infection in Ethiopians. In addition, the relationship of soluble markers with previously established cell surface activation markers was assessed. Soluble CD27 (sCD27) is proposed to be the most suitable marker for monitoring HIV-1 disease progression in Ethiopians, when facilities for enumerating $CD4^+$ T-cells are not present.

MATERIALS AND METHODS

Subjects

The study population consisted of three groups of Ethiopians; 52 HIV-negative healthy individuals (designated "HIV^-"), 32 HIV-positive individuals with CD4 counts $>200/\mu l$ and no AIDS defining conditions (designated "HIV^+"), and 39 HIV^+ patients with CD4 counts $<200/\mu l$ and/or exhibiting AIDS defining conditions based on the WHO staging system for HIV-1 infection and disease (9) (designated "AIDS"). The HIV^- and HIV^+ groups are factory workers participating in a prospective cohort study performed by the Ethio-Netherlands AIDS Research Project (ENARP) in Akaki, a village 15 km to the southeast of Addis Ababa, the capital of Ethiopia. This cohort study started in February 1997, after approval by both the National Ethical Committee and the EHNRI Ethical Committee. Cohort participants and their direct dependants are offered free medical care, according to the standards of the country. Subjects of the AIDS group are patients hospitalized in Addis Ababa, who were enrolled in order of their admittance to the hospital. HIV tests were performed by ENARP, upon request by the hospital clinicians, on coded blood samples. The blood samples were accompanied by a form detailing clinical information, gender, and age only. HIV test results were returned to the hospital using the same codes. Patients in the hospital were pre- and posttest counseled and gave their informed consent, according to the hospital's policy. Samples were not included in the study when the quality of the sample was very poor or when, according to the clinical information or whole blood CD4 T-cell counts, the criteria mentioned above were not met. No clinical history of the patients designated "AIDS" was available. Details of the subjects analyzed in this study are as published (8).

Plasma Isolation and Viral Load Determination

Whole blood was collected in 10-ml EDTA Vacutainer tubes (Becton–Dickinson, U.S.A.) and plasma was isolated by centrifugation for 10 min at 300*g* (1640 rpm) at room temperature, followed by storage at $-80°C$. HIV-1 RNA was isolated from the plasma as described (10).

Viral load was determined by quantifying the amount of HIV-1 RNA using a Nucleic Acid Sequence Based Amplification (NASBA) kit: NUCLISENS, Organon Teknika BV, Boxtel, The Netherlands). In our hands the detection limit of this kit was 80 RNA molecules/ml of plasma. The NASBA methodology was previously shown to give quantitatively reliable results on HIV-1 subtype C plasma samples (11).

Measurement of Soluble Immunological Markers

The following soluble immune markers were determined by commercial ELISAs: sCD27 (Pelikine Compact human soluble CD27 ELISA kit, CLB, Amsterdam, The Netherlands), sTNFR-II (sTNF-R, 80 kDa ELISA, Bender MedSystems, Vienna, Austria), IL12 (Cytoscreen Immunoassay kit, BioSource Int. Inc., California, U.S.A.). The levels of b_2m, IgG, IgE, and IgA were measured in plasma by immunonephlometric methods (DADE Behring, Marburg, Germany, and CLB, Amsterdam, The Netherlands). All tests were performed according to the instructions of the kit manufacturers.

Three-Color Immunophenotyping of Lymphocyte Subsets

Naïve and activated $CD4^+$ and $CD8^+$ T-cells were measured using combinations of CD4/$CD8^+$CD45RA and CD27 monoclonal antibodies (mAbs) or CD4/$CD8^+$CD38 and HLA-DR mAbs, as described (8). Naïve and activated T-cells were defined as $CD45RA^+CD27^+$ and $CD38^+HLA\text{-}DR^+$, respectively.

Statistical Methods

Statistical analyses were performed using the STATA program (Stata Corp., Texas, U.S.A.). The level of significance, for the comparison of soluble markers between the three groups, was adjusted using Bonferroni correction ($\alpha = 0.033$). The association between several soluble immune markers and $CD4^+$ T-cell count was determined using a multivariate regression analysis. Correlation coefficients were calculated by the Spearman's test.

RESULTS

Plasma Levels of Soluble Immune Markers and Viral Loads

Figure 1 summarizes the plasma levels of various soluble immune markers as measured on the three groups of Ethiopian subjects: HIV^- ($n = 52$), HIV^+ ($n = 32$), and AIDS ($n = 39$). In addition, $\log_{10}$ viral load values are shown for the HIV^+ and AIDS groups. The levels of sCD27, b_2m, and IL12 were significantly and progressively increased when comparing HIV^- to HIV^+ to AIDS groups (Figs. 1A–1C). Plasma sCD27 median values were 338 (278–392) U/ml (HIV^-), 406 (364–447) U/ml (HIV^+), and 761 (624–951) U/ml (AIDS). Plasma b_2m values were 1 (1–2) mg/L (HIV^-), 3 (2–4) mg/L (HIV^+), and 5 (3–7) mg/L (AIDS) and plasma IL12 were 74 (57–88) pg/ml (HIV^-), 134 (82–257) pg/ml (HIV^+), and 197 (151–365) pg/ml (AIDS), respectively.

The other four soluble immune markers studied (IgE, IgG, IgA, TNFR-II) were significantly increased in HIV^+ versus HIV^- individuals, but did not show the above progressively increased values when comparing the three groups of Ethiopians (HIV^-, HIV^+, AIDS). As shown in Fig. 1D, plasma IgE concentration was significantly increased in the HIV^+ group [985 (98–7038) IU/ml], compared to HIV^- Ethiopians [481 (177–1126) IU/ml], but also to AIDS patients [370 (174–1000)].

As shown in Fig. 1E, the plasma levels of IgG were significantly increased in both the HIV^+ [30 (21–36) mg/ml] and AIDS [25 (12–31) mg/ml] groups, compared to HIV^- Ethiopians [(14 (12–17) mg/ml], with no significant difference between the two HIV-1-infected groups. Figure 1F demonstrates that the increase in IgA concentration in the individuals with AIDS [3 (2–5) mg/ml] was significant, compared to the HIV^- group [2 (2–3) mg/ml], but not when compared to the HIV^+ group [2 (2–4) mg/ml]. Finally, as shown in Fig. 1G, the levels of sTNFR-II were also significantly increased in the Ethiopians with AIDS [15 (10–21) ng/ml], compared to the HIV^- [5 (4–7) ng/ml] and HIV^+ [5 (3–6) ng/ml] groups. However, there was no significant difference in plasma sTNFR-II concentration between HIV^- and HIV^+ Ethiopians.

As shown in Fig. 1H, a significantly increased $\log_{10}$ viral RNA copy number was detected in the Ethiopians who developed AIDS [median 3.949 (1.903–5.122) $\log_{10}$ RNA molecules/ml plasma)] compared to the HIV-1-infected asymptomatic Ethiopians [median 3.421 (1.903–4.384) $\log_{10}$ RNA molecules/ml plasma].

Association of Soluble Markers with Each Other and with Cell Surface Activation Markers

We investigated correlations of soluble markers with proportions of naïve and activated T-cell subsets. Table 1 indicates that the plasma levels of sTNFR-II, sCD27, b_2m, IL12, and IgG were inversely correlated with the number of $CD4^+$ T-cells of the three groups, with proportions of naive $CD8^+$ T-cells and with proportions of $CD8^+$ cells expressing CD28. However, plasma levels of these soluble molecules were positively correlated with proportions of activated $CD4^+$ and $CD8^+$ T-cells.

The HIV-1 RNA load in plasma showed a tendency to negatively correlate with the number of $CD4^+$ T-cells. It also showed a moderate but positive correlation with proportions of activated $CD4^+$ and $CD8^+$ T-cells. We performed regression analyses to estimate the association of each of the soluble immune markers with the number of $CD4^+$ T-cells and plasma viral load in the HIV-1-infected individuals (HIV^+ and AIDS). Univariate analysis showed a significant association for sTNFR-II, sCD27, and b_2m with $CD4^+$ T-cells (Table 2), but plasma viral load was only significantly associated with sTNFR-II (data not shown). Multivariate analysis revealed that sCD27 was the best independent marker of a decrease in the $CD4^+$ T-cell count. However, plasma viral load was not associated with any of the soluble markers in multivariate analysis. Spearman's correlation coefficients were also calculated to determine the association of the soluble markers with each other. Significant positive correlations were observed when plasma levels of sTNFR-II, b_2m, sCD27, IL12, and IgG were correlated to each other (Table 3).

DISCUSSION

Several studies have reported the accelerated progression to AIDS in HIV-1-infected Africans (6, 7). Furthermore, recent reports more specifically pointed toward a possible more rapid HIV-1 disease progression in African women infected with subtype C viruses (12). Other reports did not detect a difference in HIV-1 disease progression when comparing HIV-1 subtype-C-infected Ethiopians to HIV-1 subtype-B-infected Caucasians in the same environment of the industrialized world (13, 14).

In order to address the controversial issue of HIV-1 disease progression in Africans, surrogate markers need to be defined, which have prospective value in this context. Relatively little is known about surrogate markers in Africa (8, 15–17). Several soluble laboratory markers, which are easily detectable in serum, have been shown to have a strong predictive value for HIV disease progression in other populations. Some of them were indicated to be as good as or sometimes even more accurate than numbers of $CD4^+$ T-cells as prognostic factors for the occurrence of AIDS (18–23). In this study we assessed the plasma levels of soluble immune markers in HIV-1-infected and noninfected Ethiopians and we also investigated the association of these markers with proportions of various T-cell populations.

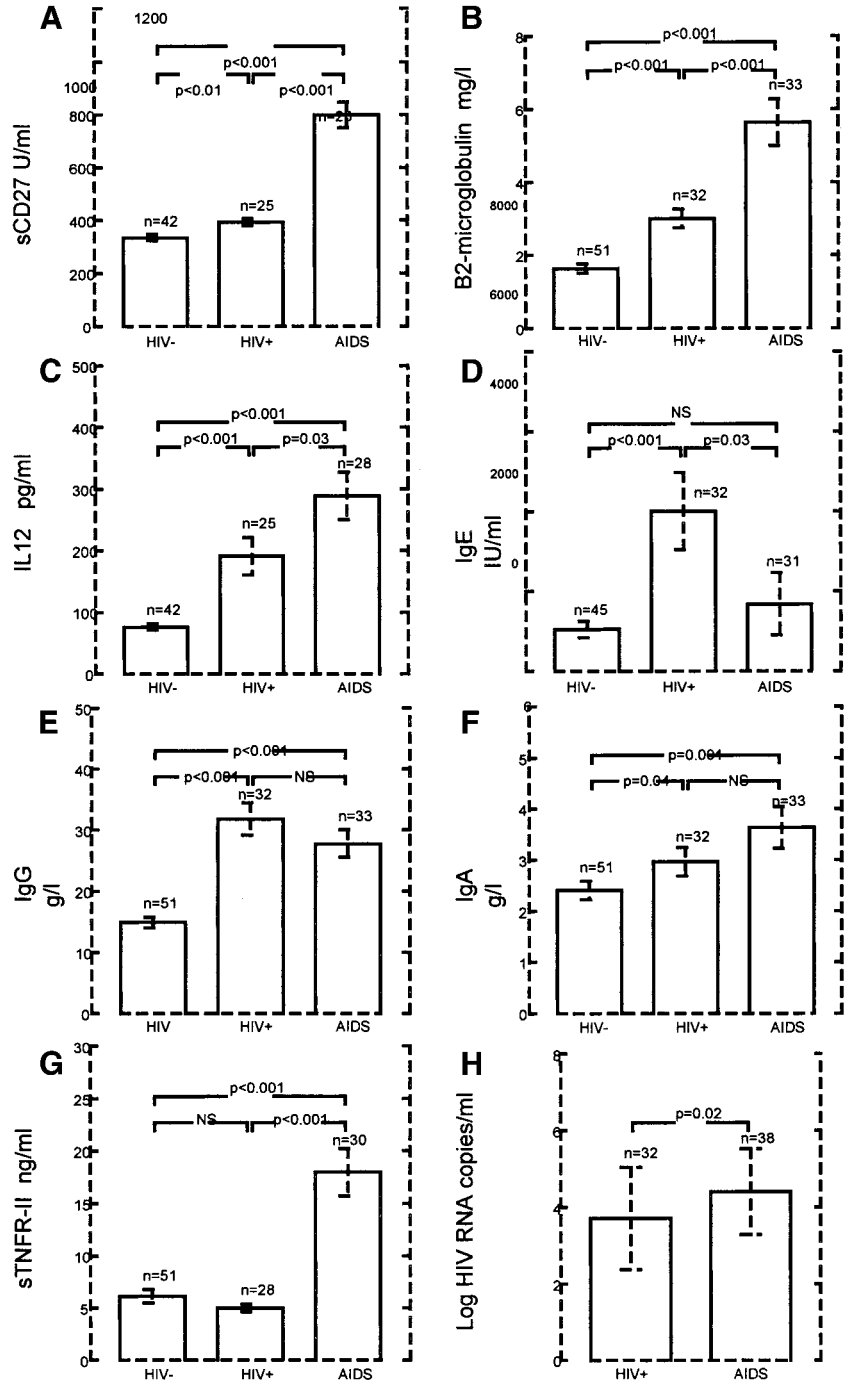

FIG. 1. Comparison of plasma levels of soluble immune markers between HIV^-, HIV^+, and AIDS groups. (A) sCD27, (B) b_2m, (C) IL12, (D) IgE, (E) IgG, (F) IgA, (G) sTNFR-II, (H) $\log_{10}$ viral loads. Data are expressed in 50 percentiles, with vertical bars indicating 25 and 75 percentiles. The number of subjects tested is indicated, as well as the *P* values of the various comparisons.

Soluble Markers Progressively Increasing with HIV Status

The levels of sCD27 in plasma showed a progressive increase in HIV-1-infected Ethiopians. Membrane-bound CD27 is expressed and released in a soluble CD27 form by activation of predominantly T-cells (24). Although sCD27 is also present in biological fluids of healthy individuals, highly increased levels of sCD27 are detected in the serum and urine of CMV patients,

TABLE 1

Spearman Correlation Coefficients (and *P* Values) of Comparisons between Soluble Markers, Log_{10} Viral Loads (HIV^+ and AIDS Groups, $n = 71$), and Various T-Cell Subsets (HIV^-, HIV^+, and AIDS Groups, $n = 123$)

T-cell subsets	Viral load	sTNFR-II	sCD27	b_2m	IL12	IgG
Number of $CD4^+$ T-cells	−0.25 (0.03)	−0.53 (<0.001)	−0.64 (<0.001)	−0.66 (<0.001)	−0.59 (<0.001)	−0.53 (<0.001)
% $CD8^+CD28^+$	−0.09 (NS)	−0.40 (<0.001)	−0.40 (<0.001)	−0.44 (<0.001)	−0.33 (<0.01)	−0.31 (<0.01)
% Activated $CD4^+$ T-cells	0.34 (0.004)	0.48 (<0.001)	0.69 (<0.002)	0.73 (<0.001)	0.69 (<0.001)	0.66 (<0.001)
% Activated $CD8^+$ T-cells	0.31 (0.01)	0.46 (<0.001)	0.69 (<0.001)	0.73 (<0.001)	0.70 (<0.001)	0.63 (<0.001)
% Naïve $CD4^+$ T-cells	−0.23 (0.05)	−0.08 (NS)	−0.10 (NS)	−0.06 (NS)	−0.003 (NS)	−0.09 (NS)
% Naïve $CD8^+$ T-cells	−0.29 (0.01)	−0.39 (<0.01)	−0.54 (<0.001)	−0.44 (<0.01)	−0.43 (<0.01)	−0.33 (<0.01)

in the cerebrospinal fluid of multiple sclerosis patients, and also in the serum and synovial fluid of patients suffering from rheumatoid arthritis (25, 26). The strong association of sCD27 levels with the decline in the number of $CD4^+$ T-cells indicates that it could also be used as prognostic marker of HIV disease progression in addition to its previously described potential as a disease progression marker in systemic lupus erythrematosus (SLE) and B-cell leukemia (27, 28). Elevated levels of sCD27 were also reported recently in HIV-1-infected non-African subjects (29).

The progressive increase of plasma b_2m when comparing the Ethiopian HIV^-, HIV^+, and AIDS groups is in accordance with literature, where b_2m is described as a useful prognostic marker for HIV-1 infection in Africa (16, 17); b_2m functions as the light chain of class I histocompatibility molecules (30). It is shed from the cell membrane during the continuous turnover of HLA molecules and serum and urine concentrations are increased in various states of immune activation (31).

IL12 has been described as a Th1 driver cytokine (32, 33). In HIV-1 infection a shift from Th1 to Th2 cytokine production has been indicated and a role for IL12 in disease progression has been postulated. Surprisingly, in our study, the level of IL12 in plasma was significantly and progressively increased in HIV-1-infected Ethiopians compared to the HIV-1-negative group of Ethiopians. This is not in support of IL12 mediating the postulated Th1 to Th2 shift (34).

Similar to what has been reported by Bentwich *et al.* (35), we observed highly elevated plasma IgE levels in HIV-1-negative Ethiopians, compared to HIV-1-negative Dutch subjects (data not shown). In HIV-1-infected Ethiopians the plasma concentration of IgE was found to be again higher than that in HIV-1-negative subjects. This increase could be explained by the previously described association of elevated IgE levels in plasma with T-cell dysregulation and the high prevalence of opportunistic infections in patients with AIDS (36). Also, the nutritional status of people in the early stages of HIV-1 infection could play a role here (37). Although we observed an increase in IgE in the HIV-1-infected Ethiopians, there was no correlation between numbers of $CD4^+$ T-cells and levels of IgE or IgA in plasma. Thus, the increase in IgE and IgA levels is most likely the result of polyclonal activation of B-cells during HIV-1 infection independent of the numbers of $CD4^+$ T-cells in the blood. However, the level of IgG, which was found to be increased in HIV-1-infected Ethiopians, was inversely correlated with the number of $CD4^+$ T-cells and positively correlated with the other soluble immune markers.

The plasma concentration of sTNFR-II was significantly increased in the Ethiopians with AIDS compared to the HIV^+ and HIV^- groups. The ligand for TNFR-II, TNFalpha, has been strongly implicated in the pathogenesis of HIV infection (38). The levels of TNFa in HIV-1-infected Ethiopians are much higher

TABLE 2

Univariate and Multivariate Regression Analyses of Soluble Immune Markers and $CD4^+$ T-Cell Counts in HIV-Infected Ethiopians (HIV^+ and AIDS Groups)

	Univariate			Multivariate		
Markers	Coefficient	95% CI	*P* value	Coefficient	95% CI	*P* value
IgE	0.008	−0.003–0.02	NS	0.004	−0.008–0.01	NS
IgG	0.17	−4.0–4.4	NS	−6.5	−13.8–0.7	**0.07**
IgA	−17.0	−46.0–11.8	NS	−29.0	−73.6–15.4	NS
b_2m	−21.8	−40.2–3.4	**0.02**	15.6	−18.4–49.8	NS
sCD27	−0.40	−0.62–0.18	**0.001**	−0.42	−0.81–0.03	**0.03**
sTNFR-II	−8.7	−14.1–3.2	**0.002**	−5.5	−13.3–2.3	NS
IL12	−0.16	−0.53–0.20	NS	0.22	−0.20–0.66	NS

TABLE 3

Spearman Correlation Coefficients (and *P* Values) of Comparisons among Soluble Markers (HIV^-, HIV^+, and AIDS Groups ($n = 123$) and Log_{10} Viral Loads (HIV^+ and AIDS Groups, $n = 71$)

	sCD27	b_2m	IL12	IgG	Viral load
sTNFR-II	0.79 (<0.001)	0.58 (<0.001)	0.55 (<0.001)	0.23 (NS)	0.42 (0.001)
sCD27		0.80 (<0.001)	0.67 (<0.001)	0.55 (<0.001)	0.31 (0.02)
b_2m			0.72 (<0.001)	0.70 (<0.001)	0.06 (NS)
IL12				0.63 (<0.001)	0.30 (0.02)

than those in HIV-1-infected Caucasians and these levels further increase upon the development of AIDS (39). Overexpression of TNFalpha mRNA and spontaneous secretion of high levels of TNFalpha in culture were observed in PBMCs isolated from AIDS patients (5, 40). However, TNFa is rapidly cleared from the circulation and it is therefore frequently undetectable (41). In contrast to TNFalpha, soluble TNFRs are very stable. Soluble TNFRs, which are shed from neutrophils, activated T-cells, and monocytes as a result of higher levels of TNFa, have been postulated to serve as binding proteins for transportation of TNFalpha and for stabilization and prolongation of its bioactivity. A strong correlation between levels of sTNFR-II and TNFalpha was shown (5). Although an elevated concentration of sTNFR-II is not specific for HIV-1 infection and is observed in many different disease processes such as sepsis, malignancies and autoimmune disorders, the increase of sTNFR, especially of type II in serum, has been found to be a strong indicator of progression to AIDS in non-African populations (4, 19). The significant increase of sTNFR-II in the Ethiopians with AIDS may not only reflect immune activation but also have pathophysiological significance related to the higher occurrence of opportunistic infections in this group.

The soluble immune markers we tested were significantly correlated with each other. Furthermore, we observed significant inverse correlations with the number of $CD4^+$ T-cells, with proportions of naïve $CD8^+$ T-cells, and with proportions of $CD8^+$ T-cells expressing CD28. Also, a positive correlation was observed between soluble markers and proportions of $CD4^+$ and $CD8^+$ T-cells expressing CD38 and HLA-DR. Although some of the soluble markers are not specific for HIV infection, the observed associations between soluble markers and T-cell subsets may suggest that they all reflect the immunologic response to HIV infection. However, plasma viral load showed only a tendency to be correlated positively with activated and negatively with naïve $CD4^+$ and $CD8^+$ T cells. A moderate positive correlation was also observed between viral load and sTNFR-II as well as sCD27. The low correlation of viral load with soluble and cellular markers observed in this study may indicate that HIV viremia is not central to immune activation and HIV disease progression in Ethiopians.

Our results indicate that measurements of soluble immune markers in fresh or stored plasma, which are relatively easy to perform, could be good alternatives to quantification of T-cell subsets when flow cytometry is not available. Although sTNFR-II, sCD27, and b_2m were negatively associated with the number of $CD4^+$ T-cells in univariate analysis, sCD27 showed the best independent association with $CD4^+$ T-cell count in multivariate analysis. It has been shown previously that activation markers on $CD8^+$ T cells have a stronger prognostic value than HIV-1 viral load. This is in agreement with our observation that sCD27 levels correlate better with CD4 decrease than viral load.

Although sCD27 does not play a direct role it might reflect the ongoing immune activation in HIV-1 disease progression. Thus, serial measurement of this marker in prospective studies may contribute to our understanding of HIV-1 pathogenesis and also may give useful prognostic information for HIV disease progression in Ethiopians. In conclusion, the present study is meant to provide information as to which potential immune soluble markers (in addition to the T-cell subsets that we previously described) are useful in predicting HIV disease course in Ethiopians. The results of this study will help to select soluble markers that could be candidates for predicting HIV disease progression in an Ethiopian context, but which subsequently should be validated in prospective studies.

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