Duration of Sample Storage Dramatically Alters Expression of the Human Immunodeficiency Virus Coreceptors CXCR4 and CCR5

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Expression of the chemokine receptors CXCR4 and CCR5 was monitored using EDTA-anticoagulated whole blood held for different time periods prior to fluorescent-antibody staining. When left overnight CXCR4 expression on leukocytes was substantially increased, whereas CCR5 expression was reduced. The results were similar when heparin and acid-citrate-dextrose were used as anticoagulants.

The G protein-coupled chemokine receptors, CXCR4 and CCR5, are the major human immunodeficiency virus (HIV) coreceptors that, in addition to CD4, are utilized for viral entry (5–7). These receptors play an important role in maintaining an effective immune response through chemotaxis in response to their specific ligands; CXCR4-expressing cells migrate in response to SDF-1α (1), while CCR5-expressing cells are recruited by MIP-1α, MIP-1β, and RANTES (4). Therefore, alterations in receptor expression in vivo could lead to dysregulation of cellular trafficking and alterations in cell permissiveness for HIV type 1 (HIV-1).

Over the past few years, a number of studies have evaluated the expression of CXCR4 and CCR5 using whole blood. It has been demonstrated that CXCR4 expression is decreased in HIV-1-infected persons compared with uninfected controls (9, 12). Antiretroviral therapy significantly increases the cellular expression of CXCR4 and decreases that of CCR5 compared with pretherapy levels (9). CCR5 density on CD4+ T cells has further been shown to be a determining factor of virus load in HIV-1-infected individuals (13). A recent study, but using isolated peripheral blood mononuclear cells, has shown that thalidomide can reduce the upregulation of CXCR4 and CCR5 induced by bacterial and mycobacterial antigens (11). Flow cytometric analysis of these chemokine receptors is therefore a valuable tool, particularly in HIV-1 disease, and is likely to be utilized in the future for predictive purposes and for monitoring the success of various therapies.

As part of a study to investigate the effect that infection with HIV-1 has on CXCR4 and CCR5 receptor expression, we had stained EDTA-anticoagulated blood samples from a cohort of HIV-1-infected patients (n = 9); these samples had been delayed due to transportation difficulties. To control for possible effects due to standing time, we compared these results with those from a cohort (comparable with respect to age, sex, race, CD4 cell count, and viral load) where samples were stained within 6 h (n = 11). Using two- and three-color staining, different subsets of mononuclear cells expressing either CXCR4 or CCR5 (CXCR4-phycocerythrin [PE] and CCR5-PE; Pharmingen, San Diego, Calif.) were identified with markers that distinguish T cells (CD3-peridinin chlorophyll protein [PerCP]), CD4+ and CD8+ T cells (CD4-PerCP, CD8-PerCP, CD3-fluorescein isothiocyanate [FITC]), B cells (CD19-FITC; Becton Dickinson, San Jose, Calif.), monocytes (CD14-FITC; Coulter, Hileah, Fla.), and CD16+ CD56+ CD3− natural killer (NK) cells (CD16-FITC [Becton Dickinson] and CD56-PE [Serotec]). B cells and NK cells were analyzed after gating for lymphocytes based on CD45 staining and forward scatter (FSC) and side scatter (SSC) properties. CD4+ and CD8+ T cells were identified by initial gating on total CD3+ cells and SSC. Monocytes and granulocytes were identified using their FSC and SSC properties and the presence or absence of CD14 staining, respectively. In order to control for nonspecific staining, quadrants were set using isotype-matched controls immunoglobulin G1 (IgG1)-PerCP and IgG2a-PE (Becton Dickinson), IgG1-FITC (Dako), and IgG2a-FITC (Serotec). Ten thousand events were acquired per sample. Calibrite beads (Becton Dickinson) were run on a weekly basis to ensure the stability of the flow cytometer. When we compared CXCR4 staining of whole blood within 6 h (t = 0) with staining after overnight (ON) incubation there were significant increases in CXCR4 expression on all cell subsets, as reflected in both the percentage of fluorescing cells (except for on polymorphonuclear neutrophils) and in the mean fluorescence intensity (MFI). In contrast, there was a trend toward a decrease in CCR5 expression in the samples that were stained after ON incubation compared with those stained within 6 h (data not shown).

In order to confirm these observations, longitudinal analysis was performed on whole blood samples obtained from six...
FIG. 1. Longitudinal analysis of CXCR4 (A) and CCR5 (B) receptor expression on cellular subsets from healthy individuals (normal donor group) and HIV-1-infected individuals (HIV group) stained within 6 h ($t = 0$) (open bars) or left ON (hatched bars). The results are expressed as the percentage of positive cells as well as the MFI (fluorescence intensity). The values represent the mean ± the standard error of the mean. The Wilcoxon test for related samples was used to determine whether the ON samples were significantly different from the samples at $t = 0$. *, $P < 0.05$. 
healthy individuals (normal donor group) and from seven HIV-1-infected individuals (HIV group). These samples were stained within 6 h (t = 0) after blood was drawn and again after being left at room temperature ON. As shown in Fig. 1A, delaying the sample staining resulted in a significant upregulation of CXCR4 expression on all cell types, as shown in both proportions of cells expressing CXCR4 as well as in the MFI. Exceptions were the proportions of CXCR4-expressing polymorphonuclear neutrophils and MFI of CXCR4 on NK cells in the HIV group. Conversely, CCR5 expression tended to be reduced within all cell subsets evaluated except for CD14+ monocytes (MFI) in the HIV group (Fig. 1B). However, significance was only attained for intensity of CCR5 fluorescence on CD3+, CD4+, and CD8+ lymphocytes in both groups; proportions of CCR5-expressing CD14+ monocytes in the normal donor group; and proportions of CCR5-expressing CD3+ lymphocytes in the HIV group. Figure 2 shows representative dot plots of data (CXCR4 and CCR5 expression on CD3+ cells) from a normal donor.

These findings raised the question as to whether the use of anticoagulants other than EDTA would give similar results. We used CD3+ cells as the representative subset for the comparisons since these cells showed significant alterations in both CXCR4 and CCR5 expression in the cross-sectional and the longitudinal analyses. As shown in Fig. 3A, CXCR4 upregulation occurred over the times indicated with all three anticoagulants evaluated, with the most dramatic increase in fluorescence intensity occurring after overnight incubation. In contrast to the upregulation of CXCR4, CCR5 expression was again downregulated with respect to the proportions of CD3+ cells expressing CCR5 as well as in the MFI (Fig. 3B).

The extent of the modulation of these receptors with time was surprising. The rapid upregulation of CXCR4 expression may be due to the fact that various leukocytes have been shown to contain large intracellular stores of this receptor (8). CXCR4 is localized in endosomal compartments from where it can recycle to the cell surface (14), and CXCR4 has been shown to be increased on the surface of lymphocytes after only a few hours of culture (2). Studies on the effect of activation of peripheral blood leukocytes on CXCR4 expression have been contradictory, with phytohemagglutinin being shown by some groups to decrease CXCR4 expression (2, 8, 14), while others have reported a rapid upregulation of expression (3).

In summary, our results have clearly demonstrated that CXCR4 and CCR5 expression was reciprocally altered with sample standing time. This has been confirmed in another study (10). What was also apparent was that CXCR4 was more easily modulated, with significant alterations being found for
most cell subsets, while CCR5 expression was most significantly altered in CD3⁺, CD4⁺, and CD8⁺ cells. Furthermore, CXCR4 and CCR5 were modulated similarly in both the normal-donor and HIV cohorts. Moreover, these alterations in receptor expression occurred with all three anticoagulants examined. Since it is often impossible to stain patient samples immediately due to logistical constraints, we recommend using 6 h as a cutoff time in which samples should be processed. In conclusion, the data presented here demonstrate that, in order to avoid compromising the accuracy of results due to ex vivo effects, careful consideration of time of venesection should be taken, particularly in the case of longitudinal samples from the same patient that are to be compared. It should be further emphasized that awareness in this regard would preclude inconsistencies in findings of receptor expression that are likely to occur within and between different laboratories.

REFERENCES


