The accurate measurement of T cell-associated CC chemokine receptor type 5 (CCR5) and CXC chemokine receptor type 4 (CXCR4) expression, including expression of CCR5 and CXCR4 mRNA as an immune measure of immunologic response to highly active antiretroviral therapy (HAART) and newer agents, including entry inhibitors, is essential. Previous studies have reported alterations in lymphocyte cell membrane CCR5 expression that were related to blood collection and cell separation media. Clinical trials often require the transport of specimens to central laboratories for evaluation, resulting in significant time delays between specimen procurement and analysis. This study shows that CCR5 expression on naïve and memory T cells is influenced by blood collection media and specimen age. Peripheral blood collected in Streck Vacutainer tubes containing a cell stabilizer and fixative was found to improve detection of CCR5 expression compared to specimens collected in K2 EDTA anticoagulant. The selection of flow cytometry gating strategies for the identification of naïve and memory T-helper cells can also significantly influence the sensitivity of detection of CCR5 expression. Procedural methods are described that allow for the optimal measurement of naïve and memory T-helper cell CCR5 and CXCR4 expression as well as the quantitation of CCR5 and CXCR4 mRNA.

The evolution of highly active antiretroviral therapy (HAART) over the past decade has led to marked increases in survival rates for those infected with human immunodeficiency virus type 1 (HIV-1) (12). Along with these successes has come the challenge of confronting an ever-increasing occurrence of drug resistance (2, 4, 11). In order to meet this challenge, novel approaches to the treatment of HIV-1 infections have emerged that focus on the viral integrase (5), as well as viral coreceptors essential for viral cell entry (8, 10, 16). The mechanism by which these coreceptors facilitate HIV-1 viral entry into host cells involves specific binding sites within the V3 region of the gp120 envelope protein of HIV-1 (3). Changes in HIV-1 cell tropism (R5 to X4) are associated with point mutations involving single amino acid substitutions within the V3 region of gp120 at positions 304 and 322 (14). Naïve and memory T cells play a role in the transition from R5 to X4 tropism, with R5 viruses preferentially infecting memory T-helper cells (13). HIV CC chemokine receptor type 5 (CCR5) coreceptor antagonists (maraviroc and vicriviroc) in combination with other antiretroviral agents enhance treatment outcomes in HIV-infected adult subjects (6, 15). The ability to accurately measure T-cell CCR5 and CXC chemokine receptor type 4 (CXCR4) expression at the protein and gene levels will provide important immune measures of patient response to CCR5 antagonist therapy, particularly in cases of virologic failure. CCR5 and CXCR4 expression on naïve and memory T cells has been shown to be sensitive to in vitro manipulations (1). These flow cytometry-based studies showed that simple cell isolation procedures, such as Ficoll-Hypaque density gradient sedimentation, resulted in reduced expression of CCR5 on T cells compared to the evaluation of whole-blood samples followed by red cell lysis and fixation. These studies evaluated freshly collected blood samples and did not evaluate the influence of specimen age on CCR5 expression. This is an important consideration in the setting of clinical trials that often require specimen transport to central laboratories for evaluation, resulting in the study of blood samples that can often be as much as 24 h old.

Our study evaluated the impact of blood collection media, specimen age following blood draw, and flow cytometry gating strategies on the measurement of CCR5 and CXCR4 cell membrane expression on whole-blood-derived naïve and memory T cells. This study also evaluated individual variation in CCR5 and CXCR4 expression over time using whole-blood-derived T cells obtained from healthy adult control subjects, with samples collected at four time points over 3 weeks. HIV-1-positive subjects on maintenance antiretroviral therapy (ART) were evaluated at a single time point. Parallel studies of variability in CCR5 and CXCR4 mRNA expression in peripheral blood mononuclear cells (PBMC) were performed for the same cohorts using a previously described real-time reverse transcription (RT)-PCR assay (9).

MATERIALS AND METHODS

Study subjects, blood collection media, and specimen treatments. This study was approved by the Institutional Review Board of The Children’s Hospital of Philadelphia Research Institute. Peripheral whole-blood samples collected in K2 EDTA or Cyto-Chex BCT (a Vacutainer tube containing a proprietary cell stabilizer and fixative; Streck Laboratories, Omaha, NE) were obtained from an established donor pool of consenting healthy adult subjects and from consenting HIV-1-infected subjects on maintenance ART who are routinely followed in the University of Pennsylvania Infectious Disease Clinic. Depending on experimental objective, blood samples were evaluated within 6 h of blood draw or held at

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Flow cytometry CCR5 and CXCR4 immunophenotyping. Three- and four-color flow cytometry protocols were developed for the measurement of CCR5 and CXCR4 expression on CD3+CD4+, CD3+CD8+, sodium (CD45RO+) and naive (CD45RO−) T cells, including memory (CD45RO−/CD8+). The final gating strategy (see Fig. 3) was based on preliminary studies of the impact on CCR5 and CXCR4 measurements using T-helper cell anchor gating employing either CD3-peridinin chlorophyll protein (PerCP)/CD4-allophycocyanin (APC), CD3-PerCP/CD4-Alexa Fluor 647, or CD45-fluorescein isothiocyanate (FITC)/CD3-PerCP/CD4-Alexa Fluor 647 (gating on the CD3+CD4+ cells) or CD3-PerCP/CD8-APC, CD3-PerCP/CD4/CD8-APC, CD45-FITC/CD3-PerCP/CD4-Alexa Fluor 647, or CD45-FITC/CD3-PerCP/CD8-Alexa Fluor 647 (gating on the CD3+CD8+ cells). Naive and memory T-helper cell subsets were differentiated based on CD45RO-FITC-positive and -negative fluorescence signals. Becton Dickinson FACSCalibur and BD LSR II flow cytometers were used for data acquisition and analysis employing Cell Quest PRO or FACs Diva analytical software, respectively (Becton Dickinson, San Diego, CA). The fluorochrome-conjugated antibodies used were CD3-FITC, CD3-PerCP, CD4-PerCP/CD8-APC, CD8-Alexa Fluor 647, CD45RO-FITC, CCR5-phycocerythrin (PE), and CXCR4-PE (BD PharMingen, San Diego, CA). One hundred-microliter aliquots of whole-blood samples were stained using a 1× wash procedure (BD Biosciences, San Diego, CA). CCR5 and CXCR4 expression on naive and memory T-helper cells was evaluated using PE-conjugated, mouse anti-CCR5 antibody (clone 2D7) and PE-conjugated, mouse anti-CXCR4 antibody (clone12G5; BD PharMingen, San Diego, CA). Samples stained with PE-conjugated mouse IgG2a isotype control antibodies were used to establish cursor settings that allowed for the differentiation of positive and negative fluorescence signals. The same lot of PE-conjugated control and epitope-specific antibody was used throughout the study to allow longitudinal comparisons. After staining of samples for 30 min at 4°C, red blood cells were lysed with the remaining white cells fixed in 2% (wt/vol) paraformaldehyde. The samples were stored at 4°C in dark until evaluated for fluorescence intensity by flow cytometry. Prior to the flow cytometric evaluation of patient samples, the instruments were optimized with the use of fluorescence compensation using FACSComp software for the BD FACS Calibur flow cytometer and FACS Diva software for the BD LSR II instrument (Becton Dickinson, San Diego, CA). A total of 2,500 events were accumulated for each target cell population. Geometric mean values for the fluorescence distribution and percent positive cells for both the isotype control and CCR5- and CXCR4-specific, PE-conjugated antibodies were recorded for total T-helper cell as well as T-helper cell naive and memory subsets.

Quantitation of CCR5 and CXCR4 mRNA by real-time RT-PCR. Real-time RT-PCR was used for the quantitative measurement of CCR5 and CXCR4 mRNA copy number in RNA extracts prepared from peripheral blood mononuclear cells as previously described (9). RNA was extracted from freshly isolated, whole-blood-derived PBMC by routine Ficoll-Hypaque density gradient centrifugation (9). RNA was treated with RNase-free DNase I before reverse transcription (RT) with the Superscript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Reverse transcription was followed by 40 cycles of amplification using the SYBR Green Supermix (Applied Biosystems, Foster City, CA) and the ABI 7000 Sequence Detection System (Applied Biosystems). The primers used were CCR5 forward, 5′-GCTTGTCTGGGCCCAAGCTG-3′; CCR5 reverse, 5′-ACGGCCACTGCTTCCAGTCA-3′; CXCR4 forward, 5′-CACTCTGGAGCTGTGGGCTG-3′; CXCR4 reverse, 5′-CTCAGGTCTGGCTGTTGCTG-3′. The volume of PCR reactions was 10 μl with 2 μl of cDNA template and 8 μl of reaction mix. The PCR was performed at 50°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence was acquired at the end of each elongation step, and the threshold cycle was determined for each sample. The threshold cycle for the calibrator sample was used as the baseline for calculation of the relative expression level of the target RNA of interest (10). All calibrations were performed in triplicate. The significance of this increase in CCR5 mRNA levels measured at week 3 is unclear, as it was not reflected in increases in CCR5 protein levels. In healthy adult controls (Fig. 5) and HIV-positive subjects (Fig. 6), the level of CXCR4 expression at both the protein and mRNA levels was consistently higher than the CCR5 levels.

Influence of specimen age on the flow cytometric evaluation of K2 EDTA anti-coagulated whole-blood-derived T-helper cell CCR5 and CXCR4 expression. Figure 7 illustrates the results of the evaluation of CCR5 and CXCR4 expression on K2 EDTA anti-coagulated whole-blood-derived T-helper cells from healthy control subjects collected at 4 time points over a
FIG. 1. Influence of flow cytometry T-helper cell anchor gating strategies on the measurement of CCR5 expression based on CD3⁺/CD4⁺ staining (A and D), CD3⁺/CD8⁺ staining (B and E), and CD45⁻/CD3⁺/CD8⁺ staining (C and F). Evaluations were performed on fresh (evaluated within 6 h of blood draw) K2 EDTA anti-coagulated whole blood obtained from an HIV-infected subject (A to C) and a healthy adult control subject (D to F). CCR5-positive fluorescence signals were collected in single- and dual-parameter histograms, with percent positive cells indicated.
A 3-week period. Figure 8 illustrates the findings of identical studies performed on blood samples collected at a single time point from HIV-infected adult subjects. In order to assess the influence of specimen age on coreceptor expression, each specimen was evaluated within 6 h of blood draw and again between 24 and 30 h after collection. In each case, specimens were held at ambient temperature until evaluated.

The evaluation of freshly collected specimens (less than 6 h post-blood draw) resulted in significantly higher percentages of CCR5-positive cells and fluorescence intensity than those in the parallel evaluation of specimens held at room temperature between 24 and 30 h. Unlike CCR5 expression, specimens held between 24 and 30 h yielded higher percentages of CXCR4 positivity, with a marked increase in fluorescence intensity.

Influence of blood collection media on whole-blood-derived memory T-helper cell CCR5 expression. The observed specimen age-related variability in T-cell CCR5 and CXCR4 expression using K2 EDTA anti-coagulated whole blood prompted our comparative evaluation of blood samples collected in K2 EDTA and a proprietary blood collection medium containing a cell stabilizer shown to preserve a number of lymphocyte markers using specimens held for as many as 2 weeks at room temperature (Streck Cyto-Chex BCT blood collection tubes; Streck Laboratories, Omaha, NE). Figures 8 and 9 illustrate the results obtained in the evaluation of memory T-helper cell CCR5 expression using healthy adult control blood collected in both K2 EDTA and Streck blood collection tubes, with evaluations performed within 6 h and between 20 and 24 h of blood draw for both blood collection media. An additional evaluation was performed between 44 and 48 h using blood collected in the Streck tubes only, as blood samples collected in K2 EDTA are not suitable for flow cytometric studies when held beyond 30 h prior to evaluation. As noted in our studies of total T-helper cell CCR5 expression, blood samples collected in K2 EDTA and held between 20 and 24 h prior to evaluation yielded significant reductions in CCR5 expression within the memory T-helper cell subset. Cells collected in Streck tubes and held between 44 and 48 h prior to evaluation yielded no significant reductions in memory T-helper cell CCR5 expression. Blood samples were obtained at four time points over a 3-week period. Data points are color coded to allow discrimination of individual subjects over the period of evaluation.
CCR5 expression compared to levels observed with blood samples drawn in K2 EDTA and evaluated within 6 h of collection. Whole-blood samples collected in Streck tubes revealed no significant differences in the percentage of memory T-helper cells expressing CCR5 for the three time points evaluated (Fig. 10A). There was a significant reduction in CCR5 mean fluorescence intensity observed between <6 h and <24 h, with no significant reductions observed between <6 h and <48 h (Fig. 10B). However, there were no significant differences in either percent CCR5-positive cells or CCR5 fluorescence intensity for all three time points compared to levels obtained with whole blood collected in K2 EDTA anticoagulant (Fig. 9 and 11).

DISCUSSION

The combined requirement of CD4 and the HIV coreceptors CCR5 and CXCR4 for R5 and X4 viral host cell entry suggests that these three cell surface molecules are most likely topographically in close proximity to each other. Further, antibodies directed against any one of the three molecules could conceivably by steric hindrance block each other’s binding to their targeted epitope. This hypothesis is supported by our findings of enhanced detection of CCR5 expression using a CD3+/CD8+/anchor gating strategy to target T-helper cells. Although CD45 is expressed at very high levels on CD4+ T-helper cells (Fig. 1), adding CD45 to the CD3+/anchor gating strategy had minimal influence on the measurement of CCR5. This observation adds support for the interpretation that the observed reduction in CCR5 expression using a CD3+/CD4+ gating strategy is the result of interference of the CCR5 binding antibody by the anti-CD4 binding antibody. The demonstration of enhanced CCR5 detection by first staining with CCR5-specific antibodies prior to staining with T-helper cell-specific antibodies (Fig. 2) adds further support for this hy-
pothesis. In addition, the reduced level of cell surface expression of CCR5 in blood samples held for 24 to 30 h prior to evaluation is accompanied by an increase in the detection of CXCR4. This conclusion is also supported by fluorescence resonance energy transfer imaging studies that demonstrated colocalization of CD4 and CCR5 on the plasma membrane of viable CD4-yellow fluorescent protein (YFP)- and CCR5-cyan fluorescent protein (CFP)-transfected HEK293T cells following treatment with a V3-containing HIV-1 gp120 core (7, 17). Using CD3+/CD8− T-helper cell gating allows for increased detection of CCR5 in total, naive, and memory T-helper cells, particularly in the evaluation of cells from HIV-positive subjects (Fig. 4). These results provided the basis for the selection of CD3+/CD8− T-helper cell anchor gating for the measurement of CCR5 and CXCR4 expression on naïve and memory T cells. The gating strategy described in our study couples T-helper cell gating with additional gates that allow for the differentiation of naïve and memory phenotypes based on CD45RO-positive and -negative signals (Fig. 3).

K2 EDTA anti-coagulated whole blood has been the blood collection medium of choice for flow cytometry-based studies of lymphocyte cell surface markers and has been used extensively in AIDS clinical trials, in which such evaluations, including T-cell CD4 percentages and absolute counts, are important end points for the measurement of response to various therapeutic interventions. T-cell markers, including CD4 and CD8, have been shown to be relatively stable in K2 EDTA anti-coagulated whole blood over a 24- to 30-h period, while other markers such as CD62L and CCR5 are sensitive to various in vitro manipulations (1). Our studies have extended the findings of Berhanu et al. (1) by showing that the level of detection of T-helper cell CCR5 and CXCR4 expression using K2 EDTA anti-coagulated whole blood is dependent on a number of factors, including the appropriate selection of T-helper cell anchor gating and the age of the specimen prior to analysis.

This study has also demonstrated the practicality of measuring CCR5 and CXCR4 expression at both the protein and mRNA levels. The accurate measurement of whole-blood-derived T-cell CCR5 expression in transported specimens of various ages has become an important capability in clinical studies of CCR5 antagonists as adjuvants to antiretroviral therapies. Our study has identified a number of factors that minimize variation in T-cell CCR5 expression that allow for specimen transport within at least a 48-h window. The repetitive analysis of T-helper cell CCR5 measurements using peripheral blood samples collected in Streck Cyto-Chex BCT Vacutainer tubes and held at room temperature for up to 48 h was found to yield more reproducible results than specimens collected in K2 EDTA. These findings will allow for the transport of clinical specimens to central laboratories having the expertise to perform such studies not available at many local clinical sites, thus providing important measures of immune function in response to therapy.

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REFERENCES