Analytical and Biological Considerations in the Measurement of Cell-Associated CCR5 and CXCR4 mRNA and Protein

D. E. Campbell,* J. P. Lai, N. B. Tustin, E. Riedel, R. Tustin III, J. Taylor, J. Murray, and S. D. Douglas

The Children’s Hospital of Philadelphia Research Institute, Philadelphia, Pennsylvania

Received 28 December 2009/Returned for modification 2 March 2010/Accepted 3 May 2010

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) co-receptor 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-refix 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

* Corresponding author. Mailing address: Division of Allergy and Immunology, The Children’s Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104. Phone: (215) 590-2353. Fax: (215) 590-3044. E-mail: campbelld@email.chop.edu.

† Published ahead of print on 12 May 2010.
room temperature between 20 and 24 h and between 44 and 48 h prior to evaluation.

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+ (based on CD3+/CD8− staining) T cells, including memory (CD45RO+) and naive (CD45RO−) subsets. 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/CD6-APC, CD3-PerCP/CD8-Alexa Fluor 647, or CD45-FITC/CD3-PerCP/CD8-Alexa Fluor 647 (gating on CD3−/CD8+ cells). Naïve and memory T-helper cells 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 CD5-FITC, CD3-PerCP, CD4-PerCP, CD4-Alexa Fluor 647, CD8-PerCP, 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 lysing wash procedure (BD Biosciences, San Diego, CA). CCR5 and CXCR4 expression on naïve 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 allow 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 optimally aligned with optimization 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 cells, as well as T-helper cell naïve 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 PicoPure Hpyhaque density gradient sedimentation. The assay employed CCR5 and CXCR4 up and down primers designed specifically to amplify a 189-bp segment of the CCR5 gene and a 100-bp segment of the CXCR4 gene. The molecular beacon probe was labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with quencher 4-(6-aminohexyl)-amino phenylalano benzoic acid. The assay has a dynamic detection range of 10⁵ to 10⁸ molecules. Data were calculated as copies of CCR5 and CXCR4 mRNA per 10⁶ copies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analyses. The statistical analyses of differences in the levels of CCR5 and CXCR4 protein expression on T-cell subsets between the various treatment groups were performed by the Student two-tailed t test for paired observations using GraphPad Prism version 5.01 software (GraphPad Software, Inc., La Jolla, CA). The Student two-tailed t test for unpaired observations was used in the analyses of CCR5 and CXCR4 expression between control and HIV-infected subjects.

RESULTS

Influence of flow cytometry T-helper cell anchor gating on CCR5 expression. Flow cytometry was based on preliminary three- and four-color flow cytometry gating methods on the measurement of whole-blood-derived T-helper cell CCR5 expression using T-helper cell-positive (CD3+/CD4+) and -negative (CD3−/CD4−) gating strategies. CD3+/CD8− staining consistently yielded higher T-helper cell CCR5 percentage and mean fluorescence intensity (MFI) values than those for CD3+/CD4+ -stained cells. The effect of staining cells with CCR5-specific antibodies before and after staining with positive T-helper cell-specific antibodies (CD3+/CD4+) on CCR5 measurements was also investigated. Marked increases in both the percentage and MFI of CCR5 expression was observed when cells were first stained with CCR5-specific antibodies before staining with T-helper cell-specific antibodies (Fig. 2). Taken together, these data suggest that T-helper cell CD4 and CCR5 are in close proximity on the cell surface and antibodies directed against one or the other of the molecules may interfere with antigen antibody binding presumably by steric hindrance. Figure 3 shows the gating strategy selected for the optimal flow cytometric evaluation of CCR5 and CXCR4 expression on naïve and memory T-helper cells using a CD3+/CD8+ gating strategy. Figure 4 summarizes the influence of T-helper cell gating strategies on the flow cytometric detection of positive CCR5 signals within total, naïve, and memory whole-blood-derived T-helper cells from healthy adult control subjects and HIV-infected adult patients. CD3+/CD8− anchor gating consistently yielded higher levels of CCR5-positive signals than a CD3−/CD8+ gating strategy.

Longitudinal variation in T-helper cell CCR5 and CXCR4 mRNA and protein expression in a healthy adult cohort as assessed by flow cytometry and RT-PCR. A three-color flow cytometry protocol using a CD3+/CD8+ T-cell anchor gating strategy was first applied to the longitudinal evaluation of T-helper cell CCR5 and CXCR4 expression in a healthy adult cohort. K2 EDTA anti-coagulated whole-blood samples were collected at 4 time points over a 3-week period and were evaluated within 6 h of collection. Parallel studies of CCR5 and CXCR4 mRNA copy number as assessed by RT-PCR were also performed using RNA extracted from PBMC isolated within 6 h of blood draw from K2 EDTA anti-coagulated whole blood. Figure 5 illustrates the individual subject variability in the measurement of T-helper cell CCR5 and CXCR4 expression as assessed by flow cytometry (Fig. 5A) and RT-PCR (Fig. 5B). The percent coefficient of variation for the flow cytometric measurement of T-helper cell CCR5 and CXCR4 expression as percent positive cells ranged from 7.7% to 24.9% and 1.0% to 5.2%, respectively. The percent coefficient of variation for the measurement of PBMC-derived CCR5 and CXCR4 mRNA ranged from 4.4% to 32.3% and 18.3% to 43.7%, respectively. No significant differences were observed in CCR5 and CXCR4 protein levels over the four time points evaluated. In addition, no significant differences were observed in CCR5 and CXCR4 mRNA levels between week 0, week 1, and week 2. Minor increases in CCR5 mRNA levels were observed at week 3 compared to week 0, week 1, and week 2. 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.
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

**FIG. 2.** Influence of sequence of anti-CCR5 and anti-CD3/CD4 antibody staining on CCR5 measurements by three-color flow cytometry. Freshly drawn K2 EDTA anti-coagulated whole-blood samples were sequentially stained with either anti-CD3 or anti-CD4 anchor gating antibodies followed by anti-CCR5 antibodies (fluorescence histograms A1 to D1) or with anti-CCR5 antibodies followed by anti-CD3 and anti-CD4 antibodies (histograms A2 to D2). Each fluorescence histogram indicates the resulting anchor-gated percent CCR5-positive signals.

**FIG. 3.** Flow cytometry CD3⁺/CD8⁻ gating strategy for the measurement of CCR5/CXCR4 cell membrane expression on whole-blood-derived naïve and memory T lymphocytes.

**FIG. 4.** Influence of flow cytometry CD4-positive (CD3⁺/CD4⁺) and CD8-negative (CD3⁺/CD8⁻) T-helper cell gating strategies on the measurement of CCR5 expression on total, naïve, and memory T-helper cells. NS, not significant.

**FIG. 5.** Longitudinal variation in the measurement of CCR5 and CXCR4 expression in a healthy adult cohort as assessed by flow cytometry and RT-PCR. Three-color flow cytometry was used to evaluate fresh (evaluated within 6 h of blood draw) K2 EDTA anti-coagulated whole-blood-derived T-helper cell CCR5 and CXCR4 expression (A), while RT-PCR was used to evaluate CCR5 and CXCR4 mRNA copy number in freshly isolated (RNA extracted within 6 h of blood draw) PBMC (B). 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⁺/CD8⁺ 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-
Using CD3⁺/CD8⁻/CD45RO⁻ CCR5 expression (% positive cells [A] and mean fluorescence intensity [B]) in whole-blood samples collected in Streck Vacutainer tubes. Data points are color coded to allow discrimination of individual subjects over the period of evaluation.

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.

ACKNOWLEDGMENTS

Overall support for the International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT) was provided by the National Institute of Allergy and Infectious Diseases (NIAID) (U01 AI068632), the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), and the National Institute of Mental Health (NIMH) (AI068632). This work was supported by the Statistical and Data Analysis Center at Harvard School of Public Health, under the National Institute of Allergy and Infectious Diseases cooperative agreement number 5 U01 AI41110 with the Pediatric AIDS Clinical Trials Group (PACTG) and number 1 U01 AI068616 with the IMPAACT Group. Support of the sites was provided by the National Institute of Allergy and Infectious Diseases (NIAID) and the NICHD International and Domestic Pediatric and Maternal HIV/AIDS Clinical Trials Network funded by the NICHD (contract number N01-DK-9-001/HHSN267200800001C). This work was performed by the Children’s Hospital of Philadelphia IMPAACT Specialty Laboratory. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

REFERENCES
1. Berhanu, D., F. Mortari, S. C. DeRosa, and M. Roederer. 2003. Optimized lymphocyte isolation methods for analysis of chemokine receptor expression. J. Immunol. Methods 279:199–207.
2. Cane, P. A. 2009. New developments in HIV drug resistance. J. Antimicrob. Chemother. 64(Suppl. 1):i37–i40.
3. Cardozo, T., T. Kimura, S. Philpott, R. Weiser, B. Burger, and S. Zolla-Pazner. 2007. Structural basis for coreceptor selectivity by the HIV type 1 V3 loop. AIDS Res. Hum. Retroviruses 23:415–426.
4. Chen, T. K., and G. M. Aldrovandi. 2008. Review of HIV antiretroviral drug resistance. Pediatr. Infect. Dis. J. 27:749–751.

5. Croxall, J. D., and S. J. Keam. 2009. Raltegravir: a review of its use in the management of HIV infection in treatment-experienced patients. Drugs 69:1059–1075.

6. Gulick, R. M., J. Lalezari, J. Goodrich, N. Clumeck, E. DeJesus, A. Horban, J. Nadler, B. Clotet, A. Karlson, M. Wohlfeiler, J. B. Montana, M. McHale, J. Sullivan, C. Ridgway, S. Felstead, M. W. Dunne, E. van der Ryst, and H. Mayer, for the MOTIVATE Study Teams. 2008. Maraviroc for previously treated patients with R5 HIV-1 infection. N. Engl. J. Med. 359:1429–1441.

7. Huang, C.-C., M. Tang, M.-Y. Zhang, S. Majed, E. Montabana, R. L. Stanfield, D. S. Dimitrov, B. Korber, J. Sodroski, I. A. Wilson, R. Wyatt, and P. D. Kwong. 2005. Structure of a V3-containing HIV-1 gp120 core. Science 310:1025–1028.

8. Kondru, R., J. Zhang, C. Ji, T. Mirzadegan, D. Rotstein, S. Sankuratri, and M. Dioszegi. 2008. Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. Mol. Pharmacol. 73:789–800.

9. Lai, J.-P., J.-H. Yang, S. D. Douglas, X. Wang, E. Riedel, and W.-Z. Ho. 2003. Quantification of CCR5 mRNA in human lymphocytes and macrophages by real-time reverse transcriptase PCR assay. Clin. Diagn. Lab. Immunol. 10:1123–1128.

10. Lederman, M. M., A. Penn-Nicholson, M. Cho, and D. Mosfer. 2006. Biology of CCR5 and its role in HIV infection ad treatment. JAMA 296:815–826.

11. Little, S. J., S. Holte, J.-P. Routy, E. S. Daar, M. Markowitz, A. C. Collier, R. A. Koupr, J. W. Mellors, E. Connick, B. Cowen, M. Kilby, L. Wang, J. M. Whitcomb, N. S. Hellmann, and D. D. Richman. 2002. Antiretroviral-drug resistance among patients recently infected with HIV. N. Engl. J. Med. 347:385–394.

12. Murphy, E. L., A. C. Collier, L. A. Kalish, S. F. Assman, M. F. Parra, T. P. Flanigan, P. N. Kumar, L. Mintz, F. R. Wallach, and G. J. Nemo, for the Viral Activation Transfusion Study Investigators. 2001. Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease. Ann. Intern. Med. 135:17–26.

13. Ribeiro, R. M., M. D. Hazenberg, A. S. Perelson, and M. P. Davenport. 2006. Naive and memory cell turnover as drivers of CCR5-to-C4CR4 tropism switch in human immunodeficiency virus type 1: implications for therapy. J. Virol. 80:802–809.

14. Rosen, O., M. Sharon, S. R. Quadt-Akabayov, and J. Anglister. 2006. Molecular switch for alternative conformations of the HIV-1 V3 region: implications for phenotype conversion. Proc. Natl. Acad. Sci. U. S. A. 103:13950–13955.

15. Su, Z., R. M. Gulick, A. Krambrink, E. Coakley, M. D. Hughes, D. Han, C. Flexner, T. J. Wilkin, P. R. Skolnik, W. L. Greaves, D. R. Kuritzkes, J. D. Reeves, and AIDS Clinical Trials Group A5211 Team. 2009. Response to vicriviroc in treatment-experienced subjects, as determined by an enhanced-sensitivity coreceptor tropism assay: reanalysis of AIDS clinical trials group A5211. J. Infect. Dis. 200:1724–1728.

16. Tsibris, A. M. N., and D. R. Kuritzkes. 2007. Chemokine antagonists as therapeutics: focus on HIV-1. Annu. Rev. Med. 58:445–459.

17. Yi, L., J. Fang, N. Isik, J. Chim, and T. Jin. 2006. HIV gp120-induced interaction between CD4 and CCR5 requires cholesterol-rich microenvironments revealed by live fluorescence resonance energy transfer imaging. J. Biol. Chem. 281:35446–35453.