The enumeration of specific lymphocyte subsets (flow cytometric immunophenotyping) has become a routine and indispensable procedure in the evaluation, prognosis, and diagnosis of a variety of clinical conditions. Over the past 20 years we have witnessed remarkable changes in the technology of “flow cytometric immunophenotyping.” Major advances have included the development of monoclonal antibodies which recognize specific human lymphocyte subsets (8), the development of new fluorochromes and their direct conjugation to the monoclonal antibodies, development of user-friendly flow cytometers and software, the adoption of whole-blood methodologies, and the development and characterization of antibodies to more than 200 different cell-associated molecules (cluster determinants). The identification of specific cell surface markers led inevitably to the characterization of specific “lymphocyte subsets,” which led naturally to the investigation of their numbers and functions in specific disease entities. Other than the investigation of lymphocyte subsets in autoimmunity, inherited immunodeficiency diseases, and hematologic neoplasia, lymphocyte subset enumeration was a relatively infrequent event relegated predominantly to specialized laboratories. Propelled by the discovery of lymphocyte subset abnormalities in human immunodeficiency virus (HIV)-infected individuals in the early 1980s, lymphocyte immunophenotyping has become an essential and widely adopted clinical procedure. Measuring CD4 counts in HIV-positive patients remains the single most important immunological parameter measured in HIV-infected individuals for the evaluation of their prognosis, immune deficiency status (5), response to therapy (4), and diagnosis of AIDS (2).

Initially, the procedures for measuring CD4+ T cells by flow cytometry were highly variable between laboratories; some laboratories used only a single light scatter parameter to identify (gate) the lymphocytes and a single marker to identify CD4- or CD8-positive T cells, many used indirect immunofluorescence techniques with a variety of fluorochromes, and most laboratories measured the percentage of CD4+ cells in isolated peripheral blood mononuclear cell (PBMC) preparations. PBMC isolation has been replaced with whole-blood preparations (7), lymphocyte identification (gating) has been improved to incorporate light scatter and fluorescence (CD45) parameters (9), and indirect immunofluorescence has been replaced with directly conjugated monoclonal antibody reagents, permitting multiple monoclonal antibody-color combinations in a single tube. With only a single marker, the ability to differentiate CD4+ monocytes from CD4+ T cells and CD8+ NK cells from CD8+ T cells was problematic. With multiple monoclonal antibodies in a single tube, the CD4+ T cells are now more accurately identified by the coexpression of both CD4 and CD3 (T-cell receptor complex) and the CD8+ T cells are more accurately identified by the coexpression of CD8 and CD3. It is now relatively common to combine up to four monoclonal antibodies and colors in order to rapidly measure multiple lymphocyte subsets in a single tube (11).

As the utility of measuring lymphocyte subsets gained acceptance and the procedures began to be adopted in clinical trials and routine clinical settings, it was recognized that better quality control, including standardized procedures and proficiency testing programs, needed to be adopted in order to improve the reliability of the results. In 1992, the Centers for Disease Control and Prevention (CDC), building on recommendations developed previously by the Association of State and Territorial Public Health Laboratory Directors, the National Committee for Clinical Laboratory Standards, and the National Institute of Health’s AIDS Clinical Trials Group, developed and published guidelines for performing CD4+ T-cell determinations on specimens from persons with HIV infection (3). This guideline provided specific information about performing the test, with recommendations for a monoclonal antibody panel, quality control procedures, information about lymphocyte gating, and reporting requirements. With the adoption of the CDC guidelines (including amendments published in 1994 and again in 1997) and the enrollment in proficiency testing programs such as the National Institute of Allergy and Infectious Disease (NIAID) Division of AIDS Quality Assurance (QA) Program, the precision of measuring CD4 percentages (within and between laboratories) has improved significantly (6).

Given these dramatic improvements in the technology and quality assurance of clinical immunophenotyping for lymphocyte subset percentages, it is very ironic that as we enter the new millennium, most laboratories still require two other procedures (not on the flow cytometer and not well controlled) to obtain absolute lymphocyte subset counts. Currently, the most common practice for obtaining an absolute CD4+ T-cell count requires (i) the percentage of lymphocytes expressing CD4 from the flow cytometer, (ii) the complete blood count or white blood cell count from a hematology blood counter, and (iii) a lymphocyte differential (percent lymphocytes), also most commonly obtained on an automated hematology instrument.

Originally, hematology instrumentation provided the white blood cell counts, and the lymphocyte differential was performed manually on stained slide preparations. Newer hema-
ology instruments have been developed which generate automated differentials, but the methods used for identification of major cellular components vary with the manufacturer. The age of the blood contributes to the accuracy of the determination, with manual differentials requiring blood less than 6 h old and newer instrumentation requiring blood less than 18 h old. Direct comparisons of hematology results between different types of instruments in different laboratories are challenging because of the time constraints inherent in shipping blood to multiple locations. Within the last 2 or 3 years, proficiency testing programs have collected information about absolute CD4 cell counts on shipped whole blood. The vast majority of the results are derived from the standard hematology methodology. As alluded to above, many hematology instruments are not validated for blood older than 18 h, and since most proficiency testing specimens must be shipped large distances from the proficiency testing vendor to the laboratory, the accuracy of these results is not known.

Within the context of the NIAID flow cytometry QA program, Dr. Gelman (personal communication) has examined the components of the CD4 count results generated on shipped whole blood in order to examine how much of the variability was due to the hematology component and how much was due to the flow cytometry component of the procedure. Her group observed that the between-laboratory lymphocyte count percent coefficient of variation (%CV) was 1.5 times higher than the percent CD4 %CV. As suspected, this observation indicates that the hematology results contribute more to the variation in absolute CD4 counts than the flow cytometer. The increased variability due to the hematology procedures suggests that the removal of the hematology component to obtain absolute CD4 T-cell counts, i.e., measuring CD4+ T-cell counts directly off of the flow cytometer, would result in a significant improvement in the assay precision.

In this issue of Clinical and Diagnostic Laboratory Immunology, two multicenter evaluations of flow cytometry-based absolute lymphocyte subset enumeration (single platform) conclusively and convincingly demonstrate that single-platform methods significantly improve the variability of CD4 and CD8 T-cell counts (both within and between laboratories) compared with conventional methods that use flow cytometry plus hematology. Both evaluations were designed by a subcommittee of the NIAID Flow Cytometry Advisory Committee and the New Technologies Evaluation Group and were completed in collaboration with Becton Dickinson Biosciences, Beckman Coulter Corporation, and laboratories certified by the NIAID Division of AIDS Flow Cytometry QA Program (10, 11). A third study designed by the New Technologies Evaluation Group and published in 1997 (10) also reported significantly improved precision in CD4 and CD8 T-cell counting on single-platform compared with conventional flow cytometry plus hematology.

Each of the above studies evaluated one single-platform technology at a time versus conventional flow cytometry, i.e., CD4 and CD8 T-cell counts from within a single technology were compared with conventionally obtained CD4 and CD8 T-cell counts. In a recent study published by the United Kingdom National External Quality Assessment Schemes for Leukocyte Immunophenotyping group, the between-laboratory variability of different single-platform technologies was compared with the between-laboratory variability observed for the “predicate” multiplatform technology (1). In this study, the precision of absolute CD4 counts obtained on completely different single-platform technologies (volumetric cytometry as well bead-based single-platform flow cytometry systems) was also shown to be significantly improved compared with predicate multiplatform technology (mean %CV = 13.7 and 23.4%, respectively).

The improved precision of absolute lymphocyte subset measurements obtained on single-platform instruments (both within and between instruments and within and between laboratories) compared with conventional flow cytometry plus hematology suggests that the single-platform measurements may also be more accurate than the current conventional methods. Reimann et al. (11) assessed the accuracy of the single-platform measurements compared with flow cytometry plus hematology. In some of the laboratories, consistent differences were observed between the absolute CD4 T-cell counts generated by the multiplatform method in that laboratory and the median of the CD4 T-cell counts generated by all five laboratories without any appreciable differences in the percentage of CD4 T cells. They concluded that the consistent differences observed between the median CD4 counts and the individual CD4 counts were due to biases in the absolute lymphocyte counts generated by some of the hematology instruments (because the percent lymphocyte subset differences were minimal and there was no bias in the lymphocyte subset percentages). In the study by Schnizlein-Bick et al. (12), the differences between absolute counts obtained with the two technologies balanced out because some laboratories generated higher absolute counts with the conventional method and some generated lower absolute counts with the conventional method. As in the study by Reimann et al. (11), consistent differences in individual laboratories were observed between the single-platform absolute counts and the conventionally obtained absolute counts. Since these differences (bias) were not observed in the lymphocyte percentages obtained between the two technologies, Schnizlein-Bick et al. also concluded that the site bias in absolute count was determined by the hematology instrumentation (12). Gelman (personal communication) has reviewed the absolute CD4 T-lymphocyte counts derived from different types of hematology instruments generated over 17 months by 74 laboratories in the NIAID QA program and compared the counts obtained with each different instrument with the counts obtained on the same samples with the Coulter STKS hematology instrument (most common hematology instrument utilized by these groups). There were clear biases, with seven machines generating significantly higher absolute CD4 counts and three machines generating lower counts than the STKS. Gelman’s analyses were performed on hematology data generated on samples which were older than the recommended 18 h. In support of these findings, however, both of the current studies (11, 12) observed that a consistent within-laboratory bias existed between the single-platform and conventional lymphocyte counts generated on fresh samples and on the samples that had been held over for more than 24 h prior to analysis.

In fact, both studies showed little change in the accuracy or precision of CD4 and CD8 counts obtained on fresh versus aged whole blood with either the single-platform flow cytometry system or conventionally derived counts (11, 12). There was a trend in both studies which showed that lymphocyte subset counts decreased with age when analyzed by the single-platform technologies, whereas lymphocyte subset counts increased with age when analyzed by conventional flow cytometry plus hematology. It is very unlikely that the number of lymphocytes increased in the EDTA tubes overnight. The increase is most likely due to an increase in the absolute lymphocyte count generated by some of the hematology instruments. It should also be noted that when a sample failed hematology (e.g., a specimen was flagged for a problem with the lymphocyte differential) or flow cytometry
quality control, all data on that sample were discarded from the analysis. Although not formally evaluated, more data were discarded because of problems with the hematology procedures than because of problems with flow cytometry. It is our belief that the adoption of the single-platform technologies will allow absolute lymphocyte subset count determinations to be made on currently problematic aged blood samples (compared with flow cytometry plus hematology), although this has been difficult to validate.

It should be noted that both of the evaluations of the single-platform technologies reported in this issue of Clinical and Diagnostic Laboratory Immunology (11, 12) conclusively demonstrated that when lymphocytes are gated using correlated CD45 (so-called fluorescent gating) and a light scatter parameter, the precision of lymphocyte subset percentage measurement is significantly improved over lymphocyte gating using only correlated light scatter parameters.

It is anticipated that the single-platform technologies will be widely adopted. This will allow the ability to properly control the measurement of absolute lymphocyte subset counts and an ongoing analysis of the precision and accuracy (using known standards) of absolute lymphocyte subset count determinations made within and between all of the different single-platform instruments within quality assessment programs. Up to now, absolute CD4 count determinations were not amenable to true quality control because no reagent which controlled both the hematology components and the flow cytometry component was available. With the recent commercial availability of absolute-count controls and the development of single-platform technology, flow cytometrists can now control all aspects of absolute lymphocyte subset count determinations.

The development of the single-platform technologies for absolute lymphocyte count determinations (with the incorporation of correlated fluorescence and light scatter lymphocyte gating) represents a significant advancement in the precision and accuracy of lymphocyte subset immunophenotyping and should be widely adopted for the enumeration of CD4+ and CD8+ T cells in HIV-infected patients. Laboratories wishing to switch from hematology-derived absolute counts to single-platform absolute counts should be aware of a potential bias (depending on their hematology equipment) between the old and new technology and take the appropriate measures to implement the change.

REFERENCES