Absolute CD4 Counts Obtained by a Three-Color Flow-Cytometric Method without the Use of a Hematology Analyzer

THOMAS S. ALEXANDER*

Department of Pathology, Summa Health System, Akron, Ohio 44309

Received 1 August 1997/Returned for modification 23 September 1997/Accepted 25 November 1997

We evaluated the Ortho TRIO-Cytorong absolute system for determining absolute CD4 counts. The CD4 counts in our blood specimens from 100 individuals ranged from 3 to 1,962; the percent CD4 ranged from 1.3 to 62.2, respectively. The TRIO system was biased toward lower absolute counts than a combination of flow cytometry and hematology but showed no bias in percent CD4 calculations.

The stage of human immunodeficiency virus (HIV) disease is determined by the number of CD4-positive T cells in an individual’s blood. This value is normally obtained by determining the percentage of lymphocytes which coexpress CD3 and CD4 by flow cytometry and multiplying that value by the absolute lymphocyte count, as determined by a hematology analyzer (dual-platform method). Single-platform flow-cytometric systems which directly determine absolute CD4 counts have been introduced. These systems include the Ortho TRIO panel, BD FACScout and TrueCount, and Coulter XL. Nicholson et al. (6) recently reviewed the TrueCount system and found that this system provided CD4 counts which averaged 11.3% higher than those obtained by a dual-platform method. The TRIO panel, along with the Ortho Cytorong absolute flow cytometer, is a Food and Drug Administration (FDA)-approved method for determining absolute CD4 counts (3). This method was recently validated in a multicenter trial with specimens from healthy individuals and found to produce precise CD4 counts averaging 15% lower than those obtained by a dual-platform method (2). The Cytorong absolute cytometer is a syringe-driven cytometer, which may be calibrated for determining the total absolute lymphocyte count (3). The software multiplies the total lymphocyte count by the percentage of the desired subset, e.g., CD4-positive T cells, obtained by immunofluorescence analysis. We evaluated the ability of the TRIO-Cytorong absolute system to determine absolute CD4 counts in HIV-infected individuals, non-HIV-infected transplant recipients, and control patients who had not been tested for HIV.

We collected peripheral blood specimens from 100 individuals into both acid-citrate-dextrose (ACD) and EDTA tubes. Our sample consisted of patients who were performing assays which physicians had ordered on the board approval was not necessary for this study because we were performing assays which physicians had ordered on the patients.

Specimens collected in ACD (our standard anticoagulant for two-color immunophenotyping) tubes were stained with the two-color antibodies (Coulter Corp., Hialeah, Fla.) listed in Table 1. This panel yields CD4 results comparable to those of the six-tube CDC panel for both healthy and HIV-infected individuals (1, 4). We incubated whole blood with antibody for 20 min at room temperature. Erythrocytes were lysed and leukocytes were fixed by using the Q-prep instrument (Coulter).

TABLE 1. TRIO and two-color antibody panels

| Antibody combination | FITC | PE   | Cy-5PE |
|----------------------|------|------|--------|
| TRIO-1               | IgG1 | IgG1 | IgG1   |
| TRIO-2               | CD4  | CD8  | CD3    |
| TRIO-3               | CD16 | CD19 | CD3    |
| Two-color-1          | IgG1 |      |        |
| Two-color-2          | CD3  | CD4  |        |
| Two-color-3          | CD3  | CD8  |        |
| Two-color-4          | CD19 |      |        |
| Two-color-5          | CD16 |      |        |

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; Cy-5PE, allophycocyanin 5–phycoerythrin; IgG1, immunoglobulin G1.
FIG. 1. Absolute CD4 counts obtained by the TRIO method and the dual-platform method. The equation and $r^2$ value represent the results of linear regression analysis.

FIG. 2. Absolute CD4 count bias plot.

CD4 Absolute Count, 2 color plus hematology analyzer

FIG. 2. Absolute CD4 count bias plot.
idation beads. Lymphocytes were detected by both light scatter and immunoscatter gates. Immunoscatter gates use an immunofluorescence marker and right-angle scatter to define a population (3, 5). Isotype controls were used for both the two- and three-color methods to set positive-negative discriminators at a 2% background level. The TRIO panel is validated by using four levels of quality control (QC), including tube-to-tube consistency, Immunosum (lymphosum), T sum, and background fluorescence. We had five specimens initially fail a QC parameter; four were corrected by regating, and one was reanalyzed by using new tubes and was probably due to a pipetting error. The TRIO panel does require accurate, precise pipetting for all QC parameters to be acceptable.

Our sample included 63 specimens with absolute CD4 counts of <500 by both methods, and 37 specimens with absolute CD4 counts of ≥500 by at least one method. The overall range of absolute CD4 counts was 3 to 1,962/mm³ (Sysmex). The absolute CD4 counts obtained by the Cytoron system correlated well with those obtained by the dual-platform method ($r^2$ value of 0.99 [Fig. 1]), although the Cytoronabsolute cytometer showed a bias of −9.5% compared to the combination of flow cytometry and hematology (Fig. 2). This bias ranged from −7% for CD4 counts of >500 to −11% for CD4 counts of <100. The percentages of CD4 by the two methods correlated well ($r^2$ value of 0.95 and bias of <1% [Fig. 3 and 4]).

Our results indicate that the three-color TRIO panel provides CD4 percentages identical to those by a two-color flowcytometric method. The percentages for other lymphocyte phenotypes also correlated well with those by the two-color method (data not shown). The absolute counts delivered by the single-platform Cytoronabsoluted system, however, have a bias toward lower numbers compared to results with the dual-platform method. These data confirm and extend data presented by Connelly et al. (2). They showed absolute CD4 counts determined by the Cytoronabsolute cytometer in specimens from healthy individuals were routinely 15% lower than those obtained by a combination of flow cytometry and hematology (2). We have shown less bias across a wider range of CD4 counts and included specimens from HIV-infected individuals. The difference in bias values may be due to different hematology analyzers being employed in Connelly’s study.

It is impossible to determine if either a single-platform or dual-platform result is “right” or “wrong” due to different methods of determining total lymphocyte count with the hematology instrument and the Cytoronabsolute cytometer. The Sysmex analyzer determines absolute counts by using direct-current measurements for cell size and volume and radio wave scatter for cell complexity. The flow cytometer determines total lymphocytes by light scatter and immunofluorescence characteristics on a specimen in which erythrocytes have been lysed. Technical errors, as described by Nicholson et al. (6), cannot be ruled out completely; however, all instruments and pipettors were calibrated, and the assays were performed by experienced technologists. When we switched from the dual-platform method to the single-platform method for our clinical reporting, we provided both sets of numbers on patients we had been monitoring by the combination method. This allowed the clinician to have a number from the dual-platform method to compare to previous values for guiding current treatment and a new baseline number for our single-platform method which would be used to follow the patient from that time forward. An alternative method would be to constantly correct reported values for the observed bias, as previously described by Nicholson et al. (5).
In conclusion, the TRIO-Cytoronabsolute system does provide CD4 percentages which correlate well with those by a two-color method across a wide range of CD4 levels. The single-platform method does provide absolute counts biased toward a lower number than those by the combination of flow cytometry and hematology. The software provides extensive QC, including lymphosum, T sum, and tube-to-tube variation analysis (3). A laboratory switching to a single-platform method must perform in-house experiments to determine if bias is present (6) and to ensure that reports to physicians account for that bias during the transition period.

(Part of this work was presented at the 8th Annual Meeting of the Association of Medical Laboratory Immunologists.)

I thank Marlys Martter, Dianne Terlecki, John Haprian, Diana Neff, Ray Johnson, and Jody Gillis for excellent technical assistance and Joe DiPersio for critically evaluating the manuscript.

This work was supported in part by the Summa Health System Foundation. The presentation of part of this work at the 8th Annual Meeting of the Association of Medical Laboratory Immunologists was done with assistance from Ortho Diagnostics.

REFERENCES

1. Alexander, T., R. Balaj, and L. Define. 1994. Flow cytometric CD4 cell determinations based upon CD3 gating in HIV infected individuals. Cytometry 18:172.
2. Connelly, M. C., M. Knight, J. V. Giorgi, J. Kagan, A. L. Lansay, J. W. Parker, E. Page, C. Spino, C. Wilkening, and T. J. Mercolino. 1995. Standardization of absolute CD4+ lymphocyte counts across laboratories: an evaluation of the Ortho CytoronAbsolute flow cytometry system on normal donors. Cytometry 22:200–210.
3. Mercolino, T. J., M. C. Connelly, E. Meyer, M. D. Knight, J. W. Parker, G. T. Stelzer, and G. DeChirico. 1995. Immunologic differentiation of absolute count with an integrated flow cytometric system: a new concept for absolute T cell subset determinations. Cytometry 22:48–59.
4. Mullins, D., and T. Alexander. 1991. Comparison of single vs multiple parameter flow cytometric analysis of lymphocyte subsets. Sixth Annual Clinical Applications of Cytometry Meeting, Charleston, S.C.
5. Nicholson, J. K. A., M. Hubbard, and B. M. Jones. 1996. Use of CD45 fluorescence and side scatter characteristics for gating lymphocytes using the whole blood lysis procedure and flow cytometry. Cytometry 26:16–21.
6. Nicholson, J. K. A., D. Stein, T. Mai, R. Mack, M. Hubbard, and T. Denny. 1997. Evaluation of a method for counting absolute numbers of cells with a flow cytometer. Clin. Diagn. Lab. Immunol. 4:309–313.