Laboratory Hematology 5:131-136
© 1999 Carden Jennings Publishing Co., Ltd.

ISLH
Official Publication

Automated Enumeration of CD4 and CD8 Lymphocyte Counts: A Multisite Evaluation of the IMAGN 2000 Microvolume Fluorimeter

DALE F. HIRSCHKORN,[a] MARY SHAW,[b] SALLY H. FORDAN,[c] COLLEEN DE BARONCELLI,[d] DAVID YONG,[e] BERRY BENNETT,[c] TERRY C. CROCKETT,[d] ANA PRYZBYLKOWSKI,[b] RONALD GOLDING,[f] STEPHEN J. MARTIN,[d] IMELDA IGNACIO-HERNANDO,[c] DEBRA J. COATES,[b] LISA CRITCHETT,[e] EBERHARD W. FIEBIG,[a] DELENE K. JOHNSON,[a] COLIN STEPHEN SCOTT[g]

[a]*Blood Centers of the Pacific–Irwin Center, San Francisco, California;*
[b]*Kennedy Health System–Cherry Hill, Cherry Hill, New Jersey;*
[c]*Florida Bureau of Laboratories, Jacksonville, Florida;*
[d]*Louisiana Office of Public Health–Division of Laboratories, New Orleans, Louisiana;*
[e]*Sonoma County Department of Health Services, Public Health Laboratory, Santa Rosa, California;*
[f]*Ventura County Medical Center, Ventura, California;*
[g]*Biometric Imaging Inc, Mountain View, California*

ABSTRACT

This study reports a comparison of microvolume fluorimetry (MVF) and various flow cytometry (FC) techniques for the enumeration of CD4 and CD8 counts from 521 individual samples from 6 participating institutions in the United States. The range of CD4 and CD8 counts for the 521 samples as determined by FC were 1-326 and 48-3842 cells/μL, respectively. MVF and FC CD4 counts correlated very well (r^2 = 0.95), with a slight constant intermethod bias of +6 CD4 cells/μL with MVF. Similar analyses of CD8 data also revealed a good correlation (least squares r^2 = 0.90), although there was a combined constant (+50 CD8 cells/μL) and proportionate (slope 0.92) bias for MVF results compared with FC. To exclude the influence of FC method variations on the basic delineation of CD4 and CD8 populations, the data were also examined as CD4:CD8 ratios, which showed an almost perfect correlation (r^2 = 0.97). This particular observation indicates that FC and MVF procedures define the same lymphocyte populations and that observed differences in the absolute counts are primarily due to the method by which flow-proportionate data are converted to absolute counts. This study established equivalence between MVF and FC methods for the determination of absolute CD4 and CD8 counts. ***Lab Hematol.*** **1999;5:131-136.**

Address correspondence to C.S. Scott, Brownberrie Cottage, Brownberrie Lane, Horsforth, Leeds LS18 5HF, England; e-mail: steven.scott1@virgin.net
Received July 21, 1999; accepted September 16, 1999

KEY WORDS: **Lymphocyte subsets · Immunophenotyping · Human immunodeficiency virus · Instrumentation · Microvolume fluorimetry**

INTRODUCTION

The value of CD4+ lymphocyte counts in the study of immunodeficiency, and in the clinical monitoring of HIV in particular, is well established. However, the generally routine implementation of CD4 counts has created a high demand for flow cytometry (FC), highlighting its limited availability and the need for alternative methods that do not require technically sophisticated resources with respect to instrumentation and staff. To date, various novel procedures have been tried [1-3], but these have not gained widespread acceptance because of perceived method limitations and poor comparisons with FC. Included among these are microtiter plate immunoassays (eg, Trax CD4, T-Cell Diagnostics, Cambridge, MA; Zymmune Monitoring Assay, Zynaxis Inc, Malvern, PA) and microbead scatter measurements by hematology analyzers (eg, STKS/Gen-S, Beckman-Coulter, Fullerton, CA).

Microvolume fluorimetry (MVF; sometimes referred to as volumetric capillary cytometry) is a recently introduced alternative measurement procedure for the automated counting of CD4 and CD8 cells [4,5]. The instrument used for MVF is the IMAGN 2000 (Becton Dickinson, Franklin Lakes, NJ). This device uses principles similar to those of FC, such as laser light excitation of fluorochrome-labeled monoclonal antibodies. However, it differs in that the sample of interest is scanned in stasis rather than in a flow stream.

TABLE 1. **Details of the Reference Flow (FC) Methods Used for Determination of CD4 and CD8 Counts**

Site	CD4/CD8 Reference Method	Fluorochromes	Reference Procedure for Absolute Counts
A (n = 193)	FACSCount (Becton Dickinson)	Proprietary (2-color CD3/CD4; CD3/CD8)	Internal bead standard
B (n = 21)	FACSCount (Becton Dickinson)	Proprietary (2-color CD3/CD4; CD3/CD8)	Internal bead standard
C (n = 110)	FACSCount (Becton Dickinson)	CD3-FITC + CD4-APC + CD8-PE	Internal bead standard
D (n = 33)	FACSCount (Becton Dickinson)	Proprietary (2-color CD3/CD4; CD3/CD8)	Internal bead standard
E (n = 30)	Epics XL (Coulter)	CD3-PE + CD4-FITC CD3-PE + CD8-FITC	STKS hematology analyzer (Coulter)
F (n = 134)	FACScan (Becton Dickinson)	CD3-FITC + CD4-PE + CD45-PerCP CD3-FITC + CD8-PE + CD45-PerCP	Internal bead standard

This study represents the first multicenter comparison of MVF with FC and comprises CD4 and CD8 data for 521 samples from 6 participating institutions in the United States. The study's aims were to validate the MVF approach and to document the level of agreement between FC and MVF for the enumeration of CD4 and CD8 lymphocytes in the immune monitoring of patients with immune deficiencies in general and HIV in particular.

MATERIALS AND METHODS

Samples Studied

A total of 521 samples were entered into this multisite comparative study of FC and IMAGN 2000 MVF procedures for the determination of absolute CD4 and CD8 counts. Details of the 6 participating sites and the number of analyzed samples are given in Table 1 as well as the CD4/CD8 procedures used by each participating laboratory. All samples were routinely collected into ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes and analyzed within 12 hours of collection.

Analytical Methods

Three of the 6 sites compared MVF results with those from the FACSCount (Immunocytometry Systems, Becton Dickinson) analyzer; 2 sites used a 3-color FC (FACSCalibur and FACScan, Becton Dickinson) method with an internal bead standard, and 1 site used a 2-color FC (Coulter Epics XL) method with an STKS hematology analyzer.

More complete details of the optical and immunologic principles for the IMAGN 4T8 assay may be found in earlier publications [4,5]. In brief, these are as follows. The MVF analysis of CD4 and CD8 cells is achieved through the use of an integrated cartridge, which contains both optimized reagents and the mechanisms to facilitate sample mixing, monoclonal antibody incubation, dilution, and automated capillary filling. The reaction cartridge contains 2 separate processing capillaries, each with the individual immunologic reagent combinations (CD3 plus CD4 or CD3 plus CD8). In both capillaries, the CD3 antibody is labelled with Cy5 (emission maximum 674 nm), whereas the CD4 and CD8 reagents are labeled with Cy5.5 (emission maximum 695 nm). For the determination of $CD4^+$ (or $CD8^+$) cells with the 633-nm helium-neon laser, coincident peaks of Cy5 and Cy5.5 collected by 2 independent photomultiplier tubes are required to define $CD3^+CD4^+$ (or $CD3^+CD8^+$) fractions. The IMAGN then automatically sets fluorescence intensity discrimination gates and takes into account the scan volume and sample dilution factors before directly reporting CD4 and CD8 cells per µL of whole blood. In addition, the analytical results include a measurement of background (free) fluorescence and the average staining intensity of the positive cell components. These values, which are referred to as BLI and RCI, respectively, provide analytical consistency in confirming that each sample is stained with an appropriate amount of conjugated antibody and that the staining of $CD3^+CD4^+$ and $CD3^+CD8^+$ fractions is optimal.

Statistical comparisons of data were made by Passing and Bablok regression [6], least squares regression, Bland-Altman bias plots [7], and the Wilcoxon signed ranks test.

RESULTS

Descriptive Statistics

The range of CD4 and CD8 counts for this series of 521 samples, as determined by FC, were 1-1326 and 48-3842 cells/µL, respectively. The mean CD4 counts for the FC and MVF data sets were 359.5 and 386.3 cells/µL, respectively, and the FC and MVF mean CD8 counts were 860.7 and 854.9 cells/µL.

Absolute CD4 Cell Counts

Comparison of MVF and FC CD4 counts by least squares regression revealed an excellent correlation (r^2 = 0.95) with a slight constant intermethod bias (Passing and Bablok [6]) of +6 CD4 cells/µL with MVF (Figure 1). Bland-Altman analysis of the data after logarithmic transformation revealed that most of the outliers were confined to samples with CD4 counts lower than 20/µL (Figure 2). The 5 samples with mean CD4 values exceeding 100/µL that fell outside the 95% confidence limits were not associated with any particular site. When the ratio of MVF to FC CD4 count was determined and compared with the mean CD4 count (Figure 3), 23 of 31 samples with an MVF:FC ratio exceeding 1.5 were found to have mean CD4 counts of <50/µL, with 20 of these being below 25/µL. For comparison, only 2 of the 521 samples had MVF:FC CD4 ratios of less than 0.5. Examination of individual participant trends specifically for those samples with CD4 counts of <600/µL showed good to excellent regression statistics for all participating laboratories (Table 2). It is notable, however, that sites C and F, both of which used a 3-color FC method with an internal bead standard, showed a more obvious tendency for the FC CD4 counts to be lower than those obtained by MVF. Nevertheless, the correlation between FACSCount and MVF CD4 results was consistently good (Figure 4).

Absolute CD8 Cell Counts

Similar statistical analyses of the CD8 data also revealed a good correlation (least squares r^2 = 0.90; Figure 5), although there was a combined constant (+50 CD8 cells/µL) and proportionate bias

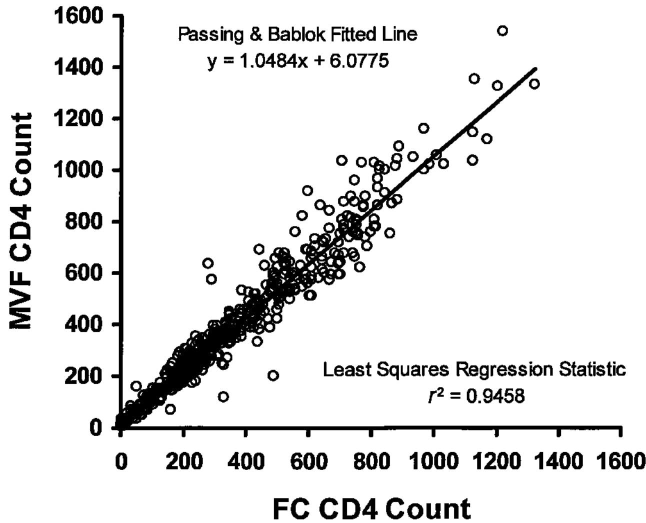

FIGURE 1. Passing and Bablok regression analysis of absolute CD4 counts (μL) obtained by FC and MVF for 521 peripheral blood samples from 6 sites.

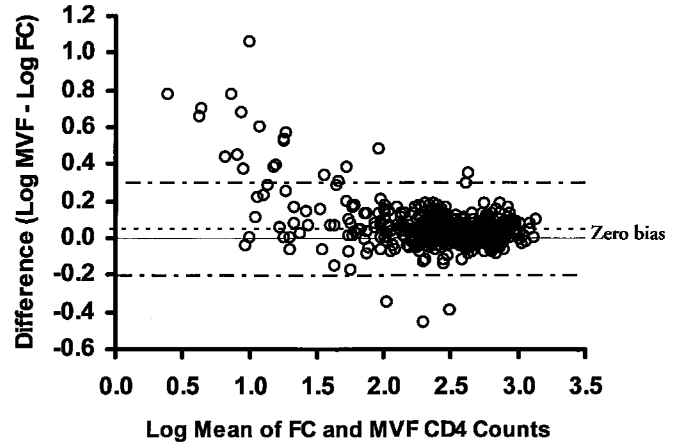

FIGURE 2. Bland-Altman bias plot (mean log absolute CD4 counts of both methods versus the log difference between the 2 methods) for 521 peripheral blood samples analyzed by 6 participating sites. The zero bias (continuous) line is shown together with the actual mean bias (center dotted line) and the 95% confidence limits for CD4 count agreement. Note that there is no visual evidence of significant intermethod bias for CD4 counts obtained by FC and MVF except at very low CD4 counts, for which the MVF values are consistently higher than FC.

(slope 0.92) for MVF results compared with FC. The Bland-Altman plot of logarithmic transformed data confirmed the good agreement between MVF and FC CD8 results and further suggested that the mean bias between FC and MVF methods was actually smaller than suggested by the regression analysis (Figure 6). Most discrepancies (19 of 521 analyzed) occurring outside the 95% limits of agreement were due to MVF results that were significantly higher than those obtained by FC methods. In a pattern similar to that of the CD4 data, these particular discrepancies were mainly associated with sites C (12/19) and F (5/19). Examining the data in a different form, in which the ratio of MVF to FC CD8 counts were compared with the mean CD8 count (Figure 7), only 10 of the 521 samples had a ratio exceeding 1.5 (predominantly from sites C and F), whereas none was less than 0.5.

Supplementary Statistical Analyses

All participants in this study obtained the absolute CD4 and CD8 numbers through reference to either a hematology analyzer white blood cell count (site E) or internal bead standards (sites A to D and F). To exclude the influence of FC method variations on the basic delineation of CD4 and CD8 populations, the data were examined as CD4:CD8 ratios. This revealed an almost perfect correlation (r^2 = 0.97; Figure 8) between the ratios of CD4 and CD8 cells as determined independently by FC and MVF. This analysis strongly suggests that the FC and MVF procedures were measuring the same cell populations with the same efficiency and that the observed differences in the absolute counts were primarily a consequence of the method by which "raw" FC proportionate data were converted to absolute counts.

The effect of this variation on clinical decision points is illustrated in Table 3. Complete decision point agreement was found with 469 of 521 (90%) of the samples in this multicenter study, 39 of 521 samples had slightly higher CD4 values by FC, and 12 of 521 samples showed slightly higher values by MVF. The only

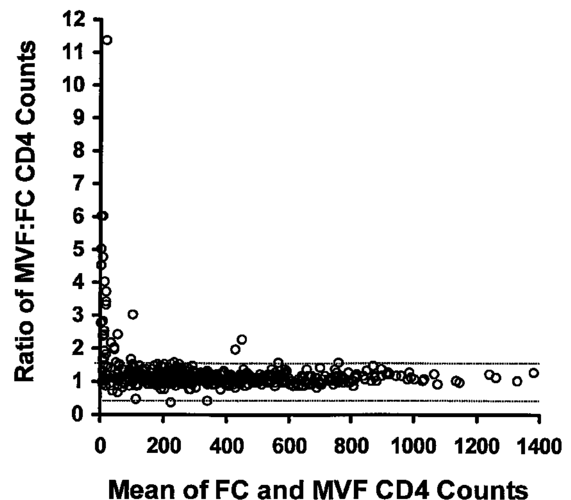

FIGURE 3. By relating the mean values with the ratio of MVF to FC CD4 counts, this plot is designed to ascertain differences between CD4 counts obtained by FC and those obtained by MVF. Exact agreement would equate to a ratio of 1.0, whereas significant disagreement is arbitrarily defined as an MVF:FC ratio of more than 1.5 or less than 0.5. This graph shows that the main discrepancies are primarily confined to the lowest (<50/μL) CD4 counts and that the discrepancy is consistent in that the FC CD4 values are typically lower.

TABLE 2. **Intermethod (FC Versus MVF) Comparisons for CD4 Absolute Counts According to Individual Sites**

Site	Number of Samples	Mean Bias*	Relation†	Regression (r^2) Statistic‡
A	170	+3.3	MVF = 0.96*x* + 5.02	0.90
B	16	−1.3	MVF = 0.92*x* + 9.40	0.98
C	94	+29.9	MVF = 1.09*x* + 14.2	0.92
D	29	+17.3	MVF = 0.96x + 23.5	0.97
E	26	−0.2	MVF = 1.02*x* + 1.00	0.97
F	101	+57.6	MVF = 1.11*x* + 16.2	0.92

Comparisons are specifically for samples with CD4 counts of <600/µL as determined by FC.
*Mean bias for all samples as determined by Bland-Altman plot where positive values indicate higher CD4 counts by MVF and negative values indicate lower CD4 counts by MVF.
†Equation for intermethod agreement as determined by the method of Passing and Bablok where *x* represents the reference (flow cytometry) analysis.
‡Least squares regression method.

significant discrepancy was a sample in which the CD4 count by MVF was 198/µL compared with 489/µL by FC.

DISCUSSION

MVF is a new procedure that can be applied to the enumeration of CD4 and CD8 lymphocytes in peripheral blood samples. This method complies with the CDC requirement for simultaneous determination of CD3 and CD4 (or CD3 and CD8) [8], but it differs from FC in some important respects. First, in MVF the cells of interest are analyzed in stasis within a fixed-precision capillary. Thus, in contrast to FC, MVF does not require cells to be suspended in a flow stream. Second, the cells under analysis that are held within the capillary are scanned by a laser and, because the scan dimensions are known, the quantification of cells can be directly provided per unit volume of sample without reference to an absolute leukocyte/lymphocyte count or an internal bead standard. Third, the laser used for MVF is helium-neon rather than argon, providing a number of advantages relating to cost, laser stability, and system heat output. Finally, the IMAGN MVF analyzer was designed to analyze samples without the need for operator intervention. After a whole-blood sample is introduced into a reaction cartridge, the subsequent scanning process, including the data acquisition and gating procedures, is fully automated and the operator needs only to confirm that internal system and reagent control values (printed with each sample result set) are within predefined limits.

Previous single-site studies have demonstrated MVF versus FC (4-color) correlations (*r*) of CD4 and CD8 counts exceeding 0.97 [9] and suggested good analytical equivalence among MVF, FACSCount, and Cytoron Absolute (Ortho Diagnostic Systems, Raritan, NJ, USA) methods [10]. This study's aim was to extend these earlier and relatively limited comparisons with a much larger data set obtained from 6 laboratories in the United States.

There are many advantages to determining analytical correlations in a combined multisite comparison, but this approach can also be complex if the number of procedural variations is significant or if the reagent sources or the reference methods used to convert raw analytical data to absolute cell numbers are not consistent. Nevertheless, such a study can be highly informative when one of the methods used in the comparison is fully standardized and procedurally identical for all the participating sites. This was the case in this study, in which a fixed MVF method was evaluated against various FC procedures in 6 sites. The constancy of the MVF method allowed the comparative analyses to consider not only the overall result correlations, but also the intersite variability of the FC methods themselves and the effect this may have had on intermethod MVF/FC bias.

In undertaking this analysis, it was important to regard the FC data as reference results, against which the MVF data would be compared, while at the same time making no a priori assumptions

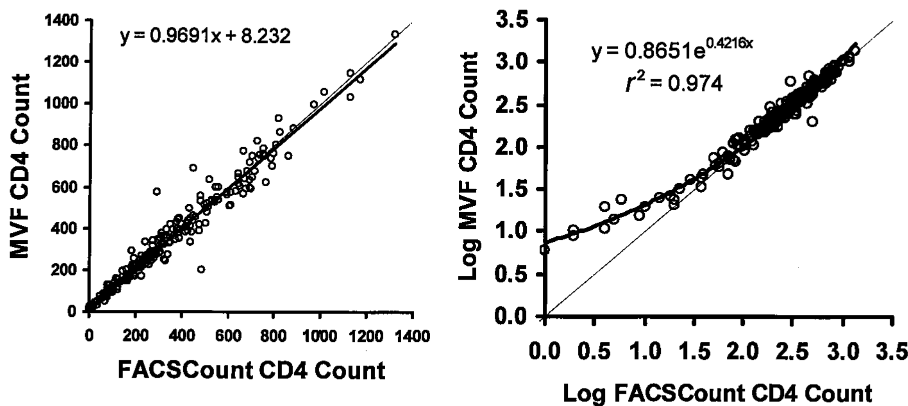

FIGURE 4. Correlations between FACSCount and MVF CD4 counts (sites A, B, and D combined; n = 247). The left plot shows the Passing and Bablok equation for the line of best fit (thick continuous line) superimposed on the line of identity (thin continuous line). The plot on the right represents the log FCASCount CD4 count versus the log MVF CD4 count and their exponential relationship (thick continuous line). Note that this plot clearly shows almost exact agreement between the 2 methods for CD4 counts higher than 50/µL, whereas for CD4 counts lower than 50/µL, the MVF values are consistently higher than the FACSCount.

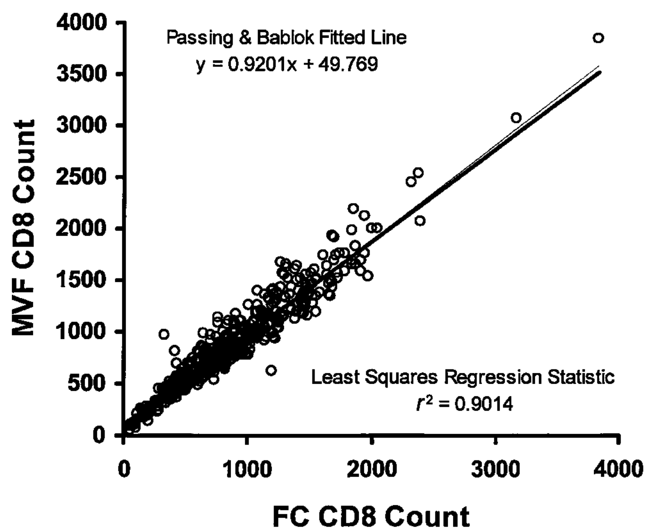

FIGURE 5. Passing and Bablok regression analysis of absolute CD8 counts (/μL) obtained from FC and microvolume fluorimetry MVF for 521 peripheral blood samples from 6 sites.

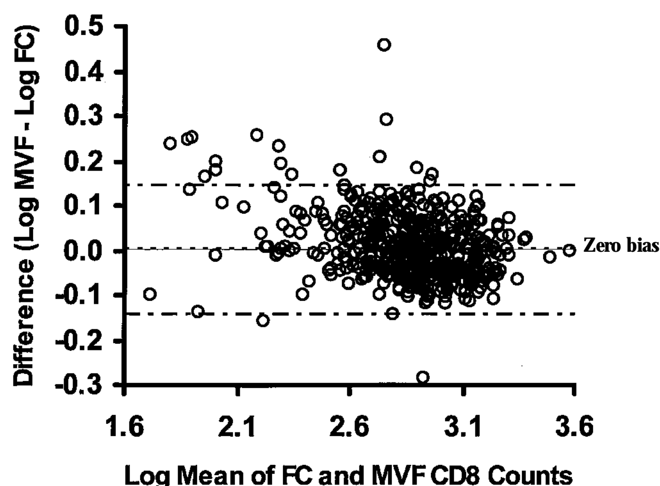

FIGURE 6. Bland-Altman bias plot (mean log absolute CD8 counts of both methods versus the log difference between the 2 methods) for 521 peripheral blood samples analyzed by 6 participating sites. The zero bias (continuous) line is shown together with the actual mean bias (center dotted line) and the 95% limits for confidence for CD8 count agreement. Note that there is no visual evidence of significant intermethod bias for CD8 counts obtained by FC and MVF.

about the accuracy of either. For this reason, the statistical approaches utilized both Passing and Bablok regression plots to determine the degrees of method association and Bland-Altman plots to assess intermethod bias. The least squares regression method was not used to obtain correlation equations because (a) it does not allow an assessment of bias, (b) it does not take account of the individual errors of each method, (c) it gives 2 regression lines (x versus y and y versus x), and (d) it should be used only when a new method is being compared with a series of reference results that are confidently known to represent "true" values. In contrast, the Passing and Bablok regression equation (a) computes the slopes of all straight lines between any 2 points, (b) gives only 1 regression line, (c) is largely unaffected by different degrees of method error at different measurement levels, and (d) is not unduly biased or skewed by extreme values. However, the least squares regression statistic (r^2) was used as an indicator of intermethod association. Finally, the Bland-Altman plots of the means of FC and MVF values against the differences between FC and MVF values were found to be particularly useful for visually assessing intermethod bias.

Site E in this study used a technique that required reference to a hematology analyzer. The remaining sites all used procedures that derived the absolute cell numbers by reference to internal bead standards, even though the methods themselves showed notable differences in the reagent combinations, fluorochromes, and instrumentation. Despite these variations, the correlations between MVF and FC estimates of CD4 and CD8 numbers were very impressive. Moreover, one observation of particular relevance was that the MVF CD4:CD8 ratio was, in almost every case analyzed, the same as the FC CD4:CD8 ratio. Because the line of best fit for this particular correlation was almost identical to the theoretical line of identity, it can be concluded that the methodological identification of each lymphocyte population ($CD3^+CD4^+$ and $CD3^+CD8^+$) by FC and MVF measurement procedures is essentially the same.

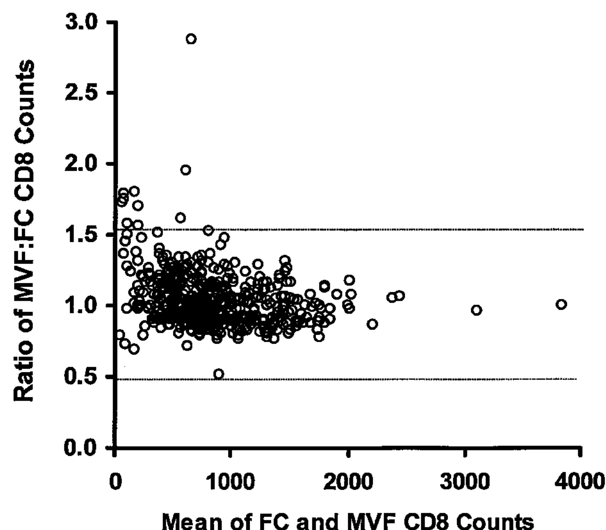

FIGURE 7. By relating the mean values with the ratio of MVF to FC CD8 counts, this plot is designed to ascertain differences between CD8 counts obtained by FC and those obtained by MVF. Exact agreement would equate to a ratio of 1.0, whereas significant disagreement is arbitrarily defined as an MVF to FC ratio of more than 1.5 or less than 0.5. This graph shows that the main discrepancies are primarily confined to the lowest CD8 counts and that the discrepancy is consistent in that the FC CD8 values are typically lower.

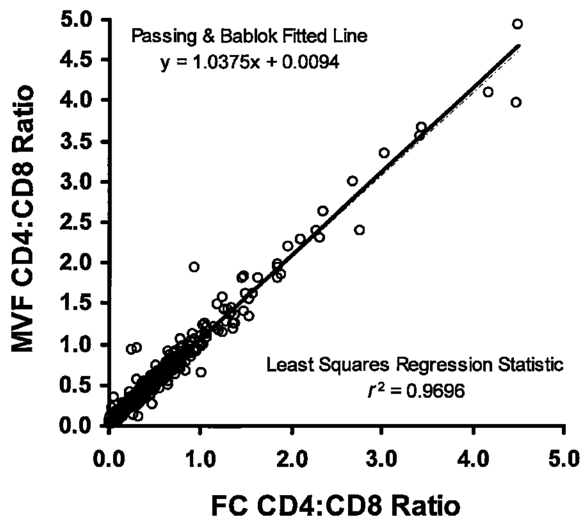

FIGURE 8. Passing and Bablok regression analysis of the FC CD4:CD8 versus MVF CD4:CD8 ratios for 521 peripheral blood samples from 6 sites. Only very occasional outliers are noted with the results confirming that FC and MVF methods are essentially defining the same immunologic subpopulations of lymphocytes.

If it is accepted that the measurement principles of both techniques are detecting the same cells, then the clear inference is that any discrepancies that occur almost certainly result from an independent factor or factors in the analysis. Indeed, in a large study such as this, occasional outliers in the comparative data are to be expected. Such discrepancies can be reasonably interpreted in situations such as this study, in which most of the data are in good general agreement, to reflect errors induced by operational factors or sample quality or both. A potential source of error in 2-platform flow estimates may be introduced by inaccuracy in the absolute lymphocyte count (derived directly by automation or through calculation of absolute WBC × manual % lymphocytes). However, this did not appear to be reflected by the results from patient samples in site E. For those participating sites where internal bead standards were used in the FC methods to determine absolute CD4/CD8 counts, the gating itself could have influenced the final results. This possibility is supported by the findings that the 2 sites using a simultaneous 3-color FC method showed the same tendency for CD4 and CD8 counts to be lower than MVF. In contrast, no obvious consistent trends were found for the other 3 sites using the FACSCount instrument. An important point arising from these findings is that better intersite consistency may result from analytical approaches that are "dedicated" and operator-independent.

This study has established equivalence between MVF and FC methods for the determination of absolute CD4 and CD8 counts. In laboratories with limited availability of a FC or no FC expertise, MVF may well have distinct merits in terms of operational simplicity.

TABLE 3. **Decision Point Comparisons of CD4 Cell Counts as Determined by FC and IMAGN 2000 MVF**

	FC <200/μL	FC 200-400/μL	FC >400/μL	Total
MVF: <200/μL	129	7	1	137
MVF: 200-400/μL	23	152	5	180
MVF: >400/μL	0	16	188	204
Total	152	175	194	521

κ statistic for agreement = 0.85.

REFERENCES

1. Johnson D, Hirschkorn D, Busch MP. Evaluation of four alternative methodologies for determination of absolute CD4+ lymphocyte count. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1995;10:522-530.
2. Cordiali Fei P, Solmone M, Vanacore P, et al. CD4 lymphocyte enumeration: comparison between flow cytometry and enzyme immunoassay. *Cytometry.* 1995;22:70-74.
3. Machin SJ, Pollard Y, Grant D, Chavda N. CD4+ and CD8+ lymphocyte immunophenotyping using antibody-bound microspheres on the Coulter STKS analyzer. *Lab Hematol.* 1997;3:60-65.
4. Dietz LJ, Dubrow RS, Manian BS, Sizto NL. Volumetric capillary cytometry: a new method for absolute cell enumeration. *Cytometry.* 1996;23:177-186.
5. O'Gorman MR, Gelman R, Site Investigators and the NIAID New CD4 Technologies Focus Group. Inter- and intrainstitutional evaluation of automated volumetric capillary cytometry for the quantitation of CD4− and CD8-positive T lymphocytes in the peripheral blood of persons infected with human immunodeficiency virus. *Clin Diagn Lab Immunol.* 1997;4:173-179.
6. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. *J Clin Chem Biochem.* 1983;21:709-720.
7. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986;i:307-310.
8. Centers for Disease Control and Prevention (CDC). Revised guidelines for the performance of CD4 T-cell determinations in persons with human immunodeficiency virus (HIV). *MMWR Morb Mortal Wkly Rep.* 1997;46:No. RR-2.
9. Robinson JE, Blum S, Koch T. Performance of the IMAGN 2000 system as compared to four color flow cytometry [abstract]. Proceedings of 1995 CAC Meeting, 1995, South Carolina.
10. Bergeron M, Mandy F, Chabot C, Lacroix F, Planeuf S, Rud E. Spatial cytometry is an option available for reporting absolute CD4+ T-cell numbers [abstract]. Proceedings of 1995 CAC Meeting, 1995, South Carolina.