Determination of CD4 and CD8 Lymphocyte Subsets by a New Alternative Fluorescence Immunoassay

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The purpose of this study was to evaluate a new alternative fluorescence immunoassay method (Zymmune CD4/CD8 Cell Monitoring Kit; Zynaxis, Inc., Malvern, Pa.) for determining the absolute CD4$^+$ and CD8$^+$ T-lymphocyte concentrations in whole blood. The investigation was performed as a two-site comparison of the reference whole blood flow cytometric method with the Zymmune method. In this investigation, a total of 166 patient samples were evaluated of which ~20% were from human immunodeficiency virus-positive individuals. The mean value for samples performed by the Zymmune CD4 assay was 1,094 (range, 74 to 2,586) cells per µl, while the reference method yielded a mean of 890 (range, 35 to 2,033) cells per µl. The correlation coefficient for regression analysis was 0.940. The mean value for samples performed by the Zymmune CD8 assay was 700 (range, 212 to 1,813) cells per µl, while the reference method yielded a mean of 546 (range, 82 to 2,158) cells per µl. The correlation coefficient for regression analysis was 0.921. No site-specific differences or trends in CD4 or CD8 values were seen when the data were analyzed by site of collection. The average precision of the CD4 assay varied from 6 to 14%, corresponding to the high and low concentration ranges. For CD8, the average precision varied from 8.3 to 16% over the respective high to low concentration ranges. We conclude that the Zymmune CD4/CD8 Cell Monitoring Kit method provides absolute CD4$^+$ and CD8$^+$ T-lymphocyte concentrations which are equivalent to those given by the reference flow cytometric method.

During the last decade, as the AIDS epidemic expanded, work focused on developing laboratory markers of human immunodeficiency virus (HIV) disease progression (7, 9, 11, 17). Enumeration of the CD4$^+$ T-helper lymphocytes has evolved into a reliable cell-based marker for determining the stages of HIV-infected individuals and evaluating therapeutic intervention (5, 6, 8, 10, 15, 17, 18–20, 27). In addition, CD8$^+$ T-suppressor lymphocyte counts aid in evaluating whether low CD4$^+$ counts are due to selective depletion or general reduction in all lymphocytes.

The standard methodology used to measure T-helper lymphocyte levels has relied on the identification of CD4 receptors expressed on the surface of this subset of T lymphocytes (12). A panel of fluorochrome-conjugated monoclonal antibodies that recognize the CD4 receptor and other lymphocyte surface markers (i.e., anti-CD3, anti-CD4, anti-CD8, etc.) is commonly used to label and distinguish one immune subset from others present in a patient's peripheral blood (1, 3, 4, 16, 24). The use of a panel of monoclonal antibodies which distinguish and enumerate all peripheral blood lymphocytes is necessary to ensure the quality of flow cytometric analysis (1, 3, 4, 16, 21). Once labeled, the cells are analyzed to determine the relative number of lymphocytes expressing the phenotype of interest. Although relative lymphocyte subset values were used alone in the past, most current clinical guidelines specify that the results of an immune subset analysis also be expressed as the absolute number of cells per microliter (2, 22). To obtain this number, automated hematology analysis is performed to provide an absolute lymphocyte count. Appropriate multiplication of the

flow cytometric relative values and the absolute lymphocyte count is required. Each of these methods has inherent variations, leading to a combined variability expressed in the absolute CD4 and CD8 values. Improvements in technology have occurred, and several guidelines have been issued to standardize reagent panels and other variables that are inherent in these assays (1, 3, 4, 21), resulting in a method which provides identification of the major types of immune regulatory cells. It would be useful if a simpler and potentially more cost-effective method, with equivalent or improved performance characteristics, were available for evaluation of absolute T-cell subset levels. Furthermore, to meet the global need, it would be desirable if such a method offered expanded availability to individuals infected with HIV in developed and developing nations.

The purpose of this study was to evaluate a new alternative fluorescence immunoassay method (Zymmune CD4/CD8 Cell Monitoring Kit; Zynaxis, Inc., Malvern, Pa.) for determining absolute CD4$^+$ and CD8$^+$ T-lymphocyte concentrations in whole blood. The Zymmune assay format combines a mixture of magnetic and fluorescent microspheres, each targeted to the same antigen. The magnetic particles constitute the separation system for the assay, while the fluorescent particles provide the detection system. During the assay incubation, the magnetic and fluorescent particles bind to and form rosettes with the target cells (CD4$^+$ or CD8$^+$ T lymphocytes). Discrimination between CD4$^+$ (or CD8$^+$) T lymphocytes and monocytes (or NK cells) is provided by antigen density differences, the antigen density being high on the target cells and low on the contaminating cell types. Shear forces generated during the incubation mediate the selection of high-antigen-expressing CD4$^+$ or CD8$^+$ T-lymphocyte populations over low-antigen-expressing monocytes or NK cells (24).

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MATERIALS AND METHODS

Subjects and blood samples. An initial correlation study was performed at two different sites and included a total of 166 samples of peripheral blood obtained from 120 healthy adult donors, 27 HIV-positive donors, and 19 donors with other clinical conditions. At site 1 (University of Medicine and Dentistry of New Jersey-New Jersey Medical School Center for Laboratory Investigation), 82 volunteers (52 healthy, 14 HIV positive, and 16 with other clinical conditions) were recruited for this study. At site 2 (Zynaxis, Inc.), specimens were obtained from three sources as follows: 21 specimens from healthy adult volunteers recruited internally, 13 HIV-positive specimens obtained from Graduate Hospital (Philadelphia, Pa.), and 50 specimens (47 from healthy persons and 3 from persons with other clinical conditions) obtained from a commercial source (Biological Specialty Corporation, Lansdale, Pa.). The racial and/or ethnic distribution of adult donors who were healthy or had non-HIV clinical conditions was as follows: 36 (26%) Black, 9 (6%) Hispanic, 86 (62%) Caucasian, and 8 (6%) Asian. In this group, there were 71 males and 68 females. The racial and/or ethnic distribution of the HIV-positive donors was as follows: 12 (44%) Black, 2 (7%) Hispanic, and 13 (48%) Caucasian. In this group, there were 21 males and six females. The median age of the healthy adult donors and those with non-HIV clinical conditions was 34 (range, 17 to 63) years, and that of the HIV-positive donors was 39 (range, 1 to 71) years. An additional single-site correlation study, conducted at site 1, included an additional 80 samples, of which 75 were from HIV-positive donors and 5 were from healthy adult donors. The distribution of age, sex, and ethnicity of the samples used in the follow-up studies was similar to what was described above for the HIV-positive donors. Studies aimed at evaluating potential interference of monocytes and NK cells on the Zymmune assay were performed with 10 samples from healthy adult donors, while specimen stability studies were performed with 10 samples from healthy adult donors.

This study was approved by the institutional review boards at both sites. Leukemia and lymphoma patients were excluded from this investigation. No other exclusion criteria were applied to the patient population.

All blood samples were obtained by venipuncture and collected in tubes containing K_3EDTA anticoagulant.

Determination of subsets by the Zymmune CD4/CD8 Cell Monitoring Kit method. The Zymmune CD4/CD8 Cell Monitoring Kit method allows 12 patient specimens to be simultaneously evaluated in a 96-well microplate format in under 35 min and can independently measure the absolute number of CD4$^+$ and CD8$^+$ lymphocytes per microliter of unmodified whole blood. The assay reagents consist of magnetic and fluorescent particles to which workshop-tested CD4 (or CD8) monoclonal antibodies are attached.

In preparation for performing this assay, working mixtures of CD4, CD8, and immunoglobulin G beads must be prepared by combining equal volumes of the appropriate magnetic and fluorescent bead reagents. Volumes of each bead mixture, consistent with the number of specimens to be analyzed, were made up within 3 h of performing the assays and were stored at 2 to 8°C. Wash buffer was also made up from a 10× concentrate in a volume consistent with the specimen load. The assay was then performed at each site in accordance with the manufacturer's instructions.

Briefly, 50 µl of wash buffer was added to all assay wells. For each specimen, 25 µl of whole blood was added to each of three assay wells in positions designated by the manufacturer. Finally, the three assay wells corresponding to a specimen received 25 µl of the CD4, CD8, or immunoglobulin G bead mixture, one bead type in each well. The plate was then covered and placed on a Titertek plate shaker (ICN Biomedicals, Inc., Costa Mesa, Calif.) at a speed setting of 8 (1,250 rpm; 1.5-mm orbit) for 5 min at room temperature. At the end of the shaking incubation, 100 µl of wash buffer was added to each assay well with a standard eight-channel micropipet. The bead-rosetted cells and excess magnetic beads were then separated by magnetic sedimentation by placing the assay plate on a manufacturer-supplied magnet for 90 s. All unbound cells and excess fluorescent beads were removed from the wells with the eight-channel pipet. The plate was removed from the magnet, and 200 µl of fresh buffer was added to each assay well with the eight-channel pipet. This wash procedure was repeated three more times. After the final aspiration, 100 µl of wash buffer was added to each assay well. A set of instrument calibrators were added to the appropriate wells of each plate, as specified in the manufacturer's instructions. Just prior to being read, each plate was placed on the plate shaker for 15 s to ensure uniform distribution of the labeled specimens. Each completed plate was then placed in a Fluoroskan II fluorescence microplate reader (Labsystems Oy, Helsinki, Finland) and read with filter set 2 (fluorescein filter set).

For the two-site correlation study, the absolute cell concentrations were calculated in accordance with the manufacturer's directions by using the following equations:

$$CD4^+ \text{ cells per microliter} = \frac{(CD4 \text{ signal} - IgG \text{ signal}) \times CD4 \text{ factor}}{\text{slope of calibration curve}} \quad (1)$$

$$CD8^+ \text{ cells per microliter} = \frac{(CD8 \text{ signal} - IgG \text{ signal}) \times CD8 \text{ factor}}{\text{slope of calibration curve}} \quad (2)$$

The calibration curve mentioned in the denominators of equations 1 and 2 consists of a series of four instrument calibrators added to the assay plate whose function is to verify instrument linearity and act as a quantitative standard for enumerating the absolute cell counts. The CD4 (or CD8) factor is a calibration factor which relates the measured fluorescence intensity to the absolute cell concentration and is provided in each kit.

After completion of the two-site correlation study, an Autoreader F (Zynaxis, Inc.), which reads and automatically calculates the CD4 and CD8 cell counts for each specimen, was supplied by the manufacturer. This instrument was used throughout the single-site study of predominantly HIV-positive specimens.

The reportable cell concentration range specified in the Zymmune CD4/CD8 product insert is 25 to 8,000 cells per µl for both CD4 and CD8 assays. However, the working assay range is 50 to 2,000 cells per µl. The extended reportable range is achieved by assaying a double blood volume when the target cell concentration is below the working assay range and diluting the specimen 1:2 or 1:4 when the target cell concentration is above the working assay range. Consequently, when assay results for either assay were below the 50 cells per µl cutoff of the assay range, the specimen was reanalyzed by using twice the volume of blood (50 instead of 25 µl) and the resulting cell concentration was divided by 2, in accordance with the manufacturer's instructions for use. If the resulting cell concentration value was ≥25 cells per µl, the results were reportable and were therefore included in the correlation calculations. Likewise, when assay results for either assay were above the 2,000 cell per µl limit of the working assay range, the specimen was diluted by a factor of 2 (or 4) with assay wash buffer and reanalyzed. In this case, the resulting cell concentration was multiplied by 2 (or 4), as specified by the manufacturer. If the resulting cell concentration value was ≤4,000/µl (or 8,000/µl for 1:4 dilution), the results were included in the correlation analysis.

For this method, all specimens were analyzed within 6 h of collection, with the exception of some of the analyses performed as part of the specimen stability study.

Determination of subsets by the reference flow cytometric method. The term "reference flow cytometric method" is used throughout this report to refer to the method by which the absolute cell concentration (CD3, CD4, CD8, etc.) is calculated as the product of the absolute lymphocyte count, determined by automated hematological analysis, and the relative subset level (percent CD3, percent CD4, percent CD8, etc.), determined by flow cytometric analysis. The hematology and flow cytometry methods are described below.

Hematological analysis was performed on a Sysmex E-2500 instrument (TOA Medical Electronics, Kobe, Japan) at site 1 and on a Coulter MAXM instrument (Coulter Corp., Miami, Fla.) at site 2. For specimens which were rejected or "flagged" by the instrument, a manual differential was reviewed to confirm the results. However, the instrument-derived differential was used in the final data analysis.

Flow cytometric analysis was performed by direct, two-color immunofluorescent staining of whole blood by using the antibody panel and methods recommended for immunophenotyping by the Centers for Disease Control and Prevention (3, 4). The following commercially available murine anti-human monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or RD-1 were used: CD45-FITC/CD14–RD-1, Isotype-FITC/ Isotype–RD-1, CD3-FITC/CD4–RD-1, CD3-FITC/CD8–RD-1, CD3-FITC/ CD19–RD-1, and CD3-FITC/CD16-PE+CD56-PE. Monoclonal antibodies were obtained from Coulter Immunology (Miami, Fla.); except for the CD3/ CD16+CD56 reagent, which was obtained from Becton-Dickinson Immunocytometry Systems (San Jose, Calif.). One hundred microliters of EDTA-anticoagulated whole blood was mixed with 10 µl (Coulter) or 20 µl (Becton-Dickinson) of monoclonal antibody reagent, and samples were incubated at room temperature for 15 min in the dark. At site 1, Coulter Immuno-Lyse (Coulter Corp.) was used to lyse erythrocytes. The samples were washed with Hanks balanced salt solution and fixed in a 1% buffered paraformaldehyde solution. At site 2, samples were processed on the Coulter Q-Prep Immunology Workstation (Coulter Corp.), which lyses erythrocytes and then stabilizes and fixes the samples. The percentage of fluorescently labeled cells was determined by multiparameter analysis with a FACScan flow cytometer (Becton-Dickinson) at site 1 and a Coulter EPICS Profile I flow cytometer (Coulter Corp.) at site 2. Optical alignment and calibration checks were performed daily on the flow cytometers at both sites. Compensation was optimized to eliminate spectral overlap of FITC with RD-1 or PE. A gate was established on the lymphocyte cluster by forward and 90° light scatter. The validity of this scatter gate was assessed by using the distribution of cells labeled with the CD45 (FITC) and CD14 (RD-1) antibody combination. Only cell preparations demonstrating scatter gate purity of more than 85% CD45$^{+(bright)}$CD14$^-$ labeled cells were accepted. At least 2,500 gated events were analyzed. Integration boundaries were set by using isotype reagents and maintained for analysis of each lymphocyte subset. Results were expressed as the percentage of cells positive for each phenotype after correction for the purity of the lymphocyte gate.

For this method, all specimens were prepared for flow cytometry and hematology analysis was performed within 6 h of collection. Flow cytometry data were acquired within 24 h of the labeling, lysing, and fixation procedures.

Depletion of T lymphocytes. Evaluation of potential interfering effects was carried out by performing assays on specimens in which a majority of the target T lymphocytes had been predepleted. For these experiments, magnetic beads were coated, at Zynaxis, Inc., with an antibody directed to the T-cell receptor (CD3). A 30-fold excess of magnetic beads, relative to target cells, was added to
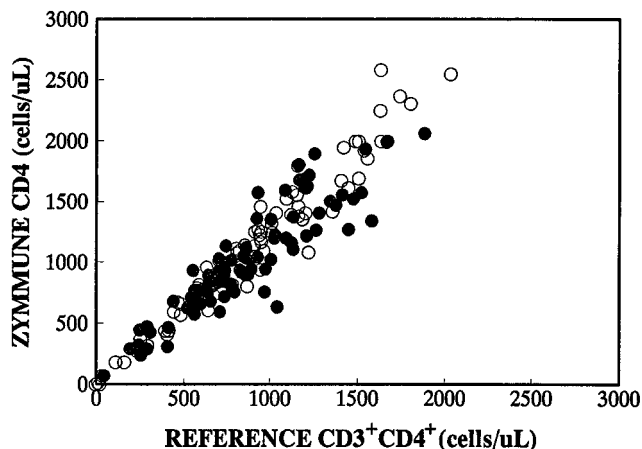
FIG. 1. Correlation plot comparing the Zymmune CD4 assay results with those of the reference flow cytometric method at sites 1 and 2. The total number of specimens analyzed was 166, resulting in an overall correlation coefficient of 0.940 and respective slope and intercept values of 1.19 and 34 cells per μl.
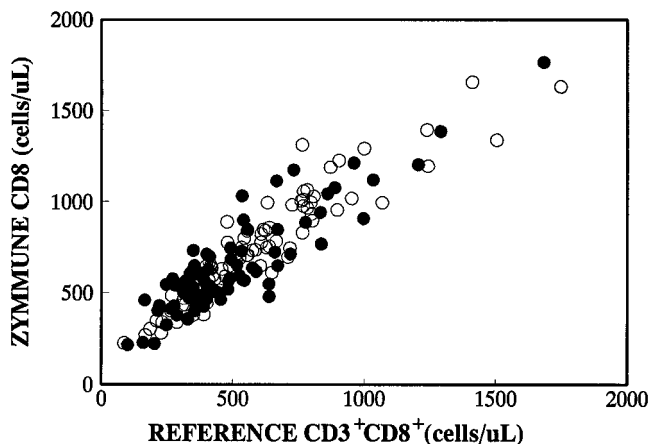

FIG. 2. Correlation plot comparing the Zymmune CD8 assay results with those of the reference flow cytometric method at sites 1 and 2. The correlation coefficient for this assay was 0.921, and the respective slope and intercept values were 0.89 and 216 cells per μl.

1.3 ml of each specimen. This mixture was agitated gently at 4°C for 30 min; this was followed by magnetic sedimentation of the T lymphocytes. The T-lympho-cyte-depleted specimens (supernatants) were then transferred to clean test tubes, and the absolute CD4$^+$ and CD8$^+$ lymphocyte concentrations were determined by the reference flow cytometric cell counting method described above. The percent depletion of the CD4 and CD8 T lymphocytes, monocytes, and NK cells was then calculated from the pre- and postdepletion counts for each cell type.

RESULTS

Two-site studies comparing Zymmune with reference flow cytometric CD4-CD8 values. A correlation plot comparing the Zymmune CD4 assay results with those of the reference flow cytometric method for CD3$^+$-CD4$^+$ lymphocyte subsets is shown in Fig. 1. The mean value for samples tested by the Zymmune CD4 assay was 1,094 (range, 74 to 2,586) cells per μl; the reference method yielded a mean of 890 (range, 35 to 2,033) cells per μl. Linear regression analysis resulted in a slope of 1.19 and an intercept of 34 cells per μl when data from the two methods were compared. The correlation coefficient for this regression analysis was 0.940. No significant differences or trends were seen in these values when the data were analyzed by site of collection.

Shown in Fig. 2 is a similar correlation plot comparing the Zymmune CD8 assay results with those of the reference flow cytometric method for CD3$^+$-CD8$^+$ lymphocyte subsets. The mean value for samples performed by the Zymmune CD8 assay was 700 (range, 212 to 1,813) cells per μl; the reference method yielded a mean of 546 (range, 82 to 2,158) cells per μl. The slope and intercept of the regression line fitted to the data were 0.89 and 216 cells per μl, respectively, and the correlation coefficient was 0.921 when data from the two methods were compared. No significant differences or trends were seen in these values when the data were analyzed by site of collection.

Figure 3 presents a comparison of the CD4/CD8 ratio calculated from the Zymmune CD4 and CD8 results with the corresponding value calculated from the reference flow cytometric method results. The overall mean CD4/CD8 ratio for the Zymmune method was 1.79 (range, 0.015 to 4.712), while that of the reference method was 2.07 (range, 0.003 to 7.219). Regression analysis yielded a correlation coefficient of 0.933 with respective slope and intercept values of 0.71 and 0.32 when data from the two methods were compared. As with the

individual CD4 and CD8 assays, no significant differences or trends were observed in data stratified to the site of collection.

The initial two-site correlation study included approximately 20% HIV-positive individuals. Because it is likely that a primary use for this method would be to monitor the immune status of HIV-positive patients, a second single-site study was performed in which greater than 90% of the specimens ($n = 80$) were obtained from HIV-positive individuals. The correlation results for the CD4 assay (slope, 1.03; intercept, 60 cells per μl; $R = 0.968$), the CD8 assay (slope, 0.84; intercept, 108 cells per μl; $R = 0.898$), and the CD4/CD8 ratio (slope, 1.16; intercept, 0.04; $R = 0.930$) all compared favorably to those of the initial two-site correlation study. The mean CD4 and CD8 absolute values also compared favorably to those of the initial study (data not shown).

Effects of monocytes and NK cells on the respective Zymmune CD4 and CD8 values. Potential sources of interference with the Zymmune CD4 and CD8 assays could be due to cell types, other than T lymphocytes, which express either CD4 or CD8 (i.e., monocytes or some NK cells). To test this hypoth-
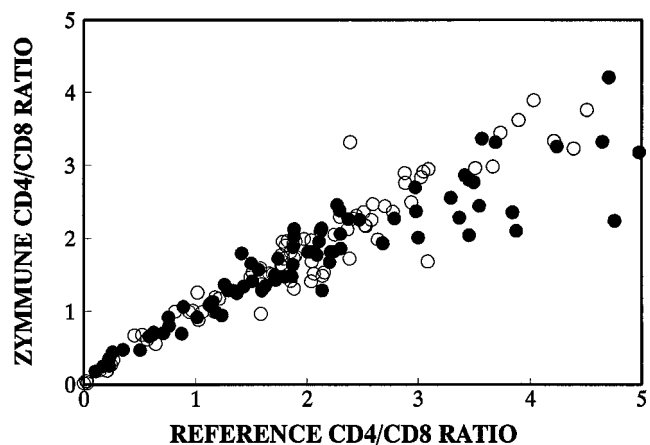

FIG. 3. Correlation plot comparing the CD4/CD8 ratios measured with the Zymmune method and the reference flow cytometric method at sites 1 and 2. The correlation coefficient for this measurement was 0.933, and the respective slope and intercept values were 0.71 and 0.32.
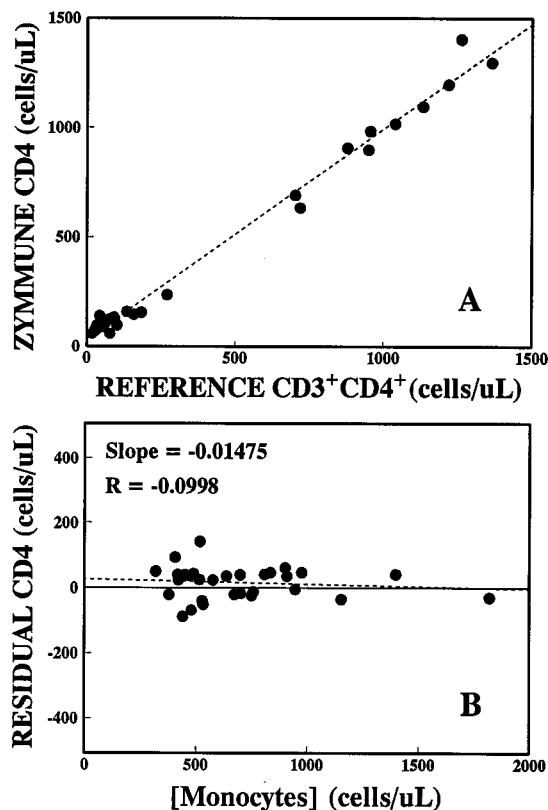
FIG. 4. (A) Correlation plot comparing the Zymmune CD4 results with those of the reference flow cytometric method with 10 specimens before and after T-cell (CD3) depletion. Combined regression statistics for these experiments resulted in slope and intercept values of 0.96 and 39 cells per μl, respectively, and a correlation coefficient of 0.990. (B) Correlation plot comparing the residual Zymmune CD4 signal (Zymmune CD4 minus reference CD4) with the absolute monocyte concentration. A near-zero slope of −0.1475 and a correlation coefficient of −0.0998 are indicated.
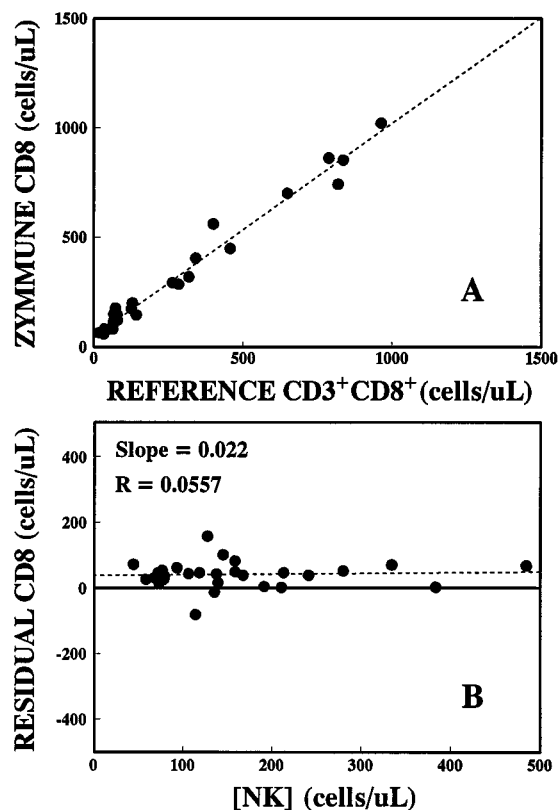
FIG. 5. (A) Correlation plot comparing the Zymmune CD8 results with those of the reference flow cytometric method with the same 10 specimens from Fig. 4 before and after T-cell (CD3) depletion. Combined regression statistics for these experiments resulted in slope and intercept values of 1.01 and 38 cells per μl, respectively, and a correlation coefficient of 0.987. (B) Correlation plot comparing the residual Zymmune CD8 signal (Zymmune CD8 minus reference CD8) with the absolute NK cell concentration. The resulting slope is 0.022, and the correlation coefficient is 0.0557.

esis, whole blood specimens from 10 normal donors were depleted of the majority of their T lymphocytes. The average levels of depletion of the CD4 and CD8 T lymphocytes were 94 and 93%, respectively, while an average of only 3% of CD4-expressing monocytes and 5% of CD8-expressing NK cells were depleted. Further, in the T-lymphocyte-depleted specimens, the average monocyte concentration exceeded the CD4 T-lymphocyte concentration by greater than 10-fold. Similarly, the average NK cell concentration exceeded that of the CD8 T lymphocytes by greater than threefold. To maximize the assay sensitivity at low cell concentrations, the depleted blood samples were assayed as both single and double blood aliquots (25- and 50-μl aliquots were evaluated), as described in Materials and Methods.

Assays were performed on both whole blood and CD3 T-lymphocyte-depleted samples, and the results were compared with those of the reference method to determine whether interfering effects could be detected. Figure 4A is a plot showing the relationship between the Zymmune and reference CD4 results for both whole blood (predepletion) and T-lymphocyte-depleted specimens, including the single- and double-aliquot analyses at the low end of the curve. The reference CD4 values (x axis) were then subtracted from the corresponding Zymmune CD4 values (y axis) to determine the residual signals for each specimen. Figure 4B is a correlation plot comparing the calculated residual signals with the absolute monocyte concen-

trations. The average residual CD4 signal for these 10 specimens was 16 ± 98 cells per μl, well below the minimum reportable concentration for the assay, whereas the monocyte concentration range went from 319 to 1,818 cells per μl, as determined by the reference method. A regression analysis was performed on these 30 data pairs, resulting in a near-zero slope of −0.01475 and an insignificant correlation coefficient of −0.0998. Since the residual parameter represents the difference between two methods, the 95% confidence interval of 98 cells per μl for the average residual in this analysis represents the combined variability of the reference and Zymmune methods.

Figures 5A and B present an analysis of potential NK cell-induced interference with the Zymmune CD8 assay similar to the monocyte analysis presented in Fig. 4A and B. Figure 5A compares the Zymmune and reference CD8 results for both pre- and post-T-lymphocyte depletion specimens, including the single- and double-aliquot analyses at the low end of the curve. Figure 5B is a correlation plot comparing the calculated residual Zymmune CD8 minus reference CD8 signals with the absolute NK cell concentrations. The average residual CD8 signal for these 10 specimens was equivalent to 41 ± 80 cells per μl (± 2 standard deviations), whereas the NK cell concentration range extended from 45 to 384 cells per μl, as determined by the reference method. Regression analysis of the 30 data pairs resulted in a near-zero slope of 0.022 and an insignificant correlation coefficient of 0.0557.

TABLE 1. Zymmune CD4/CD8 assay precision study[a]

Location and cell type	Mean level (cells/μl) ± SD, coefficient of variation (%)		
	Low	Normal	High
Site 1			
CD4	196 ± 29, 15.0	984 ± 81, 8.2	1,664 ± 97, 5.8
CD8	166 ± 31, 18.9	785 ± 61, 7.7	1,039 ± 85, 8.1
Site 2			
CD4	412 ± 53, 12.9	914 ± 118, 12.9	3,381 ± 240, 7.1
CD8	158 ± 21, 13.1	465 ± 30, 6.4	1,090 ± 93, 8.5

[a] Site within-day precision study performed at three different cell concentration ranges for both CD4$^+$ and CD8$^+$ T lymphocytes. CD4$^+$ T-lymphocyte concentrations were low at 100 to 549 cells per μl, normal at 550 to 1,300 cells per μl, and high at 1,301 to 2,000 cells per μl, while the corresponding CD8$^+$ T-lymphocyte concentrations were low at 100 to 249 cells per μl, normal at 250 to 900 cells per μl, and high at 901 to 2,000 cells per μl. Different patient specimens were used within each cell concentration range and at each site.

Zymmune CD4/CD8 assay precision. The within-day precision of the CD4$^+$ and CD8$^+$ T-lymphocyte counts obtained by using the Zymmune CD4/CD8 Cell Monitoring Kit method was determined at each evaluation site. Each laboratory selected three donor specimens whose cell counts were targeted for low, normal, and high levels of CD4$^+$ and CD8$^+$ T lymphocytes, as specified in the footnote to Table 1. Each specimen was processed by one technician in three separate runs during the course of a day, for a total of 10 replicates.

Table 1 summarizes the results by site for each cell type and each cell concentration range. The average precision of the CD4 assay varied from 6 to 14%, corresponding to the high and low cell concentration ranges, respectively. Likewise, the average CD8 assay precision varied from 8.3 to 16% over the respective high to low cell concentration ranges.

Evaluation of specimen stability. The performance of the Zymmune CD4/CD8 Cell Monitoring Kit was assessed periodically with 10 normal specimens which were kept at room temperature (18 to 25°C) in closed EDTA blood collection tubes. The evaluation was begun at 4.5 h postphlebotomy (specimen transport and handling time) and completed at 72 h postphlebotomy. Table 2 is a summary of the average specimen stability results in which the assay values are reported as a percentage of the value for the initial time point (4.5 h). The ranges of the means observed over the entire 72-h time period were 111.3 to 93.8% for CD4$^+$ cell counts and 109.3 to 94.4% for CD8$^+$ cell counts.

DISCUSSION

The initial two-site comparison of the Zymmune CD4/CD8 Cell Monitoring Kit method with the reference flow cytometric

TABLE 2. Zymmune CD4/CD8 assay specimen stability study[a]

Time (h)	% of CD4 cells	% of CD8 cells
4.5	100	100
9	104.4	105.7
20	111.3	109.3
24	104.7	103.7
33	104.8	104.9
48	96.9	94.4
72	93.8	102.2

[a] The stability-hold time of 10 normal specimens was evaluated. Initial measurements were performed at 4.5 h postphlebotomy, and each value represents the average of the 10 specimens' assay results for either CD4 or CD8 cells expressed as a percentage of the 4.5-h result.

method resulted in strong correlation coefficients for all of the parameters that were evaluated (CD4, CD8, and CD4/CD8 ratio), indicating that both methods monitored the same target cells. Since both the Zymmune and reference methods were performed at each site and the pooled data from both sites resulted in high correlation coefficients (0.921 to 0.940), it can be concluded that each method was performed in a consistent fashion at each site and that there was no significant method bias between sites.

The slope of the regression line fitted to the CD4 correlation data is 1.19, with an insignificant y intercept of 34 cells per μl. The slope is approximately 20% above the ideal, which correlates well with the 22% difference between the mean sample values for the Zymmune CD4 assay (1,094 cells per μl) and the reference method (890 cells per μl). These discrepancies were found to be primarily a function of differences in the hematology instruments used in the calibration and evaluation of the Zymmune kit (14). The manufacturer used a Coulter T-540 single-parameter hematology instrument (Coulter Corp.) as part of the reference method to calibrate the kits used in the multicenter study, while Sysmex 2500 (TOA Medical Electronics, Kobe, Japan) and Coulter MAXM (Coulter Corp.) automated hematology instruments were used in the reference methods to evaluate the same kits. It is concluded that minor variations in the hematology method from site to site can have a significant impact on the slope of the regression line fitted to the data at each site (14). In a multicenter comparison of methods in which data are to be pooled, such hematology method-induced slope variations can influence the correlation coefficient.

The slope and y-intercept values for the CD8 assay were 0.89 and 216 cells per μl, respectively. It is apparent in Fig. 2 that the two-site CD8 comparison shows a minor degree of curvature towards reduced Zymmune values or increased reference values at high CD8 cell concentrations. Because the linear regression analysis gives higher weight to values at the high end of the curve, the result is a reduced slope with a significant intercept. The effect of curvature on the linear regression analysis obscures the effect of the hematology method on the slope of the assay comparison seen with CD4 cells. However, the mean CD8 sample values differed by 28% between the Zymmune (700 cells per μl) and reference (546 cells per μl) methods, again consistent with the effect of variation in the hematology method on the multicenter correlation.

It appears that the curvature in the CD8 assay result seen in the high cell concentration range is also a function of the hematological analysis, primarily of abnormal blood specimens. This can be understood by careful examination of the CD4/CD8 ratio comparison, which, for the reference method, is derived from flow cytometry results only (percent CD4/percent CD8). For the data in Fig. 3, it was found that the specimens which had high CD8 T-lymphocyte concentrations generally had medium to low CD4 T-lymphocyte concentrations (more severely ill) and therefore had low CD4/CD8 ratios (data not shown). In this low range (CD4/CD8 < 2), the CD4/CD8 ratio correlation was very high ($R = 0.96 - 0.97$) and the slope (0.89) and intercept (0.09) values were nearly ideal. We therefore conclude that the Zymmune method correlated well with the reference method, which combines flow cytometry and hematology, and that the minor deviation of the CD8 correlation in the high cell concentration range is due to abnormal hematology.

These conclusions were borne out in the single-site study performed on predominantly HIV-positive patients. In this study, the correlation statistics for the CD8 assay (slope, 0.84; intercept, 108 cells per μl; $R = 0.898$) were consistent with the

first study. The abnormal hematological effects were also reproducibly indicated by the CD4/CD8 ratio results (slope, 1.16; intercept, 0.04; $R = 0.930$).

It is well known that peripheral blood monocytes and NK cells express CD4 and CD8, respectively, and that these cells can interfere with and affect the accuracy of the flow cytometric measurement of $CD4^+$ and $CD8^+$ T lymphocytes. Consequently, this method generally employs a T-cell (CD3)-specific antibody to discriminate between target cells and contaminants (3, 4). In this investigation, experiments were performed to confirm that the Zymmune reagents were also able to discriminate between the target cell populations and contaminants expressing the same target antigen. For these studies, 10 specimens were depleted of the majority of their T lymphocytes. These modified specimens had an average monocyte concentration 10-fold higher than the CD4 lymphocyte concentration and an NK cell concentration 3-fold higher than that of the CD8 lymphocytes. To examine the issue of potential interference from monocytes, the $CD4^+$ T-lymphocyte concentration determined by the reference method was subtracted from the Zymmune CD4 value. This residual CD4 signal represents that fraction of the total signal which cannot be accounted for by the CD4 T-lymphocyte concentration. If monocytes significantly interfere with the Zymmune CD4 assay, then any residual signal should correlate significantly with the absolute monocyte concentration in the samples. The resultant near-zero slopes and correlation coefficients for both the CD4 and CD8 assays clearly indicated that monocytes and NK cells do not significantly interfere with the Zymmune method. It is significant that the only restriction placed on the specimens was that leukemias and lymphomas were not evaluated. Specimens were not eliminated on the basis of other disease states or medications. No interfering effects from these sources have been noted.

The precision of the Zymmune CD4 and CD8 assays was tested at three different cell concentration levels, corresponding to the low, normal, and high ranges for each cell type. As expected, the precision was found to be dependent on the cell concentration range within which the measurement was made (3, 4, 9, 13, 14, 19, 26). Both assays performed approximately equivalently with respect to precision, resulting in an average value of 8.8% in the normal range of the assays.

The average stability of 10 normal specimens over a period of 72 h was examined. With the exception of the CD4 value at the 20-h time point, all average assay results were within 10% of the initial 4.5-h time point. Also, no apparent trends over time were noted in the data; this finding is consistent with no appreciable changes in the specimens. The relatively small change in cell counts and the absence of a significant trend towards reduced cell numbers indicate the stability of the specimens analyzed over the 72-h time period.

In this investigation, a total of 166 patients' samples were evaluated ($\sim$20% HIV-positive donors) by both the Zymmune and reference methods. We concluded that the Zymmune CD4/CD8 Cell Monitoring Kit method provides absolute $CD4^+$ and $CD8^+$ T-lymphocyte concentrations which are equivalent to those given by the reference flow cytometric method. These results can be used to derive equivalent estimates of the CD4/CD8 ratio, as well as the value of each immune subset expressed as a percentage of the total T lymphocytes. These results are in agreement with several recent studies comparing the Zymmune and reference methods (13, 23, 25). Up to 12 patient specimens could be processed simultaneously in a 96-well microplate in under 35 min, most of which was hands-on time. A plate washer currently under development will substantially reduce the hands-on time and

increase throughput over that of the manual method. This method should be useful for monitoring of $CD4^+$ and $CD8^+$ T-lymphocyte levels in patients at risk for immunodeficiency disorders. It should also be useful in circumstances under which rapid results and/or parallel processing of multiple specimens is desired.

REFERENCES

1. **Calvelli, T., T. N. Denny, H. Paxton, R. Gelman, and J. Kagan.** 1993. Guideline for flow cytometric immunophenotyping: a report from the National Institute of Allergy and Infectious Diseases, Division of AIDS. Cytometry **14:**702–715.
2. **Centers for Disease Control.** 1992. Recommendations for prophylaxis against *Pneumocystis carinii* pneumonia for adults and adolescents infected with human immunodeficiency virus. Morbid. Mortal. Weekly Rep. **41**(RR-4)**:**1.
3. **Centers for Disease Control.** 1992. Guidelines for the performance of $CD4^+$ T-cell determinations in persons with human immunodeficiency virus infection. Morbid. Mortal. Weekly Rep. **41**(RR-8)**:**1.
4. **Centers for Disease Control and Prevention.** 1994. Revised guidelines for the performance of $CD4^+$ T-cell determinations in persons with human immunodeficiency virus (HIV) infection. Morbid. Mortal. Weekly Rep. **43**(RR-3)**:**1.
5. **Connor, E., M. Bagarazzi, G. McSherry, B. Holland, M. Boland, T. Denny, and J. Oleske.** 1991. Clinical and laboratory correlates of *Pneumocystis carinii* pneumonia in children infected with HIV. JAMA **265:**1693–1697.
6. **De Wolf, F., M. Roos, J. M. A. Lange, J. T. M. Houweling, R. A. Continho, J. V. Noordua, P. T. Schellekens, and J. Goudsmit.** 1988. Decline in $CD4^+$ cell numbers reflects increase in HIV-1 replication. AIDS Res. Hum. Retroviruses **4:**433–440.
7. **Fahey, J. L., J. M. G. Taylor, and R. Detels.** 1990. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. N. Engl. J. Med. **222:**166–172.
8. **Fischl, M. A., D. D. Richman, H. Hansen, A. C. Collier, J. T. Carey, M. F. Para, W. D. Powderly, D. Allan, B. Wong, T. C. Merigan, V. J. McAuliffe, N. E. Hyslop, F. S. Rhame, H. H. Balfour, S. A. Spector, P. Volberging, C. Pettinelli, J. Anderson, and the AIDS Clinical Trials Group.** 1990. The safety and efficacy of zidovudine in the treatment of subjects with mildly symptomatic human immunodeficiency virus 1 (HIV) infection. Ann. Intern. Med. **112:**727–737.
9. **Gelman, R., S.-U. Cheng, P. Kidd, M. Waxdal, and J. Kagan.** 1993. Assessment of the effects of instrumentation, monoclonal antibody and fluorochrome on flow cytometry immunophenotyping: a report based on two years of the NIAID DAIDS flow cytometry quality assessment program. Clin. Immunol. Immunopathol. **66:**150–162.
10. **Giorgi, J., P. Nishanian, I. Schmid, L. Hultin, H. Cheng, and R. Detels.** 1987. Selective alterations in immunoregulatory lymphocyte subsets in early HIV (human T-lymphotropic virus III/lymph-adenopathy-associated virus) infection. J. Clin. Immunol. **7:**140–150.
11. **Giorgi, J. V.** 1986. Lymphocyte subset measurements: significance in clinical medicine, p. 236–246. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
12. **Jackson, A., and N. L. Warner.** 1986. Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes, p. 226–235. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
13. **Johnson, D., D. Hirschkorn, and M. K. Busch.** Unpublished data.
14. **Koepke, J. A., and A. L. Landay.** 1989. Precision and accuracy of absolute lymphocyte counts. Clin. Immunol. Immunopathol. **52:**19–23.
15. **Kovacs, A., T. Frederick, J. Church, A. Eller, M. Oxtoby, and L. Mascola.** 1991. CD4 T-lymphocyte counts and *Pneumocystis carinii* pneumonia in pediatric HIV infection. JAMA **265:**1698–1703.
16. **Landay, A., B. Ohlsson-Wilhelm, and J. V. Giorgi.** 1990. Application of flow cytometry to the study of HIV infection. AIDS **4:**479–497.
17. **Lang, W., H. Perkins, R. E. Anderson, R. Royce, N. Jewell, and W. Winkelstein, Jr.** 1989. Patterns of T-lymphocyte changes with human immunodeficiency virus infection: from seroconversion to the development of AIDS. J. AIDS. **2:**63–69.
18. **Liebovitz, E., M. Rigaud, H. Pollack, R. Lawrence, S. Chandwan, K. Krasinski, and W. Borkowsky.** 1990. *Pneumocystis carinii* pneumonia in infants infected with the human immunodeficiency virus with more than 450 CD4 T-lymphocytes per cubic millimeter. N. Engl. J. Med. **323:**531–533.
19. **Malone, J. L., T. E. Simms, G. C. Gray, K. F. Wagner, J. R. Burge, and D. S.**

Burke. 1990. Sources of variability in repeated T-helper lymphocyte counts from human immunodeficiency virus type 1 infected patients: total lymphocyte count fluctuation and diurnal cycle are important. J. AIDS **3:**144–151.

20. **Masur, H., F. P. Ognibene, R. Yarchoan, J. H. Shelhamer, B. F. Baird, W. Travis, A. F. Sufferedini, L. Deyton, J. A. Kovacs, J. Fallon, R. Davey, M. Polis, J. Metcalf, M. Baseler, R. Wesley, V. J. Gill, A. S. Fauci, and H. C. Lane.** 1989. CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. Ann. Intern. Med. **111:**223–231.

21. **National Committee for Clinical Laboratory Standards.** 1992. Clinical applications of flow cytometry: quality assurance and immunophenotyping of peripheral blood lymphocytes. Tentative guideline Doc H42-T. National Committee for Clinical Laboratory Standards, Villanova, Pa.

22. **National Institutes of Health.** 1990. Recommendations for zidovudine: early infection. JAMA **263:**1606–1609.

23. **Nicholson, J. K. A., W. M. Velleca, S. Jubert, T. A. Green, and L. Bryan.** Evaluation of alternative CD4 technologies for the enumeration of CD4 lymphocytes. J. Immunol. Methods, in press.

24. **Nordon, R. E., B. K. Milthorpe, K. Schindhelm, and P. Slowiaczek.** 1994. An experimental model of affinity cell separation. Cytometry **16:**25–33.

25. **Saez, M. J., M. de Frutos, P. Martinez, and V. Soriano.** 1994. Evaluation of a new fluorescence immunoassay for CD4$^+$ and CD8$^+$ T-cell counts in clinical samples. Vox Sang. **67:**86–87. (Letter to the editor.)

26. **Shenker, E. L., L. E. Hultin, K. B. Bauer, J. Ferbas, J. B. Margolick, and J. V. Giorgi.** 1993. Evaluation of a dual-color flow cytometry immunophenotyping panel in a multicenter quality assurance program. Cytometry **14:**307–317.

27. **Volberding, P. A., S. W. Lagakos, M. A. Koch, C. Pettinelli, M. W. Myers, D. K. Both, H. H. Balfour, R. C. Reichman, J. A. Bartlett, M. S. Hirsch, R. L. Murrphy, W. D. Hardy, R. Soeiro, M. A. Fischl, J. G. Bartlett, T. C. Merigan, N. E. Hyslop, D. D. Richman, F. T. Valentine, L. Corey, and the AIDS Clinical Trials Group of the National Institute of Allergy and Infectious Diseases.** 1990. Zidovudine in asymptomatic human immunodeficiency virus infection: a controlled trial in persons with fewer than 500 CD4-positive cells per cubic milliliter. N. Engl. J. Med. **322:**941–949.