Comparison of Absolute CD4⁺ Lymphocyte Counts Determined by Enzyme Immunoassay (TRAx CD4 Test Kit) and Flow Cytometry

MARK W. MOSS,¹ ANTHONY V. CARELLA,¹ VIERA PROVOST,³ AND THOMAS C. QUINN¹,²,*

Division of Infectious Disease, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore,¹ and Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, Bethesda,² Maryland

Received 11 December 1995/Returned for modification 14 February 1996/Accepted 8 April 1996

Currently, CD4⁺ lymphocyte counts are one of the most widely used surrogate markers for monitoring disease progression and in initiating therapy for human immunodeficiency virus-infected individuals. However, the process of obtaining lymphocyte subset counts can be complex and expensive, often rendering the test inaccessible to many patients. In contrast to standard laser-based flow cytometry, the TRAx CD4 Test Kit utilizes an enzyme-linked immunoassay format to provide CD4⁺ lymphocyte counts by a simple and more cost-effective means. In order to evaluate the utility of the TRAx CD4⁺ assay in comparison with flow cytometry, heparinized blood samples were drawn from 188 infected and uninfected adult patients and 24 infected pediatric patients and evaluated by both assays. The correlation coefficient for all adult individuals tested was 0.94, and the mean absolute counts (in cells per microliter, ± standard deviation) were 510 ± 358 for TRAx and 480 ± 361 for flow cytometry. The correlation for the pediatric group was 0.93, with mean absolute counts of 956 ± 767 for TRAx and 1,521 ± 1,438 for flow cytometry. Overall the TRAx CD4 Test Kit performed well in comparison to flow cytometry, and its lower cost and ease of use make it an encouraging alternative for the routine determination of CD4⁺ lymphocyte counts.

Currently, enumeration of absolute numbers of CD4⁺ T lymphocytes in patients with human immunodeficiency virus (HIV) infection is the most commonly used immunological marker for the evaluation of disease progression and for the initiation and regulation of antiretroviral therapy (3). Among HIV-infected patients, progression toward AIDS is characterized by a decline in CD4⁺ cell counts, which is often associated with a corresponding increase in immunosuppression and clinical symptoms (4, 8). Unfortunately, the determination of these markers with flow cytometry in a variety of patient populations at various stages of HIV infection, is not easily obtained in many settings, often for financial or logistical reasons. Many sites around the world simply do not possess the funds, equipment, or technical training necessary for performance of flow cytometry, the current standard for determining CD4⁺ cell counts (2). A simplified and more cost-effective procedure might place this important immunodiagnostic tool within the reach of areas of the world which, because of the rising prevalence of HIV infection, are already severely burdened in their attempts to monitor infected populations.

Several alternatives to flow cytometric analysis of CD4⁺ cell counts have recently been developed (1, 5, 9). A manual latex microsphere assay using light microscopy (CD4 Cytosphere Assay; Coulter Corporation, Hialeah, Fla.), a magnetic and fluorescent microsphere assay (Zymune; Zynaxis, Malvern, Pa.), an automated fluorescence method using capillary action within a disposable plastic cassette (Imagn2000; Biometric Imaging, Mountain View, Calif.), and a single-purpose variation on the standard flow cytometry system that can be used to determine absolute CD4⁺, CD8⁺, and CD3⁺ cell counts (FACSCount System; Becton Dickinson Immunocytometry Systems, San Jose, Calif.) have all been recently developed. The TRAx CD4 Test Kit (T Cell Diagnostics, Cambridge, Mass.) uses a microtiter plate, enzyme immunoassay format to quantitatively determine the amount of CD4 protein in whole blood and subsequently arrive at a corresponding CD4⁺ cell count. We initiated a study to evaluate the capability of this new assay to determine numbers of CD4⁺ cells in comparison with flow cytometry for TRAx CD4 assays within 24 h. Whole blood was mixed in a 1:6 dilution with a detergent-based lysis reagent, and the treated samples were either assayed within 1 h or frozen at −70°C. To perform the assay, 50 μl of the treated sample was added to 200 μl of a specimen diluent. A 50-μl volume of this mixture was added to a microtiter plate well coated with a murine anti-CD4 antibody containing an equal volume of a second, and different, anti-CD4 antibody conjugated to horseradish peroxidase. A predetermined set of standards and controls were loaded onto the plate, which was then incubated on a rotary platform (−150 rpm) at room temperature for 3 h. The plate was washed and a substrate consisting of o-phenylenediamine dihydrochloride was added and allowed to incubate for 30 min. The reaction was stopped with H₂SO₄, and the A₄₀₅ was read. Once a standard curve using predetermined standards was generated, sample values in cells per microliter could be determined by interpolation from a curve generated by the known standards of CD4 molecules.

**MATERIALS AND METHODS**

**Patients.** Blood samples were drawn from consenting patients attending several different clinic sites, including an HIV-AIDS care clinic at The Johns Hopkins Hospital and the Baltimore City Health Department sexually transmitted disease (STD) clinic; volunteers enrolled in several phase I and II HIV vaccine evaluation trials at Johns Hopkins University; and pediatric patients at The Johns Hopkins Hospital. All patients and volunteers provided informed consent, and the study was approved by the Johns Hopkins University Institutional Review Board. Whole-blood samples obtained by standard venipuncture were collected using K₂ EDTA as an anticoagulant and were processed for both flow cytometry and TRAx CD4 assays within 24 h. Whole blood was mixed in a 1:6 dilution with a detergent-based lysis reagent, and the treated samples were either assayed within 1 h or frozen at −70°C. To perform the assay, 50 μl of the treated sample was added to 200 μl of a specimen diluent. A 50-μl volume of this mixture was added to a microtiter plate well coated with a murine anti-CD4 antibody containing an equal volume of a second, and different, anti-CD4 antibody conjugated to horseradish peroxidase. A predetermined set of standards and controls were loaded onto the plate, which was then incubated on a rotary platform (−150 rpm) at room temperature for 3 h. The plate was washed and a substrate consisting of o-phenylenediamine dihydrochloride was added and allowed to incubate for 30 min. The reaction was stopped with H₂SO₄, and the A₄₀₅ was read. Once a standard curve using predetermined standards was generated, sample values in cells per microliter could be determined by interpolation from a curve generated by the known standards of CD4 molecules.

**Flow cytometry and hematology.** CD4⁺ T lymphocytes were measured by flow cytometry using methods previously described. Whole-blood samples were incubated with fluorochrome-labeled monoclonal antibodies to specific cell surface antigens. Once processed, a sample was analyzed by using a Coulter Epics Profile flow cytometer (Coulter Corporation) and the following monoclonal antibody panel: a murine serum immunoglobulin G isotype control, CD4-CD45, CD8-CD3, CD4-CD5, and CD20. Total leukocyte counts with a lymphocyte differen-
RESULTS

Overall, the correlation of TRAx to flow cytometry was excellent (r = 0.94; 95% CI, 0.92 to 0.95) for all groups analyzed (Fig. 1). The highest individual level of group correlation (r = 0.95; 95% CI, 0.91 to 0.97) was seen with those patients attending an HIV clinic at Johns Hopkins University, followed closely by those attending the Baltimore City STD clinic (r = 0.94; 95% CI, 0.90 to 0.96) and the pediatric patients (r = 0.93; 95% CI, 0.85 to 0.98). The demographics for the patients attending the STD clinic and vaccine trials were unknown, although the HIV clinic patients were predominantly African-American and male (61 of 64). The pediatric group was primarily African-American and 50% male, 50% female, with a mean age of 34 months. While the pediatric samples showed a high degree of correlation, the relationship between the TRAx result and flow cytometry result did not appear to be in direct 1-to-1 proportion, with flow cytometry generally yielding a higher result, especially as CD4+ cells per μL increased above 1,400 cells per μL. This is evidenced by the fact that the slope of the regression line for samples with flow cytometry counts of less than 1,400 cells per μL was 0.72, while it was only 0.52 for the group with greater than 1,400 cells per μL. Also, this group was analyzed by ranking the patients according to increasing age, breaking the group into quartiles based on these ages, and then comparing the mean absolute differences between flow cytometry and the TRAx assay. There was a mean absolute difference of 1,394 cells per μL for the first quartile ending at 8.9 months of age, 485 cells per μL for the second middle quartiles ending at 43.5 months, and 179 cells per μL for the last quartile.

Our study demonstrates that the TRAx CD4 Test Kit had a high level of correlation with flow cytometry in determining absolute counts of CD4+ T lymphocytes. The correlation coefficient for all groups analyzed was 0.94. When the study was broken down into individual populations, the three HIV-infected populations, composed of adult and pediatric patients attending a hospital clinic and those attending a city STD clinic, maintained the highest correlations, at 0.95, 0.93, and 0.94, respectively. There was, however, a noticeable drop in the correlation for the vaccine patients at 0.81, which, when the group was broken down further by infection status, yielded lower correlation coefficients of 0.62 for infected and 0.85 for uninfected individuals. Subsequent investigation of this group could be insightful in determining whether the difference in correlation in this group could be attributed to a biological basis or merely statistical variation due to population size and CD4+ count distributions. Further analysis of the pediatric population could also prove interesting, since while the group r value was high, the correlation of individual samples tended to become skewed as the number of CD4+ cells increased. This observation was accompanied by the fact that as the children increased in age, there was a decreasing trend in the mean absolute differences and standard deviations for CD4+ counts between the two assay formats. This suggests that the TRAx assay might be more clinically useful with older children. However, this will require testing a much larger sample population to determine an age cutoff at which there is a higher confidence of clinical accuracy. Although the manufacturer’s recommendations suggest linearity in a range of 100 to 2,000 cells per μL,
infants under 1 year of age can often have CD4 counts of greater than 2,000 cells per μl, and this information can be important in the early diagnosis of and intervention in pediatric HIV disease (6). Also, there was concern that some of the differences between the TRAx and flow cytometry results might be due to the additional CD4 from monocytes, but separate testing performed at other sites indicates that a large monocyte population does not contribute significantly to the overall CD4 measurement (10).

Important factors in the introduction of an assay such as TRAx are its availability, cost-effectiveness, and ease of use compared with flow cytometry. After performing reliably in clinical trials, TRAx was granted Food and Drug Administration approval and joined Coulter’s CD4+ Cytosphere Assay, Zynaxis’ Zymmune, Biometric Imaging’s Volumet, and Becton Dickinson’s FACSCount in gaining approval for in vitro diagnostic use in determinations of CD4+ cell counts. This widens its availability from research applications only to clinical use as well. TRAx utilizes equipment designed for standard enzyme-linked immunosorbent assay (ELISA)-format assays, such as a microplate washer and reader ($8,000 to $12,000), while flow cytometry relies on more expensive and complex equipment, including a flow cytometer and hematology system ($80,000 to $120,000). Also, assay reagent and material costs for TRAx are substantially less ($15 to $20 per test) than the relatively high cost of fluorescence-labeled monoclonal antibodies and sample processing techniques for flow cytometry ($100 to $200 per test). Whole-blood samples for TRAx can also be stored at 25°C for up to 5 days, at which point they may be processed into lysates and stored at −70°C for up to 1 year until batch analysis can be performed. Another important issue is the fact that the TRAx assay derives the CD4+ value from a single source of measurement, whereas flow cytometry uses a dual instrument platform (flow cytometer and hematology system) and is therefore subject to magnification of error when CD4+ percentages are multiplied by absolute lymphocyte counts (7). Finally, the amounts of training and expertise of personnel required for TRAx are commensurate with those already present in many areas worldwide where ELISA-based serological tests are currently performed, especially in developing nations.

In summary, we found the TRAx CD4 Test Kit to be an accurate and reliable alternative method for the determination of CD4+ T-lymphocyte counts in patients infected with HIV. Its cost and ease of use also make it a practical consideration for laboratories in those areas in which researchers and health care providers are currently limited in their use of this immunological marker for the monitoring and treatment of such patients.

REFERENCES