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NOTES

Effect of Specimen Storage on Absolute CD4 Counts

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We have evaluated the effect of specimen storage on absolute CD4 counts by a commercially available manual assay. This assay utilizes latex particles coated with CD4 monoclonal antibodies that are mixed with lymphocytes in whole blood. Thirty blood samples were analyzed on days 1, 2, 4, and 7 postcollection. Linear regression analysis and Pearson correlation coefficients were used to determine the relationship between the absolute CD4 count and the storage time after sample collection. There was a significant decrease in absolute CD4 counts from baseline over time, dropping 3.6% at day 2, 10.1% at day 4, and 18.8% at day 7. However, the standard error of the *B* coefficient was constant [SE (*B*) = 0.031] up to day 4, indicating that reliable estimates of the baseline CD4 counts could be made from the CD4 counts determined up to day 4 from the time of sample collection. In addition to being simple, rapid, and inexpensive, the manual assay is capable of giving a reliable absolute CD4 count after specimen storage of up to 4 days. The application of this assay in the limited facilities of developing countries' laboratories is attractive.

An absolute CD4 count is one of the best markers for the assessment of immune deficiency in human immunodeficiency virus type 1 (HIV-1) infection and for the prediction of progression to AIDS (4, 5). CD4 counts are also used in patient management and for stratification of HIV-1-seropositive patients into various treatment protocols.

In the United States and Europe, CD4 counts are calculated by determining the percentage of CD4 lymphocytes by flow cytometry and multiplying this percentage by the absolute lymphocyte count as determined by an automated hematology analyzer. This method is tedious and requires expensive equipment and technical maintenance. These drawbacks have limited its use in developing countries.

Coulter Corporation (Miami, Fla.) has recently developed a simple manual method (Cytospheres) for determining absolute CD4 counts (2). In this technique, latex particles (0.6-μm diameter) coated with MY4 antibody are mixed with 100 μl of peripheral blood to bind monocytes. Latex particles (2.0-μm diameter) coated with anti-CD4 antibody are then added and mixed with the 100 μl of blood. The erythrocytes are then lysed, and a sample is placed in a hemocytometer for counting of lymphocytes tagged with the larger anti-CD4 latex particles. The assay takes approximately 13 min and is easy to read. The only equipment required is a pipettor, a hemocytometer, and a light microscope.

In a recent comparative study, the correlation coefficient between this manual method and flow cytometry for determination of absolute CD4 counts was 0.912 in the evaluation of 382 blood samples from HIV-1-seropositive and -seronegative patients in the United States and Uganda (2). The clinical utility of this assay is potentially very attractive in developing countries where HIV-1 prevalence is high and funds for expensive flow cytometers and service are limited.

Unfortunately, specimens cannot always be tested on the day of collection because of specimen transportation difficulties, a temporary shortage of supplies, or specimen collections on weekends or late afternoons when technologists are not available to analyze them. Therefore, a study was designed to evaluate the reproducibility of absolute CD4 measurements on patient blood specimens that are held at room temperature and tested on days 1, 2, 4, and 7 postcollection.

Whole-blood specimens collected in 7-ml EDTA Vacutainer tubes from 30 different patients were obtained from the Clinical Immunology Laboratory at the University Hospitals of Cleveland on the day they were collected. Absolute CD4 lymphocyte counts of these specimens had already been determined with an EPICS Profile II flow cytometer (Coulter) and an automated hematology analyzer (Sysmex N-8000; Baxter) as part of routine patient care. The absolute CD4 lymphocyte counts of these 30 patients, as determined by flow cytometry, ranged from 160 to 1,480/μl (mean = 441/μl). The HIV-1 serologies of these patients were not known, although at least half of the specimens were from the AIDS Clinical Trials Unit at Case Western Reserve University.

On the first day, 100 μl of EDTA-anticoagulated, well-mixed whole blood was added to the bottom of a tube (12 by 75 mm), and then 10 μl of 0.6-μm beads coated with MY4 (CD14) (Coulter Corporation) antibody which binds to monocytes and partially or completely blocks the binding of anti-CD4-coated particles was added. The whole-blood–particle mixture was shaken for 2 min, 10 μl of anti-CD4-coated particles (2.0 μm) (Coulter Corporation) was added, and the entire mixture was shaken for an additional 2 min. Ten microliters of the mixture was then transferred to a second tube containing 100 μl of a lysing solution (2% acetic acid and 0.025% crystal violet stain). The tube was shaken for 15 s to lyse the erythrocytes. A 10-μl aliquot was placed into each of the chambers of a hemocytometer. With a light microscope and a magnification of ×400,

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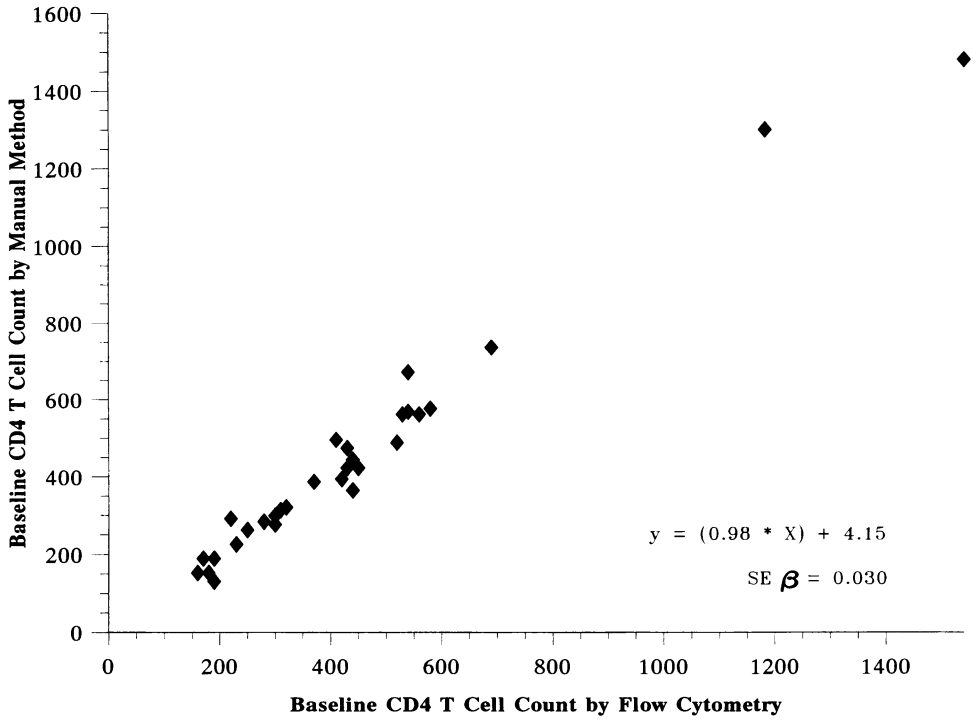

FIG. 1. Scattergram of 30 CD4 counts determined by the manual method and flow cytometry for 30 specimens at baseline. SE, standard error.

cells in all nine major squares of each chamber were counted. Cells with three or more adherent 2.0-μm beads were counted as CD4 lymphocytes. Cells with MY4 and CD4 particles were not counted. An absolute $CD4^+$ cell count per microliter was obtained by multiplying the total number of positive cells counted in both chambers by 7.3, a factor derived from the product of the dilution and transforming volume factors. After this procedure, the specimens (both the whole-blood and the lysed-blood–particle mixtures) were left at room temperature, and the procedure was repeated on these specimens on days 2, 4, and 7 postcollection.

Descriptive statistics were initially done to ensure the normal distribution of absolute CD4 counts. The paired *t* test was used to identify significant changes over the 7 days of storage and at baseline between the results of the manual method and flow cytometry. Linear regression analysis and Pearson correlation coefficients were used to determine the relationship between the time from sample collection and the absolute number of CD4 cells at baseline as measured by the flow cytometer (1). All analyses were run with SPSS/PC+ V4.0 (3).

Absolute CD4 lymphocyte counts were determined for approximately 30 independent samples at 1, 2, 4, and 7 days postcollection. The correlation between the CD4 counts obtained by the two methods, the manual method and flow cytometry, at baseline was very strong (r^2 = 0.97), with an average difference between methods of 4.15 cells for each sample (Fig. 1). As a function of storage time, the absolute CD4 counts, as measured by the manual method, demonstrated significant changes from the baseline manual CD4 measurement of the same sample (Table 1). Over time, the percent difference from baseline steadily increased, with decreases of 3.6% (16.4 cells per μl) at day 2, 10.1% (45.5 cells per μl) at day 4, and 18.8% (84.0 cells per μl) at day 7. These significant differences were less dramatic among the lysed samples, with decreases of 2.7% (12.0 cells per μl) from the baseline at day 2, 5.4% (24.0 cells per μl) at day 4, and 5.6% (25.0 cells per μl) at day 7.

The significant differences between measured CD4 counts by the manual method for stored samples and absolute counts determined by flow cytometry at baseline were calculated by linear regression (regression equation) for each day postcollection (Table 2). Each regression equation demonstrated a significant slope, indicating a significant drop in CD4 count over time. However, the standard error of the *B* coefficient up to day 4 was constant [SE (*B*) = 0.031], suggesting that the results at day 4 are comparable to those of earlier days and as reliable as estimates at baseline or day 2.

This study demonstrates that samples which cannot be analyzed for absolute CD4 lymphocyte determinations on the day of sample collection can be analyzed within 4 days of collection and still produce a reliable estimate of the absolute count at day 1. The technologist would perform the assay as usual and then use the equation appropriate for the number of days since collection to determine the baseline CD4 count

TABLE 1. Absolute CD4 count at room temperature by manual method and day since blood was drawn

Day	Whole blood			Lysed blood		
	n	Mean absolute CD4 count (± SD)[a]	% decrease from baseline	*n*	Mean absolute CD4 count (± SD)[a]	% decrease from baseline
1 (baseline)	30	446 ± 298				
2	30	430 ± 287	3.6	30	434 ± 303	2.7
4	29	401 ± 258	10.1	30	422 ± 297	5.4
7	29	362 ± 212	18.8	27	421 ± 277	5.6

[a] All experimental values are significantly different from the baseline value ($P < 0.01$, paired *t* test).

TABLE 2. Equations for whole- and lysed-blood absolute CD4 counts over storage time with baseline

Sample type	Day	Regression equation[a]	SE of *B*
Whole blood	1 (Baseline)	$y = 0.98 \times$ day 1 ct + 4.15	0.030
	2	$y = 1.01 \times$ day 2 ct + 5.08	0.032
	4	$y = 1.14 \times$ day 4 ct − 6.88	0.031
	7	$y = 1.34 \times$ day 7 ct − 35.16	0.077
Lysed blood	2	$y = 0.96 \times$ day 2 ct + 22.72	0.028
	4	$y = 0.98 \times$ day 4 ct + 26.99	0.030
	7	$y = 1.06 \times$ day 7 ct + 16.09	0.049

[a] y = CD4 count (ct) by flow cytometry at baseline; $P < 0.001$ for slope (B) estimate.

(Table 2). Since there is no equation for day 3, we suggest use of the equation for day 4, which will most likely overestimate the actual absolute count.

If analysis is deferred because of the late arrival of a specimen in the laboratory, the specimen may be processed up to the lysed stage and then stored for reading the following day. In general, the loss in CD4 cells over time was demonstrably less in lysed samples than in whole-blood samples.

This study was done in a laboratory in a developed country under constant ambient conditions. In tropical areas, temperatures and humidity can be extremely high inside laboratories, and so a study comparable to ours which will replicate these results under those conditions is needed. In addition to being simple, rapid, and inexpensive, this manual assay is capable of giving a reliable absolute CD4 count after specimen storage of up to 4 days. The application of this assay in the limited facilities of developing countries' laboratories is attractive.

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