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Guidelines for the Performance of CD4+ T-Cell Determinations in Persons with Human Immunodeficiency Virus Infection

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Summary

This document has been developed by CDC to give guidance to laboratories performing lymphocyte immunophenotyping assays in human immunodeficiency virus-infected persons. The recommendations in this document reflect current technology in a field that is rapidly changing. The recommendations apply to laboratory safety, specimen collection, specimen transport, maintenance of specimen integrity, specimen processing, flow cytometer quality control, sample analyses, data analysis, data storage, data reporting, and quality assurance.

INTRODUCTION

The pathogenesis of acquired immunodeficiency syndrome (AIDS) is largely attributable to the decrease in T-lymphocytes bearing the CD4 receptor (CD4+) (1-5). Progressive depletion of CD4+ T-lymphocytes is associated with an increased likelihood of clinical complications. Because of this association, the measurement of CD4+ T-cell levels has been used to establish decision points for initiating Pneumocystis carinii pneumonia prophylaxis (6) and antiviral therapy (7) and for monitoring the efficacy of treatment. CD4+ T-lymphocyte levels are also used as prognostic indicators in patients with human immunodeficiency virus (HIV) disease (8).

The U.S. Public Health Service (PHS) has recommended that CD4+ T-lymphocyte levels be monitored every 3-6 months in all HIV-infected persons (6). The need for CD4+ T-cell testing services has increased and is expected to continue to increase. Moreover, CD4+ T-lymphocyte levels are being considered for inclusion as a criterion for classifying HIV-infected persons by the newly revised CDC HIV classification system and the proposed AIDS surveillance case definition for adults and adolescents. *

The measurement of absolute CD4+ T-cell levels in whole blood is the product of three laboratory techniques: the white blood cell (WBC) count; the percentage of WBCs that are lymphocytes (differential); and the percentage of lymphocytes that are CD4+ T-cells.

The process of measuring the percentage of CD4+ T-lymphocytes in the whole blood sample is referred to as ``immunophenotyping by flow cytometry'' (9-14). Immunophenotyping relies on detecting specific antigenic determinants on the surface of WBCs by antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome, such as phycoerythrin (PE) or fluorescein isothiocyanate (FITC). The fluorochrome-labeled cells are analyzed by flow cytometry, which categorizes individual cells according to size, granularity, fluorochrome, and fluorochrome intensity. Size and granularity, detected by light scattering, characterize the types of WBCs (granulocytes, monocytes, and lymphocytes). Different fluorochromes are used to distinguish the subpopulations of a given type of WBC.

Flow cytometric immunophenotyping is a highly complex and relatively new technology. As the technology has moved from research laboratories to clinical laboratories, the need for standardization has increased. In response to this need, several sets of guidelines addressing aspects of the CD4+ T-lymphocyte testing process -- in particular, quality control, quality assurance, and reagents for flow cytometric immunophenotyping of lymphocytes -- have been developed (15,16, and National Institute of Allergy and Infectious Diseases (NIAID)/AIDS Clinical Trials Group: Guidelines for hematologic and flow cytometric analysis of ACTG specimens, 1992 **).

To assure the accuracy and reliability of CD4+ T-lymphocyte test results obtained within individual laboratories and to assure the comparability of results between laboratories, standard methods for performing the test, as well as guidelines for quality control and quality assurance, are desirable. This document provides CDC recommendations for immunophenotyping by flow cytometry. The recommendations apply to laboratory safety, specimen collection, specimen transport, maintenance of specimen integrity, specimen processing, flow cytometer quality control, sample analyses, data analysis, data storage, data reporting, and quality assurance.

RECOMMENDATIONS

A. Laboratory safety

    1. Use universal precautions with all specimens (17).

    2. Establish the following safety practices (18-24):

B. Wear laboratory coats and gloves when processing and analyzing specimens, including reading specimens on the flow cytometer. b. Never pipette by mouth. Use safety pipetting devices. c. Never recap needles. Dispose of needles and syringes in puncture-proof containers designed for this purpose. d. Handle and manipulate specimens (aliquoting, adding reagents, vortexing, and aspirating) in a class I or II biological safety cabinet. e. Centrifuge specimens in safety carriers. f. Wash hands with soap and water after working with specimens in the laboratory and when leaving the laboratory. g. For stream-in-air flow cytometers, follow the manufacturer's recommended procedures to eliminate the operator's exposure to any aerosols or droplets of sample material. h. Disinfect flow cytometer wastes. Add a volume of undiluted household bleach (5% sodium hypochlorite) to the waste container before adding waste materials so that the final concentration of bleach will be 10% (0.5% sodium hypochlorite) when the container is full (e.g., add 100 mL undiluted bleach to an empty 1000-mL container).

    i. Disinfect the flow cytometer as recommended by the manufacturer. One method is to flush the flow cytometer fluidics with a 10% bleach solution for 5-10 minutes at the end of the day, then flush with water or saline for at least 10 minutes to remove excess bleach, which is corrosive. j. Disinfect spills with household bleach (10% solution) or an appropriate dilution of mycobactericidal disinfectant. k. Fix all samples after staining and lysing but before analysis with buffered (pH 7.0-7.4) 1%-2% paraformaldehyde or formaldehyde. Note: The time required to inactivate cell-associated HIV and other viruses after fixation with paraformaldehyde and formaldehyde is not well established (25-27). Cell-free HIV can be inactivated with 1% paraformaldehyde within 30 minutes (28). Therefore, all samples should remain in fixative and should not be washed to remove it.

B. Specimen collection

1. Select the appropriate anticoagulant for hematologic testing

and flow cytometric immunophenotyping.

a. Anticoagulant for hematologic testing:

i. Use tripotassium ethylenediamine tetra-acetate (K3EDTA, 1.5 plus or minus 0.15 mg/mL blood) and perform the test within 6 hours (29,30).

ii. Reject a specimen that cannot be processed within 6 hours unless the hematology instrumentation is suitable for analyzing such specimens. Note: Some hematology instruments are capable of generating accurate results beyond 6 hours from specimen collection. Laboratories using such instruments are justified in extending the period between specimen collection and hematologic testing only after they have validated on their instrument the maximum time that specimens from both normal and HIV-infected persons show no significant variation in count from time zero. b. Anticoagulant for flow cytometric immunophenotyping, depending on the delay anticipated before sample processing:

iii. Use K3EDTA, acid citrate dextrose (ACD), or heparin if specimens will be processed within 30 hours.

iv. Use either ACD or heparin, NOT K3EDTA, if specimens will not be processed within 30 hours. Note: K3EDTA should NOT be used for specimens held for greater than 1 day before testing because the proportion of some lymphocyte populations changes after this period (CDC: unpublished data).

v. Reject a specimen that cannot be processed within 48 hours and request another.

2. Collect blood specimens by venipuncture (31) into evacuated tubes containing an appropriate anticoagulant, completely expending the vacuum in the tubes.

a. Draw pediatric specimens in pediatric tubes.

b. Mix the blood well with the anticoagulant to prevent clotting.

3. Draw the appropriate number of tubes:

a. When hematology and flow cytometric immunophenotyping will be performed in the same laboratory on the same specimen, use one tube containing K3EDTA.

b. In all other circumstances, draw two separate tubes (K3EDTA for hematologic determinations and K3EDTA, ACD, or heparin for flow cytometric immunophenotyping).

4. Label all specimens with a unique patient identifier, date, and time of collection.

a. Assure that patient information and test results are accorded confidentiality.

b. Provide on the submission form pertinent medications and disease conditions that may affect the immunophenotyping test (Appendix 1).

C. Specimen transport

1. Maintain and transport specimens at room temperature (18-22C)

(32,33). Avoid temperatures less than 10 C and greater than 37 C. In hot weather, it may be necessary to pack the specimen in a container with insulating material around it and place this container inside another that contains a cold pack (ice pack) and absorbent material. This method will help retain the specimen at ambient temperature.

2. Transport specimens to the immunophenotyping laboratory as soon as possible.

3. For transport to locations outside the collection facility but within the state, follow state or local guidelines. One method for packaging such specimens is to place the tube containing the specimen in a leak-proof container, such as a plastic bag, and pack this container inside a cardboard canister containing sufficient material to absorb all the blood should the tube break or leak. Cap the canister tightly. Fasten the request slip securely to the outside of this canister with a rubber band.

4. For interstate shipment, follow federal guidelines (34) for transporting diagnostic specimens. Note: Use overnight carriers with an established record of consistent overnight delivery to ensure arrival the following day. Check with these carriers for their specific packaging requirements as well.

5. Obtain specific protocols and arrange appropriate times of collection and transport from the facility collecting the specimen.

D. Specimen integrity

1. Inspect the tube and its contents immediately upon arrival.

2. Take corrective actions if the following occur:

If the specimen is hot or cold to the touch but not obviously hemolyzed or frozen, process it but note the temperature condition on the worksheet and report form. Abnormalities in light-scattering patterns will reveal a compromised specimen. b. If blood is hemolyzed or frozen, reject the specimen and request another. c. If clots are visible, reject the specimen and request another. d. If the specimen is greater than 48 hours old before processing, reject the specimen and request another.

E. Specimen processing

1. Hematologic testing

Perform the hematologic tests within 6 hours after collecting the blood specimen (29). (See Note under B.1.a.ii.) b. Perform an automated WBC count and differential, counting 10,000 to 30,000 cells (35). If the specimen is rejected or ``flagged'' by the instrument, a manual differential of at least 400 cells can be performed. If the flag is not on the lymphocyte population and the lymphocyte differential is reported by the instrument, the automated lymphocyte differential should be used.

2. Immunophenotyping

a. For optimal results, perform the test within 30 hours, but no later than 48 hours, after drawing the blood specimen (36,37).

b. Use a direct two-color immunofluorescence whole-blood lysis method. Use the ``stain, then lyse'' procedure.

c. Use the monoclonal antibody panel in Table 1, listed by CD nomenclature (38) and fluorochrome. The results for the panel provide data useful for defining the T-cell population and subpopulations; determining the proportion and purity of the lymphocytes in the gate; setting cursors for positivity; accounting for all lymphocytes in the sample; monitoring tube-to-tube variability; and monitoring T-cell, B-cell, and natural killer (NK)-cell levels in sequential patient specimens. The following internal controls are included in the panel:

i. CD3 monoclonal antibody in tubes 3-6 serves as a control for tube-to-tube variability and is also used to determine T-cell populations. Note: All CD3 values should be within 3% of each other. If the CD3 value of a tube is greater than 3% of any of the others, that tube should be repeated (new aliquot of blood labeled, lysed, and fixed).

ii. Monoclonal antibodies that label T-cells, B-cells, and NK-cells are used to account for all lymphocytes in the sample (11). Note: An abbreviated panel should only be used for testing specimens from patients for whom CD4+ T-cell levels are being requested as part of sequential follow-up and then only after consulting with the requesting clinician. The greatest danger in using an abbreviated panel is that the internal controls (noted above) are no longer included. For this reason, the immunophenotyping results should be reviewed carefully to ensure that T-cell levels are similar to those determined previously with the full recommended panel. When discrepancies occur, the specimens must be reprocessed using the full recommended monoclonal antibody panel. d. Use premixed two-color monoclonal antibodies at concentrations recommended by the manufacturer. Note: If, instead, two single-color reagents are combined, each must be titered with the other to determine optimal concentrations for use (10 uL antibody A with 5 uL antibody B; 5 uL antibody A with 10 uL antibody B; etc.). e. Use a volume of blood that assures the proper ratio of lymphocytes to monoclonal antibody and lysing reagent. For processing specimens containing greater or fewer lymphocytes than normal adults levels, refer to methodologies provided by the manufacturers of monoclonal antibodies. f. Maintain centrifugation forces between 300 and 400g for 3-5 minutes for wash steps. g. Vortex sample tubes to mix the blood and reagents and break up cell aggregates. Vortex samples immediately before analysis to optimally disperse cells. h. Include a source of protein (e.g., fetal bovine serum, bovine serum albumin) in the wash buffer to reduce cell clumps and autofluorescence.

iii. Incubate all tubes in the dark during the immunophenotyping procedure. j. Before analysis on the flow cytometer, fix all sample tubes after staining and lysing with 1%-2% buffered paraformaldehyde or formaldehyde. (This should be done even if the commercial lysing/fixing reagents contain a fixative.) Note: The characteristics of paraformaldehyde and formaldehyde may vary from lot to lot. They may also lose their effectiveness over time. Therefore, these fixatives should be made fresh weekly from electron microscopy-grade aqueous stock. k. Immediately after staining, store all stained samples in the dark and at refrigerator temperatures (4-10 C) until analysis.

F. Negative and positive controls for immunophenotyping

1. Negative (isotype) reagent control

Use this control with each specimen to determine nonspecific binding of the mouse monoclonal antibody to the cells and to set markers for distinguishing fluorescence-negative and fluorescence-positive cell populations. b. Use a monoclonal antibody with no specificity for human cells but of the same isotype(s) as the test reagents. Note: In many cases, the isotype control may not be optimal for controlling nonspecific fluorescence because of differences in fluorochrome/protein (F/P) ratio and antibody concentration between the isotype control and the test reagents. At this time there is no solution to this problem.

2. Positive methodologic control

a. Use this control to determine whether procedures for preparing and processing the specimens are optimal. This control is prepared each time patient specimens are prepared.

b. Use a whole blood specimen from a control donor. Ideally, this control will match the population of patients tested in the laboratory (see Section K.4.).

c. If this control falls outside established normal ranges, determine the reason. Note: The purpose of the methodologic control is to detect problems in preparing and processing the specimens. Biologic reasons

that cause only this control to fall outside normal ranges do not invalidate the results from other specimens processed at the same time. Poor lysis or poor labeling in all specimens, as well as the methodologic control, invalidates the results.

3. Positive control for testing reagents

a. Use this control to test the labeling efficiency of new lots of reagents, or when the labeling efficiency of the current lot is questioned. Prepare this control only when needed (when reagents are in question), in parallel with lots of reagents of known acceptable performance. Note: New reagents must demonstrate similar results to those of known acceptable performance.

b. Use a whole blood specimen or other human lymphocyte preparation (cryopreserved or lyophilized lymphocytes).

G. Flow cytometer quality control (15)

1. Align optics daily. This assures that the brightest and

tightest peaks are produced in all parameters. Note: Some clinical flow cytometers can be aligned and others can be aligned only by qualified service personnel.

a. Align the flow cytometer using stable calibration material (such as microbeads labeled with fluorochromes) that has measurable forward scatter, side scatter, PE, and FITC peaks.

b. Align the calibration particles optimally in the path of the laser beam and in relation to the collection lens, so the brightest and tightest peaks are obtained.

c. Align stream-in-air flow cytometers daily, at a minimum, and stream-in-cuvette flow cytometers (most clinical flow cytometers are this type) as recommended by the manufacturer.

2. Calibrate daily. This ensures that the flow cytometer is performing optimally each day and that its performance is the same from day to day.

a. Select machine settings that are optimal for fluorochrome-labeled whole blood specimens.

b. Use microbeads or other stable calibration material to place the scatter and fluorescence peaks in the same scatter and fluorescence channels each day. Adjust the flow cytometer as needed.

c. Maintain records of all daily calibrations. Monitor these to identify any changes in flow cytometer performance.

d. Retain machine calibration settings for the remaining quality control procedures (sensitivity and color compensation) as well as for reading the specimens.

3. Determine sensitivity daily. The flow cytometer must differentiate between the dim peak and autofluorescence in each fluorescence channel (PE and FITC).

a. Evaluate calibration material or cells that have low-level fluorescence that can be separated from autofluorescence (e.g., microbeads with low-level and negative fluorescence or CD56-labeled lymphocytes).

b. Establish minimal acceptable distance between peaks, monitor this difference, and correct any daily deviations.

4. Compensate for spectral overlap daily. This step corrects the spectral overlap of one fluorochrome into the fluorescence spectrum of another.

a. Use either microbead or cellular calibration material containing three populations: no fluorescence, PE fluorescence only, and FITC fluorescence only.

b. Analyze the calibration material and adjust the electronic compensation circuits on the flow cytometer to place the PE and FITC populations in their respective fluorescence quadrants with no overlap into the double-positive quadrant (Figure 1). Take care to avoid overcompensation.

c. If calibration particles (microbeads) have been used to set compensation, confirm this by using lymphocytes labeled with FITC- and PE-labeled monoclonal antibodies that recognize separate cell populations but do not overlap. These populations should have the brightest expected signals. Note: If a dimmer-than-expected signal is used to set compensation, suboptimal compensation for the brightest signal can result.

d. Reset compensation when photomultiplier tube voltages or optical filters are changed.

5. Repeat all four instrument quality control procedures whenever instrument problems occur or if the instrument is serviced during the day.

6. Maintain instrument quality control logs, and monitor them continually for changes in any of the parameters. In the logs, record instrument settings as well as peak channels and coefficient of variation (CV) values for optical alignment, calibration, sensitivity, and spectral compensation. Reestablish fluorescence levels for each quality control procedure when lots of beads are changed.

H. Sample analyses

1. Analyze the sample tubes of each patient in the following

order:

a. The tube containing CD45 and CD14 (gating reagent): read this tube first so that gates can be set around the lymphocyte cluster.

b. Isotype control: set cursors for differentiating positive and negative populations so that less than or equal to 2% of the cells are positive.

c. Remaining tubes in the panel.

2. Count at least 2,500 gated lymphocytes in each sample. This number assures with 95% confidence that the result is less than or equal to 2% (standard deviation(SD)) of the ``true'' value (binomial sampling). Note: This model assumes that variability determined from preparing and analyzing replicates is less than or equal to 2% SD. Each laboratory must determine the level of variability by preparing and analyzing at least six replicates of the last four tubes in the recommended panel. Measure variability when first validating the methodology used and again when methodologic changes are made.

3. Examine light-scattering patterns on each sample tube. Determine whether lysis or sample preparation, which can affect light scattering, is the same in each sample tube of a patient's specimen. Deviation in a particular tube usually indicates sample preparation error, and the tube should be repeated (a new aliquot of blood is stained and lysed).

I. Data analysis

1. Reading from the sample tube containing CD45 and CD14, draw

lymphocyte gates using forward and side light-scattering patterns and fluorescence staining.

a. Define populations on the following basis:

i. Lymphocytes stain brightly with CD45 and are negative for CD14.

ii. Monocytes and granulocytes have greater forward and side light-scattering properties than lymphocytes.

iii. Monocytes are positive for CD14 and have intermediate intensity for CD45.

iv. Granulocytes are dimly positive for CD14 and show less intense staining with CD45.

v. Debris, red cells, and platelets show lower forward scattering than lymphocytes and do not stain specifically with CD45 or CD14. b. Based on the above characteristics, draw a gate around the lymphocyte population (based on low forward- and side-scattering patterns) (39).

2. Verify the lymphocyte gate by determining the lymphocyte proportion within the gate and the purity of the gate.

a. Definitions

i. The lymphocyte proportion within the gate is the percentage of lymphocytes in the sample that are within the gate.

ii. The lymphocyte purity of the gate is the percentage of cells within the gate that are lymphocytes. The remainder may be monocytes, granulocytes, red cells, platelets, and debris. b. The lymphocyte proportion within the gate should be at least 90%. c. The lymphocyte purity of the gate should be at least 85%. d. Optimal gates include as many lymphocytes and as few contaminants as possible. e. The lymphocyte purity of the gate is determined by calculating the percentage of bright CD45+CD14- cells in the light-scattering gate. f. The proportion of lymphocytes within the gate can only be determined by backgating. Note: Some instrument software packages do this automatically; others do not. Backgating should be done on those flow cytometers that allow it.

iii. Backgate by identifying the lymphocytes by CD45 and CD14 reactivity and gating on the lymphocyte fluorescence.

iv. With forward and side light-scattering analysis regions set around the lymphocytes, determine the number of cells that fall within this region compared with the total number of lymphocytes. Note: The light-scattering analysis region should be adjusted so that it is the same as the light-scattering gate. g. If the recommended proportion and purity of lymphocytes within the gate cannot be achieved, redraw the gate. If minimum levels still cannot be obtained, reprocess the specimen. If this fails, request another specimen.

3. Set cursors using the isotype control so that less than 2% of cells are positive.

4. Analyze the remaining samples with the cursors set based on the isotype control. Note: In some instances, the isotype-set cursors will not accurately separate positive and negative staining for another sample tube from the same specimen. In such cases, the cursors can be moved on that sample to more accurately separate these populations (Figure 2).

5. Analyze each patient or normal control specimen with light-scattering gates and cursors for positivity set for that particular patient or control.

6. Where spectral compensation of a particular specimen appears to be inappropriate because FITC-labeled cells have been dragged into the PE-positive quadrant or vice-versa (when compensation on all other specimens is appropriate), repeat the sample preparation, prewashing the specimen with phosphate-buffered saline (PBS), pH

7.2, to remove plasma before monoclonal antibodies are added.

7. Include the following analytic reliability checks:

a. Optimally, at least 95% of the lymphocytes should be within the lymphocyte gate. Minimally, at least 90% of the lymphocytes should be within the gate.

b. Optimally, at least 90% lymphocyte purity should be observed within the lymphocyte gate. Minimally, at least 85% purity should be observed within the gate.

c. Optimally, the sum of the %CD3+CD4+ and %CD3+CD8+ cells should equal the total %CD3+ cells within plus or minus 5%. Minimally, this variability should be less than or equal to 10%.

d. Optimally, the sum of the %CD3+ (T-cells), %CD19+ (B-cells), and the %CD3-(CD16 and/or CD56)+ (NK-cells) should equal the purity of lymphocytes in the gate plus or minus 5%. Minimally, this variability should be less than or equal to 10%. If the data are corrected for lymphocyte purity (see K.2.), the sum should equal 95%-105% (or a minimum of 90%-110%).

J. Data storage

1. If possible, store list-mode data on all specimens

analyzed. This allows reanalysis of the raw data, including redrawing gates. At a minimum, retain hard copies of the lymphocyte gate and correlated dual histogram data of the fluorescence of each sample.

2. Retain all primary files, worksheets, and report forms for 2 years or as required by state or local regulation, whichever is longer. Data can be stored electronically. Disposal after the retention period is at the discretion of the laboratory director.

K. Data reporting

1. Report all data in terms of CD designation, with a short

description of what that designation means. Note: CD4+ T-cells are T-helper cells. The correct cells to report for this value are those that are positive for both CD3 and CD4 (determined from tube 3 in the suggested panel). Similarly, CD8+ T-cells are T-suppressor/cytotoxic cells, and these are positive for both CD3 and CD8 (tube 4 in the panel). It is important not to include other cell types (non-T-cells) in CD4 and CD8 determinations.

2. Report data as a percentage of the total lymphocytes and correct for the lymphocyte purity of the gate. For example, if the lymphocyte purity is 94% and the CD3 value is 70%, correct the CD3 value by dividing 0.70 by 0.94 and then multiply the result by 100 to give a T-lymphocyte value of 74%.

3. Report absolute lymphocyte subset values when an automated complete blood cell (CBC) count (WBC and differential) has been performed from blood drawn at the same time as that for immunophenotyping.

a. Calculate the absolute values by multiplying the lymphocyte subset percentage (from flow cytometry data) by the absolute number of lymphocytes (from WBC and differential). Note: The hematology laboratory providing the CBC (WBC and differential) must perform satisfactorily in a hematology proficiency testing program approved by the Health Care Finance Administration (HCFA) as meeting the requirements of the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88).

b. Report both percentages and absolute counts when these are available.

4. Report data from all relevant monoclonal antibody combinations with corresponding reference limits of expected normal values (e.g., CD4+ T-cell percentage, absolute number of CD4+ T-cells). Reference limits for immunophenotyping test results must be determined for each laboratory. See reference 15 for methods for

determining these limits.

L. Quality assurance

1. Assure the overall quality of the laboratory's CD4+ T-cell

testing by monitoring and evaluating the effectiveness of the laboratory policies and procedures for the preanalytic, analytic, and postanalytic testing phases:

a. Methods for collecting, handling, transporting, identifying, processing, and storing specimens.

b. Information on test request and report forms.

c. Instrument performance, quality control protocols, and maintenance.

d. Reagent quality control protocols.

e. Review and proper reporting of results.

f. High-quality employee training and education:

i. Basic training by flow cytometer manufacturers and additional training in hands-on workshops for flow cytometer operators and supervisors.

ii. Education of laboratory directors in flow cytometric immunophenotyping through workshops and other programs.

iii. Continuing education in new developments for all flow cytometric immunophenotyping personnel through attendance at meetings and workshops.

iv. Adherence to federal and state regulations for training and education. g. Assurance of satisfactory performance. Laboratories must successfully participate in a performance evaluation program. When proficiency testing programs are approved by HCFA as meeting the requirements of CLIA `88 (none are currently approved for CD4+ T-cell testing), laboratories must satisfactorily participate. h. Review and revision (as necessary, or at established intervals) of the laboratory's policies and procedures to assure adherence to the quality assurance program. All staff involved in the testing should be informed of any problems identified during the quality assurance review and the corrective actions taken to prevent recurrences.

2. Document all quality assurance activities.

COMMENTARY

To date, no defined standard exists for immunophenotyping by flow cytometry. This article provides recommendations for laboratory standardization. Proficiency testing and performance evaluation programs are also needed to assess laboratory performance and to measure the level and sources of testing variability between and within laboratories.

Three test components contribute to intra- and interlaboratory variability when lymphocyte populations are measured by immunophenotyping: a) whole blood immunophenotyping preparation and analysis; b) total WBC count; and c) leukocyte differential. Analytic and biologic variability exist in all three test components.

The analytic variability (CV) in determining the WBC count using an automated leukocyte counter ranges from 2.2% to 7.7% and from 9.3% to 17.6% using a hemocytometer. The lymphocyte differential varies from 1.9% to 5.3% for automated counts and from 12.5% to 27% for manual counts (35). Therefore, the variability in the absolute number of lymphocytes in the blood reflects the combined variability of the WBC count and the

lymphocyte differential. Biologic variability is even greater: about 10% diurnally and 13% week to week (40).

Estimates of interlaboratory variability (SD) in flow cytometric immunophenotyping results have been derived from proficiency testing data (41; College of American Pathologists (CAP) Flow Cytometry Survey: unpublished data; CDC: Model Performance Evaluation Program (MPEP), unpublished data). An analysis of data from a recent CAP survey of nearly 500 laboratories showed that SD of the percentage of CD4+ T-cells was 3.5% to 5%, regardless of the actual percentage of CD4+ T-cells in the specimen. For duplicate measurements, SD of the percentage of CD4+ T-cells was about 3% when the specimen contained 45% CD4+ T-cells. The results furnished to CDC by 280 laboratories participating in the MPEP for T-lymphocyte immunophenotyping in March 1991 were similar. For samples of CD4+ values in the range of 1% to 16%, SD of the percentage of CD4+ T-cells was about 2.5%; for samples with CD4+ values between 16% and 24%, SD was about 3.4%.

Limited information is available on the degree of interlaboratory variability in CD4+ T-cell counts. In a multicenter proficiency testing study (42) of seven laboratories for the year 1987, interlaboratory CV for the percentage and absolute number of CD4+ T-cells on normal specimens were 6% and 29.4%, respectively. This study has been ongoing and, through rigorous quality assurance and training, CV values have been reduced each year. Subsequently, in 13 laboratories in 1991, CV for the percentage and absolute number of CD4+ T-cells on normal specimens were 4.1% and 8.4%, respectively (Rickman WJ: unpublished data).

Variability in the absolute number of CD4+ T-cells can be reduced in the following ways: a) performing replicate analyses; b) standardizing the test protocol; c) improving technology; and d) increasing the skills and knowledge of testing personnel, including those responsible for specimen collecting and handling. The above methods for reducing variability, if implemented, will increase the reliability of this important laboratory measurement.

Clinicians and others who choose laboratories for testing patient samples need ways of evaluating the performance of flow cytometric immunophenotyping laboratories. Criteria that can be helpful in this process include: a) laboratory accreditation, licensure, or certification by a recognized professional organization or governmental agency; b) laboratory participation in a recognized proficiency testing/performance evaluation program; and c) laboratory use of CDC or other published guidelines for flow cytometry.

The recommendations in this document reflect current technology in a field that is rapidly changing. Revisions in these recommendations will be required as techniques, skills, and knowledge improve. Evaluation of testing practices is a tool that will provide information for making sound changes in these recommendations.

The determination of CD4+ T-cell counts has greatly contributed to the health management of persons infected with HIV, to public health HIV/AIDS prevention programs, and to epidemiologic and clinical research in HIV infection. These recommendations should enhance the ability of laboratories to provide the high quality of testing needed to support these services.

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