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# The ISHAGE Guidelines for CD34+ Cell Determination by Flow Cytometry

D. ROBERT SUTHERLAND,[1] LORI ANDERSON,[2] MICHAEL KEENEY,[2] RAKASH NAYAR,[1] and IAN CHIN-YEE[2]

## ABSTRACT

**The increased use of Peripheral Blood Stem Cells (PBSC) to reconstitute hematopoiesis in autotransplant and, more recently, allotransplant settings has not been associated with a consensus means to quality control the PBSC product. Since the small population of cells that bear the CD34 antigen are thought to be responsible for multilineage engraftment, graft assessment by flow cytometric quantitation of CD34+ cells should provide a rapid, reliable, and reproducible assay. Unfortunately, although a number of flow cytometric assays for CD34 enumeration have been described, the lack of a standardized method has led to the generation of widely divergent data. Furthermore, none of these assays has been validated as to interlaboratory reproducibility and suitability for widespread clinical application. In early 1995, the International Society of Hematotherapy and Graft Engineering (ISHAGE) established a Stem Cell Enumeration Committee, the mandate of which was to validate a simple, rapid, and sensitive flow cytometric method to quantitate CD34+ cells in peripheral blood and apheresis products. We also sought to establish its utility on a variety of flow cytometers in clinical laboratories and its reproducibility between transplant centers. Here, we describe the four-parameter flow methodology adopted by ISHAGE for validation in a multicenter study in North America.**

## INTRODUCTION

### *Peripheral blood and marrow transplants*

A VARIETY OF STUDIES performed over the past several years have established that the 1%–3% of cells in the bone marrow that express the cell surface antigen CD34 (1,2) are capable of reconstituting long-term, multilineage hematopoiesis after intensive therapy (3–5). CD34+ cells are also found in the peripheral circulation of normal individuals but are extremely rare (consensus range 0.01%–0.1%). CD34+ cells, however, can also be mobilized from marrow to the peripheral circulation in greater numbers by chemotherapy or hematopoietic cytokines or both (6,7), and peripheral blood stem cell (PBSC) autografts are increasingly used to reconstitute hematopoiesis after intensive, potentially marrow-ablative therapy (8,9). More recently, a number of studies have been initiated using PBSC grafts in an allotransplant setting.

The increased use of PBSC versus marrow for autografting has been driven by a number of clinically relevant factors, including the notion that mobilized PBSC harvests might provide a graft with less tumor contamination than autologous marrow (reviewed in 10). The availability of an increasing number of cytokines (used either singly or in combination) to mobilize CD34+ cells into the peripheral circulation has facilitated the harvest-

[1]Oncology Research, The Toronto Hospital, Ontario, Canada.
[2]Department of Hematology, The London Health Sciences Centre, Victoria Campus, London, Ontario, Canada.

ing of PBSC to the point where it is considered a suitable alternative to marrow for autologous transplantation. Time to engraftment is generally shorter with PBSC transplants, resulting in significant cost savings, and earlier concerns regarding the long-term engraftment potential of PBSC grafts, although not yet fully resolved, are no longer considered a major obstacle to their use (8–11). Harvesting of PBSC is less traumatic, and the side effects of reinfusion of the smaller volume of the PBSC product are reduced. The PBSC product is also more amenable to ex vivo procedures, such as CD34+ cell selection (4,12), tumor purging (13), and genetic manipulation (14,15).

### *Graft Assessment by colony-forming cell (CFU) assays*

Although some centers may still cling to the assumption that an increase in the peripheral white cell count will indicate that an adequate collection can be obtained, in the recent past, the most widely used approach to assess the quality of PBSC grafts relied on colony-forming cell (CFC) assays for relatively late lineage-committed hematopoietic progenitors, such as colony-forming units–granulocyte-macrophage (CFU-GM). A reliable and rapid means to assess more primitive cells that mediate long-term engraftment was lacking. Furthermore, because of differences in the way these assays were performed in various centers, including widely differing culture conditions, it was difficult to make meaningful comparisons between data from different institutions (16). The most serious limitation of CFC assays remains the 10–14 day interval required for assay readout. This characteristic alone precludes a clinically useful role for this assay both in determining the number of apheresis collections required and in real-time analysis of PBSC content to optimize timing of apheresis.

### *Graft quality assessment by CD34+ cell enumeration*

Since reinfusion of purified CD34+ cells results in hematopoietic recovery following myeloablative therapy (3–5), graft assessment by quantitation of CD34+ cells would appear to overcome the aforementioned limitations and should generate data that are more directly comparable between different transplant centers (16). Since the CD34+ population is thought to encompass the most primitive stem cells, as well as maturing, lineage-committed progenitors of all lineages (1,2,17), a multiparameter flow cytometric approach should be able to address not just quantitative issues but also the qualitative composition of the PBSC product.

Whereas most studies have reported a significant correlation between CD34+ cell levels and the frequency of circulating CFC (16,18,19), recommendations for minimum required numbers of CD34+ cells for long-term engraftment have ranged from 2 to $5 \times 10^6$/kg, in the absence of any standard assay to enumerate CD34+ cells (19,20). Several groups (21–24) have described flow cytometric assays for CD34 enumeration, but not all investigators used the same CD34 antibodies, some of which fail to detect CD34+ cells in some clinical samples and cell lines (2,17, and unpublished data). Furthermore, when used in the traditional manner to establish the positive cell analysis region, isotype-matched control antibody staining of minor populations of cells can often mask true staining of rare populations. Sample variability is a significant problem with respect to numbers of red cells, platelets, platelet aggregates, nonspecifically stained adherent cells, and other cellular debris, all of which can be faithfully recorded by flow cytometers.

To date, none of these flow cytometric assays have been validated regarding interlaboratory reproducibility and suitability for widespread clinical application. The goal of this document is to provide clinically acceptable guidelines for the enumeration of CD34+ cells in peripheral blood and apheresis products by flow cytometry.

A routine clinical assay for hematopoietic stem/progenitor cells must meet the following criteria.

1. Simplicity—to allow widespread applicability
2. High sensitivity—since hematopoietic stem cells are rare events
3. Accuracy—to provide clinically relevant results
4. Reproducibility—to provide clinically reliable results
5. Speed—to provide real-time and on-line analysis

To meet these criteria, we selected a flow cytometric assay using whole blood or apheresis products, commercially available, directly conjugated fluorochrome-labeled monoclonal antibodies, routine staining/whole blood lysing procedures, and a simple gating strategy using light scatter and two-color immunofluorescence analysis using a pan-CD45 FITC/pan-CD34 PE monoclonal antibody combination (25). This procedure meets the requirements as a simple assay that can be performed in clinical flow cytometry laboratories.

## BACKGROUND AND RATIONALE

Determination of the absolute CD34+ cell count in peripheral blood and apheresis products requires an accurate quantitation of the CD34+ cell number in a specimen as determined by flow cytometry, as well as an accurate nucleated cell count. In the case of apheresis products, an accurate determination of the product volume is also required.

*Selection of parameters*

The detection of rare events by immunophenotyping amplifies the problems of nonspecific binding of antibodies to cellular debris or dead cells, autofluorescence, and discrimination between true negative and true positive events. Although it is desirable to use as many differentiating cell characteristics as possible (e.g., light scatter properties and multiple antigen expression) to identify these rare events, a clinical guideline must recognize the cost limitations of reagents and the complexity/interpretation of results, as well as the limited use of multiparameter analysis within the clinical flow cytometry laboratory setting. We acknowledge that the antibody combinations (with or without third fluorescence channel gating of live versus dead cells) and gating procedures developed by other investigators may offer advantages, but these more complex methods may not be feasible in many clinical flow cytometry laboratories. We have, therefore, simplified the identification of CD34+ cells to light scatter properties and two-color immunofluorescence using CD45 FITC/CD34 PE, as this combination of parameters can be used to provide a clinically relevant reflection of the peripheral blood stem/progenitor cell compartment (25–27).

*Selection of monoclonal antibodies*

*CD34 Antibodies.* The CD34 antigen is a heavily glycosylated mucin-like structure (reviewed in 2), and many CD34 antibodies detect glycosylation-dependent and, in particular, sialic acid-dependent epitopes thereon. Epitopes recognized by the seven CD34 antibodies designated at the IVth International Leukocyte Differentiation Antigens Workshop (1) have been divided into three classes depending on their sensitivities to neuraminidase and the O-sialo-glycoprotease from *Pasteurella haemolytica* (28), a unique protease that exhibits a highly substrate-restricted specificity for O-glycosylated mucin-like glycoproteins, such as CD34 (29). Epitopes detected by class I antibodies, e.g., MY10, B1.3C5, 12.8, and ICH3, were sensitive to both enzymes. Those detected by class II antibodies, e.g., QBEnd 10, were sensitive only to the glycoprotease, and those detected by class III antibodies, e.g., 115.2 and Tuk3, and the nonworkshop antibody 8G12 (30) were sensitive to neither enzyme. At the most recent workshop, where a number of new CD34 antibodies were similarly classified, it was additionally shown that the enzyme chymopapain also cleaves class I and class II epitopes (17).

Due to their dependence on negatively charged carbohydrate moieties (sialic acids) and their consequent inability to detect all glycoforms of the CD34 antigen (whose fine structure varies on different glycoforms of the CD34 molecule), class I antibodies fail to detect CD34 on some leukemias and leukemia-derived cell lines (2,17,28). Their somewhat lower avidity and general inability to retain reactivity after conjugation with the negatively charged fluorochrome, FITC, further reduce their use for enumeration of CD34+ cells in PBSC samples and in protocols involving immunoselection of CD34+ cells.

In order to develop a reliable flow cytometric assay for CD34+ cells, it is, therefore, important to use CD34 antibodies that, in the hands of most investigators, detect all glycosylation variants of the molecule, that is, class II or class III antibodies. To detect rare events, such as CD34+ cells, it is also advantageous to use an antibody conjugated to the brightest fluorochrome excitable using an argon laser-based flow cytometer, for example, phycoerythrin (PE). The latter issue is especially important for molecules, such as CD34, that exhibit high net negative charge due to the large number of sialic acid residues found in their mucin-like domains. For example, whereas unconjugated and PE-conjugated forms of the sialic acid-independent class II CD34 antibody QB End10 bind avidly to the CD34 molecule, negatively charged FITC conjugates of QBEnd10 bind much less well. This is because the QBEnd10 epitope is probably nested between the clusters of negatively charged O-linked carbohydrates in the amino-terminal domain of the CD34 molecule (unpublished data). These observations underline the importance not only of selecting an appropriate CD34 antibody clone but also of selecting one that retains high avidity binding after conjugation to the designated fluorochrome.

After parallel analysis of a large number of samples of normal blood, mobilized peripheral blood, cord blood, and normal marrow, as well as of CD34+ cell lines that fail to express some class I CD34 epitopes, it is our experience that PE conjugates of QBEnd10 (class II, Immunotech or Southern Biotechnology Associates), 8G12 (HPCA2, class III, Becton Dickinson), and the recently available 581 (17) class III, Immunotech) all work equally well in the current ISHAGE protocol (see Fig. 3). If the basic protocol is modified to incorporate a third conjugated antibody in a three-color analysis (26,27) and FITC conjugates of CD34 antibodies have to be used, class III reagents, such as 8G12 and 581, can be recommended. However, for the reasons outlined, the use of FITC conjugated class II CD34 antibodies is not recommended.

*CD45 Antibodies.* A monoclonal antibody to the CD45 antigen is used in combination with CD34 to provide an additional parameter for identification of the CD34+ cell population. Primitive blast cells characteristically express CD45 at low to intermediate levels. This feature, in combination with relatively low side-angle light scatter properties, is useful in positively identifying normal and leukemic blast cells (31–33), and a variation in this blast

cell gating approach (33) was instrumental in our basic methodology to identify true CD34+ cells (25). The CD45 staining pattern is also useful as an indicator of the effectiveness of the red cell lysis step, as well as distinguishing white blood cells (WBC) from contaminating events, such as platelets, platelet aggregates, and other debris that can bind low levels of PE-conjugated CD34 antibodies.

Selection of the appropriate CD45 conjugate is also important, since not all epitopes of CD45 are expressed on all WBC. Because of the differential splicing of the single CD45 pre-mRNA species, a variety of CD45 isoforms (polypeptides) can be synthesised and each one differentially glycosylated to produce a large number of unique glycoforms (34,35). Restricted epitopes (CD45 R) are not expressed on all leukocytes. We have recently identified an apparent pan-CD45 reagent (IOL1b, Immunotech) that depends on sialic acids for its binding, and it too is variably expressed on leukocytes (unpublished observations). Thus, pan-CD45 antibodies that detect not just all isoforms but also all glycoforms of this structure are required.

FITC-conjugates of pan-CD45 antibodies are widely available from a number of commercial and other sources. For the ISHAGE protocol outlined below, we restricted our assessments to HLe-1 (Becton Dickinson), J33 (Immunotec), and KC56 (Coulter, Hialeah FL) because these reagents are all manufactured to the highest current standards of good manufacturing practice and were found to work well in the protocol. The J33 reagent is particularly good due to its high signal-to-noise ratio in PBSC samples.

Other investigators (36,37) have used combinations of antibodies, such as CD34 PE, versus a cocktail of lineage-committed antibodies conjugated with FITC, including CD3, CD11b, CD14, CD20, and glycophorin A. Although different cocktails of antibodies may have utility in specific circumstances, such as discriminating the most primitive noncommitted precursors and true stem cells from the more mature, lineage-committed CD34+ progenitors (37), the additional expense of reagents for a clinical assay concerned only with quantitation of total CD34+ cells does not justify their routine use.

*Isotype Controls and Titration of Antibodies.* As noted, in rare event analysis, such as CD34+ cell enumeration, it is possible for isotype control staining (a monoclonal antibody to an irrelevant antigen of the same isotype as the CD34 antibody employed) to mask the staining of rare CD34+ cells. Unfortunately, every fluorochrome-conjugated monoclonal antibody has slightly different nonspecific binding characteristics to the next antibody. Thus, the control reagent should be carefully assessed before it is selected for routine use. Consequent to the gating strategy outlined in this article, however, it is our view that isotype controls are of far less critical importance in establishing a positive analysis region with the ISHAGE procedure. Instead, a PE-conjugated isotype control is combined with CD45 FITC to enumerate false positive signals in the PE fluorescence region. The events that appear in region R4 are then subtracted from the average total number of events identified with the CD34/CD45 combination.

The isotype control must nevertheless be titrated to give the same level of staining on a CD34− leukocyte population as exhibited by the CD34-PE reagent. Lymphocytes are the target population of choice for this purpose due to their generally lower levels of nonspecific and Fc receptor-dependent antibody binding and lower autofluorescence. At such a concentration, staining histograms of lymphocytes generated by the isotype PE and the CD34 PE reagents should be very similar.

### *Gating strategies*

The purpose of sequential gating is to select the population of interest and simultaneously minimize interference from debris and mature cells to which antibodies can bind nonspecifically. A gating strategy based on CD45 staining versus side-angle light scatter properties is suggested as a primary gate due to its utility in separating WBC from red cells, platelets, and other debris. Although nucleated red cells and fully mature plasma cells are not detectably stained by CD45-FITC, these cells are not generally found in significant numbers in PBSC preparations and so do not affect the utility of CD45+ events to generate a stable denominator in the calculation of the absolute CD34+ value. From this primary gate, CD34+ cells are identified by their expression of CD34, their characteristic low to intermediate CD45 antigen expression, and their characteristic light scatter properties.

To include all nucleated cells in the denominator, some investigators have used nuclear dyes, such as LDS-751 (38) and SY-III-8 (39), in three-color analyses. Nucleated cells can be distinguished from nonnucleated cells and other events based on fluorescence. Whereas LDS-751 fluorescence is detected in the FL3 channel and is compatible with the procedure and the fluorochrome-labeled antibodies outlined later, SY-III-8 is detected in the FL1 channel, and the CD45 reagent must be conjugated to a fluorochrome that emits in the far red (FL3 channel) (39).

The gating strategy described in this document does not control for the presence of nonviable cells per se. Since live cells cannot be reliably distinguished from dead cells based on light scatter properties alone, some authors have used nuclear dyes that pass freely through the disrupted membranes of dead cells to identify and exclude this population. Nuclear dyes employed include 7-AAD (22,40), which emits in the far red spectrum and can be used in combination with the CD45-FITC and CD34-PE reagents suggested here. However, the FL3-

intended to be used to provide accurate, reproducible CD34+ cell determinations on fresh peripheral blood and apheresis products. Although the effects of storage on CD34+ cells in these samples have not been determined, specimen storage and integrity for the purpose of CD3/CD4 immunophenotyping have been well described for peripheral blood (43). On receipt, the specimen should be checked for temperature. If the specimen is cold on arrival, it should be checked for hemolysis and freezing. A sample that has been exposed to high temperatures also may be hemolyzed.

*Quality Control.* Laboratory facilities must maintain instrument quality assurance records as well as acceptable quality control procedures for the hematology and flow cytometry analyzers. All reagents must be tested for acceptable reactivity.

### *CD34+ cell enumeration by flow cytometry*

Quantitation of the absolute number of circulating CD34+ cells in whole blood depends on the accurate determination of two values.

1. The number of CD34+ cells meeting all fluorescence and light scatter criteria. This number is derived from taking the average of two acceptable replicate CD34+ determinations, as assessed by CD45-FITC/CD34-PE specific staining, less the number of events representing nonspecific staining, as determined by the CD45-FITC/isotype-PE control.
2. The absolute leukocyte count. *Note*: For apheresis products, the total volume in the apheresis pack(s) also must be determined.

*Monoclonal Antibodies*: anti-CD45-FITC, anti-CD34-PE, and isotype control-PE. Acceptable reactivity and appropriate titration should be established before use. A limited screen of commercially available isotype IgG1-PE reagents on a number of apheresis samples occasionally revealed striking differences between them. The isotype control should thus be selected only after appropriate testing. All monoclonal antibodies should be stored at 4°C in the dark.

*Whole Blood Lysing Reagents.* A number of proprietary red cell lysing reagents also contain fixatives. Recent comparative studies (S. Serke et al., personal communication) have demonstrated that these lyse/fix reagents can alter the fluorescence intensity of stained cells, in addition to causing light scatter changes (shrinkage) of cells over time. Thus, if fixed samples are not to be analyzed immediately, it may be necessary, at the time of analysis, to alter the forward scatter threshold or light scatter parameter settings of the cytometer or both before data acquisition. Alternatively, the light scatter data can be acquired in logarithmic mode.

### *Determination of total leukocyte count and differential*

Specimens should be analyzed on an automated hematology analyzer for determination of the LKC (required for the calculation of absolute numbers of CD34+ cells). The test must be performed within the time frame determined by the hematology instrument manufacturer and validated by the hematology department. Specimen integrity must conform to the quality assurance standards of the hematology department for CBC/differential analysis. As the nucleated leukocyte count on some blood and apheresis samples is often higher than can be reliably counted by the instrument, samples may need to be prediluted, and the dilution factor must be taken account of in the final calculation.

### *CD34+ cell quantitation by flow cytometry*

*Sample Preparation.* Prior to staining with monoclonal antibodies, ensure that the LKC is no greater than $10 \times 10^9$/L. If necessary, dilute the sample with medium that is compatible with the lysing system being used. Next, label three (or four) tubes as follows: (*1*) CD45-FITC/CD34-PE; (*2*) CD45-FITC/CD34-PE; (*3*) CD45-FITC/Isotype control-PE; (*4*) Optional: Isotype-FITC/Isotype-PE. Tube *4* is used predominantly to determine the positive analysis region for CD45. This test is generally not required once the operator is confident with the staining pattern of the CD45-FITC reagent in use.

Add 100 μl blood to each tube, and add the volume of monoclonal antibody to the appropriately labeled tubes according to the previously determined antibody titration experiments. Incubate tubes at 4°C for 20–30 min. Although not generally a problem, if the sample is old or contains nonviable cells, binding of fluorochrome-conjugated antibodies can be reduced by preincubating the samples with IgG concentrates. Next, Lyse red cells or lyse/fix samples and resuspend cells in an adequate volume for flow cytometric analysis according to routine laboratory practice.

*Flow Cytometric Analysis.* CD34+ cells in the peripheral blood are considered rare events. In normal individuals, their incidence rarely exceeds 0.1% of the total nucleated cell count. Other cells (monocytes and neutrophils) present in specimens have greater autofluorescence and a greater propensity to bind antibodies nonspecifically than do CD34+ cells. A gating strategy that uses light scattering parameters and CD34/CD45 fluorescence will aid in their accurate identification and enumeration.

True CD34+ cells (a) express CD34 antigen, (b) express CD45 antigen with staining intensity characteristic of blast cells (i.e., readily detectable but at lower levels

emitting nuclear dye, propidium iodide, is not recommended for three-color analysis on single laser instruments because it is difficult to compensate out the near red emissions that spill over into the FL2 channel. Thus, although theoretically desirable to include all nucleated cells in the denominator and a live/dead cell discriminator, the addition of a third color reagent in the form of a nuclear dye requires an appropriately equipped and compensated flow cytometer and may not be practical for routine clinical immunophenotyping. As a compromise, we suggest that viability of all samples should be checked routinely by trypan blue exclusion before samples are run, and an appropriately titrated isotype control should be used as a means of detecting nonspecific antibody staining of viable and nonviable cells. In practice, we have found the viability of most specimens to be well preserved over a 24 h period (>98%). The cell viability should nonetheless be recorded for every specimen.

Furthermore, by retaining the third fluorescence channel of three-channel cytometers for a third antibody conjugate of interest, we have been able to generate additional information about the composite antigenic phenotype or relative level of differentiation/maturation of the gated CD34+ cells (26,27,29).

*Cell separation and lysis techniques*

A whole blood lysis technique is recommended for peripheral blood and apheresis products, as previous studies have shown recovery of cell populations is improved over cell separation techniques (18,21,22). Additionally, processing time is significantly reduced.

*Number of events analyzed*

CD34+ cells are rare events in the peripheral blood and are best described as a Poisson distribution. The standard error is determined by the square root of the number of positive events counted. Therefore, to maintain precision, we recommend that a minimum of 100 CD34+ events and at least 75,000 CD45+ events be collected. Depending on the proportion of CD34 cells in the peripheral circulation, the total number of cells counted may be even greater if the percentage of CD34 is 0.1% or less. We also suggest that this test be performed in duplicate and that the number of CD34+ cells fall within 10% of the mean for the replicate samples.

*Denominator*

A critical determinant in the calculation of the proportion of CD34+ cells is the use of an appropriate denominator. Different denominators employed may include the total number of events, the number of events meeting light scatter criteria of lymphocytes, or the total number of mononuclear cells. In peripheral blood and apheresis products, we recommend using the total number of CD45+ events above a noise discriminator (threshold) set on forward angle light scatter. This discriminator must not exclude any viable WBC; that is, it must be set below the forward scatter of small lymphocytes. As a simple check, the lymphocyte population can be readily identified by their bright CD45 staining and low side scatter characteristics on a CD45 versus side scatter dotplot. The gated lymphoid cells are then analyzed by forward versus side scatter to confirm that the forward scatter threshold has been set correctly. This quick procedure will also indicate whether the forward and side scatter gain parameters of the instrument are optimally set.

*Automated total nuclear cell count*

The absolute leukocyte count (LKC) required for determination of absolute CD34+ cells in the PBSC samples must be obtained from an automated hematology analyzer. Studies have confirmed that coefficients of variation for automated LKC are significantly less than those for manual counts (41). A flow cytometric technique designed to calculate the absolute value without the need for an automated LKC may soon be commercially available (42) but has yet to be validated in clinical laboratories.

## GUIDELINES FOR CD34+ CELL DETERMINATION IN BLOOD AND APHERESIS PRODUCTS USING FLOW CYTOMETRY

*Safety.* Universal precautions should be in effect, and appropriate safety procedures should be employed (see CDC guidelines for specific recommendations, 43).

*Specimen Collection.* Acid citrate dextrose formula A (ACDA) is the anticoagulant used in apheresis procedures. This anticoagulant allows both an automated LKC and flow cytometric evaluation to be performed on the same specimen. Individual laboratories must verify the reliability of their hematology analyzer to perform an accurate LKC on blood/apheresis samples drawn into ACDA. EDTA is the anticoagulant of choice for peripheral blood samples.

*Specimen Labeling.* As a minimum, specimens should be labeled with the patient's full name, patient identification number, date, and time the samples were collected.

*Specimen Transport.* Specimens should be transported at room temperature (18°C–22°C) in containers that meet all federal, state, and provincial guidelines. Packing should ensure that the sample neither freezes nor overheats (>37°C).

*Evaluation of Sample Integrity.* These guidelines are

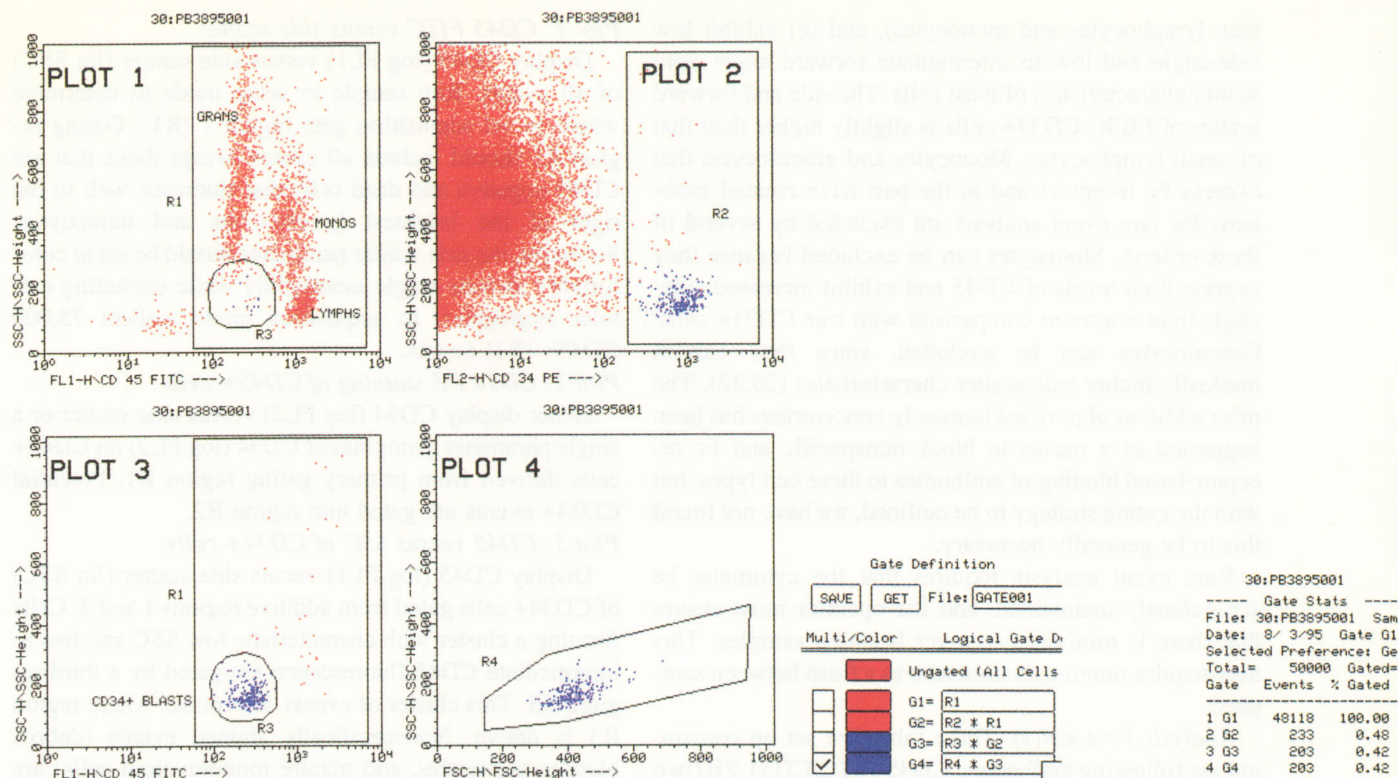



**FIG. 1.** Enumeration of CD34+ cells in apheresis sample cells using CD45 FITC/CD34 PE. Precise details of gating strategy are described in the text.

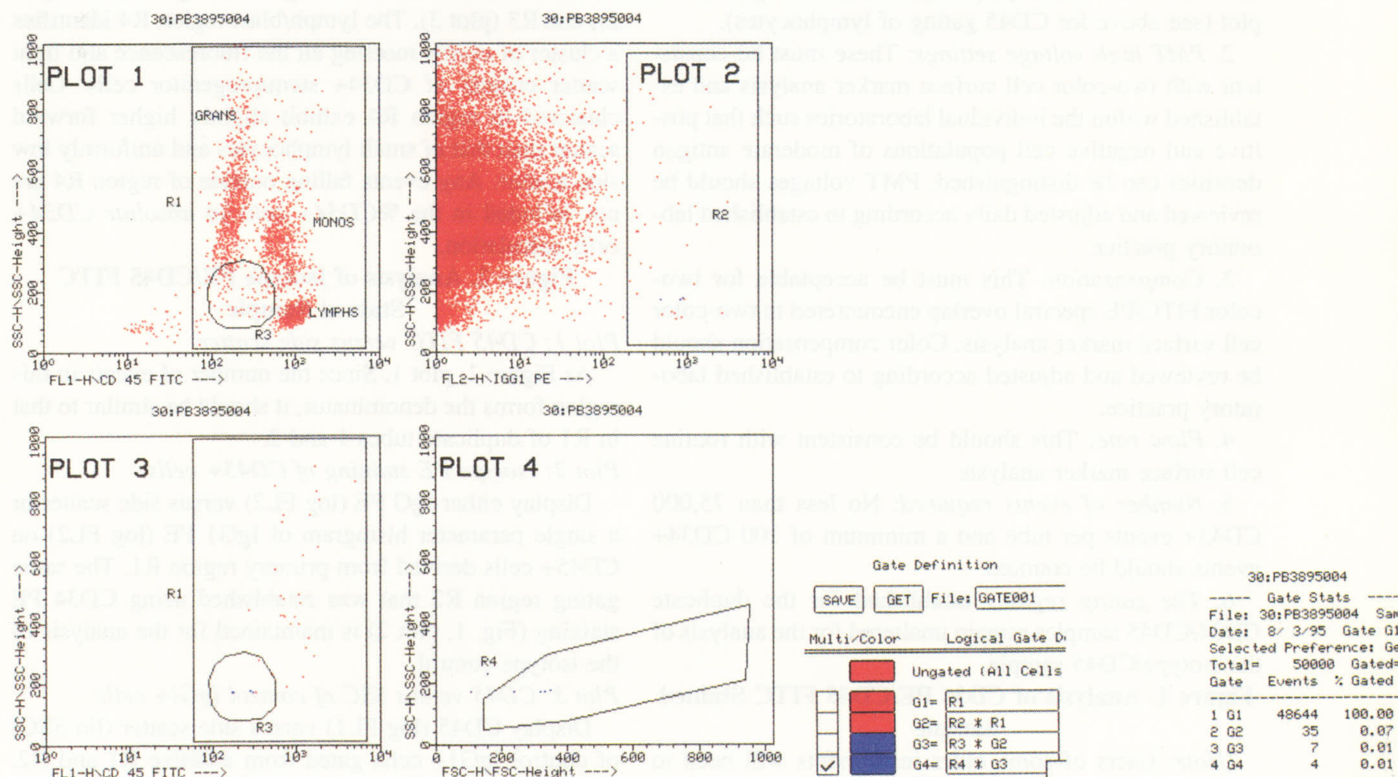



**FIG. 2.** Enumeration of events from apheresis sample stained by CD45 FITC/Isotype IgG1 PE. Identical gating regions to those shown in Figure 1 were used to analyze the staining of the same apheresis sample with the control reagents.

than lymphocytes and monocytes), and (c) exhibit low side-angle and low to intermediate forward angle light scatter characteristics of blast cells. The side and forward scatter of PBSC CD34+ cells is slightly higher than that of small lymphocytes. Monocytes and granulocytes that express Fc receptors and in the past have created problems for rare event analysis are excluded by several of these criteria. Monocytes can be excluded because they express high levels of CD45 and exhibit increased side-angle light scatter in comparison with true CD34+ cells. Granulocytes can be excluded, since they exhibit markedly higher side scatter characteristics (25,32). The prior addition of purified human Ig concentrates has been suggested as a means to block nonspecific and Fc receptor-based binding of antibodies to these cell types, but with the gating strategy to be outlined, we have not found this to be generally necessary.

Rare event analysis requires that the cytometer be scrupulously maintained, and the operator must ensure that there is minimal carryover between samples. This may require running a saline tube as a flush between samples.

*Analysis Protocol(s).* Three tubes are set up containing the following antibodies: CD45-FITC/CD34-PE (two tubes), and CD45-FITC/Isotype-PE (one tube).

1. *Discriminator/threshold*: The forward angle light scatter threshold is set to exclude debris (low forward scatter) but not small lymphocytes from the light scatter plot (see above for CD45 gating of lymphocytes).

2. *PMT high voltage settings*: These must be consistent with two-color cell surface marker analysis and established within the individual laboratories such that positive and negative cell populations of moderate antigen densities can be distinguished. PMT voltages should be reviewed and adjusted daily according to established laboratory practice.

3. *Compensation*: This must be acceptable for two-color FITC/PE spectral overlap encountered in two-color cell surface marker analysis. Color compensation should be reviewed and adjusted according to established laboratory practice.

4. *Flow rate*: This should be consistent with routine cell surface marker analysis.

5. *Number of events required*: No less than 75,000 CD45+ events per tube and a minimum of 100 CD34+ events should be counted.

6. *The gating regions* established for the duplicate CD34/CD45 samples remain unaltered for the analysis of the isotype/CD45 sample.

**Figure 1. Analysis of CD34 PE/CD45 FITC Stained Sample**

(*Note*: Users of some older instruments will need to collect data in listmode and perform off-line analysis to use the sequential gating strategy outlined in this document.)

*Plot 1: CD45 FITC versus side scatter*

Display CD45 (log FL1) versus side scatter (lin SSC) of all events. Run sample in setup mode to determine where to set acquisition gate region 1 (R1). Gating region R1 should include all events except those that are CD45 negative and dead cells that fluoresce well to the right of the brightest lymphocytes and monocytes. Similarly, the side scatter parameter should be set to comfortably include single neutrophils while excluding cellular aggregates. In acquisition mode, collect 75,000 CD45+ (R1) events.

*Plot 2: CD34 PE staining of CD45+ cells*

Either display CD34 (log FL2) versus side scatter or a single parameter histogram of CD34 (log FL2) on CD45+ cells derived from primary gating region R1. Potential CD34+ events are gated into region R2.

*Plot 3: CD45 versus SSC of CD34+ cells*

Display CD45 (log FL1) versus side scatter (lin SSC) of CD34+ cells gated from additive regions 1 and 2. Cells forming a cluster with characteristic low SSC and low to intermediate CD45 fluorescence are gated by a third region, R3. This cluster of events determines where region R3 is drawn. Nonspecifically stained events (debris, platelet aggregates, and mature monomyeloid cells) are excluded from this region.

*Plot 4: Light scatter characteristics of CD34+ cells*

Display forward scatter versus side scatter (FSC vs SSC) of cells from additive regions R1 (plot 1), R2 (plot 2), and R3 (plot 3). The lymph/blast region R4 identifies a cluster of events meeting all the fluorescence and light scatter criteria of CD34+ stem/progenitor cells. Cells clustered in region R4 exhibit slightly higher forward scatter than that of small lymphocytes and uniformly low side scatter. Any events falling outside of region R4 are not included in the *%CD34+ cells* or *absolute CD34+ cells* calculation.

**Figure 2. Analysis of Isotype PE/CD45 FITC Stained Sample**

*Plot 1: CD45 FITC versus side scatter*

As Figure 1, plot 1. Since the number of events in this region forms the denominator, it should be similar to that in R1 of duplicate tubes 1 and 2.

*Plot 2: Isotype PE staining of CD45+ cells*

Display either IgG PE (log FL2) versus side scatter or a single parameter histogram of IgG1 PE (log FL2) on CD45+ cells derived from primary region R1. The same gating region R2 that was established using CD34 PE staining (Fig. 1, plot 2) is maintained for the analysis of the isotype control.

*Plot 3: CD45 versus SSC of control IgGi+ cells*

Display CD45 (log FL1) versus side scatter (lin SSC) of control IgG1+ cells gated from additive R1 and R2. Cells nonspecifically stained by the IgG1 isotype control should scatter outside of the stem/progenitor region R3 that remains **unaltered** from Figure 1, plot 3.

*Plot 4: Light scatter characteristics of IgG1+ cells*

Display forward scatter versus side scatter (FSC vs SSC) of cells from additive regions R1 (plot 1), R2 (plot 2), and R3 (plot 3). Events falling within R4 represent nonspecifically stained cells. Since the majority of nonspecifically stained cells are excluded from R3 (plot 3), it is rare to find any events in R4. If any events do fall in R4, they are subtracted from the average number of CD34+ events from the replicate tubes containing anti-CD34 PE cells.

*Data Analysis.*

**Figure 1**

*Plot 1*

The average total number of CD45+ events of the replicate CD45 FITC/CD34 PE samples is used as the denominator in all calculations of the %CD34+ cells or the absolute number of CD34+ PBSC. The proportion of CD45+ events must be consistent (within 3%) from tube to tube. Variability may be an indication of irregular instrument flow or incomplete red cell lysing. Resolution of the problem may be as simple as rerunning an inconsistent tube (after examining the instrument for proper function) or may require preparing another tube for analysis.

*Plot 2*

This dot plot or histogram is used to define the region of fluorescence for CD34+ events. The region boundaries should initially be set wide enough to include all potential CD34+ cells. Contained in this region are all true CD34+ cells together with nonspecifically stained events (debris, platelet aggregates, and mature monomyeloid cells). Once plots 3 and 4 are established and the gate statistics for all four regions are shown, the placement of R2 can be adjusted (narrowed). This eliminates nonspecifically stained events that fall outside the clustered events gated by R3 and R4 without reducing the numbers of events that fall within the latter. Because of variations in analysis software used in different laboratories, it is usually more convenient to use a CD34 staining versus side scatter dot-plot to gate CD34+ events. This variation may be of particular utility when bone marrow is the subject of analysis or a FITC-conjugated CD34 antibody has to be used in a three-color analysis (26,27).

*Plot 3*

This is a two parameter dot-plot of CD45 fluorescence versus side scatter of cells gated by R1 and R2. True CD34+ cells contained in R2 form a cluster exhibiting low CD45 fluorescence and low side scatter, and it is this cell cluster that defines the limits of R3. Indeed, it is the ability to detect these events that determines the sensitivity of the protocol. Events from R2 that do not fall within R3 represent nonspecifically stained events and are thereby excluded. Relative to true CD34+ cells, lymphocytes are excluded from R3 by their high CD45 expression, monocytes by their high CD45 expression, and increased side scatter and granulocytes by their high side scatter.

*Plot 4*

This is a two parameter dot-plot of forward angle versus side-angle light scatter and is used to confirm the light scatter characteristics of true CD34+ cells. Events gated by R1, R2, and R3 form a cluster exhibiting a size slightly larger than small lymphocytes and generally uniform low side scatter. A lymph/blast analysis of R4 is established to include events with these characteristics. The number of events falling within this region is used in all calculations of the absolute CD34+ cell count (numerator) and should be closely similar to the number of events falling in R3 of plot 3. As already noted, once R3 and R4 have been established, it is usually possible with all but the most difficult of samples to optimize the placement of R2 to achieve similar numbers of events falling into R3 and R4 without significantly reducing the number of events gated in R4. If it is difficult to show similar numbers of events in R3 and R4, it usually means that the lower limit of R2 has been set too low, and moving this to the right (i.e., reducing the number of weakly stained events) generally obviates this problem.

**Figure 2. Isotype control**

Cells stained with CD45-FITC and IgG1 PE are analyzed using identical gates defined by R1–R4 for the CD34 PE-stained samples. In our experience, it is unusual for a significant number of events to appear in R4, even when the isotype stains more events in R2 than were stained by the CD34 reagent. Any events that do appear in R4 are subtracted from the average CD34+ cell numbers derived from plots 1–4 of Figure 1.

*Calculation of Absolute CD34+ Cells.* The number of nonspecific events in R4 from the isotype control tube is subtracted from the average total number of events in the CD34-stained sample tubes. This corrected number of CD34+ cells is the numerator, and the average total number of CD45+ events from the CD45 FITC/CD34 PE replicate samples represents the denominator. This value is multiplied by the absolute LKC as determined by the automated hematology analyzer to calculate the absolute CD34 stem/progenitor cell numbers in the sample and by the pack volume to derive the absolute CD34+ cell count per pack.

$$\frac{\text{Absolute LKC } (\times 10^9/\text{L}) \times \text{(corrected average total no. of CD34+ events)} \times 1000^*}{\text{(average total no. of CD45+ events}} = \text{absolute CD34+ cells} \times 10^6/\text{L}$$

*The equation is multiplied by a factor of 1000 to convert from $10^9$/L to $10^6$/L, for example

421 (corrected average total CD34+ events)
75,000 (average total CD45+ events)
$5.2 \times 10^9$/L (absolute LKC)

*Example*
$$5.2\ (\times 10^9/L) \times \frac{421 \times 1000}{75{,}000} = 29.1 \times 10^6/L$$

To determine the absolute CD34+ cell number per apheresis pack, the above number is multiplied by the pack volume in liters, for example

376 (corrected average total CD34+ events)
75,000 (average total CD45+ events)
$221.2 \times 10^9/L$ (absolute LKC)
54 ml (= 0.054 L) pack volume

*Example*
$$\frac{221.2 \times 376 \times 1000 \times 0.054}{75{,}000}$$

Absolute CD34+ cell count per pack = $59.9 \times 10^6$

*Data Storage.* Where facilities are available, list mode data should be stored on all specimens. A hand copy printout of gating and analysis histograms, with sufficient identification to allow subsequent review, all worksheets, reports, and related material must be stored for a period consistent with state or provincial regulatory requirements (minimum 2 years). Files may be stored electronically. The identification of the personnel responsible for analysis must also be recorded.

*Data Reporting.* Results should be reported as both percentage CD34+ events and absolute number $\times 10^6/L$ (peripheral blood) or the absolute number $\times 10^6$ (apheresis products).

## USING THE ISHAGE PROTOCOL

### *In a clinical setting*

Although the validation studies of the ISHAGE protocol currently underway in nine North American centers have not been completed, our own experience over the last 2 years (26,27), together with the data already available from the early phases of the study, suggest that this procedure is very sensitive, being capable of detecting 1 CD34+ cell in 10,000. It is highly specific using the appropriate pan-CD45 and pan-CD34 antibodies that detect all CD34 glycoforms and is reproducible. The procedure is quick and simple and can be performed on a variety of single laser flow cytometers, with only basic software being required for data analysis. Sample variability with respect to the numbers of red cells, platelet aggregates, and other cellular debris can be avoided by the inclusion of CD45+ cells as a denominator, making this procedure particularly suitable for routine analysis in flow cytometry laboratories. The sequential gating strategy used in the ISHAGE protocol allows us to use the isotype control solely to enumerate any nonspecifically stained events that exhibit the other fluorescence and light scatter characteristics of true CD34+ cells. However, as indicated, we rarely find more than a few such events, particularly in blood or apheresis samples.

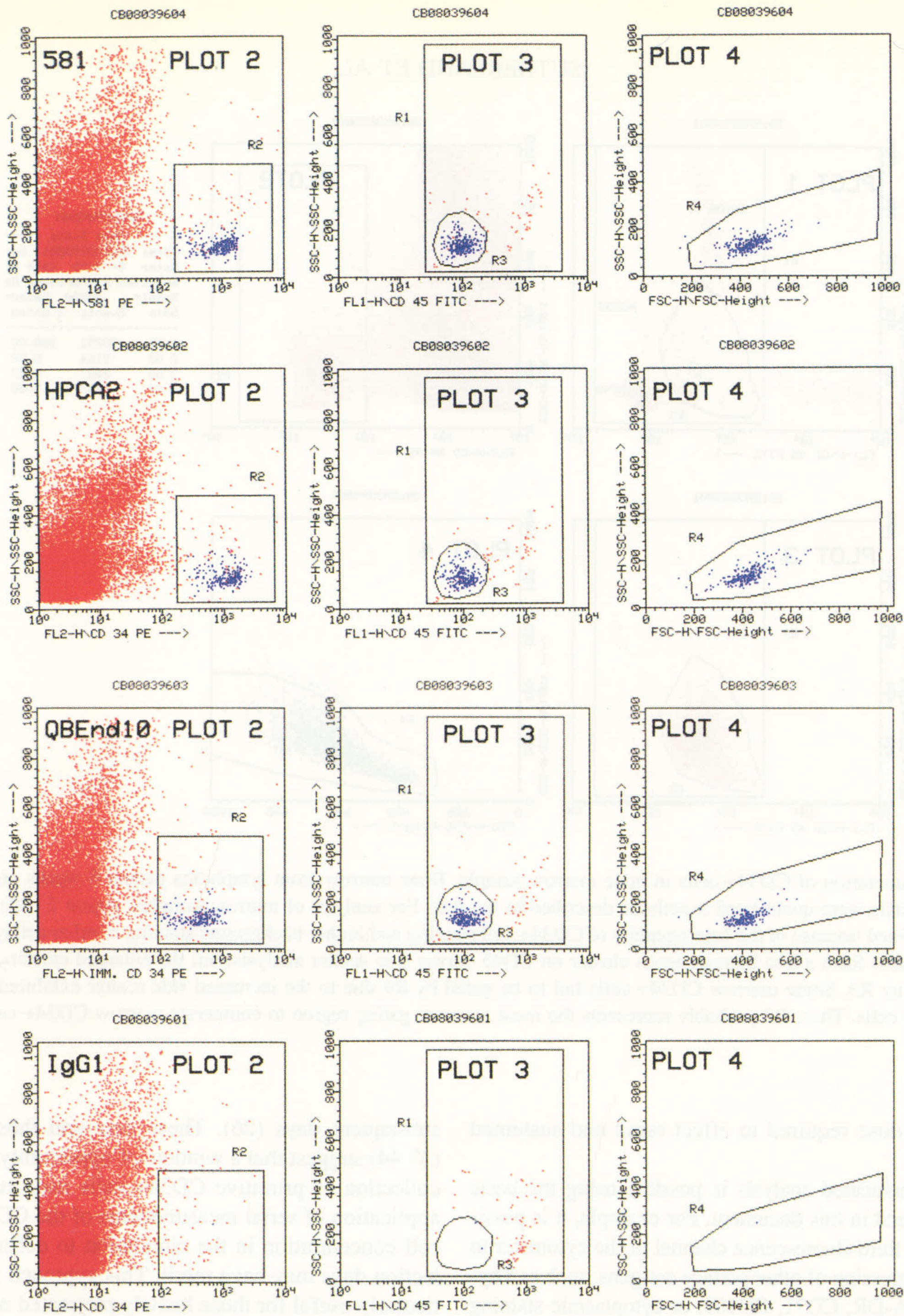
### *In the research laboratory*

Although this article is primarily concerned with the enumeration of CD34+ cells in PBSC collections for clinical purposes, we have used the ISHAGE protocol in a number of research laboratory situations. Indeed, it was originally developed to accurately quantitate CD34+ cells in bone marrow mononuclear cell fractions (25) from which we were selecting CD34+ cells (2,29). A number of centers are using marrow and cord blood for transplantation, whereas others are using CD34+ cells selected from these sources, for in vitro expansion studies and potential gene therapy protocols. Clearly, when any positive selection technique is employed to purify CD34+ cells from marrow, cord blood, or mobilized PB, the ability to accurately enumerate the CD34+ cells as a percentage of total WBC is of critical importance in calculating the efficiency and yield of the particular purification technique employed. It is more difficult to quantitate CD34+ cells in marrow aspirates because of the more heterogeneous size, granularity (side scatter), and staining characteristics of marrow CD34+ cells (Fig. 4). Due to this heterogeneity, the cluster that defines R3 is usually a little larger than for blood products. It is nevertheless our experience that the basic two-color ISHAGE methodology can accurately detect CD34+ cells in all types of normal blood and marrow samples (25,27,30) and also can be used to detect specific perturbations in the CD34+ cell compartment in some preleukemic syndromes and atopic individuals.

## FUTURE DIRECTIONS

Although the development of comprehensive guidelines for CD34+ cell quantitation may well be subject to change and must eventually address all aspects of a clinical laboratory procedure, we have focused on the procedures involved in the accurate determination of the relative percentage of CD34+ cells by flow cytometry and its utility in determining the absolute number of CD34+ cells in blood and apheresis collections. This is an essential prerequisite if studies are to be undertaken to determine optimal timing of apheresis procedures.

Comparison of results generated from different transplant centers using different mobilizing regimens should provide more reliable data, allowing clinical guidelines to be developed to address the minimum number of

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----- Gate Stats -----
File: CB08039604  Sample:
Date:  3/ 8/96  Gate G1=
Selected Preference: Ari
Total=   50000  Gated=
Gate   Events  % Gated
--------------------------
1 G1    42201   100.00
2 G2      300     0.71
3 G3      249     0.59
4 G4      247     0.59
```

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CB08039602
----- Gate Stats -----
File: CB08039602  Sample:
Date:  3/ 8/96  Gate G1=
Selected Preference: Ari
Total=   50000  Gated=
Gate   Events  % Gated
--------------------------
1 G1    42978   100.00
2 G2      279     0.65
3 G3      224     0.52
4 G4      222     0.52
```

```
CB08039603
----- Gate Stats -----
File: CB08039603  Sample
Date:  3/ 8/96  Gate G1=
Selected Preference: Ari
Total=   50000  Gated=
Gate   Events  % Gated
--------------------------
1 G1    42794   100.00
2 G2      268     0.63
3 G3      218     0.51
4 G4      217     0.51
```

```
CB08039601
----- Gate Stats -----
File: CB08039601  Sample
Date:  3/ 8/96  Gate G1=
Selected Preference: Ari
Total=   50000  Gated=
Gate   Events  % Gated
--------------------------
1 G1    42329   100.00
2 G2       69     0.16
3 G3        2     0.00
4 G4        2     0.00
```

**FIG. 3.** Enumeration of CD34+ cells in cord blood sample using CD45 FITC and class II or class III CD34 PE reagents. Sequential gating strategy was performed as described for Figures 1 and 2 (see text). The CD45 versus side scatter dot-plot (plot 1) for each sample is not shown. However, all events shown in plot 2 for each PE conjugate were derived from the corresponding plot 1. **Top row.** CD34 PE clone 581 (Immunotech). **Second row.** CD34 PE clone 8G12 (HPCA2, Becton Dickinson). **Third row.** CD34 PE clone QBEnd10 (Immunotech). **Bottom row.** Isotype control IgG1 PE (Becton Dickinson). Gate statistics show the number of CD34+ cells detected as a percentage of CD45+ events for the CD34 clones and the IgG1 control. **Left to right.** 581 (0.59%), 8G12 (0.52%), QBEnd10 (0.51%), and isotype control (0.00%).

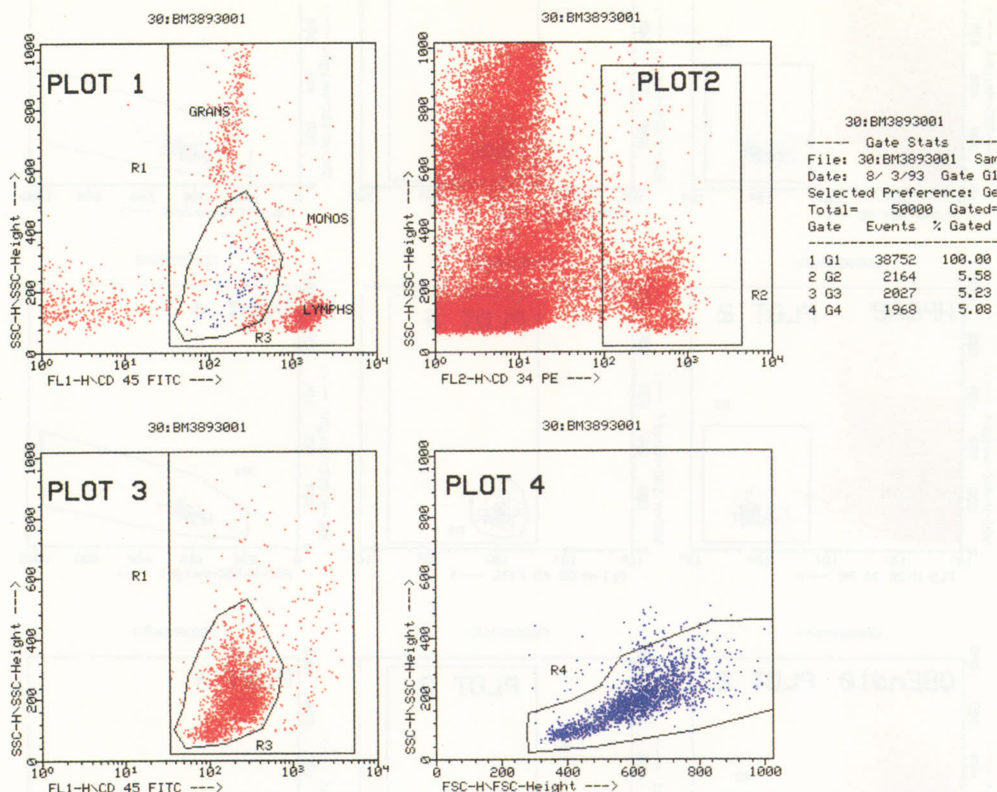


**FIG. 4.** Enumeration of CD34+ cells in bone marrow sample. Bone marrow from lymphoma patient 8 weeks postchemotherapy. CD34+ cells were quantitated exactly as described in the text. For analysis of marrow samples, region 2 (R2) is somewhat less easily defined because of the heterogeneity of CD34+ cell staining and higher background staining of other cell types. Marrow CD34+ cells also form a less homogeneous cluster on CD45 versus side scatter analysis, but the enlarged cluster can nevertheless be gated by R3. Some marrow CD34+ cells fail to be gated by R4 due to the increased side scatter exhibited by maturing monomyeloid cells. Thus, R3 probably represents the most accurate gating region to enumerate marrow CD34+ cells.

CD34+ cell dose required to effect rapid and sustained engraftment.

More sophisticated analysis is possible using the basic method outlined in this document. For example, it is possible using the third fluorescence channel of the cytometer to assess the expression of other surface antigens, such as Thy-1, CD38 HLA-DR, CD71, CD109, or cytoplasmic staining with rhodamine 123. Candidate stem cells in marrow and mobilized peripheral blood exhibit the $CD34^{hi}$Thy-1+CD109+$CD38^{lo}$HLA-$DR^{lo}$$CD71^{lo}$$rho^{lo}$ composite phenotype. Recently, it was shown that the Thy-1+ subset of CD34+ cells in PBSC samples was also enriched for the most primitive CFC currently detectable in long-term cultures, and this subset was also capable of multilineage engraftment of human hematopoiesis in immunodeficient mice (37).

We used this modified approach in 12 myeloma patients in a recent study, and our data indicate that the level of Thy-1+ cells in the CD34+ fraction is highest even before collection would normally take place, based on increased WBC counts or timed collections, and drops on subsequent days (26). These data and those of others (37,44) suggest that a window of opportunity for optimal collection of primitive CD34+ cells may exist, and the application of serial measurements of the CD34+Thy-1+ cell concentration in the circulation to define early collection days may have merit. This approach may be particularly useful for those heavily pretreated myeloma patients with a low likelihood of successful collection, especially given the recently reported increase in malignant cell contamination of apheresis products noted on later collection days (45). In these cases, it may be possible to identify the most primitive and most clinically relevant subsets of CD34+ cells required for long-term engraftment even though the total CD34+ cell dose may be considered suboptimal under current protocols.

Analysis of our results indicated that the frequency of circulating CD34+ cells not only best predicted the apheresis CD34+ and CFU-GM levels but was the only predictor of the apheresis CD34+Thy-1+ cell count. Thus, serial analysis of peripheral blood CD34+ cells most accurately predicts the progenitor/stem cell content

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Address reprint requests to:
*D. Robert Sutherland*
*Chair, ISHAGE Stem Cell Enumeration Committee*
*Assistant Professor, Department of Medicine*
*University of Toronto*
*Oncology Research, The Toronto Hospital*
*CRCS 67 College Street*
*Toronto*
*Ontario, M5G 2M1*
*Canada*

of the apheresis collection, and this simple modification of the basic ISHAGE protocol also allows for optimal collection of candidate stem cell subsets of CD34+ cells on an individual patient basis. Studies have shown that it may be the dose of such stem cells that determine not just the long-term engraftment potential of the graft but also the speed of engraftment (46). Similar studies could be undertaken to determine the optimal growth factor or growth factor combinations required to optimize the mobilization of the most primitive CD34+ cell subsets.

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