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Original Articles

Analysis of Variation in Results of CD34+ Hematopoietic Progenitor Cell Enumeration in a Multicenter Study

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A workshop was held in The Netherlands and Belgium with the aim of investigating whether or not the use of a standard protocol vs. local protocols for flow cytometric enumeration of CD34+ hematopoietic progenitor cells would reduce interlaboratory variation. The standard protocol consisted of a three-color, whole-blood staining technique based on fluorescein isothiocyanate (FITC)-labeled CD34, and phycoerythrin (PE)-labeled CD14 and CD66e monoclonal antibodies (reactive with monocytic and myeloid cells, respectively), followed by erythrocyte lysis, washing, fixation, and selection of nucleated cells during data acquisition on the basis of their positivity for LDS-751 (staining DNA and RNA). Data analysis guidelines included the elimination of nonspecific antibody binding by monocytes and myeloid cells by gating on the CD14-,66e- cells, followed by setting a window on a CD34 vs. sideward light scatter (SSC) plot around the CD34+, SSClow cells. The FITC-labeled isotype control was analyzed with the same gate and window settings, and the false-positive events were subtracted from the CD34 result. Four samples (i.e., peripheral blood and apheresis product from two patients) were sent out. Results were received on patient 1 (2) from 36 (38) laboratories. Data obtained by 24 (26) laboratories after correct application of the standard protocol revealed that the median percentage of CD34+ cells of the four samples ranged between 1.1% and 3.7% and the CVs between 18% and 30%. Incorrect performance of the standard protocol by 12 laboratories, mainly resulting from gating errors, yielded a larger variation (CVs ranging between 50% and 82%). CD34 enumeration using local protocols by 29 (34) laboratories yielded median percentage of CD34+ cells ranging between 1.2% and 3.9% and CVs ranging between 34% and 106%. We conclude that correct application of this standard protocol was effective in reducing the interlaboratory variation in percentage of CD34+ cells assessments.   Cytometry 30:109–117, 1997.   © 1997 Wiley-Liss, Inc.

Key terms: CD34; hematopoietic stem cells; enumeration; flow cytometry; quality control; standardization

CD34 represents the only cell-surface antigen whose expression on hematopoietic cells is restricted to early progenitors of all lineages (5,23). Purified CD34$^+$ cells can fully reconstitute hematopoiesis in experimental animals and man (3,4). CD34$^+$ hematopoietic progenitor cells (HPC) can be mobilized into the peripheral blood by combinations of cytotoxic drugs and cytokines, allowing their collection by apheresis in quantities sufficient for transplantation procedures (13,18). The rapidly widening application of such peripheral stem cell transplants for the treatment of hematological malignancies and solid tumors has been parallelled by a strongly increased use of flow cytometry for quantification of CD34$^+$ HPC, for timing of the apheresis procedures, for monitoring of CD34$^+$ cell yield in apheresis products, and recently for quality assessment of ex vivo-processed stem cell transplants, i.e., enrichment of HPC and/or depletion of tumor cells (13). Monitoring of the absolute number of peripheral blood CD34$^+$ cells during HPC mobilization is widely used for timing of the apheresis procedure (10,13,16). Assessment of the number of CD34$^+$ cells is a critical quality-control step in stem cell transplants (19), particularly after ex vivo processing for the removal of tumor cells. The threshold for the number of reinfused CD34$^+$ cells to ensure rapid reconstitution of neutrophils and platelets after myeloablative therapy is believed to range between 0.5 and 5×10^6 CD34$^+$ cells/kg body weight of the patient (13); the lack of standardization of CD34$^+$ cell quantification, among other factors, has prevented a more exact definition of this threshold thus far (2). The need to compare clinical and laboratory data in multicenter trials calls for standardization of CD34$^+$ cell enumeration. To this end, several workshops on this issue have been held in Europe and the United States during the past 5 years (7,11,12,14,15,20,26).

A workshop on flow cytometric enumeration of CD34$^+$ cells was held in September, 1995, as an introduction to a biannual quality-assessment scheme in The Netherlands and Belgium. During this workshop, we introduced a standard protocol aimed at resolving the major technical difficulties in the assay. The participants were requested to perform the standard protocol in parallel with their own protocols on centrally distributed samples in order to explore whether the use of this standard protocol would reduce interlaboratory variation. The features of the standard protocol were: 1) whole blood staining, lyse-and-wash methodology; 2) elimination of nonnucleated cells during data acquisition by gating on events positive for LDS-751; 3) exclusion of monocytic (CD14$^+$) and mature myeloid cells (CD66e$^+$) during list-mode data analysis, in order to eliminate interference by their Fcγ receptor-mediated monoclonal antibody (mAb) binding and relatively high autofluorescence with CD34 enumeration; and 4) placement of a window on a CD34 vs. sideward light scatter (SSC) dot plot around the CD34$^+$,SSC^{low} cell cluster in order to restrict the analysis to true CD34$^+$ HPC (Fig. 1).

Although most investigations had at that time not yet addressed the use of gating reagents to improve enumeration of CD34$^+$ cells (7,11,19,20,26), results of the first study using CD45 as a gating reagent and identification of

the HPC on the basis of bright CD34 positivity, dim CD45 positivity, and low SSC signals (Fig. 2) were available (21). However, we were primarily concerned with the high level of Fcγ receptor-mediated binding of mAb by monocytic and myeloid cells, because the expression and activity of these receptors to bind murine IgG can be up-regulated by cytokines used to mobilize HPC, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 25). Monocytic and myeloid cells can be identified by CD14 and CD66e, respectively, and the interference by CD14$^+$ and CD66e$^+$ cells with quantification of CD34$^+$ cells is shown in Figure 1G,H. Hence, we included CD14 and CD66e mAb as gating reagents in our assay. Moreover, we observed that the definition of bright CD34$^+$, dim CD45$^+$, and low SSC did not unambiguously resolve HPC from a cell population with intermediate forward light scatter (FSC) and SSC signals of which a small fraction was dimly CD34$^+$ (depicted red in Fig. 2B,D-H). We observed this population in particular in apheresis products, and its reduction by gating on the CD14$^-$,66e$^-$ population (Fig. 1E,F) suggests that these cells are immature myeloid elements. We chose fluorescein isothiocyanate (FITC)-labeled CD34 mAb and phycoerythrin (PE)-labeled CD14 and CD66e mAb because no good FITC-labeled CD66e mAb was available to us at that time. Finally, we did acknowledge the need to eliminate the influence of unlysed red cells, reticulocytes, platelet aggregates, and debris on CD34$^+$ cell enumeration. For that purpose, we turned to LDS-751, a DNA and RNA stain excitable at 488 nm, with peak emission at 670 nm (24), which is similarly in effectiveness to CD45 mAb in this respect (cf. Figs. 1B and 2B), but does so at negligible cost (i.e., _ US$0.01 per staining).

MATERIALS AND METHODS
Study Design

The workshop was held under the coordinated auspices of the Foundation for Immunophenotyping in Hemato-Oncology (SIHON), the Foundation for Quality Control in Medical Immunology (SKMI), the Foundation for Quality Control of Hospital Laboratories (SKZL; all in The Netherlands), and the Belgian Association for Cytometry (BVC/ABC), and was designed as a single send-out study to all Dutch and Belgian laboratories that performed flow cytometric enumerations of CD34$^+$ cells. The aim of the workshop was to investigate whether the use of a standard protocol vs. local protocols would reduce interlaboratory variation. The participants were provided with a standard protocol for flow cytometric enumeration of CD34$^+$ cells and the required reagents and were requested to process paired peripheral blood and apheresis product samples from two patients according both to the standard protocol and to their local protocols. The coordinating laboratory (Daniel den Hoed Cancer Center) obtained and aliquotted the samples and had them dispatched by overnight express mail at ambient temperature (i.e., 15-25°C) to arrive at the participating laboratories the following day at 9 AM, i.e., within 24 h after completion of the apheresis proce-
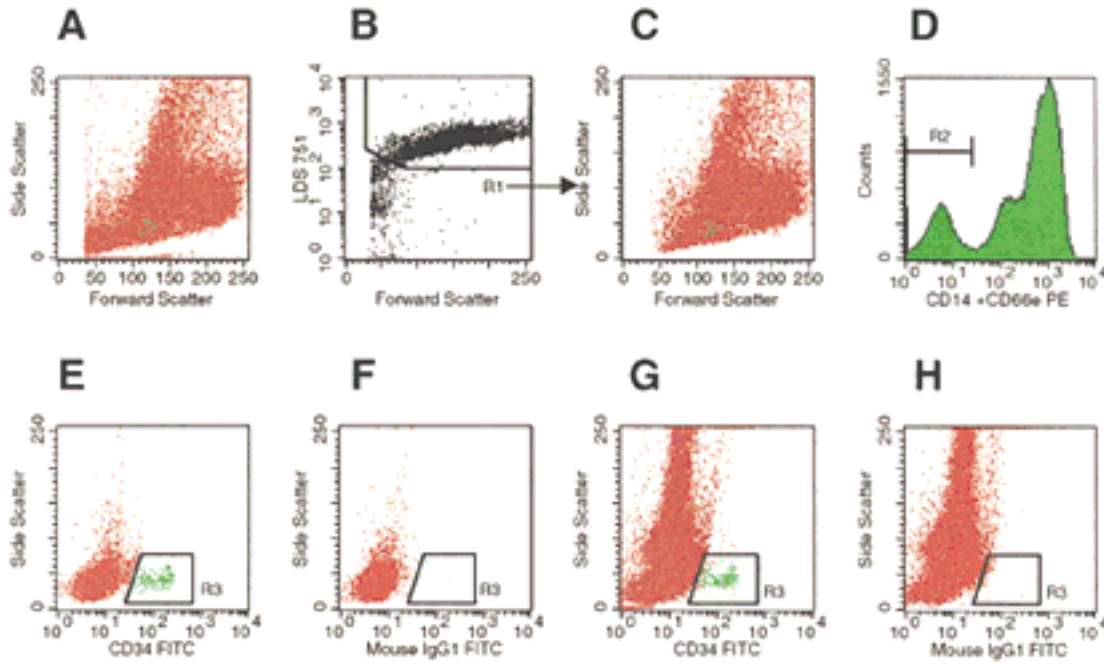
FIG. 1. Standard protocol for flow cytometric analysis of percentage CD34$^+$ cells in peripheral blood and apheresis products. Data on an apheresis product are shown as example. **A:** FSC vs. SSC dot plot of ungated data. **B:** Placement of a "live gate" on nucleated (LDS-751$^+$) cells using an FSC vs. LDS-751 dot plot display (labeled "R1"). Cellular debris, erythrocytes, and platelets are characterized by negative to intermediately positive staining with LDS-751 and low FSC signals. **C:** FSC vs. SSC dot plot of events in gate R1. Note the elimination of FSC^{low} events. **D:** Placement of a histogram gate (labeled "R2") on list-mode data of 50,000 nucleated cells, selecting the CD14$^-$,66e$^-$ cells. **E:** Assessment of the percentage CD34$^+$ cells (0.44% of nucleated cells) by setting a window (labeled "R3") around the CD34$^+$,SSC^{low} cluster on a CD34 vs. SSC dot plot after activation of gate R2. **F:** Nonspecific mAb binding (0.01% of nucleated cells) is analyzed using the same gate R2 and window R3 on the FITC-labeled isotype control mAb-stained sample. Events fulfilling the criteria of gates R1 and R2 and window R3 are depicted green in A,C,E-H. **G** (CD34 mAb-stained sample) and **H** (isotype control mAb-stained sample) illustrate the interference by background FITC fluorescence of CD14$^+$ monocytic and CD66e$^+$ myeloid cells with accurate CD34 quantification if gate R2 on CD14$^-$,66e$^-$ cells is not activated. Of the nucleated cells, 0.59% and 0.19% are reactive with CD34 and isotype control mAb, respectively. The events that are depicted bright green in A,C,E-H fulfill the criteria of R1 and R2 and R3.
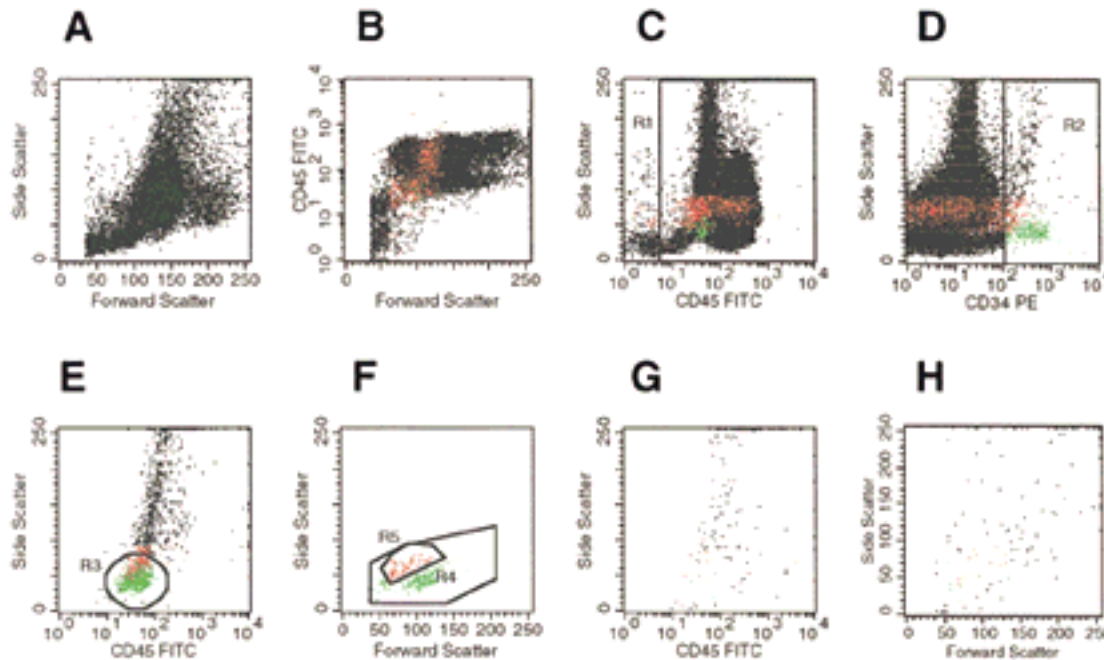
FIG. 2. A sample of the same apheresis product as shown in Figure 1 was processed according to the ISHAGE protocol (25), i.e., a whole blood staining, lyse-and-wash method. We used anti-HPCA-2/PE as CD34 mAb (A-F), mouse IgG1/PE as isotype control mAb (G,H) and anti-HLE-1/FITC as CD45 mAb (both from BDIS). Regions R1 to R4 were defined as described by Van de Winkel and Capel (25). **A:** FSC vs. SSC dot plot of ungated data. **B:** FSC vs. CD45 dot plot of ungated data. **C:** CD45 vs. SSC dot plot of ungated data. R1 excludes the CD45$^-$ events. **D:** CD34 vs. SSC dot plot of events selected by gate R1. R2 selects the CD34$^+$ events. **E:** CD45 vs. SSC dot plot of events selected by gates R1 and R2. R3 selects the dim CD45$^+$, low SSC events. **F:** FSC vs. SSC dot plot of events selected by gates R1 and R2 and R3. R4 defines the events meeting all the FL and light scatter criteria of CD34$^+$ HPC according to Van de Winkel and Capel (25; i.e., 0.55% of CD45$^+$ cells). R5 defines a subset of cells with intermediate FSC and SSC signals; 0.12% of CD45$^+$ cells in the CD34 mAb-stained sample fulfill the criteria of both R4 and R5. **G,H:** As in E,F, 0.01% of CD45$^+$ cells are in R4. The events that are depicted in bright green in D-H fulfill the criteria of R1 and R2 and R3 and R4 but not R5. The events that are depicted in red in B,D-H fulfill the criteria of R5.

dure. The participants had to keep the samples at room temperature and process them on the day of receipt. After reporting of the results to the coordinating laboratory and completion of the analyses by that laboratory, every participant was sent a confidential report with comments on his own results in comparison to those of the anonymous other participants. In addition, the results of the comparisons between standard and local procedures were presented and discussed at a plenary meeting held 2 months after the deadline for data submission.

Standard Protocol

Instrument setup. Each participant was provided with one QC Windows kit of microbeads (Flow Cytometry Standards Corp., San Juan, Puerto Rico), consisting of microbeads triple-labeled with FITC, PE, and PE-Cy5, named *QC3*, and nonfluorescent microbeads, named *Certified Blank*. In addition, centrally prepared suspensions of mononuclear cells from a healthy donor that were unstained or had been stained with CD4/FITC, CD8/PE, or LDS-751 were supplied for adjustment of electronic compensation for spectral overlap and adjustment of FSC photodiode and SSC photomultiplier settings. Instruments had to be set up as previously described (8). In brief, standardized positioning in "sample space" (i.e., the characteristics of the sample and their relationships, which are independent of the instrument) of the instrument's "window of analysis" (i.e., the portion of sample space analyzed by the flow cytometer) had to be achieved for each parameter by placement of the respective FL signals of $CD4^+$ lymphocytes (FSC and SSC parameters) or QC3 microbeads (all FL parameters) into predefined target channels. Thereafter, electronic compensation for spectral overlap was adjusted and activated.

Sample preparation. Each participant was provided, per cell donor, with 1 ml heparin-anticoagulated venous blood and 0.5 ml acid citrate dextrose-anticoagulated apheresis product (1:10 diluted in PBS), ready-to-use mAb mixtures (CD34/FITC + CD14/PE + CD66e/PE for $CD34^+$ cell assessment; mouse IgG1/FITC + CD14/PE + CD66e/PE for isotype control), FACS lysing solution (10 × stock; Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA), and 1 mg/ml LDS-751 stock solution (Exciton, Dayton, OH). The CD34/FITC (anti-HPCA-2), CD14/PE, and mouse IgG_1/FITC mAb were from BDIS; CD66e/PE is commercially available from CLB (Amsterdam, The Netherlands). One hundred microliters of undiluted blood or (1:10 diluted) apheresis product had to be added to 30 ml mAb mixture, incubated for 20 min at room temperature (RT), followed by erythrocyte lysis during 10 min at RT and a single washing step using PBS supplemented with 1% bovine serum albumin (PBS + BSA). After the washing step, the cell pellet had to be resuspended in 0.5 ml PBS containing 1% paraformaldehyde and 0.04 µg/ml LDS-751.

Flow cytometry. Flow cytometry had to be performed within 2 h. For data acquisition, a live gate had to be set on the nucleated cells ($LDS-751^+$), as visualized in an FSC vs. LDS-751 dot plot, to acquire data on 50,000 nucleated cells in list mode (Fig. 1B). For analysis of the list-mode data of

each sample, an FL histogram gate had to be set on the events negative for CD14/PE and CD66e/PE in the CD34/FITC-stained sample, in order to exclude mature myeloid and monocytic elements from further analyses (Fig. 1D). Subsequently, the percentage of $CD34^+$ cells had to be assessed using an analytical window around the CD34/$FITC^+$ cells on a CD34 vs. SSC dot plot (Fig. 1E). Finally, the same PE histogram gate and FITC vs. SSC window were used to quantify the percentage of cells that had bound the isotype control mAb to a similar level of FITC intensity as the CD34 mAb (Fig. 1F). We have chosen this strategy to avoid the interference by Fcg receptor-mediated binding of mAb by myeloid and monocytic cells (25) and by their relatively high autofluorescence with the enumeration of $CD34^+$ cells (Fig. 1G,H). The lower limit of detection of the standard procedure was set at 0.2% (i.e., 100 ÷ 50,000 events, in order to achieve a standard error of maximum $\sqrt{100} = 10$ events).

Data reporting. The participants were requested to submit printed output of list-mode data analyses and to report on a questionnaire form for each sample 1) percentage of $CD34^+$ cells (uncorrected), 2) percentage of isotype control mAb-binding cells, 3) the difference (1 minus 2; i.e., the "net" percentage of $CD34^+$ cells), all expressed as fractions of total nucleated cells (i.e., $LDS-751^+$). The assessment of absolute numbers of $CD34^+$ cells was not investigated in this workshop.

Local Procedures

The participants were requested to submit a summary of the methodology used and printed output of list-mode data analyses and to report on a questionnaire form for each sample the results of assessments of 1) percentage of $CD34^+$ cells, 2) percentage of isotype control mAb-binding cells, and 3) the "net" percentage of $CD34^+$ cells, according to their own methods with respect to instrument setup, sample preparation, and flow cytometry.

Data Processing and Statistical Analysis

Data submitted on questionnaires were checked for inconsistencies and entered into a computer database by the coordinating laboratory. When necessary, comparisons with printed output of list-mode data analyses were made, and incorrect data entries were corrected after consultation with the submitting laboratory. Data processing and statistical analyses were performed using SAS-PC (Statistical Analysis Systems, Cary, NC) and Stata (Stata Corporation, College Station, TX) software. For the analysis of performance of individual laboratories in assessing percentage of $CD34^+$ cells according to standard and local protocols (see Fig. 4), the average results per sample and per protocol were subtracted from each individual result. The absolute value of that difference represented for each laboratory per protocol the CD34 residual, expressed as percentage of the mean result of all laboratories. Standard statistical methods used are specified in Results and in the figure legends. Prior to analyses of variance (one-way ANOVA and main effects model), the data were trans-

formed to their square root or natural logarithm in order to achieve normal distributions.

RESULTS
Sent-Out Samples and Response Rate

Forty-four laboratories (listed in the title page footnote) registered for the workshop at a cost of US$125 per laboratory. Two patients agreed, after informed consent, to donate 50 ml venous blood and 2.5 ml apheresis product per person. Both patients were treated for multiple myeloma with cyclophosphamide (4 g/m² on day 0) and G-CSF (5 µg/kg/day from days 0 to 11) in order to mobilize their hematopoietic stem cells, which were collected on days 10-12 by apheresis for cryopreservation and autografting. The paired blood and apheresis samples of each patient were obtained and shipped on two occasions within 9 days. Results were submitted by 36 laboratories (82%) on patient 1 and by 38 laboratories (86%) on patient 2. The remaining laboratories either reported logistical problems (lack of personnel) at the time of the workshop or did not perform the test because of unacceptable delay in delivery of the samples (i.e., > 1 day).

Standard Protocol

The standard protocol was correctly performed on the paired samples (i.e., peripheral blood and apheresis product) of patient 1 by 24 laboratories and on those of patient 2 by 26 laboratories. The median values and CVs (in parentheses) of the percentage of CD34$^+$ cells were 1.1 (30%), 2.0 (25%), 1.1 (21%), and 3.7 (18%; labeled "Correct" in Fig. 3). Twelve laboratories performed the standard protocol incorrectly, i.e., data acquisition without selection of LDS-751$^+$ events (patient 1, n = 7; patient 2, n = 6); list-mode data analysis without gating on CD14⁻,66e⁻ events (n = 3 for both patients); or incorrect instrument setup (patient 1, n = 2; patient 2, n = 3). Here, the median values of the percentage of CD34$^+$ cells were lower and the CVs larger, i.e., 1.0 (50%), 1.2 (82%), 1.0 (52%), and 3.1 (53%; labeled "Fault" in Fig. 3). Incorrect performance of the standard protocol added to the variation in results of the apheresis products in particular, as shown by their increased interquartile ranges compared to those obtained with a correctly performed standard protocol (Fig. 3). A comparison between uncorrected and "net" percentage CD34 revealed that subtraction of the percentage isotype control mAb binding cells from the uncorrected percentage CD34 had no effect on the magnitude of the interlaboratory variation (data not shown).

We then studied the effects of "nontechnical" factors (i.e., type of material and patient) and the quality of performance of the standard protocol in a main effects model on the total data set (i.e., all patients and types of material). That analysis (Table 1) revealed that 40% of the variation in percentage CD34$^+$ cells using the standard protocol was explained by type of material (i.e., blood vs. apheresis product) and 6% by patient (i.e., patient 1 vs. 2), whereas 10% was explained by the quality of technical performance (i.e., "correct" vs. "no LDS-751$^+$ live gate"

vs. "no CD14⁻,66e⁻ analysis gate" vs. "incorrect instrument setup"). The apheresis products yielded higher percentage CD34$^+$ cells than the peripheral blood samples (Table 1) because mononuclear cells (containing the CD34$^+$ fraction) had been selectively obtained during the apheresis procedure. As for technical performance, omission of activating the live gate on nucleated (LDS-751$^+$) cells led to contamination of the list-mode data set with nonnucleated cells (erythrocytes, platelets, cellular debris), resulting in lower percentage CD34$^+$ cells (Table 1). Failure to exclude CD14⁻ and CD66e⁻ cells during list-mode data analysis prior to setting a window around the CD34$^+$ cell cluster on the CD34 vs. SSC dot plot led to higher percentage isotype control mAb-binding cells (see, e.g., Fig. 1H), resulting in lower net percentage CD34$^+$ cells (Table 1). The deficiencies in instrument setup were heterogenous: Two laboratories had not set up their instruments according to the standard protocol at all, and one laboratory had not placed the PE and Cy5 signals of the QC3™ microbeads into the required initial target channels. Thus, 46% of the variation in percentage CD34$^+$ cells using the standard protocol was due to "nontechnical" factors, 10% was due to documented flaws in its performance (mainly gating errors), and 44% of the variation remained unexplained.

Inspection of the results of the individual laboratories revealed that results of percentage CD34$^+$ cell assessments with CD34 residuals 50% could be considered as outliers (Fig. 4, top). Twelve laboratories recorded at least one outlier. Five (laboratories 17, 21, 24, 29, and 43) had no gate activated on LDS-751$^+$ events. Two (laboratories 10 and 15) had not placed a gate on CD14⁻,66e⁻ events. One (laboratory 44) had set up its instrument incorrectly. The remaining four (laboratories 1, 6, 16, and 30) had made none of these errors.

Local Protocols

The paired samples (i.e., peripheral blood and apheresis product) of patient 1 were analyzed according to local procedures by 29 laboratories and those of patient 2 by 34 laboratories. Four laboratories did not routinely perform CD34 enumerations and performed only the standard protocol. The median values and CVs (in parentheses) of the percentage CD34$^+$ cells were 1.2 (34%), 2.2 (40%), 1.2 (106%), and 3.9 (52%), respectively (labeled "Local" in Fig. 3). Thus, the interlaboratory variation obtained with local protocols was, in particular for the apheresis products, clearly larger than obtained that with correctly applied standard protocols. This conclusion remained valid after exclusion of the outliers (individually plotted in Fig. 3) from this analysis.

A summary of some features of the local protocols is shown in Table 2. All laboratories used whole blood staining, lyse-and-wash techniques. Most reporting laboratories (19 of 34; 56%) used FITC-labeled CD34 mAb, whereas 13 (38%) used PE-labeled CD34 mAb and two used both conjugates in a double-staining technique. All but one of the laboratories used the group III CD34 mAb anti-HPCA-2 (BDIS). Sixteen of the thirty-two laboratories
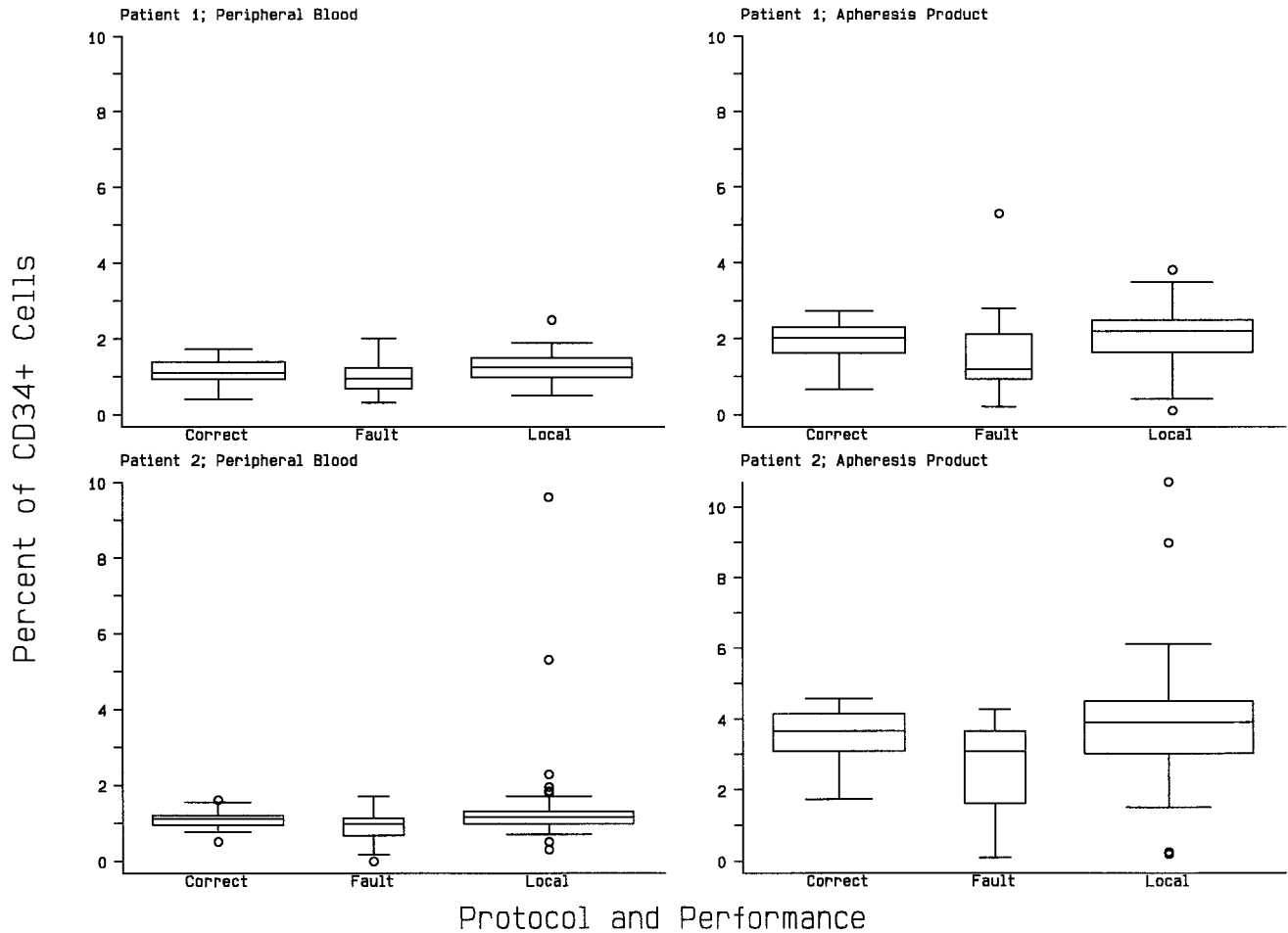
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FIG. 3. Percentages of CD34$^+$ cells on peripheral blood (left panels) and apheresis products (right panels) of patients 1 (top panels) and 2 (bottom panels). Data shown were obtained after correct (labeled "Correct") and incorrect (labeled "Fault") application of the standard protocol and by using local protocols (labeled "Local"). The boxes extend from the 25th (p25) to the 75th (p75) percentile; the line in the middle represents the median. The whiskers extend to the upper and lower adjacent values, which are defined as $1.5 \times (p75 - p25)$, rolled back to where there are data. Outliers, more extreme than the adjacent values, have been individually plotted. The width of the boxes is proportional to the number of observations (see text).

that used either FITC- or PE-labeled CD34 mAb employed (a combination of) counterstaining reagents in two- or three-color techniques to select cells of interest or to exclude unwanted cells during analysis in order to obtain more accurate assessments of percentage CD34$^+$ cells. These strategies included (combinations of) the following reagents: CD14 for the exclusion of monocytes (n = 9 laboratories), CD66e or CD67 to exclude mature myeloid cells (n = 7), CD45 to select leukocytes (n = 6), LDS-751 to select nucleated cells (n = 5), 7-AAD to exclude dead cells (n = 1), and CD3 to exclude T lymphocytes (n = 1). We studied the effects of "nontechnical" factors (i.e., type of material and patient) and the documented features of the local protocols (i.e., CD34 fluorochrome, gating reagents) in a main effects model on the total data set (i.e., all patients and types of material). That analysis revealed that 26% of the variation in percentage CD34$^+$ cells using local protocols was explained by "nontechnical" factors, i.e., type of material (21%) and patient (5%), whereas the influences of the

analyzed technical features, such as CD34 mAb fluorochrome (FITC vs. PE vs. a combination of FITC- and PE-labeled mAb; 2%) and the use of gating reagents [none vs. CD45 only vs. at least one myeloid or monocytic marker (Table 2); <1%], were only minor.

Inspection of the results of individual laboratories revealed that 17 recorded at least one outlier (i.e., CD34 residual 50%; Fig. 4, bottom). The median value of the CD34 residuals of four laboratories was exceptionally high (i.e., 50%). Three of them (laboratories 5, 25, and 44) had only processed the samples of Patient 2. Of note, laboratories 43 and 44 had also recorded exceptionally high median CD34 residuals with the standard protocol.

DISCUSSION

The variation in results of assessments of percentage CD34$^+$ HPC is determined by a large array of factors. Apart

Table 1
Assessment of Percentage CD34⁺ Cells According to Standard Protocol: Documented Sources of Variation

Source of variation	n[a]	Median	Range	P value[b]
Type of material (40%)[c]				
Peripheral blood	74	1.1	0.0–2.0	0.0001
Apheresis product	74	2.5	0.1–5.3	
Patient (6%)[c]				
1	72	1.4	0.2–5.3	0.02
2	76	1.5	0.0–4.6	
Technical performance (10%)[c]				
Correct	100	1.5	0.4–4.6	0.002
No LDS-751⁺ live gate	26	1.1	0.0–3.9	
No CD14⁻, 66e⁻ analysis gate	12	1.2	0.3–4.3	
Incorrect instrument setup	10	1.7	0.9–5.3	

[a]Number of observations (types of material and/or patients pooled).

[b]The Wilcoxon's two-sample and Kruskal-Wallis tests were used to assess the significance of differences between the two types of material, four groups of technical performance, and two patients.

[c]The contribution of each parameter to the total variation was assessed using a main effects model.

from "nontechnical" factors, such as type of material (e.g., peripheral blood, apheresis product) or cell donor (e.g., patient, healthy donor), which were responsible for most of the variation that could be explained by documented parameters in our study, a range of technical sources of variation can be relevant. These may have accounted for up to 54% of the variation observed with the use of the standard protocol and for up to 74% of the (even larger) variation observed with local protocols, and can be summarized as follows.

1. **Artifactual variation between samples:** Central processing and aliquotting of the blood samples prior to transport excluded these factors as sources of variation. However, some degree of sample deterioration during transport and storage may have occurred, which is inherent to this type of send-out study. Major deterioration of a sample would have resulted in outliers with both standard and local techniques. That situation was observed only in the case of laboratories 43 and 44, which had poor results anyway (Fig. 4). Hence, we do not consider transport and storage as a cause of gross sample deterioration in this study. The use of preserved samples would have minimized the risk of deterioration during transport and storage; cryopreserved samples have been used in one survey (15). Drawbacks with the use of cryopreserved samples are the high costs of shipment on dry ice and the variation caused by the thawing procedures in the participating laboratories. The proprietary technique to stabilize whole blood as used in the UK NEQAS surveys for lymphocyte subset enumeration and leukemia/lymphoma immunophenotyping (1) may be an alternative for future CD34 surveys.

2. **Variation in sample processing:** The participants were provided with a detailed protocol for sample prepara-
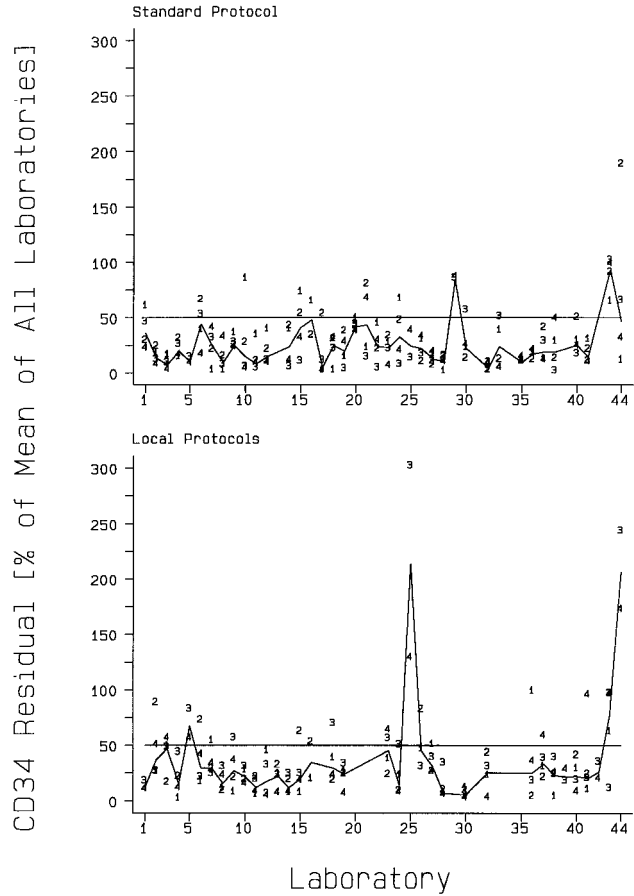

FIG. 4. Interlaboratory variation in results of assessments of percentage CD34⁺ cells. The CD34 residual, computed per sample and per laboratory for standard and local protocols, was used as a parameter to illustrate the variation of individual laboratories (for computation, see Materials and Methods). The median values of the CD34 residuals per laboratory have been connected with a line. The horizontal lines were plotted at 50% and serve as reference to identify outliers.

tion, ready-to-use mAb mixtures, and stock lysing solution and reagent for selection of nucleated cells during data acquisition (LDS-751). Still, variation arising from sample processing according to the standard protocol may have originated from differences in performing the staining, lysing, washing and fixation procedures, and storage of the stained cells prior to flow cytometric data acquisition. The fact that most laboratories had no experience with the standard protocol adds even more to the relevance of this source of variation.

3. **Instrument setup and performance:** The few observations recorded with instruments that had been set up incorrectly or not at all (Table 1) allow little to be said about the impact of standardized instrument setup. The instruments' performance of quantitative FL measurements (17) was not evaluated in this study. All but one of 39 instruments, 28 of which were included in the present study, that had been evaluated by us in a survey performed 1 year before, functioned well in this respect (8). Hence, we do not consider incorrect setup and poor performance

Table 2
Summary of Local Procedures

Parameter	Number of laboratories
Fluorochrome conjugated with CD34 mAb	
FITC	19
PE	13
FITC and PE	2
Gating reagents used for counterstaining CD34	
No	18
Yes	16
If yes, types of gating reagents	
CD45	5
CD45 + CD14	1
CD14	2
CD14 + CD66e	1
CD14 + 7-AAD[a]	1
CD14 + CD66e + LDS-751	3
CD14 + CD67 + CD3	1
CD66e + LDS-751	2

[a]7-AAD, 7-aminoactinomycin D.

of the instruments as major sources of variation in this study.

4. Flow cytometric data acquisition and analysis: Major errors in the performance of the standard protocol pertaining to data acquisition and analysis could be traced back from the reports and accounted for ~10% of the variation: failure to select nucleated cells during data acquisition (i.e., no LDS-751$^+$ gate) and failure to exclude myeloid and monocytic cells from data analysis (i.e., no CD14$^-$,66e$^-$ gate). Both failures led to an underestimation of percentage CD34$^+$ cells. We did not analyze minor differences in placing gates and CD34,SSC windows between laboratories, which will have made additional contributions to the observed variation.

This study revealed that the interlaboratory variation in percentage CD34$^+$ cells was reduced by correct application of a standard protocol compared to the use of local protocols. Correct application of the standard protocol yielded fewer outliers and smaller interquartile ranges than the correctly applied standard protocol (Fig. 2). Documented errors, mainly in the selection of events during data acquisition and analysis as per the standard protocol, were responsible for 10% of the total variation associated with the standard protocol (Table 1) and led to larger variation in results, particularly of the apheresis products (Fig. 2). The effect of standardization of the technique of assessing percentage CD34$^+$ cells is also illustrated by the reduction of the contribution of factors other than type of material and patient to the total variation from 74% (local protocols) to 54% (standard protocol). The observed reduction in interlaboratory variation is the more remarkable when taking into account that most of the participants had no experience with the standard procedure. The introduction of a standard protocol did not reduce interlaboratory variation consistently compared to local protocols in a similar study of lymphocyte immunophenotyping (27).

The CVs on two peripheral blood samples and two apheresis products ranged between 18% and 30% with correct application of the SOP vs. 34-106% as obtained using local protocols. The latter was of the same order of magnitude as those observed in three other workshops held in the United Kingdom (14,15) and in the Nordic countries (12), in which CD34 assessments were performed according to standard guidelines but without centrally provided reagents. In one U.K. workshop (14), 15 laboratories, each analyzing five fresh samples per round, obtained in two rounds CVs of 62% and 100%. In another U.K. study (15), 12 laboratories studied 12 cryopreserved apheresis products without guidelines for assessment of percentage CD34$^+$ cells and obtained CVs between 50% and 235%. After implementation of such guidelines, interlaboratory CVs on a subsequent set of 12 specimens ranged between 23% and 127%. In the Nordic workshop (12), 24 laboratories provided with guidelines established in their previous workshop (11) obtained CVs between 27% and 156% on three fresh peripheral blood and three fresh apheresis product samples. Subsequently, this variation was reduced to only 9–12% when all participants assayed two apheresis products during a hands-on workshop at a single location. The combined results of the British workshops (14,15), the Nordic workshop (12), and our workshops show that the large interlaboratory variation of percentage CD34$^+$ assessments can be reduced by the implementation of a standard protocol. Importantly, the Nordic study (12) shows the importance of hands-on training for the achievement of a significant reduction of interlaboratory variation.

The approach for assessing percentage CD34$^+$ cells based on the use of CD34/PE and CD45 mAb has recently gained wide acceptance (9,14,15,22). Identification of the HPC on the basis of bright CD34 positivity, dim CD45 positivity, and low SSC signals (Fig. 2) has been incorporated in the ISHAGE (22) and German (9) guidelines for CD34 enumeration. We by no means claim that our protocol is better, but we believe that the results of this study warrant a comparative study between these approaches. Prior to such a study, we will switch to the use of PE-labeled CD34 mAb in line with most protocols (7,9,11,12,14,15,22). The current availability of a good FITC-labeled CD66e mAb allows us to replace the PE-labeled CD14 and CD66e mAb with FITC-labeled mAb.

We have restricted ourselves in this study to the technical problems associated with the assessment of percent CD34$^+$ cells. The clinically relevant *absolute number* of CD34$^+$ cells only adds an extra source of variation, the concentration of cells per unit of volume.

Most laboratories derive the absolute number of CD34$^+$ cells in peripheral blood or apheresis product samples from the flow cytometrically assessed percentage of CD34$^+$ cells within the leukocytes, combined with assessment of the absolute leukocyte count using a hematology analyzer (i.e., dual-platform methodology). Only three participants in our study had flow cytometers that allowed direct volumetric assessment of number of CD34$^+$ cells (6), i.e., single-platform methodology. The absolute cell count in

dual-platform techniques contributes significantly to the interlaboratory variation of number of lymphocyte subsets (6) and will undoubtedly do the same in assessments of number of CD34$^+$ cells. To make single-platform methodology independent of the instrument, internal calibrators in the form of predefined amounts of reference beads are added to blood samples (Flowcount; Coulter Electronics, Hialeah, FL) or vice versa (TruCOUNT; BDIS). The latter method has recently been incorporated in a kit for single-platform absolute number of CD34 enumeration (ProCOUNT; BDIS). The effectiveness of these approaches in reducing interlaboratory variation has not yet been established.

The widening diversification of flow cytometry hardware (e.g., data acquisition based on volumetry vs. fixed numbers of events in conventional instruments), software (i.e., specialized for certain applications and reagents), and commercial assay kits renders it increasingly difficult to achieve standardization based on narrowly defined protocols. Therefore, we believe that the most effective approach to reducing interlaboratory variation in CD34 enumeration consists of the adherence to consensus protocols formulated in general terms, combined with real-time evaluation of performance by the organizations responsible for external quality assurance.

ACKNOWLEDGMENTS

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