

Quantitative Fluorescence Flow Cytometry: A Comparison of the Three Techniques for Direct and Indirect Immunofluorescence

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Received 8 June 1998; Accepted 10 June 1998

Three types of microbead calibrators available for quantitative fluorescence flow cytometry have been studied in parallel using a variety of monoclonal antibodies (MoAbs). The QIFI kit is designed for indirect immunofluorescence (IF), and both the Quantum Simply Cellular (QSC) assay and the Quanti-BRITE assay are designed for direct IF. Because of the different nature of the respective ligands, epitopes on cells versus F'ab-portions on QSC beads, large differences in titration curves for a large number of CD MoAbs were noted between QSC beads and cells. Use of the QSC assay and fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugates of the same CD reagent revealed substantially different numbers of cellular binding sites. Numbers of cellular binding sites as determined by direct IF using the Quanti-BRITE assay and by indirect IF using the QIFI kit were similar. We also found that erythrocyte (RBC)-lysing reagents cause varying and sometimes

substantial reduction in the fluorescence intensity (FI) of cells stained directly with CD34 MoAb conjugates, but the RBC-lysing reagents had no effect on the FI of cells stained indirectly with the same CD34 MoAbs. This report defines a number of variables critical for standardized quantitative flow cytometry. We conclude that the choice of calibrators, fluorochrome conjugates, staining methods, and modes of sample processing can effect the determination of cellular binding sites to MoAbs. Direct immunofluorescence using the Quanti-BRITE assay and indirect IF using the QIFI kit appear to yield comparable results for the standardized determination of numbers of cellular binding sites to MoAbs. *Cytometry* 33:179–187, 1998. © 1998 Wiley-Liss, Inc.

Key terms: quantitative fluorescence flow cytometry; QSC beads; QIFI kit; Quanti-BRITE

The basic current application of flow cytometry is to enumerate the relative frequencies of cell populations characterized by the expression of antigens identified by specific monoclonal antibodies (MoAbs). In this setting, a single cutoff is positioned to separate cells with presumably specific staining (positive cells) from those cells with presumably no staining or with only limited background staining (negative cells). Although the typical software also reports one or more measures of fluorescence intensity (FI), this parameter is not widely utilized. The reasons for the reluctance of flow cytometry users to take into account this feature of multiparameter flow cytometry routinely are numerous. First, the settings of flow cytometers, in terms of optical alignment and of voltage on the photodetectors, are not standardized. Second, the sensitivity of the photodetectors of different devices vary (1); third, the algorithms active in the log amplifiers of the different flow cytometers presumably differ. Fourth, the given MoAb and the given fluorochrome of any manufacturer and the processing mode, for example incubation time, as chosen by the operator, must be added as further variables with

effects on the parameter "FI of the positive cells." This is further complicated in the setting of antigens gradually expressed at different levels on different cell-subsets. With regard to CD33 expression, we have shown previously that six substantially differing positions of the cutoff "positive/negative" were placed by six different colleagues to whom we had sent list mode data files on floppy diskettes, when analyzed according to their routine in-house approach (2). Notably, files containing measurements with the convenient isotypic control MoAbs had also been provided.

To overcome some of the different variables referred to above, one tool to standardize quantitation of FI of cells consists of constructing calibration curves on the basis of measurements of microbeads with known numbers of binding sites to antibodies or with known numbers of

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fluorochrome molecules. The FI of these beads will provide then reference values to which the staining of the cells then can be referred. Three different technologies are currently marketed: the QSC assay (Quantum Simply Cellular beads) (3), the QIFI kit (Quantitative Immunofluorescence Intensity beads) (4), and the Quanti-BRITE assay (5). The QSC assay is designed for direct immunofluorescence (IF), and goat anti-mouse immunoglobulin (Ig) G coupled onto the beads will bind any fluorochrome-labeled murine MoAb. The QIFI kit is designed for indirect IF, and mouse IgG MoAb coupled onto the beads will bind any fluorochrome-labeled anti-mouse antibody. The Quanti-BRITE assay is designed for direct IF using PE-labeled MoAbs, and these beads bear different numbers of PE molecules. In a comparison, we have tried to define the performance characteristics of these technologies.

MATERIALS AND METHODS

Cells

Blood samples were obtained from healthy donors at our institution. For the determination of CD34-expressing cells, blood and cytopheresis samples were from patients on filgrastim mobilization. Samples were processed within 60 min of being drawn.

Microbeads

Throughout, only one lot of QIFI kit, 017(101) (Dako, Hamburg, Germany), containing equal numbers of beads with 4,000, 17,000, 50,000, 250,000, and 490,000 MoAbs was used. Only for one single experiment, one other lot of QIFI kit, 018(101), containing equal numbers of beads with 3,600, 16,000, 56,000, 190,000, and 470,000 MoAbs was used.

Throughout, only one lot of QSC-kit (Flow Cytometry Standards Corporation, San Juan, PR) containing equal numbers of beads with binding sites for 4,525, 14,611, 56,848, and 175,520 MoAbs (A-060697) was used. Only for one single experiment, another lot containing equal numbers of beads with binding sites for 3,503, 12,079, 33,271, and 101,649 MoAbs (A-060297) was used.

The single lot of Quanti-BRITE (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, PR) contained equal numbers of beads carrying 1,400, 14,000, 36,600, and 182,000 PE molecules.

Reagents

MoAbs (throughout, only one single lot used in this study) purchased from BDIS (San Jose, PR) were as follows: CD3, unconjugated, FITC and PE conjugates; CD4, unconjugated, FITC and PE conjugates; CD45, unconjugated, FITC conjugate; CD34 (8G12), unconjugated, FITC and PE conjugates.

MoAbs purchased from Coulter-Immunotech (Marseille, France) were as follows: CD33, unconjugated, FITC and PE conjugates; CD34 (IMMU-133), unconjugated and PE conjugate; CD34 (QBEND/10), unconjugated, FITC and PE conjugates; CD34 (581), FITC and PE conjugates; CD45, unconjugated, FITC and PE conjugates.

MoAbs purchased from Dako were as follows: CD34 (Birma-K3), FITC and PE conjugates. MoAb ICH-3 (PE conjugate) was purchased from Medac (Hamburg, Germany).

Cell and Bead Preparation

Three different red blood cell (RBC)-lysing reagents (FACS Lysing Solution [BDIS], ORTHOmune lysing reagent [Ortho, Raritan, NJ], and ImmunoPrep [Coulter, Hialeah, FL]) were used in the Multi-Q-Prep device (Coulter). The protocols as given by the manufacturers were strictly followed.

Unless stated otherwise, beads or blood samples were incubated with the MoAb for 90 min. After staining, the samples and the beads were subjected to RBC lysing and pelleted (250 g, 5 min, +4°C), and the pellet was washed once again in 4 ml of phosphate-buffered saline (PBS) and finally resuspended in 400 μ l PBS. In indirect IF, the blood sample was first incubated with the unconjugated MoAb for 90 min, and the sample was then washed twice with PBS, and it was resuspended in 200 μ l PBS containing 5 μ l of rabbit anti-mouse IgG FITC for 45 min, with the QIFI beads stained in parallel. No further fixative was added, but samples were kept on crushed ice until performance of flow cytometry. Specimens were measured on the flow cytometer within 45 min of final pelleting.

Flow Cytometry

All measurements were performed on a single FACScan (BDIS) instrument equipped with the LYSYS II software (BDIS). A resolution of 256 channels was chosen throughout. Constant settings of the fluorescence parameters were controlled using Calibrite beads (BDIS). When measuring beads, at least 5,000 beads per single determination were measured. When measuring cells, 10,000 cells were measured to analyze leukocyte subsets, and at least 25,000 cells were measured to analyze CD34-expressing cells. The calibration curves were constructed with the respective dedicated software supplied by the manufacturers of the beads. Statistical analyses were performed with STATGRAPH software.

RESULTS

Different Amounts of MoAbs Needed to Saturate QSC Beads and to Saturate Cells: Titration Studies

The specific cellular binding site for the fluorochrome-labeled MoAb is the respective epitope, whereas the specific binding site for the MoAb on the QSC beads is the F'ab portion of the goat anti-mouse IgG coupled to the QSC beads. Thus, substantially different binding properties can be expected. To evidence any such differences, titration studies were performed on blood samples and on QSC beads with FITC and PE conjugates of a large series of MoAbs using MoAbs at 10 μ l, 20 μ l, 40 μ l, and 80 μ l. With only six exceptions, study of the 19 MoAbs revealed that the QSC beads were not saturated by 80 μ l of MoAb, given a "20 μ l per test" as recommended by the manufacturers. This amount of 80 μ l of MoAb per single test was felt to be the maximum tolerable amount to work within the terms of economical expenses. The respective calibration curve

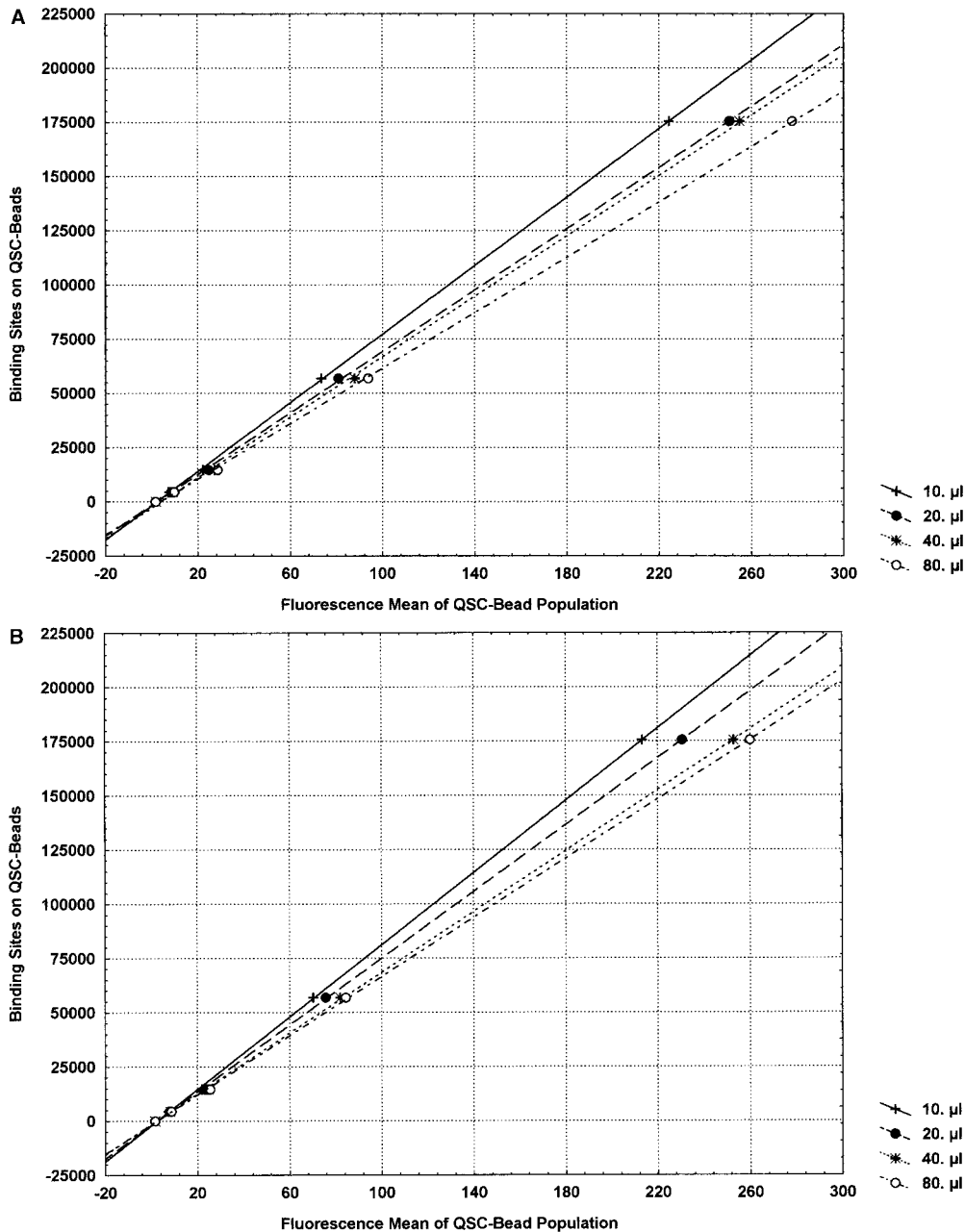


FIG. 1. **A:** Effects of staining of QSC beads for 90 min with various concentrations of CD45-FITC MoAb (marketed as 100 µg/ml and recommended as "20 µl per 100 µl blood") on the construction of the calibration curve. **B:** Effects of staining QSC beads for 90 min with various concentrations of CD66b-FITC MoAb (marketed as 100 µg/ml and recommended as "20 µl per 100 µl blood") on the construction of the calibration curve.

as determined with a "nonsaturating MoAb" is shown in Figure 1A, and that with a "saturating MoAb" is shown in Figure 1B. In contrast, cellular binding sites were almost generally saturated with 40 µl of MoAb. Interestingly, with regard to the CD45-FITC MoAb, different amounts of MoAb were necessary for the saturation of lymphocytes/monocytes and neutrophils. In fact, with increasing amounts of CD45-FITC MoAb added, a slight decrease of fluorescence on monocytes, and on lymphocytes to a much smaller extent, was observed, whereas fluorescence steadily increased on neutrophils (Fig. 2). In contrast, the CD66b-FITC MoAb effectively saturated the QSC beads with 40 µl, whereas the cellular binding sites on neutrophils were not saturated with even 80 µl (Fig. 2).

As an alternative to staining the QSC beads with the very high amounts of MoAbs for 90 min, we studied whether prolonged incubation times would lead to a saturation of the QSC beads. Figure 3, however, shows that even prolonged incubation times did not lead to a saturation of the QSC beads.

Similar Numbers of Cellular Binding Sites Using QIFI Kit and Quanti-BRITE, and Discrepant Numbers of Cellular Binding Sites Using QSC Beads

Using the concentrations of MoAbs needed for saturation of the QSC beads, 10 samples from normal donors were studied in parallel with the three techniques described, using both FITC and PE conjugates for the QSC

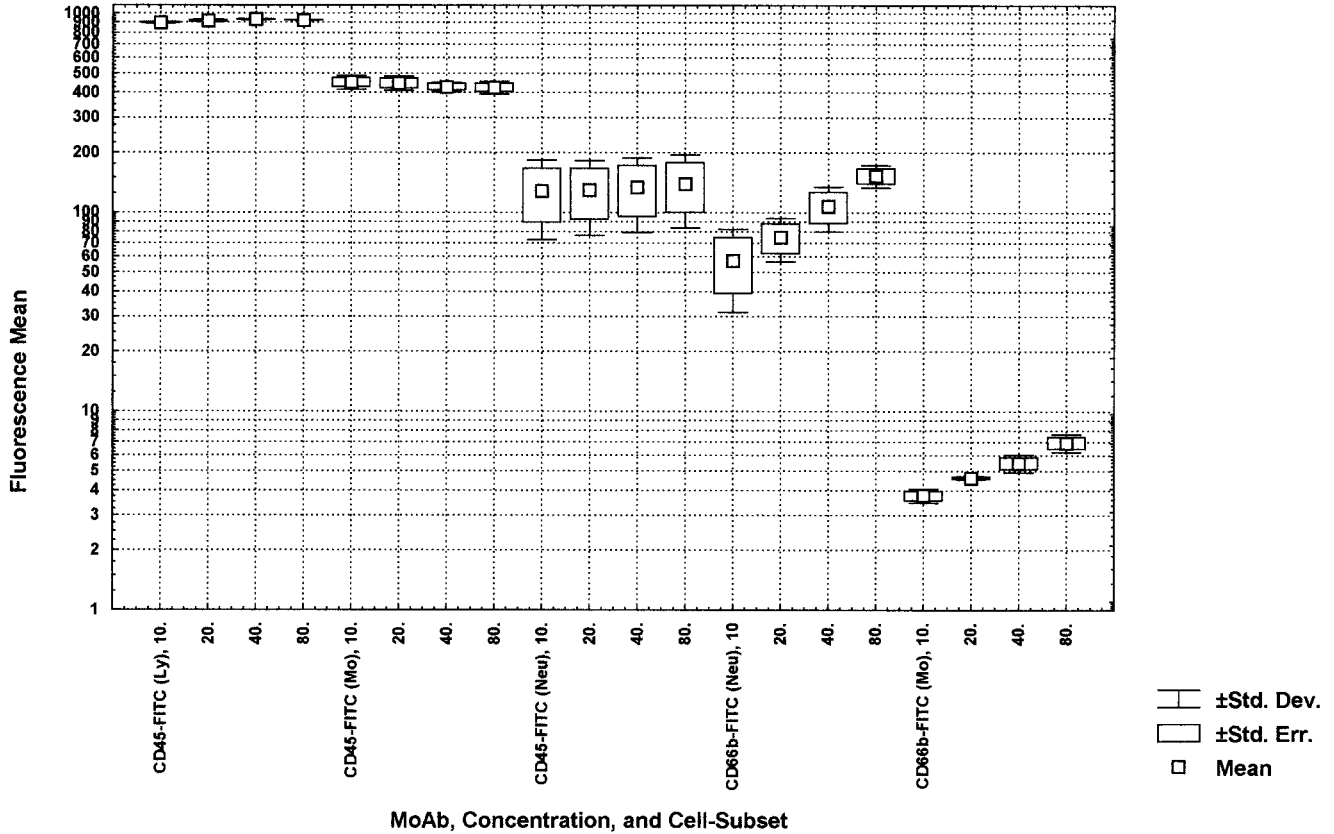


FIG. 2. Effects of staining of cells for 90 min with various concentrations of CD45-FITC MoAb and of CD66b-FITC MoAb (marketed as 100 µg/ml, and as 50 µg/ml, respectively; recommended as "20 µl per 100 µl blood"); shown are fluorescence intensity means from five different samples.

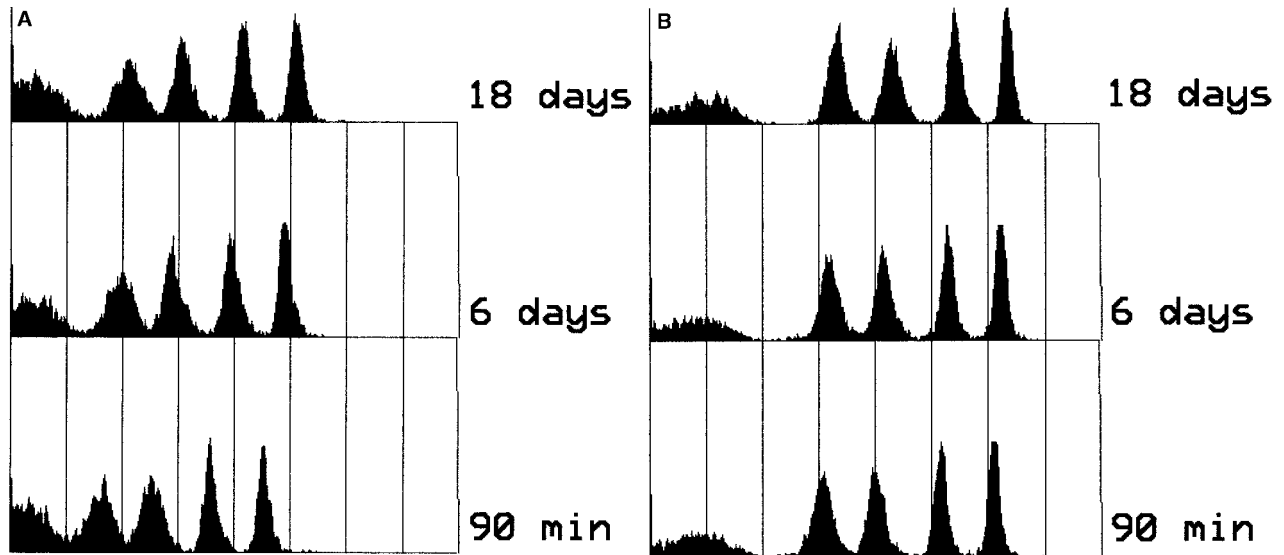


FIG. 3. Effects of incubating QSC beads for prolonged periods of time; comparison of staining CD45-FITC MoAb (A) and with CD4-PE MoAb (B). Overlay single-parameter histograms. Bottom: staining for 90 min. Middle: staining for 6 days. Top: staining for 18 days.

technique. Table 1 shows that numbers of cellular binding sites as determined by the QIFI technique and by the Quanti-BRITE technique were fairly similar, whereas those determined by the QSC technique were discrepantly

higher throughout. Table 1 also shows for comparison the respective data as reported in the literature.

Additionally, we have determined that FITC and PE conjugates of one single MoAb clone yielded different

Table 1
Number ($\times 1,000$) of Cellular Binding Sites to Monoclonal Antibodies as Reported in the Literature and in This Report*

CD	Cell subset	(7) QIFI kit	(8) QSC kit	(9) ^a	This report		
					QIFI kit	QSC assay	Quanti-BRITE
3	Lymphocytes	57 \pm 7	104 \pm 21	141	50 \pm 5	304 \pm 18	60 \pm 4
4	Lymphocytes	47 \pm 14	48 \pm 4	92	39 \pm 10	173 \pm 13	51 \pm 4
	Monocytes	17 \pm 5	6.5 \pm 1.4	ND	4.2 \pm 0.9	24 \pm 4	3.6 \pm 0.7
8	Lymphocytes	145 \pm 29	233 \pm 8	129	ND	ND	ND
16	Lymphocytes	79 \pm 30	12.5 \pm 10	ND	ND	ND	ND
33	Monocytes	19 \pm 6	ND	ND	4.4 \pm 2.2	16 \pm 8	4.5 \pm 2.8
	Neutrophils	10 \pm 5	ND	ND	1.4 \pm 0.7	6.3 \pm 2.2	0.9 \pm 0.6
45	Lymphocytes	217 \pm 64	ND	ND	119 \pm 4	565 \pm 37	70 \pm 3
	Monocytes	103 \pm 44	ND	ND	42 \pm 4	315 \pm 33	44 \pm 6
	Neutrophils	36 \pm 16	ND	ND	18 \pm 2	124 \pm 11	13 \pm 3

*QSC Determinations were based on staining with FITC conjugates. Data are from 10 blood samples and shown as means \pm 1 SD.

^aMedians reported.

Table 2
Number ($\times 1,000$) of Cellular Binding Sites to CD34 Monoclonal Antibodies*

CD34 MoAb	QIFI kit	QSC assay		Quanti-Brite
		FITC conjugate	PE conjugate	
IMMU133 (class I)	5.7 \pm 0.4	ND ^a	34 \pm 3	4.9 \pm 0.4
QBend/10 (class II)	21 \pm 2	41 \pm 3	210 \pm 12	21 \pm 2
8G12 (class III)	36 \pm 3	217 \pm 7	159 \pm 4	28 \pm 2
Birma-K3 (class III)	34 \pm 2	126 \pm 3	95 \pm 3	14 \pm 1

*Data are from 2 blood and 2 cytopheresis samples. Processing was performed with ORTHOmune RBC-lysing reagent. Data given as means \pm 1 SD.

^aNo FITC conjugate of IMMU133 is marketed.

numbers of cellular binding sites on the basis of calibration curves determined on QSC beads. These differences were minor for CD3 on lymphocytes (323,000 versus 306,000), for CD4 on monocytes (29,000 versus 24,000), and for CD33 on neutrophils (5,800 versus 6,300). These differences, however, were substantial for CD4 on lymphocytes (269,000 versus 173,000), for CD33 on monocytes (29,000 versus 16,000), and for CD45 on lymphocytes (303,000 versus 565,000), on monocytes (188,000 versus 316,000), and on neutrophils (57,000 versus 124,000). These findings may explain partially the different numbers of cellular binding sites as reported in the two published papers on quantitative flow cytometry using the QSC technique (8,9). As a further variable in this setting, the QSC beads themselves must be noted. Studying in parallel and simultaneously the two different lots of QSC beads, substantially discrepant calibration curves, differing by a factor of 1.4, have been determined (data not shown).

Determinations of the numbers of cellular binding sites to four different CD34 MoAbs again revealed the discrepancies of the three techniques aimed for standardized quantitative fluorescence flow cytometry (Table 2).

Effects of RBC-Lysing Reagents on the Staining With a Variety of FITC- and PE-Labeled CD34 MoAbs

Previously it has been shown that RBC-lysing reagents are not innocent bystanders in sample processing (6,10,11).

In view of the recent interest created with regard to CD34-expressing cells as the single cellular parameter for timing of harvest and for quality assessment of haemopoietic stem and progenitor cell harvested (12), we were interested to know the impact of the three RBC-lysing reagents most widely used on the FI as determined with a variety of fluorochrome-labeled CD34 MoAbs.

Figure 4 illustrates the finding that the two fixative-containing RBC-lysing reagents, FACS Lysing Solution and ImmunoPrep, effected substantial decreases in the FI of the cells regarded as CD34-expressing cells in terms of typical patterns with regard to CD34-expression and sideward light scatter (6). Interestingly, although the class I and class II CD34 MoAbs were affected to the greatest extent, the CD34 class III MoAb Birma/K3 was also affected substantially, as is shown in Table 3. Notably, as is shown in Table 3, these effects of the RBC-lysing reagent were observed with both FITC and PE conjugates of the CD34 MoAbs.

To confirm that the effects of the various RBC-lysing reagents were caused by direct effects on the cellular antigens/epitopes and not due to alterations of the fluorochrome label of the CD34 MoAb, all CD34 MoAbs were studied also on QSC beads, processing the QSC beads with the three RBC-lysing reagents. In this setting, only very minor differences in the fluorescence mean values for each of the CD34 MoAbs were determined (data not shown).

Our finding of little if any effects of the fixative-containing RBC-lysing reagents on the FI of fluorochrome-labeled CD34 MoAbs on QSC beads suggest a direct effect of the fixative-containing RBC-lysing reagents on the epitopes that the fluorochrome-labeled CD34 MoAbs are binding to.

To further corroborate this hypothesis, we performed indirect IF with the CD34 MoAb and rabbit anti-mouse IgG FITC. In this setting, samples were first allowed to react with the unlabeled CD34 MoAb for 60 min. The samples were then washed twice with with PBS and bovine serum albumin; the cell pellet was then resuspended in PBS and bovine serum albumin, and rabbit anti-mouse IgG FITC was added for 45 min. Finally, aliquots of the stained samples were subjected to the three RBC-lysing reagents.

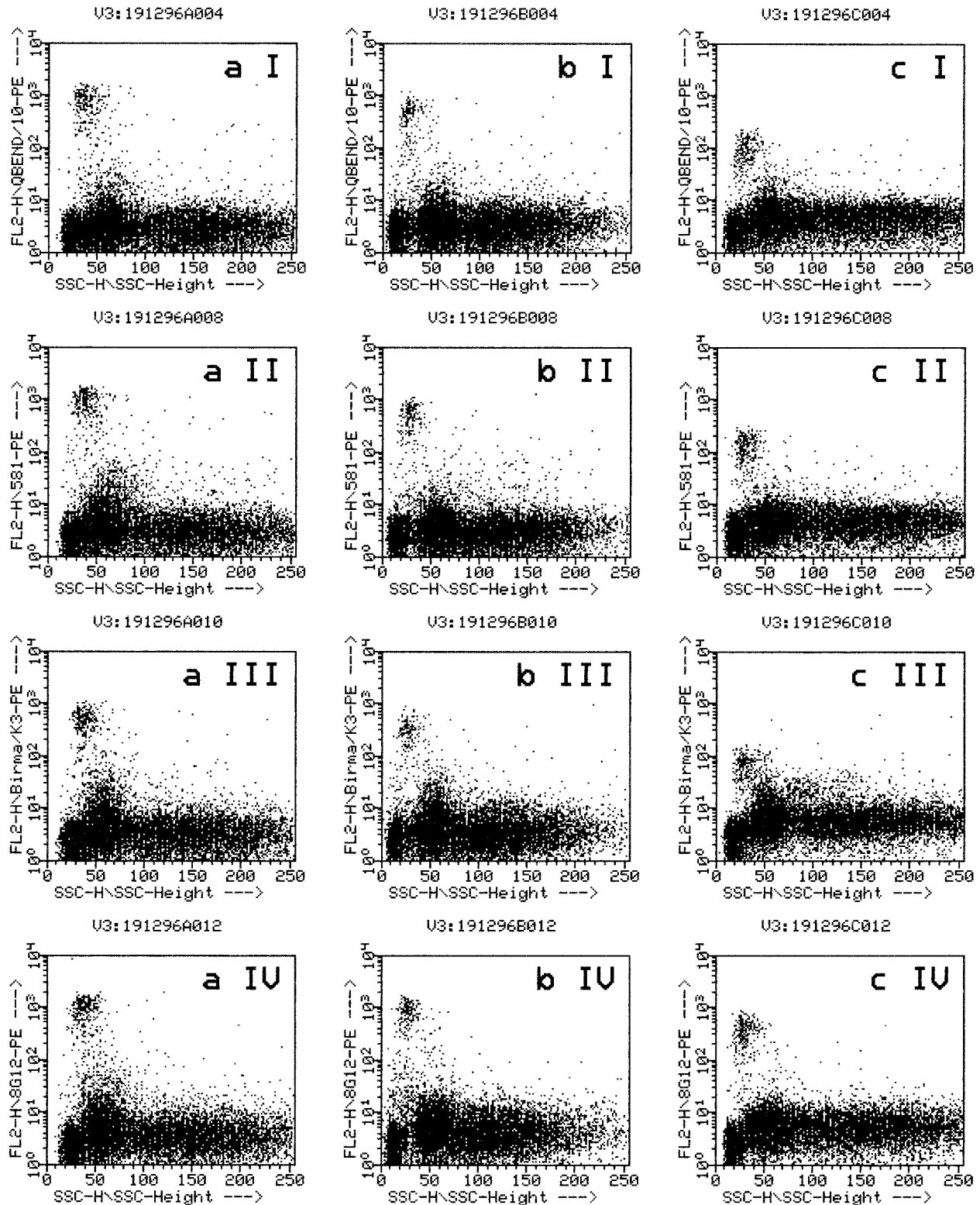


FIG. 4. Depiction of the effects of 3 RBC-lysing reagents on the fluorescence intensities of CD34-expressing cells as determined with one class II CD34 MoAb (Qbend/10) and with 3 class III CD34 MoAbs (8G12, 581, Birma/K3); PE conjugates of all antibodies. Determinations on aliquots of one single cytopheresis sample containing about 1.5 % CD34-expressing cells as determined with the reference antibody and the reference RBC-lysing reagent (8G12-PE and ORTHOmune); 12,500 cells per single determination; all cells shown; x-axis, sideward light scatter; y-axis, CD34-expression (PE emission). First column (a): processing with ORTHOmune RBC-lysing reagent. Second column (b): processing with FACS RBC-lysing reagent. Third column (c): processing with ImmunoPrep RBC-lysing reagent. First row (I): staining with Qbend/10. Second row (II): staining with 581. Third row (III): staining with Birma/K3. Fourth row (IV): staining with 8G12.

Table 3
*Fluorescence Mean Values of CD34-Expressing Cells as Identified by CD34-Expression Correlated to Sideward Light Scatter**

CD34 MoAb	Processing reagent		
	Ammonium chloride	FACS lysing	ImmunoPrep
PE conjugates			
ICH-3 (class I)	174/155/166/124	44/28/33/24	79/86/72/65
IMMU-133 (class I)	354/378/404/380	78/95/105/90	112/128/140/125
Qbend/10 (class II)	528/550/568/525	205/197/211/185	102/110/98/120
8G12 (class III)	450/485/525/553	176/221/255/203	337/345/404/384
581 (class III)	525/478/460/503	175/202/218/196	105/114/126/110
Birma/K3 (class III)	298/313/325/286	167/148/123/119	61/74/59/78
FITC conjugates			
Qbend/10 (class II)	56/63/48/54	28/21/33/36	32/28/35/35
8G12 (class III)	81/104/52/70	79/81/67/83	53/48/61/55
581 (class III)	148/104/101/127	68/57/50/60	80/56/62/69
Birma/K3 (class III)	89/72/76/70	43/31/49/50	46/40/51/44

*Processing was performed with 3 different RBC-lysing reagents. Individual data are given, each value representing the mean from triplicate determinations from 4 different samples with more than 1.0% CD34-expressing cells; at least 25,000 cells per determination.

Figure 5 shows that the effects of the fixative-containing RBC-lysing reagents in reducing the FI of CD34-expressing cells were extremely small with indirect IF staining. Table 4 shows the numerical data from the three samples studied. Of additional interest, these findings, which directly demonstrate a differential sensitivity of the various class III CD34 epitopes towards the actions of the fixative-containing RBC-lysing reagents, clearly point to a pronounced biochemical heterogeneity of the epitopes currently collectively referred to as CD34 class III MoAbs (13).

DISCUSSION

The capability of multiparameter flow cytometry to measure the FI of stained cells and thus to gain information about the estimated numbers of cellular binding sites to the respective MoAb remains largely unexploited. There are a number of reasons, but the heterogeneity of the available fluorochrome conjugates and the variety of the currently marketed flow cytometers are thought to be the major reasons. If FI is reported, terms referred to typically are "dim," "intermediate," and "bright" (14). As an alternative, a number of tools aimed at standardized, and thus reproducible, quantitation of FI which largely overcome the differences of flow cytometers and their operators, and of reagents (3-5), have been made commercially available. These currently marketed tools are the QIFI kit, QSC beads, and the Quanti-BRITE kit. Notably, identical numbers of cellular binding sites to MoAbs have been determined using the QIFI kit and indirect IF and a non-flow cytometric reference method (15).

To the best of our knowledge, this report is the first to describe an on-site comparison of the three flow cytometric techniques. Our data support the view that indirect IF and the use of the QIFI kit and direct IF using the Quanti-BRITE assay appear to be the more convenient tools for the standardized quantitative determination of cellular binding sites to MoAbs. Our data, which are supported by the discrepant data reported in two publications using the QSC beads (8,9), are another warning to

compare QSC bead-based data only if these have been obtained using a single strictly uniform approach.

We have identified a series of variables that appear to set limitations on standardized quantitative fluorescence flow cytometry. First, the concentration of fluorochrome-labeled MoAbs needed for saturation is not the same on QSC beads and on cellular antigens/epitopes. Generally, much higher concentrations of fluorochrome-labeled MoAbs are needed to saturate the QSC beads. The high concentrations of fluorochrome-labeled MoAbs needed to saturate the QSC beads, however, are not at all the optimal concentrations to stain cells. Surprisingly, some FITC-labeled MoAbs result in lower FI values on cells compared with when they are used at very high concentrations (as needed to saturate the QSC beads). Because lymphocytes show extremely high FI when stained with a CD45-FITC MoAb, the phenomenon of decreasing FI upon staining with increasing concentrations of CD45-FITC MoAb might be attributed to some kind of "self-quenching" of large numbers of very dense FITC molecules (16).

Interestingly, however, we have identified some fluorochrome-labeled MoAbs that will not saturate cellular antigens/epitopes with even extremely high concentrations (4× the manufacturer-recommended concentration). It should be noted here, however, that subsaturating staining of cells usually provides FI high enough to allow for proper recognition of stained cells.

This evidences some dichotomy between practical flow cytometry and quantitative fluorescence flow cytometry. Fully saturating concentrations of MoAbs are mandatory for quantitative fluorescence flow cytometry. In practical flow cytometry, subsaturating concentrations of MoAbs will not adversely affect the correctness of determinations involving percent cells or pattern recognition.

All these differences are not surprising, because the fluorescent ligands (MoAbs) using direct IF have two completely different targets: complex antigens/epitopes (on cells) and goat anti-mouse IgG antibodies (on QSC

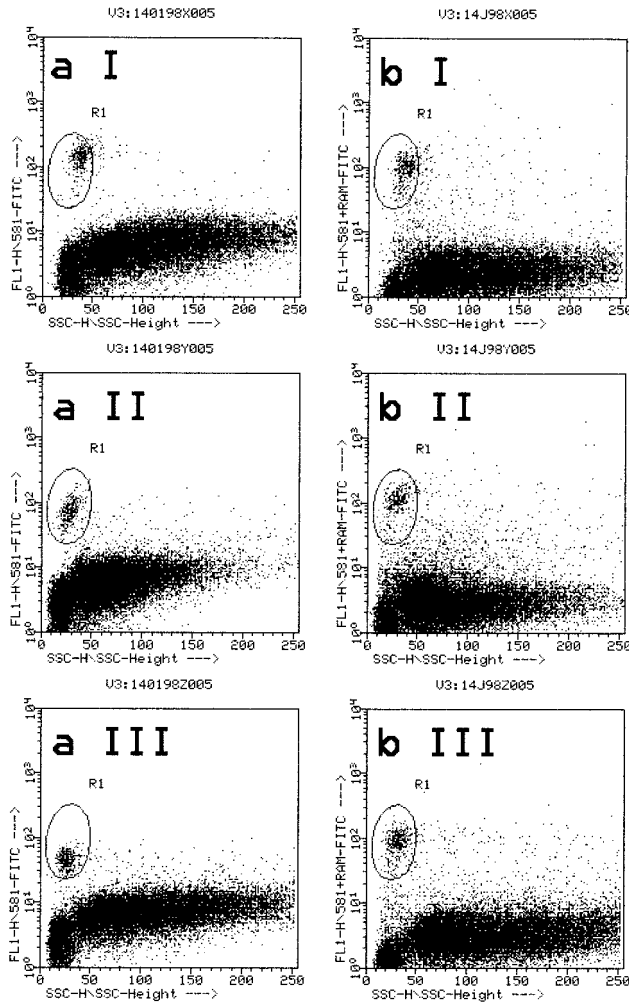


FIG. 5. Effects of processing with 3 RBC-lysing reagents on the fluorescence mean value of CD34-expressing cells; comparison of direct IF and of indirect IF; staining with class III CD34 MoAb 581; cytopheresis sample, about 1.4% CD34-expressing cells; 25,000 cells per determination; the region R1 encircles the CD34-expressing cells (one single region R1 for better direct comparison). First column (a): direct immunofluorescence (581-FITC). First row (I): processing with ORTHOmune; fluorescence mean of R1 cells, 129. Second row (II): processing with FACS lyse; fluorescence mean of R1 cells, 70. Third row (III): processing with ImmunoPrep; fluorescence mean of R1 cells, 43. Second column (b): indirect immunofluorescence (581 + rabbit anti-mouse IgG FITC). First row (I): processing with ORTHOmune; fluorescence mean of R1 cells, 82. Second row (II): processing with FACS lyse; fluorescence mean of R1 cells, 81. Third row (III): processing with ImmunoPrep; fluorescence mean of R1 cells, 75.

beads). In this setting, it is worth emphasizing that the Quanti-BRITE assay is subject to variability only in terms of optimized titration of the MoAbs on cells and a ratio of MoAb to PE of about 1:1.

Second, the QIFI kit is marketed with one single manufacturer-defined fluorescent ligand, facilitating the manufacturer's in-house calibration of the QIFI kit. In contrast is the claimed flexibility of the QSC assay; to use this assay for MoAbs with any fluorochrome label appears problematic in view of our data showing discrepant results with FITC and PE conjugates of a single CD MoAb. Again,

Table 4
Effects of the Three RBC-Lysing Reagents on the Fluorescence Mean Values of CD34-Expressing Cells*

CD34 MoAb	Processing with		
	ORTHOmune	FACS lyse	ImmunoPrep
Qbend/10			
Direct IF	54/48/60	22/28/24	26/26/24
Indirect IF	69/58/64	68/64/60	54/50/52
8G12			
Direct IF	58/70/64	54/72/62	31/58/54
Indirect IF	108/112/102	104/108/102	91/94/88
581			
Direct IF	129/100/104	70/68/82	43/58/54
Indirect IF	82/74/72	81/76/70	75/70/69

*Comparison of direct (FITC conjugates) and indirect (unconjugated monoclonal antibody + rabbit anti-mouse IgG FITC) IF. Shown are fluorescence mean values (means from triplicate determinations) from 3 different samples; only Qbend/10 (class II), 8G12 (class III), and 581 (class III) CD34 MoAbs were studied.

this is not surprising considering the differences, for example, in terms of size of the MoAb-fluorochrome complex and the electrical charge of FITC- and PE-conjugated MoAbs.

Finally, we have identified that the three most commonly used RBC-lysing reagents have substantial impact on the binding of fluorochrome-labeled CD34 MoAbs. Notably, the FI of some fluorochrome-labeled MoAbs were reduced drastically. The differences observed even among the three different CD34 class III MoAbs clearly indicates that these MoAbs detect different subepitopes, although we have shown previously (13) that by cross-blocking studies they are mutually competitive. Furthermore, we have shown that the effects of the fixative-containing RBC-lysing reagents were direct effects on the complex of cellular antigens/epitopes plus fluorochrome-labeled MoAb, because the FI of CD34-expressing cells stained by indirect IF was almost independent from the type of RBC-lysing reagent used.

The critical issues described above are evidence that indirect IF using the QIFI kit and direct IF using the Quanti-BRITE assay are associated with a smaller number of variables that need to be taken into account when trying to determine cellular binding sites to MoAbs in a standardized way, unlike direct IF using the QSC assay.

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