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DIFFERENCES IN PHYCOERYTHRIN- OR FLUORESCEIN-ISOTHIOCYANATE CONJUGATED 8G12 ON CD34+ CELL EVALUATION

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ABSTRACT

To evaluate the equivalence of 8G12 conjugated with either phycoerythrin (PE) or fluorescein isothiocyanate (FITC), duplicate fluorochrome-8G12 labelling was carried out in 229 samples. Additionally, 53 samples were simultaneously immunostained with FITC-8G12 and PE-8G12. Significantly higher values (p<0.001) were observed in PE-CD34+ cells when compared with FITC-CD34+ cells both in duplicate and simultaneous analysis. Our data suggest that the choice of fluorochrome is relevant in the measurement of CD34+ cells with 8G12.

Key words: CD34, fluorescein isothiocyanate, phycoerythrin, fluorochrome, flow cytometry

CD34 cell measurement is essential in the context of peripheral blood stem cell transplantation (PBSCT).[1] Some protocols for CD34 determination in PBSCT recommend the use of phycoerythrin (PE)-conjugated 8G12,[2,3] whereas a number of studies involving stem cell quantification use fluorescein isothiocyanate (FITC)-conjugated 8G12.[4,5] With the aim of determining whether FITC and PE-8G12 MoAbs are equivalent and of comparing the affinity of FITC and PE MoAb conjugates for the epitope against which the antibody is directed, we carried out a prospective study of duplicate and simultaneous FITC-8G12 and PE-8G12 CD34 staining and measurement.

Patients and Methods

Twenty-nine patients underwent mobilization during steady-state hematopoiesis with either rhG-CSF (n=22) or a chemotherapy + rhG-CSF schedule (n=7). A 10-L blood leukapheresis was performed daily after the third dose of the cytokine (rhG-CSF priming) or after WBC reached > 1.0×10^9/L (chemotherapy + rhG-CSF priming), using a CS-3000 PLUS blood cell separator (Fenwal, Deerfield, IL, USA). Evaluation of hematopoietic progenitor cell concentration involved 154 peripheral blood (PB) samples obtained before and on days 1-6 after rhG-CSF administration (rhG-CSF priming) or after WBC reached > 1.0×10^9/L (chemotherapy + rhG-CSF priming), and 75 different leukapheresis product samples. Fifty-one PB samples from healthy blood donors (HDS) were considered for CD19+ B-lymphocyte evaluation and represented the control group.

Processing and analysis of samples were performed following recommendations from the *International Society for Hematotherapy and Graft Engineering* (ISHAGE).[6]

For immunofluorescence analysis, mononuclear cells were labelled with a single MoAb or combination of MoAbs in the following manner: A, FITC-conjugated CD34 (anti-HPCA-2); B, PE-conjugated CD34 (anti-HPCA-2), FITC-conjugated CD14 (anti-LeuM3) and Tricolor-conjugated (TC)-CD45 (anti-CD45) and C, isotype controls. In addition, 53 samples, 27 from PB and 26 aliquots of apheresis collections, were stained simultaneously in a single tube for each sample with FITC-conjugated and PE-conjugated CD34 (anti-HPCA-2). In a like manner, 51 HDS were labelled at the same time with FITC-conjugated and PE-conjugated CD19 (anti-Leu12). All MoAbs were obtained from Becton-Dickinson (BD, San José, CA, USA), except CD45, which was purchased from Caltag (Caltag Laboratories, San Francisco, CA, USA). Data acquisition was carried out on a FACScan (BD) flow cytometer using LYSYS 2.1 software. Device sensitivity and linearity were controlled using Calibrite (BD) and a mixture of stained cells which spanned the signal intensity range of cells in the assays (HDS labelled with FITC-CD3, PE-CD19 and TC-CD4). Photomultiplier (PMT) voltages were adjusted and maintained so that light scatter signals were in the mid scale region of the instrument, and 99% of the autofluorescence signal of the cells was included within the first logarithmic decade of the instrument. A weekly calibration check was carried out. Data analysis was performed with the Paint-A-Gate Plus software, and the percentage of nonspecific events was substracted from positive events in stained samples. In tube B only $CD34^+/CD45_{low\ to\ high}$ events were considered.

To assess for statistical differences, repeated measure analysis of variance (MANOVA) with a hierarchic factorial design was applied to the duplicate FITC-CD34 and PE-CD34 samples. A two-tailed paired Student's t-test was applied to compare simultaneous FITC-CD34/PE-CD34 measurement and FITC-CD19/PE-CD19 (dual stained samples) estimation. Results are given as mean±SEM, median and range values. A significance level of $p < 0.01$ was chosen.

Results

Duplicate CD34 measurement

In the analysis of all 229 samples, we found significantly lower concentrations of FITC-CD34+ cells (358.3±48.9/μL) vs PE-CD34+ cells (448.1±59.1/μL) and vs PE-CD34+/FITC-CD14- cells (385.7±52.2/μL) ($p < 0.001$ in all comparisons).

As regards the analysis of positivity of cells for each fluorochrome separately considering either PB or apheresis samples, the concentration of FITC-CD34+ cells was also significantly lower ($p < 0.001$) than that of PE-CD34+ and PE-CD34+/FITC-CD14-

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Table 1. Analysis of CD34+ cell concentration in samples incubated separately with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated 8G12.

	*PE-34+	PE-34+/FITC-14-	FITC-34+
Peripheral blood	12.8±1.1*	11.5±1.0*	9.8±1.2
n = 154	7.1 (0.5-71.1)	6.2 (0.4-67.3)	5.4 (0.3-133.2)
Leukapheresis	1348.2±129.1*	1159.5±117.0*	1078.7±110.4
n = 75	1204.1 (51.1-6197.3)	909.8 (42.5-5164.5)	732.6 (21.1-3866.8)
Total	448.1±59.1*	385.7±52.2*	358.3±48.9
n = 229	17.1 (0.5-6197.3)	15.7 (0.4-5164.5)	11.8 (0.3-3866.8)

*Results are expressed in number of cells/μL. Results are given as mean±SEM, median and range values.*Significant difference (p < 0.001) compared to FITC-34+ cells.*

cells (Table 1). A similar statistically significant difference was observed when the mobilization protocol factor was considered in all 229 samples: a) rhG-CSF, 391.8±57.8/μL FITC-CD34+ vs 493.0±70.5/μL PE-CD34+ alone and 425.8±62.3/μL PE-CD34+/ FITC-CD14- cells; b) for chemotherapy plus rhG-CSF: 226.4±79.8/μL FITC-CD34+ vs 271.3±86.0/μL PE-CD34+ alone and 228.3±75.4/μL PE-CD34+/ FITC-CD14- cells; or when it was considered independently in PB or apheresis samples (data not shown).

Simultaneous FITC-CD34 and PE-CD34 measurement

The concentration of PE-CD34+ cells was significantly greater (305.1±49.8/μL, median 98.9/μL, range 4.6-1246.3/μL) than that of FITC-CD34+ cells (287.5±48.4/μL, median 120.3/μL, range 2.3-1203.9/μL), p < 0.001.

Simultaneous FITC-CD19 and PE-CD19 measurement

As a control to analyze the affinity of both dyes, PB samples were also sensitized simultaneously with FITC- and PE-labelled 4G7 directed against the CD19 antigen. The staining of cells with FITC-CD19 antibody was significantly lower (247± 20.2/μL, median 217.2/μL, range 14.0-835.8/μL) than that of PE-CD19 labelled cells (249.4±20.3/μL, median 218.2/μL, range 16.7-848.9/μL), p < 0.001.

Discussion

Although a number of rational protocols and recommendations for CD34 cell measurement have been published,[2,3,6] there is, to date, no standardized method for this analysis. Different studies evaluating low precursor cell concentrations use either FITC- or PE-labelled 8G12 for CD34+ cell measurement;[7,8] furthermore, several papers dealing with CD34 cell estimation do not indicate clearly whether FITC- or PE-8G12 conjugates were used.[9,10] Therefore there is an assumption that both conjugates are equivalent, though this may not be the case.

In the present study, we assessed FITC- and PE-8G12 immunostaining in a number of PB and apheresis samples. We found lower FITC-CD34 counts when comparing FITC-CD34+ vs PE-CD34+ cells and FITC-CD34+ vs PE-CD34+/FITC-CD14- cells, when considering either apheresis or PB samples independently, and when taking into account the mobilization schedule. These results suggest that the discrepancy in PE- and FITC-8G12 staining is independent of nonspecific binding of the PE-8G12 antibody to monocytic Fc receptor (as shown by CD14 MoAb), of the CD34 antigen concentration in the samples, and of the cell population distribution in the collected material and in the mobilized peripheral blood cells.

We then estimated the positivity of the MoAb labelled with both fluorochromes in the same sample using a single tube. Within this group of samples, significantly higher values were found for PE-8G12. Double CD19+ cell measurements with PE- and FITC-labelled 4G7 also showed a greater number of PE-positive events. Conclusions from our study indicate that fluorochrome selection is relevant at least in the estimation of CD34+ cells with 8G12 class III MoAb, and CD19+ cells with 4G7 MoAb. Because information concerning measurement of CD34+ cells may have clinical implications and investigational relevance,[10] data regarding the fluorescent dye used for the staining of cells has to be taken into consideration for 8G12 CD34+ cell evaluation.

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