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Assessment of distribution of CD34 epitope classes in fresh and cryopreserved peripheral blood progenitor cells and acute myeloid leukemic blasts

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ABSTRACT

Background and Objectives. **So far several reports have described changes in the expression of surface antigens in progenitor cells and blasts following cryopreservation. However, there are no data on the effects of cryopreservation on the expression of the three CD34 epitope classes, and on their relationship with the clonogenic capacity of PBPC collected by leukapheresis.**

Design and Methods. **In order to analyze the effects of freezing / thawing procedures (–80°C storage for 3 months) and use of dimethylsulfoxide (DMSO) on the immunophenotype profile and colony production of peripheral blood progenitor cells (PBPC) in apheresis products derived from 20 patients with stage 0-III non-Hodgkin's lymphoma (nHL), a flow cytometry study was undertaken using different CD34 monoclonal antibodies (MoAbs) capable of recognizing the 3 epitope classes of CD34 molecule (class III: HPCA-2 / FITC, HPCA-2 / PE, 581 / FITC, 581 / PE; class II: Q-Bend 10 / PE; class I: ICH3 / PE, BI3C5-PE, Immu-133-PE). CD34 epitope expression was also analyzed in thawed CD34$^+$ blasts obtained from 14 patients with acute myeloid leukemia (AML), who were analyzed using a larger number (#17) of CD34 epitope class I, II, and III reactive MoAbs.**

Results. **Under our experimental conditions it was found that class III and class II CD34 epitopes (differentially resistant to enzymatic cleavage with neuraminidase, chymopapain and glycoprotease) are better preserved than class I epitope – sensitive to degradation – after cell exposure to cryoprotectant DMSO and the freezing- thawing procedures. Results further showed a concomitant decrease in class I CD34$^+$ counts and in BFU-E colony production. A significant increase in CD34 antigen expression levels (i.e. antibody binding capacity, ABC) by cryopreserved cells stained with CD34 epitope class III, and class II reactive MoAbs was also documented, while no changes after cryopreservation was noted using class I-reactive MoAbs. The slight increase in the percentage of CD34$^+$ cells detected after frozen storage was correlated to a concomitant decrease in the number of more mature myeloid cells (CD15$^+$, CD13$^+$, CD33$^+$). Compared to pre-cryopreservation values, a slight reduction in class I CD34 epitope expression was also found in thawed CD34$^+$ AML blasts.**

Interpretation and Conclusions. **As far as the reduction of class I CD34 epitope is concerned, it may be hypothesized that the freezing procedure, use of DMSO, and / or lysis methodology may either damage a CD34 subset, or induce distinct alterations of the CD34 glycoprotein, possibly determining a reduction in their immunoreactivity with some CD34 MoAbs. In conclusion, this study has shown that exposure to the cryoprotectant DMSO and the freezing / thawing procedures modifies the distribution of CD34 epitopes as well as the clonogenic capacity of PBPCs from nHL patients, and CD34$^+$ blasts from AML. These findings need to considered when selecting CD34 MoAbs for enumeration and positive selection of stem / progenitor cells for research and clinical purposes.**

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The CD34 molecule is a one-pass transmembrane glycoprotein with a molecular weight of 105-120 kilodalton in either the reduced or unreduced form. The full length CD34 protein is composed of 385 amino acids, and contains nine sites for N-glycosylation and a number of sites for O-glycosylation, that are essential constituents of the three epitopes of the molecule.[1-3] More recently, a truncated form of CD34 protein has been characterized, which contains only 323 amino acids. While the intracellular cytoplasmic domain is shorter in the CD34 splice variant, the extracellular and the transmembrane regions of the two forms of CD34 are identical.[4,5] CD34 is also rich in sialic acid, and its biochemical composition suggests a mucin-like structure and resembles in some respects leucosialin (CD43).[1-5]

CD34 antigen is expressed on hemopoietic stem and progenitor cells, small vessel endothelial cells, and a subset of fibroblasts. Seven CD34 MoAbs have

been clustered at the 3rd and 4th *Workshop on Leukocyte Differentiation Antigens* (Oxford, 1986 and Vienna, 1988), and a further 25 MoAbs were verified as recognizing the CD34 molecule during the 5th and the 6th *International Workshops on Leukocyte Differentiation Antigens* (Boston, 1983; Osaka, 1996), the most direct evidence being reactivity with cells transfected with CD34 cDNA and binding to CD34 protein.[2-5] Based on current knowledge, the epitope specificity of CD34 MoAbs could be classified into three distinct groups according to the different sensitivities of the epitopes to enzymatic cleavage (which has been performed using neuraminidase, chymopapain and glycoprotease), reactivity with fibroblasts and high endothelial venules, and cross-blocking experiments.[2,5,6] We know, in fact, that glycoprotease from *Pasteurella haemolytica* specifically cleaves only proteins containing sialylated O-linked glycans, while neuraminidase cleaves sialic acid residues. On the basis of these data, it can be speculated that class III epitopes are more proximal to the extracellular side of the cell membrane than the class I and class II epitopes.[6]

Over the few last years, PBPC have been increasingly used to provide rapid and durable hemopoietic reconstitution following high dose chemo- and radiotherapy.[7] However, the quality of PBPC apheresis products has to be monitored in all the processing steps, which include cryopreservation, and subsequent thawing and reinfusion. Traditionally, either the CFU-GM test or flow cytometry measurement of CD34-expressing cells has been used to evaluate the potential of PBPC autografts for restoring hematopoiesis after high-dose therapy.[8,9] There are accumulating data showing a significant correlation between numbers of transplanted $CD34^+$ cells/kg and hemopoietic reconstitution.[10] So far several studies have investigated the effects of cryopreservation on the expression of surface antigens in progenitor cells and blasts. Most of the data have focused on the analysis of CD7, CD13, CD33 and CD34 molecules during the freezing/thawing procedures in the presence of cryoprotectants such as DMSO.[11-17] However, there are no data on the effects of cryopreservation on the distribution of expression of CD34 epitopes or on its relationship with the clonogenic capacity of PBPC collected by leukapheresis.

In order to analyze the effects of short-term cryopreservation (3 months at –80°C using 10% DMSO and 4% human serum albumin as a cryoprotective agent without rate-controlled freezing) on the immunophenotype profile and colony production of PBPC apheresis products from 20 patients with stage 0-III non-Hodgkin's lymphoma (nHL), a flow cytometry study was undertaken using different CD34 MoAbs capable of recognizing the 3 epitope classes of CD34 molecule. The effect of cryopreservation on the expression of different CD34 epitopes was also evaluated through the use of a flow cytometer and 17 distinct CD34-reactive MoAbs on $CD34^+$ ($HPCA\text{-}2^+$) blasts taken from 14 patients with acute myeloid leukemia (AML).

Design and Methods

Subjects

Non-Hodgkin's lymphoma. Fresh and cryopreserved peripheral blood mononuclear cells (PBMNC) obtained from 20 consecutive patients with stage 0-III nHL (aged between 16 and 56; mean: 35 years) were investigated within 1 hour from their collection and processing. Diagnosis of lymphoma was based on morphologic, immunologic, cytochemical, cytogenetic, and molecular genetic criteria. No bone marrow contamination of lymphoma cells was documented at the time of the study. All patients achieved a remission state at the time of PBMNC collection. The mobilization regimen was cyclophosphamide 7 g/m^2 plus rhG-CSF (filgrastim, 5-15 µg/kg/day). PBMNC were collected by apheresis. Written informed consent for PBPC collection and autologous transplantation was obtained from each patient.

Acute myeloid leukemia. Light density (< 1.077 g/mL) peripheral blood cells obtained from 14 patients with $CD34^+$ (HPCA-2/PE) AML were investigated before and after cryopreservation. Diagnosis of leukemia was based on morphologic, cytochemical, cytogenetic, molecular genetic, and immunologic criteria. According to the FAB classification, the distribution of AML subtypes was as follows: M0: 1pt; M1: 5 pts; M2: 5 pts; M4 3. AML patients had a blast percentage higher than 60% in all specimens analyzed (mean 74%). The $CD34^+$ acute leukemia samples included in this study were selected using the HPCA-2-PE MoAb.[18,19] Using HPCA-2/PE MoAb, the range of CD34 positivity on leukemic cells was 45-82%. AML patients were treated with a DAT (daunorubicin, cytosine arabinoside, thioguanine)-based induction regimen, followed by consolidation chemotherapy.

Collection of PBPC, processing and cryopreservation

PBMNC were collected by apheresis from nHL patients in remission state during hematologic recovery which followed the mobilization regimen administered (cyclophosphamide 7 g/m^2 plus rhuG-CSF (filgrastim, 5-15 µg/kg/day). PBPC were collected by means of apheresis using CS-300 Plus blood cell separator (Omnix TM, Baxter, Milan, Italy). Each apheresis session consisted in processing 10-15 liters of blood. Acid citrate-dextrose (ACD) was used as anticoagulant. Samples were collected immediately after apheresis and analyzed by flow cytometry with saturating concentrations of CD34 MoAbs and irrelevant isotype-matched MoAbs.

Peripheral blood mononuclear cell samples were mixed with an equal volume of cryoprotective solution consisting of 20% DMSO and 8% human serum albumin. The final cell concentration was adjusted to $< 50 \times 10^9$/L. The suspensions were frozen in cryo-

preservation bags (Cryocyte freezing container, Baxter) without rate-controlled freezing in an electrical freezer at –80°C (Haraeus-Votsch).

Twenty specimens from each donor were stored for 3 months in order to study the effect of cryopreservation on the immunophenotypic profile of stem-progenitor cells.

For rapid thawing, freezing tubes were taken from the freezer and placed in a 37°C water bath, they were then diluted slowly 10 fold with a calcium- and magnesium-deprived PBS buffer. Samples were also taken from freezing tubes, before and after cryopreservation, for trypan blue dye exclusion tests.

Cell counting and leukocyte differential was performed using an automated hematology analyzer (Technicon H3, Bayer, Milan). The white cell differential was also determined microscopically on May-Grünwald-Giemsa stained films.

Flow cytometry analysis of class I, II, and III CD34 epitopes

PBMC apheresis products and light density cells obtained from AML samples were analyzed before and after cryopreservation with a Facscan flow cytometer (Becton Dickinson, San Josè, CA, USA) equipped with a 15 mW argon-ion laser. The instrument was calibrated with FITC (fluorescein isothyocyanate) and PE (R- phycoerythrin) beads provided by Becton Dickinson. Data were analyzed with negative controls using Lysis II and Cell Quest research software. As negative control, we used an appropriate and similarly titered isotype control.[20] Over 50,000 viable cells (containing at least 100 $CD34^+$ cells) were analyzed for each sample at a flow rate of approximately 300 particles per second.

The enumeration and the quantitative analysis of the expression levels of the various $CD34^+$ MoAbs was performed on fresh and cryopreserved PBMNC apheresis products using a modified version of the Milan protocol.[8] This is a *no-lyse and wash*, dual platform technique based on direct CD34 immunofluorescence and assessment of leukocyte absolute counts using a separate electronic counter (Techicon H3, Bayer). Erythrocytes were lysed by NH_4Cl Ortho-mune lysing solution, according to the manufacturer's instructions. Viable cells were identified using a nucleic acid dye, as described in detail later on.

Since the ABC calculation for cell samples stained simultaneously with CD34 and CD45 MoAbs was unreliable (data not shown), we decided to follow the Milan protocol instead of the ISHAGE method, which is known to provide better results for the analysis of $CD34^+$ cells in some samples. However, the concomitant use of CD34 MoAbs and a nucleic acid dye significantly improved the sensitivity and specificity of our method.

The CD34 assay used in this study consisted of the following steps:

1. nucleated cells were distinguished from unlysed erythrocytes, debris and platelets by their relatively high FSC (forward scatter) signals (gate R 1). Propidium iodide as a nucleic acid dye was used to select nucleated cells during data analysis and counting the number of dead cells. CD34 class I, II and III reactive MoAbs were counterstained with propidium iodide to identify the number of $CD34^+$ cells positive for the nucleic acid dye. For the analysis of CD34 PE-conjugates, we used 7-amino-actinomycin-D (7-ADD) as a nucleic acid dye. This compound has been shown to be useful in the discrimination of live versus dead cells in a multiparametic analysis;[22]
2. the gated viable cells were then analyzed for their CD34 expression by looking at the dot plot generated by combining the side scatter (SSC) parameter and fluorescence 1 or 2, as appropriate (gate R2);
3. the events included in gate R1 and R2 were then displayed on the dot plot, forward scatter vs side scatter, to verify that the light scatter characteristics of the $CD34^+$ cell cluster were typical for stem/progenitor cells;
4. an isotype control MoAb was used to set the marker discriminating between positive and negative fluorescence signals. The percentage value of positive events of the isotype control was subtracted from the $CD34^+$ counts.

Flow cytometry data were expressed as percentage of positivity (using the standard marker approach), as antibody binding capacity (ABC) units, or as molecules of equivalents of soluble fluorochromes (MESF) values.[18,24]

Based on the analysis of the minimum detection threshold of our flow cytometer, and on variation in ABC values for CD34 MoAbs in the specimens analyzed (evaluated within and between different specimens), we estimated that the lower limit of quantification was between $0.5\text{-}0.7\times10^3$ MESF/cell (indirect staining) and between $0.9\text{-}1.1\times10^3$ ABC/cell. Below this limit the enumeration of antigenic determinants was considered unreliable and not reproducible.[18]

Monoclonal antibodies to CD34

A large series of CD34 epitope class I, II, and III reactive MoAbs were used in this study. PBMNC apheresis products from nHL were investigated by using 6 different CD34 MoAbs: HPCA-2/FITC (class III, BDis), HPCA-2/PE (BDis), 581-FITC (class III, Caltag), 581/PE (Caltag), Q-Bend 10-PE (class II, Immunotech); ICH3-PE (class I, Caltag), Immu-133 (class I, immunotech). For the analysis of class I, and class II CD34 epitopes, we used only PE conjugates, since recent data have indicated that FITC-labeled class I and class II CD34 MoAbs provide less reliable results than the PE-conjugated forms.[25-27]

The expression of CD34 on leukemic cells was assessed by using 17 different unconjugated CD34

MoAbs (Class I: Immu-133, Immu-409, 14G3, BI3C5; Class II: Q-Bend 10, 43A1, MD34.1, MD34.3, MD34.2, 4A1, 9066, 9069; Class III: CD34-9F2, 8G12, 581, 553, 563). This series of CD34 MoAbs was provided by Prof. M. Greaves (London, UK), and were intended for analysis during the fifth *Workshop on Leukocyte Differentiation Antigens* held in Boston, November 1993.[2,4]

Negative controls were used in all experiments. As negative controls, appropriate and similarly titered isotype-matched non-relevant MoAbs (mouse IgG1, IgG2a, IgM; Dakopatts, Silenus, Becton, Caltag) were employed. Light density cells from AML samples were washed twice in PBS before their incubation with CD34 MoAbs. Human AB serum was added prior to antibody incubation to avoid non-specific binding of MoAbs to Fc receptors. All samples were further incubated for 20 minutes with a lysing buffer (Facslysing solution, Becton Dickinson) to eliminate red blood cells.

Purified CD34 MoAbs (including the isotype controls) were utilized using an indirect immunofluorescence technique. As a second step we employed FITC-conjugated goat F(ab') Ig fragments specific for mouse Ig (fluorochrome/protein ratio: 2.3 (Dakopatts).[20]

ABC calculation (direct immunofluorescence)

To evaluate the number of antigenic determinants per cell for the different CD34 MoAbs, we used Quantum Simply Cellular Beads (Flow Cytometry Standard Corp., purchased from Walter Occhiena srl, Turin, Italy). *Quantum Simply Cellular* is a mixture of four highly uniform microbead populations of the same size which have varying capacities to bind mouse monoclonal IgG antibodies. A blank population of microbeads which has no specific binding capacity for mouse IgG is included in the mixture. These beads mimic cells with different antigen densities and they serve as a set of standards to calibrate the fluorescence scale of the flow cytometer in units of antibody binding capacity (ABC).[18,19] The calculation of ABC is based on the evaluation of the peak channel expressed by cells and different population of microbeads. The minimum detection threshold of the instrument corresponds to the ABC value for the blank beads, and provides information on the sensitivity of the flow cytometer in detecting fluorescence signals.[24]

For each sample, the ABC value of the isotypic control was subtracted from the ABC value of the positive cell population.

MESF calculation (indirect staining)

To evaluate CD34 positivity in AML samples, we used quantitative microbead calibration standards (Quantum 26p beads, Flow Cytometry Standard Corp., purchased from Walter Occhiena srl, Turin, Italy). The calculation of MESF is based on the evaluation of the peak channel expressed by cells and different population of microbeads. The value for the slope, intercept, and correlation coefficient allow the calculation of a regression line, which gives information on the linearity and stability of the instrument response, the noise level of the flow cytometer, the MESF values of the stained and unstained cells, the resolution indices for fluorescence, and the coefficient of variation (CV) of microbeads' fluorescence.

For each sample, the MESF value of the isotypic control was subtracted from the MESF value of the positive cell population.

CFU assays

The colony-forming unit granulocyte-macrophage (CFU-GM), CFU- GEMM, and BFU-E assays were performed before and after cryopreservation by plating 1×10^5 mononuclear cells/plate. Cells were cultured in 35 mm diameter standard tissue culture dishes (Flow) containing 0.9% methylcellulose (MethoCult H4230; Stem Cell Technologies, Vancouver, Canada), 30% pretested FBS, 1% pre-tested BSA, 10^{-4} M 2-mercaptoethanol, supplemented with 10% supernatant of the 5637 cell line and 1 U/mL human urinary erythropoietin. The cells were cultured for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air. After 14 days of culture, colonies of more than 50 cells were scored under an inverted microscope. All cultures were performed in triplicate.

Assessment of cell viability and FAS antigen expression by flow cytometry

The assessment of cell viability was made with a Facscan flow cytometer (Becton-Dickinson). Cells were stained with either propidium iodide solution (50 µg/mL) or 7-ADD nucleic acid dye.[22]

Expression of FAS antigen was evaluated on PBMNC using the DX2-FITC MoAb (anti-FAS from Bender, Austria) in combination with PE-conjugated class I, II and III CD34 MoAbs. Cell viability and FAS expression were evaluated before and after cryopreservation.

Statistical analysis

Expression of class I,II and III CD34 epitopes and counts of CFU-GM, CFU-GEMM, BFU-E and of other biological parameters were compared using parametric and non-parametric statistics (paired Student's t test, Wilcoxon's test, linear regression model, multivariate regression analysis). When pertinent one way analysis of variance (ANOVA) and chi square or Fisher's tests were used to compare means and frequencies.

Results

Determination of CD34+ counts for epitope class I-, II-, and III-reactive MoAbs in fresh and cryopreserved PBPC

Effects of short-term cryopreservation (90 days storage) on the expression of CD34 antigens for class I, II, and III reactive MoAbs are shown in Table 1. Flow cytometry results showed that class III and class II $CD34^+$ counts were higher in cryopreserved PBPC

Table 1. Distribution of CD34 epitope classes in PBPC before and after cryopreservation (direct immunofluorescence).*

MoAb	*Pre-cryo-preservation*	*p value*	*Post-cryo-preservation*
HPCA-2 (FITC) class III	2.9±3.2	< 0.01	4.7±5.6
HPCA-2 (PE) class III	3.0±3.3	< 0.01	5.0±5.7
581 (FITC) class III	2.6±3.1	< 0.05	4.4±4.6
581 (PE) class III	2.7±3.3	< 0.05	4.6±4.7
QBEND-10 (PE) class II	2.7±3.3	ns	4.2±4.8
BI3C5 (PE) class I	1.7±0.7	< 0.05	1.1±1.4
ICH3 (PE) class I	2.3±2.7	ns	2.0±3.6
IMMU-133 (PE) class I	1.7±1.0	ns	1.8±1.6

**Percentage of positive cells expressed as mean±standard deviation (SD) ns = not significant (paired test).*

preparations than fresh cells, while a significant reduction was observed in class I CD34+ counts resulting from freezing/thawing procedures and use of two out of three class I-reactive MoAbs, as compared with pre-cryopreservation values (Table 2). The number of positive events detected in the negative controls samples was less than 1% in all cases examined. As previously mentioned (see *Design and Methods* section: flow cytometry analysis of class I, II,and III CD34 epitopes), viable cells were identified using a nucleic acid dye staining method (propidium iodide or 7-AAD) (Figure 1).

Table 2. Percent variation of CD34 cell positivity for the various CD34 MoAbs after cryopreservation (direct immunofluorescence).

Class III				*Class II*	*Class I*		
HPCA-2 (FITC)	*HPCA-2 (PE)*	*581 (FITC)*	*581 (PE)*	*QBEND-10 (PE)*	*ICH3 (PE)*	*BI3C5 (PE)*	*Immu-133 (PE)*
+62%	+66%	+69%	+70%	+55%	- 8%	- 35%	no variation

The quantitative analysis of CD34 antigens showed that the cryopreservation procedures induced a significant increase in ABC values for class

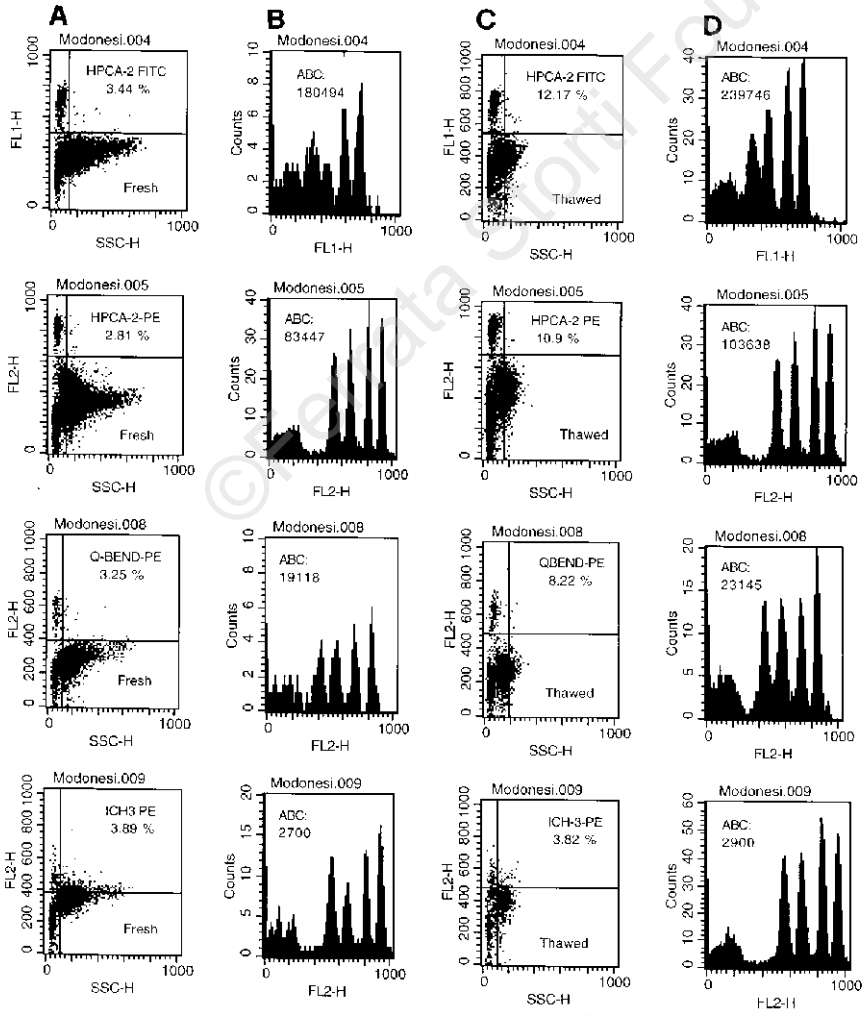

Figure 1. Flow cytometry analysis of fresh (A, B) and cryopreserved (C,D) PBSC obtained from patients with non-Hodgkin's lymphoma. Viable CD34+ cells were identified and quantified using the cell viability dye 7-AAD in combination with CD34 epitope class III, II, and I- reactive MoAbs. *Simply Cellular* beads were used for the calculation of ABC units, i.e. number of antigenic determinants expressed by CD34+ progenitors stained with the various CD34 MoAbs.

Table 3. Flow cytometry quantification of antigen expression of CD34 epitope class I, II, and III- reactive MoAbs in fresh and cryopreserved PBPC (direct immunofluorescence).

MoAbs	*Pre-cryo-preservation (ABC/cell x 10³)*	*Post-cryo-preservation*	*p value*
HPCA-2 (FITC) class III	140±48	223±76	p < 0.05
HPCA-2 (PE) class III	60±25	96±31	p < 0.05
581 (FITC) class III	53±14	81± 85	ns
581 (PE) class III	13.1±5.7	14±6.2	ns
QBEND-10 (PE) class II	20±7.8	25±11	ns
BI3C5 (PE) class I	54± 25	50±38	ns
ICH3 (PE) class I	3.5±1.6	5.0±2.5	ns
IMMU-133 (PE) class I	9.4±4.2	8.7±2.6	ns

Data are expressed as ABC (Antibody Binding Capacity) values (mean±SD)ns = not significant (paired test).

Table 4. Clonogenic capacity (mean ± SD) of PBPC before and after cryopreservation.

	Pre-cryo-preservation	*p value*	*Post-cryo-preservation*
CFU-GM/10^5 cells	144±47	ns	115±64
CFU-GEMM/10^5 cells	12±9	ns	9±11
BFU-E/10^5 cells	113±79	ns*	73±81

*Data are expressed as mean±SD; ns = not significant value (paired test). *p < 0.01 comparing a patient subgroup (#12 cases), characterized by a significant decrease in the expression of two class-I reactive MoAbs (ICH3 and BI-3C5).*

III and class II CD34 MoAbs (Table 3). However, compared to baseline values (pre-cryopreservation), the antigen density expressed by thawed PBPC labeled with class I CD34 MoAbs was only slightly increased (ICH3 MoAb) or even decreased with the remaining two class I-reactive MoAbs (BI3C5 and Immu-133 MoAbs) (Table 3) (Figure 1).

The clonogenic capacity of PBPC before cryopreservation and after thawing was evaluated by means of standard 14-day culture assays. These data are shown in Table 4. A comparative analysis of the effects of cryopreservation on CD34 epitope expression and numbers of CFU derived from PBPC apheresis products showed a concomitant decrease in class I $CD34^+$ counts and in BFU-E colony production (Table 4).

The slight increase in the percentage of $CD34^+$ cells detected after cryopreservation was correlated to a concomitant decrease in the number of more mature myeloid cells ($CD15^+$, $CD13^+$, $CD33^+$) (data not shown).

Effects of cryopreservation on cell viability were evaluated by flow cytometry using a multiparameter staining approach. The mean cell viability (±SD) decreased from 99.3±2.1% (pre-cryopreservation values) to 84.2±14.2% after three months of cryopreservation (Table 5). Table 5 also shows results derived from the analysis of either FAS (CD95) antigen expression or DNA staining in CD34-expressing cells labeled with CD34 epitope class I, II, and III-reactive MoAbs.

Table 5. Fas expression and number of dead cells in CD34-expressing cells stained with class I, II and III-reactive MoAbs.

MoAbs used	*% of double-positive cells**
FAS-FITC/HPCA-2-PE	8.1±7.2
HPCA-2-PE/ nucleic acid dye (7-AAD)	7.3±9.2
FAS-FITC/Q-BEND 10- PE	13.4±12.8
Q-BEND 10- PE/nucleic acid dye (7-AAD)	11.5±9.9
FAS-FITC/IMMU-133- PE	21.3±16.6
IMMU-133- PE/nucleic acid dye (7-AAD)	20.0±17.7

Data are expressed as percent of the total CD34-expressing cells (mean±SD).

Determination of $CD34^+$ counts for epitope class I-, II-, and III-reactive MoAbs in fresh and cryopreserved AML blasts

Compared to pre-cryopreservation values, a reduction in class I CD34 epitope expression was also found in thawed $CD34^+$ blasts obtained from 14 patients with acute myeloid leukemia (AML), who were analyzed using a larger series (#17) of CD34 epitope class I, II, and III reactive MoAbs (Table 6). In fact, the reactivity of two out of 4 class I-reactive MoAbs was decreased after frozen storage at –80°C, thus confirming data derived from the analysis of the effects of thawing procedures on the reactivity of PBPC towards the various CD34 MoAbs. In contrast, the cytofluorimetric analysis of acute leukemic cells after frozen storage at –80°C for three months showed that class III $CD34^+$ counts increased in comparison to those found on fresh cells.

The quantification of CD34 antigens showed that our flow cytometry analysis on frozen-thawed AML cells was associated with a significant increase in ABC values for several class III and class II CD34 MoAbs. An increase in ABC values was also observed in thawed AML cells stained with three out of 4 class I CD34 MoAbs, as compared with corresponding results from fresh aliquots. Antigen density was decreased in cryopreserved cells labeled with 14G3 class I MoAb, in comparison to those obtained from the fresh samples (Table 6).

Discussion

In this paper we evaluated the effects of short-term cryopreservation (3 month storage) on class I, II and III CD34 epitope expression in a series of leukapheresis products from lymphoma patients and leukemic sam-

Table 6. Flow cytometry analysis of CD34 epitope class I,II, and III reactive MoAbs in fresh and cryopreserved acute leukemic blasts obtained from CD34+ acute leukemias (indirect immunofluorescence).

MoAb (epitope class)	*pre-cryo-preservation% * MESF/cell x10³ °*	*post-cryo-preservation% * MESF/cell x10³ °*	*p value*
CD34-9F2 (III)	38.9±34.4 149±384	35.9±24 172±200	ns ns
8G12 (III)	54.8±30.7 248±414	51.7±22.3 237±462	ns ns
581 (III)	58.3±31.6 395±678	62.4±22.8 462±478	ns p < 0.05
553 (III)	58.2±31.8 409±669	63.4±22.4 427±405	ns ns
563 (III)	60.0±30.6 437±697	64.9±22.6 446±460	ns ns
QBEND-10 (II)	55.0±29.5 347±603	60.6±23.1 448±501	ns ns
43A1 (II)	27.9±34.7 6.0±16.1	28.8±29.5 94±214	ns p < 0.05
MD34.3 (II)	56.8±30.3 290±561	61.5±25.0 329±363	ns ns
MD34.1 (II)	52.6±28.9 108±193	52.9±24.6 225±232	ns p < 0.01
MD34.2 (II)	56.8±30.3 276±531	62.2±24.6 335±373	ns ns
4A1 (II)	54.8±30.2 135±237	54.8±25.8 124±193	ns ns
9066 (II)	50.5±30.7 119±207	54.5±23.4 119±168	ns ns
9069 (II)	48.3±30.4 92±175	50.2±25.7 105±150	ns ns
Immu-133 (I)	50.4±30.0 115±172	46.4±20.2 290±340	p < 0.05 p < 0.01
Immu-409 (I)	14.5±32.1 1.4±3.2	4.7±13.6 22±9	p < 0.05 p < 0.05
14G3 (I)	53.7±30.4 163±336	53.4±19.2 119±145	ns p < 0.05
BI3C5 (I)	34.4±32.1 6.4±9.2	36.7±23.4 147±205	ns p < 0.01

**Data are expressed as mean percentage±SD; °data are expressed as mean±SD; MESF = molecular equivalent of soluble fluorochrome; ns = not significant (paired test).*

ples from CD34+ acute myeloid leukemia.

In previous studies, many groups including ours have shown a great variability in results obtained within and between the CD34 MoAb classes in progenitor cells taken from bone marrow, PBPC, umbilical cord blood, and acute leukemia samples.[19, 28-35] On the basis of these data, many authors have postulated that the classification into three epitope classes could underestimate the extent of antigenic, epitopic diversity on the extracellular domains of CD34 molecule. Moreover, data derived from cross-blocking studies using several CD34 MoAbs, and the finding that some class II CD34 MoAbs (ICH3, My10, MD34.1, MD34.2, MD34.3, and 9069) were slightly sensitive to neuraminidase, provide evidence of further epitope variation within the three major classes defined by enzymatic cleavage.[2,6] Recent data have also shown that high endothelial venules selectively lack class I CD34 epitope, while class II and class III epitopes are expressed.[2,6] Since the biological significance of the distribution of class I, II and III epitopes on different CD34+ subpopulations is still uncertain, we thought it worthwhile to see whether cryopreservation modifies the distribution of different CD34 epitope classes in both normal progenitors and acute myeloid leukemic blasts.

In this study we have demonstrated that class III and class II CD34 epitopes (differentially resistant to enzymatic cleavage with neuraminidase, chymopapain and glycoprotease) are better preserved than class I epitope — sensitive to degradation — after cell exposure to cryoprotectant DMSO and the freezing-thawing procedures. CFU assays have also shown a concomitant decrease in class I CD34+ counts and in the BFU-E colony production, suggesting that the class I epitope recognized by both the CD34 MoAb ICH3 and BI3C5 may identify a subset of hemopoietic progenitors committed to erythroid lineage. From a theoretic point of view, cell sorting of class III+/class I-negative CD34+ cells and their subsequent culture would give evidence that BFU-E and class I expression are linked. However, it must be said that these cells cannot be reliably identified using a cell sorter or other techniques for a number of reasons, as reviewed elsewhere.[2,37]

A reduction in class I and class II CD34 epitope expression was also found in CD34+ blasts obtained from 14 patients with acute myeloid leukemia (AML), who were analyzed using a larger series (#17) of CD34 epitope class I, II, and III reactive MoAbs.

The slight increase in the percentage of CD34+ cells after cryopreservation was correlated to a concomitant decrease in the number of more mature myeloid cells (CD15+, CD13+, CD33+). As far as the increase in the antibody binding capacity (ABC) observed in cryopreserved cells stained with CD34 epitope class III reactive MoAbs is concerned, many explanations have been offered by authors. Recent studies have shown that class I and class II MoAbs cannot be labeled with charged fluorochromes such as FITC, because of the presence of a large number of acid residues near the binding site of the antibody. Furthermore, class I and class II MoAbs have a strong negative charge, and their conjugation to negatively charged fluorochromes such as FITC induces a significant reduction in the binding affinity of the antibody.[37] As a consequence, class I and class II-reactive MoAbs should be labeled with fluorochromes such as PE in order to obtain the most reliable results. The fact that FITC is the most negatively charged fluo-

rochrome may explain the finding that higher ABC values were detected in samples analyzed using FITC-conjugated class III MoAbs, than in those using PE-labeled CD34 MoAbs.[37]

The large variation in antigen density observed in frozen-thawed CD34+ cells could be due to a number of additional factors, such as poor reproducibility and unreliability of *Simply Cellular* beads in detecting CD34 surface antigen expression in cryopreserved cells, the differences in antibody binding affinities, as well as the lysis procedure used in this study. Recent data have shown that binding of CD34 MoAbs could be affected by lysis and fixation procedures, and that the binding of the class I antibody Immu-133 was most markedly decreased.[38-40] In a study by Basso *et al.* the percentage of CD34 positivity obtained with NH_4Cl or with Q-Lyse lysing solution was significantly higher than that obtained with FACS lysing solution (up to eight times higher in cord blood).[39] On the basis of these data, it cannot be excluded that the lysis methodology may interfere with the binding of CD34 MoAbs to the cells examined in our study. As far as the antibody binding affinities are concerned, it should be take into account that the three epitope classes are chemically and physically distinct, and that the binding capacities of the various CD34 MoAbs, either before or after conjugation with different fluorochromes, rely on the form of the MoAb and on the type of the specimen analyzed.[37] It is noteworthy that class II MoAbs are considered by some authors to be the reagents of choice for positive selection of CD34 progenitors, since the epitopes detected by these MoAbs are very close to the NH2 terminus of CD34 protein, and are contained on a stretch of linear amino acid sequence that does not directly involve sugars.[6]

Regarding the reduction of class I CD34 epitope, it can be hypothesized that the freezing procedure and/or use of DMSO may either damage a CD34 subset, or induce distinct alterations of the CD34 glycoprotein, possibly causing a reduction in their immunoreactivity with some CD34 MoAbs. Additional factors which might affect the reactivity of class I-reactive MoAbs may be lysis/fixation procedures, as well as differences in antibody binding affinities of some class I-reactive CD34 MoAbs (BI3C5, ICH3).

In conclusion, this study has shown that exposure to the cryoprotectant DMSO and freezing/thawing procedures modify the distribution of CD34 epitopes as well as the clonogenic capacity of PBPCs from nHL patients, and CD34+ blasts from AML. These findings need to considered when selecting CD34 MoAbs for enumeration and positive selection of stem/progenitor cells for research and clinical purposes.[41]

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FL and GLC were responsible for the conception of the study. FL wrote the paper. SM and LF contributed to the flow cytometric analysis of leukemic samples. BC, FM, and MD performed the CD34 cytofluorimetric assay of PBSC. AL, NP, GL and RR were involved in the processing and cryopreservation of PBSC. AL contributed to the cytofluorimetic analysis of FAS antigen. MD, DC, and AB carried out the clonogenic assay.

The criteria for the order in which the authors appear are based on the amount of work performed in this study.

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Disclosures

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