Flow Cytometry Measurement of the Labile Iron Pool in Human Hematopoietic Cells

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Abstract
Iron is important for many biological processes, and its deficiency or excess is involved in pathological conditions. Although most iron is firmly bound (e.g., in hemoglobin), some, the labile iron pool (LIP), is bound to low-affinity ligands. The level of LIP is regulated to meet the cell's requirements for iron but prevent excess. We describe herein a multiparameter flow cytometry procedure for measuring LIP in various human hematopoietic cells. Peripheral blood and bone marrow (BM) cells were loaded with calcein-AM, washed, and then incubated with or without the high-affinity iron-chelator Deferiprone (L1). Specific cell subpopulations were identified based on side-light scattering and expression of surface antigens. LIP was determined based on the ability of L1 to bind and remove iron from calcein and thereby increase the fluorescence emitted by the cells. Blood cells differ in their LIP content in the order monocytes > PMN > RBC > lymphocytes. Analysis of BM cells indicated a similar tendency among precursors of the different lineages. The results also showed that among myeloid precursors, LIP increases along cell maturation. Flow cytometry might be useful for evaluating LIP in various diseases and for studying the efficacy of iron-chelators.

Key terms
flow cytometry; iron; hematopoietic cells

Iron has a crucial role in many cellular biological processes. Deficiency or excess of iron are involved in pathological conditions, iron-deficient anemia or iron-overload (e.g., hemochromatosis, hemoglobinopathies). Iron is mostly bound to various cellular components such as hemoglobin, heme, ferritin, and various enzymes. Some iron, the labile (or chelatable) iron pool (LIP) is bound to low-affinity ligands varying in composition and quantity under different physiological settings. LIP is localized primarily, but not exclusively, in the cytosol and, as such, is regarded as the crossroad of cellular iron traffic. The level of the pool is regulated and maintained within a restricted range that meets the cell's requirements for iron but prevents excess from developing and triggering cellular damage (1).

The LIP can be quantified based on its ability to bind to cell-permeable chelators, such as calcein acetoxymethyl ester (CA-AM). Upon entering viable cells, CA-AM undergoes hydrolysis by esterases to calcein (CA) and becomes fluorescent (2,3). Its fluorescence is quenched upon binding to cellular LIP, in a stoichiometric fashion. The addition of a nonfluorescent, high-affinity chelator, such as salicylaldehyde isonicotinoyl hydrazone (SIH), which removes iron from its complex with CA, increases the fluorescence emitted by the cells (4,5). The difference in the cellular fluorescence before and after incubation with a high-affinity chelator (ΔF) reflects the amount of LIP (6). In these experiments, the cell fluorescence was measured by a spectrofluorometer. A major disadvantage of this technique is that the results are expressed as the mean of the whole cell population. The method has been applied successfully for measuring LIP in homogenous cell lines such as K562. However, in reality (in vivo),
cells are always present as heterogeneous mixtures. For example, the peripheral blood (PB) and bone marrow (BM) are composed of different cell types. Since different cells may vary significantly in their LIP, mean values may skew the results when heterogeneous populations are being studied. It is therefore crucial in such cases to use a technique that can discriminate among different cell subpopulations and yield results relevant to each cell type.

In this article, we describe a multiparameter flow cytometry (FLC) procedure for measuring LIP in specific hematopoietic subpopulations from human PB and BM. This technique is based on identifying the subpopulations by their characteristic granularity [side-light scattering (SSC)] and expression of surface antigens. LIP is determined based on its ability to quench the fluorescence of CA and the ability of high-affinity specific chelators such as Deferiprone (L1) (7,8) to bind and remove it from CA and thereby increase the fluorescence emitted by the cells. The results of the analysis of PB cells indicated that blood cells differ in their LIP content: monocytes contained the highest amount of LIP, followed by polymorphonuclear leukocytes (PMN), red blood cells (RBC), and lymphocytes. Analysis of BM cells indicated a similar tendency among the precursors of the different lineages. The results also showed that among myeloid precursors, LIP increased with cell maturation. The methodology presented herein suggest that FLC, a standard equipment in most hematological laboratories, might be useful for evaluating LIP in various diseases and for studying the efficacy of iron-chelators.

**MATERIALS AND METHODS**

The following human leukemia cell lines were used: K562—a line established from a patient with chronic myeloid leukemia in blast crisis (9); KG-1a—a line established from a patient with acute myeloid leukemia, and the cells have the morphology of undifferentiated promyeloblasts (10); HL-60—a line established from a patient with acute promyelocytic leukemia (11); DAMI—established from a patient with megakaryocytic leukemia (12); and LK—a cell line established in our laboratory from the PB of a patient with acute myelomonocytic leukemia. The cells were maintained by subculturing twice weekly at about 2 × 10^7/ml in alpha-minimal essential medium supplemented with 20% fetal bovine serum (both from Biological Industries, Beit-HaEmek, Israel). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

PB cells were obtained from normal donors and BM cells from hematological patients in remission. Samples were obtained according to the regulations of the Helsinki Committee of the Hadassah—Hebrew University School of Medicine. PB was diluted 1:1 with phosphate-buffered saline (PBS), mixed with an equal volume of 3% gelatin (Sigma, St. Louis, MO), and left to stand for 30 min at room temperature. The supernatant, containing RBC, leukocytes and platelets, was collected and the cells washed and stained. BM cells, obtained in heparin, were stained directly.

In some experiments, cells were preincubated with iron-containing compounds: Hemin (Sigma-Aldrich, Rehovot, Israel), ferric-ammonium-sulfate (FAS) or ferric-ammonium citrate (FAC) (both purchased from B.D.H., Pool, England). In other experiments, cells were preincubated with iron chelators: Deferiprone (L1)—a water-soluble, bidentate Fe(III) chelator with hydrophobic characteristics (Apotex, Weston, ON, Canada); Deferoxamine (DF)—a water-soluble, hexadentate Fe(III) chelator (8), purchased as the methane-sulfonate salt Desferal (Novartis, Basel, Switzerland); Bathophenanthroline disulfonic acid, disodium salt (bathophen) (13) (Dojin, Tokyo, Japan); SIH—a lipid-soluble, tridentate Fe(II) and Fe(III) chelator (4,14); (2-hydroxy-1-naphthylaldehyde isonicotioyl hydrazone (311) and pyridoxal 2-chlorobenzoyl hydrazone (108)—two novel iron chelators of the pyridoxal isonicotinoyl hydrazone class. SIH, 311 and 108 were obtained from Dr. Premsyl Ponka, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada.

Cellular LIP was measured as following (unless otherwise stated): Cells were washed twice with saline and incubated at a density of 0.5 × 10^6 to 1 × 10^6 per ml for 15 min at 37°C with 0.5 μM CA-AM (Sigma, St. Louis, MO), unless otherwise indicated. Then, the cells were washed twice and treated with L1 (100 μM) or left untreated. Following staining, and washing with PBS, cells were analyzed by a flow cytometer (FACS-Calibur®, Becton-Dickinson, Immunofluorometry Systems, Mountain View, CA). Cells were passed at a rate of about 1,000/s, using saline as the sheath fluid. A 488-nm argon laser and a 635-nm red diode laser were used for excitation. CA was excited at 488 nm and fluorescence was measured at 530 nm, using the FL1 PMT with logarithmic amplification as previously described (15). Cell debris and platelets were excluded from acquisition based on forward light scatter (FSC) by setting an appropriate “threshold.” Cell clusters were removed by passing the cell suspension several times through a 26-G needle.

PB and BM cells were stained at 37°C for 15 min simultaneously with PerCP-conjugated antibody (Ab) to CD45 (Becton-Dickinson, San Jose, CA) (10 μl/100 μl cell suspension).
and CA-AM (final concentration 0.5 μM), followed by washing and treatment for 1 h with L1 (100 μM) or other iron chelators. RBC, lymphocytes, monocytes, and PMN were gated based on their granularity (SSC) and size (FSC) as well as CD45 expression as routinely used in hematological immunophenotyping (16). In preliminary experiments, the identity of each cell population was verified by staining with PE-conjugated anti-glycophorin A, APC-conjugated-Abs to CD3 and CD19, APC-conjugated anti-CD14, and PE-conjugated anti-CD15 specific for erythroid cells, lymphocytes, monocytes, and PMN, respectively. In some experiments, BM cells were stained simultaneously with APC-conjugated Ab to CD33, PE-conjugated Ab to CD11b, and PerCP-conjugated Ab to CD45. Abs were purchased from IQ Products (Groningen, The Netherlands) or Becton-Dickinson (BD Biosciences, San Jose, CA). In double-staining experiments, spectral overlapping was compensated using cells stained with fluorochrome as routinely performed in flow cytometry. The mean fluorescence intensity (MFI) of at least 20,000 cells of each population was calculated by the CellQuest® software (Becton-Dickinson). For each experiment, unstained cells served as controls; their MFI was <7.

**Results**

Human hematopoietic cell lines were used to establish the technique of LIP measurement. Figure 1 depicts fluorescence intensity histograms of a representative experiment where K562 cells were loaded with CA-AM, washed, and then treated for 1 h with the iron chelators L1 or SIH. Live cells were gated based on their FSC. The procedure did not induce cell death or apoptosis as demonstrated by simultaneous staining with CA-AM and propidium iodide, a cell viability indicator (see Supplementary Material) or annexin-V which stains external phosphatidylserine, a membrane marker of apoptosis (data not shown). The MFI, which was <1 in unstained cells, increased to 27 in CA-AM loaded cells (basal fluorescence) and was further increased to 112 and 126 by SIH and L1,
respectively. The difference in the MFI before and after treatment with L1 ($\Delta F$) was used in this article to reflect the amount of LIP.

The effects of various experimental conditions on the cellular CA-associated fluorescence are depicted in Figure 2. The kinetics of CA-AM uptake by cells, reflected by their time-dependent increase in basal CA fluorescence, is shown in Figure 2A. Figure 2B shows the effect of duration of incubation with CA-AM on LIP, reflected by the difference in fluorescence ($\Delta F$) of cells treated or untreated for 1 h with L1. The results show that $\Delta F$ plateaued after 15-min incubation with CA-AM. The effect of CA-AM concentration was determined by loading cells for 15 min with different doses of CA-AM. Basal cell CA fluorescence was dose-dependent (not shown); $\Delta F$ (Fig. 2C), however, after an initial dose-dependent increase, plateaued at CA-AM concentrations above 0.25 µM. Taken together, these results indicate that CA-AM uptake and the basal CA fluorescence were dependent on CA-AM concentration and time of incubation. $\Delta F$, however, was independent on the intensity of the basal CA fluorescence. Moreover, only a small proportion of the basal CA fluorescence could be dequenched following addition of L1, indicating that the amount of cellular chelatable iron was constant and limited.

We next determined the effect of extracellular iron uptake on LIP (Fig. 2D). Cells were preincubated for 2 h with iron-containing compounds, loaded with CA-AM, followed by 1-h incubation with or without L1. The $\Delta F$ results show that transferrin, FAC, FAS, and hemin, at concentrations that provided 8 µM iron, increased cellular LIP ($P < 0.05$).

The effect of iron chelators on CA-loaded cells is presented in Figure 2E. L1 and SIH, which are known to efficiently penetrate cells and chelate their iron (17), dequenched CA fluorescence most effectively, whereas DF and 108, which enter cells poorly, did not have a significant effect.

The above experiments were carried out in both K562 and HL60 cells with similar results. We next determined LIP in additional human hematopoietic cell lines (Fig. 2F). The results indicate that K562, an erythroleukemia cell line, contained more LIP than KG1A—an undifferentiated myeloblast cell line, HL60—a promyelocytic cell line, DAMI—a megakaryoblast cell line, and LK—a monoblast cell line.

**LIP Content in PB and BM Cells**

PB and BM cells were stained simultaneously with CA-AM and a PerCP-conjugated anti-CD45 Ab, and then treated with or without L1. CD45 is an antigen present at different intensities on all nucleated hematopoietic cells (18). Various cell populations were gated based on CD45 and granularity (SSC) and analyzed for CA fluorescence. Figure 3A shows the gates set for PB RBC, lymphocytes, monocytes, and PMN, while Figure 3B depicts the histograms of cell distribution with respect to basal fluorescence as well as their MFI. The results of seven such experiments, carried out with cells from different donors, indicated that PB cells differ in their basal CA fluorescence. Analysis of these samples showed LIP ($\Delta F$ of the MFI in CA-stained cells before and after treatment with L1) to be 39.5 ± 4.5, 28.2 ± 2.6, 59.6 ± 7.8, and 41.8 ± 5.5 (average MFI ± SD) for RBC, lymphocytes, monocytes, and PMN, respectively.

Analysis of BM cells in six experiments indicated that the differences in basal fluorescence and LIP of the cell lineages are similar to that in the PB (not shown). Figures 4A and 4B depict a representative experiment where BM cells were stained simultaneously with CA-AM and an Ab to CD45, and then treated with or without L1. Gates were set on RBC, monocytes, lymphocytes, and PMN based on the intensity of CD45 and granularity (side light scatter). The distribution histogram and MFI of each cell population with regard to CA-staining in a representative experiment are indicated.
expression. The cells were stained in parallel with APC-conjugated Ab to CD33, PE-conjugated Ab to CD11b, and PerCP-conjugated Ab to CD45. During myeloid differentiation, CD33 expression decreases while CD11b expression increases (19). Analysis of the gated cells in the same six experiments with respect to expression of CD45, CD33, and CD11b (Fig. 4C) confirms that maturation of myeloid precursors is associated with increasing in their CD45; cells in G1 are the most immature while in G6 the most mature ones. Taken together, these results show an increase in LIP during myeloid maturation.

DISCUSSION

We used flow cytometry to measure the cytosolic pool of chelatable iron in hematopoietic cells. Cells were first loaded with CA-AM—a large hexadentate ligand that gains entry into viable cells via its acetoxymethyl ester. Once hydrolyzed, it becomes trapped in the cytoplasm and emits intense green fluorescence (2,3). The CA-AM uptake and CA content depend on the membrane properties of the cells, their esterases content and activity as well as other factors that affect efflux and degradation. In the cells, various metal ions bind to CA and quench its fluorescence, depending on the ion concentrations and binding affinity (20–22); among these metals, iron is by far the most abundant (20). Thus, the basal CA fluorescence depends on the intracellular CA content and the amount of the labile (chelatable) iron pool (LIP). To measure LIP, CA-loaded cells were incubated in parallel with or without the iron chelator—L1. The difference in CA fluorescence ($\Delta F$) between the two samples, with or without the chelator, reflects the LIP, i.e., iron that could be removed from CA by the chelator. The measured LIP was independent of the factors that affect the basal CA fluorescence. Several studies have indicated that CA is expelled out of cells by the multidrug resistance machinery (23,24). To rule out potential effect of L1 on this machinery, cells were labeled simultaneously with CA-AM and rhodamine 123, followed by 1-h treatment with or without L1. No difference in rhodamine 123 fluorescence was observed in cells that were treated or untreated with L1 (data not shown). Our results (Fig. 2E) indicated that L1 is comparable to SIH, an iron chelator used previously for LIP measurement (25). The specificity of the assay was tested by preincubating cells with various iron-containing compounds; the results indicated that iron uptake was associated with an increase in LIP.

The advantage of flow cytometry of LIP in PB or BM cells is the ability to measure LIP in specific subpopulations based on multiparameter cell-based analysis. The various subpopulations can be identified based on their characteristic size (FSC), granularity (SSC), and expression of surface antigens. Although the LIP data are expressed in arbitrary fluorescence units, MFI, rather than molar concentrations, it is useful for comparative purposes.

The results of analysis of PB cells indicated that blood cells differ in their LIP content: Monocytes contained the highest amount of LIP, followed by PMN, RBC, and lympho-
cytes. Analysis of BM cells indicated a similar tendency among the precursors of the different lineages. The results also showed that among myeloid precursors, LIP increased with cell maturation. Preliminary experiments suggest that in erythrocytic cells LIP is altered under different physiological (e.g., maturation) and pathological (e.g., iron-overload) conditions (not shown). Because FLC is a standard methodology in most clinical hematological and immunological labs (for diagnostic immunophenotyping of immunological and hematological diseases), the results suggest that it can also be useful for evaluating LIP in various diseases and for studying the efficacy of chelators.

Literature Cited