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ACKNOWLEDGEMENTS. We thank Prof. Sunil Bajpai, Director, Birbal Sahni Institute of Palaeobotany (BSIP), Lucknow for providing facility and encouragement. We also thank Prof. I. B. Singh, Geology Department, University of Lucknow for constructive sedimentological discussions that helped improve the manuscript. We also thank Drs Rahul Garg, Vandana Prasad, Biswajeet Thakur of BSIP for help.

Received 29 May 2013; revised accepted 3 October 2013

Heterogeneity of reticulocyte population in mouse peripheral blood

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Reticulocytes constitute about 1–6% of total blood erythrocyte in mice and their numbers may be upregulated markedly in a variety of situations like treatment with erythropoietin and induction of anaemia, etc. Reticulocytes originate in the bone marrow from erythroblasts by the process of nuclear extrusion, and are released into the blood where they further mature into erythrocytes. From blood cells, reticulocytes may be isolated using discontinuous Percoll density gradient (DPDG) fractionation. Highly enriched reticulocyte preparations are obtained from the low buoyant density cellular fraction from DPDG and such enriched cell preparations have as such been used extensively as a source of purified blood reticulocytes in many studies. The possibility of presence of reticulocytes in other cell fractions of higher buoyant densities has, however, not been examined. In the present study, we have fractionated mouse blood cells on a five-layered discontinuous DPDG and the presence of reticulocytes was monitored in each fraction by staining for Ter-119 (transferrin receptor) and CD71 markers that together are specific markers for blood-derived reticulocytes. Our results indicate that only 16% of the blood reticulocytes were present in the low buoyant density DPDG fraction, the rest being distributed in heavier DPDG fractions. Expression levels of some important functional and phenotypic reticulocyte markers like CD47 (integrin associated protein), CD147 (basigin), cellular calcium levels as well as RNA contents were compared for reticulocyte populations derived from different DPDG fractions. Our results show significant differences in the expression of these markers in reticulocyte populations of different buoyant densities and indicate that the reticulocytes isolated from low buoyant density fractions of blood cells may represent only a minor subpopulation of blood reticulocytes.

Keywords: Buoyant density, erythrocytes, mouse blood cells, reticulocytes.

INBRED C57Bl/6 male mice (8–12 weeks old, 20–25 g body wt) were used throughout this study as the source of blood cells. Animals were obtained from the National Institute of Nutrition, Hyderabad and maintained in the animal house facility at Jawaharlal Nehru University (JNU), New Delhi using standard environmental conditions. All the experimental protocols were approved by JNU Institutional Animal Ethics Committee. For preparing a discontinuous Percoll density gradient (DPDG), Percoll (Sigma-Aldrich, India) solutions with mean buoyant densities 1.06500, 1.06805, 1.07465, 1.08200 and 1.08705 g/ml were prepared in accordance with the manufacturer's instructions[1]. Discontinuous five-step gradients were prepared by superimposing 2 ml of each density layer in 17×100 mm polypropylene centrifuge tubes. Blood was collected in PBS in the presence of EDTA (5 mM) and washed three times with ice-cold normal saline containing 10 mM HEPES buffer (pH 7.4) and 1% FBS. Mouse erythrocytes (1 ml, 20% hematocrit) were layered on the Percoll gradients followed by centrifugation (4100 rpm, 30 min at 4°C). Six fractions were collected carefully starting from the top. The cells in each fraction were washed with PBS, suspended in 1 ml of PBS and cell recovery determined by cell counting on hemocytometer.

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Unfractionated blood cells as well as the six cellular fractions separated on the five-layered DPDG were double-stained with anti-CD71 and anti-Ter-119 monoclonal antibodies and analysed on a flow cytometer. Results in Figure 1 show that virtually all cells in all fractions were Ter-119$^+$, that is, an erythroid lineage marker, and there are very few events in the Ter-119 negative quadrants. This is expected since 99.9% of all blood cells are erythrocytes, including reticulocytes and only about 0.1% cells belong to the leukocyte family. Blood leukocytes, therefore, do not significantly interfere with the flow cytometry results on blood erythrocytes. About 2% cells in the unfractionated blood cells were Ter-119/CD71 positive reticulocytes. In F1 (fraction-1), that is, the lowest buoyant density fraction, about 43% of the cells were reticulocytes, which is a 21-fold enrichment of these cells as compared to the unfractionated blood cells. The percentage of reticulocytes was lower in heavier DPDG fractions (F2–F6), but significant numbers of reticulocytes were nevertheless detected in all fractions (Figure 1).

DPDG fractionation of mouse blood cells was repeated five times and combined data from these experiments is given in Table 1. The top DPDG fraction (F1) was enriched in reticulocytes by about 21-fold, whereas the next two fractions (F2 and F3) were enriched by 7- and 3.6-fold respectively. Quantitative recovery of reticulocytes from the top F1 fraction (14.66×10^6) was, however, only 16.7% of the combined reticulocyte recovery from all fractions (87.83×10^6 cells). Maximum absolute recovery of reticulocytes (55%, i.e. 48.17×10^6 out of a total recovery of 87.86×10^6 reticulocytes) was from F5, which coincides with the fraction with highest recovery of erythrocytes. Since significant numbers of reticulocytes were detected in all DPDG fractions, it was important to determine whether reticulocytes of the top DPDG fraction and those in other fractions were significantly different in characteristics. For this purpose, the expression of some important phenotypic markers as well as some functional markers was examined for reticulocytes derived from various DPDG fractions.

Figure 2 *a* shows that the mean fluorescence intensity (MFI) of CD47 expression on reticulocytes fell gradually from F1 to F6. Overall, the decline in CD47 expression was about 40% from F1 to F6. CD47 is a marker that protects cells from being phagocytized by macrophages[2,3]. Lower CD47 expression on high buoyant density reticulocyte may result in greater susceptibility of this heavier sub-population of reticulocytes to macrophages. In contrast to the CD47 expression, relative expression of CD147, a marker that regulates the recirculation of erythrocytes from spleen[4], was lowest on F1 reticulocytes, increased significantly till F4 and then declined marginally (Figure 2 *b*). Thus, for quantitative expression of both CD47 and CD147 markers, reticulocytes from different DPDG fractions were found to differ significantly.

Cellular levels of calcium and residual RNA were also examined for reticulocytes in all DPDG fractions. Cellular Ca^{2+} contents were highest in the reticulocyte fraction of lowest buoyant density (MFI 440 and 253 respectively, in F1 and F2) and fell gradually in reticulocytes from F3

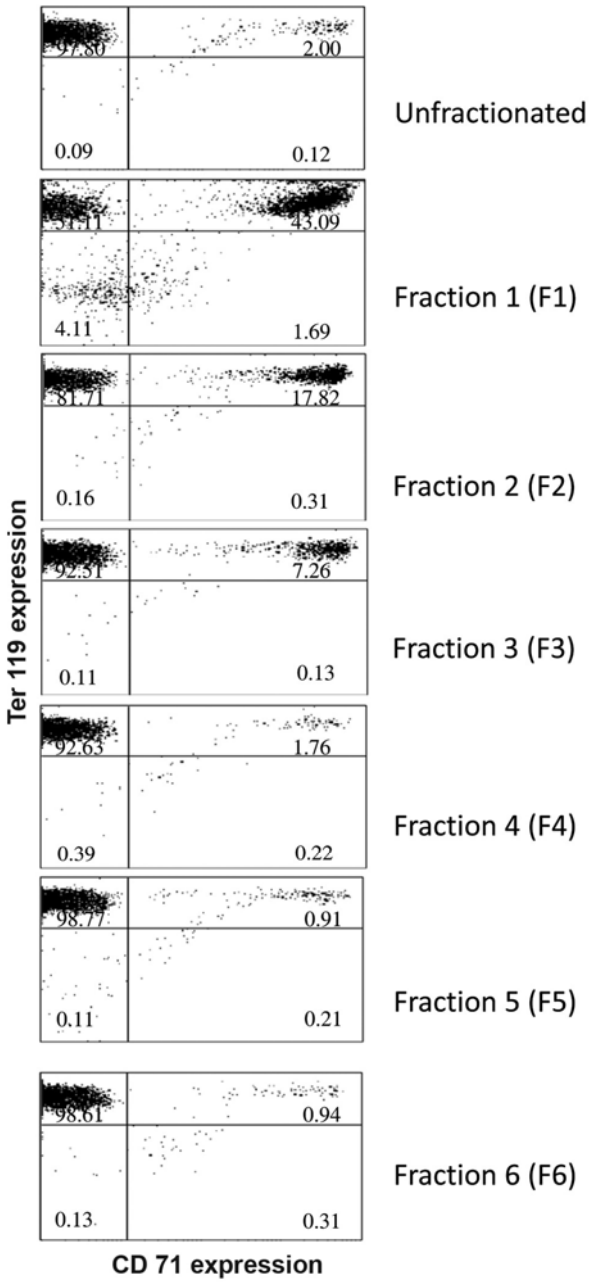

Figure 1. Enumeration of reticulocytes in different discontinuous Percoll density gradient (DPDG) fractions. Mouse blood cells were fractionated on five-layered DPDG and the cells obtained from six resulting cell fractions (F1–F6) were stained with anti-mouse Ter119 and CD71 monoclonal antibodies and analysed on flow cytometer. For flow cytometric studies, 1 million cells from each fraction were washed and resuspended in pre-warmed HEPES buffer supplemented with 2% FBS and incubated with 1 µg monoclonal antibody for 30 min. Representative quadrant plots depict the staining pattern of blood cells with CD71 and Ter119 monoclonal antibodies. Values in each quadrangle show the percentage of cells.

Table 1. Percentage and absolute recoveries of reticulocytes in different discontinuous Percoll density gradient (DPDG) fractions. Cells obtained from various DPDG fractions were stained with anti-mouse CD71 and Ter119 antibody and analysed on flow cytometer. Each value represents mean ± SEM of data from five mice

Fraction	Buoyant density (mg/ml)	Total cells recovery ($\times 10^6$)	Reticulocytes (%)	Reticulocytes absolute number ($\times 10^6$)
F1	<1.06500	35.43 ± 3.44	41.52 ± 5.67	14.66 ± 2.03
F2	1.0650–1.0711	45.53 ± 5.12	12.93 ± 2.58	5.98 ± 1.18
F3	1.0711–1.0782	100.50 ± 28.71	4.62 ± 0.61	4.60 ± 1.06
F4	1.0782–1.0858	589.00 ± 60.18	1.92 ± 0.30	11.01 ± 1.33
F5	1.0858–1.0883	2577.5 ± 44.98	1.88 ± 0.23	48.17 ± 5.03
F6	>1.08830	153.88 ± 22.04	2.32 ± 0.34	3.41 ± 0.29
Unfractionated		5400.00 ± 99.58	2.43 ± 0.22	129.08 ± 15.28

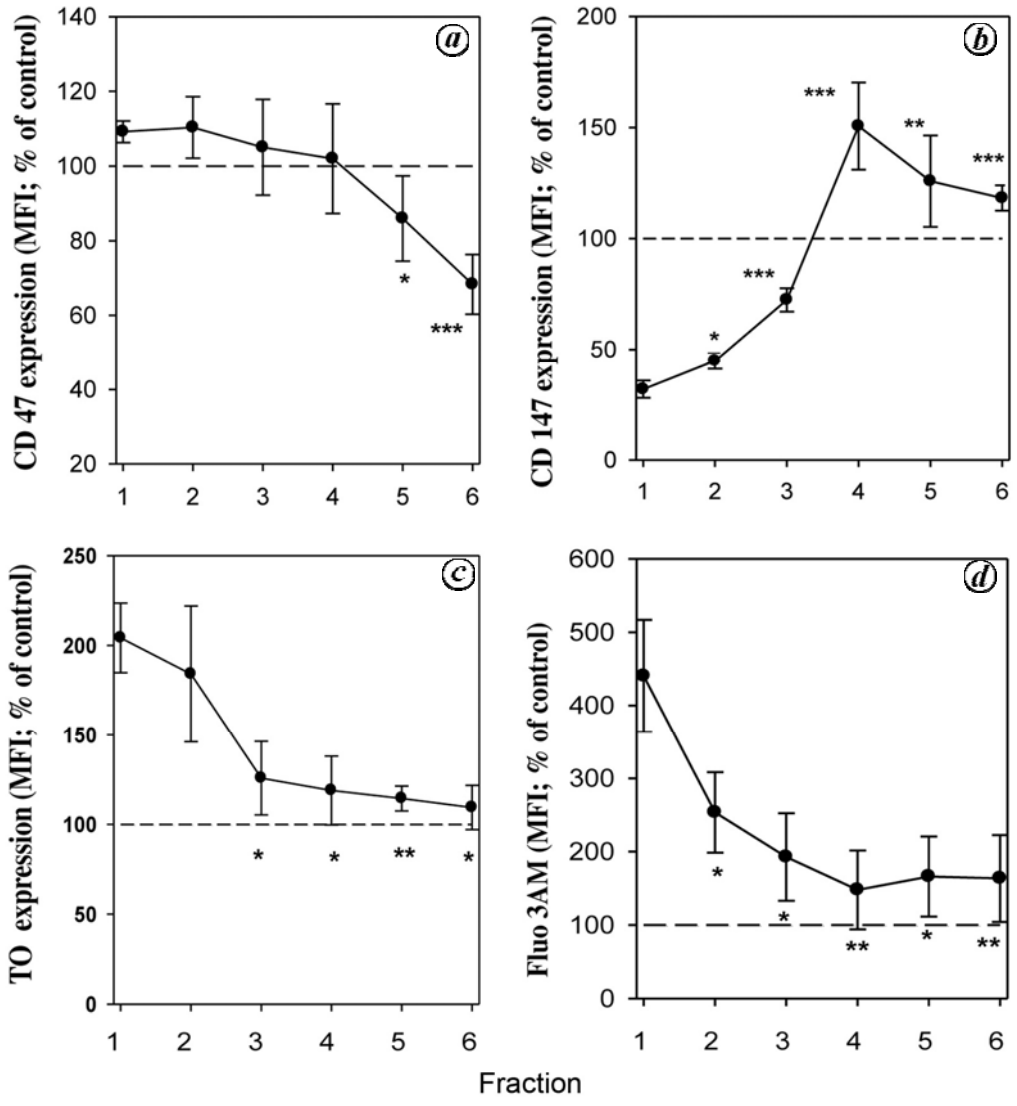

Figure 2. Expression of CD47 and CD147 markers and intracellular calcium and RNA contents in reticulocytes in six fractions (F1–F6) of cells derived from the DPDG fractionation. The cells were double-stained with antimouse CD71 along with anti CD47 mab (***a***), or anti CD147 mab (***b***), Fluo3AM (***c***), and thiazole orange (TO) dye for intracellular RNA staining (***d***), and analysed on a flow cytometer. Staining for CD47 and CD147 markers was carried out as described in the legend to Figure 1. Fluo3AM and TO staining procedures were as described in the literature[5,7]. In each case, the cells were gated based on CD71 expression and expression of other markers (CD47, CD147, Fluo3AM and TO dye) examined on reticulocytes. Mean fluorescence intensity (MFI) of markers (CD47, CD147, Fluo3AM and TO dye) for cells in different fractions has been depicted as percentage of MFI for reticulocytes in unfractionated blood cells. Each plotted value is depicted as mean ± SEM of results from five independent determinations. Dotted line in each graph shows the expression of the respective markers on unfractionated reticulocytes taken as 100. ***$P < 0.0005$, **$P < 0.005$, *$P < 0.05$, etc. (Student's *t*-test, comparisons of MFI values of reticulocytes in F1 with reticulocytes in other fractions, F2–F6).

to F6 (Figure 2 *c*). It has previously been shown that the maturation of reticulocytes into erythrocytes is associated with a decline in intracellular levels of calcium ions[5,6]. A progressive decline in cellular calcium could result from a decreased permeability of calcium ions as reticulocytes mature. Maturation of reticulocytes is associated with progressive loss of residual cellular organelles and a lower intracellular calcium level may be a consequence of loss of endoplasmic reticulum.

Since we find that the calcium ion concentration in reticulocytes varies inversely with the buoyant density of blood reticulocytes, it is tempting to speculate that the maturation of reticulocytes in blood is also associated with increase in the cellular buoyant density. This proposition was further examined by assessing the cytoplasmic levels of residual RNA in reticulocytes isolated from different DPDG fractions. It is a well-established fact that the level of residual RNA assessed by staining with thiazole orange dye, falls progressively as reticulocytes mature to the final stage of erythrocytes that have no cellular RNA[14]. Our results indicated that the cytoplasmic levels of RNA declined progressively in reticulocytes derived from F1 to F6; the total decrease being about 50% (Figure 2 *d*). Our results are, thus, concurrent with the proposition that reticulocytes of higher buoyant density may represent more mature stages of the blood cells.

Taken together, the first part of our study shows that reticulocytes are present in significant numbers in all fractions of blood cells fractionated on the basis of buoyant density. Reticulocyte fraction with the lowest buoyant density though significantly enriched in reticulocytes, represents only 16% of the total reticulocytes in blood. While using cells in this low buoyant density fraction as representative blood reticulocytes, it should be kept in mind that these cells comprise only a minor portion and do not represent the whole blood reticulocyte population. Indeed, reticulocytes in heavier buoyant density fractions of DPDG are significantly different in several important properties and expression of functional markers. From our results, it is also tempting to speculate that the maturation of reticulocyte released from bone marrow to the final stage of maturity, i.e. erythrocyte, may be associated with progressive increase in buoyant density of reticulocytes. On the basis of this proposition, it may be predicted that in acute anaemia when there is a rapid influx of reticulocytes in blood, it should be accompanied with at least a transient increase in relative proportion of F1 reticulocytes that represent the earliest phase of immature reticulocytes in blood (F1). These propositions would, however, require further validation.

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ACKNOWLEDGEMENTS. We thank DST, New Delhi for financial support. N.B. thanks UGC, New Delhi for a junior research fellowship.

Received 9 April 2013; revised accepted 25 September 2013