J Clin Pathol 1994;47:736–739

Reticulocyte maturity as an indicator for estimating qualitative abnormality of erythropoiesis

K Watanabe, Y Kawai, K Takeuchi, N Shimizu, H Iri, Y Ikeda, B Houwen

Department of Laboratory Medicine, School of Medicine, Keio University, Tokyo, Japan
K Watanabe
Y Kawai
K Takeuchi
N Shimizu
H Iri

Department of Medicine, Keio University
Y Ikeda

Department of Pathology, School of Medicine, Loma Linda University, Loma Linda, California, USA
B Houwen

Correspondence to: Dr Kiyoaki Watanabe, Department of Laboratory Medicine, School of Medicine, Keio University, 35, Shinanomachi, Shinjuku-ku, Tokyo, Japan 160

Accepted for publication 11 January 1994

Abstract

***Aims*—To determine the maturity of reticulocytes in patients with anaemia as a result of various haematological disorders including those with qualitative abnormalities such as ineffective erythropoiesis or dyserythropoiesis.**

***Methods*—The number of mature reticulocytes was measured with flow cytometry in venous blood samples from 122 patients with haematological disorders and 100 healthy controls. Reticulocytes were classified into three categories by the fluorescence intensity of auramin O staining: low fluorescence ratio (LFR), medium fluorescence ratio (MFR), and high fluorescence ratio (HFR). Immature reticulocytes were determined as the aggregate of MFR and HFR (%).**

***Results*—The mean (2SD) number of immature reticulocytes in 100 normal subjects was 9·0 (7·0)%. Significantly high mean values of immature reticulocytes with a normal or reduced reticulocyte count were shown in 90 patients with dyserythropoietic or ineffective erythropoietic conditions, such as acute myeloid leukaemia (AML) (n = 37), myelodysplastic syndrome (MDS) (n = 35), aplastic anaemia (AA) (n = 8), or megaloblastic anaemia (MA), (n = 6). Reticulocyte ratios returned to normal after successful treatment of patients with AML (n = 10) and MA (n = 3). However, high percentages of immature reticulocytes with increased reticulocyte counts were consistently observed in patients with enhanced erythropoiesis such as those with acquired autoimmune haemolytic anaemias (AIHA) (n = 4) or acute blood loss (ABL) (n = 4). Reticulocyte maturity was within the normal range in patients with reduced erythropoiesis such as occurs in chronic renal failure (CRF) (n = 11), or in iron deficiency anaemia (IDA) (n = 13).**

***Conclusions*—The evaluation of reticulocyte maturity with total reticulocyte count seems to be clinically useful for estimating the qualitative impairment of erythropoiesis, and so could help differentiate haematological disorders.**

(*J Clin Pathol* 1994;47:736–739)

An estimate of the reticulocytes present in the peripheral blood is useful for evaluating the erythropoietic activity of bone marrow,[1 2] and provides diagnostic information in cases of anaemia.[3 4]

In 1932 Heilmeyer[5] classified reticulocytes, according to the morphology of their ribosomal structures, as groups I, II, III and IV. He showed that groups I and II immature reticulocytes are released into the peripheral blood during periods of stimulated erythropoiesis. Although efforts were made to establish the usefulness of reticulocyte immaturity in the clinical laboratory, subclassification of reticulocytes has been difficult in the routine laboratory due to the large interobserver variation both in morphological identification and enumeration. Conventional microscopic methods, therefore, have not fully established the clinical usefulness of determining the presence of immature reticulocytes in peripheral blood. Their presence may reflect an increase in erythropoiesis and in red blood cell turnover.[6 7]

Flow cytometry has recently made it possible to determine rapidly the number of RNA rich immature reticulocytes,[8–12] as the reticulocyte fluorescence intensity is directly proportional to RNA content. With this method, several authors have shown the clinical utility of the determination of immature reticulocytes.[8 9 11]

Tanke *et al*[8] demonstrated that significant amounts of immature reticulocytes are detectable, even in healthy people, as a consequence of blood donation, or in patients after bone marrow transplantation. Davis *et al*[9] found that quantitation as a "reticulocyte maturity index" was useful in evaluating erythropoiesis in 20 patients treated with bone marrow transplantation. Moreover, reticulocyte immaturity has been found to be affected by iron status in those patients with iron deficiency anaemia, and to be correlated with total iron binding capacity and the serum ferritin concentration.[11]

These studies indicate that abnormal profiles of reticulocyte immaturity can result from quantitative changes in erythropoiesis. Whether changes in reticulocyte maturation also occur in patients with qualitative defects in erythropoiesis is unknown.

Methods

We evaluated 122 patients with haematological disorders, 49 females and 73 males, aged 9 to 88 years (mean 52 years). One hundred healthy Japanese subjects (50 women and 50 men, aged 20 to 57 years old (mean 37 years)) were used as controls.

Table 1 Mean (2 SD) reticulocyte maturity in 100 normal subjects

	Immature reticulocytes (%)	*Reticulocyte count ($\times 10^9$/l)*
Total (n = 100)	9·0 (7·0)	6·5 (3·8)
Women (n = 50)	8·1 (6·0)	5·6 (3·4)
Men (n = 50)	9·8 (8·0)	7·4 (4·2)

All control subjects had normal haemoglobin values (120–150 g/l for women and 140–170 g/l for men), mean corpuscular volume (80–100 fl), mean corpuscular haemoglobin concentration (320–360 g/l), leucocyte counts (4–8 $\times$ 10^9/l), platelet counts (150–350 $\times$ 10^9/l) and normal leucocyte differentials. All patients had low haemoglobin concentrations, below 110 g/l for women and 130 g/l for men.

They were categorised into four groups: group A (n = 8) with anaemia due to haemolysis or acute blood loss (ABL)—four with "warm type" acquired autoimmune haemolytic anaemia (AIHA). The remaining four patients, who had severe gastrointestinal bleeding due to gastric ulcer, were designated as anaemia caused by ABL.

Group B (n = 90) included patients with dyserythropoiesis or ineffective erythropoiesis: 37 cases of acute myeloid leukaemia (AML), subtypes M_1-M_5; 35 cases with myelodysplastic syndrome (MDS); eight cases with aplastic anaemia (AA); six cases with megaloblastic anaemia (MA); and four cases with paroxysmal nocturnal haemoglobinuria (PNH). The diagnosis and classification of AML and MDS were based on standard French-American-British (FAB) morphological and cytochemical criteria.[13,14] The patients with AML comprised nine cases of type M_1, 13 cases of type M_2, seven cases of type M_3, two cases of type M_4 and five cases of type M_5. The complete remission of AML patients was made according to the Cancer and Leukaemia Group B criteria.[15] Patients with aplastic anaemia were defined by criteria described by Camitta *et al*.[16] The six cases with megaloblastic anaemia were attributable to vitamin B_{12} deficiency. Group C (n = 11) consisted of patients with anaemia as a result of chronic renal failure (CRF). Serum creatinine concentrations in these patients were above 884·4 μmol/l. Group D (n = 13) comprised patients with iron deficiency anaemia (IDA). Criteria included serum ferritin concentrations of less than 11 pmol/l and a total iron binding capacity of more than 72 μmol/l.

EDTA-2K anticoagulated venous blood samples were residual material from specimens sent to the haematology laboratory for routine testing. Reticulocyte count and reticulocyte immaturity were determined using a fully automated reticulocyte counter, the Sysmex R-1000 (TOA Medical Electronics Co Ltd, Kobe, Japan).[17] Reticulocytes were identified in the R-1000 by fluorescence based on binding of auramine O to RNA. Staining, incubation, reticulocyte analysis and blood cell discrimination were performed automatically, without operator intervention.

Results were displayed in a two-dimensional scattergram using fluorescence intensity and laser light scatter. The reticulocyte count was expressed both as a percentage and as an absolute count. Reticulocytes were further classified into three categories according to fluorescence intensity: low fluorescence ratio (LFR); medium fluorescence ratio (MFR); and high fluorescence ratio (HFR). Immature reticulocytes displayed HFR and more mature cells, LFR. We defined immature reticulocytes as the sum of the cells of MFR and HFR (%).

Results are presented as the mean (2SD). Significance was determined using an unpaired Student's *t* test.

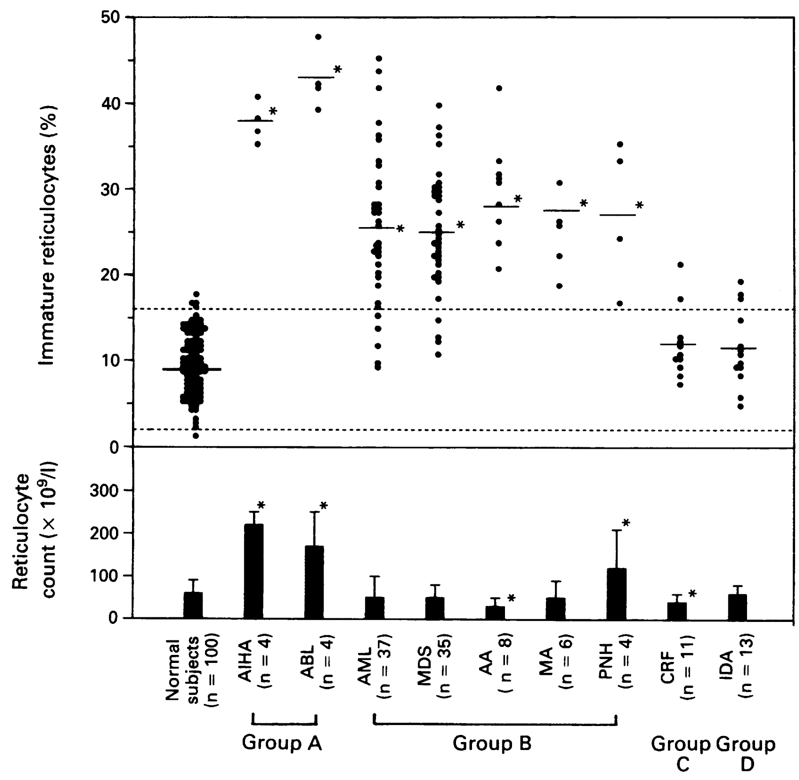

*Figure 1 Reticulocyte maturity in patients with various types of haematological disorders. Upper panel: bars indicate mean value of immature reticulocytes. Normal range (mean (2SD)) is illustrated as dotted lines (- - - - - - - -). Lower panel: reticulocyte count is shown as the mean (2SD). * Difference is significant (p < 0·001). AIHA = acquired autoimmune haemolytic anaemia, ABL = acute blood loss, AML = acute myeloid leukaemia, MDS = myelodysplastic syndrome, AA = aplastic anaemia, MA = megaloblastic anaemia, PNH = paroxysmal nocturnal haemoglobinuria, CRF = chronic renal failure, IDA = iron deficiency anaemia.*

Results

IMMATURE RETICULOCYTES

Immature reticulocytes were readily detectable in the peripheral blood of 100 normal subjects (table 1). The normal range for immature reticulocytes was calculated as 2·0% to 16·0%—that is, the mean (2SD) with a median of 9·0%. There were no significant sex related differences in the normal range ($p > 0.05$).

In Group A (fig 1) all patients with either AIHA or ABL showed a sharp increase in immature reticulocyte percentages, with an increase in the absolute reticulocyte count. There was a significant increase in the proportion of immature reticulocytes in all patients

Table 2 Mean (2 SD) reticulocyte maturity in patients with MDS subtypes

Subtype	Immature reticulocytes (%)	Reticulocyte count (× 10^9/l)
Refractory anaemia (n = 17)	24·9 (13·6)	63·8 (37·1)
Refractory anaemia with RS (n = 3)	23·0 (8·2)	39·2 (25·0)
RAEB (n = 7)	22·6 (15·0)	21·2 (18·8)
CMMoL (n = 4)	26·7 (18·1)	67·2 (23·0)
RAEB in transformation (n = 2)	29·3 (17·0)	18·6 (10·3)

RS = ringed sideroblast; RAEB = refractory anaemia with excess of blasts; CMMoL = chronic myelomonocytic leukaemia; MDS = myelodysplastic syndrome.

in group B (mean (2SD)): AML 25·3 (17·8)%; MDS 24·7 (13·4)%; AA 28·3 (8·7)%; MA 27·4 (16)%; PNH 27·4 (17)% ($p < 0·001$). Values in all the patients with AA, MA, or PNH, 32 of 37 (86%) patients with AML, and 31 of 35 (89%) patients with MDS were higher than normal.

In contrast to group A, group B patients had normal or reduced absolute reticulocyte counts, with the exception of PNH patients with high reticulocyte counts.

In patients with anaemia due to CRF (group C) immature reticulocyte values (mean (2SD) 11·8 (8·2)%) were not significantly different from those of normal subjects ($p > 0·05$), while the absolute reticulocyte count was significantly reduced ($p < 0·001$). In patients with IDA (group D) both immature reticulocytes (mean (2SD) 11·5 (9·0)%) and the reticulocyte count were not significantly increased when compared with those of normal subjects ($p > 0·05$).

Reticulocyte immaturity was evaluated in the five subtypes of MDS patients (table 2). A high percentage of immature reticulocytes combined with a normal or slightly reduced absolute reticulocyte count was consistently observed in all MDS subtypes. Similar results were obtained for subtypes of AML: immature reticulocyte values were not different among M_1, M_2, M_3, M_4 and M_5.

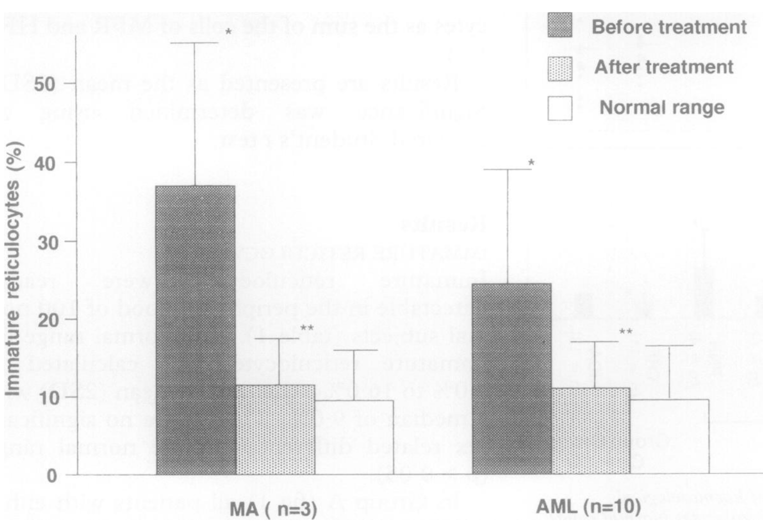

*Figure 2 Changes in immature reticulocytes in patients with AML (acute myeloid leukaemia) or MA (megalobolastic anaemia) after treatment. Each value is illustrated as the mean (2SD). * Difference is significant ($p < 0·01$); ** not significant ($p > 0·05$). Reticulocyte maturity was determined three to seven days after treatment in cases with MA and 10 to 14 days in cases with AML.*

CHANGES IN RETICULOCYTE IMMATURITY

In group B patients (fig 2) we investigated whether reticulocyte immaturity values would normalise after the abnormality had been brought under control. As it was difficult to obtain a definite improvement for the dyserythropoiesis associated with AA, MDS, and PNH, we studied three cases of MA with complete recovery after administration of vitamin B_{12}, and 10 cases of AML with complete remission after chemotherapy. The immature reticulocyte percentage was measured only when the haemoglobin concentrations of these patients became normal after treatment. In both groups the immature reticulocyte values decreased significantly and returned to the normal range after treatment ($p > 0·05$). In contrast, the immature reticulocyte percentage remained consistently abnormal in the five patients with MDS who failed to achieve remission (data not shown).

Discussion

Immature reticulocytes have been proposed as the shift reticulocytes corresponding to the Heilmeyer stage I and II.[5 7 18] Flow cytometric analysis has recently shown that the measurement of reticulocyte maturity is useful.[8-12 19]

In this study we defined immature reticulocytes as the population showing relatively high fluorescence (MFR + HFR (%)). This value was generally in accordance with that of previous reports,[12 15] although one study showed a higher range. These results do not necessarily indicate that the MFR + HFR fraction definitely consists of immature reticulocytes or shift reticulocytes.

The normal range for immature reticulocytes reported by Heilmyer,[5] using the classic microscopic methods, is calculated as about 7·5–11% of the total reticulocyte count. Although our values were similar (2–16%), it is difficult to compare the results determined by two completely different methods. The normal value for the mean fluorescence intensity of the entire reticulocyte population was recently found to be 77 (7·7)[9] or 69·7 (2·6).[11] Although determined by the same analytical procedure, it is not really possible to compare these ranges with the present results, as the mean fluorescence intensity is not directly proportional to the percentage of reticulocytes that contain a higher amount of RNA. Thus our assessment of immature reticulocytes as the number of cells in MFR + HFR (%) is presently acceptable as there is no alternative definition.

We showed that the proportion of immature reticulocytes was increased significantly, despite a normal or reduced total number of circulating reticulocytes, in patients with certain types of anaemia (group B; $p < 0·001$), including AML, MDS, AA and MA. A large portion of these patients had a higher than normal percentage of immature reticulocytes, although a limited number of patients with AML (15%) and with MDS (14%) showed an overlap of data in the normal range (fig 1). This observation strongly supports the clinical value of changes in reticulocyte maturity in patients with such disorders.

AML and MDS display clonal haematopoiesis[20 21]; the anaemia associated with these disorders arises from dyserythropoiesis due to impairment of stem cells.[22–24] Although AA is a self-perpetuating disorder characterised by a reduction or dysfunction of pluripotential stem cells,[25] recent research has shown its close association with clonally disordered haematopoiesis and dyserythropoiesis.[24 26]

MA is known to arise from an impairment of DNA synthesis which results in ineffective erythropoiesis associated with some degree of extramedullary haemolysis.[27]

It is reasonable to speculate that an increase in reticulocyte immaturity, combined with a reduced or normal reticulocyte count, might reflect either dyserythropoiesis or ineffective erythropoiesis in patients with certain haematological disorders. This is the first report, we believe, to describe an association between reticulocyte immaturity and a qualitative abnormality of erythropoiesis.

PNH is also a clonal disorder[24 28] showing dyserythropoiesis. We expected that the reticulocyte immaturity in patients with PNH would resemble that of patients with AML, MDS, or AA, but found a high degree of immaturity only in association with an increased reticulocyte count.

In haemolytic anaemia and ABL our data show that reticulocyte immaturity is increased when erythropoiesis is stimulated—as indicated by an increased absolute reticulocyte count—and are consistent with previous reports.[6 8 12 29] This increase may be produced by the enhanced stimulation of bone marrow by erythropoietin.

Reticulocyte immaturity was not increased in conditions accompanied by a decrease in erythropoietin stimulation such as CRF.[29] The immature reticulocytes measured in this study may therefore represent an increase of erythropoiesis in response to erythropoietin stimulation of a small number of erythroid precursors.

Wells *et al*[11] very recently showed that the mean fluorescence intensity of reticulocytes was correlated with the serum total iron binding capacity and ferritin concentrations, suggesting that reticulocyte immaturity is influenced by a patient's iron status. Clarification may be needed, because our patients with IDA had no significant increase in reticulocyte immaturity, with ferritin concentrations of less than 11 pmol/l and a total iron binding capacity exceeding 72 μmol/l.

In conclusion, the quantitation of immature reticulocytes can be clinically useful for estimating qualitative changes in erythropoiesis, and may provide a diagnostic aid in the differential diagnosis of patients with haematological disorders.

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