ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2000) 18: 23-27

Reticulocyte Analysis in Systemic Lupus Erythematosus and Chronic Renal Failure Using Flow Cytometry

Punnee Butthep, Sumalee Jindadamrongwech, Preyarat Kaewkethong, Boonreun Panyayutho, Raewadee Wisedpanichkij and Ahnond Bunyaratvej

SUMMARY **The number and maturation of circulating reticulocytes were measured in patients with systemic lupus erythematosus (SLE) and chronic renal failure (CRF) using an automated hematological analyzer (Technicon H*3 RTX) for their erythropoietic activities. Both SLE and CRF patients had increased reticulocyte numbers with a low degree of maturation. The SLE patients had no changes in mean reticulocyte corpuscular volume (MCVr) as compared to normal subjects (110.20 ± 15.43 fl. in SLE and 110.39 ± 5.09 fl. in normal), whereas CRF patients had significantly increased mean corpuscular reticulocyte volume (MCVr = 120.99 ± 8.09 fl., *p*-value = 0.0019 as compared with normal). Three cases of SLE with nephrotic syndrome (NS) had high degree of MCVr (113.4, 125.0 and 133.1 fl., respectively). The renal involvement in SLE patients and CRF patients may associate with increased reticulocyte corpuscular volume.**

The reticulocyte analysis in peripheral blood samples is a non-aggressive and simple method for evaluating erythropoietic activity of the bone marrow. Advanced automated flow cytometry using RNA-binding fluorochromes has greatly improved accuracy and precision and provides more detail for analysis although its cost is elevated.[1-3] Another advantage of the flow cytometry technique is the ability to measure the degree of maturation within the reticulocyte population based on the proportion between the fluorescent intensity and the amount of RNA present in the cell.[4-6] The Technicon H*3 automated flow cytometer can simultaneously measure the volume and hemoglobin concentration of red blood cells and reticulocytes and has valuable diagnostic utility for reticulocyte counting in anemic patients with microcytic and macrocytic red cells.[7] The clinical approaches of reticulocyte monitoring were also studied in recombinant human erythropoietin treatment,[8] bone marrow transplantation,[9] and cancer chemotherapy.[10] Since SLE is a multi-system autoimmune disorder associated with a variety of hematological complications such as anemia especially in patients with lupus nephritis, the anemia of chronic renal failure (CRF) may be present. In CRF anemia develops gradually; however, many factors may complicated the anemia of CRF causing it to increase in severity. The hemoglobin varies within the range 5-12 g/dl depending on the complicating factors. It is normochromic normocytic in character, but in addition, burr cells and marked poikilocytosis are seen on the blood film.[11-13] We report here H*3 reticulocyte evaluation to assess the anemic status and erythropoietic activity in CRF and systemic lupus erythematosus (SLE) patients compared with normal subjects.

PATIENTS AND METHODS

Patients and subjects

Reticulocytes were analyzed in 26 anemic patients, 12 with CRF and 14 with SLE and compared with 25 normal healthy

From the Hematology Division, Department of Pathology, and Blood Disease Diagnostic Center, Sirikit Medical Center, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
Correspondence: Punnee Butthep

subjects. The criteria for diagnosis of SLE followed the 1982 American College of Rheumatology revised criteria for the diagnosis of SLE, which includes positive LE cell preparation.[14-15] Three of 14 patients with SLE had nephrotic involvement; however, all patients showed anemic figures.

Hematological analysis

The hematological parameters were analyzed using a Technicon H*3 automated hematology analyzer within 4 hours after venous blood collection using EDTA as an anticoagulant. The Mile H*3 (Mile, Diagnostics Division, Tarrytown, NY) blood analyzer combines the capabilities of a routine complete blood count (CBC) and five-part differential blood analyzer with a reticulocyte analyzer. The H*3 includes flow cytometric analysis of cells with laser light scattering to quantify cell volume, hemoglobin concentration and light absorbance of cells stained with Oxazine 750 to detect reticulocytes and distinguish them from mature red cells.[16-17]

Reticulocyte analysis

Reticulocyte analysis was also evaluated using a Technicon H*3 automated hematology analyzer. Three microliters of whole blood were mixed and incubated with 3 ml of reticulocyte reagent for 15 minutes. The reticulocyte cytoplasmic RNA was selectively stained with fluorescent dye, Oxazine 750. The prepared samples were aspirated through the H*3 red cell flowcell and detected by three detectors, a low angle (2° to 3°), a high angle (5° to 15°), and an absorption detector. By these detectors, the stained reticulocytes were counted separately from unstained erythrocytes, platelets, and leukocytes. The reticulocytes can be classified according to their maturity by fluorescent intensity, which is proportional to their RNA content, into low, medium and high fluorescent absorption reticulocytes (L, M and H reticulocytes, respectively). The H*3 reticulocyte analysis can provide parameters of reticulocyte mean corpuscular volume (MCVr) in femtoliters and corpuscular hemoglobin concentration mean (CHCMr) in gram per deciliter as a similar pattern of MCV and CHCM in mature erythrocytes. The mean hemoglobin content of reticulocytes (CHr) and of erythrocytes (CH) are calculated from the volume and hemoglobin concentration of a single cell.[18-20]

Statistical analysis

Results are presented as mean ± standard deviation. Statistical differences between groups were tested using the Wilcoxon rank sum test, ANOVA and p-value less than 0.05 were significant.

RESULTS

Hematological analysis

The comparison for anemic status between SLE and CRF patients and normal subjects is shown in Tables 1 and 2. The RBC count, Hb, Hct and CHCM were significantly decreased in both types as compared with normal (p-value = <0.0001-<0.0002) whereas the significantly increased % of hypochromic red cell, % macrocytosis and red blood cell distribution width (RDW) were demonstrated in both types (p-value = <0.0001- <0.0006). There was no significant difference in the percentage of microcytosis among these patients. In addition, significantly increased MCV was found in CRF only (p-value = 0.0026) as compared with normal subjects (Table 1).

Reticulocyte analysis

The reticulocyte parameters of CRF patients and SLE

Table 1 Mean ± SD of hematological analysis from H*3 hematological analyzer in SLE and CRF patients compared with normal subjects

Types	RBC (10^6/μl)	Hb (g/dl)	Hct (%)	MCV (fl)	CHCM
Normal	4.94 ± 0.42	14.31 ± 1.57	43.40 ± 4.70	87.74 ± 4.86	32.90 ± 1.38
SLE (p-value)	4.06 ± 0.97 (0.0002)	7.63 ± 5.09 (< 0.0001)	33.25 ± 4.65 (< 0.0001)	84.54 ± 14.86 (NS)	29.40 ± 1.09 (< 0.0001)
CRF (p-value)	2.91 ± 0.55 (< 0.0001)	8.79 ± 1.66 (< 0.0001)	28.31 ± 4.62 (< 0.0001)	98.06 ± 7.87 (0.0026)	29.77 ± 2.10 (< 0.0001)

(NS) = Not significantly different

patients and normal subjects are shown in Table 3. The percentage of reticulocytes (Ret %) in SLE patients was significantly elevated (p-value = 0.0037) while the percentage of reticulocytes in CRF patients was not significantly different as compared to normal. The three populations of reticulocytes (L, M and H reticulocytes) that represent their maturity showed significant differences from normal in both SLE and CRF patients. The percentages of L reticulocytes in SLE and CRF were decreased compared with normal (79.66 ± 9.95 and 75.15 ± 10.5 in SLE and CRF, respectively, as compared with 91.24 ± 3.05 % in normal; p-value = <0.0001) whereas the M reticulocyte and H reticulocyte percentages were significantly increased (p-value = <0.0001 for M reticulocytes in both SLE and CRF and p-value = 0.0016 and <0.0001 for H reticulocytes in SLE and CRF, respectively). The immature reticulocytes (% M reticulocytes + H reticulocytes) also showed significant increase in both SLE and CRF patients (p-value <0.0001). A significant increase in MCVr was found in CRF only (p-value = 0.0019) whereas in 3 cases of SLE which suffered from renal involvement showed a high level of MCVr (113.4, 125.0 and 133.1 fl.), but the mean of MCVr in those patients was not significantly in-

Table 2 Comparison between the percentage of hypochromic RBC (Hypo), microcytosis (Micro), macrocytosis (Macro) and red blood cell distribution width (RDW) in SLE and CRF patients and normal subjects

Types	n	Hypo (%)	Micro (%)	Macro (%)	RDW
Normal	25	3.33 ± 1.69	0.88 ± 0.78	0.71 ± 0.19	13.02 ± 0.75
SLE	14	38.25 ± 5.66	4.53 ± 2.06	9.65 ± 3.35	17.87 ± 2.32
(p-value)		(<0.0001)	(NS)	(0.0006)	(<0.0001)
CRF	12	31.21± 7.31	1.89 ± 0.59	9.66 ± 2.68	16.91± 2.21
(p-value)		(< 0.0001)	(NS)	(< 0.0001)	(< 0.0001)

(NS) = Not significantly different

Table 3 The H*3 reticulocyte parameters measured in SLE and CRF patients compared with normal subjects

Parameters		Normal (n = 25)	SLE (n = 14)	CRF (n = 12)
Reticulocytes (%)	mean ± SD	1.16 ± 0.52	2.84 ± 3.54	2.13 ± 1.03
	(p-value)		(0.0037)	(NS)
L reticulocytes (%)	mean ± SD	91.24 ± 3.05	79.66 ± 9.95	75.15 ± 10.5
	(p-value)		(< 0.0001)	(< 0.0001)
M reticulocytes (%)	mean ± SD	6.78 ± 2.55	14.76 ± 6.96	16.12 ± 6.50
	(p-value)		(< 0.0001)	(< 0.0001)
H reticulocytes (%)	mean ± SD	1.59 ± 1.34	5.26 ± 3.12	8.77 ± 5.45
	(p-value)		(0.0016)	(< 0.0001)
M ret + H ret (%)	mean ± SD	8.36 ± 3.19	20.02 ± 9.44	24.88 ± 10.48
	(p-value)		(< 0.0001)	(< 0.0001)
MCVr (fl)	mean ± SD	110.39 ± 5.09	110.2 ± 15.43	120.99 ± 8.09
	(p-value)		(NS)	(0.0019)
CHCMr (g/dl)	mean ± SD	26.61 ± 1.41	24.72 ± 2.03	23.86 ± 1.29
	(p-value)		(0.0119)	(0.0006)
CHr (pg)	mean ± SD	28.41± 1.34	26.37 ± 4.47	27.93 ± 2.59
	(p-value)		(NS)	(NS)

(NS) = Not significantly different

creased compared with normal. The CHCMr showed a significant decrease in both SLE and CRF patients (p-value = 0.0119 and p-value = 0.0006 for CHCMr in SLE and CRF, respectively) and the CHr value also showed a decreased level but was not statistically different compared with normal (Table 3).

DISCUSSION

Anemia in CRF is multifactorial and the most common causes are chronic hemolysis and reduced red cell production related to advanced destruction of kidneys which leads to inadequate formation of erythropoietin. In SLE, anemia is a common finding and also results from several mechanisms, such as anemia of chronic disease, hemolytic anemia caused by autoantibodies to red cells, or impaired erythropoietin production by involved kidneys.[11-13] Anemia causes increased demand for erythropoiesis in bone marrow to compensate for the loss of the red cells.[21] SLE patients show significant elevation in the percentage of reticulocytes indicating that accelerated compensatory erythropoiesis leads to a reticulocytosis in the peripheral blood.

The reticulocyte maturation represented by L, M and H reticulocytes from the H*3 RTX hematological analyzer is very valuable as a marker for active erythropoiesis of the bone marrow.[4,10,22-23] The most mature reticulocytes are counted as L reticulocytes because of their low cytoplasmic RNA, thus stained low fluorescence had the normal percentage of 91.24 ± 3.05 (mean ± SD). The less mature, M and H reticulocytes are normally less present in the blood circulation in a mean percentage of 6.78 ± 2.55 and 1.59 ± 1.34, respectively. In SLE and CRF patients, the L reticulocyte percentage was decreased, but M and H reticulocyte percentages were increased significantly. These results reflected the active and compensatory erythropoiesis in bone marrow leading to the greater release of immature reticulocytes into the blood circulation, while the mature one decreases comparably to the anemic status of the patients. However, the reticulocyte percentage was increased both in the SLE and CRF patients; but a significant difference was found only in SLE patients, not in the CRF patients, which had highly variable reticulocytosis. In addition, the results from SLE patients showed that the immature reticulocytes were increased significantly. Watanabe *et al.*[6] showed significant reduction in absolute reticulocyte count and no difference in immature reticulocytes in CRF patients compared with normal, but this does not agree with our results. The MCVr of the CRF was significantly elevated, indicating that their reticulocytes were larger than the normal reticulocytes, but the CHCMr was decreased in CRF, corresponding to the insignificant change in CHr indicating that the CRF's reticulocytes contained a normal level of hemoglobin content. In SLE, there was no significant change in the mean of MCVr, although 3 of 14 cases had shown elevated MCVr in the circulation. In a nephrotic condition, blood tends to be hypercoagulable and hyperviscous, with a higher amplitude of intravascular coagulation than the non-nephrotic condition. Therefore, the response to the anemic process in a nephrotic condition should be greater than the non-nephrotic, and the finding in such may explain this phenomenon.[24]

ACKNOWLEDGEMENTS

This study was supported by a grant from NSTDA and Bencharongkul Grant Ramathibodi Foundation. The authors wish to thank Prof. Dr. Prasit Futrakul, Department of Pediatrics, Chulalongkorn Medical School Hospital for suggestions and improving the manuscript.

REFERENCES

1. Kojima K, Niri M, Setoguchi K, Tsuda I, Tatsumi N. An automated optoelectronic reticulocyte counter. Am J Clin Pathol 1989; 92: 57-61.
2. Davis B, Bigelow N. Clinical flow cytometric reticulocyte analysis. Pathobiol 1990; 58: 99-106.
3. Tichelli A, Gratwohl A, Driessen A, et al. Evaluation of the Sysmex R-1000TM: An automated reticulocyte analyzer. Am J Clin Pathol 1990; 93: 70-8.
4. Tanke HJ, Rothbarth PH, Vossen JMJJ, Koper GJM, Ploem JS. Flow cytometry of reticulocytes applied to clinical hematology. Blood 1983; 61: 1091-7.
5. Khuhapinant A, Bunyaratvej A, Tatsumi N, Pribwai M, Fucharoen S. Number and maturation of reticulocytes in various genotypes of thalassemia as assessed by flow cytometry. Acta Haematol 1994; 91: 119-25.
6. Watanabe K, Kawai Y, Takeuchi K, *et al.* Reticulocyte maturity as an indicator for estimating qualitative abnormality of erythropoiesis. J Clin Pathol 1994; 47: 736-9.
7. d'Onofrio G, Chirillo R, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. Blood 1995; 85: 818-23.
8. Tatsumi N, Kojima K, Tsuda I, Yamagami S, Itoh Y, Tanaka H. Reticulocyte count used to assess recombinant human erythropoietin sensitivity in hemodialysis patients. Contrib Nephrol 1990; 82: 41-8.
9. Kanold J, Bezou MJ, Coulet M, *et al.* Evaluation of erythropoietic/hematopoietic reconstitution after BMT by highly fluorescent reticulocyte counts compares favorably with traditional peripheral blood cell counting. Bone Marrow Transplant 1993; 91: 119-25.
10. Tanke HJ, van Vianen PH, Emiliani

FMF, *et al.* Changes in erythropoiesis due to radiation or chemotherapy as studied by flow cytometric determination of peripheral blood reticulocytes. Histochemistry 1986; 84: 544-8.
11. Bunch C, Weatherall DJ. The haematological manifestations of systemic disease. In: Hardisty RM, Weatherall DJ, eds. Blood and its disorders. 2nd ed. Oxford, Blackwell Scientific Publications, 1982.
12. Lee GR. The anaemia of chronic disease. Seminars in Haematol 1983; 20: 61-80.
13. Bagby JC. Haematologic aspects of systemic diseases. Haematology/Oncology Clinics of North America 1 (no. 2), 1987.
14. Rothfield, N.F.: Systemic lupus erythematosus: Clinical aspects and treatment. In McCarty DJ, ed. Arthritis and allied conditions: A textbook of rheumatology. 11th ed. Philadelphia: Lea & Febiger, 1989, p. 1022-48.
15. Magath TB, Winkle V. Technic for demonstrating "L.E." (Lupus erythematosus) cells in blood. Am J Clin Pathol 1952; 22: 586-7.
16. Mohandas N, Kim YR, Tycko DH, *et al.* Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. Blood 1986; 68: 506-13.
17. Bollinger PB, Drewinko B, Brailas CD, et al. The Technicon H*1TM an automated hematology analyzer for today and tomorrow. Am J Clin Pathol 1987; 87: 71-9.
18. Buttarello M, Bulian P, Venudo A, et al. Laboratory evaluation of the Mile H*3 automated reticulocyte counter a comparative study with manual reference method and Sysmex R-1000. Arch Pathol Lab Med 1995; 119: 1141-8.
19. Brugnara C, Zelmanovic D, Sorette M, *et al.* Reticulocyte hemoglobin: An integrated parameter for evaluation of erythropoietic activity. Am J Clin Pathol 1991; 108: 133-42.
20. Brugnara C, Hipp MJ, Irving PT, *et al.* Automated reticulocyte counting and measurement of reticulocyte cellular indices evaluation of the Miles H*3 blood analyzer. Am J Clin Pathol 1994; 102: 623-32.
21. Crosby WH, Finch CA, Haurani FI, *et al.* Modern methods for quantitation of red blood cell production and destruction. Erythrokinetics. Blood 1961; 18: 225-35.
22. Davis BH, Bigelow N, Ball ED, Mills L, Cornwell III GG. Utility of flow cytometric reticulocyte quantification as a predictor of engraftment in autologous bone marrow transplantation. Am J Hematol 1989; 32: 81-7.
23. Davis BH, Bigelow NC. Flow cytometric reticulocyte quantitation using thiazole orange provides clinically useful reticulocyte maturity index. Arch Pathol Lab Med 1989; 113: 684-9.
24. Futrakul P, Poshyachinda V, Poshyachinda M, et al. Nephrotic syndrome: A kinetic evaluation of hemostasis and anticoagulant therapy in children. J Med Ass Thailand 1978; 61: 309-18.