Reticulocyte Maturation Process — Experimental Demonstration of RET Channel Using Anemic Mice —

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The RET channel on the XT2000i and XE series of Sysmex automated blood analyzers is a reticulocyte measurement channel. By staining the residual RNA with the florescent dye "RETSEARCH Ⅱ" it divides reticulocytes into three fractions, high-, medium- and low- fluorescence intensity reticulocytes (HFR, MFR, LFR respectively). Counting the cells is a useful tool for understanding the status of erythropoiesis and is widely used in the diagnosis of for example anemia and bone marrow suppression. It is believed that these fractions represent reticulocytes of different maturation stages, ranging form very early and immature (HFR) to more mature reticulocytes (LFR), but individual features of the different subpopulations have not been characterized to date.
Our objectives were to isolate cells of the different stages and to analyze them for differences in morphology and RETSEARCH Ⅱ staining pattern. Furthermore, we want to compare this to the expression of the well known reticulocyte marker CD71 (Transferrin Receptor) and the new methylene blue staining and classification system.
We induced anemia in mice through intraperitoneal phenylhydrazine injection, which leads to severe reticulocytosis in the animals after five days. Anemic murine blood was collected and fractionated into reticulocyte subpopulations by a cell sorting system. The cells were fixed and examined by transmission electron microscopy (TEM) or stained and examined for specific morphology and antigen expression.
By double labeling with RETSEARCH Ⅱ and CD71-Alexa488 and subsequent FACS analysis we created a simulated Ret channel scattergram. We found that CD71 was expressed only on the more immature cells and rapidly disappeared at the differentiation step from HFR to MFR. Through TEM and confocal microscopy we confirmed that immature reticulocytes lose CD71 antigen expression at the stage of MFR. Moreover we were able to show that intracellular structures such as residual organelles disappear as the cells mature towards an RBC. This result was supported by immuno-electron microscopic analysis using CD71-gold colloid conjugate.
By electron microscopy we detected residual ribosomes in all of the immature cell fractions (LFR, MFR and HFR), albeit strongly decreasing towards the more mature stages. Since new methylene blue supravital staining, the standard method in reticulocyte analysis, targets residual ribosomes, we can conclude that reticulocytes identified by this staining corresponded to those sorted by FACS using simulated RET-channel. Moreover, this demonstrates that reticulocyte counting by the RET-channel is in accordance with that by new methylene blue supravital staining.
Many observations of the reticulocyte in vitro maturation process have been reported. This is one of the very few in vivo reports and we believe that our observations accurately reflect the in vivo maturation process.

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INTRODUCTION

Reticulocytes represent the penultimate phase of maturation of erythroid cells in the peripheral blood. The nucleus is removed from the cell usually before it enters the bloodstream. However, some of the extranuclear RNA remains in the cell in this early phase. This residual RNA is generally lost within 24-36 hours after expulsion from the bone marrow [1, 2].
Changes in reticulocyte percentage indicate bone marrow suppression or elevated erythropoiesis, therefore this percentage is clinically important and it is widely used as an indicator of recovery from a nutritional anemia or bone marrow suppression. Recent studies also have suggested that increment of younger reticulocyte percentage is useful as an index of transplantation engraftment [3].
The standard method of reticulocyte measurement in the clinical hematology laboratory is visual counting using new methylene blue supravital staining [4]. The dye used

in this method precipitates and stains the intracellular RNA, and since most of the residual RNA is localized to the ribosome new methylene blue staining is believed to represent artifactual aggregations of ribosomes [5].

In 1988, Sysmex developed the first automated reticulocyte analyzer, R-1000. To date, several companies have included reticulocyte measurement systems on their automated hematology analyzers. They employ some modifications in each model; however, their methodologies are all based on flow cytometric techniques utilizing staining with a fluorescence dye which reacts specifically with residual RNA. Using automation, reticulocyte measurement became convenient and accurate. Additionally, it enables the calculation of younger reticulocyte percentages which would be very difficult to do by visual counting.

The RET channel is the reticulocyte mode supplied with XT2000*i* and Sysmex's updated models of the XE series. The RET channel divides reticulocytes into three fractions, high-, medium- and low- fluorescence intensity reticulocyte (HFR, MFR, LFR respectively), dependent on their stainability by the RETSEARCH dye. But there are no reports concerning the individual significance of these HFR, MFR, LFR cells. We undertook morphological observations on reticulocytes classified by the RET channel, HFR, MFR, LFR and RBC cells, and characterized the reticulocytes in these fractions [6].

Two membrane-bound surface antigens have been reported as an index of immature red blood cells. Expression of CD36, known as glycoprotein IV or Thrombospondin-R, which is detected on erythroblasts [7, 8], and CD71, known as transferrin receptor, which is lost from the cell surface of reticulocytes as they mature [9, 10]. It has been shown that in *in vitro* cultured reticulocytes CD71 expression is rapidly declining [11]. We elucidated CD71 expression pattern within the reticulocyte maturation process, and compared 'reticulocyte' defined by CD71 expression with that by RET channel using RETSEARCH.

MATERIALS AND METHODS

Preparation reticulocyte-rich murine blood

Inbred ICR female mice were purchased at 20g body weight from Oriental Yeast Co. Ltd, Tokyo, Japan and were maintained in air-conditioned, specific pathogen-free animal room regulated at 20-25˚C. Commercial rodent chow (CRF-1, Oriental Yeast Co. Ltd, Tokyo, Japan) and water were available to the mice ad libitum. To obtain reticulocyte elevated murine blood for the examination, mice were injected intraperitoneally with phenylhydrazine hydrochloride (150mg/kg body weight, P-6926, SIGMA, St.Louis, United States) in PBS (pH7.4) according to the method of G. N. Zyuz'kov et al [12-14]. Five days after the treatment, reticulocyte-elevated blood was collected from the tail vein of the anemic mice in tubes containing EDTA. Blood collected from mice injected with neat PBS instead of phenylhydrazine solution in the same manner was used as control blood.

Simulation of RET channel by flow-cytometry

A RET channel scattergram was obtained from the XT-2000*i* analyzer through measurement of a whole blood sample. An aliquot of the same whole blood sample was treated with RETSEARCH dilution buffer and RETSEARCH Ⅱ staining dye using the same procedure as within the XT2000*i*: 4μL whole blood was added to a mixture of 996μL dilution buffer and 2μL staining dye. The labelled blood sample was then analysed by FACSCalibur or FACSAria flow cytometers (Becton Dickinson, New Jersey, United States). A two dimensional "simulated RET channel" scattergram was produced by plotting FSC (forward light scatter) over RETSEARCH Ⅱ fluorescence intensity.

Cell sorting and observation

Cells corresponding to the RET channel fractions, HFR, MFR, LFR and RBC, were collected independently into tubes using the cell sorter system of the FACSAria. These collected cells were fixed with 1% glutaraldehyde in PBS for 16 hours at 4˚C, and adhered to silanized glass slides by cytospin (Cytospin3, SHANDON,Thermo Fisher Scientific, Inc., Waltham, United States). Then they were post-fixed with 1% osmium tetroxide in PBS for 30 minutes at 4˚C and dehydrated in a graded series of ethanol. Following the dehydration, they were invert-embedded in Quetol 812. Ultra-thin silver-to-gold interference contrast sections (80-100nm thick) were cut using a microtome (35578, ULTRACUT UCT, LEICA, Wetzlar, Germany) and examined with HITACHI H-7500 (Tokyo, Japan) electron microscope.

Immunological staining and observation

The collected blood was diluted to 1/50 with PBS containing 1% bovine serum albumin (BSA, A-3294, SIGMA, St.Louis, United States) and blocked for 30 minutes at 4˚C. The cells were washed with PBS three times and suspended in biotin-labeled anti-mouse CD71 antibody (clone ER-MP21, prod. no. T-2114, BMABIOMEDICALS, Augst, Switzerland) solution diluted 50 times with 1% BSA in PBS. The cells were resuspended in streptavidin-Alexa488 (S-11223, INVITROGEN, California, United States) for 30 minutes at 4˚C and washed. After that the cells were restained with RETSEARCH Ⅱ for 30 seconds at 40˚C and immediately observed with a confocal laser microscope.

For the immuno-transmission electron microscopic analysis, cells were labelled with anti-CD71-biotin, washed with PBS and incubated for 30 minutes at 4˚C in streptavidin-20nm gold colloid solution (EMSTP20, BB International, Golden Gate, United Kingdon). After that the cells were fixed with 1% glutaraldehyde in PBS for 16 hours at 4˚C. The cells were attached to silanized glass slides with cytospin, post-fixed with 1% osmium tetroxide in PBS for 30 minutes at 4˚C, dehydrated in a graded series of ethanol and invert embedded in Quetol 812. Silver-to-gold sections were cut and examined with a HITACHI H-7500 (Tokyo, Japan) electron microscope.

Biotin conjugated anti-human CD42b monoclonal antibody (clone MM2/174, prod. no. CBL480B,

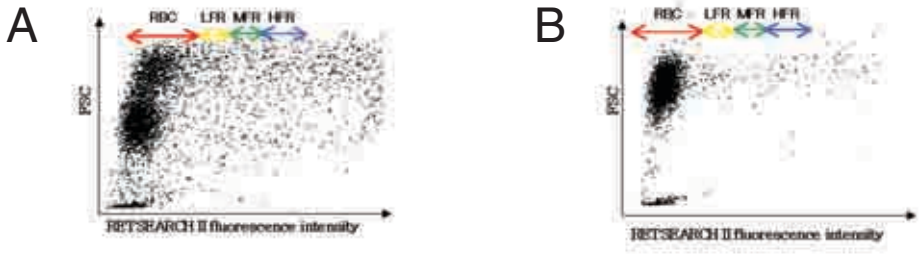

Fig. 1 *XT2000i RET channel scattergrams from anemic and control mice blood samples. Reticulocytes (HFR, MFR, and LFR) were remarkably increased in anemic mice (A) compared to control mice (B).*

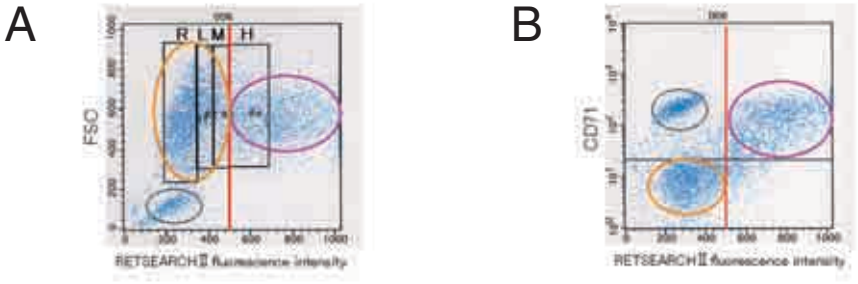

Fig. 2 *FACSCalibur analysis of anemic murine blood double stained with anti-CD71-Alexa488 and RETSEARCH Ⅱ.*
A) FACSCalibur forward scatter was plotted over RETSEARCH Ⅱ intensity to simulate a scattergram as it is generated by the RET channel. The signals were allocated to the different cell fractions (RBC:"R", LFR:"L", MFR:"M" and HFR:"H") according to their fluorescence intensity.
B) The same data, this time plotting CD71-Alexa488 signal over RETSEARCH Ⅱ intensity. The same cell populations could be identified as in A). CD71 positivity varies distinctly between the highly immature cell fractions (pink circle) and the more mature fractions RBC, LFR and MFR (orange circle).

CHEMICON, Billerica, USA) which does not cross-react with substances of other species was used as negative control for these immunochemical studies.

Double-staining with ER Tracker green and RETSEARCH Ⅱ

Murine blood was diluted with PBS at 1/50 and admixed with ER Tracker Green (prod. no. E-34251, INVITROGEN, California, United States) at a final concentration of 1μM. After incubation for 30 minutes at 37˚C, 100μL of this cell suspension was mixed with 0.1μL of RETSEARCH Ⅱ dye solution. The double-stained cells were observed by a confocal fluorescence microscopy.

RESULTS

The mice injected intraperitoneally with phenylhydrazine developed hemolytic anemia, consequently their erythropoiesis was expected to become activated. Their reticulocyte ratio, an index of erythropoiesis, began to increase within 24 hours and increased rapidly thereafter. It reached a plateau of more than 60% on day 4-7 (data not shown). From this result we decided to collect reticulocyte-rich blood on day 5 to obtain stable blood samples for the experiments.

Representative RET channel scattergrams of reticulocytosis blood and control blood were generated by analysis on the XT-2000*i* (***Fig. 1***). In these RET scattergrams, RETSEARCH Ⅱ fluorescence intensities are plotted on the horizontal axis and forward scatters on the vertical axis. The fluorescent intensities of RETSEARCH Ⅱ are shifted to the left in the scattergram as the cells mature. Reticulocytes numbers in the immature LFR, MFR and HFR fractions were increased in anemic blood compared with control blood.

To study expression kinetics of CD71 during the reticulocyte maturation process, anemic murine blood cells were double-stained with anti-CD71-Alexa488 and RETSEARCH Ⅱ, and analyzed by FACS (***Fig. 2***). We simulated a RET channel scattergram with the flow cytometer data by plotting the FSC (cell size) on the y-axis against RETSERCH Ⅱ intensity on the x-axis (***Fig. 2 A***). We assed the individual cell fractions RBC, LFR, MFR and HFR according to their RETSEARCH intensity in this chart. We then constructed another scattergram by

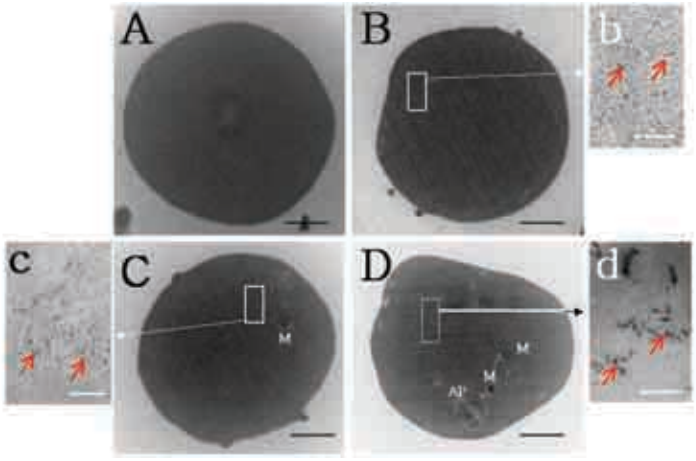

Fig. 3 *Transmission electron microscopy (TEM) images of HFR, MFR, LFR and RBC cells.*
Cells were collected using the cell sorting system on the FACSAria, fixed and analysed by TEM. The pictures show a single cell each of the RBC fraction (A), LFR (B, higher magnification in b), MFR (C and c) and HFR (D and d). Internal structures disappeared gradually during the maturation process from HFR to RBC. Residual ribosomes attached to endoplasmatic reticulum (red arrows) could be seen in all cells except RBCs, degenerative mitochondria (M) were still found in MFR and HFR whereas autophagic structures (AP) were only detectable in the most immature HFR cell fraction. Black bar = 500nm, white bar = 50nm.

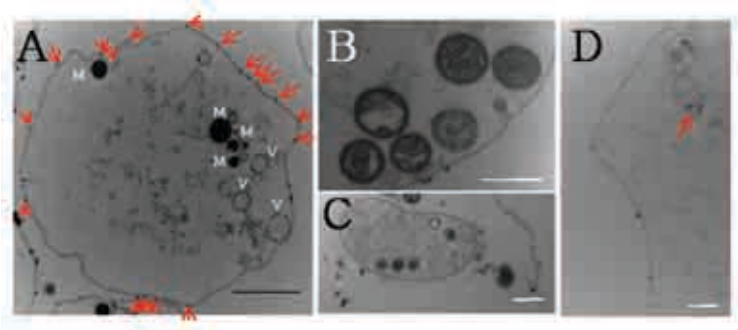

Fig. 4 *Immuno-gold electron microscopy observation of anemic murine blood cells labelled with anti-CD71-20nm gold colloid. Gold colloidal spots (red arrows) indicating CD71 expression were detectable on the cell membrane (A). Degenerative mitochondria (M), some vesicles (V) and residual endoplasmic reticulum are detectable within the whole cell (A). At a higher magnification one can detect mitochondrial degradation (B) and autophagic structures containing mitochondria (C). Anti-CD71-gold colloids are segregated in small vesicles constituting multivesicular endosomes (red arrow in D). Black bar = 500nm, white bar = 100nm.*

exchanging the FSC axis with CD71 intensity measurements of the same blood sample (***Fig. 2 B***). Concluding form the RETSEARCH Ⅱ intensities we could again allocate the signals to the different cell populations. The cells highlighted by the pink circles in both scattergram were considered to be cells of the HFR and more immature cell fraction. CD71 intensity of the examined cells decreased in accordance with the reduction of RETSEARCH Ⅱ fluorescence, i.e. maturation of reticulocyte. It notably diminished as the maturation progressed from HFR to MFR. More mature cells such as MFR and LFR, hardly developed CD71 at all as speculated from the apparent profiles of these cells (orange circle). In conclusion, one can distinguish between immature cells of the HFR fraction and beyond, and more mature cells like MFR and LFR by CD71 expression.

For morphological and structural comparison of the cells of the different maturation stages, we performed transmission electron microscopy on cells sorted by FACSAria into each maturation fraction; representative images are shown in ***Fig. 3***. Many intracellular structures such as swelling or shrunk mitochondria, hangover products from erythroblast are still present in HFR (***Fig. 3 D***). They gradually disappeared as the cells matured. However, residual ribosomes could be observed until the LFR stage. Most remarkably was the disappearance of the intracellular structures towards the MFR stage, and they were completely absent in the mature RBCs. As exclusion process of intracellular structures to the outside of the cell we could observe autophagic structures in HFR cells and exocytotic release in all reticulocytes (HFR, MFR and LFR).

The presence of residual internal structures such as mitochondria, vesicles and lysosomes, were reconfirmed morphologically by immunoelectron microscopic

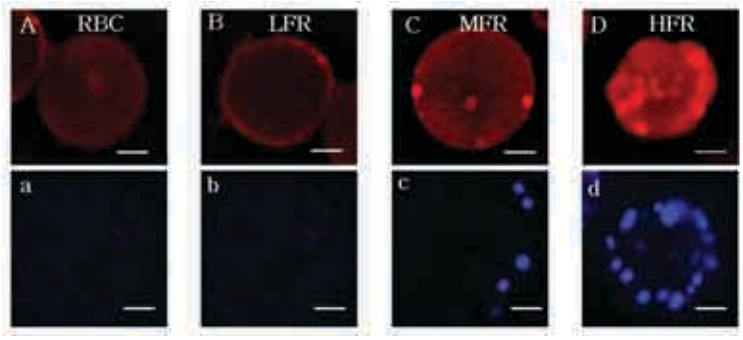

Fig. 5 *Confocal microscopy of anemic murine blood cells double stained with Anti-CD71-Alexa488 and RETSEARCH Ⅱ. Depicted are images of single cells in the four different maturation stages, taken at two different wavelengths: 660nm for RETSEARCH Ⅱ (A, B, C, D) and at 500-540nm for Alexa488 (a,b,c,d) detection. Fluorescence of RETSEARCH Ⅱ decreased remarkably as the reticulocytes mature, the cell image gradually fades out (D-A).CD71 positivity was only seen in the most immature stage HFR (d) and to a lesser extent in cells belonging to the MFR fraction (c). Bar = 500nm.*

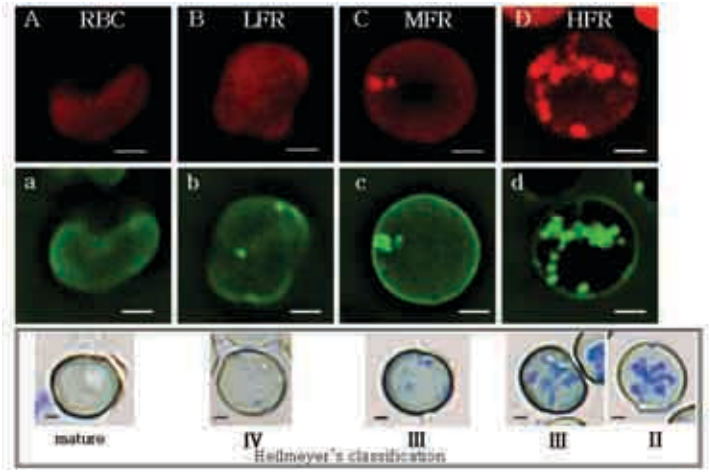

Fig. 6 *Confocal microscopy of anemic murine blood cells double stained with ER Tracker green and RETSEARCH Ⅱ. Depicted are images of single cells in the four different maturation stages, taken at two different wavelengths: 660nm for RETSEARCH Ⅱ (A, B, C, D) and at 500-540nm for ER-Tracker Green (a,b,c,d) detection. Cells were allocated to the different maturation stages according to their RETSEARCH Ⅱ staining.pattern (A-D). New methylene blue staining cells of each fraction attributed and classified according to the Heilmeyer classification (images in the double square).*

techniques in CD71-positive cells, i.e. HFR cells where anti-CD71-gold colloidal spot could be seen on cell surface (***Fig. 4***).

We next investigated the comparability of CD71 expression with RETSEARCH fluorescence intensity in single cells by double-staining with anti-CD71-Alexa488 and RETSEARCH Ⅱ. Cells of each fraction were observed by confocal microscopy at different detection wavelengths (***Fig. 5***). First of all we could confirm that RETSEARCH Ⅱ fluorescence decreased as the reticulocyte matured, the cell image gradually faded out (***Fig. 5 A-D***). We could also observe that RETSEARCH Ⅱ strongly stains intracellular structures in the HFR stage (***Fig. 5 D***). These structures move towards the cell membrane during maturation (***Fig. 5 C and 5 B***) and were finally eliminated from the RBC. We assume that this observation demonstrates the exclusion of cell organelles in the reticulocyte. From our previous finding that CD71 was expressed in the very immature reticulocytes, we conclude that strongly CD71 positive cells belong to HFR fraction (***Fig. 5 D and 5 d***). Cells with a reduced CD71 signal were considered MFR cells (***Fig. 5 C and 5 c***). A small amount of residual structures stained with RETSEARCH Ⅱ could be observed in some CD71 negative cells (***Fig. 5 B and 5 b***). Considering that the RBC cell does not have intracellular structures as shown by electron microscopy (***Fig. 3***), we attributed the cells which have few intracellular structures and no CD71 to the LFR fraction (***Fig. 5 B***). The RBC cell was considered to have no CD71 and no intracellular structures at all (***Fig. 5 A***).

The cell images stained by RETSEARCH Ⅱ, ER Tracker green known as fluorescence dye for endoplasmic reticulum (ER) and new methylene blue were shown in ***Fig. 6***. Various maturation reticulocytes were classified to each maturation stage on RET channel fraction (HFR,

MFR, LFR, RBC) from images of RETSEARCH Ⅱ staining. ER Tracker green is known to stain endoplasmic reticulum specifically and new methylene blue was reported to stain artificial aggregations of ribosomes remained in reticulocyte. So residual ribosomes must be present close to rough-surfaced endoplasmic reticulum, the image of new methylene blue can suppose to show similar image of ER Tracker green. Then we tried to determine which stage of Heilmeyer's classification (Stage 0-Ⅳ) correspond to the fraction of RET channel based on the information mentioned above. From morphological characteristics of these three staining, HFR cells speculated to mainly consist of Stage Ⅱ and Ⅲ of Heilmeyer's classification [4], and MFR and LFR cells correspond to Stage Ⅲ and Stage Ⅳ respectively. From this results, reticulocytes identified by RET channel, agreed with that by new methylene blue supravital staining, morphologically. Furthermore, staining sites of RETSEARCH Ⅱ and ER Tracker green, a ER specific dye, were in close agreement. This result means that RETSEARCH Ⅱ strongly binds to residual RNA located mainly at ribosome.

DISCUSSION

The "RET channel" in Sysmex hematology analyzers divides reticulocytes into three fractions, high fluorescence intensity reticulocytes (HFR), medium fluorescence intensity reticulocytes (MFR) and low fluorescence intensity reticulocytes (LFR), due to their different RNA contents and hence staining intensities of the fluorescence dye RETSEARCH Ⅱ. But the characteristics of the individual cells had not been investigated to date.

We first characterized the reticulocytes in the different fractions by their maturation status, which was assessed by aspects such as morphology and surface antigen expression. The membrane protein CD71 (transferrin receptor) is a well known reticulocyte marker in the biochemical field. This marker mediates intracellular iron incorporation by binding to the iron transport protein transferrin. Its binding capacity is diminished during maturation [16]. It has been demonstrated that CD71 is segregated and externalized via exosomes in maturing reticulocytes [17] and that its expression is lost from the cell surface [9, 15] at some stage. We showed that these events occurred mainly at the maturation process from HFR to MFR. Therefore various reports about reticulocytes have to be interpreted carefully since they only consider the more immature HFR (CD71 positive) fraction, but not MFR and LFR.

The observation of CD71 presence in small vesicles as show in ***Fig. 4 D*** supports the finding that a fraction of CD71 on the cell surface is internalized and incorporated in multivesicular endosomes or recycled.

The nucleus of erythroblasts is usually removed before expulsion from the bone marrow into the peripheral blood. However, some of the extranuclear RNA and internal structures remain in the cells and are lost progressively while maturation progresses.

Many studies about the reticulocyte maturation process are carried out *in vitro* by reticulocyte cell culture, however, *in vivo* reports are rare. In our *in vivo* study we observed mitochondria degradation, exocytosis and autophagy structures in HFR cells (***Fig. 4 B and C***), and residual RNA reduction in the matured reticulocyte. These results are in agreement with past in vitro studies [18-20].

According to our characterization of each fraction, the cells of HFR, MFR and LFR correspond to the cells of Stage Ⅱ - Ⅳ of Heilmeyer's classification [4]. Therefore the number of reticulocytes counted in the "RET channel" agreed with that by the new methylene blue supravital staining method which NCCLS recommends as standard method [3].

IRF is the parameter in the XE series hematology analyzers that represents the ratio of HFR and MFR to the total reticulocyte count (HFR, MFR and LFR). This parameter was reported to be the earliest sign of recovery after bone marrow transplantation. According to our findings it could be possible for a proficient medical technician, who can discriminate Heilmeyer classification stage I - Ⅲ from stage Ⅳ by new methylene blue staining, to estimate IRF by a visual count.

This is the first report about the character of the reticulocytes in each fraction of the "RET channel" in respect of the maturation process. The findings of this study could be useful for the cytological and clinical research on erythroid linage cell using "RET channel".

References

1) *Baldini M, Pammacciullii: The maturation rate of reticulocytes. Blood, 15: 614-629, 1960.*
2) *Stryckmans PA, et al.: The maturation and fate of reticulocytes after in vitro labeling with tritiated amino acids. Blood, 31(1):33-43, 1968.*
3) *J David Bessman: Reticulocytes. Clinical Methods Third Edition, 156; 735-738, 1990.*
4) *Heilmeyer L: Blutfarbstoffwechselstudien. Dtsch Arch Klin Med, 171:123-153, 1931*
5) *Haward C Mel, et al.: Reticulocyte Motility and Form: Studies on Maturation and Classification. Blood, 49(6): 1001-1009, 1977.*
6) *Product Development Division, Sysmex Corporation: Overview of Automated Hematology Analyzer XE-2100. Sysmex Journal, 22(1): 76-84, 1999.*
7) *Edelman P, et al.:Developmentally regulated expression of a 78 kDa erythroblast membrane glycoprotein immunologically related to the platelet thrombospondin receptor. Biochem J, 262(3):835-42, 1989.*
8) *Ketty Lee, et al.: The nonexpresion of CD36 on reticulocytes and mature red blood cells does not modify the clinical course of patients with sickle cell anemia. Blood, 98(4): 966-971, 2001.*
9) *Stephan D Dertinger, et al.: Enumeration of micronucleated CD71-positive humen reticulocytes with a single-laser flow cytometer. Mutation Research, 515: 3-14, 2002.*
10) *I J Woolley, et al.: Expression of Duffy antigen receptor for chemokines during reticulocyte maturation: using a CD71 flow cytometric technique to identify reticulocytes. Immunohematology, 21(1):15-20, 2005.*
11) *Stefan Serke, Dieter Huhn: Identification of CD71 (transferrin receptor) expressing erythricytes by multiparameter-flow cytometry (MP-FCM): correlation to the quantitation of reticulocytes as determined by conventional microscopy and by MP-FCM using a RNA-staining dye. British Journal of Haematology, 81: 432-439, 1992.*

12) *Dermot M F Cooper, Rosemary Jagus: Impaired Adenylate Cyclase Activity of Phenylhydrazine-induced Reticulocytes. The Journal of Biological Chemistry, 257(9): 4684-4687, 1982.*
13) *Atsuko Nagao, et al.: Application of the R-1000 Automated Reticulocyte Analyzer to Research on In Vitro Reticulocyte Maturation. Sysmex Journnal, 13(3): 407-415, 1990.*
14) *G N Zyuz'kov, et al.: Mechanisms of Regulation of Erythropoiesis during Hemolytic Anemia. Bulletin of Experimental Biology and Medicine, 10: 334-337, 2004.*
15) *Seligman PA, et al.: Automated analysis of reticulocytes using fluorescent staining with both acridine orange and an immunofluorescence technique. Am J Hematol., 14(1):57-66, 1983.*
16) *Dautry-Varsat A, et al.: pH and the recycling of transferrin during receptor-mediated endocytosis. Proc Natl Acad Sci U S A. 80(8):2258-62, 1983.*
17) *Johnstone RM, et al.: Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. Blood, 74(5):1844-51, 1989.*
18) *Gloria G, et al.: Maturation of the Reticulocyte in vitro. J. cell Sci., 71:177-197, 1984*
19) *Charles G, Aude de G, Michel V: Reticulocyte maturation : mitoptosis and exosome release. BIOCELL Review, 26(2) : 205-215, 2002.*
20) *Mark J Koury, et al.: In vitro maturation of nascent reticulocytes to erythrocytes. Blood, 105(5): 2168-2174, 2005.*