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Scanning Electron Microscopy of Reticulocytes

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Summary. The surface structure of reticulocytes was examined by scanning electron microscopy (SEM) which does not seem to have been clearly described hitherto.

Based on the results of preliminary experiments the following methods were employed. Erythrocytes were stained with 0.5 % new methylene blue (NMB) in 0.15 M phosphate buffer. NMB stain by BRECHER generally used for reticulocyte counting was found to remarkably affect the external structure of erythrocytes. Stained erythrocytes were fixed in 1 % glutaraldehyde, washed with water and dried on a middle piece cut out from a Thoma's or a Buerker-Tuerk's hemocytometer. The reticulocytes confirmed by light microscopy were identified under SEM by referring their positions on the central sectioned area and photographed. The external structures of 57 reticulocytes from 10 healthy adults were identical to mature erythrocytes with smooth surface, i.e., the majority were concave discs, some were discs and a few were swollen concave discs.

About half of 123 reticulocytes from 4 cases of reticulocytosis had a similar contour but often had some pits on the surface probably indicating BESSIS' "rhopheocytosis." The remaining were spheroid or spherical with hollows, elevations and pits. The hollows and elevations were frequently accumulated at a pole of the cells, suggesting the previous denucleation though that portion. A possible transformation from a latest erythroblast to a mature erythrocyte was presented. Reticulocytes in reticulocytosis appeared to differ morphologically between different conditions or diseases.

Reticulocytes described first by ERB in 1865 and consequently by EHRLICH in 1891 (LOEHR, 1965) are named after the characteristic reticular or granular substance—Substantia reticulo- or granulo-filamentosa—which appears in the cells when they are stained in brilliant cresylblue, new methylene blue and some other dyes (WITTERKIND and RENTSCH, 1968). This type of red blood cells has been generally thought to represent an immature stage just after denucleation of erythroblasts.

Transmission electron microscopy has revealed that the substantia reticulo-filamentosa corresponds to polyribosomes aggregated by staining (STAUBESAND, WITTERKIND and RENTSCH, 1966) and that, besides them, some vacuoles, vesicles and mitochondria usually contained in erythroblasts also occur in reticulocytes (STAUBESAND, WITTERKIND and RENTSCH, 1966; SKUTELSKY and DANON, 1967; SIMPSON and KLING, 1968). The process of denucleation, i.e., transformation from mature erythroblast to reticulocyte has also been clarified by several authors (BESSIS and BRETON-GORIUS, 1959; SKUTELSKY and DANON, 1967; SIMPSON and KLING, 1967; AKASAKA, 1971). SIMPSON and KLING (1968) demonstrated the further process of the reticulocytes losing their residual organelles to become mature erythrocytes.

Scanning electron microscopy (SEM) can reveal the three-dimensional and fine surface structure of blood cells as well as other free cells. Several investigators including the present authors (TOKUNAGA, FUJITA and HATTORI, 1969; HATTORI 1971;

HATTORI, ITO and MATSUOKA, 1971) have demonstrated by SEM the surface structure of normal mature red blood cells and some pathological ones. As for reticulocytes, however, only CLARKE and SALSURY (1967) and JENSEN (1969) presented the scanning electron micrographs of erythrocytes supposed to be reticulocytes, though this cell identification was based on no convincing proof. This paper concerns a method for identifying and examining the reticulocytes by SEM and the fine surface structure of reticulocytes of normal individuals and patients with reticulocytosis. It further demonstrates a possible process of transformation from reticulocyte to mature erythrocyte.

Materials and Methods

There are two steps in the observation of the surface structure of reticulocytes: first, one must identify a reticulocyte among other types of erythrocytes, and second, one observes the identified cell by SEM. For the first procedure we stained erythrocytes by new methylene blue (NMB), and for the orientation of the cells in the second procedure we employed hemocytometers of Thoma or Buerker-Tuerk type.

New methylene blue staining

This staining has been widely used for reticulocyte count since its introduction by BRECHER (1949). Brecher's NMB solution contains 0.5 g of NMB and 1.6 g of potassium oxalate in 100 ml. Staining is done as follows: whole blood is mixed with equal volume of the solution, left at room temperature for 10 min and finally the mixture is smeared on slide glasses for examination by light microscopy. At first we examined whether this staining might affect the surface structure of erythrocytes. The stained erythrocytes were fixed in a large amount (more than 20 times) of 1 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for several hours according to our method (HATTORI et al., 1971). Then the erythrocytes were washed once with the same buffer and then several times with distilled water and dried on small glass slides. After evaporation with carbon and gold the specimens were examined with a Hitachi SSM-2 scanning electron microscope. Compared with erythrocytes fixed without staining (Fig. 1), the stained erythrocytes were found to be markedly deformed, i.e., they appeared swollen and uneven on the surface under the SEM (Fig. 2). Even by light microscopy the swelling was recognized. Then a series of erythrocyte samples fixed at intervals (2, 4, 8, 10 and 15 min) after mixing whole blood and Brecher's solution were examined by both light microscopy and SEM. The results indicated that the deformation appeared within 2 min (Fig. 3).

Erythrocytes are well known to be readily deformed by environmental osmolarity (see HADEN, 1934). We have also demonstrated by SEM the effects of the osmolarity of both environment and fixative (TOKUNAGA, FUJITA and HATTORI, 1969; HATTORI et al., 1972 in preparation).

In this case we noted that the deformation pattern of erythrocytes stained by Brecher's method resembled that after a hypotonic fixative. This fact suggests a hypothesis that Brecher's solution though described as isotonic acts a hypotonic environment on erythrocytes. This hypothesis was confirmed by the measurement of osmolarity (Table 1). The fact that besides the substantia reticulofilamentosa the protoplasm is stained slightly bluish by NMB (BRECHER, 1949; TERADA and SUZUKI,

1966) indicates that NMB passes through the membrane of erythrocytes. This suggests that NMB is not concerned in the actual osmolarity of the dye solution at the end of staining; the solution seems to act upon the erythrocytes as a more hypotonic environment than indicated by the measurement.

Fig. 1. Normal erythrocytes prepared according to usual method. They are generally concave discs with a smooth surface. $\times 2,900$

Fig. 2. Erythrocytes fixed after 10 minutes staining by Brecher's new methylene blue solution. The erythrocytes are swollen and show an uneven surface. $\times 2,900$

Fig. 3. Erythrocytes fixed after two minutes staining by Brecher's new methylene blue solution. Deformation has begun to appear. $\times 2,900$

Fig. 4. Erythrocytes fixed after staining for ten minutes by solution B. Note that the erythrocytes have preserved their natural original form. $\times 2,900$

Thus, we made four NMB solutions (A, B, C and D as shown in Table 1) in which NMB was added to isotonic (physiological saline) or nearly isotonic (0.15 M phosphate buffer) solution to make 0.5% and 2% solutions and examined their stainability and effect on the surface structure.

Reticulocytes were counted in smears made at intervals (2, 4, 6, 8, 10, 12 and

Table 1. New methylene blue solutions

Solutions	NMB (%)	Solvent	Final osmolarity (mos.)	Final pH
Brecher's solution	0.5	potassium oxalate 1.6 g per 100 ml	240	6.9
A	2.0	0.15 M phosphate buffer pH 7.4 (327 mos.)	390	7.4
B	0.5	same buffer	336	7.3
C	2.0	physiological saline	384	2.9
D	0.5	same saline	319	3.5

15 min) after incubation with NMB solutions (Fig. 5a, b). Reticulocytes had already appeared in 2 min samples and later increased in number to reach a plateau. Reticulocyte counts of solution B were lower than those of the other solutions and almost three fourths of those of Brecher's NMB solution (Fig. 5b). It was noted that erythrocytes in both solutions C and D which contained physiological saline were stained bluish as in the case of Brecher's solution, whereas not in the cases of solutions A and B. The erythrocyte staining thus seemed attributable to lowered pH values of the solutions. On the other hand, dye precipitates were often encountered in smears from solutions A and C, (2% NMB solutions) but seldom in smears from solutions B and D (0.5% NMB solutions).

An SEM examination indicated that the erythrocytes treated with all of these

Fig. 5. Comparison of stainability of NMB solutions. **a.** A, B, C and D solution. Subject: a 47 year old male. **b.** B solution (B) and Brecher's solution (Br). Subject: a 34 year old male.

four solutions appeared similar in shape to the control erythrocytes. Deformation as in the case of Brecher's solution did not occur. As an example Figure 4 shows erythrocytes incubated with solution B.

From these results solution B was chosen for the following study.

Orientation of the cells using a hemocytometer

The central part of a hemocytometer (e.g. Thoma's) is provided with a square of the well known section pattern as shown in Figure 6a. As the four corners of the square have different patterns of line marks (A, B, C and D in Fig. 6b), one can easily identify a given portion in the square by referring to the patterns of the adjacent corner. The line marks can be clearly recognized as grooves by SEM as shown

Fig. 6. a. A schematic representation of a Thoma's hemocytometer. A small piece including the central area is cut out with a glass cutter, as indicated by long arrows. b. An enlarged schema of the central area (finely sectioned portion).

Fig. 7. A scanning electron microscopic view at low magnification of a part of the central area of a Thoma's hemocytometer. $\times 900$

in Figure 7. Thus, a cell placed at a certain site within the sectioned square can be examined by both light microscopy and SEM.

We cut out the central parts (ca 7mm square) including the sectioned square (Fig. 6a) by an ordinary glass cutter and kept them in a dust-free condition after cleaning by acetone. We also prepared for the recording of cell location paper on which an enlarged section pattern was printed.

Subjects and procedures

The subjects of this study were 10 healthy adults and 4 adult patients with reticulocytosis (3 cases of iron deficiency anemia at recovery phase after administration of iron and a case of gastric cancer with metastasis).

From the cubital vein of these subjects whole blood was drawn into a syringe containing one ninth volume of 3.8% citrate and mixed. 0.1–0.2 ml of the anticoagulated blood was incubated with an equal volume of NMB solution (solution B) and left for 10–15 min at room temperature. Then around 10 ml of fixative was added according to the method as described above. After washing with water, a drop of

cell suspension was dried on the piece prepared from a hemocytometer (*vide supra*). The first identification of reticulocytes by light microscopy was done

Fig. 8. A light microscopic photograph of the same portion as Figure 7 of the central area of a hemocytometer. The erythrocytes have been previously stained by solution B. An arrow indicates three reticulocytes which contain substantia granulofilamentosa. $\times 1,500$

Fig. 9. Scanning electron microscopic image of the same portion of Figure 8. Note the fine surface structure of the three reticulocytes which were identified in Figure 8. $\times 3,000$

using a $\times 40$ objective lens (no cover) or a $\times 100$ objective lens for oil immersion without using oil and a $\times 10$ ocular lens, and the location of the reticulocytes was recorded on the previously prepared chart. The specimens were then coated with carbon and gold by vacuum evaporation to be examined by SEM. Each reticulocyte previously judged as such by light microscopy could usually be identified by its location and photographed. In some cases, however, a reverse procedure was worthwhile: some adequate sections were photographed at lower and higher magnification by SEM and recorded on the chart and there after an identification of reticulocytes by light microscopy was attempted, this time using oil immersion. Evaporated carbon and gold did not hinder this procedure; the bluish stained precipitates were clearly visible. As an example Figure 8 is a photograph obtained in the second examination by light microscopy; three erythrocytes at the center are identified as reticulocytes whose SEM image is shown in Figure 9.

Observations

Reticulocytes of normal subjects

Observation was made on 57 reticulocytes by our combined light microscopy and SEM method. Their three-dimensional surface structures were not uniform as expected and are summarized in Table 2. It was found that they had external structures similar to usual erythrocytes (TOKUNAGA, FUJITA and HATTORI, 1969; HATTORI, 1971), i.e., the majority of them were concave discs with a diameter of about $6-8\mu$ (Fig. 10, 11). Some were flattened discs of similar diameter and a few were spheroid with less diameter. As far as we observed, their surface was smooth regardless of

Table 2. Surface structure and NMB stainability of reticulocytes

Surface structure			Normal subjects (10 adults)							Reticulocytosis (4 cases)						
			No. of reticulocytes		NMB staining				No. of reticulocytes		NMB staining					
					I	II	III	IV			I	II	III	IV		
Discoid	centrally depressed	without elevation	38						36							
		with bridge-like elevation	3	Σ46	19	21	6	0	5	Σ51	18	20	12	1		
		flat disc	5						10							
Spheroid	centrally depressed hemisphere		10						11							
			0	Σ11	2	5	4	0	3	Σ23	4	10	7	2		
		hollows and elevations at a cell pole	1						9							
Spherical	centrally depressed sphere								7							
				Σ0					5	Σ46	2	14	30	0		
		hollows and elevations at a cell pole							34							
Other forms			Σ0						Σ3	2	0	1	0			
Total			57		21	26	10	0	123		26	44	50	3		
Surface with pits			0						58		2	16	38	2		

the cell shape, except for a spheroid reticulocyte which showed a slightly uneven surface.

Fig. 10 and 11. Erythrocytes of normal subjects. Arrows indicate discoid reticulocytes with a smooth surface among mature erythrocytes. $\times 2,800$

Reticulocytes in patients with reticulocytosis

Examination was made on 123 reticulocytes. Their structures often markedly differed from those of reticulocytes in normal subjects. This is shown also quantitatively in Table 2. Besides concave shaped reticulocytes (Fig. 12), there increased in number spheroid and spherical ones, the majority of which had a few hollows (diameter about 0.5μ) and sometimes also elevations (Fig. 9, 13–16). These hollows and elevations were often accumulated at a certain portion of the cell (Fig. 13, 16): occasionally several hollows were arranged radially around one or a few process-like elevations (Fig. 9, 13), giving the impression that a part of the protoplasm had been pulled out from that portion. Besides large hollows, some pits of about 0.15μ diameter were often recognized on the reticulocytes in reticulocytosis, regardless of the shape (Fig. 9, 12–16). These pits were dispersed at random on the cell surface and appeared to be unassociated with the hollows and elevations.

Relationship between surface structure and NMB stainability

According to the intensity of NMB staining reticulocytes were divided into four classes: reticulocytes which contained 1–4 NMB-stained particles were classified as type I and thus reticulocytes which contained 5–9, 10–19 and over 20 NMB particles, were classified in order as type II, III and IV. The surface structure of reticulocytes as observed under the SEM was described in relation to this classification and summarized in Table 2. Reticulocytes in normal individuals mainly belonged to type I or II and small part of them belonged to type III. On the other hand, many reticulocytes in reticulocytosis were of type III or II and less were of type I. A tendency was noted that NMB stainability was more intense in spherical reticulocytes than in discoid ones. In the present observation type IV occurred only rarely in reticulocytosis and never in normal subjects.

Fig. 12-16. A variety of forms of reticulocytes in cases of reticulocytosis. Figure 12 shows a reticulocyte whose contour is similar to mature erythrocytes. However, note many pits on the surface. The pits are also observed on other reticulocytes. Figures 13-16: Hollows larger than pits and elevations associated with the former are usually seen on spheroid or spherical reticulocytes and they are often accumulated in one place. Fig. 12; $\times 6,700$, Fig. 13; $\times 7,000$, Fig. 14; $\times 4,500$, Fig. 15; $\times 9,000$, Fig. 16; $\times 5,300$

Discussion

The method employed in this study is expected to be applied to the investigation of the surface structure of a certain cell type which is not readily distinguished from other cells in the sample. A similar method was used by MICHAELIS et al. (1971) in their SEM study of circulating leukocytes. They used, instead of a hemocytometer, glass slide pieces which had previously been evaporated through numbered grids placed on them for the identification of the cells observed. Their specimen holders must be discarded after use, whereas our hemocytometer can be used repeatedly.

The methodological experiment has revealed that Brecher's NMB solution was inadequate for the purpose of the present study. The question why Brecher's solution did affect the surface structure of erythrocytes is, at least mainly, answered by the fact that, as described above, the solution generally believed to be of isotonic effect to the cell turned out to be considerably hypotonic according to our examination. This answer seems to be justified by the results of subsequent examination of the other four NMB solutions. However, a possibility can not be excluded that potassium oxalate itself has some metabolic effects on the shape-supporting mechanism of erythrocytes. Our examination on four NMB solutions indicated that neither NMB at the concentrations tested, nor difference of slight degree in the osmolarity of the solvent or pH of the solutions significantly influence the external structure of the erythrocytes.

Whether the pits and other fine features of reticulocytes are not artifacts induced by NMB stain is another problem in our method. An answer, though indirect, may be given by the facts that normal erythrocytes prepared in the same way revealed no such structures and that some of the erythrocytes in cases of reticulocytosis presented similar structures even when prepared without NMB staining.

The present study has revealed various types of surface structures of definitely identified reticulocytes in normal humans and in patients with reticulocytosis. Among them the spherical reticulocytes with hollows and elevations at a cell pole correspond to the erythrocytes presented as probable reticulocytes by JENSEN (1969), and the discoid reticulocytes with many pits without central depression resemble the erythrocytes which were shown by CLARKE et al. (1967a, b) as presumed reticulocytes. The present study indicated that JENSEN (1969) as well as CLARKE et al. (1967a, b) pointed out only one type of reticulocyte among many. However, it should be kept in mind that this study fails to examine reticulocytes which escape staining by the NMB solution employed, as indicated by the results of our examination on the stainability of NMB solutions described in "Materials and Methods."

Considering their dimension and distribution, the pits on the reticulocyte surface may be equivalent to the openings of pinocytotic vesicles which are known, in thin sections, to be characteristic of erythroblasts and reticulocytes (Ropheocytosis: BESSIS and BRETON-GORIUS, 1959; BESSIS, 1967; SIMPSON and KLING, 1968). This assumption is favored by the fact that they were found often on reticulocytes but seldom on ordinary erythrocytes examined.

The hollows and elevations aggregated at one pole of the cell are thought to correspond to the cytoplasmic invaginations and projections in the transmission electron microscopic image of reticulocytes (BESSIS, 1967; SIMPSON and KLING, 1967) and to be

related to the process of denucleation. Indeed, this portion is believed to be nothing but the site where the nucleus has been eliminated.

Of various forms of reticulocytes, the spherical one with a complex of hollows and elevations and with many pits throughout the surface is considered to be the most immature form. This type of reticulocyte is supposed, by losing its hollows and elevations, to become the spheroid type and then the discoid one with pits, and further to be transformed into the discoid form with a central depression and some pits, and finally into the centrally depressed discoid with smooth surface. A gross comparison of the surface appearance with substantia reticulo-filamentosa of reticulocytes appears to support this recognition.

Referring to the previous results of transmission electron microscopy (BESSIS and BRETON-GORIUS, 1959; SIMPSON and KLING, 1967; SKUTELSKY and DANON, 1967; AKASAKA, 1971) or cinematography (MIYAKE, 1969) so far, the presumptive process of transformation in external structure from the mature erythroblast through the reticulocyte to the mature erythrocyte may be demonstrated as in Figure 17.

SIMPSON and KLING (1968) elucidated, by transmission electron microscopy, the process of extrusion of mitochondria and some other organelles from reticulocytes in peripheral blood in experimentally induced anemic dogs. However, we failed to

Fig. 17. A schematic representation of a possible way of maturation in the external structure of an erythroblast at its latest stage to a mature erythrocyte. **A.** A spherical erythroblast with some protrusions and pits. **B.** An erythroblast in an early stage of denucleation. A part of the cell is swollen by the nucleus. **C.** An erythroblast in denucleation. **D-F.** Reticulocytes in an early stage which seldom appear in circulating blood under normal conditions but appear not infrequently in reticulocytosis. **G** and **H.** Discoid reticulocytes with and without pits. The latter sometimes appear in circulating blood of normal subjects. **I.** A reticulocyte in the latest stage and at the same time a mature erythrocyte.

reveal on reticulocytes any protrusions or hollows indicating this process. It may be worth-while to mention that wart-like protrusions were occasionally found on normal erythrocytes (TOKUNAGA, FUJITA and HATTORI, 1969) which may suggest that process.

The present study has shown that, under normal conditions, mature reticulocytes appear in peripheral blood, whereas under the pathological conditions examined immature as well as mature reticulocytes appear. Further, it was noted that the majority of reticulocytes in a case of gastric cancer with metastasis in the bone marrow were immature ones and those in cases of treated iron deficiency anemia were often mature in type. This difference in the nature of reticulocytes under varying clinical conditions provides a new problem to be studied. The examination of reticulocytes in bone marrow is also required.

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網赤血球の走査電子顕微鏡像

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網赤血球の立体形態を検討するために、ニューメチレンブルウ (NMB) 染色によって網赤血球と同定された赤血球を走査電子顕微鏡で観察する方法を考案し、正常人 (10名) と網赤血球増多症 (4名) について観察を行なった。一般に使われている Brecher の染色液は赤血球の形態を変化させることがわかったので、0.5% NMB 液 (0.15 M リン酸緩衝液, pH 7.4) を採用した。Thoma の血球計算盤の中央の分画部を切り出し、あらかじめ NMB 染色ののち固定、水洗した赤血球をこの上にのせ乾燥させる。血球計算盤の分画線を目標にして光顕で同定した網赤血球を走査電顕で観察した。

正常人では表面が平滑で中央がくぼんだ円板——つまり一般的な成熟赤血球の形態——をとる網赤血球が大部分であった。

網赤血球増多症ではこのような形のものが半数を占めたが、他の半数は類球状や球状で、細胞の一極に集中して陥凹や隆起がみられ、これは赤芽球の脱核直後の像と考えられた。また網赤血球増多症の多くの網赤血球の表面には、BESSIS らの Rhotheocytosis に相当すると思われる小穴がみられた。以上の成績をもとに、これまでの光線顕微鏡や透過電子顕微鏡による諸家の成績を参考にしながら赤血球から脱核をへて網赤血球らさに成熟赤血球に至る外形の経過を模式図で示した。

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