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Electron Microscopical Analysis of Surface Charge Labelling Density at Various Stages of the Erythroid Line

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Summary. With use of the positively charged, colloidal ferric oxide labelling technique for electron microscopy of sections of rabbit marrow, a reduction in labelling density on the surface of differentiating red cell precursors was demonstrated. The number of iron particles per unit length of membrane was counted. A progressive diminution in labelling density follows cell division, reaching a minimum in the orthochromatic erythroblast, from which the nucleus is expelled. A slight increase in charge density is noted in the reticulocyte, and further increase is observed with its maturation to the erythrocyte. The results indicate that biosynthesis of n-acetyl neuraminic acid stops at the earliest recognizable stage of erythroid differentiation.

As the hemopoietic stem cell differentiates into an erythroid cell, it undergoes several divisions [1, 4], up until its nucleus is expelled. During this period, biochemical and structural modifications occur in the cell development, leading toward the highly specialized red blood cell. Synthesis of hemoglobin gradually increases, while the cell and its nucleus decrease in size. There is a gradual disappearance of most of the cytoplasmic organelles. On expulsion of its nucleus, the late orthochromatic erythroblast becomes a reticulocyte which, within a short time, matures to an erythrocyte. The electric surface charge of the reticulocyte increases with maturation [7, 18, 19], and then decreases again as the red cell becomes senescent [5, 6, 10, 11, 19, 20].

The purpose of the present study was to determine whether the structural and biochemical modifications that occur during erythroid differentiation are restricted to the nucleus and to the cytoplasm and its organelles, or whether they are also expressed as differences in the surface characteristics of the cell membrane.

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The lack of a reliable method for separating homogeneous cell groups from the heterogeneous hemopoietic cell population of the bone marrow, in order to carry out electric mobility or agglutinability measurements on such groups, suggested the use of the positive colloidal ferric oxide staining technique [8], so that one could observe, on the ultrastructural level, the charge density at various stages of cell development in the bone marrow.

Materials and Methods

Cell suspension was prepared by placing small pieces of bone marrow from young rabbits in a phosphate buffer solution, and then passing the suspension repeatedly through a syringe needle [16]. The suspension was filtered through six layers of cheesecloth, and the filtrate containing the bone marrow cells was centrifuged for 7 min at $150 \times g$.

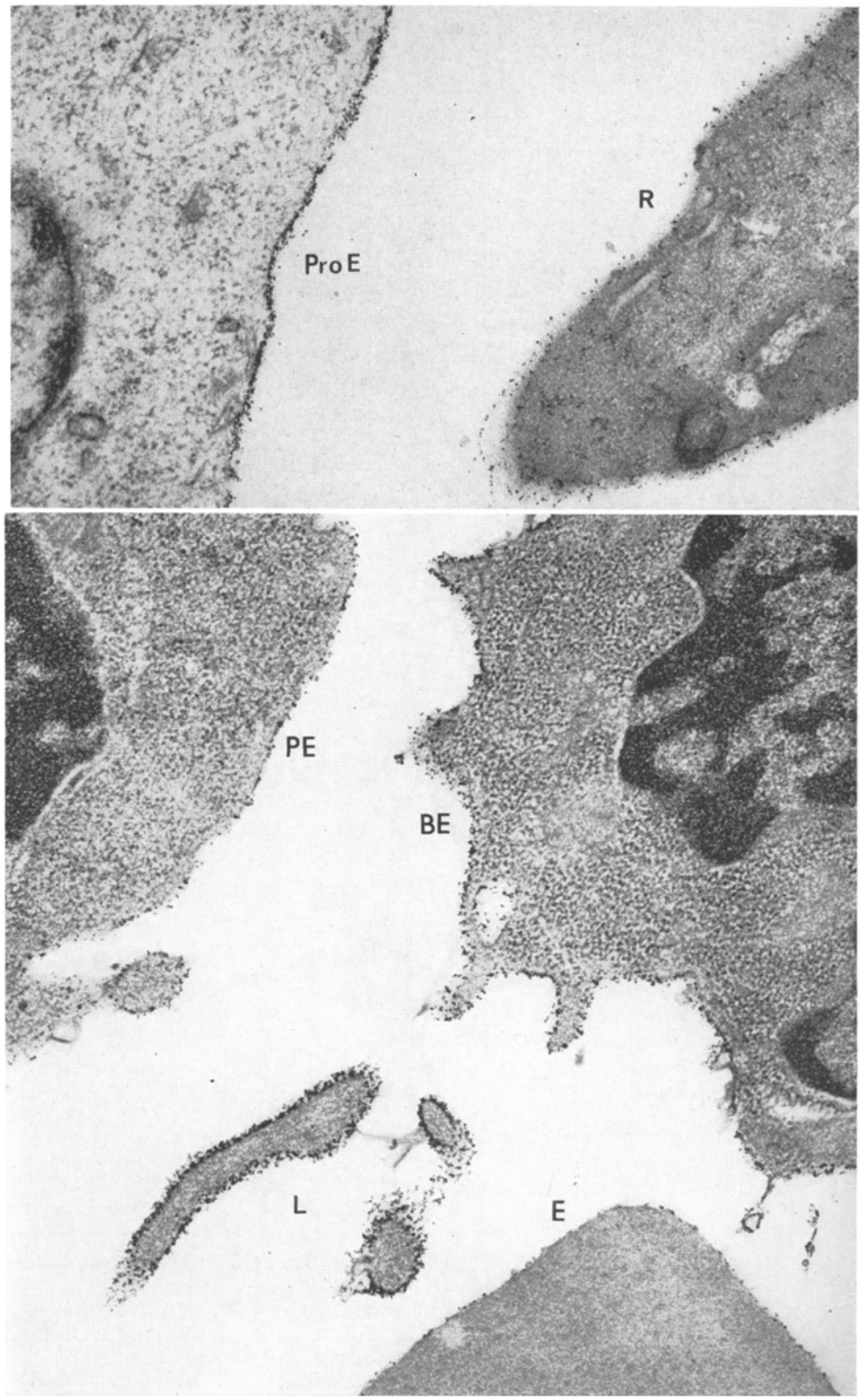
The sedimented cells were fixed by resuspension in 3% glutaraldehyde in phosphate buffer at pH 7.4 [12] for 1 hr at room temperature, washed twice in 0.1 M phosphate buffer, adjusted to isotonicity with glucose, and then allowed to stand overnight in the same buffer. The cells were then washed twice with distilled water and resuspended in positively charged colloidal ferric oxide solution, prepared according to Gasic, Berwick and Sorrentino [8]. The cells were kept in the colloidal solution, at pH 1.8, for 20 min at room temperature, washed twice with 12% acetic acid, and finally washed with distilled water. They were postfixed with 1% OsO_4 in phosphate buffer [12] for 1 hr, dehydrated, embedded in Vestopal-W [15], and then sectioned with the Danon Ultramicrotome (Yeda Research and Development Co., Rehovoth, Israel), equipped with glass knife. Sections were mounted on bare or Formvar-coated copper grids, reinforced with a thin layer of carbon, and stained on the grid with uranyl acetate and then with lead citrate [14]. Jem-7 and Jem-T7 electron microscopes were used.

In order to compare the density of the attached colloidal particles on the surface of the different cell membranes, a curvimeter (map measurer) was used to measure the length of perpendicularly sectioned membranes on which the number of black dots was counted. Micrographs at a final magnification of 30,000 were used. Pieces of such micrographs, containing the measured membranes, were laid on petri dishes and covered with a layer of 1% agar. The black dots were counted by contact with a needle using an automatic colony counter (New Brunswick Co., New Brunswick, N.J., Model 110).

Results

Ferric oxide particles appearing as black dots were observed on the membranes of all rabbit bone marrow cells. The identification of the various stages of cells of the erythroid line by electron microscopy was based on

Fig. 1. Section of rabbit bone marrow cell suspension, stained with the positively charged colloidal ferric oxide. Note the difference in the density of iron particles on the various cell membranes. The proerythroblast (*Pro E*) as well as the pseudopods of the leukoid cell (*L*) are the most heavily labelled; the basophilic erythroblast (*BE*) is less labelled; the polychromatophilic erythroblast (*PE*) is less labelled than the *BE*; and the reticulocyte (*R*) and the erythrocyte (*E*) are the least labelled. × 30,000


R
Pro E
PE
BE
L
E

previously established criteria [9, 13]. No colloidal iron particles appeared on organelles such as mitochondria or nuclei released during the preparative procedures from broken cells. In one bone marrow suspension (out of four) in which a different batch of iron hydroxide was used for the labelling procedure, the cells were found to be labelled to a lesser extent by the colloid. In the other samples, variations in labelling density of similar cell types were small. In all bone marrow suspensions, the iron particles on the surface of the cells varied in density, according to the stage of development in the erythroid line (Fig. 1). Proerythroblasts were heavily coated by the colloid. From the basophilic erythroblast stage onward, there was a progressive reduction in the density of the colloid particles, which reached a minimum in the late orthochromatic erythroblast just before expulsion of the nucleus. In the newly formed reticulocyte, a slight increase in particle density was noted, and a further increase in the density of colloidal particles was observed

Table. *Density of iron particles on the membrane surface of various erythroid cells*

Rabbit no.	Erythroid stage of development	Length (μ)[a]	Mean colloidal particle count per micron	Range for different cells of same stage
1	Proerythroblast	89.3	45	43 – 50
	Basophilic erythroblast	59.4	34	30 – 35
	Polychromatic erythroblast	156.2	18	16 – 20
	Orthochromatic erythroblast	144.5	10	9 – 11
	Early reticulocyte[b]	56.5	11	9 – 12
	Late reticulocyte	77.6	15	13 – 17
2	Basophilic erythroblast	125.3	55	50 – 63
	Polychromatic erythroblast	140.9	31	28 – 34
	Orthochromatic erythroblast	147.7	22	20 – 23
	Late reticulocyte	141.6	28	26 – 31
3	Basophilic erythroblast	88.8	64	59 – 70
	Polychromatic erythroblast	138.1	31	28 – 33
	Orthochromatic erythroblast	147.2	18	16 – 19
	Late reticulocyte	78.3	23	22 – 24
4	Basophilic erythroblast	116.4	78	70 – 90
	Polychromatic erythroblast	153.1	50	47 – 53
	Orthochromatic erythroblast	99.0	36	34 – 37
	Late reticulocyte	87.9	44	41 – 46

[a] Total length of perpendicularly sectioned membrane, at 30,000 magnification micrographs on which iron particles were counted.

[b] This additional count of iron particles on membranes of early reticulocytes to be compared with that of late ones was made to serve for the illustration in Fig. 2.

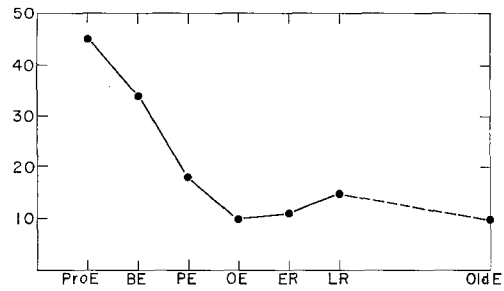


Fig. 2. Number of iron particless counted per micron length of membrane (ordinate) at various erythroid developmental stages (abscissa) in bone marrow of rabbit no. 1 (*see* Table). *Pro E*, proerythroblast; *BE* basophilic erythroblast; *PE* polychromatic erythroblast; *OE* orthochromatic erythroblast; *ER* early reticulocyte; *LR* late reticulocyte; *Old E* old erythrocyte. The estimation of the diminution of the charge of senescent red cells (*Old E*) is derived from references [5, 6, 10, 11, 20]

with maturation to the erythrocyte stage. Leukoid cells were as heavily coated by the colloid as were proerythroblasts (Fig. 1).

Counts of the number of particles per micron of perpendicularly sectioned membranes of the different stages of the erythroid line are presented in the Table. Particles coating the proerythroblasts, the most heavily labelled by the colloid, were counted only in the marrow suspension of rabbit no. 1, all the cells of which displayed a low colloidal-particle density. In the other three rabbits, counts were performed only from the basophilic erythroblast stage onward. This was necessary because when the number of iron particles exceeds 50/μ of perpendicularly sectioned membrane in sections of about 500 – 600 A thickness, overlapping of the black dots on the photograph prevents accurate counting. However, there is considerable difference in the labelling density between these two stages. It is apparent from the counts of iron particles on membranes of proerythroblasts and basophilic erythroblasts from the bone marrow of rabbit no. 1, in which labelling was about half that of the bone marrow from the other three rabbits (Table).

From the results presented in the Table, it can be seen that the density of the black dots decreases with successive stages of development of the erythroid line occurring after each cell division. This progressive reduction in surface charge is illustrated in Fig. 2, which is based on the counts of rabbit no. 1 (Table).

Discussion

The decrease in the staining capacity of positively charged colloidal ferric oxide particles, at the progressively developed stages of the erythroid line, indicates that the biochemical and structural changes which occur

during the successive division of the erythroid cells and the maturation to erythrocyte are not restricted to the cytoplasm and the nucleus. These changes are also expressed on the surface of the cell membrane. The black-dot density (Table) indicates that the decrease following every cell division results very probably from the enlargement of the overall surface of the two daughter cells, as compared with that of the mother cell. Another possible explanation would be that the reduced labelling capacity is associated with the differentiation that takes place simultaneously. The distinction between the two explanations is impossible under the present experimental conditions. The reduction in the iron-particle density continues as long as there is cell division, and reaches a minimum in the last erythroblastic stage, i.e., the orthochromatic erythroblast, before the nucleus is expelled. Furthermore, the expulsed nucleus, surrounded by a narrow rim of cytoplasm and membrane [16], lost more than half of its charge as indicated by the density of iron particles [17].

The positive colloidal ferric oxide particles adhere specifically to the n-acetyl neuraminic acid [8, 10]. It seems reasonable, therefore, to assume that the progressive reduction in the density of iron particles, subsequent to the cell division observed in this study, indicates that the biosynthesis of this molecule and/or its inclusion in the membrane structure is discontinued at an early stage after differentiation of the erythroid line.

The slight increase in iron oxide density in the newly formed reticulocyte and the further increase as it matures to the erythrocyte are in accordance with previous observations [7, 18]. This increase in surface charge, as demonstrated by the higher labelling density, is attributed to the reduction of the surface-to-volume ratio of the maturing reticulocyte previously noted [2, 3, 7].

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