

VARIATIONS IN DISTRIBUTION OF CON A RECEPTOR SITES AND ANIONIC GROUPS DURING RED BLOOD CELL DIFFERENTIATION IN THE RAT

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ABSTRACT

The distribution of receptors for concanavalin A (Con A) and anionic groups on plasma membranes of developing blood cells was investigated in the rat. Glutaraldehyde-fixed bone marrow and circulating blood cells were exposed to ferritin-conjugated Con A or positively charged ferric oxide (CI) and processed for electron microscopy. The frequency of Con A and CI binding sites varied during different erythroid developmental stages and among different leukoid cell types. There was a constant inverse relationship between Con A and CI binding sites. Among leukoid cells, Con A binding was high on plasma cells and macrophages, lower on neutrophils and lymphocytes, and still lower on eosinophils and basophils; CI binding was highest in the latter and lowest in plasma cells and macrophages. Among erythroid cells, there was a progressive increase in Con A and a decrease in CI binding after successive divisions of erythroblasts, and a progressive decrease in Con A and an increase in CI binding upon maturation of the orthochromatic erythroblast to the reticulocyte. The most pronounced modification in distribution of these sites occurred during nuclear expulsion: that portion of the plasma membrane surrounding the extruded nucleus was heavily labeled by Con A (up to four times that of the orthochromatic erythroblast) whereas the reticulocyte had considerably fewer sites. The situation was reversed with CI. The results suggest that the concentration of nonsialated glycoproteins (to which Con A binds) varies inversely to that of sialoproteins (to which CI binds) in the membrane of the differentiating erythroid cell. The remarkable changes observed at the time of nuclear extrusion suggest that there is either local modification of glycosylated groups with removal of sialyl residues from the membrane surrounding the extruded nucleus or selective segregation of membrane glycoproteins leading to a high concentration of sialoproteins (glycophorin) in the membrane of the mature erythrocyte.

Previous studies utilizing electron microscope labeling techniques have established that differentiation of the mammalian erythrocyte is associated

with significant changes in the density and distribution of binding sites (presumably glycoproteins) on the plasma membrane. Differences have been

found between various erythroid developmental stages in surface density of negative charges (34, 36), cell surface antigenicity (22, 38), and pyroantimonate (1) and Con A (2) binding. By using positively charged colloidal ferric oxide (which binds to sialic acid residues [12, 20, 42]) it was previously shown that the density of anionic groups in developing rabbit erythrocytes gradually decreases with successive divisions of the proerythroblasts, then reincreases after expulsion of the nucleus and on maturation of the reticulocyte to become a mature erythrocyte (13, 15, 36). If one assumes that the binding sites are associated with glycoproteins, these modulations in the surface charge density suggest that the rate of synthesis and insertion into the red blood cell membrane of different plasma membrane proteins varies at specific stages in the differentiation process.

It has further been shown that, in the rabbit, uneven segregation of membrane anionic groups takes place during extrusion of the nucleus from the late erythroblast (37) (accomplished by a process resembling cytokinesis [33, 35]). Anionic group density on that portion of the plasma membrane surrounding the extruded nucleus is one-third or less of that on the newly produced reticulocyte (34). The fact that this transition from high to low anionic group density is sharp and can be detected at an early stage of expulsion suggests that, by some unknown mechanism, segregation of anionic groups from other membrane components can take place in the plane of the membrane of the erythroblast.

The purpose of the present study was to determine whether the modifications described for sialoglycoprotein distribution in developing erythroid cells apply to other membrane glycoproteins as well or whether other membrane glycoproteins behave differently. Certain plant agglutinins which bind to specific hexose residues of membrane glycoproteins can be conjugated to electron-dense molecules and used for the localization of specific cell surface saccharides (27). In the present study we used ferritin-conjugated concanavalin A (Con A)¹ for analysis of the density distribution of receptors for Con A on membrane surfaces of eryth-

roid cells in the bone marrow and blood of the rat. The frequency of Con A receptors in erythroid cells has been compared to surface anionic group density as estimated by binding of positively charged colloidal ferric oxide (CI). Similar, more preliminary data on leukoid cells are also included.²

MATERIALS AND METHODS

Materials

Twice or six times crystallized horse-spleen ferritin (cadmium-free) and twice crystallized Con A were obtained from Miles Laboratories, Inc., Kankakee, Ill. Sodium heparin was obtained from Abbott Laboratories, North Chicago, Ill. *Vibrio cholerae* neuraminidase was obtained from Behringwerke, Marburg, Germany, as a solution containing 500 U/ml.

Preparation of Ferritin-Conjugated Lectins

Con A was used directly as obtained commercially. For conjugation of lectins to ferritin, the method of Nicolson and Singer (27) was carried out as follows: to a mixture containing 25 mg/ml Con A and approximately 100 mg/ml ferritin with 0.5 M NaCl in 0.05 M phosphate buffer, pH 6.8, 0.5% glutaraldehyde was added to a final concentration of 0.05%. After stirring for 60 min at room temperature, the reaction mixture was dialyzed against 0.1 M NH₄Cl for 3 h, followed by dialysis against phosphate-buffered saline (PBS). Large aggregates were removed by centrifugation at 15,000 g for 30 min. Unconjugated ferritin was removed by affinity chromatography on a Sephadex G 100 column. Elution was accomplished with 0.1 M glucose in 0.5 M PBS. Ferritin-conjugated lectins were separated from unconjugated lectins and large polymers by column chromatography on Biogel A-5M (BioRad Laboratories, Richmond, Calif.) by the techniques of Maylié-Pfenninger et al. (21). Fractions monitored for absorbance at 280 nm were tested for agglutinability of rat red blood cells and for staining characteristics by direct examination on thin sections in the electron microscope. Fractions consisting primarily of monomers and small polymers (cf. reference 21) were preferred for electron microscopical analysis of labeling density on cell membranes.

Preparation of Colloidal Ferric Oxide

Positively charged colloidal ferric oxide was prepared according to the procedure of Gasic et al. (12). Undialyzed colloid at pH 1.8 was used for labeling.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; CI, positively charged colloidal ferric oxide; Con A, concanavalin A; EDTA, ethylenediaminetetraacetic acid; KRB, Krebs-Ringer bicarbonate solution; PBS, phosphate-buffered saline.

² These findings were presented to the annual meeting of the American Society for Cell Biology and published in abstract form (32).

Collection and Fixation of Cells

BONE MARROW CELLS: Bone marrow was obtained from femurs of 120–150-g male Sprague-Dawley rats. Pieces of tissue were removed immediately after decapitation of the animal and transferred into Ca^{++} - and Mg^{++} -free Krebs-Ringer bicarbonate solution (KRB) containing 2 mM EDTA.

The tissue was then cut into small ($\sim 1 \text{ mm}^3$) blocks, incubated in KRB-EDTA for an additional 5 min at 37°C , and transferred to EDTA-free KRB medium. Bone marrow cells were released into the medium by repeated mild pipeting through a Pasteur pipet with a flame-polished tip. In several experiments, the dissociation was accomplished in Ca^{++} - and Mg^{++} -free medium without EDTA. For removal of undissociated tissue fragments, the suspension was filtered through several layers of cheesecloth. After sedimentation at 100 g for 5 min, the pellet of bone marrow cells was resuspended in fresh KRB (containing 0.1 mM Ca^{++} , 0.12 mM Mg^{++} , and 0.3% BSA).

CIRCULATING RED BLOOD CELLS: Blood was collected from decapitated animals with sodium heparin as anticoagulant (15 U/ml). After sedimentation, the pellet was rinsed twice and resuspended in KRB medium.

ALDEHYDE FIXATION: All labeling procedures were performed on aldehyde-fixed cells. Fixation was accomplished by adding cell suspension to an equal volume of dilute Karnovsky fixative (16) containing 1% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After 30 min at 25°C the cells were rinsed three times in buffer. Free aldehyde groups were reduced by incubation for 15 min at 25°C in 0.1 M borohydrate or 0.1 M glycine, before the cells were transferred to the labeling solution.

Treatment of Cells with Neuraminidase

Bone marrow or circulating blood cells were incubated at 37°C for 1 h in either (a) KRB alone, (b) KRB containing neuraminidase, 100 U/ml, or (c) KRB containing heat (65°C for 30 min)-inactivated neuraminidase (same amount).

Labeling Procedures

An aliquot of 10^7 bone marrow or circulating blood cells prefixed with aldehydes as described above was washed three times in PBS and then incubated for 30–45 min with 1 mg/ml ferritin-conjugated lectins in PBS at 25°C . For controls, cell samples were incubated with the conjugates in the presence of 0.1 M of the hexose competitor (D-methyl mannoside).

Cells to be stained with colloidal iron were washed with distilled water and then resuspended in the colloid for 5–10 min at 25°C . After a brief rinse in 12% acetic acid followed by distilled water, the cells were resuspended in acetate-Veronal buffer, pH 7.4.

Processing for Electron Microscopy

Labeled cells were washed with acetate-Veronal buffer, pH 7.4, transferred to 0.4-ml polyethylene tubes and packed by centrifugation at 10,000 g in a Microfuge 152 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) according to a method used previously for collection of polymorphonuclear leukocytes (4). The tips of the tubes containing the pellets were cut out, placed in vials, postfixed for 1 h at 4°C in 1% OsO_4 in acetate-Veronal buffer (pH 7.4), washed once with acetate-Veronal buffer, pH 7.4, stained in block for 30–60 min with buffered 0.5% uranyl acetate at 25°C (7), dehydrated in graded ethanols, and embedded in Epon (18). Sections of approximately 600-Å thickness showing gray to silver interference colors were cut with Dupont diamond knives (E. I. DuPont de Nemours & Co., Wilmington, Del.) on a Porter-Blum MT-2B Ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.). The sections were stained with lead alone or doubly stained with aqueous uranyl acetate and lead citrate (31). In some instances the bismuth subnitrate staining technique for enhancing the contrast of ferritin particles (3) was used. The sections were examined in a Siemens Elmiskop 1 or 101 operating at 80 kV with a double condenser.

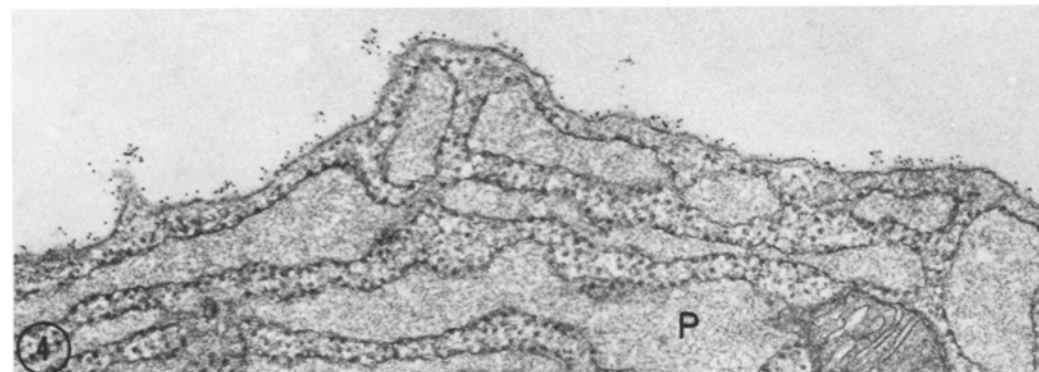
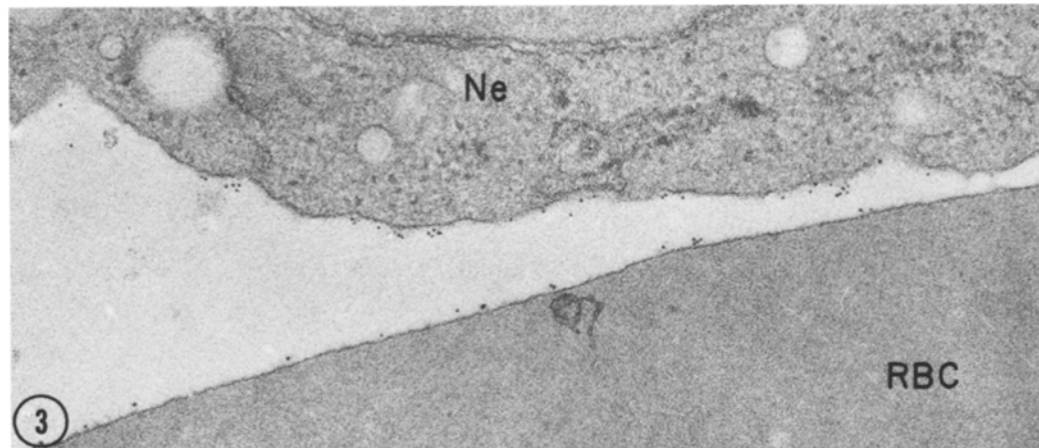
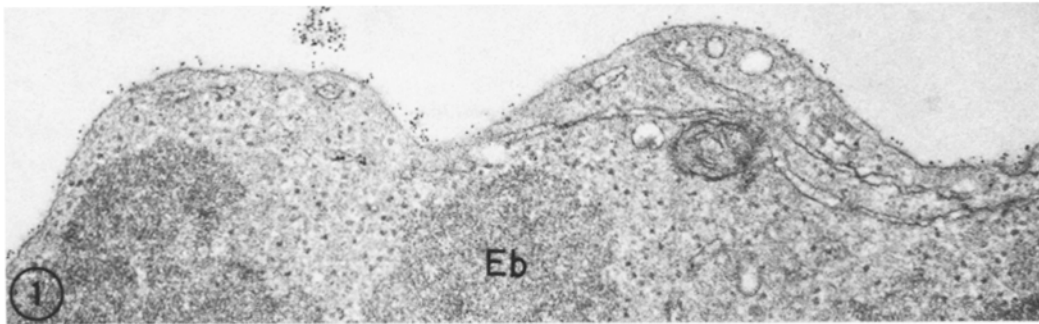
Analysis of Labeling Density

In order to compare the density of attached ferritin particles on the surface of the different cell membranes, black dots representing individual ferritin particles that were seen attached to perpendicularly sectioned membranes were counted on electron micrographs at a final magnification of 35,000. The length of the perpendicularly sectioned membranes on which the number of ferritin particles was counted was measured on the micrograph with a map measurer (36). For each leukoid cell type or erythroid developmental stage, at least 20–30 cells were counted.

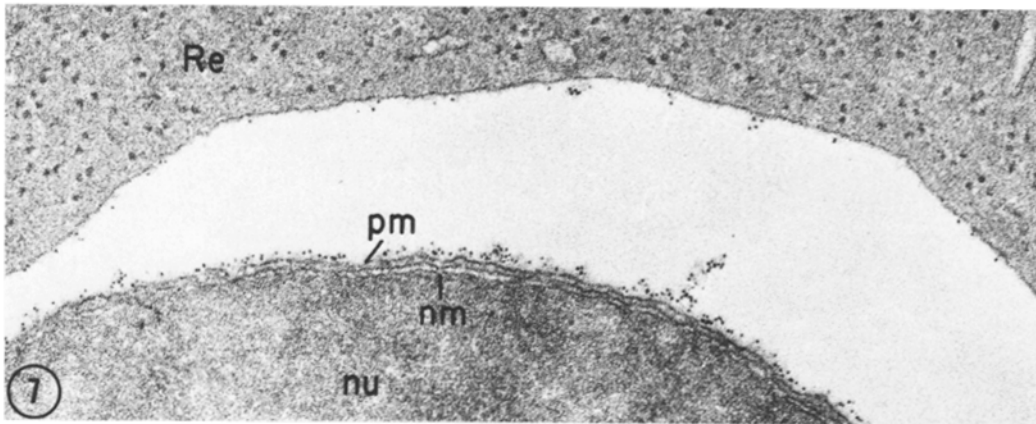
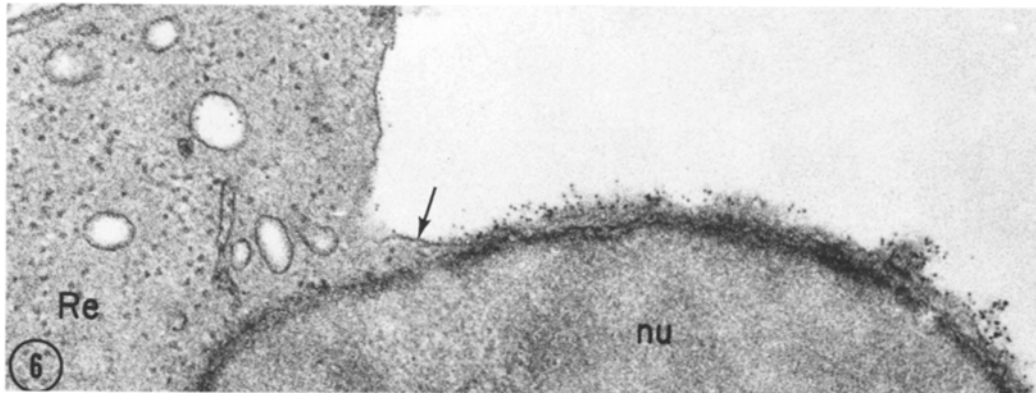
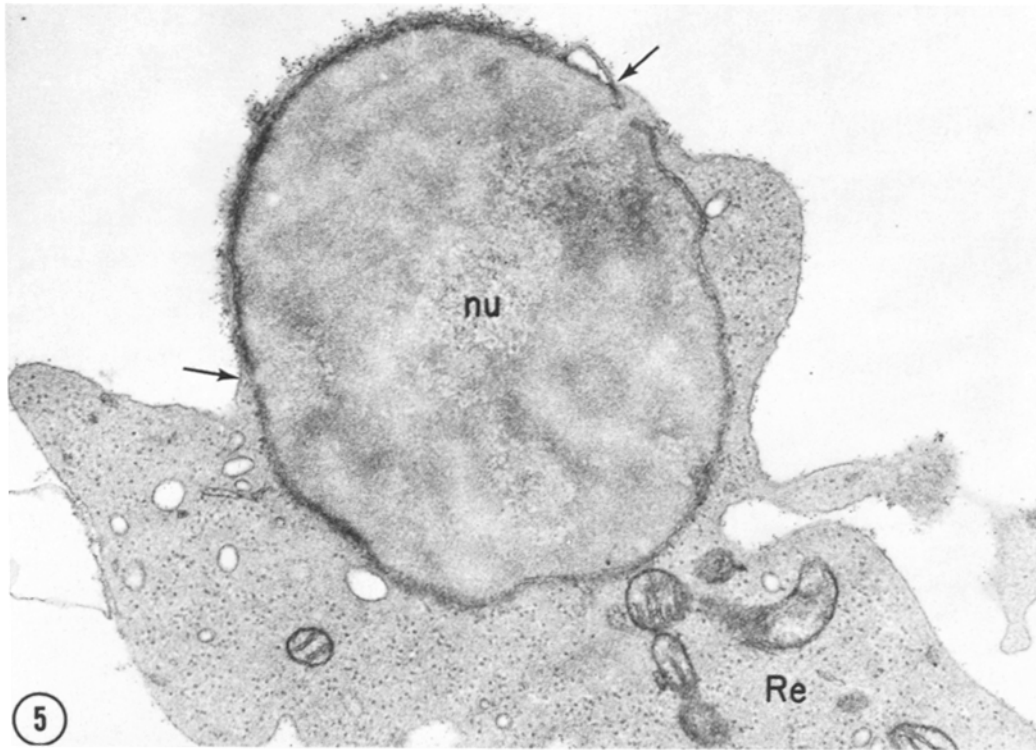
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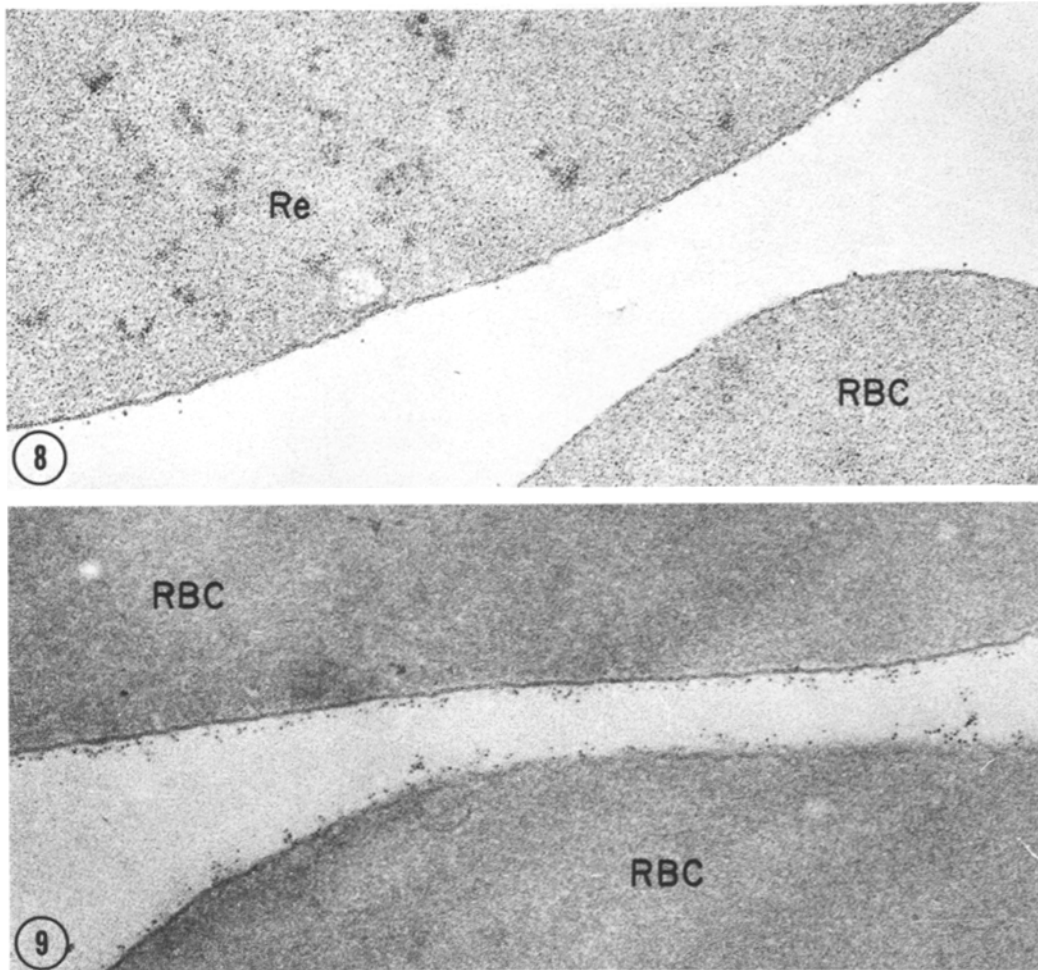
Binding of Ferritin-Conjugated Con A to Bone Marrow Cells

ERYTHROID CELLS: Reactivity of erythroid cell membranes to Con A clearly varied with the degree of maturation (Figs. 1–9). A histogram summarizing measurements made of labeling density on erythroid cells at different developmental stages is shown in Fig. 10. The data indicate that division of the proerythroblast is accompanied by a slight increase in reactivity to Con A during the basophilic erythroblast stage (Fig. 1) which peaks at the late orthochromatic stage (Fig. 2) with an average density of $20/\mu\text{m}$. The trend reverses



FIGURES 1-4 Rat bone marrow cells labeled with ferritin-conjugated Con A. Fig. 1 includes a portion of an early (basophilic) erythroblast (*Eb*) and Fig. 2 includes a late (orthochromatic) erythroblast (*Eb*), both of which show moderately heavy labeling. By contrast, a portion of an eosinophilic myelocyte (*Eo*) included in Fig. 2 shows very few ferritin molecules. Fig. 3 shows a portion of a mature erythrocyte (*RBC*) and a neutrophilic myelocyte (*Ne*) which have a low level of labeling. Fig. 4 shows a portion of a plasma cell (*P*) with its prominent dilated rough ER. Its cell membrane has large numbers of ferritin particles, indicating a high frequency of receptor sites for Con A. Figs. 1 and 4, $\times 32,000$; Fig. 2, $\times 49,000$; Fig. 3, $\times 40,000$.





FIGURES 8 and 9 Circulating erythrocytes labeled with ferritin-conjugated Con A. The cells in Fig. 9 were treated with neuraminidase before labeling, whereas those in Fig. 8 were not. Note the increased labeling of the neuraminidase-treated cells. *Re* = reticulocyte; *RBC* = mature erythrocyte. $\times 50,000$.

FIGURES 5-7 Erythroid cells from rat bone marrow stained with ferritin-conjugated Con A. Fig. 5 shows a late erythroblast in the process of nuclear extrusion. Note that the frequency of Con A receptor sites on the portion of the cell membrane surrounding the extruded nucleus (*nu*) between the arrows on the upper left is much greater than that of the remainder of the membrane surrounding the presumptive reticulocyte (*Re*). Fig. 6 is a higher magnification of a portion of Fig. 5 and shows the region of sharp transition (arrow) in the level of labeling of the membrane of the extruding nucleus (*nu*) and the presumptive reticulocyte (*Re*). Note that the portion of the cell membrane surrounding the extruded nucleus is much more heavily labeled with ferritin. Fig. 7 shows a free erythroid nucleus (*nu*) and a reticulocyte (*Re*). The heavy labeling of the membrane of the former and the sparse labeling of the membrane of the latter are evident. Note that the nucleus is not bare but is covered by a thin layer of cytoplasm with its plasma membrane (*pm*). *nm* = nuclear membrane. Fig. 5, $\times 20,000$; Figs. 6 and 7, $\times 35,000$.

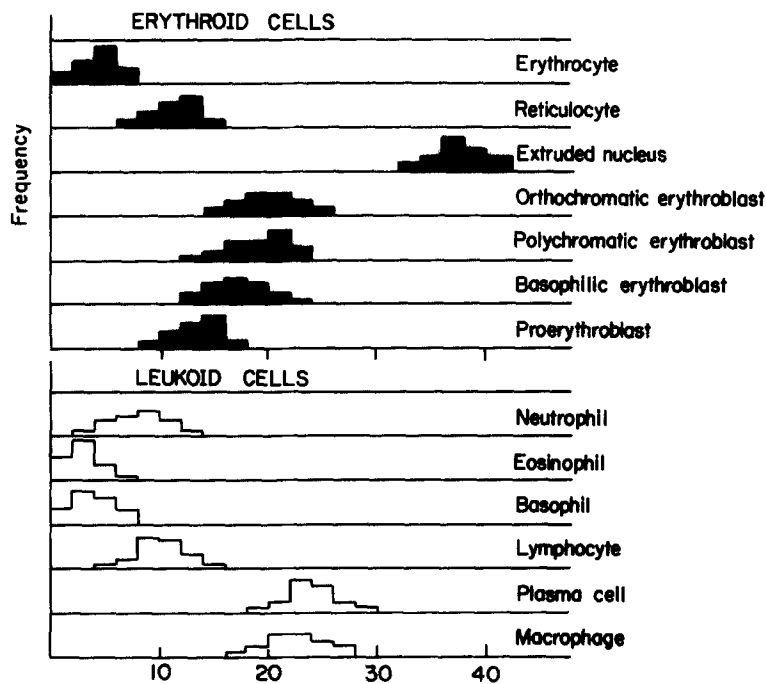


FIGURE 10 Histogram showing the frequency distribution of bone marrow cells according to reactivity of cell surfaces to Con A. *Ordinate*: frequency in percent. The scale is drawn so that the distance between the lines represents 50% of each cell population. *Abscissa*: Con A-conjugated ferritin particles counted per micrometer length of perpendicularly sectioned membrane. The cells of the erythroid line are listed according to the degree of maturation. No such separation according to developmental stage was made in leukoid lines in which the differences in binding capacity for Con A of the different developmental stages were minor.

sharply after the loss of the nucleus from the late erythroblast and formation of the reticulocyte, which presents an average density of 12 particles/ μm (Figs. 7 and 8). Further, sharp reduction in reactivity for Con A occurs on maturation of the reticulocyte to the erythrocyte which has an average particle density of only 5/ μm (Figs. 3 and 8).

CON A BINDING TO THE PLASMALEMMA SURROUNDING "EXTRUDED" ERYTHROID NUCLEI: Nuclei extruded from late erythroblasts are regularly found in bone marrow cell suspensions. Upon release, such nuclei are surrounded by a narrow rim of cytoplasm with a plasma membrane (Figs. 5-7). Progressive stages in the process of nuclear expulsion are often found in fixed bone marrow specimens (Figs. 5 and 13). Label counting on erythroblasts in the process of nuclear expulsion indicates a sharp difference between plasmalemmal domains associated with the nucleus and the rest of the plasmalemma. The average density on the former is 38 particles/ μm while on the latter it is 12 particles/ μm (Table I

and Fig. 11). The difference in distribution is detected early in the process, and the transition from high to low density domains is sharp. The ratio of particles in the transitional domain was not established as extrusion of the nucleus advances, but it would be expected to increase. The fact that the final level of Con A receptor site density on both the extruded nucleus and the newly produced reticulocyte are reached before expulsion is complete indicates that the observed differences between the two membranes are not the result of postexpulsion insertion or subtraction of binding sites but of a specific segregation of Con A receptor sites that occurs during the expulsion process.

LEUKOID CELLS: Differences in binding capacity for Con A clearly related to cell type were noticed between different leukoid cells in the bone marrow (Figs. 2-4). The data obtained on the labeling density of different leukoid cells are given in the histogram in Fig. 10. Labeling density was high on macrophages and plasma cells (Fig. 4) with an average of 24 particles/ μm length of mem-

TABLE I
Number of Attached Con A-Conjugated Ferritin Particles Counted per Micrometer Length of Membrane Surrounding Nuclei that are Being Extruded as Compared with Membrane Surrounding the Remaining Cytoplasm

Cell no.	Presumptive reticulocyte	Extruded nucleus	Ratio
1	14.9	44.0	1:2.9
2	10.4	32.0	1:3.1
3	13.0	45.3	1:3.5
4	11.0	40.8	1:3.7
5	12.8	33.6	1:2.6
6	14.1	40.2	1:2.9
7	12.2	39.0	1:3.2
8	10.7	33.0	1:3.1
9	14.8	41.0	1:2.8
10	12.8	34.8	1:2.6
Mean:	12.7	38.5	1:3.0

brane, low on cells of the eosinophilic (Fig. 2) and basophilic series with 4 particles/ μm , and slightly higher on cells of the neutrophilic series (Fig. 3) with 7 particles/ μm , and on lymphocytes with 8 particles/ μm .

CONTROLS: No binding of ferritin was seen in controls incubated with Con A-ferritin conjugate in the presence of the hexose competitor or in controls incubated in the presence of unconjugated ferritin.

Anionic Group Labeling Density on Bone Marrow Cells

ERYTHROID CELLS: Differences in the binding capacity of plasma membranes for CI were observed among different erythroid developmental stages (Figs. 12-15). Nucleated erythroid cells in the bone marrow generally presented a low reactivity, with a successive reduction after cell division of the proerythroblast, reaching a minimum in the late orthochromatic stage (Fig. 12). This trend was reversed after the expulsion of the nucleus and formation of the reticulocyte (Fig. 13). An increase in binding capacity occurs on the reticulocyte (Fig. 13) and a further, remarkable increase occurs on its maturation to an erythrocyte (Fig. 15).

In contrast to the increase in anionic group density noted on the newly generated reticulocyte, binding capacity for CI was reduced dramatically on plasma membranes surrounding nuclei extruded from late erythroblasts. Such extruded nu-

clei showed relatively few attached iron particles (Fig. 14), and those present tended to have a patchy distribution. This reduction takes place at early stages of the expulsion process and could be seen on membrane regions surrounding extruding nuclei which were still attached to the presumptive reticulocyte cytoplasm (Fig. 13).

LEUKOID CELLS: Variations in negative surface charge density were also observed between leukoid cells of different cell types. Binding capacity for CI was generally inversely related to the relative activity for Con A. Cell types presenting high reactivity for Con A, such as plasma cells (Fig. 15) and macrophages, were poorly labeled by CI. Those that were poorly reactive to Con A, such as eosinophils and neutrophils (Figs. 14 and 15), were heavily labeled by CI.

Effect of Neuraminidase on CI and Con A Binding

Neuraminidase activity was estimated by its ability to reduce binding capacity of cell membranes for CI. Erythroid cells were found to be less susceptible to the action of *V. cholerae* neuraminidase than leukoid cells. Treatment of either circulating red blood cells or bone marrow cells with neuraminidase resulted in almost complete loss of binding capacity for the positively charged colloid in leukoid cells but, in keeping with the findings of others (24, 42), resulted in only a partial reduction of such capacity in cells of the erythroid series (Fig. 16). However, treatment of circulating erythrocytes with neuraminidase resulted in a considerable increase in membrane

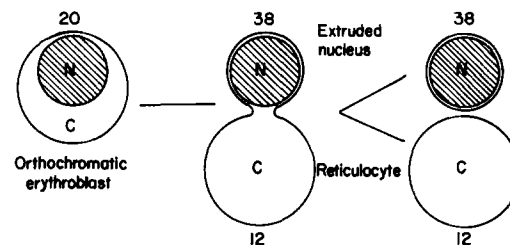
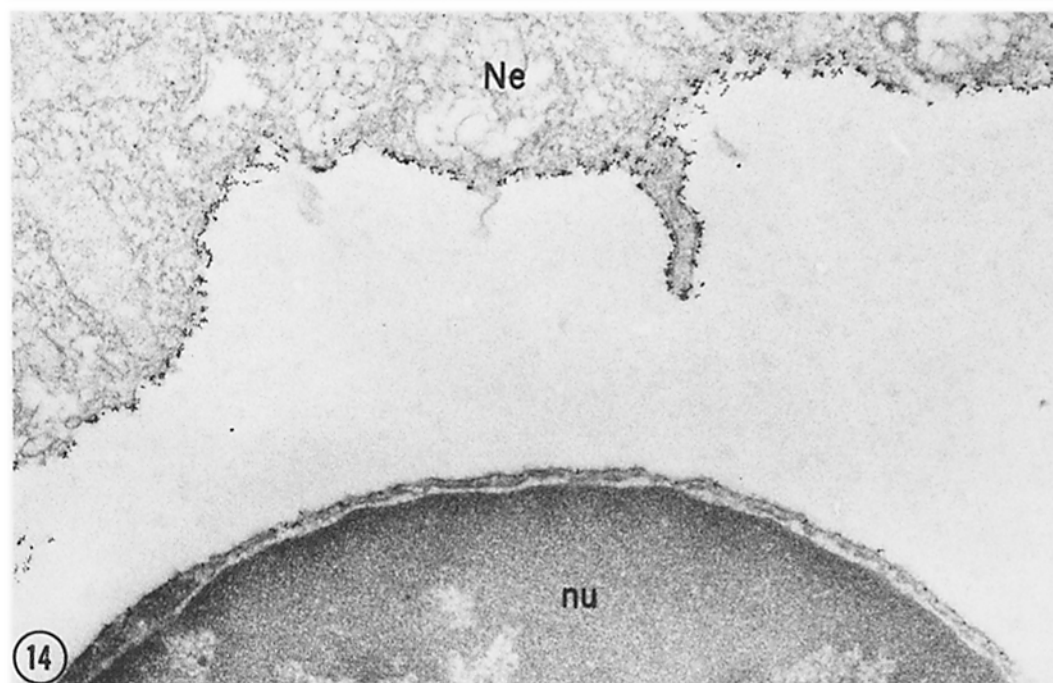
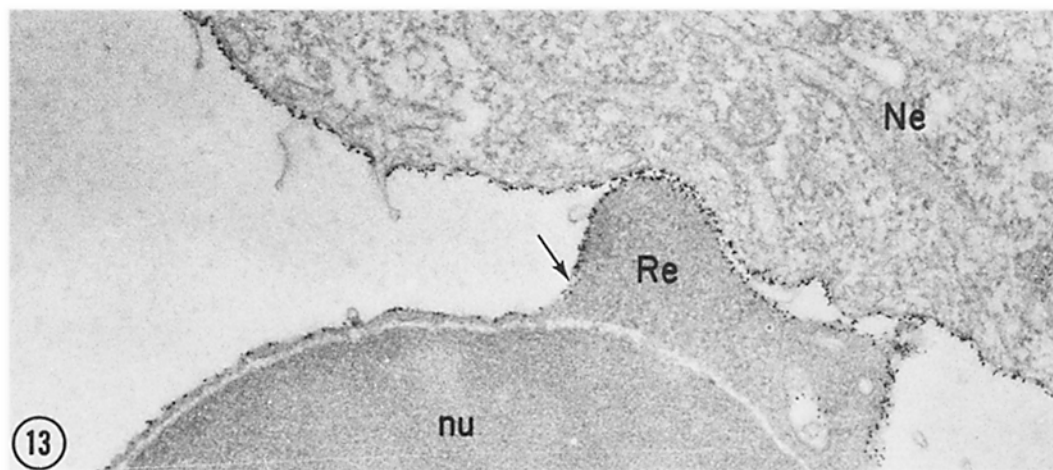
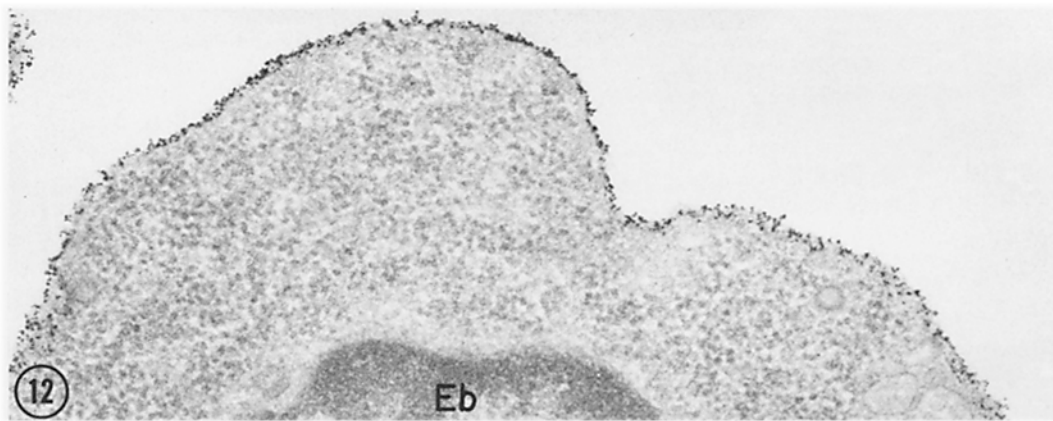


FIGURE 11 Diagram depicting the modifications in the ferritin-Con A labeling density of the plasma membrane which take place during nuclear extrusion in the late erythroblast. Note that there is a segregation of ferritin-Con A binding sites so that they are concentrated in that portion of the plasma membrane surrounding the extruded nucleus (N). The membrane of the orthochromatic erythroblast shows a labeling density of 23/ μm ; that of the reticulocyte 12/ μm , and that of the extruded nucleus 38/ μm membrane.

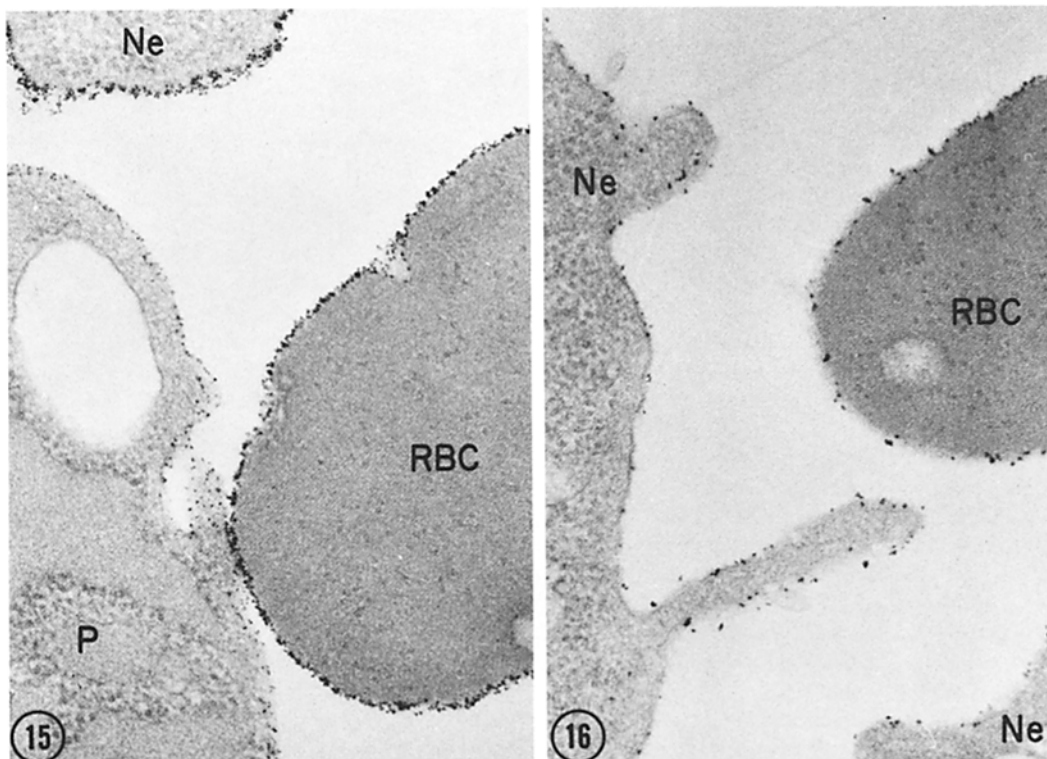


reactivity to Con A. The average Con A labeling density for membranes of neuraminidase-treated erythrocytes was $23/\mu\text{m}$ as compared with $5/\mu\text{m}$ in untreated erythrocytes (Fig. 9).

DISCUSSION

The frequency of ferritin-Con A receptor sites and CI binding sites was found to vary at different stages of differentiation in developing rat erythroid cells. Moreover, there was a consistent in-

verse relationship between the amount of Con A and that of CI binding. In cells of the erythroid series, there was a progressive increase in the receptor sites for Con A and a progressive decrease in CI binding with successive divisions of the erythroblast. The most pronounced modification in distribution of these sites occurred on the plasma membrane during nuclear expulsion and formation of the reticulocyte: the portion of the plasma membrane surrounding the extruded nu-



FIGURES 15 and 16 Rat bone marrow cells stained with positively charged colloidal iron hydroxide. The cells in Fig. 16 were treated with neuraminidase before staining, whereas those in Fig. 15 were not. Labeling density of the erythrocyte (*RBC*) and the two leukoid cells treated with neuraminidase is greatly reduced as compared to untreated cells. *P* = plasma cell; *Ne* = neutrophilic myelocyte. $\times 50,000$.

FIGURES 12-14 Rat bone marrow cells stained with positively charged iron hydroxide. Fig. 12 shows a portion of a basophilic erythroblast (*Eb*) which is moderately heavily labeled. Fig. 13 shows a late erythroblast in the process of nuclear extrusion. The portion of the plasma membrane surrounding the presumptive reticulocyte (*Re*) is heavily stained, whereas that portion surrounding the extruding nucleus (*nu*) shows very little staining and contains only a few scattered clumps of colloidal iron particles. The transition between heavily labeled and lightly labeled membrane is sharp (arrow). A portion of a neutrophilic myeloblast (*Ne*) which is moderately heavily stained is also included in the field. Fig. 14 shows a free erythroid nucleus (*nu*) and a neutrophilic myelocyte (*Ne*). The former shows little or no staining, whereas the latter is moderately heavily stained. Fig. 12, $\times 38,000$; Figs. 13 and 14, $\times 32,000$.

cleus was heavily labeled by ferritin-Con A whereas the reticulocyte had considerably fewer (one-third) labeled sites. With CI the situation was reversed: the plasma membrane surrounding the extruded nucleus was virtually unreactive, but the reticulocyte was heavily labeled.

The frequency of binding sites for ferritin-Con A and CI also varied among different leukoid cell lines, and there was also an inverse relationship in the frequency of binding sites with the two techniques. Con A binding was high in plasma cells and macrophages, lower on neutrophils and lymphocytes, and still lower on eosinophils and basophils, whereas CI binding was highest on the latter and lowest on plasma cells and macrophages.

Recently, Parmley et al. (29), and Ackerman and Waksal (2) have employed the Con A-peroxidase-diaminobenzidine technique to study the distribution of Con A receptor sites on human bone marrow cells. This technique allows a qualitative but not a quantitative assessment of Con A binding. Parmley and co-workers (29) noted differences in staining among different blood cell lines but did not detect differences among erythroid cells at different stages of maturation. However, Ackerman and Waksal (2) reported findings on erythroid cells similar to ours in that the amount of labeling decreased as the cells matured.

Positively charged colloidal ferric oxide, which binds primarily to sialic acid residues on cell membranes (12, 20), has proved to be a reliable cytochemical technique for the ultrastructural localization of anionic groups and has been used extensively to study the distribution of sialoglycoproteins in a variety of cell types including erythroid cells (34, 36) and mature erythrocytes (20, 23, 24, 26, 42). The Con A-ferritin method reliably localizes α -D-mannopyranosyl, α -D-glucopyranosyl, and α -N-D-glycosamidyl residues and has been used to study the distribution of cell surface glycoproteins (25).

In human erythrocytes >80% of the total membrane sialic acid is known (19) to be associated with glycophorin, the major red blood cell glycoprotein, whereas Con A binds primarily to Band III (10, 11). Interaction of glycophorin with Con A is negligible or absent (10, 11). Therefore, CI and Con A identify and localize basically different glycosylated compounds on the human red cell surface. The inverse relationship regularly noted between the number of Con A and of CI binding sites noted in this study suggests that the same applies to both erythroid and leukoid cells of the

rat. In a previous study (8), a similar inverse relationship between the frequency of Con A and that of CI binding sites was noted in cells of the rat anterior pituitary. Although there is no direct evidence that the Con A and CI receptors are on different integral membrane proteins in rat erythroid and leukoid cells, our results are consistent with this suggestion, and it is assumed in what follows that such is indeed the case.

The changes noted in the density distribution of sialic acid residues on developing erythroid cells during differentiation confirms in the rat the previous findings on the rabbit (34, 36). The present work demonstrates that the changes in cell membrane glycoproteins that occur during erythrocyte maturation are not restricted to sialoproteins but pertain to other glycosylated compounds as well. The fact that the density distribution of Con A receptor sites is the converse of that of CI binding capacity suggests that there is differential distribution of different components of the membrane. The increase in Con A binding capacity on erythrocytes after neuraminidase treatment suggests, however, that sialic acid residues extending to the outer surface of the membrane at least partially "mask" or interfere with binding to Con A receptor sites on the membrane. Such a masking effect will fail, however, to explain completely the remarkable increase in Con A binding capacity that has been observed on membranes surrounding extruded erythroid nuclei. The average density of ferritin-conjugated Con A particles on membranes of neuraminidase-treated erythrocytes ($23/\mu\text{m}$ membrane) was only one-half that of the labeling density on nontreated membranes surrounding extruded erythroid nuclei ($38/\mu\text{m}$ membrane).

It is of interest to consider the possible mechanism responsible for the changes in Con A and anionic group density during erythrocyte differentiation. It was previously postulated (36) that suppression of synthesis of sialated compounds at an early stage of differentiation together with the increase in membrane surface area which accompanies cell division could lead to a dilution of this component. Indeed, differential rates of synthesis of erythrocyte membrane proteins have been described (9, 17). It is likely, however, that a different mechanism exists for the changes in membrane composition noted during expulsion of the nucleus from the late erythroblast. During this process, both the decrease in anionic group density and the increase in Con A receptor site density on the plasma membrane enveloping the extruded nucleus as compared with the newly generated retic-

ulocyte can be detected at early stages of the expulsion process. In such situations, although the two parts of the membrane are still in continuity, the difference in Con A receptor site density on the two parts of the membrane is sharp and is already comparable to that of the reticulocyte and the completely extruded nucleus, respectively (Figs. 5-7). It is possible that local modification of glycosylated groups could explain this finding; however, it seems more likely that there is selective segregation of membrane proteins leading to a high concentration of sialoproteins (glycophorin) in the membrane of the reticulocyte. If so, this finding suggests that the segregation of different membrane components during nuclear expulsion starts and reaches its completion within the membrane of a single cell. The indication that the membrane proteins can move laterally within the plane of the membrane is in keeping with current concepts of membrane fluidity. Unlike phenomena such as "capping" (40, 43) in which receptor-binding, multivalent ligands are responsible for the specific aggregation of membrane components, in nuclear expulsion segregation of different membrane components occurs as an intrinsic process without the assistance of external factors.

The only other example in the literature of a case where there is evidence that selective segregation of membrane proteins may occur is in phagocytizing leukocytes. Work by Berlin and associates (5, 28) suggests that certain membrane proteins (transport systems) are selectively retained in the plasma membrane at the cell surface and that others (Con A receptors) are selectively removed and segregated in the membrane of the invaginating phagocytic vacuole. Recent findings suggest that the integral or intrinsic membrane sialoglycoproteins of the human erythrocyte, glycophorin and Band III, form an intramembrane macromolecular complex (14, 25, 30) which spans the membrane and interacts with extrinsic inner membrane surface proteins such as spectrin (6, 26, 41, 44) or actin (14, 39). Due to such an interaction, it might be expected that migration by lateral diffusion of sialoproteins in the erythrocyte membrane would be determined by their association with these extrinsic membrane proteins. If such interaction exists in the late erythroblast membrane as well as in the erythrocyte, it might provide an explanation for the mechanism involved in segregation of membrane proteins during nuclear expulsion.

Finally, one can ask what is the biological significance of the altered membrane surrounding the

extruded nucleus? In this regard, it is of interest to note that nuclei extruded from late erythroblasts are recognized by macrophages and rapidly phagocytized (37). Indeed, *in situ* macrophages are seen to surround and envelope the nuclei even before extrusion is complete. It has been postulated that the reduction in negative surface charge density on the plasma membrane surrounding the extruded erythroid nucleus (34, 36) or the unusual concentration or exposure of membrane antigens (38) which are normally either masked or highly diluted serves to distinguish the membranes enveloping the extruded nucleus from those of other erythropoietic cells. Whatever be the case, it is clear that the membrane composition is sufficiently distinctive to allow rapid recognition and removal of extruded nuclei by macrophages.

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