

# Cell-type-related Segregation of Surface Galactosyl-containing Components at an Early Developmental Stage in Hemopoietic Bone Marrow Cells in the Rabbit

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**ABSTRACT** The avidin-biotin complex was used for the selective ultrastructural labeling of terminal cell surface galactosyl residues. Rabbit bone marrow cells were treated with the enzyme galactose oxidase in the presence of biotin hydrazide. Subsequent treatment with ferritin-avidin conjugates enabled the electron microscopic visualization of terminal membrane-based galactose and/or *N*-acetylgalactosamine on these cells. All stages of erythroid development were characterized by high levels of exposed cell surface galactose, whereas all leukoid cells in the same preparations were virtually unlabeled by the above method. Modulations in the distribution of these surface determinants during differentiation and maturation of rabbit erythroid cells were found to concur in inverse fashion with respect to that of terminal sialic acids. Neuraminidase treatment, before the above labeling procedure, resulted in the exposure of additional galactosyl residues on the surface of all bone marrow cell types.

The results indicate that a galactose-bearing glycoconjugate(s) may comprise an erythroid-specific membrane constituent of rabbit bone marrow cells. The high density of galactose on the surface of even the earliest erythroid precursors may eventually enable the identification and isolation of a stem cell, which already contains the erythroid-specific galactoconjugate(s). The results suggest that variations in the spectrum of cell surface carbohydrates may serve as recognition signals in the complex set of intercellular interactions which occur during the development and maturation of the erythrocyte. The occurrence of similar but species-specific variations in the complement of surface heterosaccharides during erythroid development of humans and other mammals supports this contention.

A considerable body of evidence has been accumulated concerning the primary role of cell surface saccharides in the control of cellular behavior and fate of blood cells (1–3). For example, the integrity of surface sialic acids and their continuity with respect to the carrier molecule on the cell surface have been linked to the control of the survival of circulating erythrocytes (4–13) and the phagocytosis of extruded erythroid nuclei in most mammals (14, 15). In addition, changes in the normal level and/or distribution of surface sialic acid and terminal galactosyl residues have been implicated in the maturation, the release into circulation, and the homing of lymphoid cells (16, 17), as well as the turnover of circulating sialoglycoproteins (18) and the pathological expression in certain diseased blood cells (6, 19).

It is generally believed that cell surface sialic acids have a

“protective” role owing to a masking of cryptic recognition sites, e.g. specific saccharides (20, 21) and antigenic sites (7, 22). Since, in most cell membrane glycoconjugates, sialic acid is attached either to a galactose or to an *N*-acetylgalactosamine residue (23, 24), the masking and unmasking of these constituents are believed to be particularly important in the above-mentioned interactions.

We have shown previously that the differentiation and maturation of rabbit erythrocytes (RBC) are associated with striking modulations in cell surface sialic acids (14, 25). These changes are expressed as a reduction in the density of sialyl residues after successive divisions of erythroid precursors. After nuclear extrusion, a characteristic rise in the density of membrane sialic acid residues is observed on the newly formed reticulocyte, due to the segregation of integral membrane gly-

coconjugates (15) and spectrin (26) between the plasmalemma surrounding the nucleus and that of the remaining reticulocyte.

Using peanut agglutinin a galactose-specific lectin (21, 27), we have previously shown that, unlike other mammalian RBC studied, the circulating erythrocyte membrane of the rabbit contains exposed galactose sites capable of interaction with this lectin (21). Considering the alleged importance in the levels of membrane sialic acid versus galactose to erythrocyte survival, it was interesting to determine the possible connection between the reported modulations in surface sialic acid and the biogenesis of galactose residues in the developing rabbit RBC membrane. In addition, since a common origin is attributed to all blood cell types, i.e. the erythroid, leukoid, megakaryocytic, and lymphoid cells (28), it was also important to determine whether the changes in surface saccharide constituents are specific to the erythroid line or common to other blood cell lineages as well.

In the present study, we have used a galactose-specific method of ultrastructural staining, based on the avidin-biotin complex (6, 29–33), to analyze the density and distribution of these residues on various erythroid and leukoid precursor cells in the rabbit bone marrow. The results were compared with our previous findings regarding the modulations of surface sialyl residues on the same cells in rabbit (14, 25, 31) and in other mammalian species (15).

## MATERIALS AND METHODS

**Materials:** Horse spleen ferritin (six times crystallized, cadmium free) was obtained from Miles Laboratories Inc., Kankakee, IL, and avidin from Sigma Chemical Co., St. Louis, MO. *Vibrio cholerae* neuraminidase (free of protease, aldolase, and lecithinase C) was purchased from Boehringerwerke, Marburg, Germany. Galactose oxidase was obtained from KABI Diagnostica, Stockholm, Sweden. Biotin hydrazide was prepared as described previously (30, 32). Ferritin was conjugated to avidin via reductive alkylation as described in an earlier report (34).

**Preparation of Isolated Bone Marrow Cells:** Bone marrow was obtained from the femurs of 3-mo-old rabbits. Pieces, removed from animals anesthetized by i.v. injection of Nembutal (Abbot Laboratories, Kent, England), were transferred into  $\text{Ca}^{++}/\text{Mg}^{++}$ -free Krebs-Ringer bicarbonate (KRB) solution. The tissue was then cut into small ( $\sim 1 \text{ mm}^2$ ) blocks, and the cells were suspended by repeated mild pipetting through a Pasteur pipet with a flame-polished tip. To remove undissociated tissue fragments, we filtered the suspension through several layers of cheesecloth. After sedimentation at 100 g for 7 min, the pellet of bone marrow cells was resuspended in fresh KRB containing 0.1 mM  $\text{Ca}^{++}$  and 0.12 mM  $\text{Mg}^{++}$ .

**Treatment of Cells with Neuraminidase:** A 5% suspension of bone marrow cells was incubated in an agitating water bath for 60 min at 37°C with 0.2 U/ml neuraminidase in a medium containing 0.145 M NaCl, 3 mM CaCl<sub>2</sub>, and 4 mM NaHCO<sub>3</sub>, pH 6.5 (35). Control samples were incubated with heat-inactivated (65°C, 30 min) neuraminidase under similar conditions. At the end of the incubation period the cells were washed and transferred to a fresh solution of KRB.

**Galactose Oxidase (GO)-induced Biotinylation of Cell Surface Galactosyl Residues:** Neuraminidase-treated or untreated bone marrow cells were washed twice with veronal acetate buffered saline pH 7.4 (VBS). A 5% suspension was then agitated for 30 min at 37°C in VBS, containing 50 U/ml galactose oxidase and 2.5 mg/ml biotin hydrazide. At the end of the incubation period, the cells were washed twice with VBS and fixed for 1 h at 28°C with Karnovsky fixative (36).

**Ferritin-Avidin Labeling:** An aliquot of  $10^7$  bone marrow cells, prefixed with aldehydes as described above, was washed three times in VBS. To prevent nonspecific protein binding, we incubated the cells for 15 min with 5% bovine serum albumin in VBS. The cells were then resuspended in a solution containing ferritin-conjugated avidin (FAv) (1 mg ferritin/ml VBS) for 30 min at 25°C, washed twice in VBS, and additionally fixed for 30 min with Karnovsky fixative.

**Processing for Electron Microscopy:** Labeled cells were washed with VBS and postfixed for 1 h at 4°C in the same buffer containing 1% OsO<sub>4</sub>. After washing twice with VBS, samples were dehydrated in graded ethanol,

stained with 50% saturated uranyl acetate in 50% ethanol, and embedded in Epon (37). Sections  $\sim 60$ -nm thick, showing gray-to-silver interference colors, were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome (Ivan Sorval, Inc., Newton, CT.), mounted on naked 400-mesh copper grids, and coated with carbon. The sections were examined under a JEOL 100A electron microscope at an accelerating voltage of 80 kV.

**Analysis of Labeling Density:** To compare the density of attached ferritin particles on the surface of the different cell populations, we photographed segments of perpendicularly sectioned membranes at 30,000 times magnification and printed them at a final magnification of 75,000 times. Black dots representing individual ferritin particles attached on these membrane segments were counted on the electron micrographs. The length of the perpendicularly sectioned membrane, on which the number of ferritin particles was counted, was measured on the micrograph with a map measure. For each erythroid developmental stage or leukoid cell type, at least 20 different cells, arbitrarily selected from 10 different sections, were counted.

## RESULTS

### *Binding of FAv on Hemopoietic Bone Marrow Cells, General*

Treatment of isolated bone marrow cells with galactose oxidase and biotin hydrazide had no noticeable effect on the morphological features of the hemopoietic cells of the bone marrow. Analyses on these sections of the extent of FAv binding onto the surface of biotinylated cells revealed striking differences in the reactivity of erythroid cells to GO-induced biotinylation, compared to that of all leukoid cell types in the bone marrow (Fig. 1). In contrast to erythroid cells of all recognizable maturation stages, the latter of which were highly reactive to GO-induced biotinylation (Fig. 2*a-f*), leukoid cells of all lineages and developmental stages were virtually unreactive, as indicated by the low levels, or complete absence, of ferritin on their surfaces (Figs. 1 and 3*a, c, e, and g*). On the erythroid cells, the FAv particles were generally evenly distributed on the entire surface of the cells, except for ropheocytotic pits often found on the membrane of proerythroblasts and basophilic erythroblasts (Fig. 1). These membrane invaginations were characterized by the typical morphology of bristly coated vesicles. On the latter membrane surfaces, labeling density was considerably lower or completely absent, compared to that of the remaining plasmalemmal surfaces of the same cells (Fig. 1).

### *Variations in FAv Binding during RBC Differentiation*

Analysis of the density distribution of attached FAv particles on erythropoietic cells of different developmental stages revealed quantitative variations in the reactivity to GO-induced biotinylation according to the degree of differentiation and maturation in the erythroid series. The results of these analyses are graphically summarized in the histogram in Fig. 4. The data indicate that GO-susceptible sites are already numerous at the earliest recognizable erythroid stage, i.e. the proerythroblast (Fig. 2*a*). After successive divisions of the proerythroblast, there is a gradual increase in the labeling density at the basophilic (Fig. 2*b*) and polychromatic (Fig. 2*c*) stages, reaching a maximal density at the latest nucleated stage, the orthochromatic erythroblast (Fig. 2*d*). This trend is reversed after expulsion of the nucleus from the late erythroblast, as indicated by the decreased labeling density on the reticulocyte (Fig. 2*e*) and the further reduction on the erythrocyte (Fig. 2*f*). It is interesting to note that, in contrast to our previous observations concerning periodate-induced biotinylation of surface sialic acids in rabbit erythroid cells (31), significant modulations in

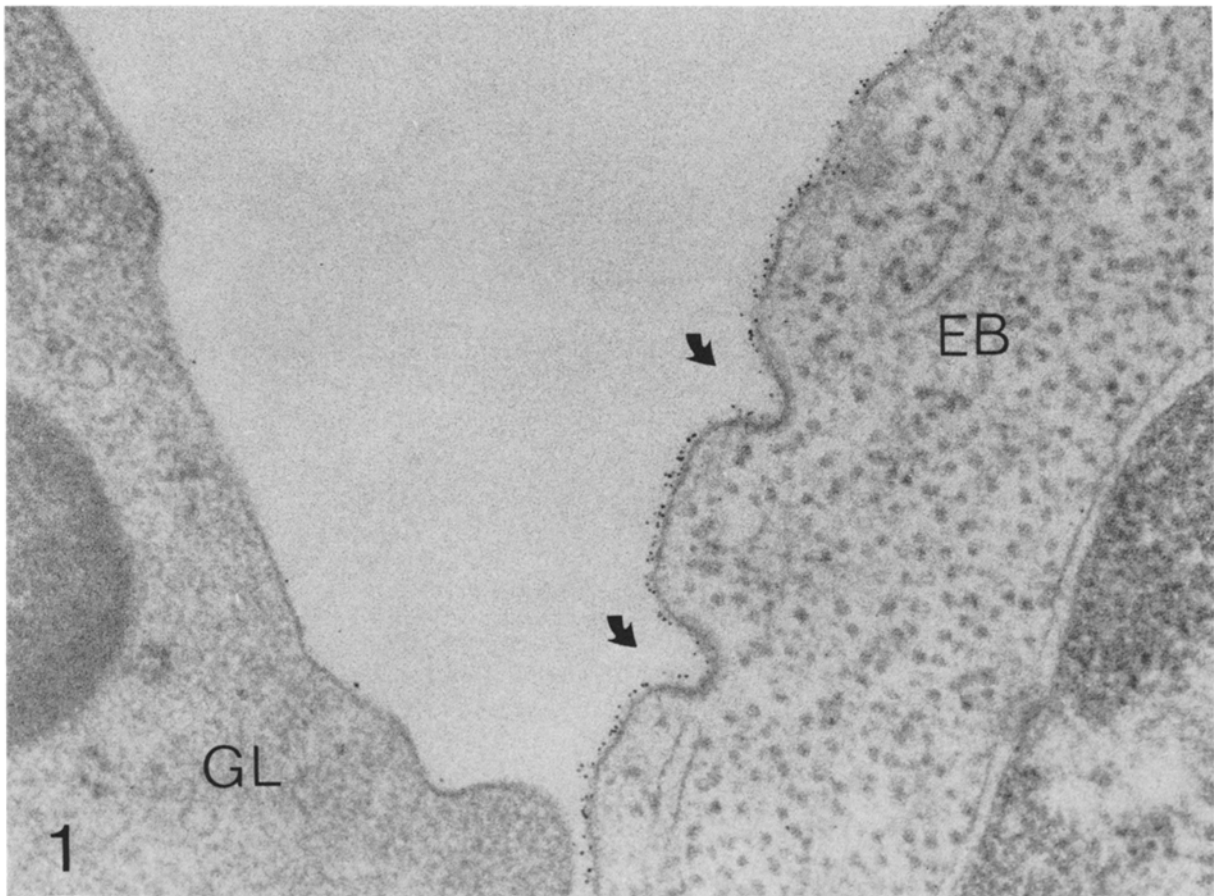


FIGURE 1 Rabbit bone marrow cells enzymatically labeled via galactose oxidase in the presence of biotin hydrazide followed by incubation with FAv. The surface of the granular leukocyte (GL) is virtually unlabeled, whereas that of the early (basophilic) erythroblast (EB) is heavily labeled by the above procedure. Note that the surface labeling of the erythroblast is more or less uniform, with the exception of the region of coated pits which are practically unlabeled (arrows).  $\times 75,000$ .

the ferritin-membrane interspace during successive stages of development were not observed as a result of GO-induced biotinylation. This topographic difference in labeling pattern might indicate that the terminal sialyl and galactosyl residues may be attached to different carrier molecules on the cell surface of the rabbit RBC.

#### *Binding of FAv on the Plasmalemmal Surface of "Extruded" Erythroid Nuclei*

"Free" nuclei, extruded from late erythroblasts, which are normally found in hemopoietic tissues surrounded by a narrow rim of cytoplasm and cytoplasmic membrane (15, 38-40), were relatively rare in the bone marrow suspension, probably due to their rapid elimination by macrophages during the cytochemical procedures. Consequently, there were too little quantitative data to enable a reliable, statistically based classification of the reactivity of extruded nuclei to GO-induced biotinylation. However, countings were performed on the few (five) extruded nuclei that were found in the treated bone marrow (Fig. 2g). The density of FAv particles on the plasmalemma surrounding the extruded nuclei ( $90 \pm 10$  mean  $\pm$  SD) was found to be considerably higher than the average density on the reticulocyte and the mature erythrocyte, and slightly higher than that on the orthochromatic erythroblast.

#### *Effect of Neuraminidase*

Neuraminidase activity was estimated by its ability to reduce

the capacity of control cell membranes to bind positively charged, colloidal ferric oxide (41) or to undergo periodate-induced biotinylation (31). Treatment of bone marrow cell samples with neuraminidase resulted in a pronounced loss in binding capacity for the latter cytochemical markers, known to be specific to cell surface sialic acids.

After neuraminidase treatment of the bone marrow cells, there was a considerable "exposure" of GO-susceptible sites on all leukoid cell types in the bone marrow (Fig. 3b, d, f, and h). In the erythroid series, neuraminidase treatment resulted in an increase in the reactivity of most erythroid cells. An equilibration of labeling densities was observed in all developmental stages reaching the highest density range, i.e. close to or slightly above that of the orthochromatic erythroblast (Fig. 4). Neuraminidase treatment (before biotinylation) of circulating erythrocytes resulted in an increase of  $\sim 75\%$  in the average density of attached FAv, compared to that of control cells not subjected to neuraminidase. Analysis of the FAv labeling density on neuraminidase-treated leukoid cells revealed considerably more-pronounced variations in the labeling densities. These differences appear to be related both to the cell type and/or to the degree of maturation within a given lineage.

#### *Controls*

No FAv binding was observed on circulating RBC treated with biotin hydrazide, fixed with aldehydes, and labeled with FAv without prior galactose oxidase treatment. Galactose oxidase alone failed to generate FAv labeling on cell surfaces.

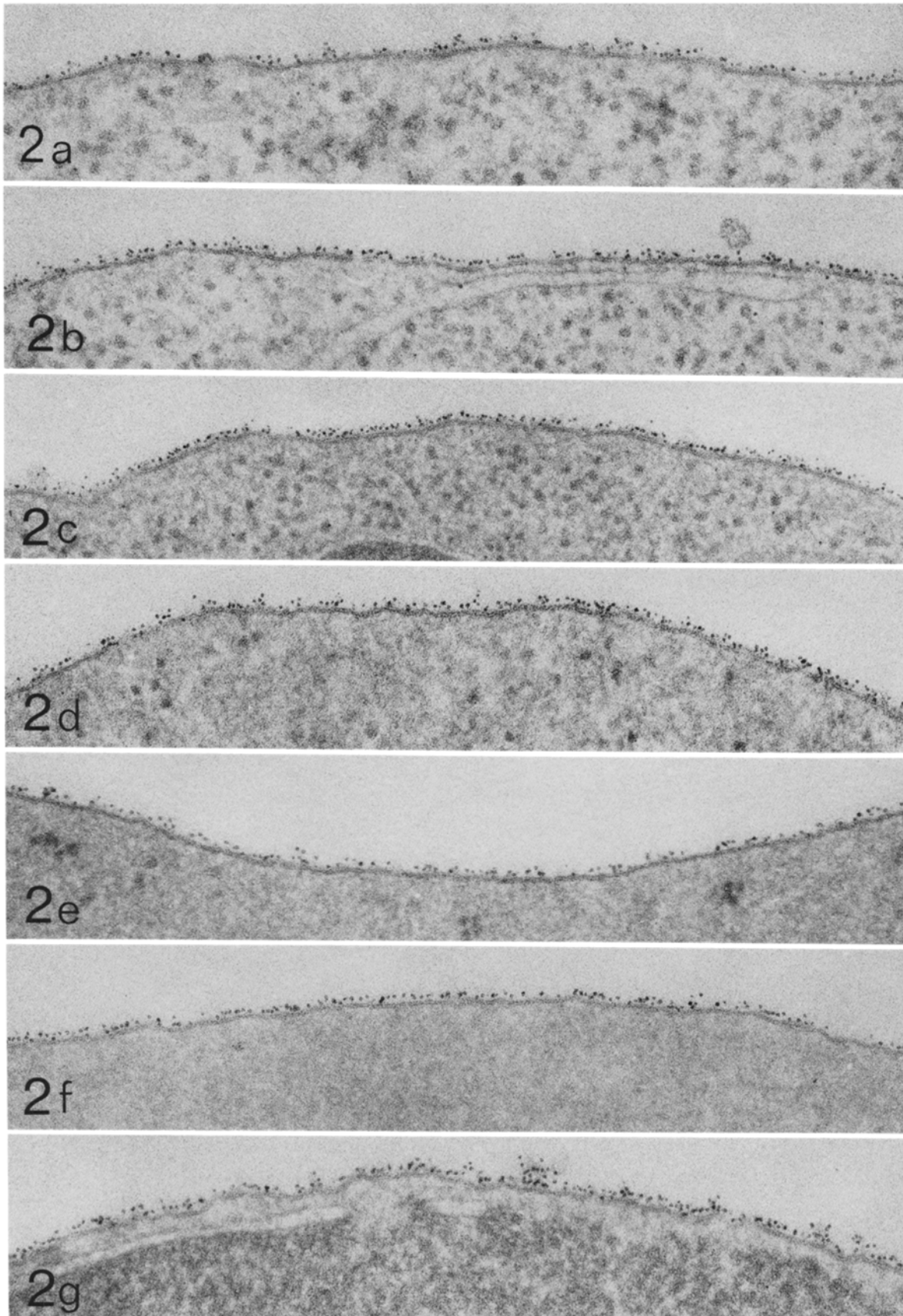


FIGURE 2 Erythroid cells from rabbit bone marrow during successive stages of development: proerythroblast (a), basophilic erythroblast (b), polychromatic erythroblast (c), orthochromatic erythroblast (d), reticulocyte (e) and erythrocyte (f). The extruded nucleus is shown in g. The cell surfaces of all erythroid cells, as well as that of the plasmalemma surrounding extruded nuclei, are heavily labeled by GO-induced biotinylation. Note that the distance separating the ferritin particles from the plane of the membrane is relatively constant throughout the developmental stages of rabbit RBC.  $\times 75,000$ .

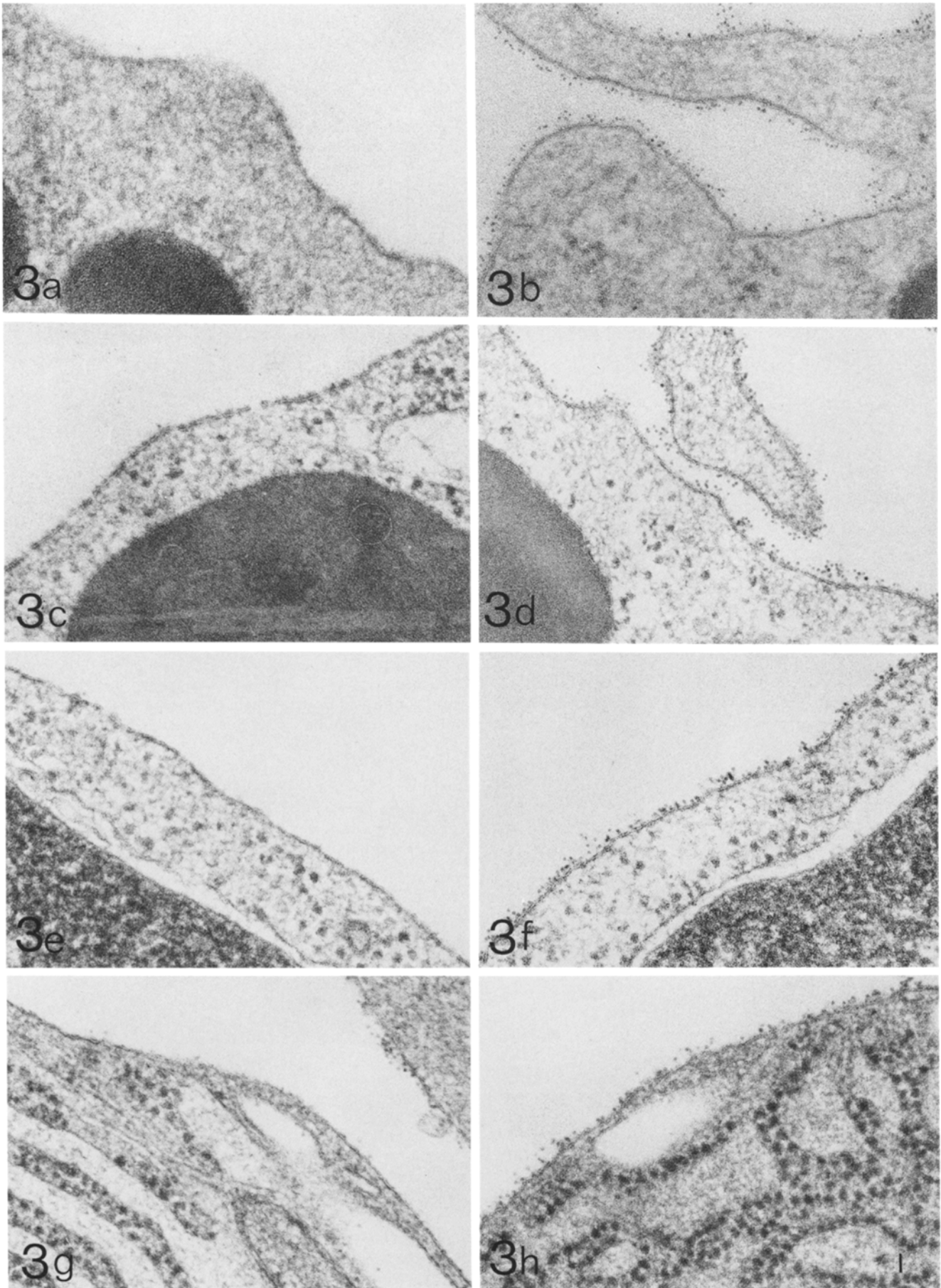


FIGURE 3 Bone marrow leukocytes treated as in Fig. 1, with or without prior neuraminidase treatment. Both granuloid leukocytes, e.g. neutrophilic myelocyte (a) and eosinophilic myelocyte (c), and lymphoid cells, e.g. lymphoblast (e) and plasma cell (g), are virtually unlabeled by GO-induced biotinylation. However, when the same cells are pretreated with neuraminidase, both cell types (b, d, f, and h; respectively) are highly labeled with ferritin molecules.  $\times 75,000$ .

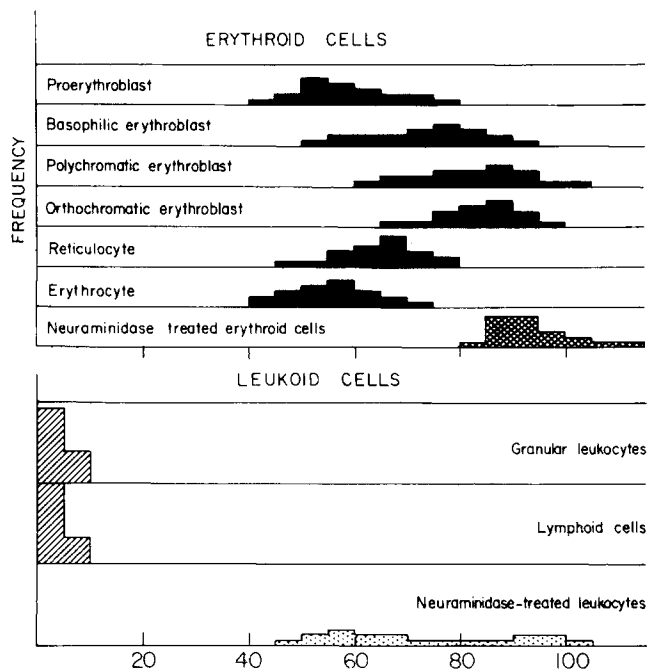


FIGURE 4 Histogram showing the frequency distribution of bone marrow cells according to reactivity of cell surfaces to GO-induced biotinylation and FAV treatments. Ordinate: frequency in percent. The scale is drawn so that the distance between the lines represent 37.5% of each cell stage for erythroid cells and 75% for leukoid cells. Abscissa: FAV particles counted per micrometer length of perpendicularly sectioned membrane.

## DISCUSSION

The results of the present study indicate that, in rabbits, exposed membrane-based galactosyl sites are present on hemopoietic bone marrow cells of all recognizable stages of erythroid development. In sharp contrast, cell membranes of the leukoid series are virtually devoid of the galactose-specific label. This distinction holds marked import regarding the development of hemopoietic bone marrow cells. First, erythroid differentiation in the rabbit is necessarily associated with the synthesis or insertion into the plasmalemma of glycoconjugate(s) which possess free terminal galactose or *N*-acetylgalactosamine residues. In view of the surprisingly high density of these latter determinants on membranes of the earliest recognizable erythroid precursor, the proerythroblast (Fig. 2*a*), one might anticipate the existence of an earlier hypothetical stage of development, characterized by moderate or high levels of surface galactose, thus enabling identification from early leukoid precursors. Since all stages in leukoid development, including the earliest recognizable stage, were unreactive with respect to GO-induced biotinylation, it is proposed that the segregation of the galactose-bearing glycoconjugate(s) occurs at an earlier, as yet morphologically unidentified precursor (28).

On the basis of the described observations and the previously described modulations in membrane sialic acid on the same cells (25, 31), the selective appearance of free galactosyl (and/or *N*-acetylgalactosaminyl) residues on rabbit erythroid cells may be interpreted within the framework of two alternative mechanisms. First, galactose-bearing glycoconjugates may represent asialoglycoconjugates, i.e. precursor molecules, analogous to the major human membrane sialoglycoprotein of the RBC, glycophorin (42) but lacking the terminal sialic acid. Alternatively, a new erythroid-specific (galactose-bearing) gly-

coconjugate(s) may be synthesized and/or inserted into the membrane at an early stage of the erythroid developmental process. Subsequent cell division would serve to propagate the newly inserted membrane component with concomitant dilution of preexisting membrane sialoproteins. Recently, Gahmberg and co-workers (43), using specific antibodies, described the biosynthesis of glycophorin during human erythroid development. It was found that glycophorin was expressed on the cell surface only at the basophilic erythroblast stage, after which a gradual rise in this membrane constituent was observed, reaching a maximum on the surface of the mature erythrocyte. Similarly, the galactose-bearing glycoconjugate(s) may represent an analogous erythroid-specific component or a spectrum of such membrane constituents in the developing rabbit RBC.

In all leukoid and erythroid cells, the removal of terminal sialyl residues by neuraminidase (Figs. 3 and 4) resulted in the exposure of large quantities of GO-susceptible sites, indicating that the latter comprise penultimate galactosyl residues normally masked by sialic acid. This pattern in rabbit blood cell membranes is apparently similar to that observed in other mammalian sialoglycoproteins, e.g. human glycophorin (23, 24) and serum glycoproteins (18). The existence of unmasked galactosyl residues on the rabbit RBC was previously indicated by the reactivity of circulating rabbit erythrocytes to peanut agglutinin (27). These results were later confirmed by Bell et al. (44), who further demonstrated that the major product obtained from intact rabbit RBC by galactosidase treatment was found to be *D*-galactose. These data may suggest that the GO-sensitive sites on the rabbit erythropoietic cells are terminal galactosyl, and not *N*-acetylgalactosaminyl, residues.

Unlike the situation in other mammalian RBC which do not have exposed galactosyl residues, those residues normally exposed on the rabbit RBC membrane apparently do not contribute as such to the recognition of desialylated rabbit RBC. This implies that the additional galactosyl residues, exposed by neuraminidase (Fig. 4), may differ biologically from those normally exposed on the same cells. In this regard, Jancik and Schauer (4) have demonstrated that, in circulating rabbit erythrocytes,  $\beta$ -galactosidase removed galactose only after enzymatic treatment with neuraminidase. In contrast, Aminoff and co-workers (8) have shown that galactose can be removed from the native erythrocyte surface of the rabbit by the action of  $\alpha$ -galactosidase without prior neuraminidase treatment. These complementary data suggest that the normally exposed galactosyl residues in the rabbit erythrocyte membrane may be structured in the  $\alpha$ -configuration, whereas the galactose residues, penultimate to sialic acid, are, at least in part, in the  $\beta$ -configuration. In both studies, *D*-galactose, and not *N*-acetylgalactosamine residues were reportedly removed from the cell surface, results consistent with our earlier studies (21).

The results presented here further indicate that during erythroid development in the rabbit an inverse correlation exists between the previously recorded changes in surface sialic acids (25, 31) and the distribution of galactosyl residues (Fig. 4). The observed interplay between these two surface determinants is particularly evident during nuclear extrusion and consequent formation of the reticulocyte from the late erythroblast. The reduction in galactosyl site density during maturation of the reticulocyte to an erythrocyte (Fig. 4) is associated with the previously reported increase in surface sialyl site density (15, 25, 31). These alterations may be attributed to the pronounced modulations in the distribution of membrane determinants upon expulsion of the nucleus from the late orthochromatic

erythroblast (14, 15, 22, 26). The contrapositive segregation of galactose versus sialic acid residues during nuclear extrusion further supports the hypothesis that galactosyl- and sialyl-bearing glycoconjugates on rabbit erythroid cell surfaces represent different classes of membrane constituents. The reciprocal relationship between these two determinants is highly reminiscent of the inverse relationship between concanavalin A binding sites and surface sialyl residues on developing erythroid cells of the rat (15). This trend, involving the contrapositive segregation of membrane glycoconjugates, might therefore reflect a general characteristic of specified surface components in the developing mammalian RBC, with species-specific variations in the spectrum of surface saccharides.

The authors are pleased to acknowledge the expert technical assistance of Mrs. Ora Asher and Mr. Stanley Himmelhoch.

Parts of this work were funded by the Recanati Fund. E. A. Bayer is the recipient of National Institutes of Health Public Health Service Award No. F32 ES5210.

Received for publication 13 October 1981, and in revised form 30 September 1982.

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