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Membrane differentiation in human erythroid cells: Unique profiles of cell surface glycoproteins expressed in erythroblasts *in vitro* from three ontogenic stages

(stem cell culture/band 3 protein/glycophorins/polylactosaminoglycan/Ii antigens)

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ABSTRACT **Human erythroblasts in culture, irrespective of the ontogenic stage of their progenitors, are characterized by: (*i*) the barely detectable amount of band 3 glycoprotein, (*ii*) the presence of two glycoproteins with molecular weights 105,000 and 95,000, (*iii*) the high concentration of glycophorin, and (*iv*) a minimum quantity of the carbohydrate chain susceptible to endo-β-galactosidase ("polylactosaminoglycan"). In contrast, mature erythrocytes, whether of fetal, neonatal, or adult origin, are characterized by a high concentration of band 3 glycoprotein, polylactosaminoglycan, and glycophorins, but do not contain 105- and 95-kilodalton-glycoproteins. Thus, the process of erythroid maturation from erythroblasts to erythrocytes is accompanied by the appearance of band 3, the disappearance of 105- and 95-kilodalton glycoproteins, and a great increase in the quantity of polylactosaminoglycan. The structure of polylactosaminoglycan may not be different between mature erythrocytes and erythroblasts from the same ontogenic stage, but it is distinctively different from one stage to the other. The profiles of oligosaccharides released by endo-β-galactosidase and immunofluorescence studies with anti-Ii antibodies indicated that a linear polylactosaminoglycan structure was present in erythroblasts as well as in erythrocytes of the fetal and newborn stage, whereas a branched polylactosaminoglycan structure was present in erythroblasts as well as erythrocytes of adult blood. Thus, two membrane characteristics are closely associated with the process of erythroid cell development; one—the membrane proteins band 3, band 4.5, and 95- and 105-kilodalton glycoproteins—determines the degree of maturation, and the other—polylactosaminoglycan—may determine the ontogenic stage of the erythroblast progenitors.**

Development and cellular differentiation may be mediated through cell surface membranes; orderly appearance and disappearance of various membrane markers such as F-9 (1), blood group ABH (2), Forssman (3), and Ii antigens (4) and hematoside (5) have been observed at various phases of ontogenesis and differentiation.

A remarkable difference in arborization of carbohydrate structure in membrane glycolipids and glycoproteins between fetal and adult erythrocyte population has been observed (6, 7); the difference is now considered to be the basis of i to I antigenic conversion (8–10); i antigens are expressed by various domains within a linear, repeating *N*-acetyllactosaminyl structure (polylactosaminoglycan‡) (8) and I antigens are expressed by various regions within a branched polylactosaminoglycan (9, 10)

Sequential membrane changes associated with the process of erythroid cell maturation are not known, although molecular changes during this process are important for understanding the role of membranes in determining cellular differentiation in general. The formation of mature erythrocytes is accomplished through an orderly sequence of events initiated at the level of morphologically unrecognizable erythroid-committed cells and terminated with the maturation changes of the morphologically recognizable nucleated erythroid cells. With the recent technical advances in cell surface analysis (13), and in clonal culture techniques for erythroid cells (15), the cell surface structure of the erythroid cell population has been studied. This paper describes the glycoprotein profile and the carbohydrate structure of human erythroblasts from three different ontogenic stages and compares them with findings in mature erythrocytes from the respective stage of development.

MATERIALS AND METHODS

Cells. *Erythrocytes* from heparinized adult peripheral blood, cord blood, and fetal blood were used after washing and buffy coat removal. *Erythroblasts* were obtained from clonal erythroid precursors from blood of adult and neonatal and fetal liver. Cells were prepared and processed for culture in methylcellulose in the presence of erythropoietin (2.0 international units/ml), as described (15, 16). After 14–16 days in culture, the hemoglobin-producing colonies (erythroid bursts) assessed by their red color in live culture plates were lifted off by a fine pipette, pooled, and suspended in Dulbecco's phosphate-buffered saline (P_i/NaCl) and immediately studied. In addition to erythroids, colonies with *granulocyte* or *macrophage* appearance grown in the same culture plates were also collected separately. The *K562 cells* (17) were kindly provided by George Klein, Karolinska Institute, Sweden, and cultured in RPMI 1640 medium containing 10% fetal calf serum.

Cell Surface Labeling. Galactose and *N*-acetylgalactosamine residues on the cell surface were labeled by the galactose oxidase/NaB^3H_4 method (18), as modified (13). Sialic acid residues on the cell surface were labeled by the modified (19) periodate/NaB^3H_4 method (20) as described (13). The labeled cells were washed three times with P_i/NaCl.

Treatment of the Surface-Labeled Cells by Endo-β-galactosidase. The tritium-labeled cells were incubated by

Abbreviations: P_i/NaCl, Dulbecco's phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$).

‡ The carbohydrate chains with a repeating *N*-acetyllactosamine (Galβ1→4GlcNAcβ1→3Galβ1→4R) and those with branched structure [Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ1→6)-Galβ1→4R] are hereby called polylactosamine or polylactosaminoglycan. This structure not only is present in glycoproteins and glycolipids of erythrocytes but also is distributed in granulocytes and monocytes as shown in this report and in various other cells (11, 12). Because the carbohydrate chain is readily degraded by endo-β-galactosidase of *Escherichia freundii*, the structure can be detected by cell surface labeling followed by treatment with endo-β-galactosidase (13). The same carbohydrate chains have previously been called erythroglycan (14).

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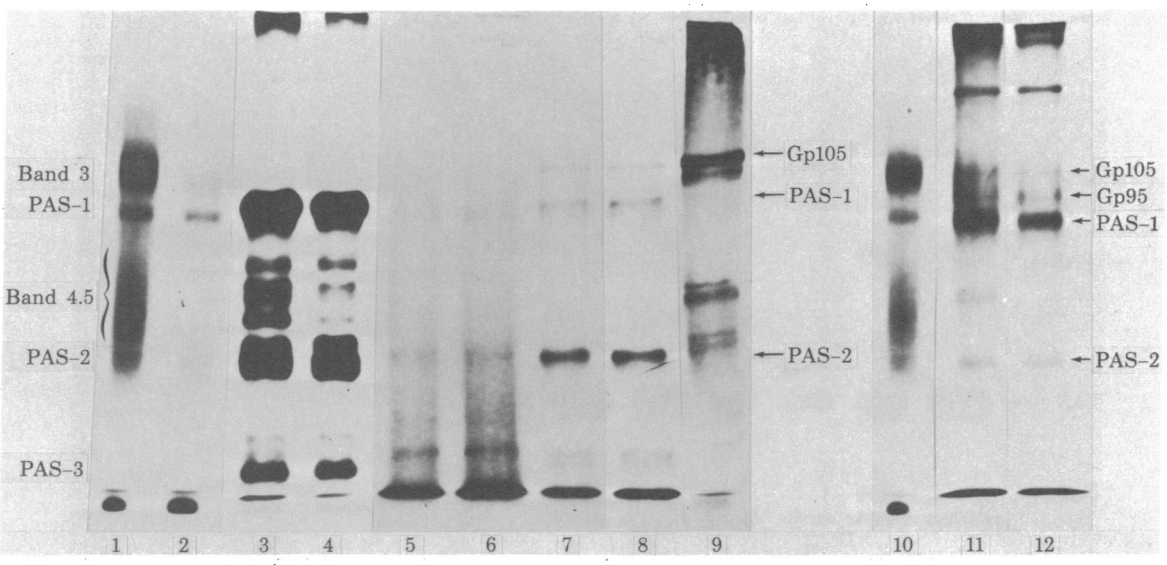

FIG. 1. Fluorogram of $NaDodSO_4$/polyacrylamide gels of surface-labeled cells. Erythrocytes from umbilical cord and erythroblasts cultured from umbilical cord blood were used. Lanes 1 and 2, galactose oxidase/NaB^3H_4-labeled erythrocytes; lanes 3 and 4, periodate/NaB^3H_4-labeled erythrocytes; lanes 5 and 6, galactose oxidase/NaB^3H_4-labeled erythroblasts; lanes 7 and 8, periodate/NaB^3H_4-labeled erythroblasts; lane 9, periodate/NaB^3H_4-labeled K562 cells; lane 10, the same as lane 1; lanes 11 and 12, galactose oxidase/NaB^3H_4-labeled erythroblasts. Lanes 1, 3, 5, 7, 9, 10, and 11 are control cells incubated without endo-β-galactosidase and lanes 2, 4, 6, 8, and 12 are cells incubated with endo-β-galactosidase. About 4.5×10^5 cells were used in lanes 5, 6, 7, and 8, and 1.2×10^6 cells were used in lanes 11 and 12. The numerical designation for the major peptides of human erythrocytes is according to Fairbanks *et al.* (27).

endo-β-galactosidase from *E. freundii* (21) with the concentration of 125 milliunits/ml at 37°C for 2 hr in 0.1 M sodium acetate buffer, pH 5.8, containing 0.07 M NaCl (13). After incubation, 1 ml of P_i/NaCl was added and supernatant and cells were separated by centrifugation. The supernatant was used for characterization of released oligosaccharide and the cell pellet was used for analysis of glycoprotein. In control experiments, tritium-labeled cells were treated in the same manner without endo-β-galactosidase.

Analysis of Cell Surface Glycoproteins. The cell pellet was directly dissolved in the sample buffer and cell surface glycoproteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis according to Laemmli (22), using 0.1% $NaDodSO_4$ and 8% acrylamide for separation gels. For fluorography, the gels were treated with dimethylsulfoxide/2,5-diphenyloxazole (23) or En^{3}Hance solution (New England Nuclear).

Immunoprecipitation. The tritium-labeled cells were dissolved in P_i/NaCl containing 0.5% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, and the solution was centrifuged at $30,000 \times g$ for 20 min. To 100 μl of the clear supernatant thus obtained, 20 μl of sheep anti-band 3 antiserum (24) or rabbit anti-glycophorin antiserum was added. Glycophorin used as immunogen was purified from the unbound fraction of an activated-thiol Sepharose column (24). After incubation on ice-water for 60 min, 200 μl of 10% *Staphylococcus aureus* suspension (25) in P_i/NaCl containing 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin was added. After incubation in ice-water for 30 min, the sample was centrifuged and the pellet thus obtained was washed with P_i/NaCl containing 1 mM phenylmethylsulfonyl fluoride and 0.1% bovine serum albumin. The pellet was boiled in 2% $NaDodSO_4$ for 3 min and centrifuged. The supernatant was lyophilized and dissolved in the sample buffer for $NaDodSO_4$/polyacrylamide gel electrophoresis.

Immunofluorescent Studies. To complement our biochemical findings, we performed indirect immunofluorescent studies employing anti-i (Den) and anti-I (Ma) sera, previously well characterized (8–10). For this purpose, slide preparations of the population of erythroblasts, after dispersion and washing, were made. Fixation and processing for anti-i and anti-I labeling were carried out as described (26). The anti-i serum (Den) used was diluted between 1:80 and 1:200, whereas the anti-I serum (Ma) was diluted between 1:100 and 1:600.

RESULTS

Cell Surface Glycoprotein Profile of Mature Erythrocytes as Compared with Erythroblasts Cultured *In Vitro*. Mature erythrocytes, irrespective of their ontogenic stage, were characterized by five major glycoproteins: band 3, band 4.5, and glycophorins (PAS-1, PAS-2, and PAS-3). In agreement with previous results (13), band 3 and band 4.5 were heavily labeled with galactose oxidase/NaB^3H_4 and the labeled carbohydrates were highly susceptible to endo-β-galactosidase (Fig. 1, lanes 1 and 2), whereas three sialoglycoproteins (PAS-1, 2, and 3) were labeled heavily with periodate/NaB^3H_4 and were not susceptible to endo-β-galactosidase (Fig. 1, lanes 3 and 4). In a striking contrast, erythroblasts *in vitro*, irrespective of their ontogenic stages, were characterized by (*i*) barely detectable amounts of band 3 and band 4.5 glycoproteins (Fig. 1, lanes 5, 6, 11, and 12), (*ii*) the presence of glycoproteins with molecular weights 95,000 or 105,000 (Gp105), which showed the same mobility as a major glycoprotein of K562 cells (Fig. 1, lanes 7–12; Fig. 2*A*), (*iii*) a minimum quantity of polylactosaminoglycan susceptible to endo-β-galactosidase, particularly that labeled by galactose oxidase (Fig. 1, lanes 6, 8, and 12; Fig. 2, lanes 2, 4, and 6), and (*iv*) the presence of major glycoproteins identified as glycophorin (PAS-1 and PAS-2)§ (Fig. 1 lanes 7, 8, 11, and 12; Fig. 2*A*).

§ Glycophorin A is usually demonstrated on $NaDodSO_4$/polyacrylamide gel electrophoresis as two bands stainable by periodic acid/Schiff reagent (PAS-1 and PAS-2), which are reversibly interconvertible depending on the protein concentration in the applied sample, $NaDodSO_4$ concentration, temperature, etc (28). At a high sample concentration, the PAS-1 band increases due to the subunit interaction. A greatly increased band of PAS-1, as demonstrated in lanes 11 and 12 of Fig. 1 as compared to lanes 5 and 6, is a typical example for such an interaction due to the increased amount of the sample applied for lanes 11 and 12.

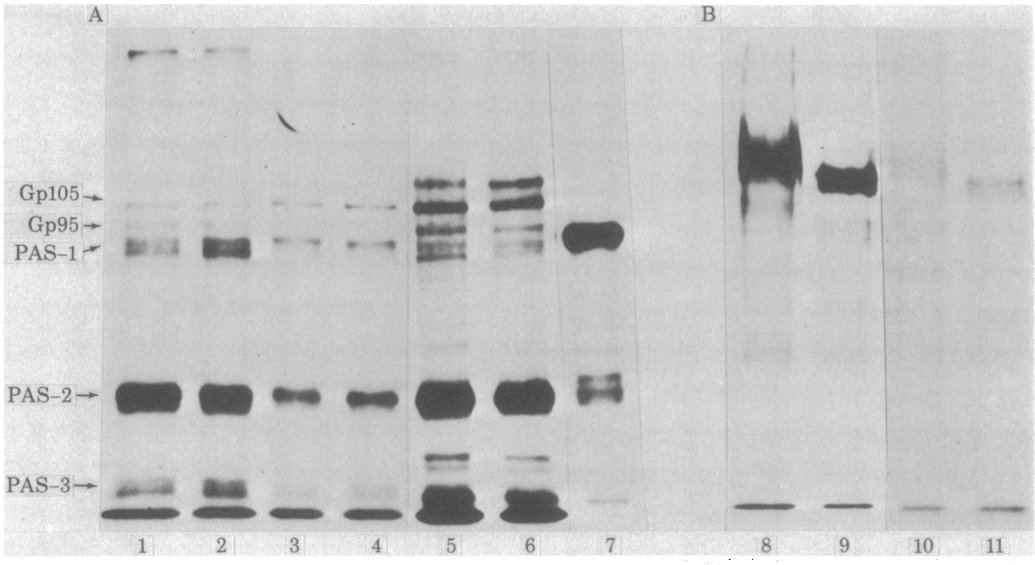

FIG. 2. (*A*) Fluorogram of NaDodSO$_4$/polyacrylamide gels of periodate/NaB^{3}H$_4$-labeled erythroblasts derived from various sources. Erythroblasts derived from fetal liver (lanes 1 and 2), from umbilical cord (lanes 3 and 4), and from peripheral blood of adult (lanes 5 and 6) were applied. Lane 7, adult erythrocytes. Lanes 1, 3, 5, and 7 are control cells incubated without endo-β-galactosidase and lanes 2, 4, and 6 are cells incubated with endo-β-galactosidase. About 8×10^5 cells (lanes 1 and 2), 5×10^5 cells (lanes 3 and 4), and 1.2×10^6 cells (lanes 5 and 6) were used. (*B*) Fluorogram of NaDodSO$_4$/polyacrylamide gels of cultured granulocytes (lanes 8 and 9) and monocytes (lanes 10 and 11) labeled by periodate/NaB^{3}H$_4$. Lanes 8 and 10 are control cells incubated without endo-β-galactosidase and lanes 9 and 11 are cells incubated with endo-β-galactosidase. Cultured cells were derived from adult peripheral blood.

Cell Surface Glycoprotein Profiles of Granulocytes and Monocytes as Compared with Erythroblasts. Granulocytes and monocytes can form colonies, though suboptimally, under the culture conditions employed in the present study. The cell surface glycoproteins of these cells were strikingly different from those of erythroid cells and showed no bands corresponding to PAS-1, PAS-2, or Gp105. In addition, major glycoproteins of these cells are partially susceptible to endo-β-galactosidase (Fig. 2*B*, lanes 9 and 11).

Immunoprecipitation of Cell Surface Glycoproteins. The absence of band 3 and the presence of glycophorin in erythroblasts *in vitro* has been further assessed by immunoprecipitation of the surface-labeled cell extract by antibodies to band 3 and glycophorin. A major band corresponding to PAS-2 and a minor band corresponding to PAS-1 was precipitated by anti-glycophorin (Fig. 3, lane 2), whereas only a faint band corresponding to band 3 was precipitated by anti-band 3 (Fig. 3, lane 8) from the erythroblast extract. No bands were precipitated from the extracts of monocytes and granulocytes (Fig. 3, lanes 3, 4, 6, and 7). In addition, rabbit anti-K562 antiserum, which contains anti-Gp105 and anti-Gp95 antibodies, precipitated no bands corresponding to Gp105 or Gp95 from mature erythrocytes.

Comparison of Oligosaccharides Released from *in Vitro* Erythroblasts of Fetal Liver, Cord, and Adult Origin. Oligosaccharides released with endo-β-galactosidase were analyzed by applying cell supernatant to a column of Sephadex G-50. As shown in Fig. 4*E*, oligosaccharides of various molecular weights were produced from adult erythrocytes, whereas oligosaccharides of the smallest molecular weight were mainly produced from fetal or cord erythrocytes (Fig. 4 *A* and *C*). The former pattern can be derived from the branched polylactosaminoglycan with I-type, whereas the latter pattern is derived from nonbranched polylactosaminoglycan characteristic of i-type, which was demonstrated previously (7, 13). The oligosaccharide pattern produced from erythroblasts of fetal liver showed a pattern essentially identical to i-type observed in fetal erythrocytes, whereas that produced from cord erythroblasts revealed a pattern basically of i-type, but with a small amount of release of higher oligosaccharides (Fig. 4 *B* and *D*). In contrast to fetal liver and neonatal erythroblasts, adult erythroblasts produced significant amounts of higher molecular weight oligosaccharides. Moreover, the amount of such oligosaccharides appear to be further increased in adult mature erythrocytes (Fig. 4 *E* and *F*).

Immunofluorescent Findings. Indirect immunofluorescence employing anti-i and anti-I sera were performed in preparations with cultured erythroblasts from fetal, neonatal, and adult sources. When anti-i (Den) serum was used in 1:100 dilution, virtually all fetal erythroblasts displayed bright fluorescence. Erythroblast preparations from cord blood cultures showed positive fluorescence with anti-i (1:100) in the majority of their cells, with a remarkable heterogeneity from erythroblast to erythroblast, similar to that observed (26) in mature cord

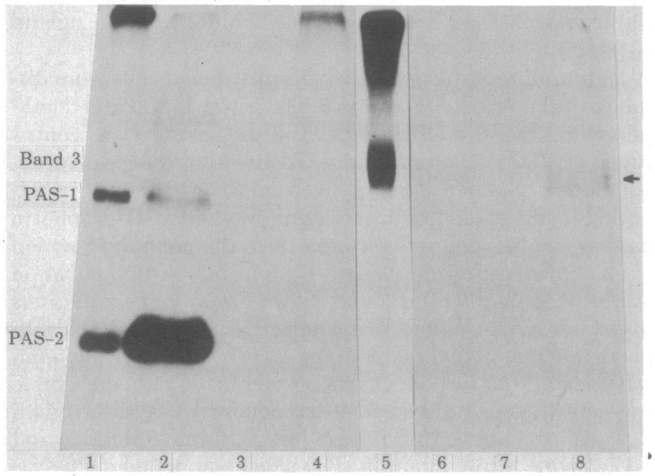

FIG. 3. Fluorogram of NaDodSO$_4$/polyacrylamide gels of immunoprecipitates with anti-glycophorin antiserum (lanes 1, 2, 3, and 4) or with anti-band 3 antiserum (lanes 5, 6, 7, and 8). Lanes 1 and 5, adult erythrocytes; lanes 2 and 8, erythroblasts; lanes 3 and 7, granulocytes; lanes 4 and 6, monocytes. Cells were labeled by periodate/NaB^{3}H$_4$. About 1×10^6 cells (lanes 2 and 8), 4×10^5 cells (lanes 3 and 7), and 8×10^5 cells (lanes 4 and 6) were used. Cultured cells were derived from adult peripheral blood.

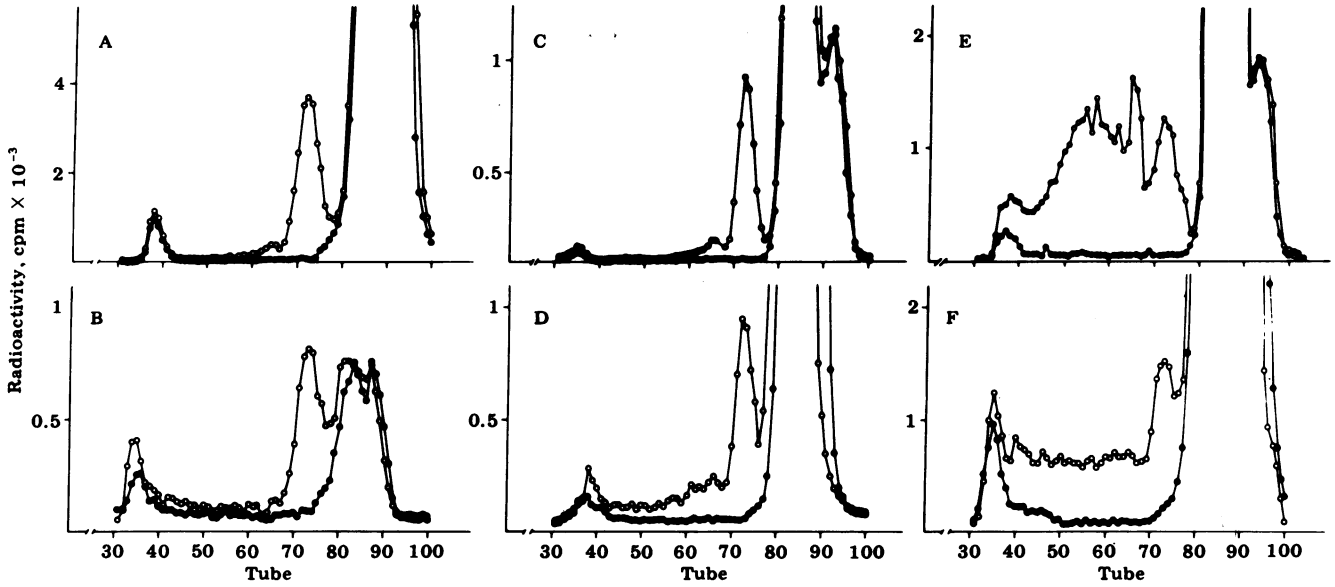

FIG. 4. Gel filtration of oligosaccharides released from periodate/NaB^{3}H$_4$-labeled cells. A Sephadex G-50 column (1 × 94 cm) was equilibrated with 0.2 M NaCl and eluted with the same solution. Fractions (0.7 ml) were collected and aliquots (50 μl for *A*, *C*, and *E* and 700 μl for *B*, *D*, and *F*) were taken for radioactivity determination. Shown are oligosaccharides from fetal erythrocytes (*A*), fetal erythroblasts (*B*), cord erythrocytes (*C*), cord erythroblasts (*D*), adult erythrocytes (*E*), and adult erythroblasts (*F*). ●, Cell supernatant after incubation without endo-β-galactosidase; ○, cell supernatant after incubation with endo-β-galactosidase. The radioactive peaks (tubes 80–100) that were observed in supernatants of both control and enzyme-treated cells are inorganic tritium salt.

blood erythrocytes before culture. In contrast to fetal and neonatal preparations, adult erythroblasts showed, after anti-i labeling, only a rare positive erythroblast. The opposite pattern was observed when anti-I serum (1:400) was used: the great majority of adult erythroblasts displayed positive fluorescence, whereas virtually no staining was observed in fetal preparations. Cord erythroblasts showed a variable amount (5–15%) of positive staining.

DISCUSSION

Differences in the glycoprotein profile between erythroblasts generated *in vitro* and mature erythrocytes have been characterized in the present study. In contrast to mature erythrocytes, erythroblasts have barely detectable amounts of band 3 and band 4.5 glycoproteins but contain glycophorin and 105- and 95-kilodalton glycoproteins. The quantity of a specific carbohydrate chain, polylactosaminoglycan, which carries blood group ABH and Ii antigenic determinants, was much higher in mature erythrocytes than in erythroblasts. The above glycoprotein profiles of erythroblasts were found irrespective of their origin—adult peripheral blood, umbilical cord blood, or fetal liver—indicating that maturation from erythroblasts to erythrocytes proceeds through a common membrane change at all three developmental stages.

Although glycophorin is present throughout these stages of erythroid lineage, band 3 and band 4.5 will appear or greatly intensify at the late stages of erythroid maturation. In contrast, it is likely that the glycoprotein with molecular weight 105,000 appears at an early stage of differentiation and maturation but declines at later stages. This notion is consistent with the presence of Gp105 as a major glycoprotein in K562 cells (29), which are presumed to be close to proerythroblast stage. Polylactosaminoglycan increases at a later stage of maturation in parallel to the increase of band 3 and band 4.5, which are characteristically labeled by galactose oxidase/NaB^{3}H$_4$.

Our results concerning glycophorins are consistent with the conclusion reported by Gahmberg *et al.* (30) that glycophorin A is present exclusively in erythroid cell lineage. Likewise the results about band 3 and its increase with maturation are analogous to findings in Friend erythroleukemic cells, which are considered to be close to the pro-erythroblast stage (31), and mature murine erythrocytes; these findings suggested that, in contrast to mature erythrocytes, a minimum amount of band 3 is present in erythroleukemic cells (32). The structure of polylactosamine chain showed a clear distinction between newborn/fetal and adult erythroblasts on the basis of oligosaccharide profile released by endo-β-galactosidase (13) and of the immunofluorescence studies with anti-I and anti-i antibodies, the reagents that can distinguish between a branched and a nonbranched polylactosamine structure (8–10). A linear, virtually nonbranched polylactosamine structure is present in fetal and newborn erythroid cells, irrespective of their degree of maturation. In contrast, a highly branched polylactosaminyl structure is present in adult erythrocytes and a somewhat less

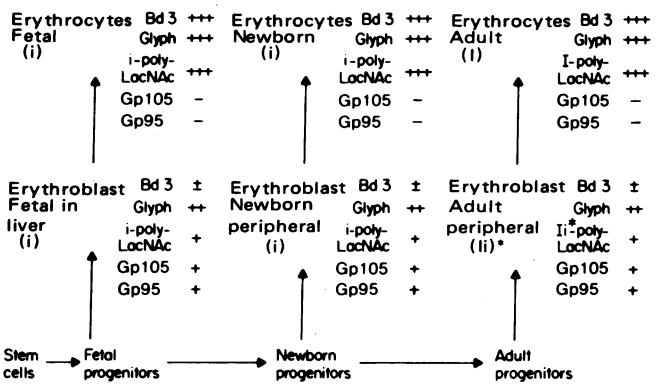

FIG. 5. Membrane differentiation of human erythrocytes. A remarkable change of protein pattern is indicated during differentiation of erythroblasts to mature erythrocytes (membrane maturation). This pattern is essentially the same for the process from erythroblasts of adult, newborn, or fetal hematopoietic tissue (liver) to their respective mature erythrocytes. The polylactosamine structure increases greatly during the maturation process, and its branching increases in adult erythroblasts; thus adult erythroblasts contain both the branched (I) and the unbranched (i) structures, as indicated by *. Bd 3, band 3; Glyph, glycophorins; polyLacNAc, polylactosaminoglycan.

but significant amount of branched structure is present in adult erythroblasts. The latter finding suggests that maturation in adult erythroid cells is accompanied by an increase in the branching of polylactosaminoglycan and thus increased expression of I-antigenic determinants. The absolute level of the coexisting small amount of i-antigenic structure in adult erythroid cells, however, cannot be measured with the present methodology. Whether membranes of erythroblasts generated *in vitro* are in some ways different from erythroblasts produced *in vivo* in bone marrow is not known.

The remarkable structural difference of polylactosamine chains observed previously between the newborn and the adult erythrocytes (7–10, 13) is now found between fetal/newborn and adult erythroblasts. It is likely that some membrane characteristics, such as the presence or the absence of band 3, band 4.5, and 105- and 95-kilodalton glycoproteins, may determine the degree of maturation from erythroblast to erythrocyte; on the other hand, other characteristics, such as the degree of branching in polylactosamine chain, may determine the stage of development, and these two membrane characteristics may operate independently. This view is summarized in Fig. 5. In this context, it was of interest to note that cord erythroblasts in culture displayed more "adult" type characteristics than cord red cells before culture. Such a finding is consistent with the view that erythroblasts *in vitro* represent the progeny of precursors with more advanced "switching," manifested also by their ability to synthesize higher levels of adult hemoglobin in culture (16).

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