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Anomalous Cell Surface Structure of Sickle Cell Anemia Erythrocytes as Demonstrated by Cell Surface Labeling and Endo-β-galactosidase Treatment

Minoru Fukuda, Michiko N. Fukuda, Sen-itiroh Hakomori, and Thalia Papayannopoulou

Department of Biochemical Oncology, Fred Hutchinson Cancer Research Center, and Departments of Pathobiology and Microbiology, University of Washington, Seattle, Washington 98104 (M.F., M.N.F., S.H.) and Department of Medicine, University of Washington, Seattle, Washington 98195 (T.P.)

Erythrocyte surface glycoproteins from patients with various types of sickle cell anemia have been analyzed and compared with those from normal individuals. By hemagglutination with various anti-carbohydrate antibodies, sickle cells showed profound increase of i antigens and moderate increase of GlcNAcβ1→3Galβ1→3 Glc structure, whereas antigenicity toward globosidic structure was unchanged. In parallel to these findings, erythrocytes of sickle cell patients have additional sialylated lactosaminoglycan in Band 3. Thus, it can be concluded that erythrocytes of sickle cell patients are characterized by an altered cell surface structure which does not appear to be due to topographical changes of cell surface membrane. It is possible that the anemia or the "stress" hematopoiesis in these patients is responsible for these changes.

Key words: sickle cell anemia, endo-β-galactosidase, cell surface labeling

During the ontogenic change from fetal to adult erythrocytes, fetal hemoglobin is mostly replaced by adult hemoglobin and the cell surface antigen i is essentially converted to I [1,2]. Recently it has been established that this i to I antigen conversion is the conversion of a linear unbranched polylactosaminyl structure, Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→R to branched lactosaminyl structure, Galβ1→4GlcNAcβ1→6 (Galβ1→4GlcNAcβ1→3) Galβ1→4GlcNAcβ1→R (Fig. 1) [3–6]. In addition, we have shown that cell surface labeling followed by endo-β-galactosidase digestion can distinguish these two forms at the cell surface, and the glycoproteins, Band 3 and Band 4.5, have been found by this technique to be the carriers of these antigens of human erythrocytes [6].

Abbreviations: $NaCl/PO_4$, 10mM sodium phosphate buffer, pH 7.0, containing 0.14 M NaCl; NaCl/acetate, 20 mM sodium acetate buffer, pH 5.8, containing 0.14 M NaCl.

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Sickle cell anemia is a prototype of a molecular disease caused by the single amino acid substitution of hemoglobin molecule, and sickle hemoglobin is responsible for morphological changes in their red cells [7]. Usually patients with sickle cell anemia have increased levels of fetal hemoglobin [8] as well as increased amounts of fetal (i) antigen, and a moderate increase of I-antigen [9–11].

It is not known yet, however, whether such increased i antigenic activity is attributed to increased exposure of Ii antigens because of topographical changes, or to an increased amount present on their red cell surface. It is also interesting to see if any particular carbohydrate structure related to Ii-antigen is increased. To approach this question we have used the new method described above and specific anti-carbohydrate antibodies to study the carbohydrate structure of erythrocyte from sickle cell patients.

MATERIALS AND METHODS

Cells

Erythrocytes from the following patients were used. Twelve cases of sickle cell anemia (SS), one double heterozygote for Hb S and Hb C (SC), and two cases of sickle β-thalasemia (STh). None of these patients was transfused at the time of study. Cord blood (newborn) was kindly provided from Group Health Cooperative of Puget Sound Hospital, Seattle, WA.

Antibodies

Anti-I(Ma) serum was donated by Dr. E.R. Giblett, Puget Sound Central Blood Bank, Seattle, WA. Anti-i (Dench) serum was donated by Dr. M.C. Crookston, Toronto General Hospital, Toronto, Canada. Antibodies against glycolipids were prepared as described previously [12].

Endo-β-galactosidase

Endo-β-galactosidase was purified from culture filtrate of Escherichia freundii as described previously [13,14].

Cell Surface Labeling and Endo-β-galactosidase Treatment

The procedure of cell surface labeling and endo-β-galactosidase treatment were described previously [6] and is mentioned briefly below. Cell surface galactose and sialic acid were labeled by galactose oxidase-NaB[^{3}H]$_4$[15] or periodate-NaB[^{3}H]$_4$ [16], respectively. The surface labeled cells (0.5 ml as packed cells, 3×10^7 cpm) were incubated at 37°C for 2 hours with or without 125 mU of purified endo-β-galactosidase in 1 ml of NaCl/acetate (pH 5.8). The cells were then suspended in 6 ml of NaCl/PO_4 (pH 7.0), and cell pellet and supernatant were separated by centrifugation. Membranes were prepared from cell pellet as described [17]. The supernatant was used for analysis of oligosaccharides released by endo-β-galactosidase (see below).

Analysis of Glycoprotein

Membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli [18]. An 8% polyacrylamide slab gel in 0.1%

SDS was used. For fluorography, the gels were treated with dimethyl sulfoxide/2,5-diphenyloxazole as described [19].

Analysis of Released Oligosaccharides

Oligosaccharides released by endo-β-galactosidase were analyzed by gel filtration as described previously [6]. The supernatant obtained by endo-β-galactosidase was applied to a column (1.0 × 96 cm) of Sephadex G-50, Superfine, which was equilibrated and eluted with 0.2 M NaCl. Aliquots of each fraction (0.7 ml) were taken for determination of radioactivity.

RESULTS

Hemagglutination of Normal and Sickle Red Cells by Anti-Carbohydrate Antibodies

Various antigenic activities which are structurally related have been studied by hemagglutination with respective antisera; anti-I, anti-i, anti-paragloboside, and anti-lacto-N-triosylceramide (see Fig. 1). While normal adult red cells are agglutinated extensively with anti-I but not with anti-i, normal cord red cells are agglutinated significantly with anti-i but not with anti-I. Sickle red cells (SS), and red cells from SC patients and from S-thalasemia patients were agglutinated to a significant degree both with anti-I and with anti-i (Fig. 2). Hemagglutinability with anti-lacto-N-triosyl structure was also found to be increased in red cells from SS and SC cells. Since all these antigens are structurally related (Fig. 1), it can be concluded that the basic common structure for Ii antigens are increased in erythrocytes from sickle cell patients. In contrast to these antigenic activities described above, the hemagglutinability with anti-

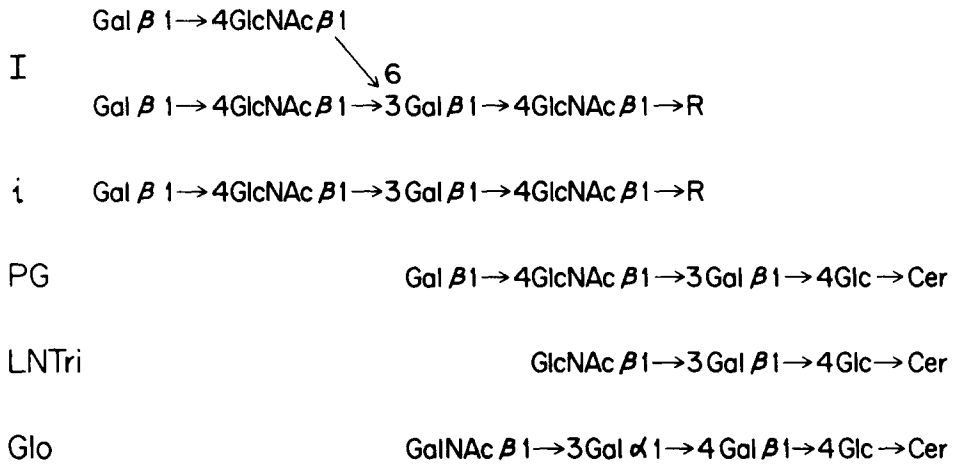

Fig. 1. Antigenic structures shown on erythrocyte surface. References of the structures shown above are as follows: I, [3,4]; i, [23]; paragloboside (PG), [24]; lacto-N-triosylceramide (LNTri), [12]; globoside (Glo), [25].

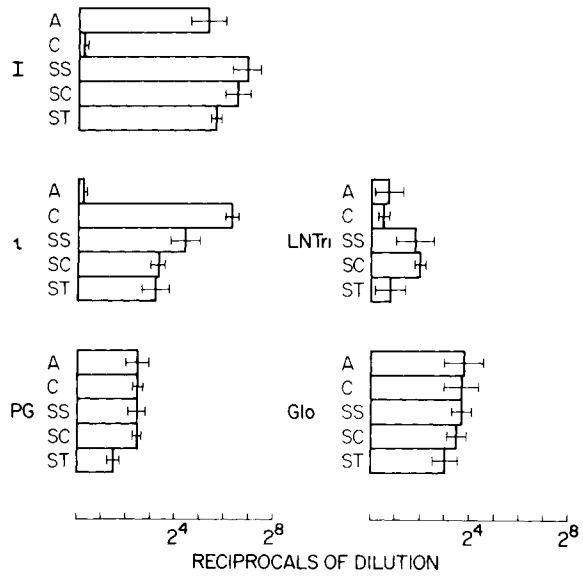

Fig 2 Hemagglutinability of normal and sickle cell anemia patient erythrocytes with anti-I, anti-i, anti-paragloboside, anti-globoside, and anti-lacto-N-triosylceramide antibodies Agglutinabilities were determined in microtiter plates with 2% erythrocytes and were expressed by reciprocals of the highest dilution of antibody that could cause obvious hemagglutination Anti-I (Ma), anti-i (Dench) were diluted to 50- and 500-fold with PO_4/NaCl (pH 7 0), respectively, and were used for hemagglutination assay A) normal adult cells, C) normal cord (newborn) cells, SS, SS (sickle homozygous) cells, SC, SC heterozygote with S and C hemoglobin cells, ST, ST heterozygote of the sickle-thalasemia cells For antigenic structures, see Fig 1

globoside antibody was not changed. Therefore, it is apparent that Ii and its related antigenic activities are specifically increased rather than carbohydrate antigens are generally increased.

Quantitative Studies on Endo-β-galactosidase Susceptible Structure on Cell Surface

Cell surface galactose or sialic acid residues were labeled by galactose oxidase-NaB[^{3}H]$_4$ or periodate oxidation-NaB[^{3}H]$_4$ methods, respectively. [^{3}H]-labeled cells were then treated with endo-β-galactosidase. Released oligosaccharides were recovered from supernatant and membranes were prepared from endo-β-galactosidase treated cells, as described previously [6].

Table I shows the radioactivity ratio of the released oligosaccharides to the total radioactivity incorporated into membrane. In all samples of red cells tested, irrespective of source of cells, higher release (40–50%) of labeled galactose than labeled sialic acid was observed, as consistent with the previous finding [6]. Although release of labeled sialic acid was not significant (5%) in the normal cells, a definitely higher release (12%) of sialosyl oligosaccharides was observed in SS, SC, and S-thalasemia red cells. This suggests a sialylated polylactosaminyl structure, which is susceptible to endo-β-galactosidase, is in-

TABLE I. The Extent of Oligosaccharide Release From Surface Labeled Cells

	Oligosaccharide release (%)[a]	
	galactose oxidase-$NaB[^3H]_4$ labeled cells	periodate oxidation-$NaB[^3H]_4$ labeled cells
Normal adult	48.9	5.0
Normal cord	49.0	4.9
SS	47.5	12.3
SC	42.0	14.0
S-Thal	44.0	17.0
Pk[b]	—[c]	5.3

[a]Oligosaccharide release is expressed as the percentage of the radioactivity of released oligosaccharides per total radioactivity incorporated in membrane.
[b]The patient who has increased reticulocyte population.
[c]Not determined.

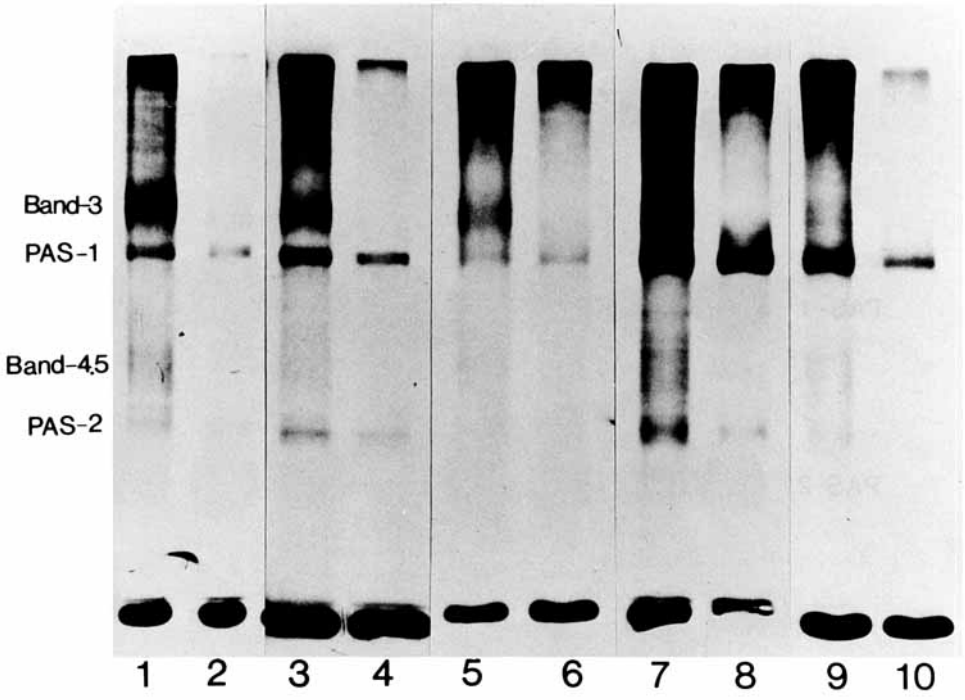

Fig. 3. Fluorogram of SDS-polyacrylamide gels of erythrocyte membrane proteins of normal and sickle cell anemia patients. Erythrocytes were labeled by galactose oxidase-$NaB[^3H]_4$ and treated by endo-β-galactosidase as described in Materials and Methods. Gels 1, 2, normal erythrocytes; gels 3, 4, erythrocytes from umbilical cord blood; gels 5, 6, SS cells; gels 7, 8, SC cells; gels 9, 10, STh cells. Gels 1, 3, 5, 7, 9, are control cells incubated without endo-β-galactosidase and gels 2, 4, 6, 8, 10 are cells treated with endo-β-galactosidase.

creased in sickle red cells. Because reticulocytes are increased in sickle cell patients, a nonsickle cell patient with increased reticulocytes was examined. The result shows that the erythrocytes from a pyruvate kinase-deficient patient released almost the same amount of sialosyl oligosaccharides as from normal erythrocytes (see Pk of Table I). Therefore the increased release of sialyl oligosaccharide is due to the intrinsic property of sickle cell erythrocytes and is not the result of the increased population of reticulocytes.

Analysis of Membrane Glycoprotein

Erythrocyte membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Both in normal and sickle red cells, the galactose oxidase-NaB[^{3}H]$_4$ method heavily labeled Band 3 and Band 4.5, as well as PAS-1 and PAS-2. After endo-β-galactosidase treatment, most of the radioactivity located in Band 3 and Band 4.5 disappeared, irrespective of source of erythrocytes (Fig. 3). On the other hand, PAS-1, -2 and -3 were labeled in normal cells by periodate-NaB[^{3}H]$_4$ method, and this label was scarcely affected by endo-β-galactosidase digestion (Fig. 4), as described previously [6]. In sickle red cells, however, Band 3 was also faintly labeled by the periodate/NaB[^{3}H$_4$]method, and the label was released by endo-β-galactosidase (compare lanes 3 and 4 of

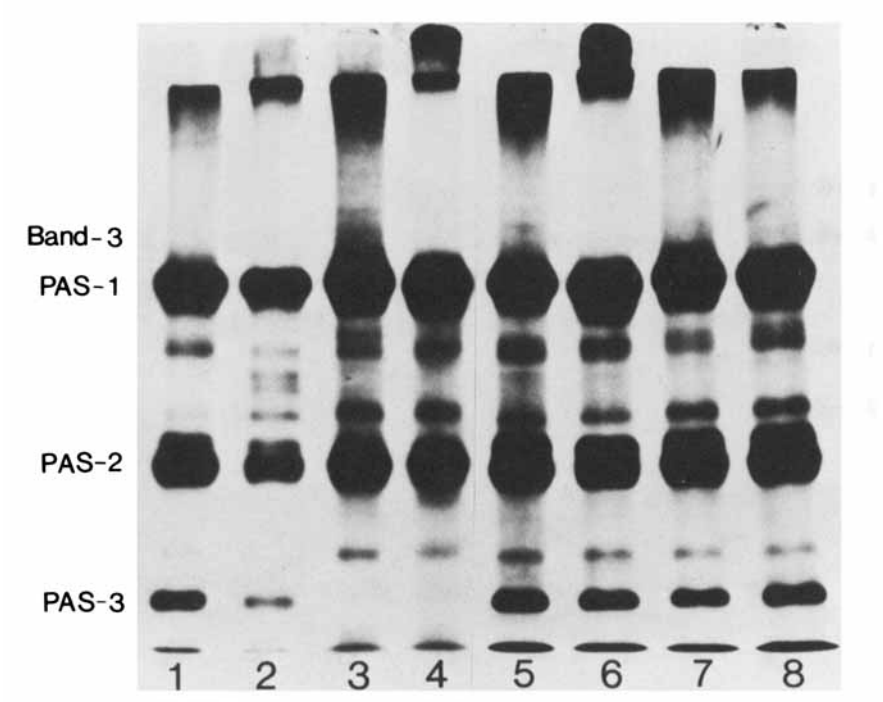

Fig 4 Fluorogram of SDS-polyacrylamide gels of erythrocyte membrane proteins Erythrocytes were labeled by periodate-NaB[^{3}H]$_4$ technique and treated by endo-β-galactosidase as described in Materials and Methods Gels 1, 2, normal erythrocytes, gels 3, 4, SS cells, gels 5, 6, SC cells, Gels 7, 8, STh cells Gels 1, 3, 4, 7 are controls incubated without endo-β-galactosidase and gels 2, 4, 6, 8 are cells treated with endo-β-galactosidase

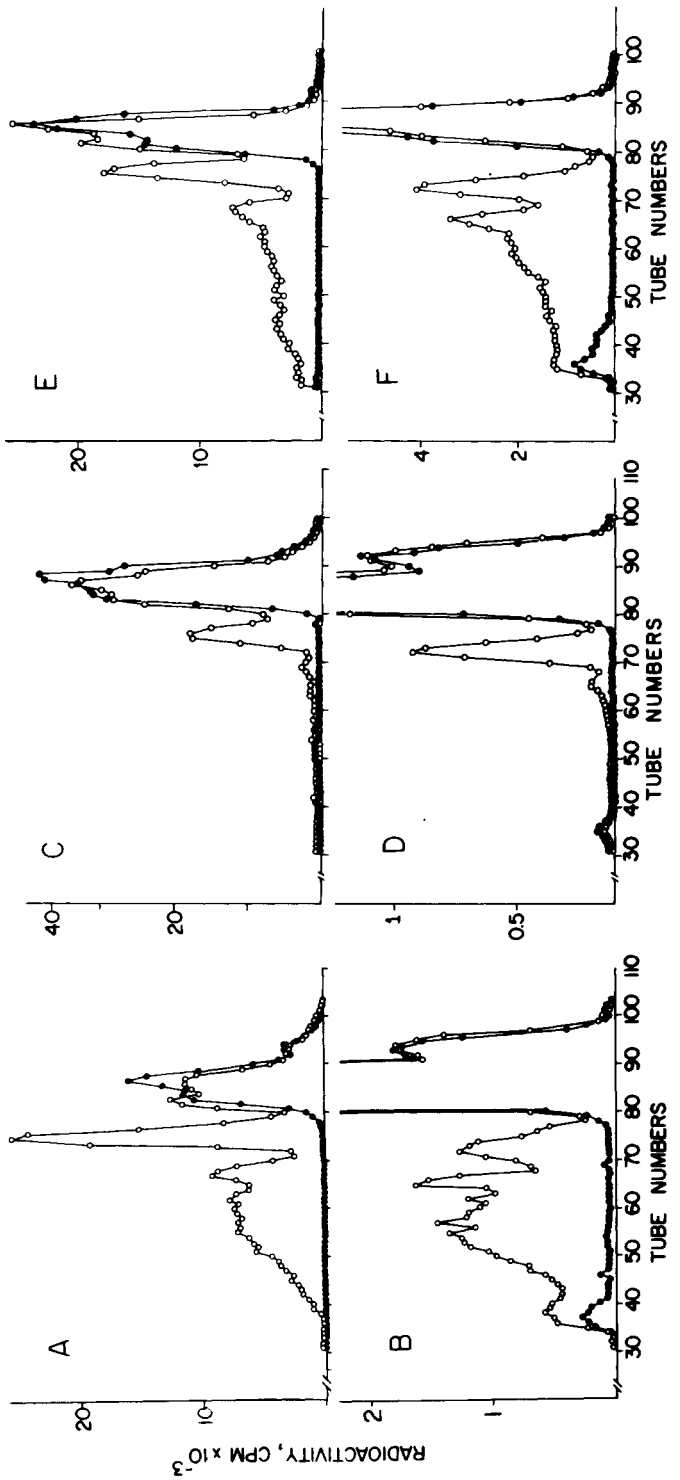

Fig. 5. Gel filtration of released oligosaccharides on a Sephadex G-50 column. Surface labeled cells were treated with endo-β-galactosidase as described under Materials and Methods. Released oligosaccharides which were separated from cells by centrifugation were applied on a column (1 × 94 cm) of Sephadex G-50 equilibrated with 0.2 M NaCl. Samples were eluted with the same solution. Fractions of 0.7 ml were collected and the radioactivity was monitored. A) Galactose oxidase-labeled normal adult. B) Periodate-labeled normal adult. C) Galactose oxidase-labeled cord. D) Periodate-labeled cord. E) Galactose oxidase-labeled SS. F) Periodate-labeled SS.

Fig. 4). Thus, it is likely that the increased amount of sialylated polylactosaminyl structure in sickle cell patients (see Table I) is partly due to sialylation of Band 3 carbohydrate chain.

It was also noticed that two of the sickle cell patients lack PAS-3 band and two other sickle cell patients have decreased amount of PAS-3 (see Fig. 4).

Characterization of Released Oligosaccharides

Released oligosaccharides from surface labeled cells by endo-β-galactosidase were analyzed by gel filtration of Sephadex G-50 column. As previously reported, i-active cells, such as cord, fetal or adult i variant red cells, gave mostly tri- and tetrasaccharides on endo-β-galactosidase treatment (Fig. 5 C, D); in contrast, I-active cells, as normal adult red cells, gave the higher molecular weight oligosaccharides with various molecular weights in addition to tri- and tetrasaccharides (Fig. 5 A, B).

Oligosaccharide profiles from SS cells showed an intermediate pattern between that from I cells and i cells (Fig. 5 E, F). As described above (Table I), increased release of sialyloligosaccharides from sickle red cells is shown again in these gel filtration profiles (Fig. 5 F). Oligosaccharide profile from SC and S-thalasemia red cells were similar to that from SS patients (data not shown).

DISCUSSION

In this paper, we showed by immunological (Fig. 2) and biochemical methods (Fig. 5) that erythrocytes from sickle cell anemia patients have an increased expression of the fetal antigen i as well as adult antigen I on their surface. The increase of Ii activities in sickle cell patient has also been reported by others using hemagglutination [9,10] or immunofluorescence techniques [11]. In addition, in the present study, antigenic activities related to Ii antigen were found to be increased in erythrocytes from sickle cell patients (Figs. 1, 2). Increased antigenic activities of GlcNAcβ1→3Galβ1→4Glc→R is particularly interesting because the increase of the structure is also observed in colon tumor [12]. In contrast to these increases, no increase of globoside structure was observed. We observed previously that after trypsin treatment of normal erythrocytes, all hemagglutinability against carbohydrate antigens such as globoside, paragloboside, and lacto-N-triosylceramide were simultaneously increased [6]. Therefore, increased Ii antigenic activity in sickle cells is due to the increase of Ii structure rather than due to the change of cryptic state of Ii antigens since no increase of globoside-antigenicity was found.

It was shown in this study that more sialylation is present at the termini of the endo-β-galactosidase susceptible carbohydrate chains in erythrocytes from sickle cell patients as compared to normal red cells. The increase of sialylation particularly concerns Band 3 carbohydrate chain (Fig. 4). It is of interest that Band 3 from neonatal red cells also contains more sialic acid than Band 3 from i-variant adult red cells [5].

Lastly, erythrocytes of some adult sickle patients were lacking in or contained only a trace amount of PAS-3. It has also been reported that some individuals of African origin lack PAS-3 [20]. Whatever the reason for this finding may be, these observations strongly suggest that PAS-3 and PAS-2 molecules are different molecules coded by different genes.

We have shown previously that the analysis of oligosaccharides released by endo-β-galactosidase gives characteristic pattern according to Ii antigenic stages of cells [6]. In this paper we showed that the same analysis is also useful to characterize carbohydrate chains present on erythrocytes of sickle cell patients. We observed that the oligosaccharide of sickle cell was intermediate between adult (I) and neonatal (i) erythrocytes (see Fig. 5 B, D, F) and the amount of sialyloligosaccharides was increased (Fig. 5 F). These results are clearly consistent with the results discussed here.

It will be interesting to know whether such anomalous cell surface structure in erythrocytes from sickle cell patients are expressed at later stage of maturation or expressed already at an early stage of maturation, such as erythroblast stage [21,22]. The work is in progress to answer such questions in our laboratory.

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