Cell Surface Modification by Endo-\(\beta\)-galactosidase

CHANGE OF BLOOD GROUP ACTIVITIES AND RELEASE OF OLIGOSACCHARIDES FROM GLYCOPROTEINS AND GLYCOSPHINGOLIPIDS OF HUMAN ERYTHROCYTES*

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Endo-\(\beta\)-galactosidase from *Escherichia freundii* has been shown to directly modify specific cell surface glycoconjugates. Antigenic changes caused by the enzyme treatment of human O1 adult, Oi-variant, and umbilical cord erythrocytes have been correlated with the altered cell surface glycoconjugate pattern and with the oligosaccharide pattern released from the cell surfaces.

The following four kinds of antigenic changes were particularly remarkable, 1) abolishment of I-antigenic activity; 2) decrease of the reactivity with anti-ABH blood group and anti-paragloboside antibodies; 3) a slight, but distinct increase of the reactivity with anti-lacto-N-trioacylceramide antibodies, and 4) no change of the reactivity with anti-globoside antibodies.

Accompanying these antigenic changes, surface-labeled carbohydrates showed the following changes through endo-\(\beta\)-galactosidase treatment: 1) about 40% of the labeled galactose was released whereas only 5% of the labeled sialic acid was released. 2) Carbohydrate moity of Band 3 and Band 4.5 glycoproteins were hydrolyzed by endo-\(\beta\)-galactosidase. In contrast, stalo-glycoproteins (PAS-1, -2, and -3) were not affected. 3) The change in glycolipids occurred mainly with long chain carbohydrates such as H\(_4\), H\(_3\), and H\(_2\)-glycolipids and in higher gangliosides. The oligosaccharide released from adult O1 active erythrocytes contained more heterogeneous higher molecular weight components than that released from adult O1, or umbilical cord O1 erythrocytes which contained relatively homogeneous lower molecular weight components. The result is therefore consistent with I-active erythrocytes containing carbohydrate chains with more branching than I-active erythrocytes.

Endo-\(\beta\)-galactosidase from *Escherichia freundii* has been shown to hydrolyze various substrates such as keratan sulfate (1), mucinous glycoproteins, milk oligosaccharides (2, 3), and glycosphingolipids (4). This enzyme has been found to hydrolyze specifically the \(\beta\)-galactosidic bond of lacto-N-glycosyl series which has the common structure \(\text{R} \rightarrow \text{GlcNAc/\(\beta\)} \rightarrow 3\text{Gal/\(\beta\)} \rightarrow 4\text{Glc (or GlcNAc)}\) (2-4). This class of oligosaccharide chain is present as antigenic determinants for blood group ABH, Lewis (5, 6), Ii (7-9), P\(_1\) (10), and p (11) and possibly for tumor-associated antigens (12-15). Thus, this enzyme is expected to be useful to study the function and structure of these antigens.

Particularly, it is interesting to know whether endo-\(\beta\)-galactosidase can directly modify cell surface antigen in situ, since the enzyme is capable of hydrolyzing the internal \(\beta\)-galactosyl linkage without removal of nonreducing sialosyl termini (4).

Furthermore, the recent development of cell surface-labeling techniques (16-19) enables us to identify the cell surface glycolipids and glycoproteins which could be modified by the enzyme treatment. Thus, it is possible to find out which glycoconjugates are responsible for those antigenicities on cell surface.

As the first step for applying the enzymatic modification of the intact cell surface, we treated the human erythrocytes by endo-\(\beta\)-galactosidase. This paper describes the antigenic change of human erythrocytes by endo-\(\beta\)-galactosidase, the characterization of an altered pattern of cell surface glycoproteins and glycolipids associated with antigenic changes, and structural characterization of released oligosaccharides from cell surface caused by the enzyme treatment. It is also found in this study that released oligosaccharide pattern from cord (new born) and O1 variant cells (20) by endo-\(\beta\)-galactosidase treatment are strikingly different from that of adult normal I cells. A part of this work has been presented preliminarily (21).

**MATERIALS AND METHODS**

Materials—Tritiated sodium borohydride (7.0 Ci/mmol) was obtained from Amersham, Arlington Heights, IL.

Cells—Erythrocytes of normal adult (blood group O, I) were regularly obtained from one of the authors (S. H.), those of umbilical cord blood vessels (blood group Oi) were obtained through the courtesy of Dr. E. Giblett (Puget Sound Central Blood Bank, Seattle, WA), and those from a very rare adult individual who is genetically deficient in I antigen (abbreviated as “adult Oi”) were obtained through the courtesy of Spectra Biological Co., Ltd., Oxnard, CA. The case number was identified as Ror-32. Outdated blood for Band 3 and glycolipid preparation was obtained from the Puget Sound Central Blood Bank.

Antibodies—Anti-I Ma serum was donated by Dr. E. R. Giblett. Anti-I Step, anti-i Dench, and anti-i Hog sera were donated by Dr. M. C. Crookston, Toronto General Hospital, Toronto, Canada. Antibodies against glycolipids were prepared as described previously (12). Anti-A and anti-B antibodies were obtained from Ortho Diagnostics, Inc., Raritan, NJ, and anti-H hemagglutinin was prepared from Ulex europaeus (22).

Glycolipids—The following glycolipids were prepared in this laboratory. Globoside (23), hematose (23), paragloboside (24), H\(_2\)-glycolipid (25), H\(_3\)-glycolipid (8), H\(_4\)-glycolipid (8), a-galactosylparaglo- bside (26), \(\beta\)-galactosa- \(\alpha\)paragloboside (27), sialosylparagloboside (24), fucoxanglioside (28), and sialylacto-\(N\)-norhexaosylceramide (9), H\(_2\)-, H\(_3\)-, and H\(_4\)-glycolipid fractions were prepared from erythrocytes by chromatography on “Iatro beads” column (Iatron Chemical Co.,
Further fractionation was achieved using a centrifugation step, and the upper phase was obtained. The upper phase glycolipids were analyzed by thin layer chromatography in a solvent system: chloroform/methanol/water (80:20:0.1, v/v). An 8% polyacrylamide slab gel in 0.1% SDS was used. For fluorography of thin layer chromatogram, a band was cut out and submitted for treatment with diethyl ether/2,5-diphenyloxazole (37).

Preparation of Band 3 Glycopeptide Labeled by Galactose Oxidase/NaB[3H]4—Band 3 glycoprotein was purified from type O adult human erythrocyte membranes as described previously (38). Band 3 (1 mg) was digested by 50 μg of porcine trypsin in 0.2 ml of 0.1 M sodium borate buffer, pH 8.0, containing 1% SDS at 37°C for 72 h. Then, the reaction mixture was heated at 100°C for 3 min to inactivate proteases. After removal of insoluble material by centrifugation, 10 Kabi units of galactose oxidase were added, and incubated at 37°C for 3 h. After incubation, the reaction mixture was titrated to pH 9.0, and 2.5 mCi of NaB[3H]4 dissolved in 50 μl of 0.01 N NaOH was added. After standing for 2 h at room temperature, 2 mg of NaBH4 was added and reduction was continued another 30 min. Reduction was stopped by adding a few drops of glacial acetic acid, and the solution was evaporated under nitrogen with repeated addition of methanol. Water-insoluble material which did not contain carbohydrate was removed by centrifugation, and water-soluble material was desalted by a Sephadex G-10 column eluted with water.

Acetylation of Oligosaccharide Alcohols—H2Glyceraldehyde, para-globoside, α-galactosylparagloboside, and β-galactosylparagloboside were labeled by galactose oxidase/NaB[3H]4 according to Suzuki and Ito (40). Each glycolipid was hydrolyzed by endo-β-galactosidase, and oligosaccharide was isolated and reduced to the alcohol by NaBH4, as described previously (4). Oligosaccharide alcohols were dried over P2O5, and acetylated in pyridine/acetic anhydride (2:1, v/v) at room temperature for 2 h. Oligosaccharides released from surface-labeled cells were reduced to oligosaccharide alcohols and acetylated in the same manner. Acetylated oligosaccharide alcohols were analyzed by thin layer chromatography in a solvent system: butylacetate/acetone/water (80:20:0.1, v/v). Fluorography of thin layer chromatography was taken after treatment with diethyl ether/2,5-diphenyloxazole (37).

RESULTS
Change of Erythrocyte Antigenicity by Endo-β-galactosidase—Changes of various antigenic activities detectable by hemagglutination with various antisera have been observed as shown in Fig. 1. While hemagglutinability by anti-A, -B, and -H was generally decreased after treatment with endo β-galactosidase, the loss of the reactivities with anti-I and anti-i antisera were most remarkable. A high agglutinability of Oi-adult cells with anti-I Ma or Step was completely abolished after the enzyme treatment. Similarly, the agglutinability of Oi-adult and Oi-cord crythrocytes with anti-i Hog and anti-i Dench was completely abolished after the enzyme treatment. An enhanced agglutinability of trypsin-treated adult erythrocytes by anti-I (Ma or Step) was not abolished, but greatly reduced. However, the enhanced agglutinability of trypsin-treated cord crythrocytes by anti-i antisera was unchanged as expected since globoside was not susceptible to endo-β-galactosidase (4).

In a striking contrast to the reactivities to anti-A, B, H, -L, -i, and para-globoside, agglutinability of trypsin-treated crythrocytes to anti-lacto-N-tetraosylceramide (LNTrI) antibodies was distinctly greater after the endo-β-galactosidase treatment. This phenomenon was consistently observed irrespective of normal adult, Oi adult, or umbilical cord Oi erythrocytes. The result is compatible with the known specificity of the enzyme, i.e. a repeating Galβ1 → 4GlcNAcβ1 → 3Gal structure.

The numerical designation for the major polypeptides of human erythrocyte is according to Fairbanks et al. (39). When type A or B erythrocytes were treated with endo-β-galactosidase, A or B blood activity was decreased also.
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Table I

| Steps* | Control cells | Endo-β-galactosidase treated cells | Control cells | Endo-β-galactosidase treated cells |
|--------|--------------|-----------------------------------|--------------|----------------------------------|
|        | 1. Surface-labeled cells | 310 | 310 | 300 | 300 |
|        | 2. Supernatant | 29 | 150 | 24 | 40 |
|        | 3. Cell pellet | 290 | 150 | 270 | 250 |
|        | 4. Membrane | 247 | 107 | 202 | 186 |
|        | 5. Chloroform/methanol extract | 142 | 111 | 35 | 15 |
|        | 6. Residue of extract | 120 | 44 | 140 | 140 |
|        | 7. Upper phase | 3.1 | 2.9 | 22 | 9 |
|        | 8. Lower phase | 84 | 94 | 4.0 | 4.0 |
|        | 9. Neutral glycolipids | 3.4 | 2.5 | 0.2 | 0.2 |
|        | 10. Gangliosides | 0 | 0 | 6.0 | 5.2 |

*The fractionation steps are corresponding to that of Fig. 2.

Table: Yield of radioactivity during fractionation procedure of surface-labeled cells

The values of radioactivity are expressed as total counts per min when 0.5 ml of packed cell was used.

| Steps | Control cells | Endo-β-galactosidase treated cells |
|-------|--------------|-----------------------------------|
| 1. Surface-labeled cells | 310 | 310 |
| 2. Supernatant | 29 | 150 |
| 3. Cell pellet | 290 | 150 |
| 4. Membrane | 247 | 107 |
| 5. Chloroform/methanol extract | 142 | 111 |
| 6. Residue of extract | 120 | 44 |
| 7. Upper phase | 3.1 | 2.9 |
| 8. Lower phase | 84 | 94 |
| 9. Neutral glycolipids | 3.4 | 2.5 |
| 10. Gangliosides | 0 | 0 |

With endo-β-galactosidase treatment, the surface galactose-labeled cell released 40% of the radioactivity (see Table I, Steps 2, 3, and 5), and the radioactivity of both protein fraction (Step 6) and glycolipid fraction (Step 5) was decreased compared to controls. Based on these data, the structure created by the enzyme was not readily accessible to antibody, therefore only trypsin-treated and endo-β-galactosidase-treated cells showed an enhanced reactivity with anti-lacto-N-triosylceramide antibodies. The time course of the decrease of I, i, and H showed the rapid decrease of hemagglutinability and reached a plateau after incubation at 37°C for 1 h with 125 milliunits of the enzyme/ml (data not shown). This rate was very similar to the hydrolysis rate of the isolated sialosyl paragloboside or H₂-glycolipid with the same enzyme concentration (4).

**Endo-β-galactosidase Treatment of Surface-labeled Cells**—The cell surface galactose residues or sialic acid residues were labeled by galactose oxidase/NaB[tH]₄ method or periodate oxidation/NaB[tH]₄ method, respectively. Labeled cells were treated by endo-β-galactosidase as described under "Materials and Methods," and cells and supernatant were fractionated as summarized in Fig. 2. The yield of radioactivity in each step of adult I cells is shown in Table I.

With endo-β-galactosidase treatment, the surface galactose-labeled cell released 40% of the radioactivity (see Table I, Steps 2, 3, and 5), and the radioactivity of both protein fraction (Step 6) and glycolipid fraction (Step 5) was decreased compared to controls. Based on these data, the structure created by the enzyme was not readily accessible to antibody, therefore only trypsin-treated and endo-β-galactosidase-treated cells showed an enhanced reactivity with anti-lacto-N-triosylceramide antibodies. The time course of the decrease of I, i, and H showed the rapid decrease of hemagglutinability and reached a plateau after incubation at 37°C for 1 h with 125 milliunits of the enzyme/ml (data not shown). This rate was very similar to the hydrolysis rate of the isolated sialosyl paragloboside or H₂-glycolipid with the same enzyme concentration (4).

**Glycoproteins of Surface-labeled Cells**—Fig. 3 showed the polyacrylamide gel electrophoresis of surface-labeled erythrocyte membrane proteins. Galactose-oxidase-NaB[tH]₄ method heavily labeled Band 3 and Band 4.5 and labeled structure as seen in lacto-N-neohexaotseramide or its analogue were hydrolyzed to yield lacto-N-triosylceramide and various oligosaccharides with this enzyme (4). However, the structure created by the enzyme was not readily accessible to antibody, therefore only trypsin-treated and endo-β-galactosidase-treated cells showed an enhanced reactivity with anti-lacto-N-triosylceramide antibodies. The time course of the decrease of I, i, and H showed the rapid decrease of hemagglutinability and reached a plateau after incubation at 37°C for 1 h with 125 milliunits of the enzyme/ml (data not shown). This rate was very similar to the hydrolysis rate of the isolated sialosyl paragloboside or H₂-glycolipid with the same enzyme concentration (4).

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A

Band-3

PAS-1

Band-4.5

PAS-2

Fig. 3. SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins of adult I cells (A), adult i cells (B), and cord cells (C) labeled by the galactose oxidase-NaB[3H]4 method. A, labeled adult cells after incubation with endo-β-galactosidase (gels 2 and 4) or without the enzyme (Gels 1 and 3). Gels 1 and 2 are Coomassie blue-stained gels and Gels 3 and 4 are fluorographs of Gels 1 and 2, respectively. B, labeled i adult cells after incubation with endo-β-galactosidase (Gels 6 and 8) or without the enzyme (Gels 5 and 7). Gels 5 and 6 are Coomassie blue-stained gels and Gels 7 and 8 are fluorographs of Gels 5 and 6, respectively. C, labeled cord cells after incubation with endo-β-galactosidase (Gels 10 and 12) or without the enzyme (Gels 9 and 11). Gels 9 and 10 are Coomassie blue-stained gels and Gels 11 and 12 are fluorographs of Gels 9 and 10, respectively.

Fig. 4. Fluorography pattern of erythrocyte membrane proteins labeled by sodium metaperiodate-NaB[3H]4 method. 1, adult I control cells; 2, endo-β-galactosidase treated adult I cells; 3, adult i control cells; 4, endo-β-galactosidase-treated adult i cells; 5, cord control cells; 6, endo-β-galactosidase-treated cord cells.

lightly PAS-1, and -2 (Fig. 3, Gels 1 and 3). Significantly, most of the radioactivity located in Band 3 and Band 4.5 disappeared after endo-β-galactosidase treatment (Fig. 3, Gel 4). Thus, the liberated oligosaccharide derived from glycoproteins were essentially derived from Band 3 and Band 4.5 carbohydrate moiety. In addition, PAS-1 and PAS-2 appear to be affected slightly to lose the radioactivity, suggesting the presence of a small population of the susceptible carbohydrate chain in PAS-1 and PAS-2.

Similar results were obtained in the case of adult i and cord cells (Fig. 3, Gels 5 to 12). Interestingly, endo-β-galactosidase-digested Band 3 migrated slightly faster than nondigested sample in SDS-polyacrylamide gel electrophoresis and showed a less broad band (Fig. 3, Gels 6 and 10).

In contrast, sialoglycoproteins which had been labeled by periodate-NaB[3H]4 method did not show obvious changes by the enzyme treatment (Fig. 4), except that two bands between PAS-1 and PAS-2 in cord cells were lost (Fig. 4, Gel 6). The short time exposed film also showed no indication of change after enzyme treatment except as described above.

Glycolipids of Surface-labeled Cells—Membrane glycolipids were extracted and fractionated as summarized in Fig. 2 and Table I. Fig. 5A shows the fluorography pattern of the thin layer chromatogram of neutral glycolipid (Step 9 in Fig. 2 and Table I) from galactose oxidase/NaB[3H]4-labeled adult I cells. It is apparent that endo-β-galactosidase hydrolyzes the carbohydrate portion of H2- and H3-glycolipid completely, and hydrolyzes some part of H1-glycolipid.

Fig. 5B shows the fluorography of the thin layer chromatogram of ganglioside (Step 10 in Fig. 2 and Table I) from periodate oxidation/NaB[3H]4-labeled adult I cells. Endo-β-galactosidase hydrolyzes one long chain ganglioside and some part of branched chain ganglioside as well. These results indicated that endo-β-galactosidase hydrolyzed the carbohydrate moiety of long chain gangliosides on cell surface. Similar results were obtained when i adult or cord cells were treated with endo-β-galactosidase (data not shown).

Characterization of Liberated Oligosaccharides—To know the size and structure of released oligosaccharide by endo-β-galactosidase, cell supernatant (Step 2 in Fig. 2 and
Table I) was applied on a column of Sephadex G-50. As shown in Fig. 6, A and B, oligosaccharides of various molecular size were observed from adult I cells. The gel chromatography profiles of the endo-β-galactosidase digest of Band 3 glycopeptides or glycolipids are shown in Figs. 7 and 8, respectively. The elution position of oligosaccharide P-1 from galactose oxidase-labeled cells (Fig. 9) is identical to the smallest size oligosaccharide peak from Band 3 glycopeptide (Fig. 7) or from glycolipids (Fig. 8). These results indicate that most of the higher oligosaccharides originated from the Band 3 carbohydrate moiety, but small size oligosaccharides (P-1 and P-2 in Fig. 6A) could have originated from Hc-, Hc-, and Hc-glycolipid as well.

The gel chromatography profile of the sialic acid-labeled oligosaccharides is similar to that of the galactose-labeled oligosaccharide, although total radioactivity of the former is

were isolated as described under "Materials and Methods." The positions of authentic neutral glycolipids are shown. Glo, globoside, GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc → Cer; Hc, Hc-glycolipid, L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer; Hc-glycolipid, L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer; Hc, Hc-glycolipid, L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer; Hc, Hc-glycolipid, similar to Hc but with an additional branching structure, exact structure undetermined. B, 1, upper phase gangliosides from adult I control cells; 2, upper phase ganglioside from endo-β-galactosidase-treated adult I cells. Cell surfaces were labeled by periodate- NaB[3H]4 method and ganglioside fraction was isolated as described under "Materials and Methods." The positions of authentic gangliosides are shown. GM3, hematoside, GNeu2α → 3Galα1 → 4Glc → Cer; SPG, sialosylparagloboside, AcNeu2α → 3Galα1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer; SHex, sialosyllacto-N-norhexaosylceramide, GNeu2α → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer; FG, fucoganglioside, L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 6(NAcNeu2α → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer.

Fig. 6. Gel filtration of released oligosaccharides on a Sephadex G-50 column. Sephadex G-50 (1 x 94 cm) was 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. The experimental conditions of endo-β-galactosidase treatment are described under "Materials and Methods." A, oligosaccharides from galactose oxidase-labeled adult I cells; B, oligosaccharides from periodate-labeled adult I cells; C, oligosaccharides from galactose oxidase-labeled adult I cells; D, oligosaccharides from periodate-labeled adult I cells; E, oligosaccharides from galactose oxidase-labeled cord cells; F, oligosaccharides from periodate-labeled cord cells. - - - , cell supernatant after incubation without endo-β-galactosidase; O --- O, cell supernatant after incubation with endo-β-galactosidase. The supernatant from approximately the same number of cells were applied. The radioactive peak (Tube 80 to 100) which was observed in both supernatants of control and enzyme-treated cells is inorganic tritium salt.
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10 times less than that of the latter (see Fig. 6, A and B). It is possible that a small amount of sialic acid in Band 3 (38) and Band 4.5 has been labeled by tritium and hydrolyzed to oligosaccharides. Small size oligosaccharides also may have originated from higher gangliosides.

Comparison of Oligosaccharides Released from Adult I, Adult i, and Cord Cells—Although oligosaccharides released from adult I cells showed various molecular sizes, oligosaccharides were released almost exclusively as smallest size from i and cord cells (Fig. 6, C to F). It appeared that carbohydrate chains of i cells could be more extensively degraded and only short oligosaccharides were produced. It is likely therefore that i cells have a different structure of oligosaccharide chains, which is more susceptible to endo-\(\beta\)-galactosidase than that of normal adult I cells.

Structural Characterization of Oligosaccharides—Since the amount of oligosaccharide released from cells was too little to be analyzed by chemical means, the oligosaccharides were characterized as follows.

The smallest size oligosaccharide (Fig. 6, P-1) from galactose oxidase labeled adult I, adult i, and cord cells was subjected to paper chromatography. In all cases, two components, P-1a and P-1b, were observed and showed the same mobility as authentic Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Gal and Fuc\(\alpha1 \rightarrow 2\)Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Gal, respectively (Fig. 9). P-1a and P-1b were eluted from filter paper after paper chromatography as described above and subjected to thin layer chromatography after derivatization to oligosaccharide alcohol acetates. As seen from Fig. 10, oligosaccharide alcohol acetates of P-1a and P-1b migrated to the positions corresponding to the acetates of Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Galol and Fuc\(\alpha1 \rightarrow 2\)Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Galol, respectively.

The release of radioactive galactose residue from each oligosaccharide by exo-glycosidase was examined by paper chromatography. From P-1a, radioactive galactose was liberated by \(\beta\)-galactosidase but not by \(\alpha\)-galactosidase. From P-1b, neither \(\beta\)-galactosidase nor \(\alpha\)-galactosidase could release radioactive galactose. However, \(\beta\)-galactosidase together with \(\alpha\)-fucosidase could release radioactive galactose from P-1b. Based on these results, the structures of P-1a and P-1b were tentatively estimated to be Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Gal and Fuc\(\alpha1 \rightarrow 2\)Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Gal, respectively.

Oligosaccharide P-2 (Fig. 6A) showed five components by paper chromatography and has not been characterized further. However, these oligosaccharides might be branched oligosaccharides since P-2 was produced only from branched H- or H\(_2\)-glycolipid but not from straight chain H\(_2\)-glycolipid (8) (see Fig. 8). The smallest oligosaccharide (Fig. 6, S-1) from sialic acid-labeled adult I, adult i, and cord cells was applied to paper chromatography and showed the same mobility as authentic AcNeu\(\alpha2 \rightarrow 3\)Gal\(\beta1 \rightarrow 4\)GlcNAc\(\beta1 \rightarrow 3\)Gal (data not shown).

![Fig. 7. Gel filtration of Band 3 glycopeptide and its digest by endo-\(\beta\)-galactosidase.](image)

![Fig. 8. Gel filtration of oligosaccharides from galactose oxidase-labeled glycolipids by endo-\(\beta\)-galactosidase. Each glycolipid fraction (H\(_2\)-, H\(_3\)-, and H\(_4\)-glycolipid fraction, see "Materials and Methods") was labeled by galactose oxidase/NaB\(_{3}\)H\(_4\) according to Suzuki and Suzuki (40). Each labeled glycolipid fraction (2 \(\times\) 10\(^4\) cpm) was incubated with 12.5 milliunits of endo-\(\beta\)-galactosidase in 100 \(\mu\)l of 0.2 M sodium acetate buffer, pH 5.8, containing 100 pg of sodium deoxytaurocholate at 37\(^\circ\)C for 2 h. After incubation, 600 \(\mu\)l of chloroform/methanol (2:1, v/v) was added and upper water phase was taken as digest of each glycolipid fraction. Each oligosaccharide fraction was applied on Sephadex G-50 column as the same manner as in Fig. 6. ---, undigested glycopeptide; O---O, endo-\(\beta\)-galactosidase digested glycopeptide.)

![Fig. 9. Paper chromatogram of smallest oligosaccharides from galactose oxidase-labeled cells.](image)
DISCUSSION

Enzymatic modification of the cell surface, particularly surface antigenicity and immunogenicity, has been a major topic in transplantation immunology. Enhanced immunogenicity of tumor cells through sialidase treatment has been reported (41-44). Conversion of blood group B erythrocytes to O erythrocytes by coffee bean α-galactosidase was reported to be useful for practical transfusion purpose (45), however, the reactivities of various sugar residues on cell surfaces to various exo-glycosidase are generally limited except to sialidase and probably to a few exceptional enzymes.

Modification of cell surface carbohydrate by endo-glycosidases could be an exciting approach if a suitable enzyme can be applied. However, no suitable endo-glycosidase has so far been reported to degrade a specific cell surface glycoprotein or glycolipid. Endo-β-galactosidase of \textit{E. freundii} has an obvious merit in that the enzyme can hydrolyze a specific β-galactosyl linkage without modification of the peripheral carbohydrate chain and its substrate specificity has been well established (4). Thus, the enzyme is ideal to modify a specific carbohydrate chain at the cell surface. This study is aimed at the antigenic modification of erythrocytes as a model with the endo-β-galactosidase and further to correlate with the changes in a specific cell surface carbohydrate to the antigenicity change caused by the enzyme treatment.

Four kinds of antigenicity changes of erythrocytes have been observed: 1) abolished Ii-antigenic activity, 2) decrease of the reactivities with antibodies to ABH and paragloboside, 3) a slight, but distinctive increase to antibodies directed to lacto-N-trioseylceramide, and 4) no change in the reactivity with anti-globoside antibodies.

Through endo-β-galactosidase treatment of surface-labeled cells, 1) about 40% of the labeled galactose was released while only 5% of the labeled sialic acid was released (Table I) and 2) the change in glycoproteins occurred mainly in Band 3 glycoprotein, the major intrinsic membrane protein. The change in Band 4.5 was also obvious (Fig. 3). In contrast, sialoglycoproteins (PAS-1, -2 and -3) were not affected by endo-β-galactosidase (Fig. 4). 3) The change in glycolipids occurred in those with long chain carbohydrate such as H₂, H₁, and H₂-glycolipids and in higher gangliosides (Fig. 5), 4) Oligosaccharides were released from the cell surface which showed characteristic patterns according to I-active adult or i-active fetal erythrocytes (Fig. 6).

From these results, it is apparent that endo-β-galactosidase modified carbohydrate chains of Band 3, Band 4.5 and long chain glycolipids resulting in the abolishment of Ii antigenic activity and the decrease of ABH and paragloboside antigenic activity. As a consequence, a new structure having N-acetylglucosamine as nonreducing terminus was created which may show the reactivity with antibodies directed to lacto-N-trioseylceramide. Thus, it can be concluded that at least some portion of carbohydrate chain of Band 3 and Band 4.5 and long chain glycolipids are responsible to express Ii-activity. This result is consistent with the recent finding that long chain glycolipids such as lacto-N-norhexaosylceramide or lacto-N-isooctaosylceramide (9, 46) and Band 3 protein are the antigenic carriers for Ii-determinants (47).

Endo-β-galactosidase hydrolyzed galactose-labeled oligosaccharide chains about 10 times more than sialic acid-labeled oligosaccharide chains. The results indicated that carbohydrate chains labeled by galactose oxidase/NaB₄[H] were much more susceptible than those labeled by periodate/NaB₄[H]. The former was represented by the Band 3 molecule which has a susceptible carbohydrate chain composed of mainly galactose and N-acetylglucosamine (38, 48), whereas the latter was represented by the carbohydrate chains of glycophorin which are mainly composed of AcNeu(α2 → 3)Galβ1 → 3(αNeu(α2 → 6)GalNAc (49). Thus, the combination of cell surface labeling and endo-β-galactosidase treatment can be a useful method to discriminate the structure of various oligosaccharide chains on cell surfaces. Recently, the glycopeptides which are mainly composed of galactose and N-acetylglucosamine were isolated from protease-released fraction of human erythrocytes and termed as erythroygycan (50). Since these glycopeptides were susceptible to endo-β-galactosidase (50), the glycopeptides appear to originate from Band 3 and Band 4.5.

Analysis of a released oligosaccharide pattern gave us useful information about the structure of the carbohydrate chain of the original molecule. A large variety of oligosaccharides with different molecular sizes were released from adult i-active erythrocytes in a striking contrast to the homogeneous small size oligosaccharides released from i-active adult, or i-active umbilical cord erythrocytes. A linear carbohydrate chain such as R → GlcNAcβ1 → 3Galβ1 → 4GlcNAc → R can be hydrolyzed readily by endo-β-galactosidase whereas the galactosidic linkage at a branched structure such as R → GlcNAcβ1 → 6(R → GlcNAcβ1 → 3)Galβ1 → 4GlcNAc was hardly hydrolyzed by this enzyme (4). Therefore, the release of oligosaccharides with various molecular sizes from adult I-erythrocytes are probably derived from the heterogeneous branched structures. In contrast, a homogeneous small size oligosaccharide can be released probably from a linear structure with little branching. This interpretation is supported by the recent structural studies on i-active and I-active glycolipids.
Cell Surface Modification by Endo-β-galactosidase

Glycolipids with short carbohydrate chains such as sialosylparagloboside, paragloboside, and H₂-glycolipids were hydrolyzed when isolated glycolipids were incubated with endo-β-galactosidase (4), but not on cell surface (Fig. 5). Only a long chain glycolipid with more than about 6 sugar residues were hydrolyzed on cell surface. This indicates that the susceptible galactosidic linkage close to glycosylceramide moiety is not accessible by endo-β-galactosidase on cell surface. Although H₂-glycolipid has a susceptible linkage at the 4th sugar residue from ceramide as shown in in vitro analysis under a high concentration of the enzyme (4), this galactosyl residue is branched and therefore is unsusceptible on cell surface. Thus, H₂-, H₁-glycolipid and their analogues are not highly susceptible to the enzyme treatment, therefore a remaining H₁-, and H₂-glycolipid may contribute H-antigenic activity after the endo-β-galactosidase treatment.

As a summary, combining cell surface labeling technique and endo-β-galactosidase digestion brought us extensive characterization of cell surface glycoproteins and glycolipids, especially those which are Ii antigenic carriers in human erythrocytes.

Human fetal and cord red blood cells have I antigenic activity but will develop to I active red blood cells in a normal adult (52). It also has been reported that the change of II blood group is associated with the tumorigenic change (15). Endo-β-galactosidase will, therefore, be an indispensable tool to analyze the developmental and oncogenic change of cell surface glycoproteins and glycolipids in these cells.

Acknowledgments—We wish to thank Dr. K. Watanabe for helpful discussions in the initial stage of this work. We also wish to thank Dr. M. Kitamikado for the kind gift of the strain of E. freundii, and Drs. E. Giblett and M. Crookston for their donation of anti-Ii sera and blood specimens.

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Cell surface modification by endo-beta-galactosidase. Change of blood group activities and release of oligosaccharides from glycoproteins and glycosphingolipids of human erythrocytes.
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J. Biol. Chem. 1979, 254:5458-5465.

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