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Exposure of Major Glycolipids in Human P^k and p Erythrocytes

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The exposure of glycolipids in P^k and p red cells was studied by the galactose oxidase/NaB^2H_4 and galactose oxidase/NaB^3H_4 surface labeling techniques. The major glycolipid in P^k cells, ceramide trihexoside was efficiently labeled when high amounts of galactose oxidase were used. In contrast, the major glycolipid in p cells, ceramide dihexoside was not oxidized by galactose oxidase. However, minor components with longer oligosaccharide chains were readily labeled in p cells by the galactose oxidase/NaB^3H_4 method.

The outer leaflet of mammalian plasma membranes contains a large variety of glycosphingolipids [1]. They may have an important function as adhesion sites for various bioactive factors. Many microbes, bacterial toxins, lectins and antibodies bind to cell surface glycolipids [1-3]. There is also evidence that glycolipids may modulate cellular functions by interacting with functional membrane proteins, such as receptors and transporters [4].

Several studies have shown that only a portion of glycolipids is accessible to external ligands [5-7]. We have determined the efficiency of glycolipid exposure by oxidizing red-cell glycoconjugates with galactose oxidase followed by reduction with NaB^2H_4. The deuterium label was quantified by gas-liquid chromatography/mass spectrometry (GLC-MS). By using this method we found that 37-66% of the major glycolipid, globoside, was exposed on human red cells. Under the same conditions, 10-15% of ceramide trihexoside (CTH) was oxidized, but no deuterium label was found in ceramide dihexoside (CDH) of normal human red cells [7].

Normal human erythrocytes contain 4-5 times as much globoside as CTH or CDH. However, there are rare individuals who lack globoside, the P antigen, on their red cells. These people belong either to the P^k or to the p blood group. The major glycolipid in P^k cells is CTH, which is lacking in p red cells. Instead, p cells have CDH as their major glycolipid [8]. We have previously studied the exposure of major glycolipids in several mammalian red cells [7, 9]. To obtain further information about factors affecting glycolipid crypticity, I now have used galactose oxidase as a tool for studying glycolipid exposure in P^k and p red cells.

Abbreviations: CDH, ceramide dihexoside, LacCer; CTH, ceramide trihexoside, $GbOse_3Cer$.

Materials and Methods

Cells

P^k erythrocytes were obtained from the Finnish Red Cross Transfusion Service, Helsinki. Cells of group p were a kind gift from Dr. Bertil Cedergren, Umeå, Sweden. All erythrocytes were treated as previously described [7].

Enzymes

Galactose oxidase (*Dactylium dendroides*) was obtained from Sigma Chemical Co. (St Louis, MO, USA). The enzyme was heated for 30 min at 56°C before use to destroy possibly contaminating proteases [6]. No loss of activity was found as a result of this treatment. Horseradish peroxidase (type II) and bovine liver catalase were from Sigma. *Vibrio cholerae* neuraminidase (1 U/ml) was obtained from Koch-Light Ltd (Haverhill, England).

Isotopes

Tritiated sodium borohydride (8 Ci/mmol) was purchased from Amersham International (Amersham, UK) and handled as described [10]. Sodium borodeuteride was from Merck AG (Darmstadt, FRG).

Labeling of Glycolipid Standard

Deuterated glycolipid standards were prepared as previously described [7].

Labeling of Erythrocytes

Labeling of erythrocytes was done as previously described [7, 11]. When neuraminidase treatment was used in the galactose oxidase/NaB^3H_4 or the galactose oxidase/NaB^2H_4 labeling, the cells were incubated with 50 µl neuraminidase/ml packed cells simultaneously with galactose oxidase. Erythrocyte membranes were prepared by lysing the cells in 5 mM Tris buffer, pH 8.0, followed by centrifugation and washings at 4°C [5].

Protein Determination

Protein was determined by the method of Lowry *et al.* [12] with bovine serum albumin as a standard.

Phosphorus Determination

Lipid-bound phosphorus was determined from chloroform/methanol extracts by the procedure of Bartlett [13].

Isolation and Analysis of Glycolipids

The glycolipid fraction was isolated by the procedure of Saito and Hakomori [14], and the glycolipids were purified by preparative TLC. The solvent system was chloroform/methanol/water, 60/30/5 by vol. The radioactive samples were counted in a Triton-X-114-based

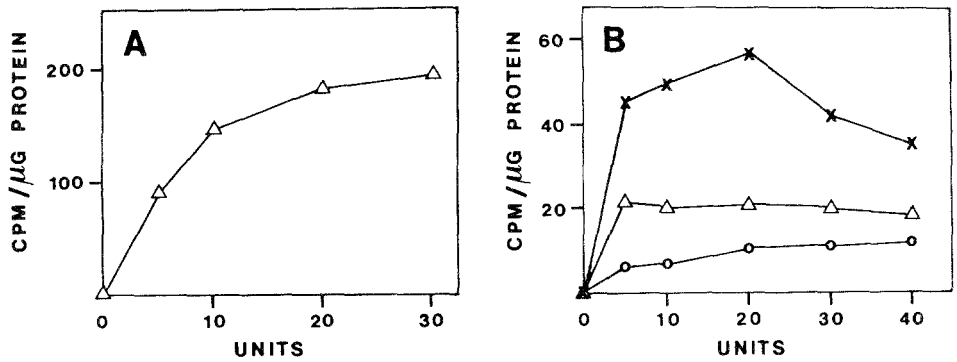

Figure 1. Incorporation of ^{3}H from $NaB^{3}H_4$ into major glycolipids of red cells after oxidation with different amounts of galactose oxidase.
A, Label in CTH from P^k cells; B, label in glycolipids from p cells. Paragloboside-globoside fraction (x); CTH △); CDH (o). 5-40 units galactose oxidase were added every two hours/ml packed cells. The total incubation time was 8 h.

scintillation fluid using a Wallac LKB 1210 Ultrobeta liquid scintillation counter. Quantification of glycolipids was done by GLC after methanolysis as described by Bhatti *et al.* [15]. The degree of deuteration of the *N*-acetylgalactosamine residues was determined as previously described by GLC-MS (Hewlett-Packard 3992 A) [7].

Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of sodium dodecyl sulfate was done according to the method of Laemmli on 8% acrylamide slab gels [16]. A ^{14}C-labeled standard protein mixture was obtained from Amersham (UK). After completion of the electrophoresis, the gels were treated for fluorography [17] and vacuum-dried. The gels were covered with Kodak RP X-Omat film and stored at -70°C until developed.

Results

Labeling of Erythrocytes with Tritium

The effect of the concentration of galactose oxidase on glycolipid oxidation was studied by labeling the erythrocytes with the galactose oxidase/$NaB^{3}H_4$ method. First the cells were incubated for eight hours with different amounts of the enzyme. After washing, the oxidized cells were reduced with $NaB^{3}H_4$. Glycolipids were isolated and analyzed by TLC. The results are shown in Fig.1. 30 Units/ml packed cells/2 h were sufficient to oxidize all available CTH in P^k cells. In contrast, very little radioactivity was incorporated into CDH in p cells, and there was no saturation when increasing amounts of the enzyme were used. It was, however, found that p cells contained minor components co-migrating with CTH and globoside standards. These minor glycolipids were effectively oxidized even by five units of galactose oxidase/ml packed cells/2 h (Fig.1B).

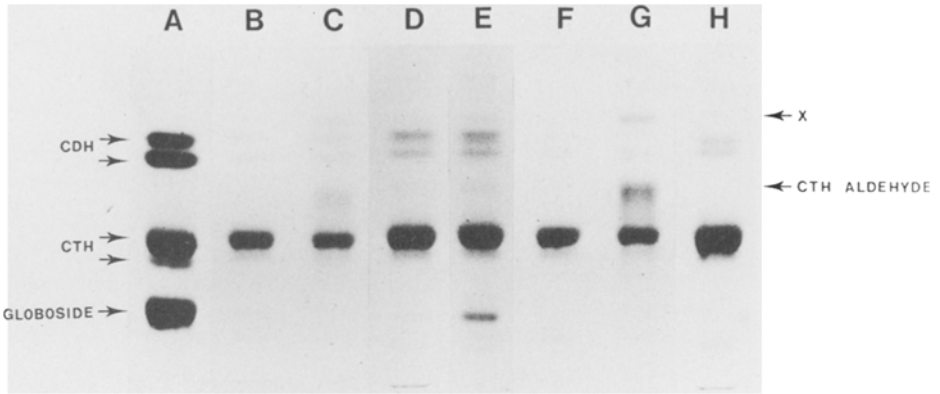

Figure 2. Thin-layer chromatography of neutral glycolipid fractions from oxidized and deuterium-labeled P^k red cells.
A, Standard glycolipid mixture; B, untreated P_2^k cells; C, cells from the same individual, oxidized with 10 units galactose oxidase/ml packed cells/2 h; D, reduction of the oxidized cells with NaB^2H_4; E, reduction of neuraminidase/galactose oxidase treated P_2^k cells; F, untreated P_1^k cells; G, cells from the same individual, oxidized with 30 units galactose oxidase/ml packed cells/2 h; H, reduction of the oxidized cells with NaB^2H_4. The bands were visualized with the aniline-diphenylamine phosphate reagent. - The unidentified band (x) may represent oxidized CTH derivative.

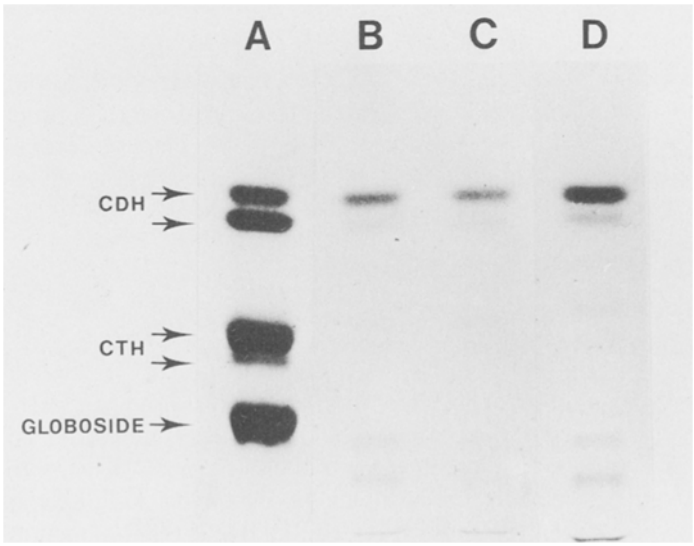

Figure 3. Thin-layer chromatography of neutral glycolipid fractions from oxidized and deuterium-labeled p cells.
A, standard glycolipid mixture; B, untreated p cells; C, oxidized p cells; D, reduction of the oxidized cells with NaB^2H_4.

Table 1. Exposure of major glycolipids in P^k and p erythrocyte membranes as determined by galactose oxidase/NaB^2H_4 labeling. Each red cell sample is from one individual.

Source of red cells	Glycolipid studied	Units galactose oxidase/ ml cells/2 h	Glycolipid content (nmol/μmol lipid P)	Percentage exposed to galactose oxidase
P_1^k	CTH	30	30.5	57
	CDH		2.9	6
P_2^k	CTH	10	24.2	24
	CDH		5.3	2
P_2^k (+ neuraminidase)	CTH	10	21.3	48
	CDH		5.4	0
p	CDH	10	17.4	1

Labeling of Erythrocytes with NaB^2H_4

In our previous studies labeling of several major red-cell glycolipids was done by using 10 units of galactose oxidase/ml cells/2 h [9]. Both p and P^k cells were also oxidized with the same amount of galactose oxidase. In addition, P^k cells were oxidized by adding 30 units of the enzyme/ml cells every two hours. In all experiments, the total incubation time was eight hours. Thereafter the cells were washed with PBS (0.15 M NaCl - 0.01 M sodium phosphate, pH 7.4) and reduced with 5 mg NaB^2H_4/ml cells. During incubation, samples of 0.5 ml were taken from the cell suspension, washed with ice-cold PBS containing 3 mg galactose/ml, then with PBS, and reduced with NaB^3H_4. The incorporation of label into CTH of P^k cells leveled off at the end of the incubation period, when 30 units galactose oxidase/ml cells/2 h were used (results not shown).

As oxidized glycolipids move faster on thin-layer chromatograms than native glycolipids, both glycolipid oxidation and reduction with NaB^2H_4 could be analyzed by TLC. Figs. 2 and 3 show glycolipid fractions from oxidized P^k and p cell samples, respectively. Glycolipid fractions from equal amount of unoxidized cells were used as controls. The oxidized cells were efficiently reduced with NaB^2H_4, as no extra bands could be seen in the glycolipid fractions from deuterium-labeled cells (Figs. 2 and 3).

Table 1 shows the results from deuterium-labeled cells. CTH in P^k cells was 2.5 times as effectively oxidized by galactose oxidase when the amount of the enzyme was increased three-fold. However, the red cell samples were from different individuals, which may have some influence on the results. CDH in P^k cells seems to be more effectively oxidized when a higher enzyme concentration was used. The shift from m/z 103 to m/z 104 is, however, so small that these lower values may not be as reliable as the higher values obtained for CTH.

Effect of Neuraminidase Treatment

Fig. 4 shows the effect of neuraminidase treatment on the labeling of p cell glycolipids. The only difference was observed in the region which corresponds to the globoside marker. As

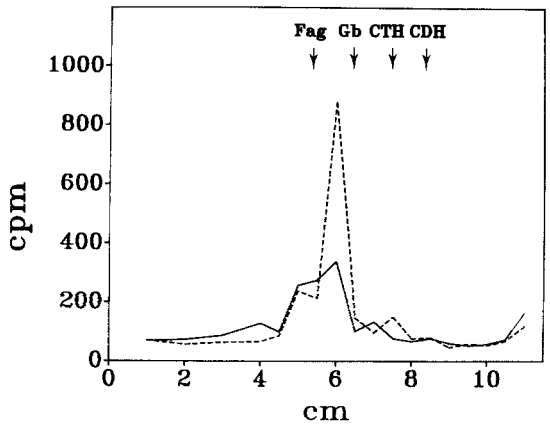

Figure 4. Effect of neuraminidase treatment on the labeling of p cell glycolipids by the galactose oxidase/NaB^3H_4 method.
The thin-layer chromatogram was fractionated from the starting line to the solvent front. Areas of 0.5 x 1.0 cm were scraped from the plate, and their radioactivity was counted. The arrows indicate the position of standard glycolipids. Dashed line: cells treated with neuraminidase and galactose oxidase; solid line: cells treated with galactose oxidase.

paragloboside moves in this area [8], it is obvious that the increased radioactivity is caused by desialylated sialoparagloboside.

After neuraminidase treatment, twice as much deuterium label was incorporated into CTH of P^k cells (Table 1). The chemical amount of the glycolipid was not increased.

Surface Labeling of Membrane Proteins

The glycoprotein pattern of galactose oxidase/NaB^3H_4 and neuraminidase/galactose oxidase/NaB^3H_4 labeled p, P^k and normal human red cells were studied by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. No differences were detected in the glycoproteins in p and P^k cells as compared to normal erythrocytes (results not shown).

Discussion

In our previous studies we have found that globosides of normal human and porcine red cells were efficiently exposed to galactose oxidase [7, 9], while CTH was poorly labeled with the galactose oxidase/NaB^2H_4 method, and no deuterium label was found in CDH from human cells. We then assumed that the length of the carbohydrate chain might limit the interaction of small glycolipids with external ligands. Galactose oxidase is also known to have a greater affinity for terminal carbohydrate residues in longer oligosaccharide chains than in short ones [18].

In this study I have found that CTH can be labeled with the galactose oxidase/NaB^2H_4 method as effectively as globoside if higher amounts of the enzyme are used (Table 1). This is in accordance with the results we had when small unilamellar glycolipid-phospholipid vesicles were labeled by the galactose oxidase/NaB^3H_4 method. Vesicles containing CTH

were oxidized four times more slowly than globoside-lecithin vesicles [19]. As a similar difference was found between the oxidation rates of globoside and CTH when tetrahydrofuran/buffer was used, it is obvious that the oligosaccharide chain of CTH is a relatively poor substrate for galactose oxidase. That may be the main reason why CTH is not labeled as well as globoside on the cell surface.

The linkage of the terminal carbohydrate residue may have some influence on the oxidation rates, and α-linked terminal galactosyl residues, as in CTH, may be poorer substrates to galactose oxidase than ß-linked residues [20]. Moreover, Forssman glycolipid, which has a terminal α-*N*-acetylgalactosamine residue, was inefficiently oxidized by galactose oxidase [9, 19].

In p cells, CDH was not oxidized by galactose oxidase even when very high concentrations of the enzyme was used. This indicates that the carbohydrate part of CDH is too close to the lipid bilayer for the enzyme to reach it [19]. Longer chain glycolipids were, however, labeled efficiently (Fig. 1B, Fig. 4). The radioactive band below CDH could represent CTH. There is evidence that this glycolipid occurs in p cells in trace amounts [8]. Moreover, Iizuka *et al.* have found that UDP-Gal:LacCer α(1-4)-galactosyltransferase, which produces CTH from CDH, exists in p lymphoblastoid cells [21].

The major component migrating just below the globoside marker consists mainly of paragloboside, but it may also contain some globoside. When a p red cell sample was studied by an overlay assay, Galα1-4Gal-recognizing *E. coli* bound to a glycolipid migrating in the same position as globoside did [22]. Thus different probes may detect compounds, which occur only in trace amounts on the cell surface.

Neuraminidase treatment of p cells did not affect the labeling of CDH by the galactose oxidase/NaB^3H_4 method. Sialoparagloboside was evidently hydrolyzed in these conditions as there was a three-fold increase in radioactivity in the paragloboside-globoside area. In contrast, the labeling of CTH in P^k cells was increased by 100% after neuraminidase treatment, when 10 units galactose oxidase/ml cells/2 h was used. Wiels and coworkers also found that CTH, which was cryptic in ARH77 cells, became exposed to a specific antibody by sialidase treatment [23]. However, when galactose oxidase is used for studying glycolipid exposure, the increased labeling after neuraminidase treatment may be caused by several factors. Glycolipids in desialylated membranes may have an altered organization and, as a result, they might be more susceptible to the enzyme. Alternatively, galactose oxidase could bind to sialic acid residues in native cells and thus be prevented from oxidizing short-chained glycolipids.

Until recently, glycolipids were thought to be located primarily at the outer aspect of the plasma membrane. However, Symington *et al.* found that in polymorphonuclear neutrophils 75% of CDH resides in intracellular compartments [24]. In erythrocytes, the plasma membrane is the only membrane, and no intracellular organelles are found. Thus red cells offer an ideal model when glycolipid organization and interactions are to be studied. By using the galactose oxidase/NaB^2H_4 method, I have shown that globoside and CTH are highly exposed on the red cell surface. As CDH is not a substrate for galactose oxidase when incorporated in the lipid bilayer [19], there is no direct evidence of its organization in the

red cell membrane. In a recent review, the globo-series glycolipids were proposed to provide a protective, non-informative carbohydrate coat at the cell surface. They should cover the membrane area not occupied by functional glycoproteins [25]. On this basis, one would assume that all major red-cell glycolipids are organized in a similar way, providing "blank space" between functional regions of the membrane.

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