External Labeling of Cell Surface Galactose and Galactosamine in Glycolipid and Glycoprotein of Human Erythrocytes*

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SUMMARY

Treatment of erythrocytes with galactose oxidase (EC 1.1.3.13) followed by reduction with tritiated sodium borohydride (NaB³H₄) at pH 7.4 allowed the labeling of galactosyl and N-acetylgalactosaminyl residues on external surfaces of cells with tritium (³H). Labeling patterns and specific activities of galactose and galactosamine were determined after separation with gel electrophoresis and thin layer chromatography. The labeling patterns of normal adult cells differed greatly from fetal cells, and were significantly altered when cell surfaces were modified by proteases and neuraminidase. The results of analysis indicated that (a) the carbohydrate moieties of two glycolipids (globoside and ceramide trihexoside) and at least three glycoproteins (molecular weight 9.5, 8.2, and 6.4 × 10⁴) were exposed to the external environment, but not ceramide dihexoside, ceramide mono- and hexoside, or other glycoproteins with higher molecular weights; (b) the specific activities of galactose and galactosamine residues in glycolipid and of galactose in glycoprotein increased after protease treatment, although total activity of galactose did not change; (c) labeling of glycoprotein was greatly enhanced by neuraminidase treatment, while that of galactose was enhanced to a lesser degree; (d) “the relative exposures” of glycoprotein and glycolipid differed greatly between normal and fetal erythrocyte surfaces. Glycoproteins of fetal cells had a very low label as compared to glycolipid.

A significant role of complex carbohydrates on cell surfaces in controlling cell division and intercellular association has been predicted based on the change of chemical and organizational structures of membrane-bound carbohydrates or related enzymes in association with “contact inhibition” (12-15), cell aggregation (16), mitotic cell cycle (17), and malignant transformation (18-20). Surface-exposed carbohydrates of cells are, therefore, of great cell sociological significance, and it has become increasingly important to elucidate the exposed chemical structures of cell surfaces. Labeling of cell surface tyrosyl residues has been developed using lactoperoxidase and radioactive iodine (21, 22), while cell surface amino groups have been labeled with ³⁵S-labeled formylmethionylsulfone methylphosphonate (23) or with ³⁴S-labeled sulfanilic acid diazonium salt (24); labeling of specific surface carbohydrates, however, has been awaiting development. Very recently, labeling of surface sialyl residues by periodate and tritiated sodium borohydride was described (25), whereby sialyl residues were converted to a 3-deoxy-5-acetamidoheptulosonic acid.

Although the majority of cellular glycosphingolipids are found in plasma membranes, direct evidence that the carbohydrate moiety of glycolipids is exposed to the external environment has not been provided. Also, nothing has been known about the relative exposures of glycoprotein and glycolipid or about possible change in exposure with change of surface function and with modification of cell surfaces by neuraminidase and proteases. In order to solve these problems, we have developed a method using galactose oxidase (26) and tritiated sodium borohydride (NaB³H₄), which allowed specific labeling of surface galactose and galactosamine residues in glycolipid and glycoprotein. Application of this method to analyze the organizational state of surface carbohydrates of human erythrocytes is reported in this paper.

MATERIALS AND METHODS

Cells and Enzymes

Human adult erythrocytes (A Rh⁺) were obtained from citrated blood by centrifugation and were washed with phosphate-buffered saline, pH 7.4. Fetal erythrocytes were obtained from an abortion case from the Division of Human Embryology, Department of Pediatrics, University of Washington.

Galactose oxidase of Dactylum dendroides (26) was obtained from Sigma Chemical Company, St. Louis, Missouri, with a described activity of 37 units per mg of protein. The enzyme
had no contamination from protease activity, which was determined using "Azo-albumin" (Sigma Chemical Company, St. Louis, Mo.) as substrate, nor any detectable neuraminidase activity, which was determined using submaxillary mucin and diisologanglioaside as substrate. Pseudo (Streptomyces griseus, 45 units per mg) and neuraminidase (Vibrio cholerae, 500 units per mg) were purchased from Calbiochem, La Jolla, California. Trinitiated sodium borohydride (100 mCi per mmole) was obtained from New England Nuclear Company, Boston, Massachusetts.

Cell ghosts (plasma membranes) of erythrocytes were prepared by lysing the cells in hypotonic PBS, pH 7.4 (isotonic PBS diluted 1:9 with water), followed by centrifugation at 10,000 rpm for 20 min. The membranes were washed three times, after which only traces of hemoglobin remained with the ghosts.

Treatment of Cells with Trypsin—Cell suspensions in PBS, pH 7.4, were mixed with twice the volume of 0.25% trypsin solution ("Gibco" Biological Company, Berkeley, California) and incubated at 37° for 30 min with continuous shaking, transferred to an ice-water bath, and washed by centrifugation with a large volume of ice-cold PBS, pH 7.0, three times.

Treatment of Cells with Pronase—To the cells was added twice the volume of 0.5 mg per ml of pronase in PBS, pH 7.4, and the cells were incubated and washed as for trypsin treatment.

Treatment of Cells with Neuraminidase—The cells were suspended in twice the volume of 0.1 M sodium phosphate buffer, pH 6.0, and 0.05 ml of the neuraminidase solution per ml of packed cells was added. The cells were incubated and washed as for trypsin treatment.

Labeling Procedure

Erythrocytes were washed with PBS, pH 7.0, and 1 to 5 ml of packed cells were mixed with 10 to 100 mg of protein of galactose oxidase dissolved in PBS, pH 7.0. The cells were incubated for varying periods of time (optimal time 3 hours) at 37° in a water bath with gentle shaking and were washed by centrifugation in PBS, pH 7.4. To the washed packed cells was added 0.05 ml of a freshly prepared tritiated sodium borohydride solution at room temperature, and the radioactivity was determined after addition of scintillation fluid.
Fig. 1. Light microscopic picture of an autoradiogram of labeled intact cells. The autoradiogram was prepared according to the method of Rieke et al. (33) by the courtesy of Mrs. Ruth Tyler, Department of Biological Structure, University of Washington, School of Medicine. Photography by Mr. Randy Jenkins.

Table I

| Sample                  | Galactose | Total cpm X 10^4 | Protein | Lipid |
|-------------------------|-----------|------------------|---------|-------|
| Intact cells            | 100       | 24.6             | 62.3    | 37.7  |
| Intact cells            | 0         | 3.5              | 35.1    | 64.9  |
| Neuraminidase-treated   | 100       | 141.8            | 80.0    | 14.0  |
| Trypsin-treated         | 100       | 27.1             | 55.4    | 44.6  |
| Pronase-treated         | 100       | 34.5             | 51.0    | 49.0  |
| Ghost                   | 100       | 101.3            | 54.2    | 45.8  |
| Ghost                   | 0         | 32.7             | 39.8    | 60.2  |

Total label in 1 ml of packed normal and fetal cells

| Sample                  | Total cpm | Protein | Lipid |
|-------------------------|-----------|---------|-------|
| Intact normal cells     | 50        | 30.1    | 57.5  | 42.5  |
| Intact normal cells     | 0         | 4.5 n.d. | n.d.  | n.d.  |
| Intact fetal cells      | 50        | 14.8    | 32.3  | 67.7  |
| Intact fetal cells      | 0         | 9.8     | 33.3  | 66.6  |

a Determined by sodium dodecyl sulfate gel electrophoresis.
b Nonspecific label by tritiated sodium borohydride alone (see Footnote 3 for text).
c n.d., not determined.

Labeling of isolated cell ghosts resulted in a much greater label than in intact cells, especially in the lipid fraction (Table I and Fig. 4b).

Nonspecific Labeling

Nonspecific labeling, i.e., a label occurring without galactose oxidase but only with tritiated sodium borohydride, was observed (Table I); the major nonspecific label, although weak, occurred in an unidentified lipid fraction, as demonstrated on gel electrophoresis (see Fig. 4c) and by lipid extraction, but some very weak nonspecific labels were also found in protein. Nonspecific labeling was not remarkable when intact erythrocytes were labeled, but was greatly enhanced when cell ghosts were labeled (Table I, “Discussion”).

Specific Labeled Activities of Galactose and Galactosamine in Glycoproteins and Glycolipids

The specific activities (counts per min per nmoles) of labeled galactose and N-acetylgalactosamine in glycoproteins, globoside, and CTH are shown in Table II, and the dependency of those activities on the amount of galactose residue is shown in Fig. 3. The specific activity was higher in the galactosyl residue for proteins and in the N-acetylgalactosaminyl residue for lipids (Fig. 3). A remarkable increase of specific activity of glycoprotein galactose was observed after treatment with proteases, although total labeling increased only slightly. The specific activities of both galactosamine and galactose in globoside increased after cells were treated with Pronase.

Labeling for both galactose and N-acetylgalactosamine in glycoprotein and for N-acetylgalactosamine in globoside increased (10 times) when the amounts of galactose oxidase added

5 Nonspecific label without galactose oxidase in lipid fractions could be aliphatic aldehydes (plasmals), ketosphingosine, and pyridinium compounds plausibly bound to lipids. They are, however, not identified. Nonspecific label for protein is unknown, but any reducible structure as have been found in collagenous protein in the form of Schiff’s base (34) can be considered. Nothing is as yet identified.
The trimethylsilyl sugar derivatives were quantified by gas liquid chromatography and the radioactivity of the peaks determined as described under “Materials and Methods.”

| Glycoprotein | Globoside | CTH |
|--------------|-----------|-----|
| Gal | Gal NAc | Gal | Gal NAc | Gal |
| Intact red cells 100 μg of galactose oxidase | 73.7 | 307 | 15.1 | 805 | 60 |
| Intact red cells 0 μg of galactose oxidase | 1.0 | 9.6 | 0 | 10.5 | 0 |
| Trypsin-treated 100 μg of galactose oxidase | 251 | 155 | 10.4 | 879 | 52 |
| Pronase-treated 100 μg of galactose oxidase | 183 | 344 | 21.4 | 1000 | 64 |

Fig. 3. Specific activities in galactose (a) and N-acetylgalactosamine (b) in glycoprotein (○—○) and globoside (●—●) obtained with varying galactose oxidase concentrations. Note that: (a) the specific activity of galactosamine was greater in glycolipid than glycoprotein, while that of galactose was greater in glycoprotein than in globoside (globoside); (b) the specific activity of galactosamine in globoside was greatly enhanced on increase of galactose oxidase added, while that of galactose in globoside did not increase much.

were increased, while labeling for the galactosyl residue of globoside increased to a lesser degree when the amount of galactose oxidase increased (Fig 3). No label was found in glucose.

Comparison of Specific Activities of Individual Glycolipids

Distribution of labels in various glycolipids is shown in Table III. The major label was found in globoside, followed by ceramide trihexoside, while no label was present in lactosylceramide. It is noteworthy that the ratio labels for ceramides with a long carbohydrate chain were also changed by treatment with neuraminidase and proteases. Some labels for ceramides with a long carbohydrate chain were also found (see footnote to Table III).

The labeling ratio between globoside, CTH, and CDH differed greatly from the actual chemical quantities of these glycolipids present in membranes (see Table III). The “degree of exposure,” as expressed by counts per min per μm amount, was quite high in globoside as compared to other glycolipids.

Relative Radioactivities of Labeled Glycoproteins and Glycolipids

The labeled glycoproteins and glycolipids were separated by sodium dodecyl sulfate gel electrophoresis (7), and the relative radioactivities of the glycoprotein peaks and of the lipid peak could be determined. This pattern of activities depends on surface properties of erythrocytes and varies according to physiological state (fetal or adult) and to modification of cell surfaces by enzyme treatment.

Intact human erythrocytes were labeled, membranes were prepared, and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Under these conditions three glycoprotein Peaks a, b, and c, corresponding, respectively, to apparent molecular weights of 9.5, 8.2, and 6.4 x 10^4, and a sharp lipid peak (L) were observed (Fig. 4a). If the cell membranes were first prepared and then labeled, the sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern was significantly different. There was a dominant Peak L and a labeled glycoprotein with high apparent molecular weight (1.5 x 10^6), in addition to Peaks a, b, and a very weak c; thus, a highly active extra peak was labeled when membranes were first prepared then labeled (Fig. 4b and "Discussion").

Labeling Pattern of Cells Whose Surfaces Were Modified by Enzymes

About 60% of the total label in intact cells was found in protein and 40% was in lipid. The total label was greatly increased by neuraminidase treatment but only slightly by protease treatment (Table I). After protease treatment, the label in glycolipid increased more than in glycoprotein (Table I). With trypsin treatment glycoproteins a and b lost some activity, but activity for the lipid peak (L) intensified somewhat (see Fig. 4c). After neuraminidase treatment an increased label was found mainly in glycoprotein with a smaller increase in glycolipid (compare Fig. 4, a and d); thus, about 85% of the total label was found in glycoprotein after treatment with neuraminidase. Neuraminidase-treated erythrocytes showed remarkable enhancement in a particular glycoprotein peak with an apparent molecular weight 8.5 x 10^4, which is probably derived from glycoprotein a, and the appearance of a new peak with an apparent molecular weight 8.5 x 10^4.
weight \(3.5 \times 10^4\). Also, the label in the lipid peak (L) was enhanced (see Fig. 4d).

Comparison of Labeling Patterns of Normal Adult Erythrocytes and of Fetal Erythrocytes

Glycolipid of fetal erythrocytes obtained from 3 months gestation period was labeled less efficiently than adult erythrocytes, although the label proportions of individual glycolipids (glloboside-CTH-CDH) is not greatly different in adult and fetal erythrocytes (Table I). A great deal of enhanced agglutinability by antigloboside antisera was demonstrated in fetal cells, however, in agreement with the previous results (9). The most remarkable label difference between normal and fetal erythrocytes was demonstrated in the ratio of activities between protein-bound carbohydrates (glycoprotein) and lipid-bound carbohydrates (glycolipid) (Table I and Fig. 4f). Fetal erythrocytes showed a very weak activity in the area of glycoproteins c and d. No activity corresponding to Peaks a and b was demonstrated, whereas the activity for lipid peak (L) was remarkably demonstrated.

**DISCUSSION**

In this study galactose oxidase from *Dactylium dendroides* has been used to obtain specific labeling of cell surface glycoproteins and glycolipids. Galactose oxidase shows a strict specificity for galactose and N-acetylgalactosamine, whose primary hydroxyl groups are oxidized to aldehyde groups (26). Oxidation by galactose oxidase followed by reduction with tritiated sodium borohydride has been used previously to label galactosyl and galactosaminyl residues in glycolipids (37-40) and glycoproteins (41, 42). This reaction has now been successfully applied to external labeling of galactosyl and galactosaminyl residues at the cell periphery. Lactoperoxidase with a molecular weight of 78,000 (22) is known to react exclusively at the red cell surface. Galactose oxidase has a molecular weight of 78,000 (26), which closely approximates that of lactoperoxidase. Therefore, penetration of this enzyme through the cell membrane should not occur. Surface labeling of erythrocyte was indicated by the autoradiograph of cells as seen in Fig. 1.

Cell ghosts, isolated then labeled, demonstrated a highly labeled protein with apparent molecular weight of 150,000, whereas such a protein is not seen in membranes of labeled intact cells (cells labeled then ghosts isolated) (see Fig. 4b). This protein, therefore, could be located on the inner surface of the plasma membrane. The results indicate that galactose oxidase cannot penetrate the cell membrane to label this protein in the intact cell.

Non-specific labeling (see Footnote 3) was not significant in intact cells, but increased to a great extent when ghosts were labeled. Enhanced lipid labeling in ghosts as seen in Fig. 4b...
is largely due to increased nonspecific labeling, i.e. the naturally occurring reducible lipid component increased when cells were lysed. The reducible materials in intact membranes can be made more accessible to borohydride by lysis of membrane, either by change of membrane conformation or by exposure of the inner surface. Although naturally occurring materials reducible by borohydride have not been identified in membrane,

This study gives direct evidence that sugars of both glycolipids and glycoproteins are exposed on the membrane surface. Two proteins have been known to be exposed on the outer surface of human erythrocytes (22, 23, 41). One of these is the major membrane glycoprotein, which carries all demonstrable sialic acid (22-24), AB and MN blood group antigens, and receptors for influenza virus and phytoagglutinins (43). There is evidence that this protein traverses the cell membrane to the cytoplasmic surface (44). The region of protein which is exposed to the outside carries the carbohydrate portion, while the COOH-terminal end is enriched in hydrophobic amino acids and possibly serves to attach the protein to the membrane (43). This idea that membrane proteins have a hydrophobic inner end and a protease-sensitive hydrophilic outer segment is also true for cytochrome b, arranged in the microsomal membrane (45) and Semiliki Forest virus membrane proteins (46), and it possibly has general significance. The main labeled glycoprotein peak (a) has an apparent molecular weight of 95,000 and no doubt corresponds to the multispecific glycoprotein (22, 23, 43). The major oligosaccharide portion of this protein has been proposed to have the structure NeuNAc α (2 → 3) Gal β (1 → 3) (NeuNAc α (2 → 6) GalNAc (47). This structure could explain the more efficient labeling after neuraminidase treatment.

We have also obtained direct evidence that some glycosphingolipids of membranes are exposed directly to the external environment. Labeling of human erythrocytes was particularly remarkable in globoside, the major glycolipid of human erythrocytes and (b) intact human erythrocytes are only slightly reactive to anti-globoside but also to immunoglobulin (see Fig. 5). Galactose oxidase can penetrate between such bushes of glycoprotein; glycolipids are located among these bushes. Galactose oxidase can penetrate between such bushes and label glycolipids, but other larger molecules like immunoglobulin cannot react with the glycolipids in normal erythrocytes as shown in a. In contrast, the model in b showed a possibility that fewer glycolipids and proteins are present on fetal erythrocytes and therefore, not only galactose oxidase but also immunoglobulin can react with globoside on fetal cell surfaces. This model is based on a provision that glycoprotein of fetal erythrocytes are qualitatively similar to that of adult erythrocytes.

in view of a recent observation by Nicolson (11) that trypsinization can cause clustering of some phytoagglutinin reactive sites on cell surfaces.

The relevant explanation for understanding labeling pattern of globoside (and other glycolipids) and glycoprotein is that globoside may be seated directly on the lipid bilayer among "bushes" of protein and glycoprotein (see Fig. 5), and that globoside is available only to galactose oxidase but not to larger macro molecules like immunoglobulin

4 B. Siddiqui and S. Hakomori, unpublished observations.

5 K. Watanabe and S. Hakomori, unpublished observations.
erythrocytes are not reactive to anti-lactosylceramide even after trypsin digestion (9).

Comparative labeling of glycoprotein and glycolipid has been observed by gel electrophoresis of labeled membranes. Labeling of glycoprotein varied greatly in contrast to a rather constant labeling of glycolipid. Absence of Peaks a or b glycoprotein in fetal erythrocytes is of great interest, which indicates either of the following possibilities: (a) fetal glycoproteins do not have any galactosyl or galactosaminyl residues so that they are not labeled; (b) galactosyl or galactosaminyl residues of fetal glycoprotein could be highly substituted by other sugar residues such as sialyl or fucosyl residues so that they are not labeled; (c) fetal erythrocytes have smaller number of glycoproteins; glycoproteins a or b are virtually absent. A tentative model based on the third possibility that a higher agglutinability of fetal erythrocytes to antigloboside can be ascribed to less steric hindrance due to the absence of some surface glycoprotein is shown by Fig. 5. Further extensive study is needed to correlate the surface structure revealed by external labeling to the immunological reactivity of cells surfaces. A study in progress (1) showed that this surface labeling procedure for sugars was based on the third possibility that a higher agglutinability of fetal erythrocytes is of great interest, which indicates either of the following possibilities: (a) fetal glycoproteins do not have any galactosyl or galactosaminyl residues so that they are not labeled; (b) galactosyl or galactosaminyl residues of fetal glycoprotein could be highly substituted by other sugar residues such as sialyl or fucosyl residues so that they are not labeled; (c) fetal erythrocytes have smaller number of glycoproteins; glycoproteins a or b are virtually absent. A tentative model based on the third possibility that a higher agglutinability of fetal erythrocytes to antigloboside can be ascribed to less steric hindrance due to the absence of some surface glycoprotein is shown by Fig. 5. Further extensive study is needed to correlate the surface structure revealed by external labeling to the immunological reactivity of cells surfaces. A study in progress (1) showed that this surface labeling procedure for sugars was found to be extremely useful to distinguish the surface properties of various normal and transformed cells as well.

Note Added in Proof—Since this paper was processed, at the stage of proofing, we noticed that T. L. Steck used galactose oxidase for surface labeling of erythrocytes ((1972) in Membrane Research, edited by C. F. Fox, p. 71 Academic Press, New York).

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