Occurrence and distribution of glycoprotein H substances in blood type O human erythrocyte membrane were studied using an α-N-acetylgalactosaminyltransferase (A enzyme) purified from milk of blood type A women.

Erythrocytes of blood group O, nonsecretors and secretors alike, contain H substances that serve as a substrate for this transferase, and the H determinants seem to occur in all three erythrocyte membrane glycoproteins (periodic acid-Schiff (PAS)-1, PAS-2, and PAS-3). Structural studies of the A glycoproteins obtained by the transferase action, suggested that all erythrocyte H glycoproteins contain only type 2 antigenic determinant.

Furthermore, structural analysis of the carbohydrate moiety of the blood group active glycoprotein was performed using the endo-β-galactosidase as described in the preceding paper (15).

EXPERIMENTAL PROCEDURES

Oligosaccharides—2'-Fucosyllactose, lacto-N-tetraose, lacto-N-fucopentaose I, lacto-N-difucohexaose I, and N-3 were isolated from human milk by the methods previously reported (16, 17), 2'-Fucosylgalactose was prepared from 2'-fucosyllactose by alkaline degradation as described by Kuhn et al. (18). GalNActo1→3(Fucα1→2)Gal was isolated from ovarian cyst mucin with blood group A activity by endo-β-galactosidase digestion as described in the preceding paper (15).

Chromatography—UDP N-acetylgalactosamine labeled with 14C at the acetyl group (5 mCi/mmol) and NaBH₄ (162 mCi/mmol) were obtained from the International Chemical and Nuclear Corp. and New England Nuclear, respectively.

Enzymes—"A-enzyme" was purified from milk of blood type A women as reported in the previous paper (14), by adopting the affinity chromatographic method reported by Whitehead et al. (19) for the purification of serum A-enzyme. Purified α-N-acetylgalactosaminidase from beef liver was a kind gift of Dr. B. Weissman, University of Illinois College of Medicine. β-N-Acetylgalactosidase from Bacillus fulminans, which hydrolyzes only Fucα1→2Gal linkage was prepared according to the procedure of Kochibe (20). The Bacillus strain was kindly provided by Dr. S. Yamamoto, National Institute of Police Science. β-N-Acetylhexosaminidase from jack bean was prepared by M. Ogata-Arakawa of our laboratory according to the method of Li and Li (21). Endo-β-galactosidase from Diplococcus pneumoniae was purified as described in the previous paper (15). Galactose oxidase and horseradish peroxidase were obtained from Worthington Biochemical Co. and Sigma Chemical Co., respectively.

Preparation of Glycoprotein—Erythrocyte ghosts were prepared from bloods of blood type O with different Lewis activities by the method of Dodec et al. (22). The ghosts were kindly donated by Dr. F. W. Dodge.
Kochibe, Gumma University School of Medicine and also obtained from Japan Red Cross Blood Center, Hyogo Branch. Glycoproteins were isolated from the ghost by the method of Marchesi and Andrews (6).

A-Enzyme Treatment of Erythrocyte Membrane Glycoprotein—Reaction mixture (50 μl) containing 0.5 μg of erythrocyte membrane glycoprotein, 0.4 mM UDP-N-[14C]acetylgalactosamine, 24 mM MnCl₂, 20 mM Tris-HCl buffer (pH 7.5), and 300 units of the A-enzyme was incubated for 40 hours at 37°C with a small amount of tolenue. The reaction was stopped by heating in a boiling water bath for 2 min, and then the reaction product was then applied to a column (1 × 95 cm) of Sephadex G-50, previously equilibrated with 0.05 M ammonium acetate, pH 7.0. Elution was carried out with the same buffer, and the eluate was collected in 2-ml fractions. An aliquot of each fraction was counted in 1 ml of Bray’s solution (25) using Packard liquid scintillation spectrometer model 3320.

Pretreatment of Glycoprotein with α-L-Fucosidase—The glycoprotein (0.5 mg) from erythrocyte with blood type O and Le(a-b+) specificities was digested with a sufficient amount of α-L-fucosidase (35 μg) from B. fulminans in 20 μl of 0.05 M Tris-HCl buffer, pH 7.5, at 37°C for 15 hours with a small amount of tolenue. After the fucosidase was inactivated by heating the reaction mixture at 80°C for 5 min, the solution was subjected to the reaction with A-enzyme under the same conditions as described above. A control experiment, in which the glycoprotein was incubated without α-L-fucosidase, was simultaneously performed.

N-Acetylhexosaminidases Treatment of N-[14C]Acetylgalactosamine-incorporated Glycoprotein—The N-[14C]acetylgalactosamine-incorporated glycoprotein (70 μg, 2 × 10⁶ cpm) incubated with 6 μg of α-N-acetylgalactosaminidase in 30 μl of 0.1 M citrate buffer, pH 4.5, at 37°C for 12 hours with a small amount of tolenue. Treatment with α-N-acetylhexosaminidase was similarly carried out at the optimum pH for α-N-acetylhexosaminidase, pH 4.0 (21).

Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Weber and O’bannon (24). The acrylamide concentration was 7.5%, and tubes of 4-mm inner diameter and 10-cm length were used. Electrophoresis was performed at 8 mA per tube for 51/2 hours. The gel was cut into 1.5-mm slices. Each slice was heated at 70°C for 2 hours with 1 ml of 1% aqueous sodium dodecyl sulfate solution in a scintillation vial, and was then shaken at room temperature for 1 day. After addition of 7 ml of Bray’s solution, the radioactivity was measured by Packard Tri-Carb liquid scintillation spectrometer. Glycoproteins on the gel were stained by periodic acid-Schiff reagent as described by Fairbanks et al. (25). The stained gel was scanned at 560 nm on Chromoscan (Joyce Loeble Co.).

Paper electrophoresis were carried out in pyridine/acetic acid buffer, pH 5.4 (26), and in 0.06 M borate buffer, pH 9.0 (27).

Paper Chromatography—Descending paper chromatography was performed using the following solvent systems: Solvent I, ethyl acetate/pyridine/water (12/5/4); Solvent II, ethyl acetate/pyridine/acetic acid/water (5/5/1/3); and Solvent III, 1-butanol/ethanol/water pH 5.4 (26), and in 0.06 M borate buffer, pH 9.5 (27).

Characterization of Reaction Product

Distribution of Acceptor—Erythrocyte membrane glycoproteins can be separated into three glycoproteins, PAS-1, PAS-2, and 3, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25). When the N-[14C]acetylgalactosamine-labeled membrane glycoprotein was subjected to sodium dodecyl sulfate-gel electrophoresis, radioactivities were detected in all three glycoproteins (Fig. 4). Comparing the intensities of PAS stain and radioactivities of each peak, PAS-2 seemed to be the best acceptor.

Alkaline Degradation—Alkaline degradation of N-[14C]acetylgalactosamine-incorporated glycoprotein was carried out in the presence of sodium borohydride (29), and the degradation product was applied to a column of Sephadex G-50. The elution profile is shown in Fig. 5. Oligosaccharides containing N-[14C]acetylgalactosamine were almost completely released from the glycoprotein, suggesting that the

RESULTS

Occurrence of A-Enzyme Acceptors in Erythrocyte Membrane Glycoprotein

Incorporation of N-[14C]Acetylgalactosamine into Erythrocyte Membrane Glycoprotein—Membrane glycoprotein isolated from erythrocyte with blood type O, Le(a-b) was incubated with A-enzyme and UDP-N-[14C]acetylgalactosamine as described under “Experimental Procedures.” When the reaction mixture was subjected to gel filtration on a Sephadex G-50 column, three radioactive peaks were obtained (Fig. 1). The major peak which was eluted from tubes 33 to 44 contained radioactive UDP-N-acetylgalactosamine, N-acetylgalactosamine-1-phosphate, and N-acetylgalactosamine. The small two peaks which were eluted from tubes 13 to 21 were not detected with control experiment in which membrane glycoprotein was omitted from the incubation mixture. The

eluting positions of these two peaks were the same as membrane glycoprotein. These results show that the membrane glycoprotein has the sugar chains which work as substrates of A-enzyme, namely H determinants.

Optimal Conditions for Incorporation—In order to determine the optimal conditions for the reaction, the effects of incubation time and molar concentrations of radioactive sugar nucleotide on the amount of radioactivity incorporated were examined.

The maximal incorporation was obtained at 40 hours (Fig. 2). When the reactions were performed for 40 hours with increasing concentrations of UDP-N-[14C]acetylgalactosamine, the amount of radioactivity incorporated into membrane glycoprotein was increased, and reached maximum value at 0.4 mM (Fig. 3). Even though the membrane glycoprotein was subjected to repeated enzyme reaction, no more incorporation was observed. This evidence assured that the transfer of N-[14C]acetylgalactosamine occurred to all the accessible acceptors. Calculated on the basis of specific activity of UDP-N-[14C]acetylgalactosamine, it was concluded that 3.4 nmol of N-acetylgalactosamine were incorporated into 1 mg of glycoprotein.

Characterization of Reaction Product

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Alkaline Degradation—Alkaline degradation of N-[14C]acetylgalactosamine-incorporated glycoprotein was carried out in the presence of sodium borohydride (29), and the degradation product was applied to a column of Sephadex G-50. The elution profile is shown in Fig. 5. Oligosaccharides containing N-[14C]acetylgalactosamine were almost completely released from the glycoprotein, suggesting that the
linkages between the H active sugar chains and the protein were alkaline labile O-glycosidic types. The released oligosaccharides were distributed in a wide range in size. They were divided into three fractions (Fractions I, II, and III) as indicated in Fig. 5. Each fraction was then subjected to paper chromatography. By comparing with the several known oligosaccharides from human milk, major components of Fractions I, II, and III were roughly estimated to be over octasaccharide, hexasaccharide, and trisaccharide, respectively (Fig. 6). Probably, the trisaccharide is a product formed by "peeling reaction," and the largest oligosaccharide will be an intact form.

Effect of Fucosidase Pretreatment on N-[^14]CAcetylgalactosamine Incorporation—As already described, high substrate specificity of A-enzyme assured that the incorporation of N-[^14]Cacetylgalactosamine occurred at the H antigenic determinant of membrane glycoprotein. For further confirmation that actually all radioactivity incorporation occurred only at the Fuc1→2Gal grouping, the membrane glycoprotein was treated with α-L-fucosidase purified from B. fulminans to remove all fucosyl residues which occurred as Fuc1→2Gal structure. This pretreatment completely extinguished the acceptor activity of the membrane glycoprotein to A-enzyme.

Linkage of Incorporated N-Acetylgalactosamine—The radioactivity incorporated into the erythrocyte membrane glycoprotein was completely released as N-acetylgalactosamine by α-N-acetylgalactosaminidase digestion. No radioactive sugar was released by jack bean β-N-acetylgalactosaminidase treatment.

These results showed that all the radioactivity incorporated into the membrane glycoprotein occurred as nonreducing terminal α-N-acetylgalactosamine residues.

Partial Structure of Carbohydrate Moiety Including N-[^14]CAcetylgalactosamine—An endo-β-galactosidase purified
from the culture supernatant of *D. pneumoniae* releases \( \text{GalNAc}^\text{ul} + 3 (\text{Fuc}^\text{ul} + 2) \text{Gal} \) and \( \text{GalLul} + 3 (\text{Fuc}^\text{ul} - 2) \text{Gal} \) from blood type A and B antigenic determinants with type 2 chain (15). This enzyme was used for the structural study of the carbohydrate moieties of \( \text{N}^-\text{\[^{14}C\]acetylgalactosamine-labeled erythrocyte membrane glycoprotein.} \)

The \( \text{N}^-\text{\[^{14}C\]acetylgalactosamine labeled glycoprotein} \) (5,000 cpm, 0.16 mg) was incubated with endo-\( \beta \)-galactosidase (0.2 milliunits) in 50 \( \mu l \) of 0.15 M citrate/phosphate buffer, pH 6.0 at 37° for 24 hours with a small amount of toluene. The reaction product was analyzed by paper chromatography using Solvent I. As shown in Fig. 7, all the radioactivity was released as a single trisaccharide with \( R_f \) value of 1.19. This product has the same paper chromatographic mobility as the authentic \( \text{GalNAc}^\text{ul} - 3 (\text{Fuc}^\text{al} - 2) \text{Gal} \) (15), with Solvents I, II and III.

Identity of the radioactive trisaccharide as \( \text{GalNAc}^\text{al} - 3 (\text{Fuc}^\text{al} - 2) \text{Gal} \) was further confirmed by the same methods used in the previous paper (15).

**Effect of Secretor Status**—In order to elucidate the relationship of the occurrence of blood group glycoproteins in erythrocyte membrane to the secretor status of the blood donor, membrane glycoproteins isolated from several blood type O erythrocytes with different Lewis activities were studied for their acceptor activities to A-enzyme.

As summarized in Table I, membrane glycoproteins obtained from Le(a+b-), Le(a-b+), and Le(a-b-) erythrocytes with different Lewis activities were studied for their acceptor activities to A-enzyme.

As summarized in Table I, membrane glycoproteins obtained from Le(a+b-), Le(a-b+), and Le(a-b-) erythrocytes with different Lewis activities were studied for their acceptor activities to A-enzyme. These results indicate that blood group ABU glycoproteins are present in erythrocyte membrane irrespective of the secretor status of the blood donor.

**Discussion**

It has been suggested by many investigators that blood group ABO active glycoproteins are present in erythrocyte membrane (3–10). However, these studies used serological inhibition tests for the detection of blood group activities, and gave rise to serious questions about the contamination of blood group glycolipids in the glycoprotein preparation used (30). The possibility of cross-reaction must also be taken into account, especially when the reactions are very weak.

The possibility of blood group glycolipids contamination can be eliminated in our study, since radioactivities of \( \text{N}^-\text{\[^{14}C\]acetylgalactosamine-labeled membrane glycoproteins} \) were completely released as oligosaccharides by mild alkaline/\( \text{NaBH}_4 \) treatment. The alkaline stability is one of the common properties of glycosphingolipids. Therefore, it can be concluded that blood group glycoproteins are positively present in erythrocyte membrane.

A few inconsistent results have been presented with respect to the distribution of blood group A, B, and H determinants among erythrocyte membrane glycoproteins. Marchesi et al. (6) reported that the glycophorin, which is equivalent to PAS-1, bore blood group ABH activity. Hamaguchi and Cleve (9) showed that ADII blood group activity was exclusively associated with GP-III (PAS-3). Fujita and Cleve (31) recently reported that some activities were also found in GP-II (PAS-2). Our results indicated that H determinants are distributed in all three glycoproteins obtained from blood type O erythrocyte, and PAS-2 seemed to have more determinants than other glycoproteins.

However, Furtmayer et al. (32) recently reported that

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**Figure 6** Paper chromatography of the alkaline degradation products. A, Fraction III as indicated in Fig. 5, for 20 hours with Solvent I; B, Fraction II, for 90 hours with Solvent II; C, Fraction I, for 90 hours with Solvent II. Standard sugars: 1, lactose; 2, \( 2'\)-fucosyllactose; 3, lacto-N-tetraose; 4, lacto-N-difucohexaose I, 5, N-3 (octasaccharide).

**Figure 7** Release of oligosaccharide containing \( \text{N}^-\text{\[^{14}C\]acetylgalactosamine} \) from \( \text{N}^-\text{\[^{14}C\]acetylgalactosamine-labeled glycoprotein} \) by endo-\( \beta \)-galactosidase from *Diplococcus pneumoniae*. The conditions of enzyme treatment were described in the text. The reaction product was spotted to Whatman 3MM filter paper, and chromatographed using Solvent I for 20 hours. ---, reaction product; ----, control without enzyme. Standard sugars: 1, \( \text{N-acetylgalactosamine} \); 2, galactose; 3, lactose; 4, \( 2'\)-fucosyllactose.

**Table I** Incorporation of \( \text{N}^-\text{\[^{14}C\]acetylgalactosamine into various glycoproteins} \)

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Types of bloods</th>
<th>Incorporated amounts (cpm/0.5 mg of glycoprotein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H, Lea+b-</td>
<td>12,100</td>
</tr>
<tr>
<td>2</td>
<td>H, Lea+b-</td>
<td>14,500</td>
</tr>
<tr>
<td>3</td>
<td>H, Lea+b-</td>
<td>15,000</td>
</tr>
<tr>
<td>4</td>
<td>H, Lea-b+</td>
<td>13,400</td>
</tr>
<tr>
<td>5</td>
<td>H, Lea-b-</td>
<td>15,500</td>
</tr>
<tr>
<td>6</td>
<td>H, Lea-b-</td>
<td>11,800</td>
</tr>
</tbody>
</table>

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aggregation and interconversion of erythrocyte membrane glycoproteins are caused by heating or chloroform/methanol extraction of the sample solution prior to electrophoresis. Although our sample of membrane glycoproteins were not subjected to such pretreatment, these evidences show that we have to be careful in discussing these points.

It is well known that the biosynthesis of blood group H antigenic determinants requires the combined action of H and Se genes in secretory tissues (33, 34). Therefore, individuals with sese genotype cannot form H determinants in their secretory glands. Since H determinants work as immediate precursors of A and R determinants, their secretions are devoid of A, B, and H antigenic mucins. This is the biochemical basis of nonsecretor phenomena. However, blood group ABH activities are detected on erythrocyte regardless of the secretor status (35). Gardas and Koscielak (8) reported that ABH glycolipids of erythrocyte are present in both secretors and nonsecretors, while A, B, and H activities in glycoprotein fraction of stroma are present only in erythrocytes of secretors. Results against their report were also presented by other workers (9, 10).

Our results conclusively showed that the blood group ABH active glycoproteins occur in erythrocyte membrane irrespective of the secretor status.

The endo-β-galactosidase from D. pneumoniae was successfully used for proving the occurrence of the actual blood group determinant in the carbohydrate moiety of erythrocyte membrane glycoprotein. As shown in Fig. 8, the structure of the product released by this enzyme was the same as expected from the substrate specificity of this endo-β-galactosidase described in the preceding paper (15), and is certainly a determinant of A specificity (36, 37). Consequently, the presence of blood group glycoprotein on human erythrocyte was proved on a structural basis. Moreover, the result obtained using endo-β-galactosidase gave us more valuable information that the carbohydrate moiety of blood group ABO glycoprotein on human erythrocyte is composed only of type 2 chains, because N-[14C]acetylgalactosamine incorporated into the membrane glycoprotein was completely liberated as GalNAcα1–3(Fucα1–2)Gal by the endo-β-galactosidase. As was demonstrated in the preceding paper (15), this enzyme releases the trisaccharide only from blood group determinants composed of type 2 chain and not from those composed of type 1 chain, and human milk A-enzyme can transfer cy-N-acetylgalactosaminyl residue to both type 1 and type 2 H determinants as well. Actually, when ovarian cyst mucin from blood group O individual, which is known to contain both type 1 and type 2 chain determinants (11), was subjected to the same treatment, only 70% of the incorporated N-[14C]acetylgalactosamine was released as the trisaccharide, and 30% remained bound to the glycoprotein (15).

As already reported by Hakomori et al. (1), and Stellner et al. (2), blood group A and H glycolipids in erythrocyte membrane are composed of only type 2 chains. Therefore, it can be concluded that all the blood group substances on erythrocyte are composed of only type 2 chains, unlike those in secretory tissues which are composed of both type 1 and type 2 chains (11, 13). It is widely accepted that the Lewis antigens on erythrocyte surface do not originate in this cell, but are adsorbed from serum. Since both Leα and Leβ antigens require type 1 chain as a part of their structures (38, 39), the inability of hematopoietic tissues to synthesize type 1 chain may be the reason for the absence of Lewis antigens in these tissues.

Alkaline degradation studies of the N-[14C]acetylgalactosamine-labeled membrane glycoprotein suggested that the carbohydrate chains with blood group ABH activities are larger than octasaccharide. Whether these sugar chains have branched structure or not remains for a future study.

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