Multiple components of blood group A and B antigens in human erythrocyte membranes and their difference between A1 and A2 status

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ABSTRACT Human type O erythrocyte membranes were converted to type A1 by purified human A1-enzyme, to type A2 by purified human A2-enzyme, and to type B by purified human B-enzyme in the presence of radioactive sugar donors (i.e., UDP-N-acetylgalactosamine for A-enzyme and UDP-galactose for B-enzyme, respectively). Type A2 erythrocyte membranes were also converted to type A1 by purified A1-enzyme. The labeled blood group antigens (A1, A2, and B) thus produced were analyzed by sodium dodecyl sulfate gel electrophoresis and by isoelectric focusing. It was demonstrated that blood group antigens on erythrocyte membranes are mostly (>90%) glycolipid, not glycoprotein. The blood group glycolipids were presumably conjugated with other membrane materials and formed three major components in type A1 and type B erythrocyte membranes. However, two of the three components were absent in type O erythrocyte membranes converted to type A2. These results indicate the heterogeneity of blood group components in erythrocyte membranes and the qualitative difference between A1 and A2 antigens on these membranes.

Since Yamakawa and lida first isolated glycolipids associated with blood group ABH activity from human erythrocyte membranes (1), structural studies of these substances have greatly advanced. It is known that blood group ABO specificity is determined by the terminal glycosyl residues attached to common carbohydrate chains of glycolipids in erythrocyte membranes (2-8). Several investigators, however, reported that glycoproteins also had blood group activity and that glycoproteins, not glycolipids, might be major blood group components (9-15). It is likely that several glycolipids and glycoproteins have blood group activity and these antigenic substances are conjugated with other constituents in erythrocyte membranes.

Blood type A is subdivided into A1 and A2, based on the agglutination reactions of the erythrocytes with anti-A1 (16, 17). The difference between the A1 and A2 antigens is largely quantitative—i.e., A1 cells have about 3 times more A sites than do A2 cells (18). But certain qualitative differences could exist between A1 and A2 because some A2 and A2B individuals form anti-A1 antibody (17). Structural differences between A1 and A2 components are not fully understood.

Type O erythrocytes can be converted to type A by an N-acetylgalactosaminyltransferase existing in blood group A subjects or to type B by a galactosyltransferase existing in blood group B subjects (19-21). Recently, blood group N-acetylgalactosaminyltransferase (A1- and A2-enzymes) and blood group galactosyltransferase (B-enzyme) were highly purified from human plasma in this laboratory (22, 23). The purified A- and B-enzymes were not contaminated with non-blood-group glycosyltransferases which also can transfer the sugars into erythrocyte membranes. Thus, it became possible to examine the A1, A2, and B antigenic components produced in type O membranes by the purified enzymes in the presence of radioactive sugar donors. In the present study, the labeled blood group components thus produced were analyzed by isoelectric focusing.

MATERIALS AND METHODS

Blood Group Transferases. Blood group N-acetylgalactosaminyltransferase (A1- and A2-enzymes) were purified about 10,000- to 100,000-fold from A1 plasma and A2 plasma by affinity chromatography with Sepharose 4B as described (22). Blood group galactosyltransferase (B-enzyme) was partially purified about 1000-fold by column chromatography with CM-Sephadex and gel filtration with Sephadex G-200 as described (23). The purified enzymes were reconstituted in a volume about 1% that of the original volume of plasma.

Erythrocyte Membranes. Ghosts were prepared from erythrocytes of phenotypes O, A2, and O8 by the method of Dodge et al. (24). The membranes were suspended in water to give a concentration of 4.8 X 10^7 ghosts per ml.

Conversion of Erythrocyte Membranes. Type O membranes were converted to type A by A1- and A2-enzymes, and to type B by B-enzyme. The reaction mixture (total, 1 ml) for O to A conversion contained 50 mM cacodylate buffer at pH 6.8, 25 mM MnCl2, 75 mM NaCl, 1 mM Na2HPO4, 0.2% bovine serum albumin, 25 μM UDP-N-acetylgalactosamine (3H-labeled; 4 μCi; 1 Ci = 3.7 X 10^10 becquerels), 500 μl of erythrocyte membrane (O, A2, or O8) suspension (i.e., 2.4 X 10^6 ghosts), and 200 μl of A1- or A2-enzyme.

The reaction mixture (total, 1 ml) for O to B conversion contained 25 mM imidazole buffer at pH 6.5, 25 mM MnCl2, 75 mM NaCl, 1 mM Na2HPO4, 0.2% bovine serum albumin, 25 μM UDP-galactose (3H-labeled; 8 μCi), 500 μl of type O erythrocyte membrane suspension, and 200 μl of B-enzyme solution.

After incubation of the reaction mixtures at 37°C for 14 hr, the erythrocyte membranes were collected and washed six times with water by centrifugation, for complete removal of unincorporated nucleotide sugars. Finally, the labeled membranes were suspended in 1 ml of water. The incubated membranes were typed by an imunoabsorption test. The converted erythrocyte membranes were mixed with anti-A1 lectin (Dolichas biflorus), anti-A antisemur, or anti-B antisemur in suitable dilutions, membranes were removed by centrifugation, and the agglutination activity remaining in the supernatants was determined by using A1, A2, or B erythrocytes.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. The suspension of freshly prepared labeled membranes was mixed with an equal volume of 0.13 M Tris-HCl, pH 6.8/4% sodium dodecyl sulfate/20% glycerol/10% 2-
zyme than into rum—i.e., and B-enzyme type lectin—i.e., anti-Al became enzyme membranes of incubated with Type A2 was reported from Amersham. Nuclear, Isoelectric (final, 1.6% at urea (final, 9.5%). Sodium sulfate/10% mercaptoethanol. The mixture was heated at 100°C for 2 min. The membranes were completely solubilized by this treatment.

Electrophoresis was carried out in 10% gels by the method of Laemmli (25). After electrophoresis, the gels were stained for protein with Coomassie brilliant blue and for lipid with Sudan Black B, as described (26). To determine the distribution of radioactivity, gels were sliced transversely into segments 2.56 mm thick. Sliced pieces were solubilized in counting vials (27) and assayed for radioactivity by a liquid scintillation counter.

Isoelectric Focusing. The suspension of freshly prepared labeled membranes was mixed with an equal volume of 4% sodium dodecyl sulfate/10% 2-mercaptoethanol and heated at 100°C for 2 min. After cooling, the sample was mixed with urea (final, 9.5 M), Nonidet P-40 (final, 8%) and Ampholine (final, 1.6% pH 5–7 Ampholine and 0.4% pH 3–10 Ampholine). Isoelectric focusing was performed by the method of O'Farrell (28). The gels were stained with Coomassie brilliant blue and radioactivity was measured as described above. Chemicals: UDP-N-Acetyl[1-3H]galactosamine was purchased from New England Nuclear, and UDP-[6-3H]galactose was purchased from Amersham. UDP-N-Acetylgalactosamine was synthesized as reported (22), and UDP-galactose and Pronase were purchased from Sigma. Standard anti-A antiserum, anti-B antiserum, and anti-A1 lectin were obtained from Ortho Diagnostics (Raritan, NJ).

Table 1. Incorporation of radioactive N-acetylgalactosamine and galactose into erythrocyte membranes by A- and B-enzymes from human plasma

<table>
<thead>
<tr>
<th>Erythrocyte membrane</th>
<th>Enzyme</th>
<th>Total radioactivity (cpm × 10^5)</th>
<th>Incorporation (cpm × 10^5)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>A1</td>
<td>36.17</td>
<td>3.92</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>35.77</td>
<td>3.81</td>
<td>10.7</td>
</tr>
<tr>
<td>A2</td>
<td>A1</td>
<td>35.50</td>
<td>3.05</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>35.78</td>
<td>2.95</td>
<td>8.2</td>
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<tr>
<td>O</td>
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<td>38.81</td>
<td>1.53</td>
<td>3.9</td>
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<tr>
<td></td>
<td>A1</td>
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<tr>
<td>O_b</td>
<td>A1</td>
<td>33.00</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>B</td>
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<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>O</td>
<td>B</td>
<td>78.70</td>
<td>12.10</td>
<td>15.4</td>
</tr>
</tbody>
</table>

Data are from membranes analyzed by isoelectric focusing shown in Figs. 4 and 5. In other conversion experiments, percentages of incorporation of radioactivity into the membranes were as follows: O→A1 (6 experiments), mean 11.5% (range, 10.6–13.6); A2→A1 conversion (4 experiments), mean 8.0% (range, 8.8–8.6); O→A2 conversion (5 experiments), mean 4.5% (range, 3.9–6.4); O→B conversion (3 experiments), mean 15.4% (range, 15.0–15.7).

RESULTS

Conversion of Type O Membranes to A\(_1\), A\(_2\), and B, and of Type A\(_2\) Membranes to A\(_1\). When type O membranes were incubated with UDP-N-acetylgalactosamine and A\(_1\)-enzyme, the membranes were converted to type A\(_1\)—i.e., the incubated O membranes were capable of adsorbing anti-A\(_1\) lectin. Type O membranes incubated with the nucleotide sugar and A\(_2\)-enzyme became capable of adsorbing anti-A antiserum but not anti-A\(_1\) lectin—i.e., the membranes were converted to type A\(_2\). Similarly, type O membranes incubated with UDP-galactose and B-enzyme became capable of adsorbing anti-B antiserum—i.e., the membranes were converted to type B.

Approximately 3 times more N-acetylgalactosamine was incorporated into type O erythrocyte membranes by A\(_1\)-enzyme than by A\(_2\)-enzyme at saturation. The amount of N-acetylgalactosamine incorporated into A\(_2\) erythrocyte membranes by A\(_1\)-enzyme was about two-thirds that incorporated into the same amount of O membranes by A\(_1\)-enzyme (Table 1). The amount of galactose incorporated into O erythrocyte membranes by B-enzyme was slightly greater than that of N-acetylgalactosamine incorporated by A\(_1\)-enzyme. When O\(_b\) erythrocyte membranes were used as sugar acceptors, virtually no incorporation of the sugar was observed. Because the only difference between type O and type O\(_b\) membranes is the absence of H in O\(_b\) membrane, the result implies that the highly purified blood group glycosyltransferase used was not contaminated with non-blood-group glycosyltransferases that can transfer the sugar into acceptor sites other than H-substance.

Analysis of Labeled Components by Sodium Dodecyl Sulfate Gel Electrophoresis. Polyacrylamide gel electrophoresis patterns of erythrocyte membranes are shown in Fig. 1. A typical distribution of labeled components in the gel is shown in Fig. 2. One major and at least three minor radioactive peaks were observed. The major peak, associated with 83% of total radioactivity, coincided with the position of the lipid fraction...
which was clearly stained by Sudan Black B (Fig. 1B). The minor peaks, associated with 17% of total incorporated radioactivity, were located in the protein region. When the labeled membranes (O membranes converted to A1, 1.5 mg) were digested by Pronase (30 μg) in 0.3 ml of 0.13 M Tris-HCl at pH 6.8 and 37°C for 2 hr and subjected to sodium dodecyl sulfate gel electrophoresis, all protein bands (i.e., Coomassie bands) disappeared. However, the major lipid band (Sudan Black band) remained unchanged and 89% of radioactivity coincided with this position.

**Analysis of Labeled Components by Isoelectric Focusing.** Typical isoelectric focusing patterns of the labeled erythrocyte membranes are shown in Fig. 3. Typical distribution profiles of radioactivity in the gel are shown in Fig. 4. The O membranes converted to type A by A1-enzyme always showed three major radioactive peaks (Fig. 4A), whereas the O membranes converted to type A by A2-enzyme always showed only one major peak (Fig. 4C). It is interesting that the A2 membranes incubated with A1-enzyme and nucleotide sugar (Fig. 4B) had the two components that were missing in the O to A2 conversion. The O membranes converted to type B by B-enzyme contained three major radioactive components which corresponded to the components found in the O membranes converted to A type by A1 enzyme (Fig. 5).

The membrane samples that were stored at -20°C or freeze-dried gave different isoelectric focusing profiles of radioactive components. Therefore, freshly prepared samples should be used for analysis by isoelectric focusing.

**Extraction of Labeled Components from Erythrocyte Membranes.** A suspension of the labeled membranes (O membranes converted to A1, 5 mg in 1 ml of H2O) was mixed with 9 ml of chloroform/methanol, 2:1 (vol/vol), and vigorously shaken for 30 min at room temperature. The aqueous phase, organic phase, and insoluble precipitate were separated by centrifugation. The aqueous phase (protein fraction) contained 13% of the total radioactivity, the organic phase (lipid fraction) contained 73%, and the remaining activity was in the insoluble precipitate.

**DISCUSSION**

Accumulating information indicates that highly glycosylated water-soluble sphingolipids, designated macroglycolipids, are major components of the ABH antigens on human erythrocytes (6-8). However, several investigators still believe that glycoproteins, not glycolipids, are major blood group components (9-13). The conflict seems to be due to the separation and quantification of the ABH antigen. In this study, we analyzed blood group A1, A2, and B antigens produced in O erythrocyte membranes by the action of purified blood group glycosyltransferases (i.e., by A1-, A2-, and B-enzymes). After completion

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**Fig. 3.** Isoelectric focusing pattern of type O membranes converted to type A1. Other types of membrane also gave an essentially identical isoelectric focusing pattern. Gel was stained with Coomassie brilliant blue.

**Fig. 4.** Distribution of radioactivity in a gel after isoelectric focusing of the labeled erythrocyte membranes. (A) O erythrocyte membranes converted to type A1 by A1-enzyme; (B) A2 membranes converted to type A1 by A1-enzyme; (C) O membranes converted to type A2 by A2-enzyme.

**Fig. 5.** Distribution of radioactivity in a gel after isoelectric focusing of O erythrocyte membranes converted to type B by B-enzyme.
of the transfer, approximately 3.25 nmol (2.75–3.75 nmol) of the sugar was incorporated into 2.4 × 10^6 O ghosts by A_1- and B-enzymes. This implies that about 8 × 10^5 molecules of sugar were incorporated into one ghost, a value comparable to the reported number of antigen sites on A_1 and B erythrocytes (6–11 × 10^6 sites per cell) (18). A smaller amount (0.58 nmol) of N-acetylgalactosamine was transferred into O erythrocytes membranes by A_2-enzyme. The number of the sugar molecules incorporated into O ghosts by A_2-enzyme (2.4 × 10^5 per ghost) is comparable to the reported number of the antigen sites on an A_2 erythrocyte (2.3–2.9 × 10^5 sites per cell) (18). These findings indicate that the A_1, A_2, and B antigens produced in vitro by the transferases are equivalent, both quantitatively and qualitatively, to the naturally existing antigens on erythrocytes.

Our results indicate that more than 80% of blood group substances migrated at the position of lipid, not protein, as demonstrated by sodium dodecyl sulfate gel electrophoresis (Fig. 1), providing evidence supporting the glycolipid nature of the ABH blood group substances. Results of Pronase digestion and extraction of the labeled components by chloroform/methanol further support this conclusion. Schwzyer and Hill (14) reported that radioactive N-acetylgalactosaminase was incorporated mostly into glycoproteins and only 15% into glycolipids of type O erythrocyte membranes by N-acetylgalactosaminyltransferase obtained from porcine submaxillary gland. The discrepancy between our results and their findings could be due to the use of enzymes from different sources.

The isoelectric focusing studies showed that there are at least three major A_1 and B components in erythrocyte membranes. The findings imply that the macroglycolipids with blood group antigenic activity form stable complexes with other components in erythrocyte membranes.

In the case of O membranes converted to A_2 type by A_2-enzyme, only one major radioactive component was detected; in the case of A_2 membranes incubated with A_1-enzyme, the two missing major radioactive components appeared. Accordingly, A_2-enzyme can transfer the sugar into only one major H-component (middle peak in Fig. 4), whereas A_1-enzyme can transfer the sugar into all three major H-components. This could be due to the difference in the interactions of the two enzymes with the three major H-components.

Hakomori, Strycharz, and Watanabe (2, 29) isolated four types of A-active glycosceramides from A_1 cells. Some of these glycosceramides lacked A_2 cells (2). These glycosceramide components may be related to the three components separated by isoelectric focusing in the present study.

It has been suggested that terminal N-acetylgalactosamine is linked to both type 1 chains (Gal β 1→3 GlcNAc) and type 2 chains (Gal β 1→4 GlcNAc) of H-substance in A_1 erythrocytes but only to type 2 chains in A_2 erythrocytes (20). However, Schachter et al. (31) demonstrated that A_2-enzyme transferred N-acetylgalactosamine into both lacto-N-fucopentaose (a type 1 chain analogue) and 2′-fucosyllactose (a type 2 chain analogue). Moreover, several investigators have reported that blood group A and B glycolipids on erythrocytes are composed of only type 2 chains (3, 4, 6). The structure of blood group glycosceramides in the three major antigenic components described in this paper remains to be examined in order to elucidate exact qualitative differences between A_1 and A_2 antigens.

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