Membrane Abnormality in Red Blood Cells With Weak Type B Expression

By A. Yoshida, H. Fujii, V. Davé, M. J. Cozant, and P. A. Morel

The mechanisms of unusually weak blood group (A and B) expressions are not yet well understood. We examined properties of blood group galactosyltransferase (B-enzyme) and characteristics of red cell membrane components obtained from family members with A1Bm character. B-enzyme activity of the A1Bm plasma is in normal range, and kinetic properties (i.e., $K_m$ for UDP-Gal, $K_m$ for 2'-fucosyllactose, and pH optima) of B-enzyme from the A1Bm subjects are identical to that of normal B-enzyme. When A1Bm red cells were incubated with UDP-Gal and B-enzyme, the cells became strongly agglutinable with anti-B. When A1Bm membranes were incubated with B-enzyme or A-enzyme (i.e., blood group N-acetylgalactosaminyltransferase) and the appropriately labeled nucleotide sugar (UDP-Gal$^3$H for B-enzyme and UDP-GalNAc$^3$H for A-enzyme), significant incorporation of the sugar was observed. The amounts of the sugar incorporated into A1Bm membranes were about 40%-50% of that incorporated into O membranes at saturation, indicating that about one-half of H-sites remained unglycosylated in A1Bm red cells. Examination of radioactive components by isoelectric focussing revealed that the labeled components of A1Bm membranes were distinctively different from that of O membranes. Therefore, one can conclude that the weak B expression is not due to direct mutation of ABO locus, but due to a secondary consequence of genetic abnormality of a membrane component (or components) associated with blood group substances.

Although human blood group ABO phenotypes are generally classified as A, A, A,B, A,B, B, and O, weak or very weak A and B expressions in red cells are occasionally observed. The mechanisms of these unusual blood group expressions are not well understood. Since the blood group A and B substances are synthesized by specific blood group glycosyltransferases, i.e., UDP-GalNAc:2'-fucosylgalactoside $\alpha$3-N-acetylglactosaminyltransferase (A-enzyme) for synthesis of A substance, and UDP-Gal:2'-fucosylgalactoside $\alpha$3-N-galactosyltransferase (B-enzyme) for synthesis of B substance, the inherited weak blood group expressions may be due to diminished activity of blood group transferases in bone marrow. It is also conceivable that membrane abnormality causes insufficient glycosylation of the acceptor sites (H-sites) or a putative abnormal glycosidase decomposes A (or B) substance, resulting in weak A (or B) activity on the red cell surface. We examined the properties of B-enzyme and of red cell membrane components from a family associated with very weak B expression, and found that the unusual B expression is due to membrane abnormality.

MATERIALS AND METHODS

Blood samples. Plasma from fresh blood (ACD anticoagulant) of the subjects and from A, B, and O controls were stored at -60°C. No significant decrease in the blood group transferase activity occurred during several months of storage.

Agglutination titer. Agglutinability of fresh washed red cells was tested using anti-A and anti-B agglutinin (Ortho Diagnostics) with serial dilutions.

Assay of blood group transferase activity. The blood group A- and B-enzymes were assayed by one of the following two methods. In method 1, O red cells were incubated with the nucleotide sugar (UDP-GalNAc for A-enzyme, and UDP-Gal for B-enzyme) and plasma (or partially purified enzyme), and the newly produced blood group substance (A or B) on O red cell surfaces was semiquantitatively assayed using anti-A or anti-B agglutinin with serial double-fold dilutions. In method 2, the transferase activities were assayed by measuring the incorporation of sugar from radioisotope-labeled nucleotide sugars into 2'-fucosyllactose, an analog of the natural sugar acceptor. The details of the assay methods have been reported.

Partial purification of A- and B-enzymes. The enzymes were partially purified from the subject's plasma and control A, and B plasma as previously described. The partially purified enzymes were reconstituted in a volume about 50 times less than the original plasma.

Incorporation of GalNAc$^3$H and Gal$^3$H into red cell membranes. Red cell membranes were incubated with the sugar donor (UDP-GalNAc$^3$H or UDP-Gal$^3$H) and the partially purified transferase (A- or B-enzyme), as previously described.

Isoelectric focussing. Solubilization and isoelectric focussing of the labeled membranes were performed as previously described. The gels were stained with Coomassie brilliant blue. To determine the distribution of radioactivity, gels were sliced transversely and subjected to measurement of radioactivity, as previously described.

Red cell membranes. Red cell ghosts were prepared by the method of Dodge et al.

Sources of the materials used, i.e., UDP-GalNAc, UDP-Gal, UDP-GalNAc$^3$H, UDP-Gal$^3$H, 2'-fucosyllactose, and ion-exchangers have been previously described.

RESULTS

Red Cell Agglutinability

The propositus's red cells had a high agglutination titer with anti-A and anti-H but a very low titer with...
Table 1. Red Cell Agglutinability and Saliva Type

<table>
<thead>
<tr>
<th>Subject</th>
<th>Blood Type</th>
<th>Original</th>
<th>Converted</th>
<th>Saliva Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>A,Bm</td>
<td>1</td>
<td>1,024</td>
<td>Le(a⁻,b⁻)</td>
</tr>
<tr>
<td>I-2</td>
<td>A⁻</td>
<td>0</td>
<td>—</td>
<td>Le(a⁻,b⁻)</td>
</tr>
<tr>
<td>II-1</td>
<td>A⁻</td>
<td>0</td>
<td>—</td>
<td>Le(a⁻,b⁻)</td>
</tr>
<tr>
<td>II-2</td>
<td>A,Bm</td>
<td>2</td>
<td>1,024</td>
<td>Le(a⁺,b⁻)</td>
</tr>
<tr>
<td>II-3</td>
<td>A,Bm</td>
<td>2</td>
<td>512-1,024</td>
<td>Le(a⁺,b⁻)</td>
</tr>
<tr>
<td>II-4</td>
<td>A,Bm</td>
<td>4</td>
<td>512-1,024</td>
<td>Le(a⁺,b⁻)</td>
</tr>
<tr>
<td>Control</td>
<td>A⁻</td>
<td>0</td>
<td>2-8</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>B</td>
<td>512-1,024</td>
<td>512-1,024</td>
<td>—</td>
</tr>
</tbody>
</table>

*Blood types were classified based on agglutination of red cells, plasma antibody, and saliva blood group substances, as described in the text.

†Agglutination titer was expressed by the highest dilution of standard agglutinin that caused detectable agglutination after 30 min of incubation.

§Washed untreated red cells.

$Washed red cells treated with UDP-Gal and partially purified B-enzyme for 4 hr, as described in ref. 5.

Table 2. Plasma Blood Group Glycosyltransferase Activities

<table>
<thead>
<tr>
<th>Subject</th>
<th>Blood Type</th>
<th>Transferase Activity Assayed by Method 1</th>
<th>Assayed by Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>A,Bm</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>I-2</td>
<td>A⁻</td>
<td>1,024</td>
<td>0</td>
</tr>
<tr>
<td>II-1</td>
<td>A⁻</td>
<td>1,024</td>
<td>0</td>
</tr>
<tr>
<td>II-2</td>
<td>A,Bm</td>
<td>1,024</td>
<td>32</td>
</tr>
<tr>
<td>II-3</td>
<td>A,Bm</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>II-4</td>
<td>A,Bm</td>
<td>512</td>
<td>16-32</td>
</tr>
<tr>
<td>Control</td>
<td>B</td>
<td>Mean 44</td>
<td>Mean 10.6</td>
</tr>
</tbody>
</table>

*Activity is expressed as the highest dilution of standard A-agglutinin that caused detectable agglutination after incubation of O red cells with UDP-GalNAc and plasma for 16 hr, as described in ref. 4.

†B-enzyme activity is expressed as the highest dilution of standard B-agglutinin that caused detectable agglutination after incubation of O red cells with UDP-Gal and plasma for 1 hr, as described in ref. 5.

§B-enzyme activity is expressed as percent of UDP-Gal transferred into 2'-fucosyllactose by the plasma under the reaction condition described in ref. 6. The A- and B-enzyme activities of phenotype A⁻ and B plasma differs widely among the samples as previously described.

Kinetic Properties of B-Enzyme of the Propositus

Enzymatic characteristics (i.e., $K_m$ for UDP-Gal, $K_m$ for 2'-fucosyllactose, and pH optima) of the propositus's B-enzyme were compared with control B-enzyme using partially purified enzyme preparations. The rate of enzyme reaction as a function of the concentrations of UDP-Gal and 2'-fucosyllactose indicated the usual Michaelis-Menten relationship. The $K_m$ values for the substrates calculated from a Lineweaver-Burk plot are given in Table 3. No significant difference was observed in $K_m$ values between the propositus's enzyme and control B-enzyme. A pH activity profile of the subject's B-enzyme was also similar to that of control B-enzyme, both having maximum activity at pH 6.5–7.5.

In Vitro Conversion of Red Cell Type

When the red cells with A,Bm phenotype (subjects I-1, I-2, II-3, and II-4) were incubated with partially purified B-enzyme and UDP-Gal, the red cells became agglutinable with B-agglutinin with high dilution.

Table 3. Michaelis Constants of B-Enzyme

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>UDP-Gal</th>
<th>2'-Fucosyllactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control B plasma (3 samples)</td>
<td>22 (16–36)</td>
<td>56 (50–61)</td>
</tr>
<tr>
<td>A,Bm (II-3) plasma</td>
<td>23 ± 4</td>
<td>70 ± 8</td>
</tr>
</tbody>
</table>

$K_m$ were calculated from Lineweaver-Burk plot. Reaction mixture contains various concentration of UDP-Gal or 2'-fucosyllactose in the reaction mixture, pH 6.5, as specified in ref. 13.
Agglutination titer of the incubated A,Bm red cells was the same as that of control B red cells, i.e., the A,Bm red cells were converted to A,B (Table 1). Type A red cells could not become strongly agglutinable with B-aggbutinin (titer lower than 8) after the same treatment. The result indicated that H-sites on A,Bm red cell surface can be galactosylated by B-enzyme and become reactive with B agglutinin.

**Incorporation of GalNAc and Gal Into A,Bm Red Cell Membranes**

When red cell membranes obtained from the propositus’s red cells were incubated with partially purified A,-enzyme and UDP-GaINAc3H, incorporation of the sugar into the membranes was observed. Similarly, when A,Bm red cell membranes were treated with partially purified B-enzyme obtained from the propositus plasma or control B plasma, significant incorporation of Gal was observed (Table 4). The amounts of the sugars incorporated into A,Bm red cell membranes were about 40%–50% of that incorporated into O red cell membranes at saturation. Very little incorporation of the sugars was observed into A red cell membranes obtained from A,O subject and B red cell membranes from BO subject.

The incubated red cell membranes were solubilized and subjected to analysis by isoelectric focussing in acrylamide gel. Isoelectric focussing patterns (as visualized by Coomassie blue) of the incubated A,Bm membranes were similar to that of the incubated O membranes (Fig. 2). However, distribution profiles of radioactivity in the gels were entirely different between the incubated A,Bm membranes and the incubated O membranes (Fig. 3).

**DISCUSSION**

The unusual weak B expressions have been found in various populations. The expressions are heterogeneous and classified into the following three major categories: (1) anti-B in serum, B of some kind in saliva; (2) no anti-B in serum, B in saliva; and (3) no anti-B in serum, H but no B in saliva. The present case belongs to the second category, which has been often designated as Bm status. The blood phenotype of the affected family members should be classified as A,Bm. Although Bm expression has been found in various populations, a majority of the Bm cases reported are Japanese. The present family is also of Japanese descent.

The mechanisms of weak A and B expressions have not yet been extensively studied at the enzymatic and molecular level and remain to be explored. The weak B (or A) expression could be due to a mutation of the gene that governs B- (or A-) enzyme formation, i.e., a mutation in ABO locus. The abnormal blood group

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**Table 4. Incorporation of GalNAc or Gal Into Red Cell Membranes by A- and B-Enzymes In Vitro**

<table>
<thead>
<tr>
<th>Source of Membranes</th>
<th>Enzyme</th>
<th>Source of Enzyme</th>
<th>Percent of Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>A</td>
<td>A</td>
<td>13.5</td>
</tr>
<tr>
<td>O</td>
<td>B</td>
<td>B</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.7</td>
</tr>
<tr>
<td>A,Bm (II-3)</td>
<td>A</td>
<td>A,Bm</td>
<td>7.6</td>
</tr>
<tr>
<td>A,Bm (II-3)</td>
<td>B</td>
<td>B</td>
<td>6.6</td>
</tr>
<tr>
<td>A,Bm (II-3)</td>
<td>B</td>
<td>A,Bm</td>
<td>6.0</td>
</tr>
<tr>
<td>A,B</td>
<td>A</td>
<td>A,Bm</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>A,B</td>
<td>B</td>
<td>B</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>A,B (from A,O)</td>
<td>A</td>
<td>A</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>B (from BO)</td>
<td>B</td>
<td>B</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>
A gel slice number

8
7
6
6.6
6.2
5.6
5.2
4.4
4.0

5
10
15
20
25
30
35

cpm/slice

Fig. 3. Distribution of radioactivity in a gel after isoelectric focussing of the labeled red cell membranes. (A) Type O red cell membranes incubated with UDP-GalH and partially purified B-enzyme. (B) Type A,Bm (propositus) red cell membranes incubated with UDP-GalH and partially purified B-enzyme.

glycosyltransferase thus produced might have diminished enzyme activity or altered enzyme properties and would not be able to efficiently synthesize B (or A) substance on O red cell surface. However, in the A,Bm cases examined, plasma B-enzyme activity is within normal range (Table 2). Moreover, no abnormal kinetic properties were found in the partially purified B-enzyme from the propositus’s plasma (Table 3). The partially purified B-enzyme from the A,Bm subject’s plasma can convert O red cells into B red cells as effectively as B-enzyme obtained from control B plasma. Consequently, the weak B expression in the present case cannot be attributed to direct mutation of the gene governing the formation of B-enzyme, producing defective B-enzyme. Most of the A- and B-enzymes in the circulatory plasma are not derived from the bone-marrow, and thus, the weak B activity of red cells could be due to suppression of the B-enzyme production (or secretion) only in bone marrow but not in other tissues and plasma. However, one can completely rule out the above possibility due to the fact that the present A,Bm red cells strongly react with anti-H and contain a large quantity of open H-sites (about 40%-50% of H-sites of O red cells), in contrast to red cells from A,O heterozygous subjects, which do not contain a significant amount of unoccupied H-sites (Tables 1 and 4).

The possibility of degradation of B substance by a putative unusual α-galactosidase in the subject’s bone marrow is also ruled out. Since, if this is the case, H-sites thus produced should be converted to A substance by the existing A-enzyme and not remain unoccupied.

The A,Bm red cells became fully agglutinable with anti-B, i.e., became converted to A,B, after the incubation with UDP-Gal and partially purified B-enzyme (Table 1), and significant amounts of Gal were incorporated into A,Bm red cell membranes (Table 4). Isoelectric focussing patterns of A,Bm red cell membranes glycosylated with the radioactive sugar are similar to that of the glycosylated O red cell membranes (Fig. 2). However, the radioactive components of the labeled A,Bm red cell membranes are distinctly different from that of the labeled O red cell membranes, as demonstrated by the unusual distribution patterns of radioactivity in isoelectric focussing (Fig. 3). As previously reported, three major radioactive components, which are presumably complexes of the blood group macroglycolipids with other membrane components, are observed in O red cell membranes converted into B (or A,) in vitro (Fig. 3). These three components are missing, and instead, two other radioactive components were found in A,Bm membranes glycosylated in vitro (Fig. 3). These results indicate that A,Bm red cell membranes contain unusual macroglycolipids with H-activity or unusual complexes of H-glycolipid with an abnormal membrane component (or components). Judging from the above findings, one can conclude that the weak B expression in the case examined is not due to direct mutation of the gene governing the blood group transferase (i.e., ABO locus). It should be due to the abnormality of red cell membrane components resulting in insufficient galactosylation of H-sites. Thus, A,-enzyme can transfer GalNAc into 50%-60% of H-sites of the present A,Bm red cells, but B-enzyme, although the enzyme itself is not unusual, cannot efficiently transfer Gal into the remaining unusual H-sites in bone marrow, presumably due to steric hindrance between the transferase and the unusual H-sites. In the presence of highly active blood group transferase (i.e., partially purified B-enzyme) and high concentrations of the UDP-Gal in vitro, the unoccupied H-sites of the A,Bm red cells are further glycosylated, and thus they can be converted into A,B type.

In order to account for some disturbance of the normal Mendelian inheritance in some families with
unusual A and B expressions, several investigators postulated an involvement of a "modifying gene" in these Bm and Ax cases.\textsuperscript{1,2} Our study indicates that the weak B expression in the present family is due to a secondary consequence of genetic abnormality of a membrane component (or components) and not due to a direct mutation of blood group ABO locus supporting the modifying gene hypothesis.

In rare expressions, such as Bm and Ax, the genetic mechanism is not necessarily identical in unrelated subjects with the abnormalities. Thus, another mechanism in weak Bm expression in other unrelated cases is not completely excluded. The biochemical approaches used in the present study may be adopted for elucidating underlying mechanisms of various types of weak A and B expressions.

\textbf{ACKNOWLEDGMENT}

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\textbf{REFERENCES}

Membrane abnormality in red blood cells with weak type B expression
A Yoshida, H Fujii, V Dave, MJ Cozant and PA Morel