Identification of Genotypes of Blood Group A and B

By Akira Yoshida

The human blood groups (ABO) are known to be determined by the terminal glycosyl residues attached to common carbohydrate chains of the red cell surface. N-Acetylgalactosaminyltransferase (A-enzyme) in blood group A persons and galactosyltransferase (B-enzyme) in blood group B persons are responsible for producing A and B substances on the red cell surface, with both enzymes absent in blood group O persons. It has been impossible, without examining their family pedigree, to determine genotypes (i.e., AA or AO, BB or BO) of group A and B persons. The blood group A-enzyme was purified to homogeneity, and rabbit antibody against A-enzyme was prepared. The existence of an enzymatically inactive, but immunologically cross-reactive protein (CRM), was demonstrated in O plasma. Immunoneutralization profiles of homozygous AA or BB plasma were clearly distinguished from those of heterozygous AO or BO plasma. In addition, the existence of CRM in heterozygous AO and BO plasma, but not in homozygous AA and BB plasma, was demonstrated by separating CRM and active enzyme (A and B) by affinity chromatography. Thus, genotypes of the blood groups can be identified with certainty by these biochemical methods. The existence of CRM in O plasma and heterozygous AO and BO plasma but not in homozygous AA and BB plasma, is incompatible with any nonallelic model for ABO locus. The genes governing the blood group expression should be truly allelic.

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### Materials and Methods

#### Blood Samples

Homohzygous $A_A$, and $B_B$ blood samples were obtained from individuals with phenotypes $A_A$ and $B_B$ who are offspring of $AB \times AB$ parents. Heterozygous $A_AO$ and $BO$ blood samples were obtained from individuals whose genotypes were identified by their family pedigree. Fresh plasma (ACD anticoagulant) was stored at $-60^\circ C$. The blood group A- and B-transferase activities in the plasma did not change during the storage period of at least 2 mo. Rabbit antiserum against purified A-enzyme was prepared as described previously. The antibody was salted out with 2 $M$ (NH$_4$)$_2$SO$_4$, and reconstituted in 0.01 $M$ Tris-Cl, pH 7.0, and stored at $-15^\circ C$. The transferase activities were assayed by measuring the incorporation of sugar from nucleotide sugar into fucosyllactose, an analog of the natural sugar acceptor. The reaction mixture contained 2 $mM$ fucosyllactose, 25 $mM$ UDP-N-acetylgalactosamine ($^3$H, 0.5 $\mu Ci$), 1 $mM$ ATP, 15 $mM$ MnCl$_2$, 0.15 $M$ NaCl, 0.2% bovine serum albumin, 0.25% Triton X-100, 1 $mM$ NaN$_3$, 40 mM cacodylate buffer, pH 6.5, and enzyme. For the B-enzyme assay, 25 $mM$ UDP - galactose ($^3$H, 0.5 $\mu Ci$) was used as a sugar donor, and 25 $mM$ imidazole, pH 6.5, was used as a buffer. The reaction mixture was incubated for 16 hr at 37°C. An aliquot (20 $\mu l$) of the reaction mixture was applied to an arrow-shaped strip of DEAE-cellulose filter paper ($2 \times 7$ cm) and eluted with water (total 2 ml). The radioactivity of the eluate was measured by a scintillation counter. In order to correct for spontaneous and catalytic hydrolysis of the nucleotide sugars, the reaction mixture minus fucosyllactose was also incubated as a control. The radioactivity of the control mixture (less than 10% in the case of crude plasma as enzyme source, and less than 3% in the case of partially purified enzymes) was subtracted from that of the complete reaction mixture.

The blood group A- and B-enzymes were partially purified as previously reported, and stored in concentrated form at $-60^\circ C$. Before use, the enzyme was appropriately diluted with 0.01 $M$ Tris-Cl, pH 7.0, containing 1% bovine serum albumin. UDP-N-acetylgalactosamine was synthesized as previously reported.$^6$ UDP-galactose was purchased from Sigma Chemical Co. UDP-N-acetylgalactosamine (galactosamine-1-3H) was purchased from New England Nuclear and UDP-galactose (galactose-6-3H) was purchased from Amersham Corp. Fucosyllactose was prepared from human milk as previously described,$^7$ and further purified by gel filtration with Biogel P-2. Purity of the preparation was better than 98% as determined by its sugar composition. Sepharose 4B (lot no.

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**Table 1. Blood Group A- and B-Enzyme Activity in Plasma**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>Enzyme Activity</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-1</td>
<td>A(<em>A)</em></td>
<td>17.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-2</td>
<td>A(<em>A)</em></td>
<td>9.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-3</td>
<td>A(<em>A)</em></td>
<td>17.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AO-1</td>
<td>A(<em>O)</em></td>
<td>8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AO-2</td>
<td>A(<em>O)</em></td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Phenotype A\(_A\)_

(9 samples) mean 6.44, range 2.8-16.8

Phenotype B

(7 samples) mean 10.6, range 5.0-17.5

An aliquot of plasma, 50 \(\mu\)l, was used for enzyme assay. The composition of the reaction mixture was described in the text. The enzyme activity was expressed as percent of sugar (N-acetylgalactosamine for A-enzyme and galactose for B-enzyme) transferred into fucosylactose under the assay conditions described in the text.

17C-0110) was obtained from Sigma Chemical Co. UDP-Sepharose 4B (1 \(\mu\)mol UDP/ml gel) was prepared as previously described.\(^{10}\)

**EXPERIMENTS AND RESULTS**

**Plasma A- and B-Enzyme Activities**

A- and B-enzyme activities in the homozygous and heterozygous plasma samples are presented in Table 1. The enzyme activities of homozygous AA-1 and AA-3 plasma were nearly twice that of heterozygous plasma, but the activity of homozygous AA-2 was only slightly higher than that of heterozygous plasma. Similarly, the B-enzyme activity of one of the homozygous plasma (BB-1) was distinctively higher than that of two heterozygous samples, but the B-enzyme activity of another homozygous plasma (BB-2) was similar to that of the heterozygous plasma.

The A- and B-enzyme activities of phenotypes A\(_A\)_ and B plasma differed widely among the samples. From the known genotype frequencies, most, if not all, of the samples were expected to be heterozygous A\(_O\)_ or B\(_O\).

**Neutralization of Plasma A- and B-Enzyme by Anti-A-Enzyme Antibody**

The plasma samples were dialyzed against 0.01 \(M\) Tris-Cl, pH 7.0, at 4\(^\circ\)C overnight. An aliquot of plasma (or plasma diluted with 1% bovine serum albumin) 40 \(\mu\)l, was mixed with various amounts of anti-A-enzyme antibody (totaling 50 \(\mu\)l) and incubated at 4\(^\circ\)C for 5 hr with shaking, then subjected to an assay of the remaining transferase activity. The immunoneutralization profiles indicated that the A- and B-enzyme activities of homozygous plasma were more strongly neutralized by the same amount of antibody than those of heterozygous plasma (Figs. 1 and 2).

Antibody, \(\mu\)l

- Fig. 1. Immunoneutralization of A-enzyme in homozygous AA and heterozygous AO plasma by anti-A-enzyme antibody. The dialyzed plasma, 40 \(\mu\)l, was mixed with various amounts of antibody (totaling 50 \(\mu\)l) and incubated at 4\(^\circ\)C for 5 hr, then subjected to an assay of remaining A-enzyme activity. Prior to mixing with antibody, the AA-1 and AA-3 plasma were diluted with 1% bovine serum albumin to result in approximately the same activity as that of AO-1 plasma. The values represent mean values of duplicate analysis taking the control (without antibody) as 100. (\(\bullet\)) AA-1; (\(\odot\)) AA-2; (\(\square\)) AA-3; (\(\bigcirc\)) AO-1; (\(\bigtriangleup\)) AO-2; (\(\triangle\)) mixture (1:1) of AA-1 and O plasma.

- Existence of Enzymatically Inactive but Immunologically Cross-Reactive Protein (CRM) in Heterozygous AO and BO Plasma but not in Homozygous AA and BB Plasma

It has been known that A\(_A\)-enzyme can be completely adsorbed with Sepharose 4B, and the adsorbed A-enzyme can be eluted with a buffer containing uridine 5'-disphosphate.\(^8\) B-enzyme cannot be adsorbed with Sepharose 4B, but it can be adsorbed with UDP-Sepharose 4B (unpublished observation). Utilizing these adsorption properties, CRM present in heterozygous AO and BO plasma was separated from A\(_A\)- and B-enzymes.

Aliquots of homozygous A\(_A\)_A\(_A\)_ plasma, heterozygous A\(_O\)_A\(_A\)_ plasma, and O plasma, 5 ml, were placed on a Sepharose 4B column (0.15 ml bed volume), and the column was washed with 1 ml of 0.1 \(M\) cacodylate buffer, pH 7.0, containing 2 \(mM\) MnCl\(_2\) and 1 \(mM\) EDTA. The eluate was dialyzed against 0.01 \(M\) Tris-Cl, pH 7.0, containing 5% glycerol and 1 \(mM\) EDTA,
and incubated at 4°C for 5 hr, then subjected to an assay of remaining B-enzyme activity. Prior to mixing with antibody, the BB-1 plasma was diluted with 1% bovine serum albumin to result in approximately the same activity as that of BO-1 plasma. The values represent mean values of duplicate analysis taking the control (without antibody) as 100. (A) BB-1; (Δ) BB-2; (C) BB-1; (Δ) BO-2; (O) mixture (1:1) of BB-1 and 0 plasma by anti-A enzyme antibody. The results represent mean values of duplicate analysis as described above. The protein solution (final volume of about 1 ml) was used for assay of CRM activity.

The A- and B-enzymes were completely adsorbed to Sepharose 4B or to UDP-Sepharose 4B, respectively, so that the nonenzyme protein (NEP) obtained by the above treatments had neither A- nor B-enzyme activity. When the antibody solution was incubated with NEP obtained from 0, heterozygous A,0, and BO plasma, the treated antibody solution lost its ability to inactivate A- and B-enzymes. In contrast, NEP obtained from homozygous A,A, and BB plasma did not react with the antibody, and the treated antibody solution retained its capacity for inactivating A- and B-enzymes (Table 2). These results indicated that heterozygous A,0 and BO plasma, as well as O plasma, contained CRM, but homozygous A,A, and BB plasma did not contain CRM.

**DISCUSSION**

It has been known that the genotypes of blood groups A and B subjects cannot be identified by the agglutinability of their blood. Blood group A and B enzyme activities differ widely among individuals with

Table 2. Content of CRM in Homozygous A,A, and BB Plasma and in Heterozygous A,0 and BO Plasma

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Antibody</th>
<th>A-Enzyme</th>
<th>B-Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Treated with buffer</td>
<td>78</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from AA-1</td>
<td>84</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from AO-1</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2</td>
<td>Treated with buffer</td>
<td>88</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from AA-2</td>
<td>80</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from AO-2</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>3</td>
<td>Treated with buffer</td>
<td>92</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from pooled phenotype</td>
<td>&lt;5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from A, plasma</td>
<td>&lt;5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from O plasma</td>
<td>&lt;5</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Treated with buffer</td>
<td>72</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from BB-1</td>
<td>66</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from BB-2</td>
<td>&lt;5</td>
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<td>88</td>
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<td></td>
<td>Treated with NEP from BB-2</td>
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<td>92</td>
</tr>
<tr>
<td></td>
<td>Treated with NEP from BB-2</td>
<td>—</td>
<td>11</td>
</tr>
</tbody>
</table>

**NEP (nonenzyme protein)** was prepared as described in the text. An aliquot of antibody, 20 µl, was mixed with 40 µl of NEP (or 0.01 M Tris-Cl, pH 7.0, as control), and the mixture was incubated at 4°C for 5 hr with shaking. An aliquot of the mixture (or Tris buffer as control), 20 µl, was mixed with 10 µl of partially purified A,- or B-enzyme and was incubated at 4°C for 5 hr with shaking. The incubated enzyme was subjected to an assay of A- and B-enzyme activity under the conditions described in the text. The results represent mean values of duplicate analysis.
the same phenotypes, and even with the same genotypes, as reported by previous investigators. Therefore, the genotypes cannot be identified by measuring their blood group enzyme activities.

The immunoneutralization profiles of homozygous AA and BB plasma were distinctively different from those of heterozygous AO and BO plasma. A- and B-enzymes were more strongly neutralized in homozygous plasma than in heterozygous plasma. These results are most readily interpreted to mean that heterozygous AO and BO plasma contain CRM in addition to the active A- or B-enzyme, while homozygous AA and BB plasma do not contain CRM.

In fact, the existence of CRM in heterozygous AO and BO plasma, but not in homozygous AA and BB plasma, was unequivocally demonstrated by separating CRM and active enzyme by Sepharose 4B (for A-enzyme) and UDP-Sepharose 4B (for B-enzyme). After this work was completed, one additional homozygous BB plasma and four samples of A,A2 plasma were examined. It was found that all these plasma did not contain CRM, confirming the above conclusion.

The genotypes of phenotype A and B subjects can be identified with certainty by examining immunoneutralization profiles of their plasma, and more directly, by examining the presence or absence of CRM in their plasma, as described in this article.

Although the inheritance of blood groups ABO is compatible with mendelian inheritance, and genes governing the ABO blood groups are generally considered to be allelic, the fundamental question, i.e., whether or not ABO genes are truly allelic, has not yet been answered. In the past, Furuhata postulated a nonallelic model involving two sites, one for A, or not A, and one for B, or not B. Later, the true allelic model was challenged by the discovery of Cis-AB expression, i.e., an AB offspring of AB × O parents. Komai considered that the Cis-AB originated from crossing-over between A and B genes that were pseudoallelic but not allelic in the strict sense. However, Cis-AB does not necessarily originate by crossing-over. In addition, crossing-over to produce a chromosome with both A and B genes is theoretically possible even between true allelic genes.

There are three conceivable models for ABO locus (ABO cistron), i.e., true allelic model, nonallelic model, and regulatory gene model (Fig. 3). The regulatory gene model assumes that structural genes for A- and B-enzymes are not allelic and that their expression is controlled by allelic regulatory genes α, β, and O. According to this model, CRM should not be produced in O subjects, since both A and B genes are shut off and silent. The demonstrated existence of CRM in O plasma is incompatible with the regulatory gene model, so the model can be rejected.

In the nonallelic model, CRM should be produced not only in O, heterozygous AO and BO subjects, but also in homozygous AA and/or BB subjects. The present results indicate that CRM does not exist in either AA or BB plasma, refuting the nonallelic model. It can be concluded that the genes governing the ABO blood groups are truly allelic.

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REFERENCES

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