Identification of an Anti-A and Anti-B Blood Group Glycosyltransferase Antibody After Incompatible Bone Marrow Transplant

By Marina Mojena and Lisardo Bosch

The occurrence of a potent antibody against plasmatic A and B glycosyltransferase activities has been characterized in a patient (blood group A1) transplanted with a bone marrow from a blood group O donor. A and B glycosyltransferases were purified to near homogeneity from plasma of A1 and B blood-group individuals. The half-maximal inhibition of both enzymes was obtained at 1 to 2 μg/mL of the post-transplant IgG fraction, prepared by protein A-sepharose chromatography. A and B glycosyltransferases were also recognized by the post-transplant IgG fraction after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrophoretic transfer to nitrocellulose membranes. 1989 by Grune & Stratton, Inc.

The CARBOHYDRATE structures of glycoproteins that confer blood-group ABO specificities are synthesized by glycosyltransferases determined by genes located in different chromosomal loci. The blood group A or B active structures are determined by the incorporation of N-acetyl-D-galactosamine or D-galactose onto a common acceptor carbohydrate chain (H substance) by the action of α-2,3-N-acetyl-D-galactosaminyl transferase (A glycosyltransferase) or the α-2,3-D-galactosyltransferase (B glycosyltransferase). ABO antigens are widely distributed among tissues and even on body fluids. However, the relationship between the amount of antigen expressed on the cell surface and the corresponding glycosyltransferase activity is a subject not well-documented and one for which more research is required.

Glycosyltransferases are released by tissues to the plasma. The A glycosyltransferase is present in the plasma of group A individuals and the B enzyme in those of group B. The tissular contribution to the plasma glycosyltransferase activities has been studied mainly after the measurement of the changes in the plasmatic activities of these enzymes in patients who underwent solid organ or bone marrow transplantation (BMT) between ABO incompatible donors.

In a previous study of the plasmatic glycosyltransferase activities after organ transplantation, an unexpected lack of plasmatic activity was found in one patient (group O) engrafted with a liver from a group A donor and in two patients (group A1) who underwent BMT from a group O donor. In all these cases of ABO major incompatibility (liver) or ABO minor incompatibility (bone marrow) organ transplant, the post-transplant sera from the patients, in addition to the absence of plasmatic glycosyltransferase activity, completely inhibited the A and B glycosyltransferase from control serum. As previously described, these patients also shared in common an episode of severe graft-versus-host disease (GVHD). However, this inhibitor was absent in cases of ABO isogroup BMT and in those of ABO incompatible BMT but without manifestation of the GVHD (two cases).

In the present investigation we purified both A and B glycosyltransferases as well as the IgG fraction of the serum and provide evidence to establish clearly the IgG nature of the glycosyltransferase inhibitory activity that occurred after incompatible bone marrow transplantation.

MATERIALS AND METHODS

Chemicals. UDP-N-acetyl-D-galactosamine, UDP-D-galactose, and 2'-fucosyllactose were from Sigma (St Louis). DE52 was from Whatman (Maidstone, UK). Sepharose 4B and protein A sepharose CL 4B were from Pharmacia (Uppsala, Sweden). Electrophoresis, blotting materials, and other ion-exchange resins were from Bio-Rad laboratories (Richmond, CA). Polyclonal anti-A and anti-B sera were purchased from Ortho Diagnostics (Raritan, NJ). UDP-[14C]galactose (155 Ci/mol) was from Amersham International (Amersham, UK). Other chemicals and biochemicals were obtained from Merck (Darmstadt, FRG) and Boehringer (Mannheim, FRG).

Control sera were aliquoted and stored at −40°C. Serum from a patient (MF, blood group A1) transplanted 26 months previously with a bone marrow from a group O donor was stored in small aliquots at −40°C. The patient suffered from chronic myeloid leukemia and in the postoperative course received doses of 3 mg/kg body weight of cyclosporine A. This serum was the same used in a previous work.

Purification of A and B glycosyltransferases. Fresh group A or B sera (450 mL) were filtered through glass-wool and centrifuged at 10,000 g for 15 minutes at 4°C. A1 glycosyltransferase activity was purified essentially as described by Nagai et al18 by sepharose 4B chromatography followed by an affinity chromatography on UDP-hexanolamine-agarose column (0.5 x 3 cm) and eluted with 20 μM UDP-N-acetyl-D-galactosamine. The activity was purified about 160,000-fold. Fractions containing activity were pooled, concentrated, and desalted by means of an Ultrasetr device (Sartorius, Göttingen, FRG) provided with a cellulose triacetate membrane with a cut-off of 30,000 daltons, and stored at −20°C in the presence of 20% glycerol (vol:vol). A glycosyltransferase was also partially purified from pooled A group sera by DE52 chromatography. In this case two peaks with enzymatic activity were observed after elution of the column by a NaCl gradient (from 50 to 300 mmol/L).

B glycosyltransferase activity was purified as described by Nagai et al18 by DE52 and hydroxyapatite chromatography. The procedure used was exactly the same except for the elution of the hydroxylapatite column, which was modified as follows: after load-
and 50 mmol/L UDP-N-acetyl D-galactosamine, group 0 red-cell suspensions were incubated in 3 mL sterile plastic tubes at 37°C in a rotatory shaking water bath (40 cycles per minute). The procedure was as follows: aliquots of 20 μL of group O red cells. The procedure was as follows: aliquots of 20 μL of serum were centrifuged at 10,000 g for 15 minutes and applied to a sepharose 4B column. After washing (buffer A: 150 mmol/L NaCl, 0.1 mmol/L EDTA, and 20 mmol/L HEPES, pH 7.2) the activity was eluted with 20 μmol/L UDP (A) followed by dialysis and ultrafiltration through a membrane with a cut-off of 30,000 daltons and the volume reduced to 2 mL. This fraction was applied to a UDP-hexanolamine agarose column and was eluted with 20 μmol/L UDP-N-acetylgalactosamine (B). For the purification of B-glycosyltransferase the serum was diluted 1:3 and applied to a DE52 column. After washing with buffer A diluted 1:3, a gradient of NaCl was applied to elute the enzyme (C). Fractions containing activity were applied to a hydroxylapatite column. After washing with 0.4 mol/L NaCl, the enzyme was eluted with a potassium phosphate gradient (D). For both enzymes, fractions containing activity were ultrafiltrated up to 2 mL and stored at -20°C in the presence of 20% glycerol (vol:vol). Arrows indicate the steps at which the addition of the ligand was produced.

Fig 1. Purification of A and B glycosyltransferase from human serum. Blood group A, serum (450 mL) was centrifuged at 10,000 g for 15 minutes and applied to a sepharose 4B column. After washing (buffer A: 150 mmol/L NaCl, 0.1 mmol/L EDTA, and 20 mmol/L HEPES, pH 7.2) the activity was eluted with 20 μmol/L UDP (A) followed by dialysis and ultrafiltration through a membrane with a cut-off of 30,000 daltons and the volume reduced to 2 mL. This fraction was applied to a UDP-hexanolamine agarose column and was eluted with 20 μmol/L UDP-N-acetylgalactosamine (B). For the purification of B-glycosyltransferase the serum was diluted 1:3 and applied to a DE52 column. After washing with buffer A diluted 1:3, a gradient of NaCl was applied to elute the enzyme (C). Fractions containing activity were applied to a hydroxylapatite column. After washing with 0.4 mol/L NaCl, the enzyme was eluted with a potassium phosphate gradient (D). For both enzymes, fractions containing activity were ultrafiltrated up to 2 mL and stored at -20°C in the presence of 20% glycerol (vol:vol). Arrows indicate the steps at which the addition of the ligand was produced.

The reaction was started by the addition of 10 to 50 μL of serum or purified A or B glycosyltransferase. To quantitate the amount of A and B antigens formed, 20 μL of the incubation mixture were taken at different times and then incubated with 50 μL of anti-A or anti-B serum for ten minutes in an ice-cold water bath. After centrifugation at 79 g for two minutes, the erythrocyte pellets were gently resuspended, and the tubes were filled with 1 mL of ice-cold 3% Dextran 40, 1% polyethylene glycol 6000, followed by centrifugation at 30 g for two minutes. Under these conditions only agglutinated erythrocytes sedimented. The absorbance of the supernatant, corresponding to the nonagglutinated erythrocytes, was measured at 540 nm. The decrease in absorbance was proportional to the enzyme activity. One unit of glycosyltransferase (agglutination assay) was defined as the amount of enzyme that produced 50% of agglutination of red cells per hour under the described assay conditions.

B-glycosyltransferase activity was also measured by the incorporation of [U-14C]galactose into the soluble acceptor 2'-fucosyllactose as previously described. One unit of glycosyltransferase (incorporation assay) corresponded to the activity that incorporated 1 pmol of galactose per minute into 2'-fucosyllactose. IgG was prepared by absorption on protein A sepharose and

Assay of A- and B-glycosyltransferase activities. A- and B-glycosyltransferase activities were measured by their capacity to incorporate N-acetylgalactosamine and galactose, respectively, onto the common carbohydrate chain (H-substance) of saline-washed group O red cells. The procedure was as follows: aliquots of 20 μL of group O red-cell suspensions were incubated in 3 mL sterile plastic tubes at 37°C in a rotatory shaking water bath (40 cycles per minute) in the presence of 150 mmol/L NaCl, 15 mmol/L MnCl₂, 0.5 mmol/L UDP-N-acetyl D-galactosamine, and 50 mmol/L HEPES, pH 6.8 (A glycosyltransferase), or 150 mmol/L NaCl, 15 mmol/L MnCl₂, 0.5 mmol/L UDP-D-galactose, and 50 mmol/L HEPES, pH 6.6 (B glycosyltransferase), in a final volume of 150 μL. The reaction was started by the addition of 10 to 50 μL of serum or purified A or B glycosyltransferase. To quantitate the amount of A and B antigens formed, 20 μL of the incubation mixture were taken at different times and then incubated with 50 μL of anti-A or anti-B serum for ten minutes in an ice-cold water bath. After centrifugation at 79 g for two minutes, the erythrocyte pellets were gently resuspended, and the tubes were filled with 1 mL of ice-cold 3% Dextran 40, 1% polyethylene glycol 6000, followed by centrifugation at 30 g for two minutes. Under these conditions only agglutinated erythrocytes sedimented. The absorbance of the supernatant, corresponding to the nonagglutinated erythrocytes, was measured at 540 nm. The decrease in absorbance was proportional to the enzyme activity. One unit of glycosyltransferase (agglutination assay) was defined as the amount of enzyme that produced 50% of agglutination of red cells per hour under the described assay conditions.

Table 1. Purification of A- and B-Glycosyltransferase Activity From Human Serum

<table>
<thead>
<tr>
<th>Blood Group</th>
<th>Step</th>
<th>Specific Activity (U/mg of Protein)</th>
<th>Purification (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Serum</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sepharose 4B</td>
<td>7,500</td>
<td>75,000</td>
</tr>
<tr>
<td></td>
<td>UDP-hexanolamine agarose</td>
<td>16,000</td>
<td>160,000</td>
</tr>
<tr>
<td></td>
<td>DE52 (100 mmol/L NaCl)</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>DE52 (200 mmol/L NaCl)</td>
<td>21</td>
<td>210</td>
</tr>
<tr>
<td>A (pooled)</td>
<td>DE52</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hydroxylapatite</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>B</td>
<td>Serum</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DE52</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Hydroxylapatite</td>
<td>16,800</td>
<td>84,000</td>
</tr>
</tbody>
</table>

The enzyme activity was measured by the agglutination assay. Serum was centrifuged at 10,000 g for 15 minutes prior to use. All purification steps were carried out at 4°C.
elution under mild-acid conditions with 150 mmol/L NaCl, 50 mmol/L acetate, pH 3.5. The eluate was immediately neutralized with 0.1 mol/L HEPES, pH 8.0. This IgG fraction was given to the original serum. Cross-linking of IgG to protein A sepharose was carried out by incubation for 15 minutes at 25°C in the presence of 150 mmol/L NaCl, 0.2 mmol/L EDTA, 20 mmol/L TRIS HCl, pH 8.2, and 1 mmol/L of dimethylsuberimidate. To determine the antigenic serum proteins from control A, B, or O individuals recognized by the MF IgG fraction, 5 mL of serum from blood group A, B, or O donors was centrifuged at 10,000 g for 15 minutes and the supernatants incubated with 1 vol of protein A sepharose (4 mg of protein A/mL). After 30 minutes at 4°C the incubates were centrifuged (2,000 g) to remove the IgG fraction of the serum. These supernatants were filtered through a column of 0.3 mL of protein A sepharose cross-linked with the IgG fraction of MF (0.3 mL of serum). After washing the column with 150 mmol/L NaCl, 0.2 mmol/L EDTA, 20 mmol/L TRIS HCl, pH 8.2, a gradient of NaCl (from 150 mmol/L to 800 mmol/L) Nonidet P-40 (from 0% to 1%; vol:vol) was applied, and fractions were collected to determine protein and A or B glycosyltransferase when appropriate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel) and the electrophoretic transfer of proteins from the gel to nitrocellulose sheets were carried out following the manufacturer’s recommendations. Protein was measured according to Bradford.

RESULTS AND DISCUSSION

To establish clearly the nature of the inhibitor present in the serum of the patient transplanted with an ABO-incompatible bone marrow, A,- and B-glycosyltransferase activities were purified from human plasma to near homogeneity following Coomassie brilliant blue staining criteria. The purifications were carried out by ion-exchange chromatography and an improved affinity chromatography on a UDP-hexanolamine agarose column. These purifications were essential to characterize the presumed antibody produced by the patient and to rule out the possibility of interfering activities in the enzyme assay (mainly hydrolases and phosphatases). Figure 1 shows the purification profile of both activities through the different steps, and Table 1 summarizes the yield in these enzyme activities. It is interesting to note that if pooled blood-group A is used as a source of the enzyme, two peaks containing activity emerge after DE52 chromatography due to the presence of various isoenzymatic forms of this activity in human populations. For this reason blood-group A serum was used to purify the enzyme.

To characterize the inhibitory activity present in the post-transplant serum of MF, purified A,- and B-glycosyl-
glycosyltransferases. The use of two different methods for the assay of the glycosyltransferase activity as well as the transferred nitrocellulose membrane were incubated with purified IgG from either A, B, or O blood groups or post-transplant IgG from MF (dilution 1:500). After appropriate washing, the nitrocellulose strips were incubated with 1:3,000 rabbit anti-human IgG peroxidase conjugate. After becoming visible the recognized antigens through the peroxidase activity, a single band was evident in strips incubated in the presence of MF IgG fraction, regardless of the type of glycosyltransferase present (Fig 2). The mol wt of these bands were 50,000 and 44,000 daltons, which are in the range of the reported subunit mol wt of these enzymes. No staining was evident in strips incubated with IgG from A, B, or O control sera.

The titration of the antiglycosyltransferase antibody from MF serum was done by using purified A and B glycosyltransferase. The results are shown in Fig 3. A progressive inhibition of the enzyme activity was observed either through the transformation of O red cells into A or B blood groups or through the incorporation of [14C]galactose into 2-fucosylactose for the B-glycosyltransferase activity. The half-maximal inhibition of the purified A and B glycosyltransferase (agglutination assay) was obtained at 1 to 2 μg/mL of IgG for both enzymes and at 1.7 μg/mL of IgG for the B glycosyltransferase assayed by the incorporation test. These results are in the line of previous work on the presence of glycosyltransferase inhibitors in minor incompatible liver transplantation and in minor incompatible BMT and with the reported immunologic homology of the human blood group glycosyltransferases. The use of two different methods for the assay of the glycosyltransferase activity as well as the use of purified enzymes guarantees the interaction between the enzymes and the antibody.

From our results on the purification of the glycosyltransferases as well as those from previous work, the concentration of enzyme in plasma may be estimated to be lower than 100 ng/mL of plasma, although continuously present in this fluid. These circumstances may be considered to explain the rise of the antibody after ABO minor incompatibility BMT.

The use of this antibody against A and B glycosyltransferases as a clinical marker of the evolution of the engrafted bone marrow (or solid organ) in cases of ABO nonisogroup identity between donor and receptor is a subject that requires additional work to establish its value.

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