Design of the blood group AB glycoconjugate

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Although the nature of the blood groups A and B has been comprehensively studied for a long time, it is still unclear as to what exactly is the epitope that is recognized by antibodies having AB specificity, i.e. monoclonal and polyclonal antibodies which are capable of interacting equally well with the antigens GalNAcα1-3(Fuca1-2)Gal (A trisaccharide) and Galα1-3(Fuca1-2)Gal (B trisaccharide), but do not react with their common fragment Fucα1-2Gal. We have supposed that besides Fucα1-2Gal, A and B antigens have one more shared epitope. The trisaccharides A and B are practically identical from the conformational point of view, the only difference being situated at position 2 of the Galα-glycoside, i.e., trisaccharide A has a NHAc group, whereas trisaccharide B has a hydroxyl group (see formulas). We have hypothesized that the AB-epitope should be situated in the part of the molecule that is opposite to the NHAc group of GalNAc residue. In order to test this hypothesis we have synthesized a polymeric conjugate in such a way that de-N-acetylated A-trisaccharide is attached to a polymer via the nitrogen in position C-2 of the galactosamine residue. In this conjugate the supposed AB-epitope should be maximally accessible for antibodies from the solution, whereas the discrimination site of antigens A and B by the antibodies should be maximally hidden due to the close proximity of the polymer. Interaction with several anti-AB monoclonal antibodies revealed that a part of them really interacted with the synthetic AB-glycoconjugate, thus confirming our hypothesis. Moreover, similar antibodies were revealed in the blood of healthy blood group 0 donors. Analysis of spatial models was performed in addition to identify the hydroxyl groups of Fuc, Galα, and Galβ residues, which are particularly involved in the composition of the AB-glycoconjugate.

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Keywords: antibodies, blood group antigens, epitope

Abbreviations: BSA, bovine serum albumine; ELISA, enzyme-linked immunosorbent assay; HRPO, horseradish peroxidase; Ig, immunoglobulin; mAbs, monoclonal antibodies; PAA, poly[N-(2-hydroxyethyl)acrylamide]; PBS, phosphate buffered saline.

Introduction

The two erythrocyte antigens A and B form four blood groups. Individuals having only the A antigen belong to group A, those who have only the B antigen belong to group B, people with both antigens constitute group AB, and individuals lacking both antigens belong to group 0(H). Antigen H, the precursor of antigens A and B, is expressed on erythrocytes of the latter group, with the exception of the ‘Bombay’ phenotype [1].

The blood group of an individual can be revealed using monoclonal antibodies (mAbs). These mAbs are usually obtained by immunization of mice with human erythrocytes; the selection of antibody-producing cells (hybridomas) is also performed using erythrocytes A and B, followed by the confirmation of epitope specificity with the help of synthetic oligosaccharides [2]. Sometimes this technique gives rise to mAbs of the so-called AB-specificity [3,4], i.e. mAbs, which recognize A and B antigens equally well, agglutinate erythrocytes of individuals with the blood groups A as well as B. The common structural motif for trisaccharides A and B (Table 1) is Fucα1-2Gal, the so-called H antigen. However, anti-AB antibodies are not directed towards this antigen, because they do not agglutinate blood group 0(H) erythrocytes. In addition, anti-H antibodies can interact with erythrocytes of all four blood groups, A, B, AB, and 0 (though interaction with A-erythrocytes is often weak because a highly expressed A antigen masks the H epitope) [1], whereas anti-AB mAbs only bind to A, B, and AB red blood
pounds (\(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2\) glycosides) were synthesized as described [7,8].

### Materials and methods

Goat anti-mouse Ig antibodies conjugated with horseradish peroxidase gam-Ig-HRPO) were obtained from Boehringer Mannheim (Germany). Goat anti-human Ig (IgM + IgG + IgA) conjugated with horseradish peroxidase (gal-Ig-HRPO) was obtained from Southern Biotechnology Associates, Inc. (USA). Poly(4-nitrophenylacrylate), m.w. \(~40\) kDa, referred herein as "activated polymer" was obtained according to [6]. 

<table>
<thead>
<tr>
<th>A trisaccharide ((A_h))</th>
<th>B trisaccharide ((B_h))</th>
<th>H disaccharide ((H_a))</th>
<th>A (type 2) tetrasaccharide</th>
<th>B (type 2) tetrasaccharide</th>
<th>H (type 2) trisaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuca1-2</td>
<td>Gal</td>
<td>Gal</td>
<td>Fuca1-2</td>
<td>Gal/(\beta1-4)GlcNAc</td>
<td>Fuca1-2Gal/(\beta1-4)GlcNAc</td>
</tr>
<tr>
<td>GalNAc(\alpha1-3)</td>
<td></td>
<td></td>
<td>Gal/(\beta1-4)GlcNAc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A polyacrylamide conjugate of the 'AB' trisaccharide, 'AB'-PAA, was synthesized by attachment of compound (1) to the activated polymer via the NH\(_2\) group of the galactosamine moiety according to the method described earlier [6]. Affinity adsorbent 'AB'-Sepharose FF was obtained by attachment of 1 to activated polymer followed by immobilization on aminated Sepharose FF (Pharmacia-Biotech, Austria) according to method [6], ligand density 0.5 \(\mu\)mol per ml.

### ELISA for monoclonal antibodies

Plates were coated with PAA conjugated saccharide (30 kDa, 20 mol% of saccharide), 10 \(\mu\)g/ml in carbonate buffer (50 mM Na\(_2\)CO\(_3\)/NaHCO\(_3\), pH 9.6) for 60 min at 37°C, followed by 4°C overnight. Between all of the following steps the plates were washed three times with PBS (0.13 M NaCl, 3 mM KCl, 8 mM Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\), pH 7.2) containing 0.1% Tween. Plates were blocked by 150 \(\mu\)l 3% BSA in PBS. After washing, the mAbs were to be tested were added in serial dilutions (1/50, 1/100, etc.) in PBS containing 0.3% BSA for 60 min at 37°C. Subsequently, the plates were incubated with gam-Ig-HRPO (diluted 1/2000) for 60 min at 37°C. Finally, color was developed by a 30 min incubation in 0.1 M sodium phosphate/0.1 M citrate buffer, containing 0.04% o-phenylenediamine and 0.03% H\(_2\)O\(_2\) and the reaction was stopped by the addition of 100 \(\mu\)l 1 M H\(_2\)SO\(_4\). Absorbance was read at 492 nm with a Multiskan MCC 340 plate reader (Labsystems, Finland). Control reactions (blank) were performed by omitting the mAbs. Each assay was performed in duplicates and the optical densities of blanks were subtracted from the respective test values to obtain corrected absorbance values.

### Inhibitory ELISA for monoclonal antibodies

Plates were coated and blocked as described above. At the next stage inhibitors were added simultaneously with antibodies in a concentration ranging from 500 \(\mu\)g/ml to 100 ng/ml. Plates were incubated for 60 min at 37°C, then developed as stated above. Percent of inhibition was calculated as \((\text{OD}_A - \text{OD}_1) \times \)
100/OD_A where OD_A was the mean value of optical density in
the absence of the inhibitor, and OD_I was the mean value of
optical density in the presence of the inhibitor.

**ELISA for human sera.** Plates were coated with PAA
conjugated saccharide (30 kDa, 20 mol% of saccharide) or
saccharide-free PAA, 10 μg/ml in carbonate buffer for 60 min
at 37°C. Between all of the following steps the plates were
washed three times in PBS containing 0.1% Tween 20. Plates
were blocked by 1% BSA in PBS for 60 min at 37°C. After
washing the pooled serum or antibodies eluted from affinity
column were serially diluted by PBS containing 0.3% BSA,
added to the plate and incubated for 60 min at 37°C followed
by incubation with gah-Ig-HRPO (diluted 1/4,000) for 60 min
at 37°C. Color development and calculation of corrected optical
density values (subtraction of blanks) were performed as
described above.

**Affinity chromatography of human sera.** Pooled sera of 12
donors of blood group 0 were incubated for 30 min at 56°C
defor decomplementation and centrifuged (13,000g). The super-
natant was diluted 1:1 with PBS (0.15 M, pH 7.3) containing
0.02% NaN_3 and run over the column with the affinity ab-
sorbent ‘AB’-PAA-Sepharose overnight at room temperature
(flow rate ~30 μl/min). The column was washed with PBS con-
taining 0.02% NaN_3, then PBS/0.02% NaN_3/0.5% Tween 20,
and finally with PBS/0.02% NaN_3. Anti-AB-antibodies were
eluted by Gly-HCl buffer (0.2 M, pH 2.9, 0.02% NaN_3). Fra-
cions containing antibodies were neutralized immediately using
Tris-HCl buffer (2 M, pH 8.6). The antibody concentration
was measured at 280 nm. Specificity of the eluted antibodies
was analyzed using a panel of PAA-glycoconjugates, see **ELISA for
human sera.**

**Computational methods.** The crystal structure of the blood
group B trisaccharide [11] was used as a template to build the
molecular models of blood group oligosaccharide derivatives.
The structures were edited with the use of the Sybyl software
program (Tripos Inc, USA) for adding additional groups. Atom
type and charges of the carbohydrate moiety were defined ac-
cording to the PIM parameters that have been derived for car-
bohydrates [12]. For additional groups, partial charges were
calculated using the MNDO Hamiltonian in the MOPAC pack-
ge. Complete energy minimization of each structure was per-
formed with the use of the Tripos force field [13]. A dielectric
constant of 80 was used in order to minimize the influence of
an intramolecular hydrogen bond.

**Results and discussion**

The aim of this study was to reveal the nature of the epitope to
which the anti-AB antibodies bind to, i.e. antibodies capable of
interacting equally well with A and B blood group antigens, but
not reacting with the common antigen fragment Fucα1-2Gal.
Conformation of oligosaccharides A and B has been thoroughly
studied [2,11,14-18], and most of the cited works provide concord-
ant results. Moreover, oligosaccharides A and B are practi-
cally identical from the conformational point of view. As men-
tioned disaccharide Fucα1-2Gal can not be the AB epitope.
Thus, it could be concluded that the AB-epitope should include
the fragments of all the three monosaccharide residues brought
close to each other by the conformation of the oligosaccharide
scaffold. The only structural difference, which exists between
the trisaccharides A and B is located at position 2 of the Galα
residue: trisaccharide A bears the NAc group, whereas trisac-
charide B has the hydroxyl group. Taking into consideration the
aforesaid, we have supposed that the AB-epitope is situated op-
positely to this site, i.e. it should be situated maximally distant
from the A vs. B discrimination site of trisaccharides A and B
(see Figure 2). To test this hypothesis experimentally, we have
used mAbs obtained from the 4th Workshop on Monoclonal
Antibodies Against Human Red Blood Cells and Related Anti-
gens (Paris, 2001) [9]. The used mAbs should, according to the
data of submitting laboratories, have true anti-AB specificity.
Anti-A and anti-B mAbs were used as controls.

The only direct approach to reveal which particular groups of
an oligosaccharide interact with Fab fragment of antibodies is
the X-ray resolution of crystal structure of antibody containing
the antigen. However, crystallization of antibodies (or fragment
of antibodies) directed against carbohydrate, appeared to be a
difficult task. At the present time, only one crystal structure of
anti-blood group A (AC1001) Fv fragment has been solved and
in the absence of the carbohydrate antigen [19]. That is why we
have used the immunochemistry approach in order to test our
hypothesis concerning the situation of the AB-glycotope. We
have synthesized trisaccharide A without the N-acetyl group
and conjugated it to a polymer via a liberated amino group. In
such a conjugate the supposed AB epitope should be maximally
accessible as it is situated oppositely to the conjugation point.
In contrast, the discrimination site of antigens A and B should
be hidden from the interaction with antibodies due to the close
proximity of the polymer (‘stealth’ effect). 30 kDa polyacry-
lamide was taken as a polymer and the method described in [6]
was used for conjugation: oligosaccharide 1 in an amount of
20% mol was attached to fully activated polyacrylic acid. The
remaining –COO-nitrophenyl groups were converted to amides
upon the action of ethanolamine. Interaction of antibodies with
the obtained synthetic ‘AB'-antigen (Figure 1) and several re-
lated antigens were studied using ELISA [20]; the results are
summarized in Table 2.

It can be seen that none of the anti-A and anti-B mono-
clonals interacted with ‘AB'-PAA conjugate. This result was
expected: the recognition site absolutely necessary for anti-A
or anti-B was shielded by the polymer chain in ‘AB'-PAA.
From the presumed anti-AB antibodies submitted to the work-
shop, six were true anti-AB according to the data provided by
the submitting laboratories, i.e. 2-30, 2-31, 2-32, 2-33, 2-36,
and 2-38, whereas the other eight (data shown only for 2-35)
were either mixtures of anti-A and anti-B or the submitters
did not provide the corresponding information [9]. All such
cocktails interacted well with A and B trisaccharides or type 2
tetrasaccharides, (type 2 tetrasaccharide being the most expressed on human erythrocytes [1]). Meanwhile, none of these cocktails interacted with the ‘AB’-epitope. In contrast, three of the six true AB-antibodies, i.e. 2-30, 2-32 and 2-38, interacted with ‘AB’-PAA, though the affinity was different in each case. Antibody 2-30 interacted with the ‘AB’-epitope much weaker than with the A and B antigens, though reliably and reproducibly. Antibody 2-32 also recognized equally well both normal A and B antigens and ‘AB’, fully corresponding to our hypothesis of the ‘AB’-epitope. Finally, the antibody 2-38 resembled the previous one, but the binding with all three antigens was weak. We have additionally tested these three antibodies, 2-30, 2-32 and 2-38, on the interaction with H disaccharide and H (type 2) trisaccharide. It can be seen from Table 2 that there were no cases of interaction with H-antigens.

Specificity of the mAbs’ interaction with ‘AB’-epitope was confirmed by inhibitory analysis (see Figure 3). It can be seen that although the inhibitory potency of ‘AB’-PAA is not as strong as that of Atri-PAA, it is potent and dose-dependent. In sum, the obtained data favors our hypothesis concerning the nature and localization of the epitope for anti-AB antibodies. The further question is what particular groups make direct contact with anti-AB antibodies.

Molecular models of trisaccharides shown in Figure 2 permit one to speculate on this matter. Hydroxy groups at C4 and C6 of αGalNAc (or αGal), and hydroxy group at C2 of fucose are presumably involved in key polar interactions of oligosaccharides with antibodies. The hydroxyl group at C6 of the βGal,
Design of the blood group AB glycoprotein

Table 2. Epitope specificity of true anti-AB mAbs, A + B 'cocktail', and some anti-A and anti-B mAbs as probed with saccharide conjugates

<table>
<thead>
<tr>
<th>mAb, Workshop #</th>
<th>Specificity as declared by submitter</th>
<th>Interaction with</th>
<th>A (type 2)-PAA</th>
<th>B (type 2)-PAA</th>
<th>‘AB’-PAA</th>
<th>H (type 2)-PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-30</td>
<td>AB (true)</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-31</td>
<td>AB (true)</td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-32</td>
<td>AB (true)</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-33</td>
<td>AB (true)</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-35</td>
<td>AB (cocktail)</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-36</td>
<td>AB (true)</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-38</td>
<td>AB (true)</td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-8</td>
<td>A</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-9</td>
<td>A</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-10</td>
<td>A</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-11</td>
<td>A</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-51</td>
<td>B</td>
<td></td>
<td></td>
<td>++</td>
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<td>++</td>
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<tr>
<td>2-53</td>
<td>B</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-54</td>
<td>B</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2-55</td>
<td>B</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

The number of ‘+’ reflects the intensity of OD in ELISA, ‘−’ absence of binding, ‘nt’ not tested.

Even tough at longer distance (6 Å) may also participate in polar contact. Additional stabilization of the antigen-antibody complex can be performed by the van der Waals interaction of CH hydrogens on carbon C3, C5 and C6 of the βGal residue and the antibody. Thus, the fragments of all three monosaccharide residues are involved in the interaction.

A question arises: why do only three antibodies of the six interacted with the ‘AB’ epitope? One explanation might be that real carbohydrate chains of erythrocyte glycoproteins and glycolipids are larger than trisaccharides and the next residue, GlcNAc, is also important for the interaction with some antibodies. Most of anti-A and anti-B monoclonals used for erythrocyte typing recognize the type 2 tetrasaccharides (see the structures in Table 1) better or even much better than the respective trisaccharides [1]. Analysis of molecular models demonstrates that some atoms of GlcNAc residue are spatially close to the supposed AB-epitope and so they can therefore be a part of it. By this we can explain the fact that not all anti-AB mAbs studied by us interacted with the synthetic ‘AB’-epitope with a high affinity: presumably, the presence of the fourth monosaccharide residue, GlcNAc, is important. Whether this is true or not can be revealed by the synthesis of the corresponding ‘AB’-tetrasaccharide. Trisaccharide ‘AB’ was designed in such a way that its aglycon group –OCH₂CH₂CH₂NHCOCH₃ mimicked the corresponding fragment in GlcNAc residue of tetrasaccharide (type 2), see Figure 4, but, obviously, this mimetic approach is too far from the real tetrasaccharide structure.

Mengwasser et al. [21] have described monoclonal antibodies recognizing both B and A (notably weaker) antigens, but did not interact with the H antigen, i.e. actually anti-AB. These mAbs displayed the ability to bind breast carcinoma cells. Hopefully, a clear understanding of epitope specificity of anti-AB antibodies will provide the possibility to reveal and identify the so-called A-like and B-like antigens observed in many carcinomas.

It was especially interesting to test whether human blood of group 0 individuals contains antibodies recognizing ‘AB’-epitope similarly to monoclonals described above, as these antibodies could play a role in immune reactions during blood transfusions and organ transplantations. This knowledge could define strategies of recipient treatment in the case of ABH-mismatched transfusions and transplantations, and would also help in the more precise selection of donors and recipients in the corresponding cases. Human blood contains a very wide repertoire of anti-carbohydrate antigens and concentrations of the sought for anti-AB antibodies was expected to be low, limiting the chance to detect these antibodies directly in serum. Therefore, we used affinity isolation using synthetic adsorbent with the ‘AB’-epitope attached to Sepharose matrix. Tests for the presence of antibodies to the ‘AB’-epitope and related oligosaccharides in pooled sera of blood group 0 donors were performed similarly to the assay of murine antibodies (see above). Antibodies were revealed in acid eluate, which was immediately neutralized. The results shown in Figure 5a display that the starting serum contains antibodies interacting with ‘AB’-PAA, though, as expected, this fraction was minimal. On the contrary, the content of affinity isolated anti-‘AB’ antibodies in the eluate was high (Figure 5b). Indeed, anti-‘AB’ antibodies bind not only AB-PAA, but also Aₙᵥ-PAA and Bₙᵥ-PAA. It should be noted that the interaction of the affinity isolated antibodies with H antigen and Fuc-PAA (which are the fragments of ‘AB’) does not exceed the background level, i.e. we in fact
observed antibodies with the specificity similar to the specificity of mAbs as described above. Thus, human blood serum in fact does contain antibodies having AB-specificity. The role, which these antibodies play in innate immunity, blood transfusion and organ transplantation should be revealed by further studies.

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References


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