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Design of the blood group AB glycotope

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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(Fucα1-2)Gal (A trisaccharide) and Galα1-3(Fucα1-2)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 Galα residue, *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-glycotope, 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-glycotope.
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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

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Table 1. Structure of oligosaccharide ligands

Fucα1-2 Gal	A-trisaccharide (A_{tri})
GalNAcα1-3 Fucα1-2 Gal	B-trisaccharide (B_{tri})
Galα1-3 Fucα1-2Gal	H-disaccharide (H_{di})
GalNAcα1-3 Gal	A (type 2) tetrasaccharide
Fucα1-2 Galα1-3 Galβ1-4GlcNAc	B (type 2) tetrasaccharide
Fucα1-2 Fucα1-2Galβ1-4GlcNAc	H (type 2) trisaccharide

cells. It should be noted that true anti-AB mAbs are individual immunoglobulins, in contrast to a mixture of anti-A and anti-B monoclonals as in some commercial AB-reagents. Similarly, true polyclonal anti-AB cannot be separated into anti-A and anti-B subfractions, *e.g.* by way of absorption/elution using erythrocytes of blood groups A or B. The existence of a common AB epitope not linked to H rises the question which spatial carbohydrate epitope (glycotope) is recognized by anti-AB antibodies. Conformations of A and B oligosaccharides are now well established [1,5] and can serve as a structural basis for design of new compounds. In this study we have synthesized a polymeric conjugate expressing a putative AB epitope and studied its interaction with monoclonal antibodies and human sera.

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 (gah-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]. Tween 20 and bovine serum albumin were from Sigma (USA). All other chemicals were analytical grade from Fluka (Switzerland). MaxiSorp 96-well microtiter immunoplates were from Nunc (Denmark).

PAA neoglycoconjugates were obtained from Lectinity (Moscow). Non-conjugated (free) saccharides as spacered compounds ($-CH_2CH_2CH_2NH_2$ glycosides) were synthesized as described [7,8].

Sera from 12 healthy adult blood donors of blood group 0 were obtained from Sklifosofsky Research Institute of Emergency Care (Moscow).

Monoclonal antibodies. mAbs with the proposed anti-A, anti-B and anti AB specificity were obtained from Section 2 of the 4th Workshop on Monoclonal Antibodies Against Human Red Blood Cells and Related Antigens (Paris, 2001) [9] as cell culture supernatants with 0.1% NaN_3.

*3-Acetamidopropyl l-3-O-(2-amino-2-deoxy-α-D-galactopyranosyl)-2-O-α-L-fucopyranosyl-β-D-galactopyranoside (**1**) – trisaccharide* '*AB*' was obtained according to [10] from protected trisaccharide (**1a**) [7] by the following way: removal of benzylidene protecting group (80% AcOH, 70°C), deacetylation and detrifluoroacetylation (Et_3N, aqueous MeOH), *N*-acetylation (Ac_2O, Et_3N, MeOH), reduction of azide group to amine ($Ph_3P/THF/H_2O$) and its trifluoroacetylation (CF_3COOMe/ Et_3N), hydrogenolysis of benzyl groups (H_2, Pd/C, MeOH), removal of trifluoroacetic protection by aq. ammoniac. ^1H NMR: Bruker 500 MHz (δ, ppm; J, Hz; D_2O): 1.2d (3H, $J_{5,6}$ 6.6, CH_3 Fuc), 1.82m (2H, CH_2), 1.97s (3H, Ac), 3.08 (1H, $J_{2,1}$ 3.42, $J_{2,3}$ 10.76, 2c-H), 3.23m (2H, CH_2N), 3.65–3.85m (12H), 3.91–3.98m (3H, 3a-H, 3c-H, 4c-H), 4.21m (1H, 5c-H), 4.27d (1H, 4a-H), 4.39m (1H, 5b-H), 4.53d (1H, $J_{1,2}$ 7.6, 1a-H), 5.21d (1H, $J_{1,2}$ 3.42, 1c-H), 5.25 (1H, $J_{1,2}$ 3.66, 1b-H).

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 μmol per ml.

ELISA for monoclonal antibodies. Plates were coated with PAA conjugated saccharide (30 kDa, 20 mol% of saccharide), 10 μg/ml in carbonate buffer (50 mM $Na_2CO_3/NaHCO_3$, 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_2HPO_4/KH_2PO_4, pH 7.2) containing 0.1% Tween. Plates were blocked by 150 μl 3% BSA in PBS. After washing, the mAbs 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_2O_2 and the reaction was stopped by the addition of 100 μl 1 M H_2SO_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 duplicate 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 μg/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 $(OD_A - OD_1) \times$

$100/OD_A$ where OD_A was the mean value of optical density in the absence of the inhibitor, and OD_1 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 with 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 the 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 for decomplementation and centrifuged (13,000g). The supernatant 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 absorbent 'AB'-PAA-Sepharose overnight at room temperature (flow rate ~30 μl/min). The column was washed with PBS containing 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). Fractions 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 according to the PIM parameters that have been derived for carbohydrates [12]. For additional groups, partial charges were calculated using the MNDO Hamiltonian in the MOPAC package. Complete energy minimization of each structure was performed 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 concordant results. Moreover, oligosaccharides A and B are practically identical from the conformational point of view. As mentioned 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 NHAc group, whereas trisaccharide B has the hydroxyl group. Taking into consideration the aforesaid, we have supposed that the AB-epitope is situated oppositely 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 Antigens (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 polyacrylamide 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 related 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 monoclonals 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 workshop, 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

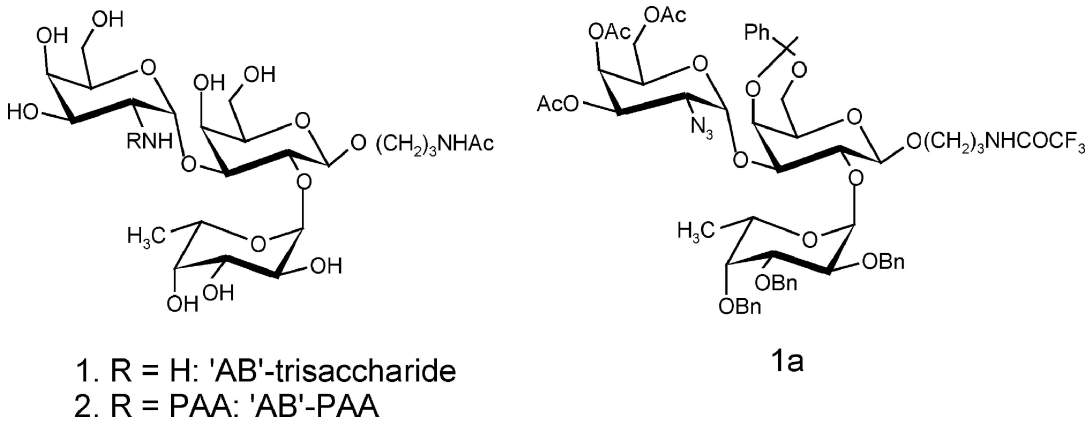

Figure 1. Structure of 'AB'-trisaccharide, its synthetic precursor and PAA conjugate.

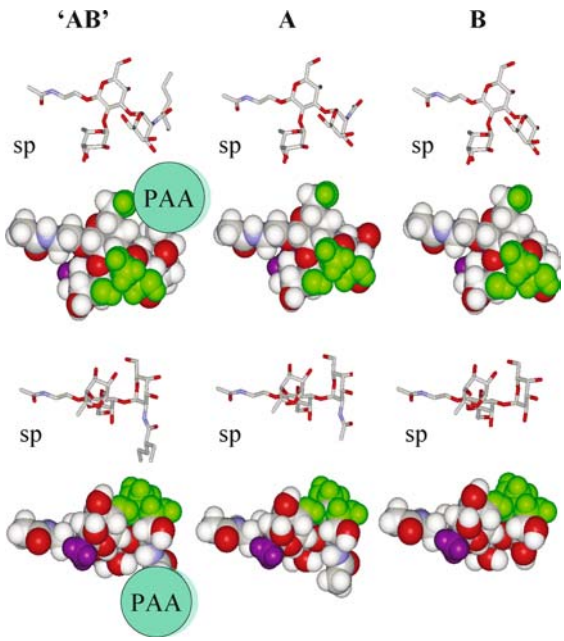

Figure 2. Molecular models of A_{tri}, B_{tri}, and 'AB' trisaccharides with atom color coded according to their type except for the methyl group C6 of fucose (violet) and the conformational 'AB' epitope (green). 'AB' trisaccharide is attached to the PAA matrix that is shown as a sphere, which masks 'normal' A or B epitopes. sp is $OCH_2CH_2CH_2NHAc$.

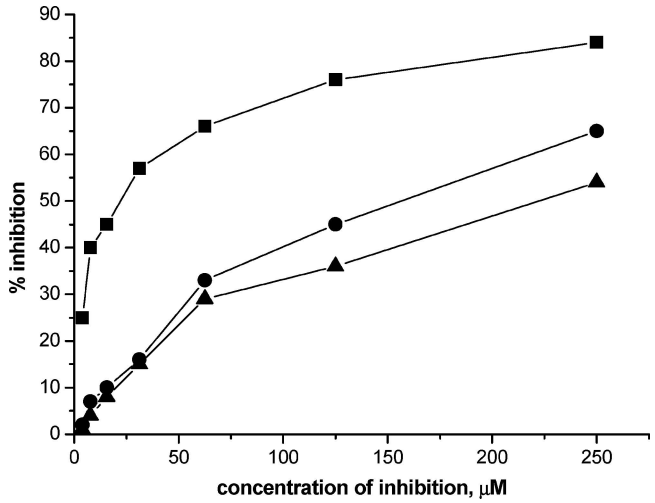

Figure 3. Concentration-dependent inhibition of the mAbs 2-32 interaction with A_{tri}-PAA (ELISA, see Experimental). Three inhibitors were tested: polymeric trisaccharide A (■), monomeric trisaccharide A (●) and polymeric 'AB'-PAA conjugate (▲).

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 A_{tri}-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,

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

mAb, Workshop #	*Specificity as declared by submitter*	*Interaction with*						
		A_{tri}-PAA	*A (type 2)-PAA*	*B_{tri}-PAA*	*'AB'-PAA*	*B (type 2)-PAA*	*H_{di}-PAA*	*H (type 2)-PAA*
2-30	AB (true)	+++	+++	+++	+	+++	–	–
2-31	AB (true)	++	++	–	–	++	–	–
2-32	AB (true)	+++	+++	+++	+++	+++	–	–
2-33	AB (true)	–	++	–	–	++	nt	nt
2-35	AB (cocktail)	++	++	–	–	–+	nt	nt
2-36	AB (true)	–	++	–	–	++	nt	nt
2-38	AB (true)	+	+	+	+	+	–	–
2-8	A	+++	+++	–	–	–	–	nt
2-9	A	–+	–+	–	–	–	–	nt
2-10	A	+++	+++	–	–	–	–	nt
2-11	A	+++	+++	–	–	–	–	nt
2-51	B	–	–	+++	–	++++	–	nt
2-53	B	–	–	+++	–	+++	–	nt
2-54	B	–	–	++	–	++	–	nt
2-55	B	–	–	+++	–	+++	–	nt

The number of '+' reflects the intensity of OD in ELISA, '–' absence of binding, 'nt' not tested.

even though 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 the 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 the 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'-*tetra*saccharide. Trisaccharide 'AB' was designed in such a way that its aglycon group $–OCH_2CH_2CH_2NHCOCH_3$ 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 antibodies 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 a 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 A_{tri}-PAA and B_{tri}-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

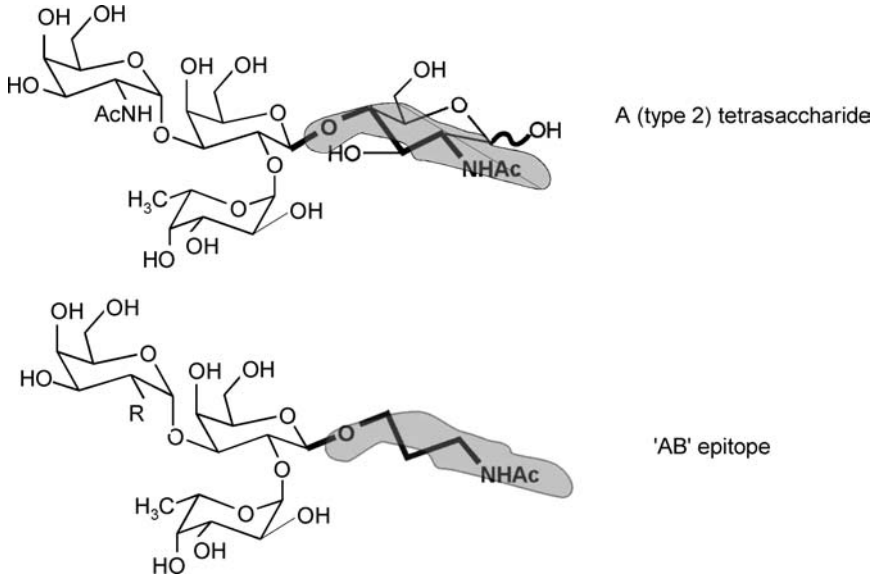

Figure 4. Rationales for the choice of 3-acetamidopropyl aglycon. Fragment of similarity is in bold.

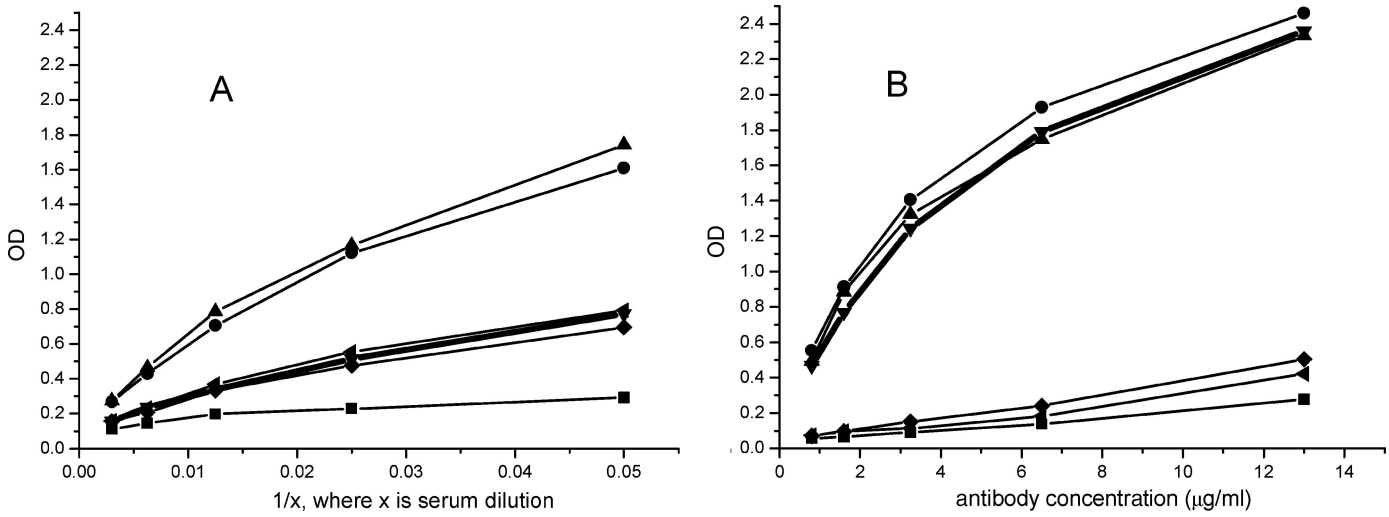

Figure 5. Binding of pooled sera obtained from 12 blood group 0 donors with carbohydrate antigens. A. Initial pool. B. Fraction eluted from 'AB'-Sepharose affinity column. The following conjugates were used for plate coating: PAA (■), A_{tri}-PAA (●), B_{tri}-PAA (▲), 'AB'-PAA (▼), H_{di}-PAA (♦), Fucα-PAA (◀).

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.

Acknowledgments

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