Original Article

ABO Blood System: Biosynthesis of Agglutinogenic Alkaline and Non-Agglutinogenic Acid Glycotopes of A and B Antigens at Different pHs of the Culture Medium

Delevsky, YP [1] ,Zinchenko, OA[2*].

1. *State Institution "Institute of Pathology of the Spine and Joints named after prof. M. I. Sitenko of the Academy of Medical Sciences of Ukraine", Kharkov.*
2. *State Enterprise "Ukrainian Scientific Pharmacopoeial Center for the Quality of Medicines", Kharkov.*

How to cite this article: **Delevsky YP, Zinchenko OA. ABO Blood System: Biosynthesis of Agglutinogenic Alkaline and Non-Agglutinogenic Acid Glycotopes of A and B Antigens at Different pHs of the Culture Medium. Archives of Razi Institute.2024;79(1):55-67.** DOI: 10.32592/ARI.2024.79.1.55

Article Info:
Received: 20 May 2023
Accepted: 18 July 2023
Published: 29 February 2024

Corresponding Author's E-Mail:
dielvna@gmail.com

ABSTRACT

The biosynthesis of agglutinogenic and adsorbing groups A and B glycotopes of the erythrocyte's membrane is mediated by the activity of specific glycosyltransferases. This study aimed to assess the nature of the biosynthesis of A and B antigenic glycotopes, depending on the pH of the medium during the cultivation of erythrocytes, and the antigenic (transferase) characteristics of the donor serum of the other group. Monoclonal antibodies (Mabs) were obtained from IGBRL under Program IV of the International Workshop on Monoclonal Antibodies and Red Blood Cell Antigens. Biosynthesis was performed using erythrocytes, fresh serum, medium 199, and an antibiotic solution. The agglutinogenic characteristics of 11 out of 33 samples changed by the end of the cultivation period due to the acquisition of additional agglutinogen corresponding to the donor serum. None of the samples lost their inherent agglutinogen due to its absence in the donor serum. Four of six samples of O(I) erythrocytes acquired the ability to be agglutinated by anti-A reagents, especially by the polyclonal anti-A, and the manifestation of agglutination depended on the reaction time. Two of the three samples with initial A(II) agglutinogenic specificity added to the donor serum with Bc'+ characteristic of the erythrocytes acquired this characteristic. However, none of the five A(II)Ac'+ samples cultured in the serum of Ac'-O(I)Ac'-Bc'+ and O(I)Ac'-Bc'- donors lost their inherent earlier Ac'+ characteristic. The investigation of the inhibitory ability of alkaline and acidic glycoconjugates isolated from membranes revealed that alkaline Alp-00 and Alp-1 glycotopes isolated from glycolipids had the highest inhibitory activity, and the degree of inhibition of polyclonal anti-A antibodies was even higher than that of monovalent BRIC-131. This study showed the possibility of the biosynthesis of specific non-agglutinogenic A and B glycotopes under the influence of a different group's serum as a source of the corresponding transferase.

Keywords: Agglutination, Blood, Detection, Erythrocyte, Transferase

1. Introduction

The ABH antigens are the best-known carbohydrate antigens given their importance for blood transfusion and transplantation. The A antigen is synthesized by the A glycosyltransferase (α1,3-N-acetylgalactosaminyltransferase) that adds N-acetylgalactosamine (GalNAc) in an α(1,3)-linkage to Gal within the H antigen. The B antigen is produced by the B glycosyltransferase (α1,3-galactosyltransferase) that adds Gal in an α(1,3)-linkage to the H antigen terminal Gal (1, 2).

Studies on the biosynthesis of glycotopes of the erythrocyte membrane are very rare since the process of antigen synthesis requires the accurate performance of all stages, with the detection of specific glycosyltransferases and the identification of carbohydrate structures. Moreover, the synthesis of ABH erythrocytes' antigens at different pH values in the cultural medium has not been investigated. Meanwhile, the activities of glycosyltransferases are known to be strongly dependent on pH values, and the estimation of their activity *in vitro* might help to elucidate the optimal conditions for the synthesis of ABH structures.

Both A and B transferases are highly specific toward the H antigen as the sugar acceptor. The enzymes react exclusively with those glycoconjugates and oligosaccharides carrying the terminal group Fuc(α1-2)Gal(β1-x). Type 1 and 2 precursor chains lacking terminal (α1-2)-linked fucose are not as active as substrates. Both transferases were found to show approximately equal affinities toward type 1 and type 2 H structures.

The enzymes are specific for the sugar nucleotide; however, it has been noticed that the B transferase, when incubated at elevated pH values, is able to transfer the A specific α-GalNAc onto H structures. A transferase may transfer some quantities of the B determinant α-galactose to low molecular weight acceptors. This could explain the fact that A1 erythrocytes contain low quantities of the B antigen, and cells from donors with extremely high B transferase activity react weakly but distinctly with anti-A antibodies. Studies on the enzyme kinetics of the A transferase yielded similar KM values for UDP-GalNAc and UDP-galactose but showed a decreased vmax value for the galactose transfer. The B transferase showed similar transfer velocities for GalNAc and galactose but a greatly increased KM value for UDP-GalNAc (3, 4).

A1 and A2 gene products found in A1 or A1B and A2 or A2B sera, respectively, are distinguishable from each other according to their kinetic properties, including the optimum pH activity (6.0 or 7.0, respectively), metal requirement (the effect of Mg++), and the *in vitro* conversion of O into A erythrocytes. A1 and A1B sera gave high erythrocytes-converted titers, whereas A2 sera led to very weak values. Unlike A2 sera, A2B sera, being a good source of A enzyme for erythrocyte conversion, suggested a possible interaction between A2 and O gene products, as well as A2 and B gene products. A simple and reproducible assay, established on pH ratio-dependent values, allows the direct recognition of A1A2 genotypes among A1 subjects, meaning that both A1 and A2 enzymes are present in heterozygote sera. The activity of Am enzymes has been reported to be 1/2 to 1/3 of that of A1 or A2 controls. In prolonged incubation times, very weak A enzyme activity (1/50 to 1/200 of controls) was found in Ay sera. Therefore, the recessive Ay gene appears to be not completely silent but blocks A enzyme synthesis in most cell lines. A3 samples are highly heterogeneous. The occurrence of A3 serum exhibiting strong A enzyme activity (1/2 to 1/3 of controls) and kinetic properties similar to those described in A1 sera was also reported. In standard or prolonged incubation times, alpha-N-acetylgalactosaminyltransferase seems to be absent in several samples of Ax, Aend, or Ael sera (5).

The mutation in α-1,3-N-acetylgalactosaminyltransferase may lead to the Ax phenotype by reducing the stability of the enzyme (4).

The isotypic labeling of blood cells is complex and is not limited to the agglutinogens identified in the agglutination test. This, in particular, is evidenced by the discrepancy between the agglutination and absorption characteristics, revealed for the first time in the system of HLA antigens (the agglutination-negative–absorption-positive [ANAP] phenomenon) (6).

The most convincing argument in favor of the dual nature of the ABH antigen labeling of erythrocytes, agglutinogens, and non-agglutinogens could be the difference in the nature of their biosynthesis. Some previous studies have considered the existence of only one type of corresponding transferases responsible for the biosynthesis of A and B antigens (7). However, it

became clear that there were not one but two types of isoelectrically different transferases. Therefore, the isoelectric point of B specificity, which determines the galactosyl transferase of one type, lies in the alkaline region (pI 8.8), while the other is in the acid region (pI 4.8) (8). For N-acetyl-D-galactosamine-transferase, which is responsible for A specificity, the maximum activity of one type is manifested at pH 5.8 and the other at pH 7.8 (9). Although it was found that the transferase that is more active at pH 5.8 is more often detected in A1 subjects and the alkaline type is mostly observed in A2 subjects, this difference was not associated with the phenomenon of erythrocyte agglutination. Meanwhile, comparison of the antigenic spectrum of A1, A2, and Ax (the cascade of falling agglutination of erythrocytes) showed that acid type A glycotopes (of lipid origin) exhibit equally expressed inhibitory anti-A Mab activity (including Ax); in other words, they are not agglutinogens (10). If we proceed from isoelectric interactions, then the alkaline type of transferase should be responsible for this acidic type of glycotope. However, this dependence was not manifested in relation to the acidic glycoconjugates of protein origin. Moreover, the decrease in the inhibitory activity of this particular type of Apr-3 glycoconjugates was most evident when comparing A1, A2, and Ax. Attempts to explain this phenomenon were based only on assumptions. All of the above substantiated the aim of this study to assess the nature of the biosynthesis of A and B antigenic glycotopes, which is predominantly acidic depending on the pH of the medium during the cultivation of erythrocytes, as well as the antigenic (transferase) characteristics of the donor serum of the other group.

2. Materials and Methods

2.1 Monoclonal Reagents

Mabs were obtained from IGBRL under Program IV of the International Workshop on Monoclonal Antibodies and Red Blood Cell Antigens (Paris, 2001). High titer polyclonal anti-A, anti-A Mab BRIC 131 (9W1), BRIC 145 (9W2), 2-4 (Workshop-IV), 2-10 (Workshop-IV), as well as 2-28 (Workshop-IV), anti-A1 2-24 (Workshop-IV), and anti-H BRIC 39 (9W7) were used.

2.2 Antigens

Glycoprotein fragments were isolated using the treatment of erythrocytes with 1% trypsin solution (Spofa, Slovakia) under the previously described conditions (11) in the second and third series of experiments with additional heating at 60°C for 30 min. The chloroform-methanol method was used to isolate glycolipids from erythrocyte membranes (12). Further isolation and purification of glycoconjugates were carried out on DEAE-cellulose (Reahnal, Hungary) with elution in an NaCl gradient and decreasing pH, and on DEAE-Sephadex A-50 (Farmacia, Fine Chemicals, Sweeden). The yield of fractions was evaluated photometrically (at 205 nm, 280 nm, and the full UV spectrum on spectrophotometers SF-26, Specord) and serologically.

2.3 Serological Studies

Hemagglutination reactions were carried out on 96-well panels according to the standard method using O, A1, B, and AB erythrocytes (13), taking into account visually (+) and under a microscope (+m) both the titer and the severity of agglutination in dilutions (Score). The score was calculated according to W.I. Marsh (14) and the previously described scale (10). The inhibition reaction of Mabs was performed in a 1% albumin solution after 60 min of contact with the antigenic substrate (glycoconjugates), followed by titration and 60 min incubation with a suspension of test erythrocytes. The decrease in their agglutinating ability (Score) was then estimated. The cell electrophoresis test was performed as previously described (15) using polyclonal group-specific antibodies and guinea pig complement.

2.4 Biosynthesis

Under aseptic conditions, 0.1 ml of the sediment of three times-washed erythrocytes, 2 ml of fresh serum, 2 ml of medium 199 (RPMI 1640 in the second and third series), and 0.1 ml of antibiotic solution were incubated in test tubes. In control samples, 0.5 ml of a 1% solution of Trilon B (EDTA) was added to the medium or serum, mixed with the medium, and then heated at 60°C for 30 min. The tubes were incubated at 37°C for two days (in the second and third series, for four days, with the change of the supernatant after 48 h).

2.5 Gas Chromatography

In sample preparation, 1 ml of the analyzed solution was placed in a tube of 1.8 ml and lyophilized at +5°C in a vacuum at a residual pressure of 1 mm Hg over freshly calcined granules of silica gel KSK. After that, 0.5 ml of purified tetrahydrofuran was added to the dry

residue of 0.5 ml of trifluoroacetic anhydride. The tubes were sealed with a rubber membrane and a fluoroplastic layer and kept at 30°C for 4 h. Next, 2 ml of the resulting solution was chromatographed on a Shimadzu GC-14B gas chromatograph with a flame ionization detector and an AOC-14 autosampler under the following conditions: 1) quartz capillary column 25 m×0.32 mm in size, 2) stationary phase/SE of 54, 3) layer thickness of 0.25 μm, 4) column temperature of 75°C (2 min) and a temperature increase at a rate of 8°C/min to a temperature of 240°C (15 min), 5) 0.95 ml/min speed of the carrier gas (hydrogen), 6) evaporator temperature of 240°C, 7) detector temperature of 250°C, and 8) flow division of 1:20 (1 min).

The ratio of the components was determined by the Chromatopak C-R7A integrator using the internal normalization method according to the following formula:

$$Ci = \frac{Si * 100}{\sum_{i-1}^{i=n} Si}$$

where Si is the peak area of the determined component, n is the number of components to be determined, and Ci is the concentration of the determined component.

The study was approved by the Ethics Committee of the institute (Protocol 4).

3. Results

The isotype characteristics of erythrocytes were analyzed after cultivation with the serum of other groups at pH 7.4. Thirty-three samples of erythrocytes were studied in the usual agglutinogenic test and in the complement-dependent test of cell electrophoresis before and after cultivation with the other group's serum. The erythrocytes and sera of donors of O(I)Ac'-Bc'-, O(I)Ac'+Bc'-, O(I)Ac'-Bc'+, A(II)Ac'+Bc'-, A(II)Ac'+Bc'+, B(III)Ac'-Bc'+, B(III)Ac'+Bc'+, AB(IV)Ac'+Bc'-, and AB(IV)Ac'-Bc+ groups were used. Table 1 presents data on the isotype characteristics of 10 samples of erythrocytes of group O(I) before and after cultivation. The same samples served as controls, for which the serum added to the medium was preheated for 30 min at 60°C or Trilon B was added to the cultivation medium.

Based on the findings, four of the seven samples of the O(I)Ac'-Bc'- group showed non-agglutinogenic complement-binding glycotopes of Ac'+ specificity, in accordance with the characteristics of the donor serum, and three demonstrated glycotopes with Bc'+ specificity, again in accordance with the features of the isotype characteristics of the serum donor. Only in one case did the studied characteristics of erythrocytes not change. Glycotopes with Bc'+ specificity appeared in one case of O(I)Ac'+ samples from the three O(I) samples of erythrocytes that initially had Ac'+ or Bc'+ characteristics after incubation with B(III)Ac'+Bc'+ donor serum. Two samples of this group of erythrocytes are of interest since they were placed in the medium with serum without a transferase responsible for the biosynthesis of Ac'+ or Bc'+ glycotopes from the O(I)Ac'-Bc'- donor. As it turned out, in these cases, there was a kind of catalysis of the corresponding glycotopes, possibly with the participation of antibodies, and erythrocytes lost their inherent antigenic specificities (Ac'+ or Bc'+) (Table 1). Two of the three samples with initial A(II) agglutinogenic specificity added to the donor serum with Bc'+ characteristics acquired this specificity. However, none of the five samples of A(II)Ac'+ cultured in the serum of a donor with Ac'-, including O(I)Ac'-Bc'+ and O(I)Ac'-Bc'-, lost their inherent Ac'+ characteristics. Three of the four samples also acquired Ac'+ specificity in the group of erythrocytes with the initial B(III)Ac'-Bc'+ characteristics in the medium with A(II)Ac'+Bc'- serum. No loss of Bc'+ specificity was observed. At the same time, three of the five samples with B(III)Ac'+Bc'+ characteristics lost Ac'+ glycotopes in the serum of the donor O(I)Ac'-Bc'-, and B(III)Ac'-Bc'+ specificity was detected in three samples.

Table 1. Biosynthesis of non-agglutinogenic complement-binding Ac´ and B c´ glycotopes on O erythrocytes at initial pH 7.4.

Initial characteristic of erythrocytes	Characteristics of the donor serum donor added to the culture medium					Inactivation of transferases
	O(I) Ac'-Bc'-	O(I) Ac'+Bc'-	A(II) Ac'+Bc'-	B(III) Ac'-Bc'+	B(III) Ac'+Bc'+	
O(I) Ac'-Bc'-		O(Ac'+Bc'-)				-
		O(Ac'-Bc'-)				Trilon B
-²-			A(Ac'+Bc'-)			-
			O(Ac'-Bc'-)			Trilon B
-²-			O(Ac'+Bc'-)			-
			O(Ac'-Bc'-)			60°C , 30'
-²-				O(Ac'-Bc+)		-
				O(Ac'-Bc'-)		60°C , 30'
-²-				B(Ac'-Bc+)		-
				O(Ac'-Bc'-)		60°C , 30'
-²-					O(Ac'-Bc'-)	-
					O(Ac'-Bc'-)	60°C , 30'
-²-					O(Ac'+Bc'+)	-
					O(Ac'-Bc'-)	Trilon B
O(I) Ac'+Bc'-	O(Ac'-Bc'-)					-
	O(Ac'+Bc'-)					Trilon B
-²-					B(Ac'+Bc'+)	-
					O(Ac'+Bc'-)	Trilon B
O(I) Ac'-Bc'+	O(Ac'-Bc'-)					-
	0(Ac'-Bc+)					Trilon B

In the group of erythrocytes with AB(IV)Ac'+Bc'-, both samples in B(III)Ac'-Bc'+ serum acquired additional Bc'+ specificity, and in the group with AB(IV)Ac'-B'+ specificity, one sample in the medium with A(II)Ac'+ serum acquired additional Ac'+ specificity. Therefore, 15 of the 17 samples of glycotopes acquired Ac'+ or Bc'+ specificity (Ac'+ specificity: 8 and Bc'+ specificity: 7). Moreover, 4 of the 16 samples lost the corresponding non-agglutinogenic complement-binding glycotopes. The agglutinogenic characteristics of 11 of the 33 samples examined changed by the end of the cultivation period due to the acquisition of additional agglutinogen corresponding to the donor serum. None of the samples lost their inherent agglutinogen due to its absence in the donor serum.

The biosynthesis of A glycotopes was performed after the cultivation of erythrocytes in an acidic medium. In the experiments of this series, the isotypic characteristics of O(I)Ac'-Bc'- erythrocytes were evaluated after cultivation with A(II)Ac'+Bc'- serum at pH 5.6 for four days (with the change of serum, medium RPMI-1640, and acetate-acetic acid buffer after the first two days). When analyzing the results, attention was drawn to the changed agglutinogenicity of the samples. Four of the six O(I) samples of erythrocytes acquired the ability to be agglutinated by anti-A reagents, especially strongly by polyclonal anti-A, and the manifestation of agglutination depended on the reaction time (Table 2).

Table 2. A agglutinogenic characteristics of O(I) erythrocytes acquired after cultivation with A(II) sera in an acidic medium.

Time	Exposure 1-3'		Exposure 10'		Exposure 15'	
RBC samples	N 1	N 3	N 1	N 3	N 1	N 3
Polyclonal anti-A	+'m	+m	+	+	+	+
Anti-A MAb BRIC 131	-	-	+'m	-'m	+	+m
Anti-A MAb BRIC 145	-	-	-	-	+m	-'m

Note: 1',3',10',15 ' - minutes.

No less interesting results of a study of the inhibitory ability of alkaline and acidic glycoconjugates isolated from membranes were obtained. The degree of inhibition of Mabs BRIC-131 and BRIC-145 and the high titer of polyclonal anti-A, alkaline Alp-00, and Alp-1 glycotopes isolated from glycolipids showed the highest inhibitory activity, and the degree of inhibition of polyclonal anti-A antibodies was even higher (indices of inhibition by glycoconjugates Alp-00: 30, Alp-1: 14) than that of monovalent BRIC-131 (indices 4 and 5, respectively). When the complex of additionally heated fractions was separated again on an anion exchanger, the elution peaks of serologically active fractions were not changed. Importantly, the acidic fraction of Alp-3, in contrast to the alkaline one, did not show A specific inhibitory properties.

Table 3 shows the results of a study on the Mab inhibitory ability of Alp-00 and Alp-1 glycoconjugates (samples A 44 and A 43) isolated after the culturing of O(I)Ac'-Bc'-erythrocytes in an acid medium with the A(II) serum.

Table 3. Inhibiting Mab ability of glycoconjugates isolated after cultivation in the acidic medium O(I) of erythrocytes with serum A (II).

Type of glycoconjugates Sample N	Monoclonal antibodies (Workshop IV)			
	2-4 anti-A	2-10 anti-A	2-24 anti-A1	2-28 anti-A
Alp-00 Sample A 44	$\bar{x}$ 1 = 26.6 ± 1.2 $\bar{x}$ 2 = 20.2 ± 1.3 **- 6.4**	$\bar{x}$ 1=55.3±6.3 $\bar{x}$ 2=51.3±4.8 **-4**	$\bar{x}$ 1=35.5±1.3 $\bar{x}$ 2=32±1.6 **- 3.5**	$\bar{x}$ 1=24.5±1.8 $\bar{x}$ 2=19±1.2 **-5**
Alp-1 Sample A 43	$\bar{x}$ 1 =26.6±1.2 $\bar{x}$ 2 =22±2.4 **-4.6**	$\bar{x}$ 1 =55.3±6.3 $\bar{x}$ 2=51.8±3.9 **-3.5**	$\bar{x}$ 1 =35.5±1.3 $\bar{x}$ 2=28.5±1.2 **-7**	$\bar{x}$ 1=24.5±1.8 $\bar{x}$ 2=20.5±2.07 **-4**

Note: Score, inhibition index $\bar{x}$ 1–2 $\bar{x}$; A2 test - erythrocytes.

Table 4. Mab inhibiting ability of glycoconjugates isolated after cultivation of O(I)Ac'-Bc'- erythrocytes in the alkaline medium with serum A(II)Ac'+Bc'- or O(I)Ac'+Bc'-.

Type of glycoconjugates Sample N	Serum in culture medium	Monoclonal antibodies			
		2-4 anti-A	2-10 anti-A	2-24 anti-A1	2-28 anti-A
Alp-3, Sample A41	A(II)Ac'+Bc'-	$\bar{x}$ 1 = 26.6 ± 1.2 $\bar{x}$ 2 = 22 ± 2.2 **- 4.6**	$\bar{x}$ 1=55.3±6.3 $\bar{x}$ 2=49.6±4.1 **-5.7**	$\bar{x}$ 1=35.5±1.3 $\bar{x}$ 2=31±1.4 **-4.5**	$\bar{x}$ 1=24.5±1.8 $\bar{x}$ 2=19±1.4 **-5.5**
Apr-3, Sample A42	O(I) Ac'+Bc'-	$\bar{x}$ 1 = 26.6 ± 1.2 $\bar{x}$ 2=20.4 ± 1.1 **- 6.1**	$\bar{x}$ 1=55.3±6.3 $\bar{x}$ 2=54.2±5.2 **-1.1**	$\bar{x}$ 1=35.5±1.3 $\bar{x}$ 2=31.5±1.1 **-4**	$\bar{x}$ 1=24.5±1.8 $\bar{x}$ 2=18.5±2.1 **-6**

Note: Score, inhibition index $\bar{x}$ 1 - $\bar{x}$ 2 ; test-erythrocytes A 1; Alp-3 - the acid type of glycoconjugates from glycolipids of membranes; Apr-3 - the acid type of glycoconjugates from glycoproteins of erythrocyte membranes.

Alp-00 was the most alkaline type of glycoconjugate isolated from the glycolipids of erythrocyte membranes. Alp-1 was an alkaline type of glycoconjugate isolated from erythrocyte membrane glycolipids.

As the data show, three of the four Mabs used were significantly inhibited by biosynthesized alkaline glycoconjugates with acquired A specificity after cultivation. A relatively low degree of inhibition is consistent with the inhibition of Mabs by glycoconjugates of this type isolated from the membranes of A(II)Ac'+Bc'- erythrocytes that have not been cultivated (16).

The biosynthesis of A and B glycotopes was performed after the cultivation of erythrocytes in an alkaline medium. After the cultivation of O(I)Ac'-Bc' erythrocytes for four days at pH 8.1 in a medium with O(I)Ac'+Bc'- donor serum, the fraction of acid glycoconjugates of the protein origin acquired an expressed anti-A inhibitory activity (Apr-3, sample A 40, BRIC-145 inhibition index: 21). The same fraction weakly inhibited the polyvalent anti-H (index: 5) and did not inhibit the monoclonal anti-H BRIC-39 (index: 0). The anti-H activity was retained in the less acidic Apr-2 fraction (Hpr-2 with a pI about 7.2), which inhibited BRIC-39 (inhibition index: 11) and polyclonal anti-H (index: 19). This fraction did not possess anti-A inhibitory activity (index with BRIC-145: 0). A serologically active fraction, Apr-3 (sample 40), was heated at 60°C for 30 min to remove the possible protein and amino acid residues after trypsinization from glycoconjugates, which are also able to change pI, and then Apr-3 was analyzed by chromatography. After repeated chromatography of this fraction, it turned out that a significant part of the inhibitory activity shifted to the more alkaline fractions, Apr-00, pI~8.0 (sample A140, inhibited polyclonal anti-A with index: 28), and Apr-2 (anti-A inhibition index: 11), while the sufficiently high serological activity of the Apr-3 acid fraction (sample A42, inhibition index: 32) was retained. A similar finding was observed after cultivating O(I)Ac'-Bc'- erythrocytes in the medium with A(II)Ac'+ serum. In this regard, the results of the biosynthesis of acidic glycotopes of lipid origin are of interest. No shift in the activity of the fractions was observed after heating and repeated chromatography for purification. Polyclonal anti-A was inhibited by the Alp-00 fraction (index: 17), the Alp-2 fraction (index: 5), and the Alp-3 fraction (index: 23).

Table 4 presents the data on Mab inhibiting ability of the mentioned glycoconjugates of the acid type isolated from the membranes of O(I)Ac'-Bc'- erythrocytes after their cultivation in alkaline medium with the sera of donors with Ac'+ markers, including A(II)Ac'+Bc'- and O(I)Ac'+ Bc'-. As can be seen in the table, the glycoconjugates in six of the eight cases showed significant inhibitory activity against Mabs Worksop IV 2-4, 2-24, and 2-28. More activity against glycoconjugates of lipid origin, Mabs 2-10, showed a certain selectivity (however, not significant).

It is important to note that under alkaline conditions, A2 transferase, which is apparently responsible for the biosynthesis of acidic non-agglutinogenic Ac'+ glycotopes, should have shown (16) and clearly has shown its activity, and this activity was revealed in glycotopes cultivated both in A(II)Ac'+Bc'- and O(I)Ac'+Bc' sera (or Ax). Therefore, it is reliably present in these sera.

Importantly, under these conditions, the biosynthesis of both acidic glycolipid epitopes (associated with the concept of non-agglutinogenic hidden ABH glycotopes) and acidic glycoprotein epitopes (on the contrary, indicators of the degree of agglutinability of erythrocytes in the A1, A2, and Ax cascades) was observed (10, 17). The dissociation of glycoprotein complexes was obtained by heating, and repeated ion-exchange chromatography made it possible to clarify this contradiction and isolate the non-agglutinogenic acid oligosaccharide component, Apr-3, which does not differ in serological activity from its acid glycolipid component.

The gas chromatography of the natural and biosynthesized acid A glycoconjugate was performed. For the qualitative and quantitative analysis of the components of glycoconjugates, two samples were obtained from donors with natural acidic A antigenic markers, including A38, donor A(II)Ac'+Bc'-, and A30, donor O(I)Ac'+Bc'-, and two were obtained as a result of biosynthesis after the incubation of O(I)Ac'-Bc'- erythrocytes in a culture medium with donor serum A(II)Ac'+Bc'- (sample A41) or in a medium with donor serum O(I)Ac'+Bc'- (sample A42) (Figure 1). Such a selection of samples allowed us to compare acidic glycoconjugates that are homogeneous in charge but different in origin (Table 5).

On the chromatograms of all the samples, five main peaks were revealed (N1, 5, 7, 9, and 11), indicating the fundamental similarity of all the main components in the compared preparations. Of these, the terminal monosaccharides that determine A and H specificity are of particular interest. Parallel chromatograms of the control samples of N-acetyl-D-galactosamine (A specificity) showed that in the studied samples, the peak of its output corresponds to the chromatograms of fraction N5 (retention time 10.848 min), quantitatively and dominantly present in all the samples and observed in the biosynthesized glycotopes even in a higher ratio

than in the samples with natural A specificity. Fraction N1 of glycoconjugates was quantitatively represented in the second place on the control chromatogram corresponding to L fucose and the terminal monosaccharide responsible for H-specificity (retention time: 4.213 min).

Noticeably, in the biosynthesized A41 and A42 samples, the relatively higher specific gravity of these two components was observed, apparently due to the higher purity of the fractions that were previously subjected to gentle heating and repeated chromatography on an anion exchanger. In general, the comparison of acid glycoconjugates with A specificity indicates the obvious similarity of samples in terms of the main structural components of glycoconjugates of both natural and biosynthetic origin, isolated from both glycoproteins and glycolipids.

It is also important to note the fact that N-acetyl-D-galactosamine-transferase activity for the biosynthesis of acidic non-agglutinogenic A glycotopes in an alkaline medium was manifested not only in the serum of the donor A(II)Ac'+Bc'- (sample A41) but also in the serum of O(I)Ac'+Bc'- donor (sample A42) (the same expression).

4. Discussion

The role of histocompatibility testing in transplantation is well recognized, as the best results are obtained with fully matched donors (18). The presence of agglutinogenic and adsorbing antigens in human cells was previously reported in the human leucocyte antigen system (HLA) (6, 19), and researchers pointed to the existing analogy with the ABH system. It was thus demonstrated that leucocyte transplantation antigens present on platelets and other tissues belong to the HLA system and are controlled by the genes on at least two closely linked mutational sites on a pair of autosomal chromosomes, which makes possible the association of antigenic factors and the inclusion of some antigens in other broader antigens. This phenomenon was reported to have parallels in erythrocyte serology. The A1 antigen is included in the A antigen and in the rhesus system, while the C and D antigens are included in the G antigen. The authors highlighted the necessity of absorption methods for the elucidation of these antigens. Absorption studies have shown that leucocytes, although not agglutinated by serum, may completely absorb all agglutinating activity. These cells are said to be ANAP and are considered to be positive for the antigen. A similar cytotoxic-negative–absorption-positive (CYNAP) phenomenon has been found in lymphocytotoxic antibodies, which may partly be associated with anti-complementary factors in the serum. The evidence suggested that the reaction-negative–absorption-positive (RNAP) phenomenon and the inclusions may sometimes be associated with cross-reactive antibodies (5). The topic under consideration deserves attention. This study discusses the second, non-agglutinogenic type of group antigen labeling in the ABO system that has been identified in recent years. The main point is not only how quickly these or those types of glycocolipids from erythrocyte membrane migrate (Ad, Ac, Ab) or not (Aa) during thin-layer chromatography in silica gel (20) but also that they are serologically active and, to a certain extent, differentiable by Mabs, allowing them to be composed in clusters (11).

The fundamental isoelectric differences are discussed, which form either a negative charge in agglutinogenic glycotopes of lipid (Alp-00, Alp-0, Alp-1) and protein (Apr-00, Apr-0) origin or a positive charge in non-agglutinogenic glycotopes Alp-3 and Apr-3 at blood pH. Enzymes are involved in the formation of these two types of structures. Two variants of N-acetyl-D-galactosamine-transferase are known that form A specificity and have an opposite charge at blood pH. Accordingly, two galactosyltransferases are responsible for B specificity. That is why under artificially created acidic or alkaline conditions of the environment, the charge and activity of one type of transferase will increase and those of the other will weaken, and consequently, the differences between the two types of isoelectrically different antigenic structures will be demonstrated and genetically determined (8, 14). N-acetylgalactosaminyltrasferase uses UDP-GalNAc to convert the H antigen to the A antigen, whereas galactosyltransferase uses UDP-galactose to convert the H antigen to the B antigen (21). The conversion of O erythrocytes into A and B erythrocytes has been described (22). O erythrocytes, incubated with UDP-N-acetyl-d-galactosamine and α-N-acetyl-d-galactosaminyl transferase of blood-group A1 individuals, were reported to acquire A2 specificity and, following continued incubation, became agglutinable by anti-A1 reagents (20). When the A1 enzyme was diluted to show a transferase activity toward the H substance equal to that of the A2 enzyme preparation, there was a significant difference between the enzymes. The speed of their conversion into A2 erythrocytes was slower with the A2 enzyme than with the A1 enzyme. Previous research demonstrated that A1 and A2 enzymes have different substrate specificities (5, 23, 24).

Table 5. Gas chromatography of natural and biosynthesized A glycoconjugates

No.	Hold-up peak time	Samples Type of glycoconjugates, erythrocyte donor, donor serum at biosynthesis			
		Sample A38	Sample A30	Sample A41	Sample A42
		Apr-3, Erythrocytes A(II)Ac'+	Apr-3, Erythrocytes O(I) Ac'+	Alp-3, Erythrocytes O(I) Ac'-Bc'-	Apr-3, Erythrocytes O(I) Ac'-Bc'-
		-	-	Serum A(II)Ac'+	Serum O(I) Ac'+ B c'–
		Component content in %%			
1	4.213	26.198	21.945	28.43	26.57
2	4.285	0.550	0.4566		
3	6.973		0.829		
4	9.868	0.262	0.331		
5	10.848	52.311	56.9065	59.151	61.11
6	11.709	3.477	1.977		0.263
7	11.995	8.962	8.412	4.912	5.206
8	16.256	0.940	0.2675		
9	16.802	4.38	4.818	6.31	5.902
10	17563	0.33	0.218		
11	19.618	1.579	2.174	0.53	0.52
12	22.028		0.309		

Note: Alp-00, Alp-0, Alp-1, Alp-3, Apr-00, Apr-0, Apr-1, Apr-3, Blp-00, Blp-0, Blp-1, Blp-3, Bpr-00, Bpr-0, Bpr-1, Bpr-3 are conditional, reflect the nature and order of elution of glycoconjugates from the anion exchanger and are characterized by differences in their isoion point. Ac'+ , Ac'- , Bc'+ , Bc'- - are conditional, reflect the presence or absence of non-agglutinogenic complement binding glycotopes A and B of specificity and supplement rather than cancel the existing symbolism.

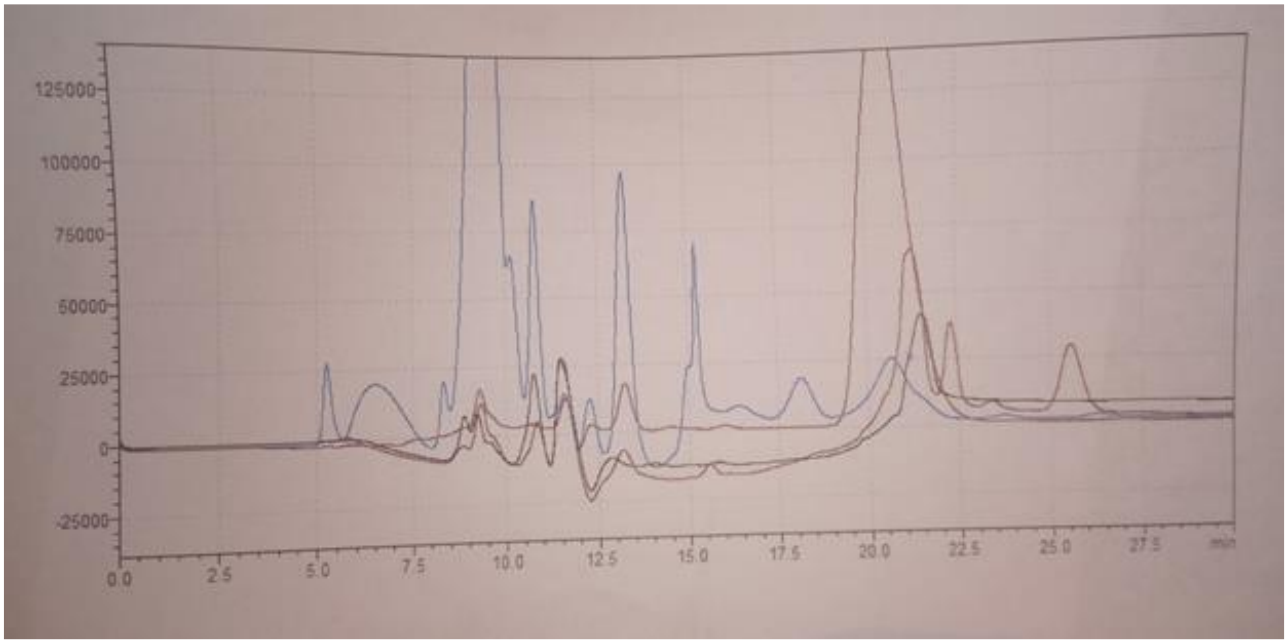

Figure 1. A substance (N-acetyl-D-galactosamine) revealed by chromatography from the persons with Ac'+ characteristics

In the present study, the fundamental possibility of the biosynthesis of non-agglutinogenic specific A and B glycotopes was manifested under the influence of a different group of serum as a source of the corresponding transferase in cell electrophoresis in the first series of experiments with two-day cultivation of erythrocytes at natural blood pH.

At the same time, agglutinogenic A and B glycotopes of the other group appeared even less often than non-agglutinogenic Ac´+ and Bc´+ specificities. Their predominant biosynthesis may be explained by the revealed slight alkalization of the medium during cultivation, taking into account the competition between the two types of N-acetyl-D-galactosamine transferases studied on acceptors of artificially created oligosaccharide structures (25). By deliberately creating acidic or alkaline conditions for cultivating erythrocytes with a different group of serum in the second and third series of experiments with a buffer, we allowed the maximum activity of one of the two types of transferases to manifest itself, and by increasing the duration of cultivation to four days and a change of the serum, nutrient medium, and buffer, more expressed biosynthesis was ensured, which made it possible to evaluate the results in cell electrophoresis, isolate active samples from erythrocytes serologically, and evaluate their inhibitory ability against Mabs. It was found that the majority of the samples of O(I)Ac'-Bc'- specificity after cultivation in an acid medium have acquired rather expressed A agglutinogenicity, and the study of A glycoconjugates isolated from the membranes revealed the highest activity of alkaline fractions of glycolipids (Alp-00 and Alp-1) in relation to both polyclonal and monoclonal antibodies.

The results of the cultivation of the same type of erythrocytes in the alkaline medium were different. Erythrocyte samples of the same O(I)Ac'-Bc'- group did not acquire A agglutinogenic properties either with A(II)Ac'+Bc'- or O(I)Ac'+Bc'- serum. On the other hand, a well-expressed ability of acidic non-agglutinogenic glycotopes (glycoconjugates) to inhibit anti-A Mabs and polyclonal anti-A antibodies has appeared. It is important that acidic glycoconjugates of both lipid and protein origins showed this ability since acidic protein glycoconjugates in their original form correlated with the degree of agglutinogenicity of A1, A2, and Ax of erythrocytes despite their acidic properties that could not be explained (10). The result was somewhat clarified by our heating and repeated chromatography of the fraction on an anion exchanger, as it turned out that a significant part of the serological A activity was shifted from the acidic area into the alkaline one. In other words, the acidic properties of Apr-3 glycoconjugates could be partly due to protein (amino acid) residues on heat-resistant carbohydrate complexes, especially since human erythrocytes' membrane glycoproteins have two groups of carbohydrate chains different in molecular size and pH with determinants of ABH blood types, and acidic carbohydrate chains differ structurally and in large sizes. Moreover, there are often discrepancies in the manifestation of these two types, as with the presence of a hidden, additional, and non-agglutinogenic type, including O(I)Ac´+, O(I)Ac´-Bc´+, A(II)Ac´+Bc´+, and B(III)Ac´+Bc´+(13), and with the absence of this acidic marker in the presence of agglutinogens, including AB(IV)Ac´-Bc´+, AB(IV)Ac´+Bc´-, and AB(IV)Ac´-Bc´- (28). In general, the data obtained help substantiate the concept of two types of group ABH antigenic labeling of blood cells and tissues in humans, based on the existence of alkaline agglutinogens and latent, undetectable agglutination, isoelectrically opposite in charge (acid), tested by the absorption of antibodies with the fixation of the complement. Each of the two types of ABH antigen labeling has its own isoelectrically opposite enzyme systems responsible for their biosynthesis and exhibiting activity in the pH area opposite to the formed glycotopes.

Acknowledgment

No funding support was provided for the study.

Authors' Contribution

Study concept and design: Delevsky. Analysis and interpretation of data: Delevsky, Zinchenko. Drafting of the manuscript: Delevsky. Critical revision of the manuscript for important intellectual content: Delevsky.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

No funding support was provided for the study.

References

1. Jajosky RP, Shang-Chuen Wu, Zheng L, Jajosky AN, Jajosky PG,Josephson C D, Hollenhorst MA, Sackstein R,Cummings RD, Connie M. A., Stowell S R. ABO blood group antigens and differential glycan expression: Perspective on the evolution of common human enzyme deficiencies iScience. 2023 Jan 20; 26(1): 105798.
2. Kajiura, H., Miyauchi, R., Kakudo, A. et al. Bombyx mori β1,4-N-acetylgalactosaminyltransferase possesses relaxed donor substrate specificity in N-glycan synthesis. Sci Rep. 2021; 11: 5505.
3. H. Schenkel-Brunner. 3.19 - Blood Group Antigens. Hans Kamerling. Comprehensive Glycoscience. Elsevier, 2007; 343-372.
4. Smolarek D, Krop-Watorek A, Waśniowska K, Czerwiński M. Molekularne podstawy układu grupowego ABO (Molecular background of the ABO blood group system). Postepy Hig Med Dosw (Online). 2008 Jan 16;62:4-17.
5. Cid E., Yamamoto M. & Yamamoto F. Amino acid substitutions at sugar-recognizing codons confer ABO blood group system-related α1,3 Gal(NAc) transferases with differential enzymatic activity. Sci Rep. 2019; 9, 846.
6. Rood JJ van, Loberiza F R Jr., Zhang M J, Oudshoorn M, Claas F, Cairo M S, Champlin R E, Gale R P, Ringden O, Hows J M, Horowitz M H. Effect of tolerance to noninherited maternal antigens on the occurrence of graft-versus-host disease after bone marrow transplantation from a parent or an HLA-haploidentical sibling. Blood, 2002; 99 (5): 1572-1577.
7. Kosyakov P. N. On the question of the “dual nature” of antigens of the AB0 system in blood cells and human tissues. Vestn. USSR Academy of Medical Sciences, 1975; 5, 87-90.
8. Kishi Koichiro, Takizawa Hisao, Iseki Shoei. Isoelectric analysis of geneassociated L-galactosyl-transferases in human serum and saliva. Proc. Jap. Acad., 1977; B-53.4.172-177.
9. Schachter H, Michaels MA, Tilley CA, Crookston MC and Crookston JH. Qualitative differences in the enzymes produced by human A1 and A2 genes. AABB-JSBT, International Transfusion Congress Proceedings of the Simultaneous Sessions, Abstracte of volunteer Papers, Washington, 1972: 16.
10. Delevsky Yu.P., Zinchenko A.A. Comparison of the antigenic spectrum of A1-, A2- and Ax-erythrocytes - inhibition of MAV by glycoconjugates of lipid and protein nature with different isoelectric properties. Clinical laboratory diagnostics, 2007; 12, 48-53.
11. Delevsky Yuri P. Serological and topographic differences in erytrocitic antigenic markers of ABH –glycolipids and glycoproteins.Medicine and., 1999;2(5) : 17-23.
12. Brockhaus M. Detection of glycolipid antigens using monoclonal antibodies In the book. Immunological research methods, M, Mir, 1988: 160-162.
13. Conrath TV Handbook of microtiter procedures. Cambrige, 1972; Dynotech corp.
14. Marsh WL Scoring of hemoagglutination reaction. Transfusion, 1972: 352-353.
15. Delevsky Yu.P. On the selection of a donor taking into account genetically determined differences in AB0 tissue differentiation. Orthopedics, traumatology and prosthetics, 1971; 9, 56-63.
16. Delevsky Yu. P. To study the nature of A1 - and A2 - antigenic differences using monoclonal antibodies - the role of glycoprotein and glycolipid epitopes in their formation. Wedge. lab. diagnostics, 2006; 10, 6-11.
17. Takasaki S, Yamashita K, Kabata A. The sugar chain structure of AB0 blood group active glycoproteins obtained from human erythrocyte membrane J. Biol.Chem. 1978; 253(17): 6086-6091.
18. Patenaude SI, Seto NO, Borisova SN, Szpacenko A, Marcus SL, Palcic MM, Evans SV. The structural basis for specificity in human ABO(H) blood group biosynthesis. Nat Struct Biol. 2002; 9(9):685-690.
19. Yamamoto M., Tarasco M.C., Cid E. et al. ABO blood group A transferase and its codon 69 substitution enzymes synthesize FORS1 antigen of FORS blood group system. Sci Rep. 2019; 9: 9717.
20. Delewski Jury. Dualität der Antigenmerkmale der Erythrozyten und des Knocnengewebes. Wissenschaftliche Zeitschrift der Humboldt-Universität zu Berlin 1974, 2/3, 219-223.
21. Anso I., Naegeli A., Cifuente J.O. et al. Turning universal O into rare Bombay type blood. Nat Commun. 2023; 14:1765.
22. Delevskiy Yu. P. To the substantiation of isotype differentiation in the AB0 system, taking into account non-agglutinogenic acid glycotopes

of lipid origin. Vestn. Russian Academy of Medical Sciences, 2008; 4, 3-10.
23. Delevsky Yu. P., Gavrilina LV, Ishkhanova MA, Khavkina LV Human serum immunosuppressive factor associated with the AB0 system. Rep. interdepartmental Sat. Immunology and Allergy, Kyiv, 1979: 49-54.
24. Delevsky Yu.P. On the dual nature of the group antigenic label of blood cells and human tissues. Vestn. USSR Academy of Medical Sciences, 1975, 5, 79-87.
25. Clausen H., Hacomori S. ABH and Related HistoBlood Group Antigens: Immunochemical Differences in Carrier Isotypes and Their distribution. Vox Sang, 1989; 56.1-20.