

Blood group A₁ and A₂ revisited: an immunochemical analysis

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Vox Sanguinis

Background and Objective The basis of blood group A₁ and A₂ phenotypes has been debated for many decades, and still the chemical basis is unresolved. The literature generally identifies the glycolipid chemical differences between blood group A₁ and A₂ phenotypes as being poor or no expression of A type 3 and A type 4 structures on A₂ red cells, although this assertion is not unanimous.

Materials and Methods Using purified glycolipids and specific monoclonal antibodies, we revisited the glycolipid basis of the A₁ and A₂ phenotypes. Purified glycolipids were extracted from four individual A₁ and four individual A₂ blood units. One blood unit from an A weak subgroup was also included. Monoclonal anti-A reagents including those originally used to define the basis of A₁ and A₂ phenotypes were used in a thin layer chromatography – enzyme immunoassay to identify the presence of specific glycolipids.

Results A type 3 glycolipid structures were found to be present in large amounts in all phenotypes. In contrast, the A type 4 glycolipid structure was virtually undetectable in the A₂ phenotype, but was present in the A₁ and A subgroup samples.

Conclusion The major glycolipid difference between the A₁ and A₂ phenotypes is the dominance of A type 4 glycolipids in the A₁ phenotype.

Key words: ABO, glycolipids, immunochemical, monoclonal antibody, subgroups.

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Introduction

For many decades, the basis of A₁ and A₂ phenotypes has been a subject of debate. Today, it is recognized that the A₁ and A₂ phenotypes have a genetic basis with the A₂ phenotype being defined by a transferase that is relatively inefficient compared to the A₁ transferase. The inefficiency is probably due to mutation in the A₂ glycosyltransferase peptide chain including the common A₂ deletion in the coding region, which creates a protein with 21 extra amino acids [1]. It is also well-established that the A₁ and A₂ transferases have different pH optimum, K_m values and ion requirements [2].

Despite resolution of the genetic and enzymatic basis, the chemical structures that define the A₁ and A₂ phenotypes still remain debated. Without doubt the major chemical difference

between A₁ and A₂ is of a quantitative nature with the A₁ phenotype expressing up to four times as many A epitopes as the A₂ phenotype [3]. Despite this, there is clear evidence as summarized in Table 1 that there also is a qualitative basis to these phenotypes [4–17]. Earlier studies have suggested that the A-trisaccharide based on type 3 (Galβ3GalNAcα) and type 4 (Galβ3GalNAcβ) chain glycolipids may be important in distinguishing the phenotypes [4–8], although this observation is not concordant (Table 1).

This article re-examines the A₁ and A₂ phenotypes from a glycolipid perspective and also reviews the literature with respect to glycolipid antigen expression in the A₁/A₂ blood group phenotypes.

Materials and methods

Blood samples

One unit blood from four individual blood group A₁ and four individual A₂ blood donors was obtained by Australian Red Cross Service (Melbourne, Australia) and the New Zealand

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Table 1 A₁-A₂ phenotype glycolipid antigen expression as interpreted from published reports

A ₁ phenotype				A ₂ phenotype				References
A		H		A		H		
Type 3	Type 4	Type 3	Type 4	Type 3	Type 4	Type 3	Type 4	
+++	++	+	(-)	(-)	-	+++	++	[4-10]
+++	++	+		++	-	++		[11]
+++	++			-	-			[12]
+++				+				[13]
+++	++	-	-	-	-	+++	++	[14]
+++	+		+	-				[15]
+++	++			(-)	(-)			[16]
+++				++				[17]

The presence and absence of glycolipid antigens on erythrocyte membranes are denoted: +++, relative high level; ++, moderate level; +, low level; (-), very low level; -, absent.

Table 2 Structurally recognized glycolipids discussed in this article

A-6-1	GalNAc α 3(Fuc α 2)Gal β 3GlcNAc β 3Gal β 4Glc β 1Cer
A-6-2	GalNAc α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
A-7-1	GalNAc α 3(Fuc α 2)Gal β 3(Fuc α 4)GlcNAc β 3Gal β 4Glc β 1Cer
A-7-4	GalNAc α 3(Fuc α 2)Gal β 3GalNAc β 3Gal α 4Gal β 4Glc β 1Cer
A-9-3	GalNAc α 3(Fuc α 2)Gal β 3GalNAc α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
A-10-2	GalNAc α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
A-11-3	GalNAc α 3(Fuc α 2)Gal β 3GalNAc α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
H-5-2	(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
H-7-2	(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
H-8-3	(Fuc α 2)Gal β 3GalNAc α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
H-9-2	(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer
H-10-3	(Fuc α 2)Gal β 3GalNAc α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer

Blood Service. Red blood cells were washed and then frozen until further processed. No samples were available for DNA analysis. Glycolipids from an A_w (A weak subgroup) sample, previously reported [11], were co-analysed.

Phenotypes

The red blood cell A₁/A₂ blood group phenotypes were determined by routine serological reagents (antibodies and lectins) by the contributing laboratories. Lewis and secretor phenotypes were determined from glycolipids by TLC-EIA (thin layer chromatography-enzyme immunoassay).

Glycolipid isolation and thin layer chromatography-enzyme immunoassay

The method used to isolate glycolipids from erythrocyte membranes was based on the method of Karlsson [18]

with modifications as reported in Svensson and co-workers [11]. The TLC-EIA method was based on the method of Schnaar [19], also reported in [11]. Chemical structures on the glycolipids discussed in this article are shown in Table 2.

The amount of glycolipids loaded onto the silica TLC plates were 20 μ g per lane for A₁ and 50 μ g for A₂ samples unless stated otherwise. A blood group A glycolipid TLC control (TLC-A) was included on all plates to allow comparison between assays and was immunostained with monoclonal anti-A (A581 - DAKO, Glostrup, Denmark and Lorne Laboratory, Reading, UK).

Relative migration (*rm*) scales are indicated on all TLC plates to allow the position of various bands on the TLC plates to be described [11]. The scale was set to have *rm* 6.0 for the A-6-2 glycolipid in the TLC-A control. The *rm* scale has interplate comparability of less than ± 0.5 units.

Monoclonal antibodies

The monoclonal antibodies (MoAb) used to analyse the A₁ and A₂ glycolipids were HH3 (anti-ALe^b), HH4 (anti-A type 2), HH5 (anti-A type 3/4), TH1 (anti-A type 3), AH16 (anti-A type 1 + 2) and AH21 (anti-A type 1) from Prof H. Clausen [4,7,8,20–22]; KB 26-5 (anti-A type 3/4) from Knickerbocker, Barcelona, Spain [23]. The specificity of TH1 antibody against A type 3 glycolipid structures and in defining the A₁ glycolipids is well-established [4], specifically reacting with A-9-3 and A-11-3 and an extended structure. From the 4th Workshop on Monoclonal Antibodies Against Human Red Cells and Related Antigens (Paris, 2001; www.ints.fr/4thworkshop/bin/workshop-reports-query.php3) [24] were MoAbs 2-26 (A1 IE3) and 2-27 (A1 3E6) from Hematological Scientific Centre (Moscow, Russia); MoAbs 2-22 (AY209), 2-24 (NaM200-16C5) and 2-39 (HW5) from ETS (Bretagne, France); MoAb 2-9 (HMR1) from Hokkaido Red Cross Blood Centre (Sapporo, Japan) and MoAb 1401 (E11 H5) from Lorne Laboratory (Reading, UK). H antigens were analysed against MoAbs BE2 (anti-H type 2) [6,25] and HH14 (anti-H type 3) from Prof H. Clausen (Denmark).

Lewis and secretor glycolipid phenotypings were undertaken by using MoAb 2-83 (17A5G8) from Ortho-Clinical Diagnostic (Raritan, NJ, USA); MoAb 33-2 (GAMA 704) from Gamma Biologicals Inc (Houston, TX, USA) and MoAb 21-5 (LM 137/264-3) from Dr R. Fraser, Glasgow and West of Scotland Blood Transfusion Service. These MoAbs have previously been shown to react appropriately with blood group glycolipids [11].

Results

Phenotypes and nomenclature

The Lewis and secretor phenotypes of the eight individual A₁/A₂ donors as determined by immunochemical staining were: samples in lanes 6 and 8 are Le(a+b-) non-secretor, samples in lanes 2 and 3 are Le(a-b-) secretor and the remainder (lanes 4, 5, 7 and 9) are Le(a-b+) secretors. The nomenclature and specific glycolipids are as described in Table 2 and selected A glycocone specificities are as follows: A type 1, GalNAcα3-(Fucα2)Galβ3-R; A type 2, GalNAcα3(Fucα2)Galβ4-R; ALe^b GalNAcα3(Fucα2)Galβ3(Fuc4)-R; A type 3, GalNAcα3(Fucα2)-Galβ3GalNAcα3(Fucα2)Galβ4-R; H type 3, Fucα2Galβ3GalNAcα3(Fucα2)Galβ4-R; and A type 4, GalNAcα3(Fucα2)-Galβ3GalNAcβ3Galα-R.

Thin layer chromatography-A control

On each TLC-EIA figure the TLC-A control, from lane 1 on each TLC plate, is stained independently with a generic anti-A reagent and used to set the *rm* scale for that plate. The

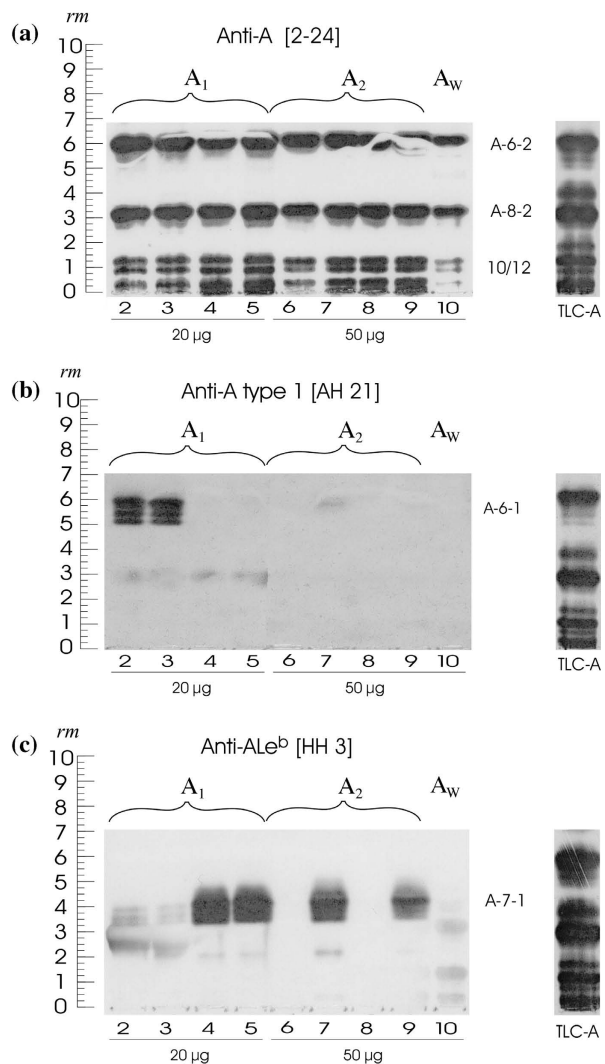


Fig. 1 Anti-A (MoAb 2-24) immunostaining (TLC-EIA) of A₁ (20 µg/lane), A₂ and A_w subgroup glycolipids (50 µg/lane). MoAb 2-24 that is reactive with both A type 1 and A type 2 structures found no significant differences between any of the subgroups of A. MoAbs AH21 (b) and HH3 (c) that detect type 1 A structures of the Lewis and secretor systems, as expected, show no correlation with the A₁ and A₂ phenotypes.

TLC-A control contains not only the normal forms of A antigen, but also has two artefacts of glycolipid preparation, due to incomplete deacetylation. These artefacts seen at *rm* 4.0 and *rm* 1.8 ± 0.5 were not observed in any test sample, thus confirming deacetylation of the test samples was complete.

A type 1, ALe^b and A type 2 glycolipids

The monoclonal anti-A used on the plate in Fig. 1 is MoAb 2-24, which reacts with most types of A and in particular the dominating A type 2 structures. The glycolipids from the A₁,

A_2 and A_w phenotypes appear to have identical patterns albeit the samples in lane 6 and 10 appear to have less extended structures. Identical results were obtained with anti-A HH4 reagent (not shown). Monoclonal reagents AH21 (plate II) and HH3 (plate III), which react with type 1 A antigens (A type 1 and ALe^b , respectively), reacted as expected and gave no bands of relevance to defining the A_1 and A_2 phenotypes. In both these plates, the characteristic multiple ceramide wide banding patterns of plasma-derived type 1 glycolipids can be seen.

A type 3 and A type 4 glycolipids

Monoclonal antibodies used to determine the A_1 and A_2 subgroups reactivity with type 3 and type 4 structures were TH1, HH5, and 2-26 (Fig. 2a-d). Against TH1 that reacts with A type 3, both the A_1 and A_2 samples showed the same reactivity, and interestingly so did the A_w sample (Fig. 2a). Bands were seen at rm 2.8, 1.0 and 0.3 corresponding to A-9-3, A-11-3 and extended type 3 structures. In contrast, MoAb HH5 (Fig. 2b) that has anti-A type 3 and 4 activity showed two major A_1 and A_2 phenotype specific bands. This antibody showed identical results with MoAb KB 26-5 (not shown). In the A_1 samples, a band could be seen at rm 4.2 corresponding with the A-7-4 glycolipid; this band was essentially absent from the A_2 samples. In the A_2 samples, a band at rm 3.3 was seen corresponding with the internal A bearing H-8-3 glycolipid; this band is also seen in the A_1 samples although to a lesser degree. Binding of MoAb HH5 with H type 3 has been seen by other research groups (H. Clausen, personal communication). The remaining A_1 and A_2 glycolipids showed the same reaction patterns as Fig. 2a. It is interesting to note that the A_w sample (Fig. 2b, lane 10) showed a profile more like the A_1 sample than the A_2 , albeit weaker expression of bands.

MoAb 2-26 (Fig. 2c) showed a similar reaction pattern as HH5 with the A_1 , A_2 and A_w samples (Fig. 2c). More intense bands were seen at rm 3.8 corresponding with H-8-3 and a more intense band is seen at rm 1.7 (immediately above the rm 1.5 band), corresponding to H-10-3. At equal glycolipid sample loadings (Fig. 2d), the H-8-3 band was clearly more intense in the A_2 than the A_1 samples, as also seen with both anti-HH5 (Fig. 2b) and to a lesser extent with anti-H type 3 (HH14) (Fig. 3a).

Thus, two differences between the subgroups were observed. The A-7-4 glycolipid structures were visible in the A_1 , but essentially absent in the A_2 samples (Fig. 2b-d) and H-8-3 and H-10-3 were present in larger amount in the A_2 samples.

Further analysis of H activity was undertaken with samples from two A_1 and two A_2 phenotypes (lane numbers retained from previous experiments) loaded at identical concentrations and tested against several monoclonal anti-H reagents (Fig. 3). Anti-H type 3, MoAb HH14 (Fig. 3a) appeared to

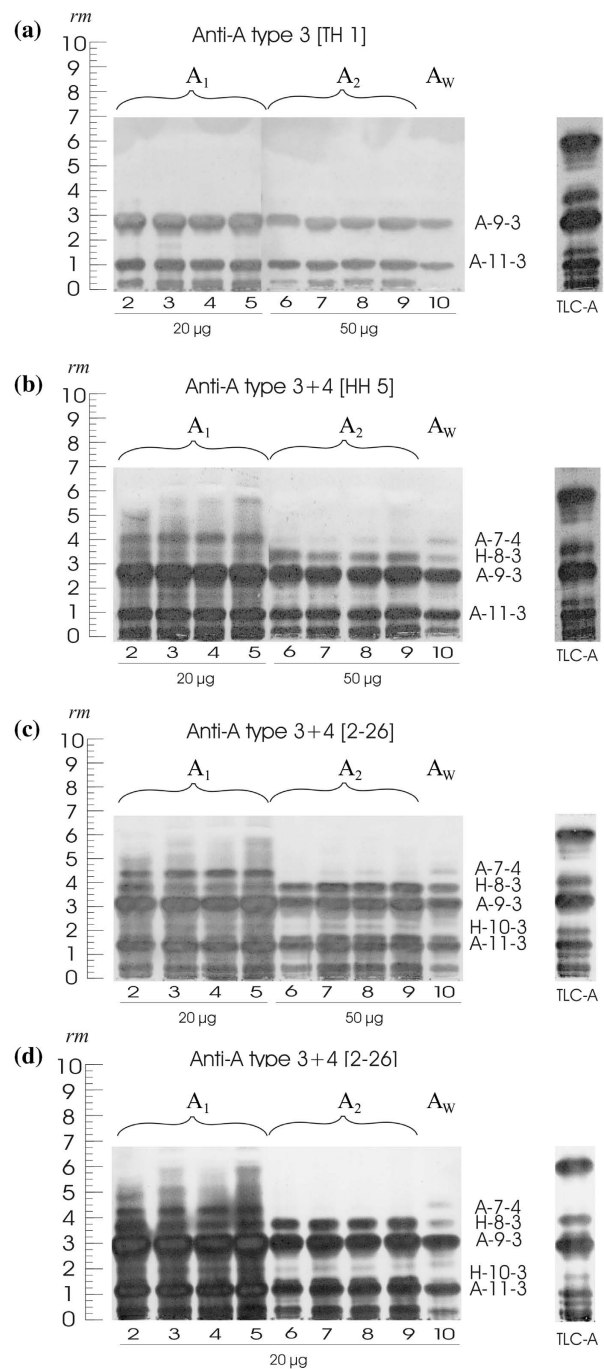


Fig. 2 Anti-A type 3 and 4 (MoAbs TH1, HH5 and 2-26) immunostaining (TLC-EIA) of A_1 , A_2 and A_w subgroup glycolipids. MoAb TH1 reacts with A type 3 but not with A type 4 glycolipids (a). No differences were visible between blood group A_1 , A_2 and A_w subgroups, except for the potential absence of an extended glycolipid (rm 0.2) in the A_w sample (lane 10). MoAbs HH5 (b) and 2-26 (c) both react with A type 3, A type 4 and H type 3 structures. The A_1 samples and the A_w sample show reactivity with A-7-4 glycolipids. No similar reactions were seen in the A_2 samples. Conversely, the A_2 samples appear to show more reactivity in the regions of H-8-3 (b, c) even when loaded at equivalent (20 μ g/lane) concentrations (d).

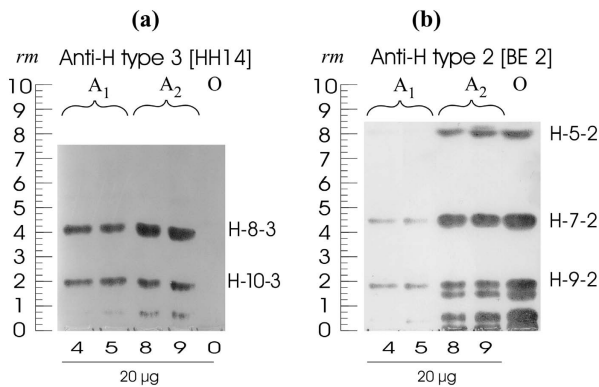


Fig. 3 Anti-H type 3 (HH14) and anti-H type 2 (BE 2) glycolipid profiles of two A₁ and two A₂ samples (lane numbers correlate with samples on previous plates) and an O sample loaded at equal concentrations (20 µg/lane).

react more strongly with H-8-3 in the A₂ than the A₁ samples. As expected, it did not react with the O sample (lane 0), as H type 3 is based on an extension of blood group A. Anti-H type 2 (MoAb BE2) reacted with multiple lactosamine extensions of H type 2. H type 2 reactivity was clearly the strongest in the O sample and stronger in both A₂ samples than in the A₁ samples.

Discussion

Several articles have been published during the mid to late 20th century, concerning the qualitative differences between blood groups A₁ and A₂ (Table 1). The consensus of these publications is that the chemical basis of the A₁ phenotype is the presence of a large amount of A type 3, moderate amount of A type 4 and lesser amounts of H type 3. Conversely, the A₂ subgroup is characterized by the absence of, or the presence of, a moderate amount of A type 3, the absence of A type 4, and presence of H type 3. The literature also notes that some subgroups of A also express A type 3 [11]. The qualitative differences reported in most text books are that A-9-3 and A-7-4 are expressed in A₁ subgroup but not in A₂ [26–30].

In order to reassess the issue, we examined glycolipids isolated from four A₁ and four A₂ individuals and a weak subgroup of A. As far as possible the same monoclonal antibodies, as used in the original reports, were used in this study.

By adjusting the glycolipid concentration of the A₂ samples to be 2.5 times more than the A₁ samples (thus artificially correcting for lower A antigen expression in A₂), we were able to show that the only substantial glycolipid difference between A₁ and A₂ glycolipids was the presence of A-7-4 in the A₁ subgroup, which was essential absent in the A₂ subgroup. Although genotyping of the samples had not been undertaken, the results were 100% concordant between serological red blood cell phenotyping and glycolipid profiling, that is, the patterns of the four A₁ samples were identical and those of the A₂ were identical to each other but

different to the A₁ samples. Surprisingly, as the weak subgroup of A (genotype A¹O^{1v}) [11] also expressed the A-7-4 antigen, this suggests that the absence of A type 4 is not a consequence of an inefficient glycosyltransferase, but instead may be due to altered A₂ transferase activity possibly due to the extension of the A₂ protein. The opportunity now exists to create a 'true' anti-A₁ monoclonal antibody, which is specific for A type 4 (and not cross-reactive with A type 3). This reagent would be expected to show A₁ specificity regardless of antibody concentration.

We were able to clearly demonstrate the presence of large amounts of A type 3 in both A₁ and A₂ (and A subgroups), thereby excluding the presence of this antigen as a basis of the A₁ phenotype. This is in accordance with many published articles, but in contrast to what is commonly reported in text books. In summary, the glycolipid difference between A₁ and A₂ phenotypes is the presence of A type 4 glycolipids in the A₁ phenotype and their absence or very low levels in the A₂ phenotype.

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