Blood group A\textsubscript{1} and A\textsubscript{2} revisited: an immunochemical analysis

L. Svensson,\textsuperscript{1,3} L. Rydberg,\textsuperscript{1} L. C. de Mattos\textsuperscript{2} \& S. M. Henry\textsuperscript{3}

\textsuperscript{1}Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg University, Gothenburg, Sweden
\textsuperscript{2}Immunogenetics Laboratory, Department of Molecular Biology, Faculty of Medicine of São José do Rio Preto, Sao Paulo, Brazil
\textsuperscript{3}Biotechnology Research Institute, AUT University, and KODE Biotech Ltd, Auckland, New Zealand

**Background and Objective** The basis of blood group A\textsubscript{1} and A\textsubscript{2} 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 A\textsubscript{1} and A\textsubscript{2} phenotypes as being poor or no expression of A type 3 and A type 4 structures on A\textsubscript{2} 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 A\textsubscript{1} and A\textsubscript{2} phenotypes. Purified glycolipids were extracted from four individual A\textsubscript{1} and four individual A\textsubscript{2} 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 A\textsubscript{1} and A\textsubscript{2} 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 A\textsubscript{2} phenotype, but was present in the A\textsubscript{1} and A subgroup samples.

**Conclusion** The major glycolipid difference between the A\textsubscript{1} and A\textsubscript{2} phenotypes is the dominance of A type 4 glycolipids in the A\textsubscript{1} phenotype.

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

**Introduction**

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

Despite resolution of the genetic and enzymatic basis, the chemical structures that define the A\textsubscript{1} and A\textsubscript{2} phenotypes still remain debated. Without doubt the major chemical difference between A\textsubscript{1} and A\textsubscript{2} is of a quantitative nature with the A\textsubscript{1} phenotype expressing up to four times as many A epitopes as the A\textsubscript{2} 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 A\textsubscript{1} and A\textsubscript{2} phenotypes from a glycolipid perspective and also reviews the literature with respect to glycolipid antigen expression in the A\textsubscript{1}/A\textsubscript{2} blood group phenotypes.

**Materials and methods**

**Blood samples**

One unit blood from four individual blood group A\textsubscript{1} and four individual A\textsubscript{2} blood donors was obtain by Australian Red Cross Service (Melbourne, Australia) and the New Zealand
Blood Service. Red blood cells were washed and then frozen until further processed. No samples were available for DNA analysis. Glycolipids from an A\textsubscript{w} (A weak subgroup) sample, previously reported [11], were co-analysed.

**Phenotypes**

The red blood cell A\textsubscript{1}/A\textsubscript{2} 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 $\mu$g per lane for A\textsubscript{1} and 50 $\mu$g for A\textsubscript{2} 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.

**Table 1** A\textsubscript{1}–A\textsubscript{2} phenotype glycolipid antigen expression as interpreted from published reports

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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(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer | A-6-2 GalNAc(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer |
| A-7-1 GalNAc(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer | A-7-4 GalNAc(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer |
| A-9-3 GalNAc(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer | A-10-2 GalNAc(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer |
| A-11-3 GalNAc(Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer | H-5-2 (Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer |
| H-7-2 (Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer | H-8-3 (Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer |
| H-9-2 (Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer | H-10-3 (Fucx2)Galβ3Galβ3βGalβ3βGlcβ3βCer |

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Monoclonal antibodies

The monoclonal antibodies (MoAb) used to analyse the A₁ and A₂ glycolipids were HH3 (anti-ALeb), 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 (HMR1) from Hokkaido Red Cross Blood Centre (Sapporo, Japan) and MoAb 1401 (E11 HS) 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 glycotope specificities are as follows: A type 1, GalNAcα3(Fucα2)Galβ3-R; A type 2, GalNAcα3(Fucα2)Galβ4-R; ALeb GalNAcα3(Fucα2)Galβ3(Fucγ4)-R; A type 3, GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ4-R; H type 3, Fucα2Galβ3GalNAcα3(Fucα2)Galβ4-R; and A type 4, GalNAcα3(Fucα2)Galβ3GalNAcβ3Galβ-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 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, ALeb 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\textsubscript{1} and A\textsubscript{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 AL\textsubscript{E} or AL\textsubscript{E}\textsuperscript{c}, respectively), reacted as expected and gave no bands of relevance to defining the A\textsubscript{1} and A\textsubscript{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\textsubscript{1} and A\textsubscript{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\textsubscript{1} and A\textsubscript{2} samples showed the same reactivity, and interestingly so did the A\textsubscript{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\textsubscript{1} and A\textsubscript{2} phenotype specific bands. This antibody showed identical results with MoAb KB 26-5 (not shown). In the A\textsubscript{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\textsubscript{2} samples. In the A\textsubscript{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\textsubscript{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\textsubscript{1} and A\textsubscript{2} glycolipids showed the same reaction patterns as Fig. 2a. It is interesting to note that the A\textsubscript{w} sample (Fig. 2b, lane 10) showed a profile more like the A\textsubscript{1} sample than the A\textsubscript{2}, albeit weaker expression of bands.

MoAb 2-26 (Fig. 2c) showed a similar reaction pattern as HH5 with the A\textsubscript{1}, A\textsubscript{2} and A\textsubscript{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\textsubscript{2} than the A\textsubscript{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\textsubscript{1}, but essentially absent in the A\textsubscript{2} samples (Fig. 2b–d) and H-8-3 and H-10-3 were present in larger amount in the A\textsubscript{2} samples. Further analysis of H activity was undertaken with samples from two A\textsubscript{1} and two A\textsubscript{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](https://example.com/fig2.png)  
**Fig. 2** Anti-A type 3 and 4 (MoAbs TH1, HH5 and 2–26) immunostaining (TLC-EIA) of A\textsubscript{1}, A\textsubscript{2} and A\textsubscript{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\textsubscript{1}, A\textsubscript{2} and A\textsubscript{w} subgroups, except for the potential absence of an extended glycolipid (rm 0·2) in the A\textsubscript{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\textsubscript{1} samples and the A\textsubscript{w} sample show reactivity with A-7-4 glycolipids. No similar reactions were seen in the A\textsubscript{2} samples. Conversely, the A\textsubscript{2} samples appear to show more reactivity in the regions of H-8-3 (b, c) even when loaded at equivalent (20 µg/lane) concentrations (d).
Glycolipid basis of A₁ and A₂ phenotypes

Fig. 3 Anti-H type 3 (HH14) and anti-H type 2 (BE2) 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).

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 A¹O¹) [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 A₄ transferase activity possibly due to the extension of the A₄ protein. The opportunity now exists to create a ‘true’ anti-A₄ 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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