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Studies of the ABO and FORS histo-blood group systems:

Focus on flow cytometric and genetic analysis

Doctoral thesis by

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Studies of the ABO and FORS Histo-Blood Group Systems: Focus on Flow Cytometric and Genetic Analysis

Abstract: ABO is the clinically most important blood group system and its antigens are carbohydrate moieties present on the surface of the red blood cell (RBC) but also on other tissues throughout the body. The *ABO* gene encodes an enzyme, a glycosyltransferase (GT),that adds a terminal monosaccharide to the precursor structure, H antigen, to define the A or B antigens. Blood group O is due to a non-functional GT that leaves the precursor unchanged. Weak expression of ABO antigens can be acquired or be due to polymorphisms in the *ABO* gene. The aim of this study was to characterise normal/weak/aberrant expression of ABO and related structures on RBCs in light of genetic variations of the *ABO* gene or acquired changes in the setting of transfusion, pregnancy or stem cell transplantation.

The O^2 allele has been proposed sometimes to give rise to weak A expression. In 40 group O blood donors heterozygous for the O^2 allele no A antigen expression could be detected nor could any GTA activity be noted in enzyme activity testing. (Paper I)

Flow cytometry was used to examine genetically-defined ABO subgroups and flow cytometric patterns were shown to correlate with many genetic changes seen at the *ABO* locus. In addition, this method was found invaluable for semi-quantification of ABO antigens in clinical samples from individuals with weak antigen expression, both acquired or inherited but genetically unexplained. (Paper II)

Thirteen new *ABO* alleles were defined based on an A^2-allelic backbone. A combination of genetics, flow cytometry and 3D-modelling gave an insight to possible mechanisms underlying the diminished GT activity in these samples. For instance, the first reported change in the important DVD motif was noted in this cohort. (Paper III)

Synthetic A- and B-glycolipids called Functional-Spacer-Linker (FSL) derivatives were used to modify group O RBCs. Different amounts of FSL were used for upload, and flow cytometry was applied to semi-quantify the acquired A or B expression. The purpose of the modification was to create RBCs that mimic the ABO antigen expression of naturally-occurring subgroups and to use these for control purposes. Certain concentrations of FSL gave similar flow cytometric patterns to genetically defined A_x and B_w RBCs included as controls in the study. (Paper IV)

The phenomenon of donor-derived group O RBCs acquiring weak A/B expression following transfusion or ABO-incompatible stem cell transplants were examined. By flow cytometry, antigen levels ranging from very low in A_1 non-secretor individuals to levels almost equivalent to the A_x control sample in secretor individuals were noted. The major role of adsorption of A/B antigen from plasma as the probable mechanism was supported but our findings also indicate a secretor-independent mechanism.(Paper VI)

The enigmatic ABO subgroup A_{pae} was examined and shown to be ABO-independent. The A-like antigen was proven by structural analysis to be the Forssman (Fs) antigen and shown to be expressed in normal haematopoietic tissue. The Fs gene (*GBGT1*) is thought to be inactive in humans but in A_{pae} individuals a missense mutation in the *GBGT1* gene was identified. Transfection studies showed a significant difference in antigen expression between the wildtype and the mutant *GBGT1*. Naturally-occurring anti-Fs exists in plasma and was shown to cause haemolysis of Fs-positive cells *in vitro*, hinting at potential risk for intravascular lysis of transfused RBCs. Furthermore, some *E. coli* strains are known to bind specifically to the Fs antigen, which suggests biological implications. (Paper V)
In summary, the combination of a sensitive flow cytometry method for semi-quantification of ABO antigens, genetic analysis and 3D-modelling provide good tools to examine *ABO* subgroups. The elucidation of the A_{pae} subgroup provided insight into the complex world of glycobiology and established a novel blood group system designated FORS.

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Blood Group, ABO, Weak expression, FORS, Flow cytometry

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Studies of the ABO and FORS histo-blood group systems:

Focus on flow cytometric and genetic analysis

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Annika K Hult

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En del av Förpacknings- och
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Till mormor och morfar

“ 'Cause people often talk about being scared of change
but for me I'm more afraid of things staying the same
'cause the game is never won
by standing in any one place for too long”
From Jesus of the moon by Nick Cave

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Abbreviations

aa	amino acid
bp	base pair
CAZy	Carbohydrate-Active enZYmes Database
HDFN	Haemolytic Disease of the Foetus and New-born
HTR	Haemolytic Transfusion Reaction
EC	Enzyme Commission
ER	Endoplasmic Reticulum
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Gb3	globotriaosylceramide
Gb4	globoside
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GDP	Guanosine diphosphate
GT	Glycosyltransferase
GT6	The glycosyltransferase gene family 6
GTA	3-α-*N*-acetylgalactosaminyltransferase encoded by the blood group A gene
GTB	3-α-galactosyltransferase encoded by the blood group B gene
iGb3	isogloboside 3
ISBT	International Society of Blood Transfusion

kb	kilo base pairs
MAb	Monoclonal antibody
MFI	Mean Fluorescence Intensity
nt	nucleotide
ORF	open reading frame
PCR-ASP	Polymerase Chain Reaction-Allele Specific Primers
PMT	Photo Multiplier Tube
RBCs	Red blood cells
SNP	Single Nucleotide Polymorphism
UDP	Uridine Diphosphate
URI	Upper respiratory infection
UTI	Urinary Tract Infection
WB	Western blot

List of Papers

This thesis is based on the following papers, which are referred to in the text by Roman numerals.

I. Yazer MH, **Hult AK**, Hellberg Å, Hosseini-Maaf B, Palcic MM, Olsson ML. Investigation into A antigen expression on *O*[2] heterozygous group O-labeled red blood cell units.

   Transfusion. 2008 Aug;48(8):1650-7

II. **Hult AK**, Olsson ML. Many genetically defined ABO subgroups exhibit characteristic flow cytometric patterns.

   Transfusion. 2010 Feb;50(2):308-23

III. **Hult AK**, Yazer MH, Jørgensen R, Hellberg Å, Hustinx H, Peyrard T, Palcic MM, Olsson ML. Weak A Phenotypes Associated with Novel *ABO* Alleles Carrying the *A*[2]-related 1061C Deletion and Various Missense Amino Acid Substitutions.

   Transfusion. 2010 Jul;50(7):1471-86

IV. **Hult AK**, Frame T, Chesla S, Henry S, Olsson ML. Flow cytometry evaluation of red blood cells mimicking naturally-occurring ABO subgroups following modification with variable amounts of FSL-A and -B constructs.

   Transfusion. 2012 Feb;52(2):247-51

V. Svensson L*, **Hult AK***, Stamps R, Ångström J, Teneberg S, Storry JR, Jørgensen R, Rydberg L, Henry SM**, Olsson ML**. Forssman expression on human erythrocytes: biochemical and genetic evidence of a new histo-blood group system.

*/** Shared first/last authorship

Blood. 2013 Feb 21;121(8):1459-68

VI. **Hult AK***, Dykes JH*, Storry JR, Olsson ML. Semi-quantification of A and B antigen levels acquired by donor-derived erythrocytes following transfusion or minor ABO-incompatible haematopoietic stem cell transplantation.

* Shared first authorship

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Background

Blood groups

Historical perspective

The modern era of transfusion medicine commenced by the Nobel-Prize-winning discovery by Karl Landsteiner of the ABO blood group system in 1900.[1,2] However, blood and blood transfusions have fascinated people through the centuries during which different species, humans in addition to e.g. dogs and lambs, have been subjected to act as donors and/or recipients of blood. The outcome of these early and quite innovative experiments varied and the first reported successful transfusion of blood between humans was performed by Dr. James Blundell in 1818.[3] In light of today's knowledge about transfusion reactions this probably included quite a portion of luck for the recipient. As well as saving some people's lives (and killing a few unlucky souls) through blood transfusions, Blundell constructed several devices to facilitate blood transfusions.[4] One reason for this was his observation that the blood needed to be infused into the recipient without delay due to the problem with clotting.

In the early years of the 20th century the use of anticoagulant made it possible to store blood for a short period of time. Some years later the first facilities for collecting, storing and distributing blood was established in the Soviet Union.[5] The well-known term commonly used today, "Blood Bank", was coined by Bernard Fantus at the Cook County Hospital in Chicago in 1937.[6]

In his pioneering discovery of the ABO blood group system, Karl Landsteiner noticed different agglutination patterns when he mixed blood from individuals working in his laboratory. This eventually led to the practice of a "crude" crossmatch between donor and recipient prior to transfusion introduced by Ottenberg[7] and made sure that the patients

would receive as compatible blood as possible for the time. In the decades to come more blood group systems were identified, the MN and P blood group systems were discovered in 1927[8] and Rh and LW in 1940.[9] New techniques to identify blood group antibodies were introduced and refined, e.g. the Coombs' test,[10] the use of proteolytic enzymes[11] and low-ionic strength solution for detection of antibodies and cross-matching.[12]

The collected endeavour by the transfusion medicine community and clinicians to ensure safe transfusions for patients of course includes more aspects than blood groups and antibodies, e.g. the transition from transfusing whole blood to using blood units divided into specific components of blood; testing blood donors for disease agents transmitted via blood transfusions and more. More than 110 years after the discovery of the ABO blood group system, the field of transfusion medicine is still an area where new, exciting discoveries can be made to promote patient safety further.

Terminology

Blood group antigens are polymorphic structures expressed on the extracellular surface of red blood cells (RBCs), defined as being able to induce an antibody production in individuals lacking the corresponding antigen. This immune response can be caused by cells/antigens introduced via transfusion, transplantation or pregnancy. A blood group system is comprised of one or more inherited antigen(s) governed by a single gene or a cluster of closely located and homologous genes.

To date there are 33 blood group systems including 297 antigens acknowledged by The International Society of Blood Transfusion (ISBT) Working Party for Red Cell Immunogenetics and Blood Group Terminology. In addition, 42 antigens do not fulfill the criteria for inclusion in a blood group system but are assigned to one of three categories: Collections (serologically, biochemically or genetically related antigens), 700 series (low prevalence antigens) or 901 series (high prevalence antigens), for details see http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/.

Historically, blood group antigens were named either after the individual who made the antibody or, rarely, the discoverer, or even both. This terminology is still used in some publications but can be somewhat

confusing. The effort of the ISBT working party (mentioned above) to find a more organised terminology has resulted in a numerically based nomenclature that genetically classifies the blood group antigens. This is mostly used for database work so the ISBT has also generated a recommended list of alternative names for antigens to facilitate the use of a more unified but still easy-to-use vocabulary for publications and everyday work.[13]

In this thesis the recommended terminology will be used, in addition to the traditional names.

For easy access to both serological features and genetic variation the Blood Group Antigen Gene Mutation Database is available and updated regularly. Unfortunately, the terminology used in this database is not completely in coherence with the recommendations from the ISBT working party. For details see http://www.ncbi.nlm.nih.gov/projects/gv/mhc/xslcgi.cgi?cmd =bgmut/home.[14] A recent textbook summarises and uses the new terminology.[15]

Antigens and antibodies

Presence on the erythrocyte, whether innate or adsorbed, is a prerequisite for a blood group antigen. Antigens of the RH and MNS blood group systems (among others) are relatively restricted to RBCs only, whereas other antigens of different blood groups e.g. ABO and P1PK are more widely distributed[15]. When expressed in different cells and tissues throughout the body the antigens are considered to belong to a histo-blood group system.[16,17] Antigens of the LE and CH/RG systems are produced by non-erythroid cells and later adsorbed to the RBC membrane where they are considered blood group antigens.[18-20]

With the introduction of molecular biology blood group genes were cloned and the genetic basis of antigens identified. Many of these are the direct product of a gene expressed in erythroid tissue *i.e.* endogenous protein-based antigens whilst other blood group genes encode enzymes, glycosyltransferases (GT), that reside in Golgi[19]. The action of such blood group gene products is to catalyse addition of a terminal monosaccharide to a defined precursor chain. The product of this enzymatic reaction then forms a carbohydrate antigen, a structure carried by glycoproteins or glycolipids on the RBC membrane.

Exposure to foreign antigens/cells can induce an immune response giving rise to alloantibodies against (histo-) blood group antigens.[21] This may occur in different settings, *e.g.* transfusion of RBCs, transplantation of various tissues or during pregnancy. The clinical relevance of these antibodies varies from insignificant to grave, and may in the worst case scenario cause fatal haemolytic transfusion reactions (HTR),[22-24] transplant rejection[25] and haemolytic disease of the foetus and newborn (HDFN)[21].

Antibodies can also be naturally-occurring and are often directed against glycan epitopes. Antibodies against the ABO, P1PK, GLOB and some other blood group system antigens may cause severe HTR resulting in a potentially lethal intravascular haemolysis while others are typically less clinically irrelevant. One of the major risks for transfusion recipients is to receive an ABO-incompatible blood unit.[22-24] Naturally-occurring antibodies are primarily IgM even if an IgG or IgA component can also be present. These so-called isoagglutinins are not present at birth but normally become detectable within the first year of life. The antibodies are not induced by exposure to RBCs carrying the foreign antigen in question but are still the product of an immune response towards a non-self antigen. In fact, they are suggested to be stimulated by microbial structures in the normal gut flora, very similar to the carbohydrate blood group antigens according to the molecular mimicry concept.[26]

Proof of this was first shown in experiments performed by Springer in the 1950s where he fed bacteria (*E. coli* strain O86 known to carry group B-like structures) to germ-free chicken. This triggered an immunological response and induced antibodies directed to blood group B antigen. These antibodies could also be neutralised by injection of *E. coli* O86 lipopolysaccharide.[27,28] In addition to these early experiments there is a more recent report including three children with different congenital immune defects. These children were dependent on parenteral feeding. Their RBCs typed as blood group A but despite normal levels of immunoglobulins they did not form the expected anti-B. The conclusion drawn was that without the exposure to normal gastrointestinal microbes via diet, none or very low levels ABO antibodies were produced.[29] The induction of a high titre anti-B by probiotics became evident when two patients had severe HTRs after receiving platelets. Prior to this donation, the donor had not been implicated in any adverse reactions but had recently started eating probiotics. Serologic investigation revealed that the donor's plasma had an anti-B titre of ~16 000.[30]

Structure and function

Many proteins, glycoproteins and glycolipids present in the membrane serve a purpose at some stage in the development of the erythrocyte, during its lifecycle or in its interaction with the surrounding environment. The function of different blood-group-carrying molecules has been elucidated in some cases, whereas others remain unknown or have been suggested a putative function.[31-34] These may be active during erythropoiesis although this will not be discussed further. Biochemical information on blood groups has become available from the 1950s and onwards and in more recent years when molecular biology became accessible, the cloning of blood group genes has added to that information. When the amino acid sequence became available (most often via the nucleotide sequence) it also became feasible to create structural models of blood group molecules. These models made it possible to compare some blood-group-bearing proteins and glycoproteins to homologous proteins with known function and hence deduce a likely physiological role. As will be evident from this thesis, enzymes capable of synthesizing carbohydrate blood group epitopes have also been crystallized and their functions dissected, partially with the help of naturally-occurring mutants.

In the two blood group systems that are considered to be the most important in clinical transfusion medicine, namely ABO and RH, the null phenotype is quite common in one (blood group O of the ABO system) whilst Rh_{null} is extremely rare. Instead, it is the deletion of one of the two RH system genes, *RHD*, which causes a null-like phenotype specifically for the RhD protein. In a selected Caucasian populations for example, approximately 40-45% of the population are blood group O and ~15% are RhD-negative. In certain Amerindian populations, group O is very common, almost reaching 100% in certain Amazon Indian tribes.[15] In the newly discovered blood group system FORS (described in this thesis), the null phenotype is by far the most common and individuals carrying the FORS1 antigen are actually very rare in the populations studied so far.[35] In other blood group systems though, individuals displaying homozygosity for an inactivating mutation giving rise to a null phenotype are uncommon. These unusual cases are the equivalent of a knock-out model and can provide information regarding the significance of the absent red cell membrane component. There are few reports of diseases directly caused by a blood group null phenotype, which suggests that overlapping/alternative

mechanisms exist for most functions of the missing molecule. For example, absence of aquaporin-1 or the erythrocyte urea transporter found in Co(a–b–) or Jk(a–b–) individuals, respectively, is not associated with any apparent pathology or morbidity under normal conditions. However, in both these two examples it can be demonstrated by stress tests that RBCs without aquaporin-1 or the urea transporter behave differently to normal RBCs. Thus, it cannot be excluded that individuals with these kinds of blood group defects may be more vulnerable in certain, perhaps extreme, situations.[36,37] On the other hand, there are exceptions where the individual can be severely affected, *e.g.* the absence of the Kx antigen, and thus the XK glycoprotein, results in a reduced survival of RBCs *in vivo* but more seriously may be associated with the McLeod syndrome where symptoms include peripheral neuropathy, cardiomyopathy, haemolytic anaemia and most often results in early death.[38] Some male individuals with the McLeod phenotype have an X-linked chronic granulomatous disease (CGD) associated with the contiguous deletion syndrome.[39]

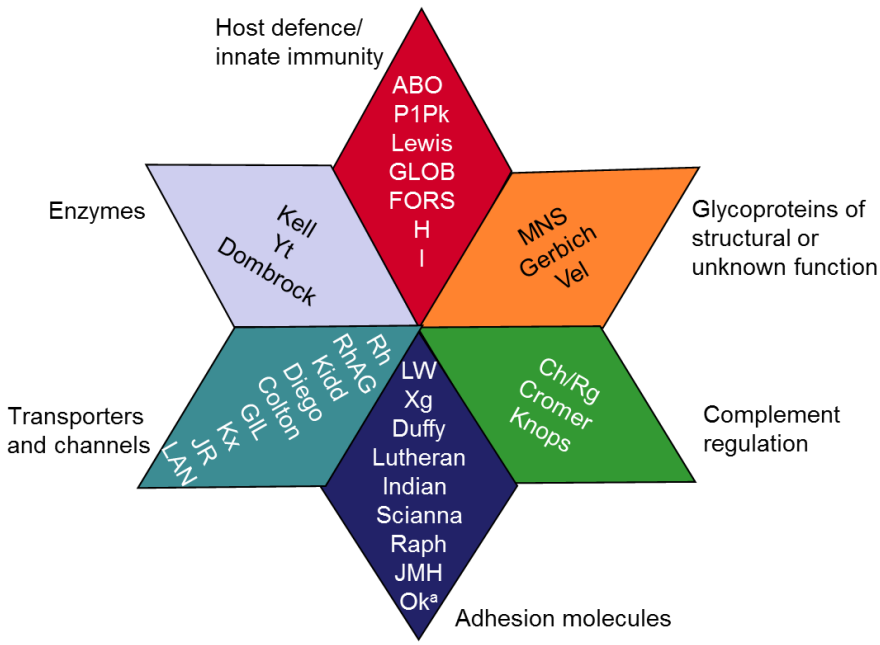

Figure 1. Functional aspects of different blood group systems. Figure courtesy Dr. Jill Storry

The functions of carrier molecules of different blood group systems include enzymatic activity, structural integrity of the erythrocyte, transport of molecules across the hydrophobic lipid bilayer, complement regulation, cell adhesion involved in cell-cell and cell-matrix interactions etc. (Figure. 1). However, the many reported polymorphisms in the blood group genes do not generally seem to affect the function of the carrier molecule.[31,32,34]

Carbohydrate blood group antigens are part of the "sugar coating" of the erythrocyte, the glycocalyx, which consists of carbohydrate moieties that contribute to the negative surface charge essential for the erythrocyte. This sugar matrix prevents aggregation of circulating RBCs and adhesion to endothelial cells, protects against mechanical damage and pathogen invasion. That said, these glycans can also function as involuntary receptors for various pathogens and infectious agents (Figure 2). It has been suggested that the rich variation in carbohydrate structures is mainly based on the herd immunity concept,[40,41] according to which differences in the population aims to make sure that susceptibility to a range of different pathogens differs between individuals in a group so that some will survive when an epidemic, or even pandemic, strikes.

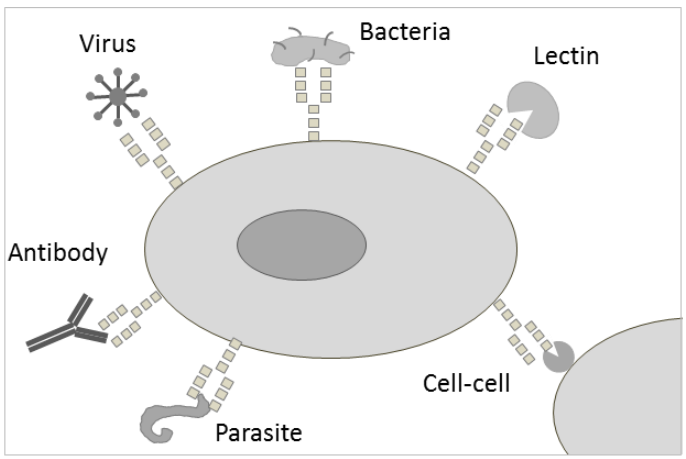

Figure 2. Schematic overview of glycocalyx interactions.

Orphan and emerging antigens

The concept of orphan and emerging antigens is relatively new and can be defined in the following way: Orphan antigens are members of the Collections, 700 series or 901 series but they have not fulfilled the criteria for inclusion in an established blood group system. This means that they have no known genetic home (locus). Often, their carrier molecule is therefore also unknown. Emerging blood group antigens are previously unrecognised blood group antigens, not previously acknowledged by ISBT and can be either newly-discovered or known structures not earlier thought to reside on RBCs, or not known to be lacking in some individuals. Depending on how well an emerging antigen has been defined, it may be included in an existing blood group system or possibly form a system of its own. Alternatively, it can be included in either one of the Collections or series. For instance, at the ISBT working party meeting in Berlin 2010, 14 new blood group antigens emerged and were added to 10 existing blood group systems.[13]

Another emerging antigen was also recognised in 2010. The x_2 glycolipid structure had been known for many years[42,43] but was put forward as a blood group antigen when it was realised that certain individuals lack it and have made antibodies against it.[13] Correlation with the proposed responsible gene has not been fully shown as yet.[44] The PX2 antigen was therefore included in collection 209, GLOB, and is now considered an orphan antigen. Another example of an emerging antigen is the carbohydrate antigen NOR, which was recently included in blood group system number 003, P1PK. Suchanowska *et al.*[45] showed that the glycosyltransferase responsible for making the P^k and P1 antigens could also make the NOR antigen, as an extension of the P (Gb4) antigen. This is due to a SNP in the *A4GALT* gene that gives rise to a glycosyltransferase with broadened acceptor specificity, hence a new antigen could be formed.

Prior to the biannual international ISBT meeting in July 2012, there were 30 blood group systems acknowledged by the ISBT but at the working party meeting, three more systems were approved: FORS (031)[46] containing an emerging antigen, FORS1, which is a story that will be reviewed in more detail in this thesis, JR (032)[47,48] and LAN (033).[49] Jr^a and Lan, high-prevalence orphan antigens previously included in the 901 series, were shown to be carried on different ABC-transporters, and the genetic mechanisms for the antigen-negative phenotypes elucidated. Thus,

they were promoted to form their own group systems. Very recently, another high-prevalence antigen, Vel, was shown to depend on a previously uncharacterized erythroid type I protein designated SMIM1.[50-52] Accordingly, Vel with its underlying gene has now been given a provisional system number (034), while waiting for the next ISBT working party meeting.

Disease associations

The most obvious association between blood groups and disease are the clinical adverse effects on patients in the context of transfusion, HTR, or in the case of alloimmunised pregnant women, the risk of HDFN.[21] In addition to this, transplant rejection can also be due to blood group incompatibility.[53]

Table 1. Relationship between selected pathogenic microorganisms and blood group antigens.

Blood groups/ antigens	Microorganism	Disease	Reference
	Parasites		
ABO, MNS	*Plasmodium falciparum*	Malaria	54-57
Duffy	*Plasmodium vivax*	Malaria	58
CR1	*Plasmodium falciparum*	Malaria	59
	Bacteria		
P1, P^k	*Escherichia coli*	UTI	60-62
P	*Escherichia coli*	UTI	63
Dr^a (Cromer)	*Escherichia coli*	UTI	60
Le^b	*Helicobacter pylori*	Gastritis	64
Lu(a-b-), AnWj-	*Haemophilus Influenzae*	URI	65
H, Le^b	*Burkholderia ambifaria*	Lung infection	66
ABO, LE	*Vibrio cholerae*	Cholera	67
	Virus		
P	Parvovirus B19	Fifth disease	68,69
P^k	HIV	AIDS	70
H/Le^b	Norovirus	Gastroenteritis	71,72
ABO LE H	Rotavirus	Infantile gastroenteritis	73,74

Attempts to associate other types of disease/trait with different blood groups have been made since the early days of transfusion medicine and are still on-going. Certain blood group phenotypes constitute risk factors for a multitude of different diseases including cancer, cardiovascular disease and numerous infectious diseases.[75,76] Blood groups, particularly carbohydrate antigens, have been found to be ligands for a variety of parasites, bacteria and viruses (Table 1). Many more disease linkage and pathogen connections have been made than the examples briefly described here.

As discussed by Weatherall,[77] Anstee[76] and Cserti and Dzik[55] among others, malaria seems to be one of the most powerful selective forces influencing blood groups in an evolutionary perspective. Studies have shown that the most virulent species of the malaria parasites, *Plasmodium falciparum*, leads to a more severe form of cerebral malaria in non-O than in group O individuals.[54] The parasite rosetting ligand PfEMP1 has been shown to bind to the A/B antigens and subsequently form rosettes where an infected erythrocyte binds to uninfected cells.[78] The rosetting is linked to severity of disease and in group O individuals, while rosettes are formed, these are smaller and more easily disrupted.[54] Recently an additional mechanism to the advantage of group O individuals in resistance to severe malaria was suggested: Wolofsky *et al.* showed that infected group O erythrocytes are more efficiently phagocytosed by macrophages than infected A and B erythrocytes which may contribute to a reduced parasite burden for group O individuals.[56] However, ABO does not seem to influence the invasion stage but instead, yet other blood group molecules serve as entry points for the parasite during infection, e.g. glycophorins A and B (*P. falciparum*) and Duffy (*P. vivax*).[57,58]

E. coli which is one of the more common reasons for urinary tract infection (UTI), adheres to cells expressing the P1, P^k, P and LKE antigens through P fimbriae and their PapG adhesins.[61-63,79] Some *E. coli* strains (including enterohemorrhagic *E. Coli*, EHEC) produce verotoxins for which the P^k antigen is known to be a ligand.[80] Human rotavirus, which can cause severe infantile gastroenteritis, has been shown to bind to either A type 1, H type 1 or Le^b depending on strain.[73,74] Studies have also found that the P^k antigen protects against HIV infection *in vitro* whilst cells from individuals with the p phenotype (lacking a functional *A4GALT* glycosyltransferase and thus not able to synthesise the P^k antigen) have a significantly increased

susceptibility to HIV-1 infection *in vitro*.[70] Also, experimental infection can be blocked by adding synthetic analogues of P^k.[81,82]

In more recent years genome wide association studies (GWAS) have become a commonly used tool to correlate increased risk for certain traits or diseases/malignancies with genetic markers *e.g.* blood groups. Examples of this are reports of higher risk of pancreatic cancer in group non-O individuals[83] and the correlation between ABO and soluble Intracellular Adhesion Molecule 1 (sICAM-1) levels. This indicates that ABO antigens may have a regulatory role on one or more inflammatory mediators that have been associated with severe conditions including myocardial infarction, stroke and malaria.[84]

Host-pathogen interactions have been said to be a major force in evolution. The polymorphisms in different blood group systems and the diverse prevalence of antigens in populations around the globe suggest a balancing selection mechanism at play (the active maintenance of one or more polymorphic gene in a population).[85] Fumagalli *et al.*[85] report that the genes encoding three blood group systems CROM, RAPH and JK respectively (some blood groups genes were excluded, among those ABO) had been subjected to balancing selection and that 11 blood group loci out of 26 had a correlation with pathogen richness (basically the calculated number of pathogen species per country). They state that their data indicate: "that blood group antigens have been playing a central role in the host-pathogen arms race during human evolutionary history and no other gene category shows similar levels of widespread selection, with the only exception of loci involved in antigen recognition".

Glycosyltransferases

The immense diversity of carbohydrate structures seen in nature is determined by many different glycosyltransferases (GTs). These constitute a large family of enzymes [Enzyme Commission (EC) 2.4.x.y] that are involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides mainly in the Golgi apparatus. The GTs are defined by their donor/acceptor specificities and the glycosidic linkages formed. More than 240 different human glycosyltransferases have been described and added to the CAZy database (Carbohydrate-Active enZYmes Database) to date (http://www.cazy.org/Glycosyl Transferases.html).[86,87]

Glycosylation

Glycosylation is a very common post-translational modification of proteins and lipids and can be seen as a way to fine-tune their function. The great diversity of carbohydrate structures can range from one simple sugar to very complex, branched structures. The possibility to create these large, intricate structures stems from the many possible combinations of different sugars in addition to the two variants of glycosidic linkage (α or β linkage); by using different carbon atom links (*e.g.* 1-2, 1-3, 1-4, 1-6 etc) and furthermore the hydroxyl groups of monosaccharides can be subject to *e.g.* phosphorylation or sulphation, which adds to the diversity.[26]

The basic core structures of glycoproteins and glycolipids are formed in the endoplasmic reticulum (ER) where the first saccharide that is attached divides/defines the glycoconjugates into classes with different properties.[26] Proteins are often glycosylated by a covalent N-linkage between *N*-acetylglucosamine (GlcNAc) and an asparagine site in the amino acid sequence. It has been estimated that approximately half of all eukaryotic proteins are glycosylated and about 90% of the glycosylation is N-linked.[88] O-glycosylation of proteins occurs through a linkage of *N*-acetylgalactosamine (GalNAc) to either a serine or threonine residue. Glycosphingolipid, which is the abundant glycolipid found in vertebrates, has a ceramide lipid base with an attached galactose (Gal) or, more relevant for glycolipid expression of blood group antigens, glucose (Glc) which with subsequent glycosylation can be classified into (neo)lacto-, globo- and ganglio-series.[16,26]

The membrane-bound GTs are present in the different Golgi compartments where they sequentially and specifically catalyse addition of one sugar to another sugar on a glycoprotein or glycolipid acceptor. Every addition of a sugar creates a new acceptor for the next GT in line in the glycosylation pathway. The donor sugar substrates utilized are often activated in the form of nucleotide sugars e.g. UDP-Gal or GDP-fucose (Fuc). These are synthesised in the cytoplasm and imported to Golgi via membrane transporters. The spectrum of glycosyltransferases present in Golgi is not static and the effect of glycosylation varies with cell type. Terminal modification of glycoproteins and glycolipids are often regulated in a tissue or cell lineage manner.[26,89]

Structure and function

Glycosyltransferases are classified into families by similarities in amino acid (aa) sequence,[90] and there are currently 91 such families listed in the CAZy database. The function and the specificity of the GTs can sometimes be predicted by the aa sequence although there are examples of GTs with very similar sequences where the specificity for either the donor or the acceptor substrate differs. Good examples are the highly homologous ABO GTs, glycosyltransferase A (GTA) and B (GTB) which use the same acceptor, the H antigen, but differ in donor specificity from *N*-acetyl-D-galactosamine for blood group A to D-galactose for blood group B despite ~99% aa homology between GTA and GTB.[91]

Glycosyltransferases are type II integral transmembrane proteins that typically have a short amino-terminal cytoplasmic tail, a hydrophobic transmembrane domain, a short stem region where a protease-cleavage site is situated and finally a large C-terminal globular catalytic domain on the luminal side of Golgi. Two main folds are known, GT-A and GT-B (not to be mixed up with GTA and GTB mentioned above) and in addition to that they can be either inverting or retaining enzymes.[26,92] Many GTs can be solubilized via the protease cleavage site in their stem region and secreted out of the cell.[89,93] These enzymes are catalytically active but lack the N-terminal and the hydrophobic transmembrane domain.[26] (Figure 3) The release of GTs to the extracellular compartment might be a way to regulate their activity in Golgi or possibly to permit them to act at the cell surface. The availability of nucleotide sugars outside the cell has been questioned but a few years ago Sesma *et al.*[94] reported that nucleotide

sugar transporters in Golgi can facilitate release of nucleotide sugar to the extracellular environment.

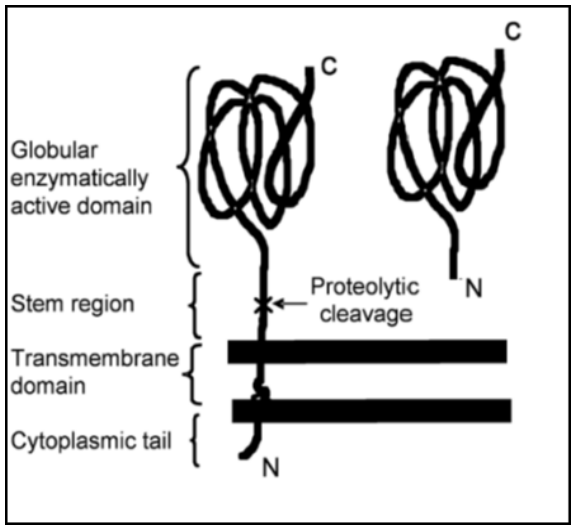

Figure 3. ABO glycosyltransferase. The topology of the transferase is shown as Golgi-localised membrane-bound enzyme with a cytoplasmic N-terminus and catalytic C-terminal domaine (modified from Paulsen &Colley)[95]. Figure courtesy Dr. Bahram Hosseini-Maaf

The catalytic domain of the GT contains an enzymatically-active cleft which has an acceptor-recognition site that binds the acceptor molecule and a donor-recognition site which binds the nucleotide-donor sugar. Many GTs (GT-A fold family) have a metal ion binding DXD motif and are dependent on a divalent cation.[26,87,92] The DXD motif is highly conserved but not invariant. It has been shown through labelling experiments to have an important role in the interaction with the nucleotide part of the donor nucleotide sugar through the coordination of the divalent cation, typically Mn^{2+} but Mg^{2+} can also be used, and interaction with phosphate groups.[96]

Dimer formation or multimeric complexes of GTs have been suggested to retain the enzymes in specific subcompartments of the Golgi. This is thought to enable them to remain in their expected order of function.[97] More lately, reports have suggested that these dimer formations increase the specificity and efficiency of the glycosylation process by physically joining together relevant GTs as reviewed by de Graffenried.[98] In 2010 Hassinen *et al.*[99] elegantly showed for the first time that a group of GTs relevant for N-glycosylation form either homodimers or functionally

relevant heterodimers in live cells. Previous experiments had also suggested that defective GTs can be rescued by functional GTs.[100]

The glycosyltransferase gene family 6

The enzymes within the glycosyltransferase gene family 6 (GT6), as defined in the CAZy database, have a high degree of sequence homology particularly in the catalytic domain suggesting a common fold. Members of this family are type II transmembrane enzymes, using a UDP donor and are metal ion-dependent as described above. The GT6 genes are homologues and probably arose from a series of gene duplication events and subsequent divergence has formed GTs with different donor and acceptor specificities.[101-103] To date there are 180 CAZy entries in the GT6 family of which the majority are from eukaryotes, 13 from bacteria and one from a virus.[104] The bacterial GT6 genes appear to be of vertebrate origin and might add to the pathogen's ability to molecularly mimic host glycans. Since there are so few GT6 genes found in prokaryotes and bacteria, it suggests a possible horizontal gene transfer between eukaryotes and these species, which is opposed to the normal evolutionary process of vertical gene transfer.[105]

The functional enzymes of GT6 catalyze the transfer of either Gal or GalNAc in α1,3 linkage to an acceptor substrate forming the A and B antigens, the Forssman (Fs) antigen, the α3-Gal epitope (i.e. the Galili xeno-antigen, also known as straight B), isogloboside 3 (iGb3) glycolipid or antigens related to these structures.[102,105] The number of GT6 genes found in different species varies extensively. In humans the only functional gene within the GT6 family is said to be the *ABO* histo-blood group gene (Table 2).[102,103,106] Other members [*GBGT1* (Fs antigen), *GGTA1* (α3-Gal epitope) and *A3GALT2* (iGb3)] have shown the presence of one or more critical mutations in the region corresponding to the catalytic domain or, as in the case of *A3GALT2*, alterations in the promoter region and aberrant splicing and hence been deemed pseudogenes.[103,107-110] However it has been suggested that the *GBGT1* gene may still be functional, albeit with an alternative specificity, because the *GBGT1* gene does not display the same degree of decay or number of mutations as seen in the other pseudogenes.[103] The recently reported expression of *GBGT1* and subsequent detection of the Fs antigen in erythroid tissue[35] will be further discussed later in this thesis.

Table 2. Characteristics of the most investigated members of the human GT6 gene family. Ch.location refers to chromosome location.

EC number	Glycosyltransferase	Donor	Acceptor	Gene	Ch. location	Tran-scribed	Trans-lated	Produced antigen	Ref.
2.4.1.37	3-α-galactosyltransferase	UDP-Gal	H antigen	*ABO*	9q34	Yes	Yes	B	91
2.4.1.40	3-α-*N*-acetylgalactosaminyl-transferase	UDP-GalNAc	H antigen	*ABO*	9q34	Yes	Yes	A	91
2.4.1.87	3-α-galactosyltransferase	UDP-Gal	Galβ1-4GlcNAc-R	*GGTA1*	9q33	Yes	No	α-Gal epitope	109 111
2.4.1.87	3-α-galactosyltransferase	UDP-Gal	Galβ1-4Glcβ1-Cer	*A3GALT2*	1p35	Yes	No	iGB3	110
2.4.1.88	globoside 3-α-*N*-acetylgalactosaminyl-transferase	UDP-GalNAc	Globoside/ P antigen/Gb4	*GBGT1*	9q34	Yes	No/Yes	Forssman	35 107 108

Expression of ABO antigens on RBCs is restricted to humans and anthropoid apes and these species also do not express the α3-Gal epitope, iGb3 or the Fs antigen.[103] It was recently proposed that this erythroid ABO expression is due to GATA-1 binding sites in intron 1 of the *ABO* gene, present in higher apes but deleted in other mammals.[112] However, ABO is expressed in various other tissues in lower mammals.[113]

Expression of Fs antigen varies among non-primate species, for example, high expression is seen on sheep RBCs and it is readily detectable on cat, dog and mouse erythrocytes however, RBCs of other higher mammals such as rats and rabbits lack the Fs antigen.[108,114]

Inactivation of human GT6 family genes is suggested to be an adaptation that confers to resistance to various pathogens.[105] These terminal glycan structures encoded by GT6 family members can act as receptors for pathogens as discussed in the section on disease association (above). The presence of naturally-occurring antibodies against the lacking glycans probably also contributes to the human defence against pathogens. The lack of the α3-Gal epitope (and to some extent iGb3[115]) in humans is the major hindrance for xeno-transplantation. The high amounts of antibodies against the α3-Gal epitope (1-3% of the immunoglobulins in human circulation) causes the hyperacute rejection of any tissue carrying the antigen.[105] It is also a likely barrier or neutralizing agent against bacteria or other pathogens displaying this epitope on their surfaces.

The ABO Blood Group System

The importance of the ABO blood group system in transfusion medicine is unquestionable and one of the major risks for fatalities is still transfusion of the wrong ABO group.[23,24] Although rare it has been reported that transfusion of RBCs expressing weaker than normal antigen levels *e.g.* A_x can cause adverse transfusion reactions.[116] Thus, correct typing of both patients and blood donors is essential. The resolution of weak and unusual phenotypes can sometimes be challenging both serologically as well as defining the genetic background. Elucidating the complex world of ABO with its many sides, serology, glycobiology, enzymology, genetic studies and so forth, has been an effort carried out during more than a century by many famous investigators. To summarise the whole (hi)story of ABO is impossible in only a few pages but the aim has been to include some of the most important discoveries and findings throughout the years.

ABO antigens

Normal expression

There are four different antigens assigned in the ABO blood group system (ISBT 001) and thus recognized by the ISBT working party. They are A, B, A1 and A,B and are accordingly defined by the corresponding antibody specificities. The ability to express these antigens is inherited in a co-dominant fashion, *i.e.* the phenotype expressed will correlate to the alleles present at the *ABO* locus.[15] The most common phenotypes with their corresponding antibodies are shown in Table 3. In addition to the common phenotypes there are many reports of weak and aberrant expression and this is in many cases due to alterations in the *ABO* gene (this will be reviewed in more detail later). In erythroid tissue ABO antigens are mainly found as N-linked glycans on RBC glycoproteins like Band 3, the glucose

transporter, RhAG and CHIP-1. In addition, some 10% of the ABO epitopes are expressed as glycosphingolipids.[15]

Table 3. Phenotypes and antibodies of the ABO blood group system

Phenotype	Main antigens*	Antibodies
A_1	A**	Anti-B
A_2	A	Anti-A1*** Anti-B
B	B	Anti-A
A_1B	A, B	-
A_2B	A, B	Anti-A1***
O	H	Anti-A, -B Anti-A,B

* H antigen is present also in the non-O phenotypes but most prominently on A_2 RBCs.
** The identity of the A1-specific antigen(s) is still under debate (see below)
***Not always present

Typing for ABO with standard serological methods is most often straightforward. Type and screen of donors/patients is performed *en masse* every day so that ABO-compatible blood units can be distributed and patients in need of transfusions receive them. In the vast majority of cases it is sufficient to type RBCs with anti-A and anti-B complemented by reverse typing, *i.e.* testing the patient's plasma with A and B RBCs to verify the presence of the expected naturally-occurring antibodies.
Even if monoclonal ABO reagents were successfully introduced during the 1980s and onwards, polyclonal ABO reagents are still available for certain purposes and lectins at the right dilution can be used to *e.g.* distinguish between A_1 and A_2 RBCs. For this purpose A-reactive lectins from *Dolichos biflorus* and *Helix pomatia* are available. Another lectin frequently used is *Ulex europaeus* that displays anti-H activity.[19]

Modification of ABO expression

Alteration of ABO antigens has been attempted in a couple of different ways and for different reasons.

By using an enzyme source, *e.g.* soluble glycosyltransferases present in human plasma or purified naturally-occurring or recombinantly synthesised enzyme it is possible to transform blood group O RBCs to cells exhibiting A and/or B antigens.[117,118] By incubating RBCs of blood group O with A, B or AB plasma, UDP-sugar and the required metal ion, the H antigens can be converted to A or B depending on what enzyme and donor sugar is used. These group O RBCs can be made to display A or B antigen levels comparable to native A and B RBCs (this was applied in Papers I, III and VI).

To do the opposite has been investigated with the hope of creating ABO-universal blood, *i.e.* enzymatic conversion of blood group A and B RBCs to O. This requires the use of enzymes that specifically cut off the immunodominant terminal GalNAc for A and Gal for B without interacting with other, similar carbohydrate structures to create *de novo* antigens. This was pioneered by Goldstein and colleagues[119] who used an α-galactosidase of GH family 27/36 from green coffee beans to convert B RBCs to O. Also other sources of principally similar enzymes were tested.[120] Problems with pH, poor kinetic properties and the large amount of enzyme needed for converting one unit of blood hampered this project even if both preclinical and clinical trials through phase II were completed.[121] However, the main obstacle was the lack of an appropriate enzyme that could convert whole units of group A RBCs to type as O,[122] even if candidates were not lacking. Some years later a new family of bacterial exoglycosidases was identified and displayed improved selectivity for blood group A and B antigens and at a neutral pH.[123] Analysis of antigen levels after enzyme treatment showed near complete removal of A and B antigens with these new enzymes.

In analogy with uptake by RBCs of glycolipids with A or B specificity from plasma, synthetic analogs of blood group glycolipids (KODE technology) can be used to modify RBCs.[124] For instance, synthetic glycolipids have been constructed to carry A or B blood group determinants (or other terminal carbohydrates of choice) which can be controllably inserted into the erythrocyte membrane. Depending on the amount added, these cells

can then mimic different variants of weak ABO expression (not taking the glycoproteins into account though) and be used for *e.g.* quality control purposes.

ABO Biochemistry

The biochemical basis of the ABO blood group system is now well understood due to the ground-breaking work in the 1950s, by Morgan & Watkins and Kabat.[125,126] The experiments were performed on ovarian cyst fluids which contain large amounts of water-soluble blood group substances, a material that was chosen due to problems to extract enough material from erythrocytes. They determined that ABO antigens were carbohydrate structures on the red cell membrane. The hypothesis of the relationship between precursor, the H antigen, and product, A and B antigens, was uncovered in 1959 by two groups.[127,128] In the 1960s, the presence of two different glycosyltransferases, the enzymes which synthesise the A and B antigens was proposed by Watkins.[129] This was later established through different experiments.[130,131] It would take until 1990 before the gene corresponding to A GT was cloned and characterised by Yamamoto *et al.*[91,132] The ABO antigens are not only present on the RBC but in many different tissues and cells and are considered histo-blood group antigens.[16]

Biosynthesis and tissue distribution

Great structural variation in the ABO antigens is seen due to the many differences in the oligosaccharide core structures. Inner core structures of glycoproteins and glycolipids are influenced by the degree of carbohydrate branching and the extent of repeating poly *N*-acetyllactosamine structures (Galβ1-4GlcNAcβ1). These chains are terminated by different variants of oligosaccharides known as peripheral core structures. They are also affected by the type of glycoconjugate, glycoproteins with N- or O-linked sugars or glycosphingolipids from different series. Several types of peripheral core structures have been identified where type 1-4 serve as the main precursors to blood group A, B or O (Table 4).[16]

Table. 4. Peripheral core types and their tissue distribution (adapted from Clausen & Hakomori[16])

Peripheral core type	Structure	Distribution
Type 1	Galβ1-**3**GlcNAcβ1-R	Endodermal tissue, secretions, plasma
Type 2	Galβ1-**4**GlcNAcβ1-R	Ectodermal and mesodermal tissue, e.g. RBCs
Type3	Galβ1-3GalNAc**α**1-R	Various tissues (O-linked mucin type) Repetitive-H/A on RBCs
Type 4	Galβ1-3GalNAc**β**1-R	Glycolipids on kidneys and on RBCs

The minimal defining epitopes of the A and B antigens are trisaccharides, GalNAcα3(Fucα2)Gal-R and Galα3(Fucα2)Gal-R, respectively, where the distinction between blood group A and B is dependent on the terminal monosaccharide. GTA and GTB are responsible for the transfer of either GalNAc or Gal, respectively, from an UDP-donor to the common precursor glycan, the H antigen (Fucα2Gal-R) (Figure 4). The H structure terminates with L-fucose (Fuc) added by 2-α-fucosyltransferase and if left unaltered normally referred to as blood group O. Individuals of blood group O do not have a functional ABO enzyme while AB individuals have both GTA and GTB in most cases.[125]

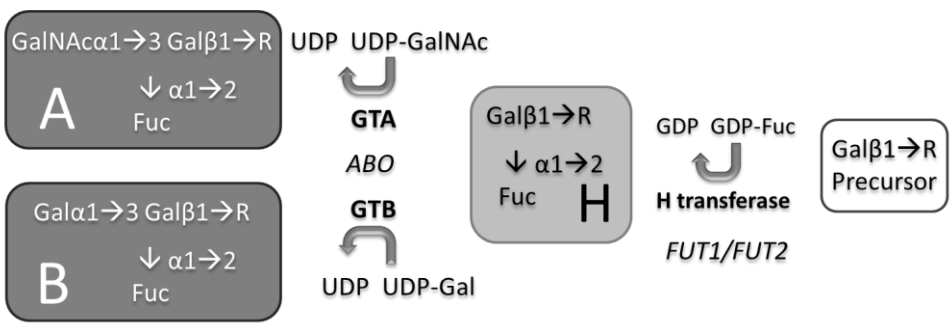

Figure 4. Schematic overview of the biosynthetic pathway for ABO(H) blood group structures.

The synthesis of H antigen is governed by two closely linked and homologous genes on chromosome 19, *FUT1*, the *H* gene and *FUT2*, the secretor gene,[133] that act independently of the *ABO* gene on chromosome 9. The gene products of these two 2-α-fucosyltransferases (α2FucT1 and α2FucT2) act on different precursor chains present in different tissues. α2FucT1 mainly synthesises H antigen on type 2 chains on the erythrocyte. The product of the secretor gene, α2FucT2, mainly acts on precursor type 1 chains present in secretions and plasma but these type 1 antigens can later be absorbed and incorporated into the red cell membrane. Both enzymes can also act on type 3 and type 4 chains. The secretor gene is active in approximately 80% of Caucasians, so-called secretor individuals.
Of the two forms of type 3 antigens (Table 4) the O-linked variant is not present on RBCs whereas the repetitive A is. The A and B structures are generally considered to be terminating the oligosaccharide chain but the type-3 repetitive structure is an exception to the rule. Type 4 ABO antigens are only present on glycolipids. In the glycolipid globo series, H type 4 antigen (globo-H) is an extension of Gb5 and in turn the acceptor for an additional monosaccharide giving rise to globo-A or -B. (Figure 5)

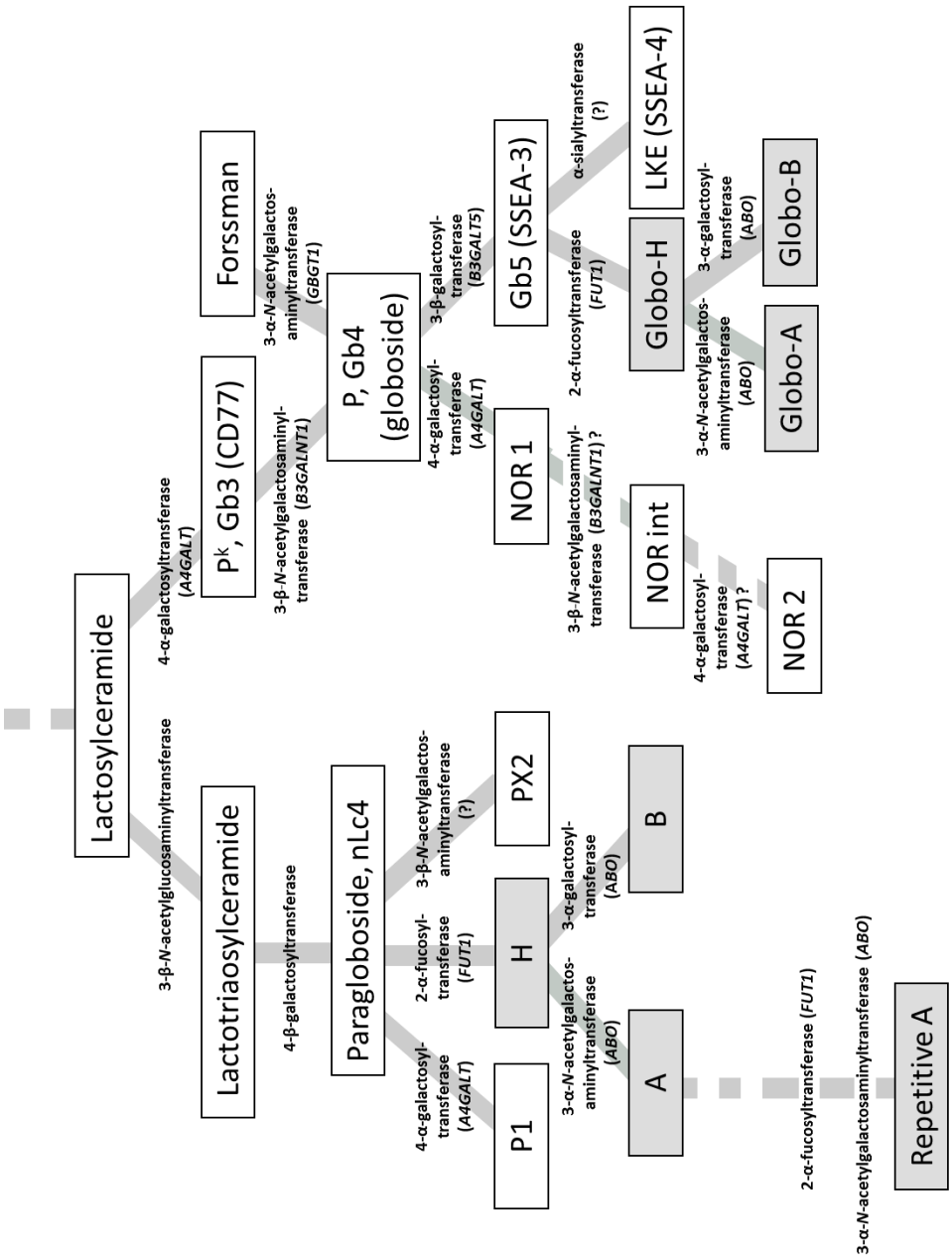

Figure 5. Oligosaccharide chains harbouring the A and B antigens (grey boxes) as well as other related structures (white boxes). If the responsible gene is known, its name is given in brackets below the implicated enzyme.

A_1 versus A_2

The two most common subgroups of A are A_1 and A_2 and they were recognized as early as in 1911 by von Dungern and Hirszfield.[134] There is a clear quantitative difference where A_1 RBCs express approximately five times the amount of A antigen than A_2 does.[20,135] The debate throughout the years has been regarding the qualitative differences of the oligosaccharide chains. It was shown in the 1970s that the A_1 and A_2 glycosyltransferases were qualitatively different with a pH optimum at 5.6 for A_1 and 7-8 for A_2. A_1 also showed a lower K_m for acceptors hence a higher affinity for the substrate. The iso-electric point also differs between the A_1 and A_2 GTs.[136,137] Investigation into the carbohydrate structures revealed presence of type 3 chains, repetitive A and type 4 chains, globo-A on A_1 erythrocytes of which neither, or at least at very low levels, could be detected on A_2 RBCs. The presence on A_2 RBCs of the precursors, H type 3 and H type 4, globo-H on A_2 RBCs suggest inability of the A_2 enzyme to convert these structures to A. This has been proposed to be the qualitative difference between A_1 and A_2 phenotypes.[138,139] Somewhat contradictory, Svensson *et al.*[140] reported in 2008 that the only significant difference in glycolipid expression between A_1 and A_2 is the absence of A type 4, globo-A, and presence of globo-H in A_2. The equal levels of A type 3, repetitive A, detected (when compensating for the lower amount of A on A_2 cells) indicates that the A_2 GT is able to covert H type 3 to A type 3 but not H type 4, globo-H. This is in contrast to previous reports so the question about qualitative difference between A_1 and A_2 is probably still open. Our own unpublished data using monoclonal anti-A type 3 (clone TH1) by flow cytometry indicate that there is much more of this epitope exposed on A_1 RBCs compared to A_2, in which it is barely detectable. The genetic differences underlying A_1 vs. A_2 phenotypes will be discussed in the next section.

The *ABO* gene

The gene

The gene encoding the glycosyltransferases of the ABO blood group system is located on the long arm of chromosome 9 (9q34). The chromosomal assignment was made in 1976[141] and later confirmed by fluorescence in situ hybridization(FISH).[142] Following isolation and purification of GTA from human lung tissue, the first cloning of cDNA representing mRNA from group A transferase was performed by Yamamoto *et al.*[91,132] The *ABO* gene consists of seven exons[142,143] spanning about 19.5 kb. The 1062 base pairs (bp) encode a 354-aa sequence corresponding to a 41-kDa protein. Exons 6 and 7 are the two longest and contain 77% of the whole open reading frame (ORF) and ~90% of the soluble, enzymatically-active GT. These two exons correspond to 274 of the 354 aa that constitutes GTA/GTB (Figure 6).

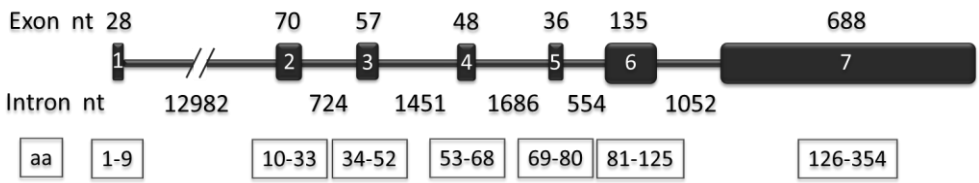

Figure 6. Organisation of the *ABO* gene. The seven exons are drawn to scale whereas the introns are not. The numerals above the boxes represent the number of nucleotides in each exon and those below correspond to the introns. The white boxes show the corresponding aa numbers.

Common alleles

There are several alleles giving rise to normal expression of ABO antigens indicating that neutral SNPs that do not affect enzyme activity are not uncommon in the *ABO* gene. In these phenotypes expressing normal levels of A or B antigen, some of the SNPs are silent, *i.e.* do not cause an amino acid changes. However, there are many examples of missense mutations but the impact on the GT, if any, does not seem significant enough to

decrease the antigen density so that it is detectable in routine blood group typing assays. After all, it is possible that also slightly suboptimized GTA or GTB will be able to convert the limited number of H antigen precursors.

The frequency of common alleles varies between different populations and world-wide, blood group O is the most common. Some populations, such as Native American Indians, are almost exclusively blood group O. *A* alleles are more frequent in populations in Northern and central Europe whereas *B* alleles are more common in Asia. The A_2 phenotype is rare in South East Asia, relatively high in Europe, Africa, South Western Asia and most common in the Sami population of Northern Scandinavia.[144] The diversity of ABO groups among different populations is, as previously discussed, probably caused by selection pressure due to pathogens.

The six most common alleles in Caucasian population groups are briefly presented here. The A^1 allele (*ABO*A1.01*) is considered the consensus allele to which all other alleles are compared (Figure 7). It can be noted that there are several other A^1 alleles reported.[15] The A^2 allele (*ABO*A2.01*) which gives rise to one of the two most common subgroups of A differ from the A^1 allele by a deletion of one of three consecutive cytosines between positions 1059 and 1061.[145] This alters the ORF and abolishes the normal stop codon resulting in an enzyme product containing an additional 21 aa and displaying qualitative differences compared to the GTA produced by the A^1 allele. In a Caucasian population the 467C>T (P156L) SNP is most often present in the A^2 allele but this aa change does probably not impact the enzyme activity in a major way. The 467C>T SNP is also present in an alternative A^1 allele (*ABO*A1.02*) that gives rise to a normal A_1 phenotype (this is the predominant A allele in Asia). The A_2 phenotype has also been reported in connection with alleles not carrying the 1061delC SNP and to a variety of genetic backgrounds.[14,15]

The *B* allele differs from A^1 in seven positions of which three are silent mutations (297A>G, 657C>T and 930G>A) and four are missense mutations (526C>G, 703G>A, 796C>A and 803G>C) leading to aa changes within the coding region.[91] In addition, an eighth substitution (1096G>A) just downstream of the stop codon but part of the mRNA has been utilized for ABO genotyping purposes.[146] The aa changes lead to altered substrate specificity for GTB compared to GTA, *i.e.* the terminal monosaccharide added is Gal instead of GalNAc. The two positions shown to be most responsible for the change in donor specificity are 796C>A (Leu266Met)

and 803G>C (Gly268Ala)[147] whereas the other two aa changes have been suggested to have a part in acceptor binding and turnover rate.[148]

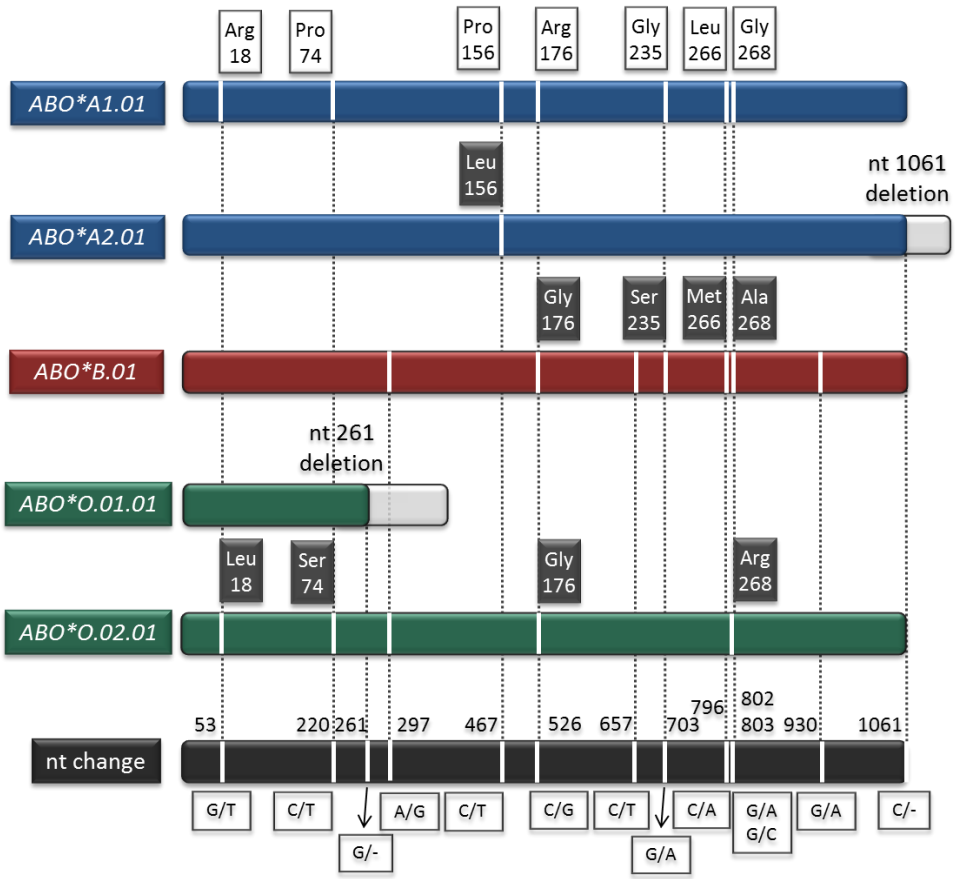

Figure 7. Schematic presentation of the ORF of five common *ABO* alleles. Light grey bars represent translated non-A^1 consensus. Amino acid changes are seen (the dark grey squares) in comparison to the corresponding residues translated by the consensus A^1 allele (top white squares). White vertical bars indicate nucleotide positions and nucleotide change are seen in the lower bar. (lower white squares).

The most common variant of the *O* allele (*ABO*O.01.01,* previously also termed O^1 or *O01*) differs only from the consensus sequence by a single nucleotide deletion in exon 6 (261delG) that results in a premature stop codon after aa 117.[91] The encoded protein (if any) is severely truncated, non-functional and lacks the globular domain with the enzymatically active

site, hence leaving the precursor (H antigen) unchanged. The other common so-called deletional *O* allele is O^{1v} (*ABO*O.01.02,* previously also termed *O02*) which has the same crippling 261delG mutation but with nine additional SNPs in exons 2 through 7.[149] These two alleles are disparate in evolutionary terms.[150]

The O^2 allele (*ABO*O.02.01,* previously also termed *O03*) does not have the critical deletion at nt 261 and belongs to a group of *O* alleles called nondeletional and was first reported in 1993.[151] This allele contains SNPs that are specific for O^2 (53G>T and 802G>A) but also *e.g.* a *B*-specific mutation (526C>G).[152] The 802G>A SNP that results in an aa change, Gly268Arg, has been shown to abolish enzyme activity[151,152] and further studies of the enzyme showed complete blocking of the donor site hence deeming it an *O* allele.[153] Although viewed as an *O* allele it has occasionally been associated with weak expression of A antigen or with O-like phenotypes without anti-A present in plasma. This phenomenon has not been explained but has been the focus of several papers[154-158] of which one is included in this thesis (Paper I). There are several other variants of *O* alleles, some of which belong to the nondeletional group. Some of them are suballeles resembling O^2 and sharing the 802G>A SNP, whilst others are less common but have independent critical alterations, *e.g.* O^3 (*ABO*O.03*),[159] O^4 (*ABO*O.04*), O^5 (*ABO*O.05*) and O^6 (*ABO*O.06*).[156]

Finally, it should also be added that the above-mentioned common allelic lineages also differ by allele-specific or allele-related SNPs in the intronic sequences.[160-162]

Gene regulation

The complex regulation of gene expression is under the control of many regulatory factors, of which many are tissue-specific, to increase or decrease the level of gene product. Several studies have been performed to clarify the regulation of *ABO* gene expression. The first regulatory region to be determined was the proximal promoter situated 5' of exon 1 between -117 and +31 within a CpG island to which the RNA polymerase binds.[143] As in many other glycosyltransferases no TATA box (a common feature in many regulatory regions of eukaryotic genes) can be detected in the *ABO* promoter region upstream of the gene. This proximal promoter also contains binding sites for the transcription factor Sp-1.[143] (Figure 8)

Disruption of this binding site has been shown to decrease promoter activity in an erythroleukaemia cell line.[163]

An alternative promoter region have been defined 5' of the CpG island and in connection to an alternate exon 1 (exon 1a).[164,165] In transcripts containing exon 1a instead of exon 1 the usual initiation ATG codon is not present. Instead a downstream alternative start codon in exon 2 is most likely used which would give rise to a functional but N-truncated glycosyltransferase.[164] If this enzyme actually exists in a natural form has not been proven but two reports[164,165] showed that about 2% of transcripts in cultured CD34+ were noted to contain exon 1a.

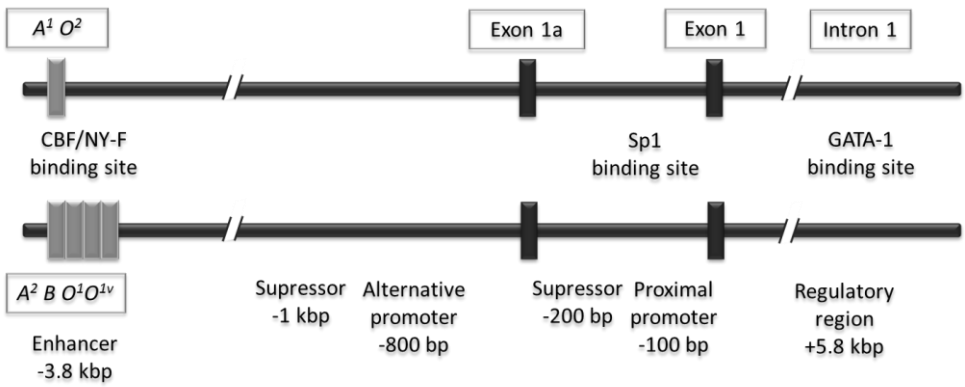

Figure 8. Schematic overview of the *ABO* promoter region of different alleles. The enhancer repeat units are shown in grey boxes and exons as dark grey boxes.

An enhancer region approximately 3.8 kbp upstream of the *ABO* gene contains four 43-bp tandem repeats to which the CBF/NF-Y transcription factor has been shown to bind.[166] Depending on what allele is present, different numbers of repeats are found. Four repeats are seen in A^2, *B*, O^1 and O^{1v} alleles whereas in A^1 and O^2 only one repeat is present.[167,168] A study by Yu *et al.*[169] demonstrated an >100-fold increase in *ABO* transcript levels in a gastric cell line when using a construct composed of four repeats compared to constructs with one unit. It has been suggested that the number of repeats influence expression and that alterations in the enhancer region might be the cause of the weak antigen expression seen in some ABO subroups.[170] However a contradictory report by Thuresson *et al.*[171] shows that despite only one repeat (due to an O^2-*B* hybrid allele) in

connection with a B^{weak} allele the levels of B transcripts are similar to the included normal B controls containing the anticipated four upstream repeats.

An erythroid cell-specific regulatory element in intron 1 was recently reported and shown to bind to the erythroid transcription factor, GATA-1.[112] A large deletion of the region including this motif was shown to cause the rare B_m phenotype which is defined as very low levels of B antigen present on RBCs but readily detectable levels of B substance in secretions. This substantiates the importance of this region in intron 1 for transcription, translation and expression of ABO antigens in erythroid tissue. In addition, comparison between species (higher primates) carrying ABO antigens on their RBCs showed that this part of the *ABO* gene was highly conserved while it is deleted in lower monkeys and other mammals with no or very low levels of ABO antigens on their RBCs. Furthermore, two very recent publications also show that point mutations in this GATA-1-binding motif are responsible for A_m and B_m phenotypes.[172,173]

The gene product

The glycosyltransferases encoded by the *ABO* gene are type II transmembrane enzymes, using a UDP donor and are metal ion-dependent as described earlier. As shown by Patenaude *et al.*[148] who solved the crystal structure of GTA/GTB, the topology of the enzymes contains two domains separated by an enzymatically active cleft. The N-terminal domain (aa 96-227, 338-345) recognises the UDP-donor while the C-terminal (aa 228-237) forms a binding site for the acceptor.

In two areas of the GTs the exact location of the amino acids has not been described. One is the internal disordered loop (aa 179-194) situated between the N-terminal and C-terminal domains. The other is the disordered loop in the C-terminus, the very last 10 amino acids (aa 345-354) of the enzyme. Many GTs become ordered upon substrate binding, however this is not the case with GTA and GTB, hence the exact location of these aa remains to be clarified, although synthetic inhibitors show promising results when it comes to locking these loops.[174]

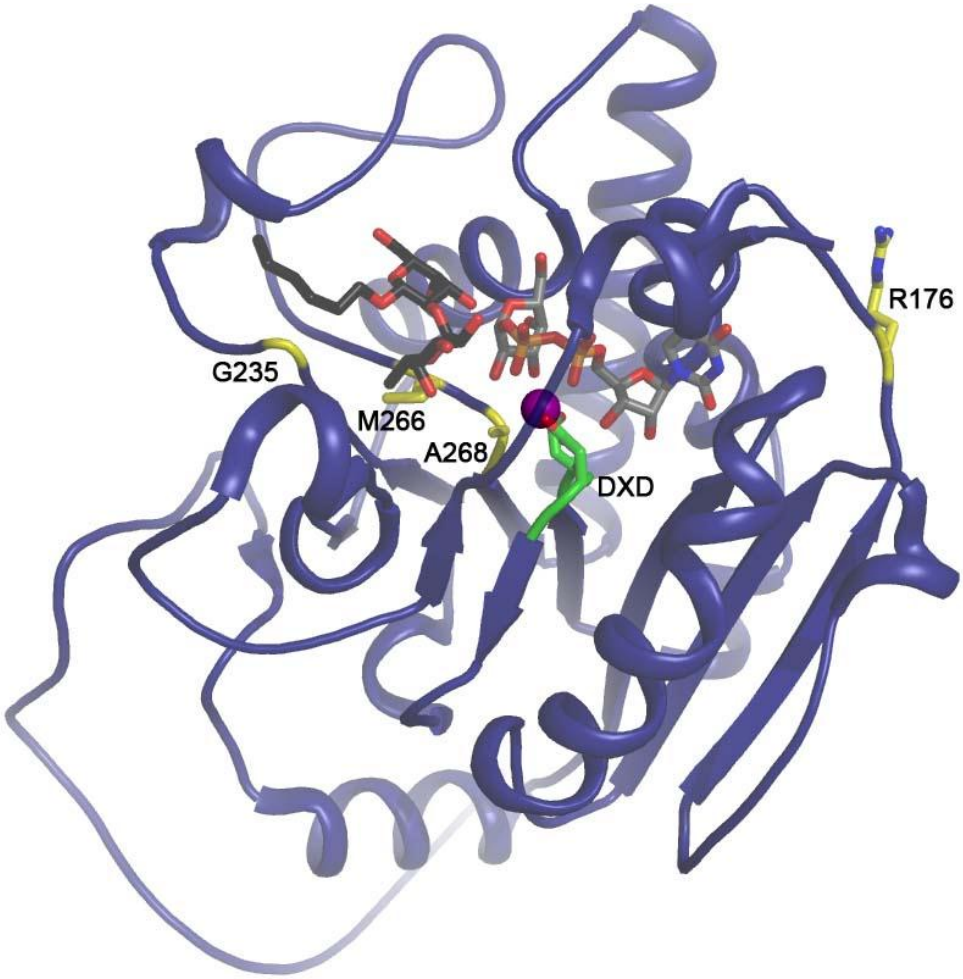

Figure 9. A 3D-model of the ABO GT showing the four amino acids differing between *ABO*A1.01* and *ABO*B.01*. This is a variant structure, **R**176G, **G**235S, L266**M** and G268**A** (GT-AABB, PDB entry 2rj7.pdb). The important DXD motif is shown in green and the Mn^{2+} ion as a purple sphere. The UDP-donor is shown in grey sticks and the acceptor H antigen in black sticks. Figure courtesy of Dr. René Jørgensen.

The important DXD motif, in the case of GTA and GTB a DVD motif, correlates to aa 211-213 and is located within the catalytic cleft (Figure 9). This motif coordinates the divalent cation (Mn^{2+}) on which the activity of these enzymes are strictly reliant. This is because the Mn^{2+} in turn coordinates the binding of the phosphate groups of the UDP part of the donor. During catalysis, binding of the UDP-donor is the first step which initiates formation of the acceptor-binding site, *i.e.* binding of the donor must precede binding of the acceptor.

Of the four aa that differ between GTA and GTB the first residue (Arg176Gly) is situated close to the disordered loop and has been suggested to be important for enzyme turnover but not for donor specificity. The next residue (Gly235Ser) seems to interact with the acceptor which assumes different conformations depending on whether glycine or serine is present. The two last residues (Leu266Met and Gly268Ala) have been shown to be involved in donor specificity[175] where 266 is the most important because of its position to interact directly with the acetamide/hydroxyl group that differs between UDP-GalNAc and UDP-Gal.[148]

Weak expression of ABO

Traditionally, weak A and B subgroups have been fully investigated and defined serologically. The different subgroup phenotypes are characterised by their degree of agglutination with anti-A, anti-B, anti-A,B and anti-H as well as various lectins. In addition, presence or absence of ABO antibodies in plasma are taken into consideration. In some cases it may be necessary to perform adsorption-elution studies and/or to detect the presence of A or B antigens in saliva. Definition of A subgroups has been more distinct, and they are easier to distinguish between than subgroups of B (Table 5). Due to this, it has previously been suggested that the different phenotypically weak B definitions, e.g. B_3, B_x, B_v etc. should not be used. A proposal was therefore put forward to call any weakly expressing B subgroup B_{weak}, *i.e.* abbreviated B_w.[176] During the last 20 years the molecular bases underlying many but not all of the A and B subgroups have been explained. The subgroup genotype has been correlated with the serological findings of different subgroups but within some subgroups there are serological and/or genetic variations and also, not every subgroup has a genetic explanation defined yet.

Weak expression of A or B antigens is often due to one or more SNPs in one of the seven exons of the *ABO* gene and the aa change affects the specificity and/or activity of the GT. To date, more than 100 different ABO alleles have been described[14,177] that give rise to weak A and B antigen expression displaying antigen strengths from almost normal to very weak. Many SNPs encoding an aa change close to catalytic site where it is graspable to understand the crippling effect an aa change can have on the enzyme. Others are found in regions where the influence of the SNP/aa change is more difficult to predict. In a paper by Seltsam *et al.*[178] they showed that a SNP situated away from the catalytic site, 668G>C (Gly230Arg) in a *B* allele, probably causes conformational changes to the enzyme. When transfected into a cell line the mutated GT, in contrast to the wild type, could not be detected within the Golgi apparatus hence aberrant intracellular trafficking was suggested to cause the weak expression of B antigen.

Table 5. Serology patterns adapted from current textbooks.

Subgroup of **A**	RBC reactions with Anti-A	Anti-A,B	Anti-A1	Anti-H	ABO substance in saliva	Anti-A1 in serum
A_1	4+	4+	4+	0	A (H)	No
A_2	4+	4+	0	4+	A, H	Sometimes
A_{int}	4+	4+	3+	3+	A, H	No
A_3	3+/- mf	3+/- mf	0	4+	A, H	No
A_x	0/+	2+/+	0	4+	H	Often
A_{el}	0*	0*	0	4+	H	Sometimes
A_{end}	+	+	0	4+	H	Sometimes
A_{finn}	+	+	0	4+	H	Yes
A_{bantu}	+/(+)	+/(+)	0	4+	H	Yes
A_m	0*/+	0*/+	0	4+	A, H	No
A_y	0*	0*	0	4+	A, H	No
Subgroup of B	**RBC reactions with Anti-B**	**Anti-A,B**		**Anti-H**	**ABO substance in saliva**	**Anti-B in serum**
B_3	mf	mf		4+	B, H	No
B_x	(+)	(+)		4+	H	Yes
B_m	0*/(+)	0*/(+)		4+	B, H	No
B_{el}	0*	0*		4+	H	Sometimes

mf= mixed field agglutination

*Anti-A/B can adsorb onto and elute from these cells

Allelic enhancement

In common phenotypes as A_1B or A_2B there is a competition between the two active GTA and GTB resulting in fewer A and B antigens than if only one of the enzymes were present. The reason for this is that the number of H acceptor sites is fixed. This has also been seen when an already weakened expression of A is further diminished when the allele *in trans* encodes a full-length GTB instead of the truncated enzyme encoded by a deletional *O* allele.[161] In the allelic enhancement phenomenon, first described in 1976,[179] the opposite seem to occur. The allele *in trans* influences the antigen expression of the weakened *ABO* allele in such way that higher antigen expression is noted when accompanied by an allele

encoding a full-length GT. The mechanism behind allelic enhancement has not been completely clarified but the favoured/probable hypothesis is that the GTs form dimers via the stem region and that this stabilises the structures, thus increasing the specificity and efficiency of the crippled enzyme.[99,100] A nice example of allelic enhancement is described in individuals carrying the A^x-4 allele (*ABO*AW31.03*). When accompanied by a deletional *O* allele, A^x-4 gives rise to an apparent O phenotype but while accompanied by an O^2 or *B* allele weak expression of A antigen is detectable.[180,181]

Weak expression due to unknown factors

Even though SNPs scattered along the ORF of the *ABO* gene explain a lot of the ABO discrepancies found in routine typing, there are still healthy individuals with weakened A or B expression on their RBCs but without unexpected alterations in the coding region of *ABO*. The reason for this remains to be discovered but may well depend on *ABO*-independent factors. However, some ABO subgroups without known causes have been shown in early family studies to be linked to the *ABO* locus and further studies are needed to understand better the complex regulation of the *ABO* locus. For instance, it may be that distant enhancers or suppressors not yet identified may act on ABO transcription, or that the long intron 1 harbours other surprises than those recently revealed.[112] Pregnancy and malignancy (especially in the haematopoietic myeloid lineage) has also been implicated in down-regulation of A and B antigen but as of now no adequate explanation to this phenomenon has been presented[19,176,182] even if methylation of the ABO promoter may be implicated in leukemic downregulation of A/B.[183]

Typing discrepancies with normal ABO expression

Discrepancies in ABO typing can arise from a serological pattern that hints toward a suspected weak ABO type, *e.g.* a mixed field reaction. In many cases this can be due to previous transfusions. Before entering into further investigations this should be ruled out. A rare phenomenon that can give a similar serological discrepancy is the occurrence of natural chimeras. These are individuals with two different populations of cells displaying different

antigen profiles. ABO antigens are expressed at a normal level but might be of different phenotypes between two different RBC populations. This mixture of cells might mimick a weak subgroup but is not. Genetically, one can find more than two alleles and the amplification of the different alleles can vary in strength depending on the mixture of cells. Chimerism in a blood donor has caused a haemolytic transfusion reaction in a patient even though the mixture of cells was not detectable with routine blood group testing.[184] RBC chimerism can also be seen in the post-haematopoietic stem cell transplant (HSCT) patient. In ABO/RhD-incompatible HCST pre-transfusion blood typing is important since detection of a RBC mixed chimerism implies selection of blood components compatible with both recipient and donor ABO/RhD-blood groups.[185]

Weak ABO expression due to weak/no expression of H antigen

The rare Bombay phenotype is caused by inactivating mutations in the *FUT1* and *FUT2* genes and these individuals lack the H antigen completely.[186] The consequence is that these individuals are also missing the A and B antigens although their *A* or *B* alleles at the *ABO* locus may be fully functional. Due to strongly reactive anti-A, -B and -H in their plasma it can be a challenge to find compatible blood for these individuals.

The para-Bombay phenotype on the other hand is due to silencing mutations in *FUT1* but an active secretor gene, *FUT2*, or possibly weakening of the *FUT1* gene product independent of *FUT2* status.[186] The *FUT2* gene encodes an enzyme that is able to synthesise H antigen on type 1 chains present in plasma which in turn, in the presence of functional *A* and/or *B* alleles, can be further extended to A and/or B antigens of type 1. These antigens can then be absorbed to the RBC membrane and give rise to a phenotype that is characterised by weak expression of A or B (A_h or B_h, respectively) in the serological absence of H antigen.
Heterozygosity for a consensus *FUT1* allele have been suggested to be a rate-limiting step in the A/B antigen synthesis in certain individuals.[187] Two samples of group A and B (with apparently normal A and B strength and common genotypes predicting blood group A and B, respectively) were surprisingly typed as H negative. For the A sample titration studies with monoclonal anti-A showed lower than normal levels of antigens compared to control A_1 and A_2 RBCs despite 4+ reaction in the routine RBC typing. Both samples were found heterozygous for novel missense mutations in the

FUT1 gene suggesting that zygosity for *FUT1* can influence the expression levels of A and B antigens.

Weak *ABO* alleles

Below, a few of the many variants of *ABO* alleles that are implicated in weak/aberrant expression of A and B antigens are described.

A^3 alleles

The A_3 subgroup is the most common among weak ABO subgroups and was first recognised in 1936.[188] The agglutination pattern, a mixed-field reaction with one agglutinate or a few large agglutinates among a large number of free unagglutinated RBCs, is very typical for this phenotype.[20] The first genetic definition of an A subgroup was published in 1993[189] where two of four samples with an A_3 phenotype displayed a SNP 871G>A (Asp291Asn) on the A^1 backbone. This allele was later named A301 (*ABO*A3.01*). To date there are 10 different alleles registered in dbRBC[14] and supposedly giving rise to the A_3 phenotype. They are based on several different backbone alleles (*ABO*A1.01*, *ABO*A1.02*, *ABO*A2.01* and *ABO*A2.06*) with one additional mutation. There have also been three publications[181,190,191] with in total 31 samples serologically defined as A_3 with no additional mutations in the consensus A^1 allele. We believe these samples to represent the actual A_3 phenotype that was described originally. In Paper II included in this thesis, A_3 samples (individuals from Sweden heterozygous for a consensus *ABO*A1.01* allele), when tested with flow cytometry, display two main populations of cells, one with high and one with low expression of A antigen but still distinctly different from chimeric/transfusion (mixed field) pattern due to RBCs with intermediate expression levels. The dual populations seen with flow cytometry are highly consistent with the classic serology for A_3 samples. When other A^3 alleles with SNPs like 871G>A were tested, this dual appearance was not found. The frequency of the A_3 phenotype has been reported to be as high as 1 in 1000 individuals[192] in Denmark so at least in Scandinavia, the true genetic background of the most common weak A subgroup remains to be elucidated.

A^x alleles

The first A^x (A^x-1, later *ABO*AW.30.01*) allele to be published contained a single SNP, the 646T>A (Phe216Ile).[193] The A^x alleles published a few years later almost all had the 646T>A SNP but with additional mutations within the ORF and where some were based on hybrid alleles.[194] However, not all A^x alleles contain the 646T>A SNP. In dbRBC[14] the total number of A^x alleles is 21 (*Ax1-Ax21*) where a recent publication added eight new A^x alleles[195] of which seven are single missense mutations and one is based on a hybrid allele (*ABO*O.01.02-ABO*A1.02*). In this paper, the authors have used *ABO*A1.02* as the consensus allele appropriate to their study population. This predominantly Asian A^1 allele carries the polymorphism 467C>T which might be confusing since the entry in dbRBC, which compares to *ABO*A1.01*, does not include 467C>T. Hence it is easy to misconstrue that these new SNPs are compared to the more commonly accepted consensus allele, *ABO*A1.01*. The different genetic backgrounds in A_x samples are gathered under a quite wide serological definition (Table 5). The variation in antigen strength in these samples can also be seen in family studies where the same genetic background can give rise to a variation in antigen expression depending on what allele is present *in trans*, *i.e.* effects of the allelic enhancement phenomenon.[180,181] In general, it is felt that serological characterisation of RBC blood samples in cases of ABO discrepancies, is a difficult task that requires long experience by reference serologists and careful selection of the appropriate reagents. The A_x definition requires that the agglutination reaction is stronger with anti-A,B than with anti-A. With the advent of mono- och oligoclonal ABO reagents, reactions are no longer necessarily like those originally reported with polyclonal reagents. There is a major risk that subgroup samples have been mislabeled for the above and other similar reasons. It is therefore difficult to know if A_x is just another weak A variant or if there are special antigens present that only react with anti-A,B reagents as previously proposed.[196]

The A_{pae} phenotype reported in 1987 by Stamps *et al.*[197] was said to be very similar to the A_x phenotype and should the authors had suggested it to be included under the A_x umbrella no objections would have been made.[20] But this turned out to be a completely different story further discussed in Paper V.

A^{finn} and A^{bantu} alleles

As opposed to the unclear serological definitions of many A subgroups, the A_{finn} and A_{bantu} subgroups were reported to be restricted to the populations reflected in the subgroup names and also to have very specific serological characteristics. As expected, this has resulted in a more homogeneous genetic background, with one allele reported for each variant.[198-201]

A^{el} alleles

A_{el} RBCs are neither agglutinated by anti-A nor anti-A,B (Table 5) but do adsorb and elute anti-A. The resulting eluate will give a strong positive reaction when tested with group A RBCs. As in many other ABO subgroups there are several different genetic explanations underlying the A_{el} phenotype. The first reported has an insertion of a guanosine at position 798-804 which is a sequence containing seven guanosines.[202] This allele is now designated *ABO*AEL.01* and its insertion causes a frame-shift that affects all aa just after aa 268, which is important for donor substrate binding, and also extends the enzyme by 37 aa. Even if it is difficult to imagine how such a serious GT alteration would allow any A antigen production at all, this is a very consistent finding true for many samples from different population groups.[203,204] In the *ABO*AEL.03* allele the same area in the nucleotide sequence is influenced but this time it is a deletion of a G that gives rise to an early stop codon resulting in a truncated GT.[205] The general feature of the alleles giving rise to the A_{el} phenotype is that they produce a GT that has a severely decreased enzyme activity.

CisAB and *B(A)* alleles

CisAB and B(A) are phenotypic phenomena where one allele encodes both GTA and GTB activity. They are either *A* or *B* alleles with additional mutations. These are often but not always located at or near the four nucleotide positions where the A and B alleles differ.[206,207] The phenotypic result for cisAB is, in general, quite normal (A_2-like) expression of A antigen but with weaker expression of B. B(A) on the other hand shows a very weak expression of A antigen and a normal expression of B.[19]

The first report describing the rare cisAB phenotype came in 1964 where a the mother typed as group AB whereas her child unexpectedly typed as group O.[208] In this paper they suggested that this phenotype " is formed by the interaction of two genes, A_2 and an atypical B gene occurring at the same locus on one chromosome". The first cisAB phenotype to be genetically defined was *cisAB-1* (*ABO*cisAB.01*) an *ABO*A1.02* allele with 803G>C (Gly268Ala). In the four positions discriminating between *A* and *B* alleles this allele can be described as AAAB.[209] This allele has turned out to be the most common *cisAB* allele in Asian populations. In a study of 16 Korean blood donors heterozygous for the *ABO*cisAB.01* allele it was shown that both GTA and GTB enzyme activity was clearly diminished. The activity was 29% for GTA and 27% in GTB in comparison to wild-type controls.[210] The second allele to be described, *cisAB-2* (*ABO*cisAB.02*), was based on an *ABO*B.01* allele with a 796A>C (Leu266Met) SNP and hence can be described as BBAB.[211] In a recent paper Cai *et al.*[195] reported a *cisAB* allele that is based on an hybrid allele (*ABO*cisAB.01-ABO*A2.05*) which can also be defined as AAAB *i.e.* the same as *ABO*cisAB.01*. No information on the crossing over position is given.

The B(A) phenotype was first detected when monoclonal ABO reagents became available. They display normal levels of B antigen and very low levels of A antigen when testing with some monoclonal anti-A.[20] The GTs encoded by these alleles have the capacity to produce normal levels of B antigen but also to some extent use UDP-GalNAc as substrate and produce detectable levels of A antigen. The *B(A)* alleles are based on *B* alleles. The first to be identified, *ABO*BA.01*, was reported by Yamamoto *et al.*[193] and can be defined as BABB where aa 235 is the A^1 consensus glycine. The second *B(A)* allele (*ABO*BA.02*) has all the four B specific aa, BBBB, with an additional mutation (700C>G, Pro234Ala) causing an aa change very close to the abovementioned 235.[212] It can be noted that also normal GTB encoded by consensus *B* allele has the capacity to synthesise A antigen in minute amounts only detectable by certain anti-A reagents[213] that have been deemed inappropriate for ABO routine typing. The corresponding (opposite) phenomenon has also been reported, i.e. small amounts of B antigen found on regular A_1 RBCs, but has not been generally accepted and is difficult to reproduce despite access to the monoclonal anti-B (BS-85) used in the original report.[214]

B^{weak} alleles

As mentioned before the classification of different B_{weak} subgroups is even more difficult than A subgroups. B variants are also scarcer, especially in a Caucasian population however, the number of B^{w} alleles are rising since more reports are published where ABO subgroups have been identified in different Asian populations.[14,195] An A_1B_3 sample with a missense mutation 1054C>T (Arg352Trp) in the *ABO*B.01* allele was the first to be genetically identified.[189] Several other mutations have since been associated with the B_3 phenotype, including an Asian allele in which a mutation at a splice-site induces transcript abnormality.[14,215] Recently a deletion of 18 nucleotides (-35 to -18) upstream of exon 1 was reported to give rise to mixed field pattern with anti-B consistent with the B_3 phenotype. Using so-called "reporter" assays the authors showed a two-fold decrease in promoter activity compared to the wild-type promoter. This is the first reported variation in the proximal promoter to give rise to a weak ABO antigen expression.[195]

The weakest expression of B antigen is seen in samples with the B_{el} phenotype. The two first B^{el} alleles, *ABO*BEL.01* and *ABO*BEL.02* reported had missense mutations 641T>G (Met 214 Arg) and 669G>T (Glu223Asp) respectively. The two latest reported B^{el} alleles[216] have different genetic "mechanisms" where *ABO*BEL.07* have a mutation early in exon 1, 7G>T (Glu3stop) that gives rise to an early stop codon. Technically this should be considered an *O* allele (in analogy with the deletional *ABO*O.01.01* allele) but adsorption/elution showed presence of very low levels of B antigen. *ABO*BEL.08* has a mutation early in intron 1, IVS1+5G>C, which possibly can cause a splicing defect with an apparently severe effect on the enzyme and hence give rise to a very weak phenotype. These observations may form the basis of a hypothesis stating that rare *O* alleles based on early premature stop codons in either *A* or *B* alleles, may escape the GT truncation by one of several proposed mechanisms including the use of downstream alternative start codons, genetic exchange with the allele *in trans* (so-called autologous chimerism[156]) and repair mechanisms in a minor fraction of all haematopoietic cells.

Some mutations have been detected in both *A* and *B* alleles and give rise to weak expression in both cases. *E.g.* 539G>A (Arg180His), that when present in an *A* allele (*ABO*A3.04*) may induce an A_3 phenotype and cause a B_{weak} phenotype when noted in a *B* allele (*ABO*BW.05*).[176,217] Also, a

Japanese sample with an B_x phenotype had the same mutation 871G>A (Aps291Asn) (*ABO*BW.01*)[218] as the first reported genetic background to an A_3 sample (*ABO*A3.01*).[193]

Hybrid alleles

Weak antigen expression can also be seen in relationship to hybrid alleles. These alleles are a recombination, a hybrid, of two known *ABO* genes.[176,203,219,220] With the extensive knowledge available on sequence variation in the polymorphic *ABO* gene, it has been possible to narrow down the area were the crossing over has taken place. In addition to the well-known polymorphisms in the coding region sequence variations for the introns as well as the up- and downstream regions have been published.[161,162,194,221,222]

The FORS blood group system

More than a century ago, when Prof. John Forssman immunised rabbits with tissue from guinea pig or horse these rabbits produced an antibody that was able to hemolyse RBCs from sheep in the presence of complement. Tissue from other species *e.g.* cow or rat were not able to induce the same immune response.[223] Due to these experiments the antigen recognized by these antisera was later named after Dr. Forssman. The structure of the Fs glycolipid was elucidated some 60 years later and shown to be GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer by Siddiqui *et al.*[224] and the structure was verified by mass spectrometry a few years later.[225] The Fs antigens are generally considered to be present in the cell membrane as glycosphingolipids but there have been suggestions that they may also be found as glycoproteins.[226,227] As in the case with naturally-occurring antibodies against the ABO blood group system antigens, human plasma is known to contain naturally-occurring anti-Fs. These antibodies are mainly of IgM class and do not cross-react with the similar A antigen structure.[228]

Species and tissue distribution

The Fs antigen has been identified in a number of mammals (and other species) and since this heterophilic antigen is expressed in some species but not others the presence or absence of the Fs antigen has classified them as Fs-positive or Fs-negative.[229] Mouse, dog, cat and sheep are examples of Fs-positive animals whilst *e.g.* rat and cow do not display any Fs expression.[114] Heterogeneity with respect to cell type expressing Fs among species is also seen. Sheep RBCs express high levels of the Fs antigen and lower expression is seen on RBCs from cats and dogs. In many species Fs antigen expression is restricted to tissue and is not found on RBCs. Purification of the Fs glycolipid from different species has been done from *e.g.* kidney tissue and intestinal mucosa.[225,227] Humans and anthropoid apes are considered to be Fs-negative but there are some reports of Fs glycolipid expression in normal human non-haematopoietic tissue.[226,230,231]

On the other hand it has been suggested that some immunological methods used may not distinguish between Fs and similar glycoconjugates *i.e.* the antibodies used might have cross-reacted with other glycolipid structures.[232]

Expression in malignancies

The presence of Fs antigen in various forms of cancers has been described.[226,230,233] Hakomori *et al.*[230] reported presence of Fs glycolipid in human gastric and colonic mucosa. Interestingly, when Fs was present in tumour tissue the surrounding normal tissue were Fs-negative whereas if present in normal tissue the Fs glycolipid was not detectable in tumour tissue. They also implied that Fs could be the incompatible A-like antigen seen in some tumors in non-group-A individuals. In a study by Mori *et al.* seven human cancer cell lines were examined and Fs glycolipid was detectable in gastric and breast cancers cell lines.[234]

GBGT1, the Forssman gene

The cDNA corresponding to the canine Fs synthase was cloned in 1996 by Haslam *et al.*[235] An open reading frame of 347 aa was predicted and the sequence had a putative cytoplasmic tail and a 22 aa hydrophobic sequence, a potential transmembrane region. The topology matched well with the structure of a type II transmembrane glycosyltransferase and the sequence showed a 45% homology to GTA and GTB.[91,110] As ABO, the Fs gene, *GBGT1*, is a member of the GT6 glycosyltransferase family previously discussed. Transfection studies performed with canine constructs in the COS-1 cell line showed expression of Fs antigen.

The human *GBGT1* equivalent was cloned in 1999 by Xu *et al.*[107] It is situated on chromosome 9 (9q34) and consists of seven exons spanning over ~11 kb (Figure 10). The ORF (1041 nts) encodes a 347 aa long protein with an estimated molecular weight of ~40 kDa. In analogy with GTA the Forssman synthase (globoside 3-α-*N*-acetylgalactos-aminyltransferase, EC2.4.1.88)[104] transfers a terminal GalNAc in an α1-3 linkage. However, instead of H antigen, the required precursor for Fs synthase is globoside (Gb4) also known as the P antigen (Figure 5).

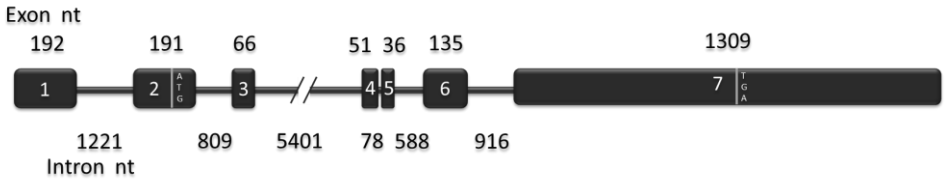

Figure 10. Genomic organisation of the *GBGT1* gene. The seven exons are drawn to scale whereas the introns are not. The numerals above the boxes represent the number of nucleotides in each exon and those below correspond to the introns. Grey, vertical lines in exon 2 and 7 indicate the start and stop codons, respecitvely.

Xu *et al.*[107] showed in transfection studies that the human Fs synthase did not possess the ability to synthesise Fs glycolipid as the canine version of the gene could. This corroborates the known expression of Fs in dogs and its absence in humans. Chimeric constructs, *i.e.* a combination of canine and human sequences showed that the human catalytic site was inactive but no precise definition as to why was defined. RNA expression in a variety of tissues was measured and transcripts were detected in all tissues examined but did not appear to give rise to Fs antigen expression. The authors hypothesised that given the high expression of RNA, this human Fs synthase might have acquired altered enzyme specificity. Others have put forward that the near identity with canine Fs synthase and presence of transcripts suggest that the human ability to synthesise Fs was lost quite recently.[236]

Forssman negativity in humans

In a paper recently published by Yamamoto *et al.*[108] the genetic basis of Forssman negativity in humans was defined. By comparing aa sequences of Fs-negative and Fs-positive species, aa conserved in Fs-positive species and differing from Fs-negative species were identified and hypothesised to be important for Fs expression. Three positions were pointed out, 536C>T (Ile179Thr), 688G>A (Gly230Ser) and 887A>G (Gln296Arg). Each of these substitutions was introduced into a mouse Fs construct (a Fs-positive

species) and subsequently expressed in the COS-1 cell line. Two positions, 688G>A and 887A>G, significantly decreased/abolished the expression of Fs antigen. When reversing these two positions in a human construct (to the mouse version) the Fs synthase activity was fully restored and was able to synthesise Fs antigen in the COS-1 cell line. The conclusion drawn was that these two aa changes in the human Fs synthase compared to Fs-positive species constitute the cause of Fs negativity in humans.

Pathogen interaction

Another part of Forssman biology is that, as many other carbohydrate structures, the Fs antigen can act involuntarily as a receptor for various microbes. *E.g*. some P-fimbriated *E. coli* strains containing the prsG adhesin recognizes Galα4Gal-containing glycolipids but preferentially bind to the Fs-terminating structure, GalNAcα3GalNAc.[237] In transfection experiments, Fs-binding *E.coli* were able to bind primate cells following introduction of the canine *GBGT1* construct.[107] On the other hand it has also been shown that high levels of Fs antigen protect against Shiga toxins (Stx) since the preferred binding site is converted into the Fs antigen. Cell lines derived from human and primate tissue were shown to be more sensitive to Stx than cell lines expressing the Fs antigen.[236]

Present Investigations

Aims for this thesis

The general aim of this thesis was to characterise the expression of ABO antigens on RBCs with a focus on flow cytometry but in combination with other established methods used in serology and molecular biology.

More specific aims were:

- To evaluate A and B antigen density and distribution in genetically defined ABO subgroups
- In cooperation with other investigators elucidate the background to the ABO subgroup A_{pae} where the donors (unexpectedly) were found to be homozygous for common *O* alleles
- To examine the low amounts of A and B antigen present on donor-derived group O RBCs in A or B recipients after transfusion or HSCT and try to establish what mechanisms are at play
- To resolve the genetic basis of uncharacterised A and B subgroups

Methodological considerations

The many different methods used in the six papers included in this thesis are established and validated methods and details on manufacturers and products can be found in the original papers. Many of the papers included in this thesis are collaborative efforts together with other researchers. Although ABO and carbohydrate biology are the common denominators these investigations are focused and specialised on different aspects of the complex ABO biology. To be able to benefit from the expertise on methods not used in our laboratory and expensive or complex equipment used for analysis collaborations have been essential in *e.g.* Paper V, the Forssman paper. Thus, no further details will be given on methods although flow cytometry in relation to ABO will be discussed, see below.

Flow Cytometry and ABO

Very briefly, flow cytometry measures several characteristics of single cells (or particles) in a stream of sheath fluid passing through a light source, a laser. Light scattering (FSC, forward scatter and SSC, side scatter) can distinguish cells of different size and granularity. When cells labelled with fluorochrome-conjugated antibodies pass the laser, the fluorochrome is excited and emits light of a different wavelength. The light is sent to different detectors known as, photo multiplier tubes (PMTs) by the use of optical filters and reflectors. The electrical pulses originating from the light signal detected by the PMTs are then processed and each event can be plotted on a graphical scale. Dot plots or histograms can be used to visualise the results. Histograms have 1024 channels that correspond to the original voltage generated by a light event detected by the PMT. Depending on the pulse height for an individual event it is "assigned" a certain channel, *i.e.* the more a cell fluoresces the higher channel number it will have when displayed in a histogram. Mean fluorescence intensity (MFI) is a measurement often used, and corresponds to the mean fluorescence of a specific cell population. It is most useful if the data are normally distributed, otherwise median fluorescence intensity is accurate and recommended for use. A multitude of fluorochromes are available and a modern flow cytometer can use up to twelve (or more) at the same

time.[238] Although when using flow cytometry in our ABO setting most often only one antibody at a time have been used *i.e.* one fluorochrome.

Flow cytometry is a technique utilised in many different settings, both in the routine laboratory and in research facilities. Many types of material can be tested, e.g. cells, DNA and bacteria. Through the years flow cytometry has been used to examine RBCs in different immunohaematology settings.[239,240] Flow cytometry has never completely found its way into the routine transfusion medicine laboratories since detection of blood group antigens is readily and quickly performed with traditional blood bank serology. Today, the main use of routine flow cytometric analysis in transfusion medicine is for quality control of blood components (leucocyte counts) and for detection of foeto-maternal haemorrhage and sometimes on limited number of samples for RBC typing in the reference laboratory setting.

For ABO, flow cytometry has previously been used for a variety of purposes; 1) to try (pre-genomic testing) to define homo- and heterozygosity for alleles exhibiting normal amounts of ABH antigens,[241] 2) to examine if alterations of ABH antigens occur during storage in the blood bank,[242] 3) to analyse the decrease of ABH antigen levels in leukaemia patients. It was reported that both loss of A and B antigens could be detected, as well as a decrease in H antigen levels.[182] The problem encountered in these reports is that the sensitivity of the assay appears to be quite low. Many of the studies did not include weak control RBCs and in the two studies where they did include A_x RBCs they were unable to detect any A antigen at all.[182,241]

In a previous study, we devised a method for detection of A and B antigen with flow cytometry optimised to detect very small amounts of antigen on RBCs, remaining after treatment with a family of newly-discovered exoglycosidases from bacteria.[123] A large number of different ABO reagents were screened and tested with a variety of secondary antibodies to find the optimal settings. Throughout the different projects presented in my thesis, this protocol has been adopted and refined as a helpful tool to define A and B antigen signatures on native RBCs as well as RBCs modified by synthetic A and B antigens.

Paper I

Investigation into A antigen expression on O^2 heterozygous group O-labeled red blood cell units

Aim

To elucidate whether GTA activity is detectable in group O donors that carries the O2 (ABO*O.02.01) allele and if their RBCs express very low levels of A antigen. This was stimulated by reports indicating that blood unit from these donors should not be transfused to group O patients.

Questions addressed

Can we detect A antigen by routine serological methods?

When testing with different ABO reagents in standard serological testing (manual gel cards and tubes) no A antigens were detected. The more sensitive adsorption-elution testing was performed with a polyclonal anti-A and came out negative in the 3 samples from donors with the O^2 allele tested. Fifteen samples were tested with a flow cytometric method optimised for detection of very low levels of A antigen. The anti-A clone used in this method can detect the very low levels of A antigen present on B RBCs but no A antigen could not be detected on the RBCs from tubes collected at the time of donation.

Is there a difference in anti-A and -A_1 titers in these O^2 donors compared to donors homozygous for deletional *O* alleles?

Titration studies were performed on plasma from three randomly chosen donors with non-deletional *O* alleles and compared to titers of three donors homozygous for *O* alleles with 261delG (*ABO*O.01.01*) allele. Testing was performed with A_1 and A_2 RBCs and the titration scores were considerably lower with plasma from these O^2 (*ABO*O.02.01*) donors compared to those from group O donors homozygous for deletional *O* alleles.

Can we detect GTA activity in plasma from donors heterozygous for the *O*^{2} allele?

Enzyme activity testing showed no GTA activity and attempts to transform blood group O RBCs (from donors homozygous for the deletional *O* allele) to A_{weak} using *O*^{2} plasma in an upload assay were unsuccessful, *i.e.* these RBCs remained negative for A antigen by flow cytometry.

Were any adverse reactions noted in blood group O recipients during or after transfusion?

When these units were transfused no reverse reactions were reported (data from Pittsburgh where part of the cohort of donors was collected).

Summary

DNA was extracted from a large number (n=779) of random blood group O donors and screened for the infrequent *O*^{2} (*ABO*O.02.01*) allele. Forty of those genotyped as heterozygous for the *O*^{2} (*ABO*O.02.01*) allele and further serological and enzyme activity testing showed no blood grouping discrepancies except for a lower titration of anti-A/A1. Accordingly, there appears to be no reason to label such units as group A or avoid giving them to group O recipients. However, it is still not understood why their anti-A/A1 titres should be lower, or why the *O*^{2} allele is sometimes found in cases of ABO typing discrepancies with weak A antigen expression.

Paper II

Many genetically defined ABO subgroups exhibit characteristic flow cytometric patterns.

Aim

To characterise A and B antigen expression by flow cytometry in samples from individuals with an A_{weak} and/or B_{weak} phenotype where the genetic background had previously been resolved.

Questions addressed

Is there consistency in the flow cytometric pattern for common ABO phenotypes?

Included in this study were a number of samples (n=80) from blood donors with common ABO phenotypes. When tested the pattern within each group was consistent and this confirmed specificity and sensitivity for the monoclonal antibodies chosen for this study.

Can we correlate the genetic background to a specific flow cytometric pattern?

The extensive cohort of rare samples [A_{weak} (n=80) and B_{weak} (n=14)] gave us an opportunity to correlate the flow cytometric pattern to genetically defined subgroups. Previously known genetic explanations had already been determined for the majority of the samples. Many of the samples displayed flow cytometric patterns that were reproducible between different individuals with the same genetic alteration and also between repeat samples from the same individual, especially within the A_{weak} cohort.

B_{weak} samples are rarer (at least in Caucasian populations) and seem to display a more general pattern with anti-B, reminding of the pattern seen

with A_x samples and anti-A, and this is consistent with serology where B_{weak} samples seem more difficult to categorise to a specific subgroup phenotype.

Does ageing, freezing and thawing influence antigen expression patterns in this system?

In an A_{finn} (*ABO*AW.44/ABO*O.01.02*) sample the very typical flow cytometric pattern were reproducible when the RBCs were tested fresh versus frozen-thawed, in repeat samples and in a samples stored at 4℃ for up to four months.

Does flow cytometry add information to weak samples with no apparent genetic explanation?

The A_3 subgroup for which no genetic explanation has been found in the Scandinavian setting, although heterozygosity for an A^1-like allele is always seen upon *ABO* genotyping, exhibited a distinct pattern with dual populations (consistent with the serological definition with a mixed field agglutination) but also clearly ruled out a chimera. To perform flow cytometry on these samples (in addition to regular serology) will define the weak expression in this inherited ABO subgroup while waiting for the genetic basis to be resolved.

Flow cytometry is well known to be a good tool to distinguish and quantify separate populations of cells. Five samples turned out to be chimeric and this had not been resolved with regular serology or genetic ABO testing.

When testing samples from pregnant women with weakened A on their RBCs, the antigen expression had a tendency to be the lowest in the last trimester and the lowest expression was seen in women with the A^2B genotype. The benefit from flow cytometry will be an easier way to semi-quantify antigen levels and the possibility to compare repeat samples.

Summary

We found flow cytometric testing of various weak ABO samples to be a valuable tool in the reference laboratory as a complement to traditional serology, time-consuming titrations, adsorption-elution studies and genomic typing.

Paper III

Weak A Phenotypes Associated with Novel *ABO* Alleles Carrying the A^2-related 1061C Deletion and Various Missense Amino Acid Substitutions

Aim

The purpose of this study was to elucidate the genetic basis of 20 clinical samples displaying weakened A phenotypes. All samples were negative in screening for known A subgroup mutations but heterozygous for 1061delC.

Questions addressed

How were the samples characterised by standard serology?

Most of the serology was performed by the referring centres and the RBCs were generally classified as A_{el}, A_x or A_{weak}. The serology results were difficult to interpret in two samples carrying the 527G>A SNP due to underlying conditions (leukaemia and/or transfusion and pregnancy). The third 527G>A (*A212, ABO*A2.12*) sample displayed an A_2 phenotype.

How were the samples characterised by flow cytometry?

Flow cytometry was performed on 16 samples where RBCs were available and they included 10 different alleles. The levels of antigen detected ranged from very low to almost normal but most of the samples displayed levels comparable to the A^x-1/O^1 (*ABO*AW.30.01*) control RBC. One sample, *Aw24* (*ABO*AW.24*), displayed an interesting pattern similar to that of a para-Bombay sample but with no other serological indications of being one. The three samples containing the 527G>A SNP demonstrated very different patterns as mentioned above, probably due to different underlying conditions.

Does plasma enzyme activity in the *A212* (190G>A,Val64Ile; 527G>A, Arg176His, *ABO*A2.12*) from a healthy donor differ from the *A201* (*ABO*A2.01*) control plasmas?

Plasma enzyme analysis was performed and, along with direct analysis of the RBCs by flow cytometry, showed only slightly decreased GTA activity in the *A212* (*ABO*A2.12*) sample compared to *A201* (*ABO*A2.01*) control plasmas, consistent with the routine serology. This once again indicates that amino acid residue 176 is probably involved in enzyme turnover rate and not in determining donor sugar specificity.

What potential effects can the amino acid changes have on the resulting GT (assessed by 3D-molecular modelling)?

In several phenotypes, a plausible molecular mechanism for the weakened A phenotype could be proposed based on the structural model. For instance:

The changes in *Aw17* (*ABO*AW.17*, 236C>T, Pro79Leu) and *Aw23* (*ABO*AW.23*, 722G>A, Arg241Gln) may interfere with enzyme dimer formation.

Aw24 (*ABO*AW.24*, Arg248Cys) was speculated to eliminate interaction of the GT with H type 2 while still being able to use H type 1 present in plasma in this secretor donor. The A antigen expression detected on RBCs may then be due to adsorption of glycolipids with A specificity from plasma, hence the para-Bombay-like flow cytometric pattern.

The *Aw22* (*ABO*AW.22*) allele features a SNP (634G>A, Val212Met) that is predicted to affect the highly conserved DVD motif which precisely coordinates the Mn^{2+} important for catalysis. Structural analysis of the enzyme encoded by *Aw22* (*ABO*AW.22*) indicated that substituting Val with a bulky Met at residue 212 would likely disrupt a hydrophobic pocket formed by 4 surrounding amino acids, thereby displacing Mn^{2+}.

Summary

In total, thirteen novel alleles were discovered and the combination of traditional serology, flow cytometry, genetic analysis and the opportunity to model *in silico* the enzyme structures gave insights into GT function.

Paper IV

Flow cytometry evaluation of red blood cells mimicking naturally occurring ABO subgroups after modification with variable amounts of function-spacer-lipid A and B constructs

Aim

Synthetic A- and B-glycolipids called Functional-Spacer-Linker (FSL) derivatives were reported from a research group in New Zealand. These FSLs can be used to upload synthetic A and B antigen onto normal group O RBCs (so-called kodecytes) and are used as weakly positive controls for ABO reagents in some countries. In cooperation with this group we evaluated these synthetic ABO subgroup RBCs by flow cytometry to asses if they have any resemblance to naturally-occurring ABO subgroups.

Questions addressed

Does the flow cytometric pattern for kodecytes resemble that of naturally-occurring subgroups of A and B?

As previously reported, ABO subgroups display a variety of flow cytometry patterns and antigen levels range from almost normal to very low. In this study kodecytes were compared to an A_x (*A^x-1/O^1, ABO*AW.30.01)* and B_w (*B^w-3/O^1, ABO*BW.03*) sample. A_x samples are often used as the "golden standard" for qualifying reagent anti-A as to what levels of A antigen an anti-A should be able to detect and the B_w sample included is quite representative for many B subgroups. The kodecytes displayed similar patterns to the A_x and B_w samples at certain concentrations of FSLs determined by titration.

Do the kodecyte patterns resemble those of para-Bombay samples?

ABO expression on RBCs from para-Bombay individuals are dependent on uptake of glycolipids with A or B specificity from plasma which is the natural analogue to kodecytes. The para-Bombay samples display a different flow cytometric pattern with a much more homogeneous and uniform cell population than kodecytes although the reason underlying the difference seen has not been explained.

At what concentration of FSL are these kodecytes most similar to naturally-occurring subgroups?

Serial two-fold dilutions of FSLs were prepared and tested, with starting concentrations of 15 µg/mL for FSL-A and 78 µg/mL for FSL-B. The kodecytes that resembled the naturally-occurring subgroup control RBCs used in this study was determined to be ~2-4 µg/mL for FSL-A and ~10 µg/mL for FSL-B.

What could be the cause for the higher concentration needed for FSL-B than FSL-A?

We postulate that the lower affinity often seen for anti-B reagents (both monoclonal and polyclonal), could explain the difference, but it may also be due to the difference in antigen structure between the A and B antigens.

Summary

In the future, it is possible that kodecytes treated with the right amounts of FSL-A and FSL-B constructs may substitute naturally-occurring subgroup RBCs, which can be difficult to access for many laboratories.

Paper V

Forssman expression on human erythrocytes: biochemical and genetic evidence of a new histo-blood group system

Aim

In this study, the aim was to elucidate the true identity of the rare ABO subgroup $A_{pae.}$ This was initiated because of the unexpected finding that two individuals with the A_{pae} phenotype were typed as homozygous for common deletional *O* alleles. Therefore, the hypothesis became that this phenotype is due to an A-like but unique glycolipid structure.

Questions addressed

Can we identify the antigenic structure giving rise to the unusual serological ABO pattern in these individuals?

Total neutral glycolipids were isolated by Lola Svensson at the University of Gothenburg from blood units from two donors with the A_{pae} phenotype and tested against a panel of ABO reagents and *Helix pomatia* by TLC-EIA. Presence of A antigen was excluded but unexpected TLC migration and unusual reactivity suggested Fs as a candidate structure. Subsequent testing with monoclonal anti-Fs showed strong reactivity with the aberrant pentaglycosylceramide. Confirmatory structural analysis was performed in Gothenburg with LC-ESI/MS-MS and ^{1}H-NMR and these methods conclusively identified the Forssman glycolipid as the A_{pae}-defining antigen.

Do A_{pae} RBCs display Fs reactivity and are they agglutinated by naturally-occurring anti-Fs in plasma?

When tested by flow cytometry using MAb anti-Fs clearly positive reactions were obtained for positive controls (sheep and dog RBCs) and A_{pae} cells whilst negative reactions were seen with all other human RBCs. Screening

of blood donor plasma against A_{pae} RBCs by tube and gel card testing showed positive reactions in approximately 20% of tested donors. Adsorption and elution testing of strongly reactive plasmas against A_{pae} cells clearly showed the eluted antibody to be anti-Fs and not crossreactive anti-A.

Does the Forssman gene (*GBGT1*) in the A_{pae} individuals deviate from the human consensus sequence?

Exons 1 through 7 were sequenced in the two A_{pae} individuals and 9 normal controls. No deviations from the GenBank consensus sequence were seen in the control samples whereas a previously unreported SNP was detected, 887G>A (Arg296Gln), in the two unrelated A_{pae} samples. Interestingly, however, two different alleles were found in the two families investigated, both including the 887G>A SNP. This alteration changes the human Fs synthase to the aa (Gln) found at this position in Fs-positive mammals.

PCR-ASP screening for 887G>A of saliva DNA from 12 family members showed concordance between the phenotype and genotype.

Are *GBGT1* transcripts present in haematopoietic tissue?

Transcripts were readily detectable in mRNA preparations from peripheral blood; similar levels were noted for the normal controls (n=4) tested and the two A_{pae} individuals. Transcript levels in cultured erythroid cells from normal human bone marrow were approximately 4 times higher. The presence of transcripts suggests that Fs synthase is expressed in erythroid progenitors and that the A_{pae} individuals with an apparently activated Fs synthase should be able to express Fs antigens on RBCs.

Does the 887G>A change induce Fs synthase activity?

Wild type (887G) and mutant (887A) constructs were used for transfection studies. The two variant constructs were co-transfected with the gene encoding P synthase (*B3GALNT1*) and the megakaryoblastic cell line MEG-01 was used. When measuring Fs antigen levels after transfection by flow cytometry, a statistically significant difference in Fs-positive cells was noted with the mutant compared to the wildtype. This indicates that the 887G>A change in the A_{pae} individuals activates the Fs synthase.

Can modeling of the Fs synthase contribute to understanding of the activating mechanism?

A 3-dimensional model was created based on the crystal structure of the ABO glycosyltransferase. The homology between the Fs synthase and the ABO GT is 45% when comparing the aa sequences. In this 3D model the aa change in the Fs synthase, Arg296Gln, corresponds to the catalytically important His301 in the ABO transferase. In ABO, His 301 forms a hydrogen bond with the glycosyl donor whereas in the Fs synthase the human consensus Arg296 is predicted to be directed away from the donor sugar and accordingly not suited to form this bond. If substituted with Gln296 there is a much more favourable configuration in which this aa bends towards the donor substrate and the critical hydrogen bond is more likely to be formed. Hence, this is the probable explanation as to why the Fs synthase is active in A_{pae} individuals.

Could there be possible biological consequences for the A_{pae} individuals?

Uropathogenic *E. coli* (containing a PrsG-encoding plasmid) were shown to bind to A_{pae} RBCs (and sheep RBCs) but not to other human control RBCs of various ABO groups. The Fs antigen has been shown to protect against some variants of Shiga toxin but to be more susceptible to others. These A_{pae} individuals could possibly be more susceptible to some pathogens whilst perhaps protected against others. The presence of Fs antigens in other tissues than haematopoietic cells that could possibly be more relevant as target for pathogens has not yet been shown.

Could there be possible clinical consequences for recipients of blood from A_{pae} donors?

A haemolysin test performed with freshly drawn sera from donors with strongly reactive anti-Fs were incubated with A_{pae} RBCs and control RBCs of different ABO types. Haemolysis was seen with both ABO-incompatible cells and the tested A_{pae} cells which indicates that the anti-Fs present in these sera may have the potential to bind complement and could possibly cause intravascular lysis of transfused Fs-positive RBCs. This may cause adverse effects in patients receiving units of blood from A_{pae} donors, although the *in vivo* effects of anti-Fs are unknown. A problem highlighted

by these studies is that Fs-positive donors will not be found if type-and-screen approaches are used as pre-transfusion tests. Currently, only direct crossmatching, lectin screening (in non-A donors) or DNA screening for 887G>A will reveal such donors.

Summary

The ABO subgroup A_{pae} was shown to be ABO-independent and the Fs glycolipid was conclusively shown for the first time to be expressed in normal haematopoietic tissue. The Fs antigen was structurally confirmed and a missense mutation in the *GBGT1* gene was found in all A_{pae} individuals but not in non-A_{pae} family members or normal donors. Transfection studies showed a significant difference in antigen expression between the wildtype and the mutant *GBGT1*. Naturally-occurring anti-Fs exists and was shown to have the potential to cause haemolysis *in vitro* of Fs-positive cells which might have implications in both transfusion medicine and potentially also organ transplantation.

Taken together these findings elucidate the true structure and genetic background to what was believed to be an ABO subgroup. In light of these data the ISBT approved the proposed new blood group system, FORS (031). The name was suggested in honour of Prof. John Forssman, the discoverer of the antigen more than 100 years ago. The Fs antigen was given the name FORS1.

Paper VI

Semi-quantification of A and B antigen levels acquired by donor-derived erythrocytes following transfusion or minor ABO-incompatible haematopoietic stem cell transplantation

Aim

To investigate the phenomenon of weak expression of blood group A or B antigens on donor-derived group O RBCs, following transfusion or minor ABO-incompatible HSCT.

Questions addressed

To what extent can group A/B antigens be acquired *in vivo* by donor-derived group O RBCs?

Blood samples from patients originally typed as blood group A, B or AB and recently transfused or transplanted with cells from group O donors were analysed by flow cytometry. The acquired A/B antigen levels detected on donor-derived group O RBCs varied, ranging from very small amounts in non-secretor individuals to almost subgroup A_x-like profiles in group A secretors. Repeat samples from group A secretors transfused with variable number of group O RBCs units at different time intervals showed a delayed onset of detectable levels of A antigen on donor-derived RBCs and a subsequent increase in antigen levels over time.

What *in vivo* characteristics may influence the weak A/B expression on donor derived O RBCs?

The patients' ABO types and secretor status were determined with established methods for ABO phenotyping and *FUT2* genotyping, respectively. Genomic typing of the *ABO* gene was performed in selected cases. In group A patients, the levels of A antigen acquired by donor-derived group O RBCs following transfusion or HSCT were greater in

secretors than in non-secretors, and comparatively low in secretor individuals who typed as group A_2 or AB. In group B secretors very low levels of B antigen were detectable on donor-derived group O RBCs following transfusion but not HSCT.

What are the possible sources of group A/B antigen available for adsorption onto group O RBCs?

In vitro experiments were designed to investigate two possible mechanisms, 1) adsorption of glycolipids with A/B specificity from plasma by incubation of O RBCs with group A/B secretor or non-secretor plasma 2) direct exchange of A/B antigens between RBCs addressed by incubation of O RBCs with A/B, secretor and non-secretor, RBCs in the absence of plasma.

Group O RBCs were found readily to acquire A antigen from donor RBCs and increasingly over time from donor plasma and at higher levels when using secretor donors than non-secretors. In contrast, B antigens were acquired by O RBCs when incubating with donor B RBCs but not from group B plasma.

Can enzymatic conversion of H antigen by GTA/GTB in plasma contribute to the A/B antigen expression?

In an *in vitro* experiment an increase in A antigens accompanied by a decrease in H antigen levels was seen after incubation with both A secretor and non-secretor plasma indicating that glycosyltransferases in plasma actively converted some of the H structures to A antigen.

Summary

In this study we have confirmed that the small amount of A antigen seen on donor-derived group O RBCs is mostly due to adsorption of glycolipids present in plasma but that at least one other mechanism may exist; most likely a very low level of conversion of H substance to blood group A antigens.

General discussion

Investigating unusual group O samples

The easy definition of an *O* allele would be to say that it is an allele that encodes a glycosyltransferase that cannot, or does not, synthesize A or B antigens on the RBC surface. Our conclusions are often based on the observation of apparent "killing" mutations in the *ABO* gene that suggest that a GT crippled by such a mutation is not able to synthesise any ABO antigens. Of course it is not as easy as that. The O^2 (*ABO*O.02.01*) allele that has the single change 802G>A encoding Gly268Arg, which has been shown to completely block the donor sugar recognition site[153] still has occasionally been associated with weak expression of A antigen or, more commonly, with O-like phenotypes without anti-A present in plasma. In one family, the same allele (*ABO*O.02.02*) was shown to give rise to an A_{weak} expression in the son whereas the father's RBCs typed as group O.[156] Deletional *O* alleles, on the other hand, have not been implicated in expression of A antigen.

In **Paper I,** blood donors heterozygous for an O^2 (*ABO*O.02.01*) allele were investigated. All had typed as blood group O in routine serology. Furthermore, no A antigens could be detected when tested by our flow cytometry protocol (with which it is possible to detect A antigen in the weakest A subgroup A_{el}). Adsorption-elution studies also failed to detect the presence of A antigen and plasma enzyme activity assays failed to show any GTA enzymatic activity. The only discrepancies noted were lower levels of anti-A in plasma compared to control plasmas from donors homozygous for deletional *O* alleles. In contrast, another study examined samples from donors in a routine setting but with more focus on samples that demonstrated discrepancies.[154] Adsorption-elution testing of both RBCs from O^2 donors and a HeLa cell line transfected with *ABO*O.02.01* constructs showed presence of A antigen although very weak results (0.5+) were obtained. This is in stark contrast to the corresponding result following adsorption-elution with A_{el} RBCs that yield eluates reacting 4+ with group A RBCs.
The lower levels of anti-A in these donors remain unexplained. One hypothesis is that the GT encoded by the O^2 allele may have an increased activity in other tissues and hence can produce levels (although low) of A

antigen that will prevent the formation of normal levels of anti-A. It can also be imagined that such synthesis occurs on an alternative oligosaccharide chain, either H or another precursor. Finally, it cannot be excluded that O_2 GT uses an alternative donor substrate, although this seems unlikely. In our investigation of donors previously typed as blood group O we could not detect any regular A antigen using very sensitive methods but we have previously encountered several samples with an A_{weak} expression that when genotyped were heterozygous for a deletional *O* allele and O^2 (*ABO*O.02*). What factors outside the coding region of the ABO gene influence the presence or absence of A antigen in individuals carrying the O^2 allele is still to be determined.

Investigating A and B subgroup samples

To date, the number of *ABO* subgroup alleles that have been genetically defined exceeds 100 and the number is still rising. In our laboratory (the Nordic Reference Laboratory for Genetic Blood Group Typing) we have had the opportunity to investigate a large number of samples referred to us for ABO discrepancies from all over the world. When investigating a patient/donor sample it is important to "see the big picture" *i.e.* to evaluate all aspects that may influence the results and which will decide ultimately what blood should be transfused if it is a patient. If it is a subgroup donor, the task is to recommend to whom his/her blood can be given safely. The serological results including flow cytometry, medical history of the patient/donor and genetic testing of the *ABO* locus are all taken in account when a report to the referring centres is issued.

The flow cytometry method has proven to be an invaluable tool in our laboratory for assessing genetically defined subgroups and samples expressing normal levels of ABO antigens (**Paper II**) The ability to semi-quantify A and B antigen levels and to be able to analyse the results as both histograms and dot plots gives the opportunity to compare results in a less subjective way than traditional serology. To compare results between samples with the same genetic background and in repeat samples from the same patient has added consistency to the evaluation of these samples.

Some samples display flow cytometric patterns that have enabled us to foresee what the genetic alteration is. A good example is the A_{finn} phenotype caused by the splice site mutation IVS6 +4a>g (*ABO*AW.44*)

which displays a very specific, almost chimeric pattern. In a variety of A_x samples, the allelic enhancement phenomenon (where antigen expression varies depending on what allele is present *in trans*) was very nicely visualised. The A_3 subgroup for which no genetic explanation has yet been found, exhibited a distinct pattern with dual populations (consistent with the serological definition with a mixed field appearance) but still clearly ruled out a chimera.

Even though some genetically defined samples display similar patterns to the weak control RBCs (*ABO*AW.30.01* or *ABO*BW.03*) always included in the test, most samples do not. Since the paper was published we have tested many more samples and new patterns keep appearing. It is interesting that some samples have a very even distribution of antigens whereas in others the RBCs seem to express antigen from almost normal levels and ranging down to very weak. The mechanism behind the allele-related variation in antigen distribution is largely unknown but very consistent.

In **Paper III** we investigated twenty samples with a suspected A_{weak} phenotype, all were based on an A^2 allelic backbone and in total, 13 novel alleles were discovered. In addition to the A^2-specific 1061delC, these alleles all carried at least one additional SNP in the coding region of the *ABO* gene. Flow cytometry was performed and most of the 16 cases investigated displayed A antigen levels comparable to the A_x (*ABO*AW.30.01*) control RBCs, although results varied from very weak to almost normal. Molecular 3D-modelling techniques were used in collaboration with the Carlsberg laboratory in Copenhagen to study the potential effects of the amino acid changes on the resulting GTs. In several phenotypes, a plausible molecular mechanism for the weakened A phenotype could be proposed based on the structural model.

The different mechanisms are discussed in detail in the paper but among the more interesting cases were two samples *Aw17* (*ABO*AW.17,* 236C>T, Pro79Leu) and *Aw23* (*ABO*AW.23,* 722G>A, Arg241Gln) which are both hypothesised to interrupt dimerization. The flow cytometry results from these two samples were very different indicating that the aa changes have different impact even though they are situated in the same area of the enzyme. It has been shown that GTs form homo- and heterodimers in live cells[99] and it is logical to think that if allelic enhancement, which is almost

certainly due to dimerization, rescues GT activity, then disruption of the dimer will diminish activity.
The flow cytometric pattern seen for *Aw24* (*ABO*AW.24*, Arg248Cys) was almost identical to that seen in para-Bombay (A_h) samples and in donor-derived group O RBCs in an A secretor recipient (see Paper VI). The A expression in those samples are mainly due to uptake of A type 1 glycolipids from plasma. In 3D-modelling it was not unequivocally demonstrated but it was speculated that the Arg248Cys change eliminates the ability of the GT to interact with H type 2. This would suggest that the A expression seen is A type 1 *i.e.* similar to the A_h para-Bombay sample.

This extensive cohort added 13 new *ABO* subgroup alleles based on an A^2 backbone to the (then) eight previously reported. In evaluating these different variants of ABO subgroup GT samples the 3D-modelling has proven a valuable addition and provided structural insight into GT function.

Investigating the source of A/B antigens acquired by donor O RBCs

In **Paper VI** we tried to establish the mechanisms causing the weak A or weak B antigen expression on donor-derived group O RBCs in patients of group A/B/AB transplanted with allogeneic hematopoietic stem cells or transfused with blood units. We monitored the ABO status of donor RBCs by flow cytometry. Higher expression was seen in secretor individuals than non-secretors. Two hypotheses were addressed as to why this phenomenon occurs: 1) passive adsorption of glycolipids expressing A or B antigen, or 2) active conversion of H substance to blood group A or B antigens by A or B GTs in plasma, respectively.

In vitro experiments were performed and support the major role of uptake of glycolipids with A/B specificity from secretor plasma but it was also shown that in blood group A non-secretors, a very low expression of antigens could be detected. In non-secretor plasma, A/B antigens are found, although only in small quantities, on type 2 chains linked predominantly to glycosphingolipids but possibly also to glycoproteins and are presumed to be derived from endothelial or haematopoietic cells.[243,244] These could be adsorbed onto RBCs and give rise to the low levels of A antigens seen in non-secretor individuals. Another possible mechanism for direct membrane glycolipid exchange between adjacent RBCs was

suggested by the *in vitro* experiments. An indication of active conversion of H substrate to A antigen was observed with the corresponding decrease in detectable H substance on group O RBCs after *in vitro* incubation with group A_1 plasma, without the addition of donor sugar. However, further studies are needed to confirm this finding, especially given the doubts regarding the sufficient presence of extracellular donor substrate.

Taken together, these data may have implications for current, post-transplantation transfusion policies in the HSCT setting. Today the general policy[245] is that the recipient receives blood components of donor ABO type. Our work supports the suggestion of plasma components compatible with both donor and recipient.

A_{pae}: from A subgroup to Forssman

In **Paper V**, the unexpected homozygosity for common *O* alleles in the rare A subgroup, A_{pae}, instigated a closer examination of the true nature of the antigen and the corresponding gene. Structural analysis revealed the supposed A antigen present on RBCs from these donors to be the Forssman glycolipid structure. A missense mutation in the *GBGT1* gene (in humans believed to be a pseudogene) was identified, giving rise to the aa change, Arg296Gln, that was hypothesised to activate the Fs synthase. Transfection studies showed a statistically significant increase in the number of Fs-positive cells when the mutated *GBGT1* construct was transfected in comparison to the wildtype.

The Fs antigen expression seen on RBCs could be due to uptake of glycolipids from plasma with Fs specificity since both these donors were genotyped as secretors. It is unlikely though since transcripts from mRNA preparations from peripheral blood and erythroid bone marrow culture were readily detectable and indicated that the Fs synthase is expressed in erythroid tissue.

SNPs that impact the function of the resulting GT in such a way that they activate a previously inactive enzyme or change the specificity of either the donor or acceptor substrate are rare. In a recent report[45] though, the authors detected a SNP in *A4GALT* that was shown to alter, or rather broaden, the acceptor specificity. The *A4GALT* gene normally gives rise to a GT responsible for the synthesis of Gb3 from the lactosylceramide precursor. The change induced an enzyme with broadened acceptor

preference able to use not only lactosylceramide (terminating with a Galβ4) but also Gb4 (terminating with a GalNAcβ3) as the precursor, and create the NOR antigen. The same precursor, Gb4, is used by the Fs synthase. In analogy with anti-Fs, naturally-occurring anti-NOR is present in most NOR-negative individuals. The antibody agglutinates NOR+ RBCs and NOR-positive individuals therefore display an inherited polyagglutination syndrome. Although limited in scope, haemolysin tests with serum containing anti-Fs indicated that the antibody can bind complement and potentially cause intravascular hemolysis. This may have implications in both blood transfusions and possibly also organ transplantation.

Of the members of the *GT6* enzyme family, the *ABO* gene is the only one said to be active in humans. However, it has been suggested that the *GBGT1* gene may still be functional, albeit with an alternative specificity, since *GBGT1* does not display the same degree of decay or number of mutations as seen in the other pseudogenes.[103] The mechanisms behind the appearance of Fs antigen in some malignant cells and the reported expression in normal tissue is not understood, but several mechanisms have been proposed. For example, overexpression of the *GBGT1* gene, splicing variation or aberrant glycosylation by another GT have been suggested.[108]

In the recent publication by Yamamoto *et al.*[108] they reported that two aa present in the human Fs synthase (Ser230 and Arg296) significantly decreased/abolished the expression of Fs antigen when compared to an Fs-positive species (mouse; Gly230; Gln296). This corresponds well to the reactivation of the Fs synthase seen in the A_{pae} donors where we identified glutamine at position 296. The Fs synthase (dog) used for comparison in our paper displays the same aa at positions 230 and 296 as the mouse.

In summary, this study has redefined the A_{pae} subgroup and formed the basis for a new blood group system, FORS (031). In addition, it has provided additional insight into to complex world of glycobiology.

Future plans

The genetic background of many ABO subgroups have been elucidated and 3D-modelling based on the crystal structure of the ABO GT has given us insight into structural changes that *e.g.* affect enzyme turnover, change the acceptor preferences or disrupt dimer formation. The allelic enhancement phenomenon is likely to be due to dimer formation of the GTs and would be interesting to pursue further. Initial discussions have been held with Kellokumpu's group in Finland, from whom the interesting report on GTs forming homo- and heterodimers in live cells was published. We would like to test different ABO constructs with their established method and to examine whether normal and variant ABO GTs form dimers and if they are localised to the Golgi membranes where they are known to function. Furthermore, it would be fascinating to be able to prove that defective mutant subgroup GTs can be rescued by the O_2 GT, as implicated by the allelic enhancement phenomenon observed (and nicely visualized by flow cytometry) in certain A_x pedigrees.

In the Forssman project there are a few questions that would be interesting to follow up on. Initial Western blot (WB) experiments were performed on RBCs from A_{pae} individuals to examine the possibility that Fs antigen can occur on glycoproteins. No reactivity was observed, which suggests that Forssman is present only on RBC glycolipids. It would also be interesting to perform WB with an anti-Fs synthase antibody to examine the presence of the GT in *e.g.* plasma from both A_{pae} individuals and normal controls.

We readily detected transcripts from mRNA preparations in both A_{pae} individuals and normal donors. Some initial testing has been performed to define the different transcripts present in haematopoietic tissue. In addition to the expected full-length transcripts, both shorter and longer transcript variants were detected. It would be interesting to characterize these further and determine their significance as well as to define the transcription start and stop sites by 5'- and 3'-RACE. As very little is known about the upstream and downstream regions of the *GBGT1* gene and how this gene is regulated this would also be interesting to probe further. Finally, we would be keen to investigate *GBGT1* polymorphism in a panel of donors from different population groups, especially non-Caucasians.

As with all research, for every answer found more new questions asked.

Svensk populärvetenskaplig sammanfattning

Genom historien har människor fascinerats av blod och blodtransfusioner och många försök har gjorts att transfundera blod från t.ex. olika djur till människor. Resultatet av dessa tidiga transfusioner resulterade ofta i allvarliga reaktioner hos individen som mottagit blodet och i vissa fall avled även patienten. Den första lyckade transfusionen mellan människor utfördes i England år 1818 av Dr. James Blundell.

Karl Landsteiner upptäckte år 1900 det första blodgruppssystemet ABO genom att han blandade blod från sig själv och sina medarbetare och noterade att i vissa fall klumpade blodet sig, i andra inte. Upptäckten av ABO gjorde att Karl Landsteiner belönades med Nobelpriset i medicin år 1930. Blodgruppssystemet ABO betraktas som det viktigaste när man ska transfundera blod från blodgivare till patienter. Idag finns det 33 olika blodgruppssystem som är godkända av International Society of Blood Transfusion (ISBT).

På ytan av röda blodkroppar finns olika strukturer, molekyler, som kan vara proteiner eller lipider, med eller utan kolhydrater (sockerstrukturer). De har många olika funktioner som gör att cellen kan fungera, t.ex. kan de vara transportörer av olika ämnen som ska in och ut ur cellen eller vara en del av immunförsvaret. Likväl som människor är olika så ser deras celler olika ut. De strukturer som finns på cellytan hos vissa kan saknas hos andra, t.ex. inom blodgruppssystemet RH så kan man vara positiv eller negativ, d.v.s. antingen så har man det så kallade RhD-proteinet eller så saknar man det helt. I andra fall så har man samma strukturer men modellen på dem skiljer sig lite åt, t.ex. inom blodgruppssystemet ABO. ABO-blodgruppsmolekylen är i princip uppbyggd av olika typer av socker som bildar en kolhydratstruktur där den sista och yttersta sockerbiten är det som skiljer mellan blodgrupp A och B. Är man blodgrupp O så saknar man den sista sockerbiten. Dessa olika varianter av strukturer på cellytan av olika blodgrupper kallas antigen.

När man ska transfundera blod måste man ta hänsyn till vilka ABO-antikroppar mottagaren av blodet har. Antikroppar är immunförsvarets verktyg för att skydda människan mot främmande ämnen t.ex. bakterier eller i fallet med transfusion, främmande röda blodkroppar. Är man blodgrupp A så har man antikroppar mot blodgrupp B och tvärtom. Är man istället blodgrupp O, d.v.s. saknar den sista sockerbiten som kan vara

antingen A eller B så har man antikroppar mot båda och är man blodgrupp AB så saknar man antikroppar. Dessa ABO-antikroppar gör det av yttersta vikt att man noga blodgrupperar både patienter och blodgivare innan man transfunderar blod. Ger man blod av fel ABO-grupp så kan allvarliga transfusionsreaktioner ske och i vissa fall kan patienten avlida. Även vid transplantation av organ som lever, njure eller hjärta måste man ta hänsyn till vilken ABO-grupp mottagaren och donatorn har.

Vilken ABO-blodgrupp man har på sina röda blodkroppar är ärftlig. I arvsmassan, DNA, finns genen för ABO och den kodar för ett protein, (ett enzym som kallas glykosyltransferas), som sedan bygger upp de kolhydratstrukturer som finns på cellytan. Genen ger på detta sätt upphov till verktyget som bygger den slutliga ABO-antigenstrukturen. Har man förändringar, mutationer, i *ABO*-genen kan detta ge upphov till strukturella förändringar i enzymet, verktyget, som gör det mindre effektivt. Detta i sin tur gör att man kan få ett lägre uttryck av ABO-antigen på cellytan vilket kan göra det svårt att bestämma vilken blodgrupp individen i fråga är, med risk för feltransfusion.

I de publikationer som ingår i denna avhandling har vi använt oss av olika metoder för att definiera förändringar av ABO-uttryck. Vi har med molekylärbiologiska och serologiska metoder bl.a. undersökt hur *ABO*-genen ser ut och hur höga nivåer av ABO-antigen det finns på cellytan. Med hjälp av tredimensionell modellering av enzymet har vi försökt förstå hur förändringar i strukturen påverkar enzymets effektivitet.

I denna mycket kortfattade sammanfattning på svenska tänker jag inte gå in på alla de sex delarbeten som ingår i avhandlingen utan fokusera på ett par av dem som jag själv har funnit särskilt intressanta att arbeta med.

I delarbete II har vi med en metod som kallas flödescytometri undersökt en stor grupp med blodprover från individer med ett normalt uttryck av ABO (80 stycken) och även prover från individer som har svagt uttryck av ABO-antigener (80 stycken A-svaga och 14 stycken B-svaga). Flödescytometri är en väletablerad metod som används för många typer av kliniska och vetenskapliga undersökningar men har traditionellt inte använts mycket för att undersöka ABO-uttryck på röda blodkroppar. Man använder sig av antikroppar som är märkta med en fluorokrom som när den skickas förbi en laser avger ljus av en viss våglängd. Ju fler antikroppar (anti-A och anti-B) som har fastnat på den röda blodkroppen desto mer ljus avges och detta är då proportionellt mot hur mycket A- eller B-antigen som finns på

cellytan. Först undersökte vi proverna med normalt ABO-uttryck för att etablera hur det flödecytometriska mönstret för dem såg ut. Efter det undersökte vi de ABO-svaga proverna där vi sedan tidigare hade undersökt *ABO*-genen och definierat den genetiska bakgrunden till varför dessa individer har lägre nivåer än normalt av ABO-antigen. I många fall gick det tydligt att korrelera det flödescytometriska mönstret med den genetiska bakgrunden. Slutsatsen är att denna metod är mycket användbar vid utredningar av svagt uttryck av ABO-antigener i kombination med traditionell serologi och genomisk typning av *ABO*-genen.

I delarbete V har vi i samarbete med forskargrupper från Göteborg, Sheffield, Auckland och Köpenhamn upptäckt ett nytt blodgruppssystem, FORS. Forssman-antigenet hittades 1911 av Professor John Forssman, verksam i Lund. Då hittades detta antigen bara hos olika djur, t.ex. får och hundar, men vi har nu för första gången visat att det finns på röda blodkroppar hos vissa (dock hittills väldigt få) människor. År 1987 rapporterades om en vad man trodde vara svag variant av A-antigenet, A_{pae}, i tre engelska familjer. Vid närmare undersökning av *ABO*-genen (som såg ut att vara blodgrupp O) hos dessa individer verkade det mycket osannolikt att det var en svag variant av A-antigen. Strukturanalys av antigen från röda blodkroppar visade att det istället var det strukturellt ganska lika Forssman-antigenet vilket dock kodas av en helt annan gen, *GBGT1*. Denna gen finns hos alla människor men i en inaktiv form. Vid analys av *GBGT1*-genen hos A_{pae}-individerna visade det sig att de hade ”backat i evolutionen” och muterat tillbaka till en aktiv version av genen som finns hos vissa djur, t.ex. hundar (som normalt uttrycker Forssman-antigenet). Då människor som saknar Forssman-antigenet i regel har antikroppar mot det bör gemene man inte få blod från blodgivare som är Forssman-positiva. Andra möjliga kliniska konsekvenser av detta är att vissa *E. coli*-bakterier kan binda till Forssman-strukturen, d.v.s. bakterier som normalt bara infekterar vissa djur skulle nu potentiellt kunna infektera även människor. Sammantaget visar våra undersökningar att denna antigen-struktur är helt ABO-oberoende och vid ISBT:s världskonferens i Cancun 2012 blev Forssman-antigenet godkänt som ett nytt blodgruppssystem, FORS (031).

Sammanfattningsvis så hoppas jag att alla arbeten inkluderade i denna avhandling har bidragit med ytterligare och förnyad kunskap om FORS och framför allt ABO-blodgruppsystemets många aspekter. I slutändan så finns det alltid en patient i behov av blodtransfusioner och det är av yttersta vikt

att vi på blodcentralen har verktygen och kunskapen för att korrekt blodgruppera både blodgivaren och patienten. För mig personligen har detta projekt varit en oerhört lärorik resa med mycket arbete, mycket glädje (blandat med lite gråt och tandagnisslan) och att ha kommit till slutpunkten känns väldigt bra om än lite sorgligt. Förhoppningsvis kan jag i någon form fortsätta att jobba med detta fantastiskt intressanta område!

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Paper I

Investigation into A antigen expression on O^2 heterozygous group O–labeled red blood cell units

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BACKGROUND: There are two principal types of group *O* alleles; deletional alleles feature 261delG leading to nonfunctional truncated protein. Nondeletional alleles have the consensus guanosine at residue 261. The major nondeletional allele, O^2, encodes full-length protein with Gly268Arg. While reports vary, O^2 has been proposed to encode weakly functional A-glycosyltransferase (GTA). The main objective of this study was to evaluate if GTA activity is detectable in O^2 donors.

STUDY DESIGN AND METHODS: Donor samples from Pittsburgh and Lund were ABO typed by automated methods. DNA was extracted from 779 group O donors whose red blood cells (RBCs) were available for transfusion. *ABO* genotyping identified those with O^2 alleles. The following tests were performed on randomly selected O^2 samples (number): adsorption-elution with anti-A (3), flow cytometry (15), plasma enzyme activity (4), and attempts to convert group O RBCs to A (2) with O^2 plasma and titration of plasma anti-A/-A_1 (3).

RESULTS: Forty O^2-heterozygous donors were identified (5.1%). Adsorption-elution and sensitive flow cytometry did not reveal A antigens on O^2 RBCs. Plasma enzyme analysis failed to show GTA activity above baseline; O^2 plasma was unable to add measurable A antigens to O RBCs. Titers of anti-A/-A_1 appeared reduced in O^2 plasma but did not cause ABO typing discrepancies. No immediate hemolysis or adverse reactions were reported following transfusion of O^2 RBCs to six evaluable group O recipients.

CONCLUSIONS: Other than lower plasma anti-A titers, GTA activity was not found in these O^2 samples. Neither automated blood grouping discrepancies nor clinical problems related to transfusing these O^2 units were observed.

The ABO blood group system is the most clinically significant in both transfusion and organ transplantation. The determinants of blood group A and B are *N*-acetylgalactosamine (GalNAc) and galactose, respectively, and they are structurally quite similar.[1] These antigens are synthesized on a common carbohydrate precursor known as H antigen. Individuals with blood group O feature an unmodified H antigen structure, while creation of A and B antigens essentially "masks" the underlying H antigen. Because these antigens are carbohydrates, the underlying *ABO* gene does not encode them directly; instead it gives rise to a glycosyltransferase (GT), which adds either the A (GTA)- or the B (GTB)-specific sugar to H antigen. The most common *O* alleles, O^1 [*O01*] and O^{1v} [*O02*], differ most significantly from the consensus A^1 [*A101*] gene by a single-nucleotide deletion, 261delG.[2,3] The O^{1v} allele has several other intronic and exonic single-nucleotide

ABBREVIATIONS: GalNAc = *N*-acetylgalactosamine; GT(s) = glycosyltransferase(s); GTA = A-glycosyltransferase; GTB = B-glycosyltransferase; SNP(s) = single-nucleotide polymorphism(s).

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polymorphisms (SNPs) differentiating it from the A^1 allele.[3] The 261delG deletion is enzymatically crippling because it induces a frameshift mutation such that the resulting protein is predicted to lack the active domain. In contrast to an earlier report,[4] recent data have indicated that O^1 and O^{1v} mRNA are not degraded faster than A^1 transcripts,[5] and these shortened proteins might actually be expressed and have been proposed to act as minor histocompatibility antigens.[6]

Between 2 and 5 percent[7] of group O donors have variant group *O* alleles that lack the 261delG SNP but consistently feature 802G>A (Gly268Arg) along with a variety of other SNPs in both exons[8,9] and introns.[10,11] Collectively they are known as nondeletional O^2 alleles, the most common of which is termed O^2-1 [*O03*]. This group of alleles encodes a full-length GT with significant homology to both GTA and GTB,[12] such that the full-length protein encoded by an O^2 allele can be bound by monoclonal antibodies to the latter two enzymes.[13] In addition, there are other nondeletional group *O* alleles that do not give rise to full-length transcripts that have also been implicated in causing ABO discrepancies.[9]

Lee and colleagues[14] analyzed the crystallographic structure of an *Escherichia coli*–optimized model of the O^2-encoded enzyme and demonstrated that the introduction of Arg268 impedes the donor sugar's access to the active site, thus severely reducing enzymatic activity to a value barely distinguishable from background levels in their system. This in vitro model of the O^2 enzyme demonstrated 6-log less activity (k_{cat}) than the wild-type GTA with UDP-GalNAc donor substance. Earlier studies revealed similar findings; when GTA and GTB constructs with Arg268 were transfected into HeLa cells and exposed to their preferred donor substance, no A or B antigens were subsequently detectable on the surface of the cells by flow cytometry.[15] Furthermore, the enzymatic activity of these GTA and GTB constructs featuring Arg268 was also undetectable.[15] Gly268Arg is therefore the most enzymatically crippling mutation in O^2 alleles and it is common to all of them. Thus, it would appear that these nondeletional *O* alleles, like their 261delG counterparts, are also null alleles in the *ABO* system.

Recent data, however, have indicated that nondeletional *O* alleles sometimes have weak GTA activity manifested by small quantities of A antigen on red blood cells (RBCs) detectable only by sensitive serologic techniques or inferred by reduced anti-A levels on reverse typing.[8,9,16] In one study, it was clear that most O^2 alleles did not give rise to a weak A phenotype, but in some cases they did.[9] It was even shown that the same O^2 allele, O^2-*2* [*O48*] could give rise to either a regular group O phenotype that included anti-A in plasma or a weak A phenotype lacking plasma anti-A in different members of the same family.[9] In a German study of ABO discrepancies caused by reduced or absent activity of either anti-A or anti-A and anti-B in group O blood donors using automated testing, nondeletional *O* alleles were present in the majority of cases.[16] A antigen was detected on the surface of HeLa cells when transfected with recombinant O^2-*1* and O^2-*4* [*O50* or *Aw08*] genes.[8] Thus, given that nondeletional *O* alleles can cause ABO discrepancies and occasionally produce weak expression of A antigen, we screened group O RBC units from our blood bank inventories for the presence of O^2 alleles to determine the impact these alleles have on automated ABO testing and performed additional analyses to determine if A antigens are expressed on RBCs from donors with O^2 alleles.

MATERIALS AND METHODS

Automated and manual donor ABO grouping

Automated forward and reverse donor ABO grouping was performed on a PK7231 (Olympus America, Inc., Irving, TX) using A_1 and B test RBCs for reverse typing (Pittsburgh, PA) and on a multisampler system (IBG Plato 3000 SI, Inverness Blood Grouping System Ltd, West Sussex, UK) using A_1, A_2 and B RBCs (Lund, Sweden). We considered an ABO discrepancy to be any situation whereby the automated typing instruments could not interpret the donor's ABO group. These discrepancies would then be resolved by using manual methods. In addition, for all donors with an O^2 allele, manual serologic testing was performed according to the routine methods in place at the two laboratories to confirm the absence of anti-A reactivity with their RBCs.

Donor selection

Between Lund and Pittsburgh, 779 group O units available for transfusion were selected for further analysis. In Pittsburgh, all RBC units were procured from an FDA-accredited blood center. This protocol was approved by the Quality Improvement Branch of the University of Pittsburgh's Institutional Review Board. In Lund, CE-approved reagents were used for serologic determination of blood unit types under a SWEDAC accreditation protocol following the European EN17025 laboratory standards.

ABO gene analysis and terminology

DNA was extracted from nucleated cells in anticoagulated (ethylenediaminetetraacetate [EDTA] or acid citrate dextrose [ACD]) tubes or segments from RBC units using either a salting-out method[17] or a commercially available kit (Qiagen, Valencia, CA). A polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method was initially employed as previously described.[18] Briefly, exon 6 was amplified using primers mo-57 and mo-46. The amplicons were incubated with *Kpn*I, which

cleaves DNA in the presence of 261delG. All specimens that demonstrated an uncleaved band after *Kpn*I digestion were amplified again and then incubated with *Bst*EII, which cleaves DNA when the consensus sequence at nucleotide 261 is present. In addition to confirming the first screening result, this second restriction enzyme step is useful to rule out incomplete digestion of *Kpn*I or the unlikely presence of inhibitors to *Kpn*I, thereby increasing the specificity of the assay for the 261delG polymorphism.

Allele-specific primer PCR to detect the presence of O^1, O^{1v}, and O^2-associated SNPs was performed as previously described[19] to prove the presence of an O^2 allele in specimens that demonstrated an uncleaved allele after *Kpn*I and *Bst*EII digestion. Furthermore, other O^2-related SNPs at nucleotide positions 53, 220, 297, 526, and 1096 were confirmed on samples identified in Lund as previously described.[9]

ABO allele nomenclature is still under consideration by the International Society of Blood Transfusion (ISBT). In this article, alleles that lack 261delG but have 802G>A (on an A^1-like allelic backbone in the exon 7 region corresponding to the catalytic site) and found in donors of group O units are categorized as O^2 alleles in the absence of an officially agreed terminology. An alternative terminology used by the Blood Group Antigen Gene Mutation Database (dbRBC) is given in square brackets the first time a certain allele is mentioned.

Additional serologic testing

In addition to the automated blood grouping methods, adsorption-elution studies were performed on three donors with an O^2 allele by previously described methods[20,21] using an in-house human-source polyclonal anti-A. Additional manual forward testing using monoclonal, human source polyclonal, and experimental anti-A reagents by saline and column agglutination techniques were also performed.

Titration of anti-A and -A_1 was performed with doubling dilutions of plasma and test RBCs phenotyped as A_1 or A_2. Reactivity was read in gel cards with or without (neutral) anti-immunoglobulin G (IgG; DiaMed AG, Cressier, Switzerland) and scored according to Marsh.[22]

Flow cytometric detection of A antigen expression

RBCs were washed three times in phosphate-buffered saline (PBS), and 10 μL of packed and washed RBCs was suspended in 400 μL of PBS. A 96-well plate (NUNC, Apogent, Denmark) was used for sample preparation: approximately 500,000 RBCs were added to each well containing a total of 50 μL of PBS. Cells were fixed for 10 minutes by the addition of 100 μL of 0.1 percent glutaraldehyde to prevent agglutination of antigen-positive cells. This was performed at room temperature under constant mixing; the plate was then centrifuged at 200 × *g* for 1 minute and the supernatant was discarded. Fifty microliters of PBS and 5 μL of primary antibody (anti-A, clone ES-15 from Serologicals Ltd, West Lothian, UK) were added to each well, incubated for 10 minutes at room temperature under constant mixing, and then washed three times with 150 μL of PBS. An additional 50 μL of PBS and 5 μL of secondary antibody (phycoerythrin [PE]-labeled rat anti-mouse Ig κ light chain from Becton Dickinson, San Jose, CA) were added, incubated for 10 minutes in darkness at room temperature under constant mixing, and washed three times with 150 μL of PBS. Finally, the RBCs were resuspended in 300 μL of PBS.

Flow cytometric analysis was performed on a flow cytometer (FACScan, Becton Dickinson) using sheath fluid (FACSflow, Becton Dickinson). From a sample volume of 300 μL, a total of 10,000 events were collected at a flow rate of 60 μL per minute. Log fluorescence data were gated on a linear forward scatter versus linear side scatter dot plot. Included in each run were samples of the ABO phenotypes A, B, and O and also two genetically defined weak controls, A^x-*1* O^1 and B^w-*3* O^1. All donors of group O control RBCs were confirmed to be homozygous for 261delG.

Plasma activity assay

GTA transferase activity was analyzed by monitoring the transfer of radioactivity from UDP-[^{3}H]GalNAc to Fucα1,2Galβ-O-$(CH_2)_7CH_3$ acceptor.[23] Reactions were carried out in 0.5-mL microfuge tubes containing 5 μL of plasma in 50 mmol per L MOPS buffer, pH 7.0, containing 20 mmol per L $MnCl_2$, 1 mg per mL bovine serum albumin, 10 μmol per L unlabeled UDP-GalNac, 0.126 μCi (280,000) dpm radiolabeled donor (American Radiolabeled Chemicals, St Louis, MO), and 200 μmol per L acceptor in a total volume of 10 μL. The mixture was incubated at 37°C for 20 hours. The reaction mixture was diluted with 500 μL of water and applied to reverse-phase cartridges (Sep-Pak, Waters, Mississauga, Canada).[23] The cartridges were rinsed with 150 mL of water to remove any unreacted donor, and then the radiolabeled products were eluted from the cartridges into scintillation vials with 3.5 mL of methanol. Radioactivity was measured in a scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA) after the addition of 10 mL of liquid scintillation cocktail (EcoLite, ICN, Costa Mesa, CA). Control reactions were carried out by replacing the plasma with water.

Conversion of group O RBCs by ABO GT in plasma

A 10 percent suspension of washed group O RBCs (from genotypically verified 261delG-homozygous donors) in autologous or allogeneic plasma from nonsecretor donors

of different blood groups was incubated at 37°C with 0.35 mmol per L final concentration of UDP-GalNAc donor substrate (Sigma-Aldrich, Stockholm, Sweden) and 10 mmol per L $MnCl_2$ (Sigma-Aldrich) for 24, 48, or 72 hours in a total volume of 250 µL (adapted from Schenkel-Brunner and Tuppy).[24] The treated cells were washed after incubation and subjected to flow cytometric analysis of A antigen expression as described above. ACD, citrate phosphate dextrose, or EDTA plasma was used as a source of ABO GT and controls included A_1 and A_2 plasma (strongly positive), B plasma (weakly positive), and plasma from genotypically verified 261delG-homozygous donors (negative). In addition, the specificity of the conversions could be confirmed by omitting either the substrate donor or the $MnCl_2$. When one of these reagents was excluded or when 261delG-homozygous group O plasma was used, no change in mean fluorescence intensity (MFI) compared to the native group O RBC control was observed. Complete inactivation of the plasma GTs could be achieved by the addition of a 10× excess of EDTA compared to the concentration in a blood-filled regular EDTA tube.

RESULTS

Population screening

Between the blood banks at Pittsburgh and Lund, we identified 40 out of 779 group O donors who were heterozygous for a nondeletional *O* allele (5.1%). Thus, the overall O^2 allele frequency among all *O* alleles was 2.6 percent. Among group O donors, the frequency of those with an O^2 allele was 4.9 percent (16/328) in Pittsburgh, and in Lund it was 5.3 percent (24/451). All of these donors demonstrated three bands upon cleavage of the exon 6 PCR amplicons with both *Kpn*I and *Bst*EII, thus confirming their heterozygosity for both a deletional and a nondeletional *O* allele. Allele-specific primer PCR for the O^2-specific SNP (802G>A) was performed on 38 of 40 donors for which additional DNA was available and all were heterozygous for the O^2 allele along with either an O^1 or O^{1v} allele. In addition to 802G>A, the donors identified in Lund were also found to be heterozygous for all tested O^2-related exon SNPs (53G>T, 220C>T, 297A>G, 526C>G, and 1096G>A). No homozygous O^2O^2 donors were identified.

Additional manual serologic testing

A antigen was not detected on the O^2 RBCs using either the manual gel card or the tube testing methods and different blood grouping reagents. Adsorption-elution studies were performed on the RBCs from three donors with the O^2 allele; anti-A was not recovered in the eluates from any of these donors. The plasma from three randomly selected donors with nondeletional *O* alleles were

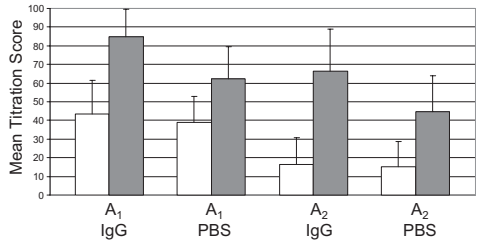

Fig. 1. Mean titration scores for plasma samples from three randomly selected O^1O^2 (white bars) and three O^1O^1 (dark gray bars) donors tested against A_1 and A_2 RBCs. The test cell phenotypes and the different kinds of gel cards utilized are given along the *x* axis. The annotation "IgG" represents gel columns containing anti-human IgG reagent while "neutral" gel cards contain buffer only (PBS) and demonstrate agglutination caused by anti-A. The error bars reflect 1 SD.

titered against A_1 and A_2 cells and compared to titers obtained using plasma from three randomly selected O^1O^1 donors (Fig. 1). The overall titration scores were much lower with the plasma from the nondeletional O donors compared to controls, and this difference was especially prominent in the anti-A titers.

Flow cytometric detection of A antigen expression on RBCs

A sensitive flow cytometry test using a monoclonal anti-A and a PE-labeled rat anti-mouse Ig κ secondary antibody capable of detecting the small quantity of naturally occurring A antigen on group B cells was performed on the RBCs of 15 random donors with the O^2 allele. None of these RBCs demonstrated levels of A antigen in excess of the control O^1O^1 cells upon visual inspection of FACS dot plots and histograms (Figs. 2A and 2B), and MFI values were identical between cells of the O^1O^1 and O^1O^2 genotypes.

Plasma enzyme activity assay

The results of these studies performed on four randomly selected heterozygous O^2 donors and three randomly selected homozygous O^1 donors are presented in Table 1. When the plasma from these donors was incubated with UDP-GalNAc donor and acceptor, only baseline levels of activity were seen for both groups. The mean activity of both groups of donors was, on average, below the values registered with the water blank control.

Conversion of group O RBCs by ABO GT in plasma

After incubation with the appropriate reagents and group A plasma samples, A antigens were readily

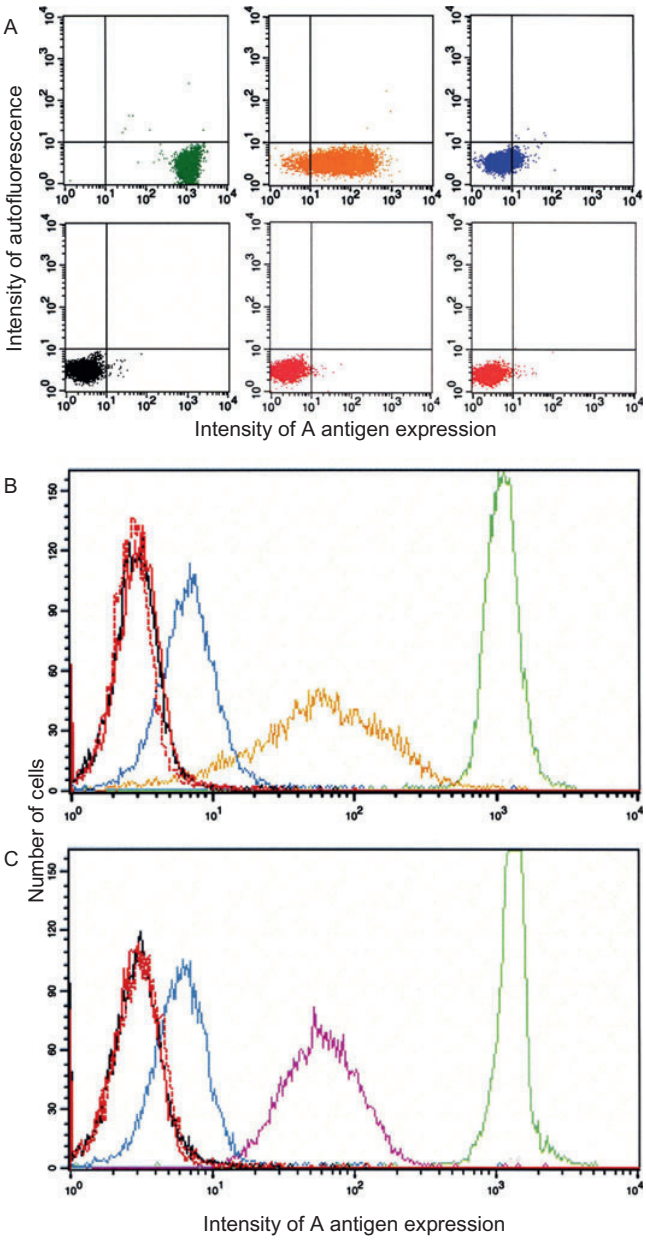

Fig. 2. Flow cytometric analysis of A antigen levels on RBCs. (A) Dot plots and (B) histogram from flow cytometric testing of native RBC samples with anti-A (clone ES-15) and PE-labeled secondary antibodies. In the dot plots, the *x* and *y* axes represent PE-derived fluorescence and fluorescein isothiocyanate (FITC), respectively, on logarithmic scales, although only PE was used in this study. The negative readout for FITC shows that any weak PE signal is not due to autofluorescence. Each dot represents the result from one cell. In the histograms, the *x* axis shows the intensity of the PE-derived fluorescence on a logarithmic scale, while the *y* axis shows the number of cells on a linear scale. Control RBCs: A_1 (green), A_x (A^x-$1O^1$; orange), and B (blue), O (O^1O^{1v}, black). Test RBCs: O^1O^2 RBCs (red; solid or dashed lines in histogram). The sensitivity in this flow cytometric protocol is shown by the detection of the small amounts of A antigen present on B RBCs (MFI 7) compared to the O^1O^{1v} control RBCs (MFI 3). The O^1O^2 RBCs show no detectable difference in MFI compared to the O^1O^{1v} control RBCs; that is, with this method, A antigen cannot be detected in two examples of RBCs from donors with the O^1O^2 genotype. (C) This histogram shows the results from flow cytometric testing with anti-A (clone ES-15) of group O (O^1O^{1v}) RBCs incubated with plasma, UDP-GalNAc, and $MnCl_2$ at 37°C and tested after 48 hours. Further incubation to 72 hours did not increase signals for O^1O^2 plasma but resulted in slight autofluorescence for all combinations of RBCs and plasma. The different plasmas used are A_1 plasma (A^1O^1, green), A_2 plasma (A^2O^{1v}, purple), B plasma (BO^{1v}, blue), O control plasma (O^1O^{1v}, black), and O test plasma (O^1O^2, red; solid and dashed lines). The A_1 (MFI 1280) and A_2 plasma (MFI 67) showed significant increases in MFI compared to the O^1O^{1v} plasma control (MFI 3), and as expected A_1 plasma showed a higher increase in MFI than A_2 plasma. BO^{1v} plasma (MFI 7) showed a slight increase in MFI compared to the O^1O^{1v} plasma control (MFI 3), while the two O^1O^2 plasma samples did not.

TABLE 1. Plasma enzyme activity from donors with deletional and nondeletional group *O* alleles using UDP-GalNAc donor nucleotide substrate

Plasma donor type	Plasma enzyme activity (dpm)
O^2 heterozygote (n = 4)	
Mean	640
Median	667
Range	507-716
O^1 homozygote (n = 3)	
Mean	942
Median	1,093
Range	639-1,095
GTA from A plasma (n = 4)	
Mean	31,256
Median	24,544
Range	7,070-68,867
Water blank*	1,061

* In the water blank control, sterile water was used in place of plasma.
dpm = disintegrations per minute.

detectable on the converted group O RBCs by flow cytometry using monoclonal anti-A after only a few hours. To increase the sensitivity of the assay, all experiments were incubated at 37°C and read at 24, 48, and 72 hours. Plasma from group B donors resulted in a small but distinct increase in the MFI value while no activity could be detected with any of the O plasma samples including those from O^2-heterozygous donors. For the positive controls, the MFI increased with time, and after 48 or 72 hours, group O RBCs incubated with A plasma expressed A antigen at a level almost comparable to that found on native group A RBCs. The low levels of A antigen detected on the group O RBCs incubated with B plasma was comparable to the small amounts of A antigen detectable on normal B RBCs. Representative results from these conversion experiments are illustrated in Fig. 2C. In summary, there was no evidence to suggest that the O^2-encoded GT in O^1O^2 plasma was able to transfer UDP-GalNAc to the H antigen present on group O (O^1O^{1v}) RBCs.

Clinical outcomes

There were 44 RBC units donated by the 40 heterozygous O^2 donors. All of these RBCs were treated as regular blood group O units based on the automated blood grouping results and in addition, for repeat donors, their historical blood group. Of the 20 O^2 RBC units transfused in Pittsburgh, 19 were administered to group O recipients without any reported hemolytic events. The remaining unit went to a group A recipient. Detailed pre- and posttransfusion laboratory variables were available for 6 of the group O recipients; all had at least the expected 1 g per dL (10 g/L) increase in hemoglobin in the 24 to 28 hours after the transfusion, and in none of these recipients were the biochemical markers of hemolysis (e.g., lactate dehydrogenase, bilirubin, haptoglobin, reticulocyte count) specifically ordered after the transfusion, which further suggests that intra- or posttransfusion hemolysis was not clinically suspected.

DISCUSSION

There have been four types of approaches that have shed light on nondeletional *O* alleles: 1) studies where samples had been referred to a reference laboratory for resolution of a typing discrepancy,[8,9] 2) studies based on large-scale automated blood donor screening with extra attention paid to samples that demonstrated discrepancies,[8,16] 3) studies based on units of group O–labeled RBCs that had not caused discrepancies during testing,[8,9] and 4) studies based on synthetic constructs involving expression of the O^2 allele.[14,15] The sample populations in the first two groups of studies were enriched for the presence of nondeletional *O* alleles. Furthermore, by studying samples known to contain ABO discrepancies, the weak GTA activity of the O^2 alleles was more likely to be apparent. The reason why some O^2-containing samples demonstrated weak GTA activity while others did not is unknown; no genetic alteration has been noted between those O^2 alleles that gave rise to a regular group O phenotype versus those that produced a weak A phenotype and/or a lack of anti-A in plasma.

In this study, we evaluated units that were already labeled as group O for the presence of nondeletional *O* alleles; while the prevalence of the O^2 allele in our population is within the range reported in the literature (2%-5%),[7] the nature of the samples we evaluated was biased toward finding those O^2 alleles resulting in relatively lower GTA activity, if such differences exist. Since the units we sampled were already labeled as group O and available for transfusion, the GTA activity of the O^2 encoded protein must have been low enough not to cause weakening of anti-A on reverse typing, much less actual expression of A antigen on the RBC surface. Indeed this was the case. Since we only screened for the presence of the main O^2-associated SNPs (including the critical 802G>A) in our donors with nondeletional *O* alleles, it is possible albeit highly unlikely that we have discovered novel nondeletional *O* alleles that feature other SNPs that further reduce its GTA activity relative to the most commonly described O^2 alleles. Given the high frequency of the O^2-1, O^2-3 [*O49*] and possibly O^2-4 (also known as O50 or A_w08) alleles among nondeletional *O* alleles,[8,9] it would be unlikely that a substantial percentage of our nondeletional O donors had a novel allele with reduced enzymatic activity. Our results beg the question, how functional are O^2 alleles?

As stated, Lee and colleagues[14] used an *E. coli*–optimized in vitro model of the O^2 enzyme spanning amino acids 63 to 354 to study its kinetics.[14] They found a 5-order-of-magnitude reduction in k_{cat} relative to the wild-type GTA enzyme when the Gly268Arg mutation was

introduced into an enzyme that already contained the Arg176Gly mutation, and a further reduction in k_{cat} when all three O^2-*1*–specific amino acid mutations were present. So how close to zero is a $k_{cat} = 3.4 \times 10^{-5}$ per second?[14] The majority of our data suggest that it is close enough, while the earlier data that showed some residual GTA activity for the O^2 allele encoded protein argue that it is not quite equal to zero, at least not in selected cases.[8,9,16]

While we did not find any A antigens on the O^2 RBCs using a very sensitive flow cytometry technique and adsorption-elution studies, and two separate assays failed to demonstrate any GTA activity in O^2 plasma, our results are not completely consistent with the notion that nondeletional *O* alleles are enzymatically inactive. Despite the small sample size, the results of the plasma anti-A and anti-A_1 titration studies seem to suggest that some A antigen might be expressed on tissues other than RBCs, causing a reduction in the levels of these naturally occurring antibodies. Perhaps the activity of the O^2 allele–encoded enzyme can be enhanced in other body tissues, but not on RBCs, thus preventing the immune system from producing normal levels of these antibodies. As O^2 mRNA transcripts have been detected in peripheral blood[4] and the O^2-encoded protein detected in previous studies of human gastric carcinoma cells with the O^1O^2 genotype,[13] it is thought that these proteins are being translated. This is also supported by synthetic expression experiments in which this protein appears stable enough for crystallographic structure studies.[14] Direct comparisons, however, between the anti-A titers in the deletional and nondeletional donors in this study might be confounded by the fact that these randomly selected donors were not specifically matched for age, sex, and ethnicity.

This dichotomy of GTA activity in the O^2-encoded protein was highlighted in a family study whereby the same allele, O^2-2, produced a weak A phenotype in the son (anti-A eluted from his RBCs and no anti-A detected on reverse typing) but a completely typical group O phenotype in his father.[9] Perhaps epigenetic factors or other factors outside the *ABO* locus can be responsible for enhancing the GTA activity of the O^2 product. Likewise, in a study of 2196 group O German blood donors, only 38 (1.7%) donors with O^2 alleles were identified;[16] if the true frequency of donors with these alleles is approximately 5 percent, then more than 100 such donors should have been identified. The fact that the authors only found about one-third of the donors that might have been expected suggests that either the frequency of O^2 donors in their population truly is lower than that seen in this study or else other factors caused donors with an O^2 allele not to manifest an ABO discrepancy and hence avoid detection. It is possible that the reduced anti-A titer may vary between individuals or even over time so that only the lowest titers cause discrepancies on automated blood grouping instruments. In addition, different automated typing systems are bound to differ in their sensitivity. Even so, none of the nearly 800 group O samples analyzed in this study gave rise to discrepant reverse typing results.

An enigmatic mechanism of increasing the enzymatic output of a weakened GT centers around the phenomenon of allelic enhancement.[25] This rare event seems to occur when certain weakened ABO GTs are coexpressed with another full-length ABO GT.[26,27] While the biochemical basis for allelic enhancement is unclear, it is possible that heterodimerization might occur when two types of full-length ABO GTs are expressed in the Golgi membrane.[14] This dimerization might then cause a conformational change in the mutated and less active GT such that it becomes more functional than would have been predicted had it been expressed by itself. Alternatively, a mutated subgroup GT may have difficulties reaching the Golgi due to defective trafficking and another full-length GT like O^2 may act as a chaperone for the crippled subgroup GT. This hypothesis is not too dissimilar to the situation reported to cause the Tn syndrome, which is due to a defective chaperone function.[28] The O^2 allele was shown to participate in allelic enhancement in a Polish propositus with a hybrid A^x subtype (A^x-4) allele; when the hybrid A^x allele was expressed with an O^1 allele, an O phenotype resulted whereas an A_x phenotype was clearly present when it was inherited in trans with an O^2 allele.[26] Allelic enhancement has not been reported in the presence of a deletional *O* allele. Earlier studies have demonstrated identical phenotypes in donors who are homozygous and heterozygous for O^2 alleles, that is, homozygosity for an O^2 allele did not cause further depression of anti-A levels in plasma or increased A antigen expression on RBCs beyond that seen in heterozygotes.[8,16] This suggests that allelic enhancement is not involved in the creation of a weak A phenotype by O^2 derived enzymes. That said, in our study we did not find any O^2O^2 homozygotes; perhaps homozygosity would have prevented the labeling of these units as group O by creating some A antigen expression. This cohort of 779 samples, however, was clearly too small to have identified a rare O^2 homozygote. Based on the frequency of O^2 alleles detected in this study (2.6% of all *O* alleles), it is estimated that one O^2 homozygote would be expected among 1480 group O donors.

In conclusion, we studied nearly 800 labeled group O RBC units that were available for transfusion in both Sweden and the United States and found that 5.1 percent of group O donors have an O^2 allele. While anti-A titration studies revealed lower levels of anti-A in the O^2 donors, automated and manual ABO agglutination tests, flow cytometry, adsorption-elution, and plasma enzyme activity studies failed to demonstrate either the presence of A antigen on the RBCs or GTA enzymatic activity in plasma, respectively. More work needs to be done to reconcile these results with earlier studies that demonstrated some activity of the O^2 allele–encoded enzyme. In the

meantime, the true nature, mechanisms of action, and consequences of O_2 GT remain unknown.

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Paper II

Many genetically defined ABO subgroups exhibit characteristic flow cytometric patterns

Annika K. Hult and Martin L. Olsson

BACKGROUND: A flow cytometric method for detection of low levels of A/B antigen had been developed previously in our laboratory. The aim of this study was to investigate if this approach could be utilized to characterize different ABO subgroups and constitute a useful tool in a reference laboratory.

STUDY DESIGN AND METHODS: Blood samples causing ABO discrepancies (n = 94) by routine serology were further analyzed by *ABO* genotyping and flow cytometry. To verify the specificity of the monoclonal anti-A and -B reagents and to establish normal flow cytometric patterns, samples from 80 blood donors with common phenotypes were also assessed.

RESULTS: Distinguishable flow cytometric patterns were detected for several subgroups but were more apparent for A_{weak} (n = 80) samples than B_{weak} (n = 14). Two subgroups, A_{finn} (n = 11) and A_3 (n = 10) displayed diagnostic features and were used to establish reproduciblity over time and between donors. In general, the consistency within subgroups was remarkable. The allelic enhancement phenomenon was clearly visualized among A_x samples (n = 10) where different alleles in *trans* resulted in high, low, or no A antigen expression. Nonsubgroup samples including O/A and O/B chimeras or A_h and B_h para-Bombay phenotypes displayed clearly distinguishable histograms. Samples from pregnant women (n = 10) displayed acquired A antigen loss, apparently accentuated during the third trimester.

CONCLUSIONS: Genetically defined ABO subgroups and other anomalous phenotypes displayed flow cytometric profiles that may contribute valuable information to the investigation of ABO discrepancies. We conclude that the presented assay may complement traditional serology and genetic analysis in the reference laboratory setting.

It is of utmost importance to determine the ABO group of donors and patients correctly since blood group incompatibility remains one of the major risks of modern transfusion therapy.[1-3] Historically, weak A and B subgroups were always discovered and investigated serologically, and a wide array of different ABO phenotypes can be differentiated phenotypically, for example, A_3, A_x, A_{finn}, A_{el}, B_3, B_x, and B_{el}. The different subgroup phenotypes are characterized by the degree of agglutination with anti-A, anti-B, anti-A,B, and anti-H as well as the presence or absence of antibodies in plasma. In some cases it may also be necessary to perform adsorption-elution studies to confirm very weak A or B antigen expression and/or to detect the presence of these antigens in saliva if the patient/donor is a secretor. Since the cloning of the *ABO* gene in 1990,[4] the molecular bases underlying many but not all of the A and B subgroups have been investigated.[5,6] Intriguingly, a certain subgroup can be associated with several different allelic variants, and certain alleles can also give rise to more than one phenotype.[7,8] The A or B expression can also be influenced by the *ABO* allele in *trans*.[9,10] In addition, apparently weak expression of A and/or B antigens on red blood cells (RBCs) may also be an acquired serologic finding associated with pathologic or physiologic conditions including

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hematologic malignancies,[10,11] bacterial infection,[10,12] and pregnancy.[13] Rare phenotypes such as para-Bombay or chimeras may also mimic weak or atypical A/B expression. The advent of molecular genotyping methods for ABO phenotype prediction has given reference laboratories yet another analytical tool for differentiation of these diverse backgrounds of ABO typing discrepancies. The interpretation of such tests is quite complex, however, and the number of *ABO* alleles is still rising from today's more than 200 documented *ABO* alleles.[14]

Flow cytometry has been used in different settings for analysis in RBC immunohematology as reviewed by Garratty and Arndt.[15] The *ABO* blood groups have been assessed by several investigators for different purposes, for example, to define homo- or heterozygosity for alleles expressing normal amounts of ABH antigens[16] to study the density profiles of A antigens in common blood groups[17] and to examine alterations in ABH antigens on aging RBCs during storage[18] or in the blood of leukemic patients.[11] So far, no study has focused on the flow cytometric patterns that may differentiate naturally occurring ABO subgroups. This may be due to sensitivity issues. Indeed, two groups were unable to detect any A antigens on RBCs from single examples of A_x subgroup samples,[11,17] although the primary purpose of one of the studies was to investigate the decreased A expression levels on RBCs in leukemia patients.[11,19]

We have developed a highly sensitive flow cytometric method to detect A and B antigens.[20] Subgroup RBCs were used as weak positive controls and A antigens present in low amounts on normal group B RBCs[21] could be detected clearly. This method was used successfully for quality assessment after the enzymatic conversion of group A or B RBCs to cells typing serologically as blood group O with all monoclonal ABO typing reagents tested.[20] We hypothesized that the same protocol could be applicable to the characterization of ABO subgroups and contribute valuable information to the investigation of ABO discrepancies in general. In this study we have therefore systematically investigated a large number of samples with ABO discrepancies by semiquantifying the levels of A and/or B antigens by flow cytometry. We asked if the antigen density plots and histograms obtained correlate to the defined genetic backgrounds underlying the variant ABO phenotypes and if this approach could be useful in the clinical reference laboratory.

MATERIALS AND METHODS

Samples

Samples from blood donors and patients exhibiting suspected A or B weak phenotypes were referred to the Nordic Reference Laboratory for Genomic Blood Group Typing in Lund, Sweden, for in-depth analysis of their ABO typing discrepancies. On arrival, the RBCs were washed three times and suspended in the low-ionic strength preservative solution CellStab (DiaMed AG, Cressier, Switzerland) for long-term storage. The buffy coat was taken for DNA preparation. In addition to the 71 fresh samples we received, our in-house ABO subgroup test cell panel of 23 frozen RBC samples with known *ABO* genotypes were thawed and deglycerolized according to standard operating procedures in use at the Blood Center and tested by flow cytometry. Blood samples from 80 blood donors with the following known common phenotypes were also included in the study cohort, mainly to ensure sensitivity and specificity of the assay: 15 A_1, 15 A_2, 15 B, 10 A_1B, 10 A_2B, and 15 group O. The samples analyzed in this study are summarized in Fig. 1.

Flow cytometric detection of A and B antigens

During the development, optimization, and evaluation of the flow cytometric method, screening of more than 20 different monoclonal and polyclonal ABO reagents with two different monoclonal secondary antibodies was performed. Test RBCs of different common *ABO* types but also genetically defined ABO subgroups and enzyme-converted group O (ECO) RBCs[20] were included in the screening (data not shown). In parallel, the ABO reagents were tested serologically with the same test RBCs. After extensive testing, anti-A IgM clone ES-15 (Serologicals Limited, West Lothian, UK) and anti-B IgM clone 9621A8 (Diagast, Loos, France), both of murine origin, were chosen because of their outstanding ability to detect weak A and B antigen expression both with flow cytometry and serologic testing, together with a phycoerythrin (PE)-conjugated rat anti-mouse immunoglobulin kappa light chain reagent (whole immunoglobulin molecule) (Becton Dickinson, San Jose, CA) selected as the secondary antibody.

RBCs were washed three times in phosphate-buffered saline (PBS), and 10 μL packed and washed RBCs was suspended in 400 μL of PBS. A 96-well plate (Nunc, Apogent, Roskilde, Denmark) was used for sample preparation: approximately 500,000 RBCs were added to each well containing a total of 50 μL of PBS. Cells were fixed for 10 minutes by the addition of 100 μL of 0.1% glutaraldehyde to reduce agglutination of antigen-positive cells. Incubation was performed at room temperature under constant mixing. The plate was then centrifuged at $300 \times g$ for 1 minute and the supernatant was discarded. Fifty microliters of PBS and 5 μL of primary antibody were added to each well, incubated for 10 minutes at room temperature under constant mixing, and then washed twice with 150 μL of PBS. Another 50 μL PBS and 5 μL of secondary antibody were added, incubated for 10 minutes in darkness at room temperature under constant mixing, and washed twice with 150 μL of PBS. Finally, the RBCs were resuspended in 300 μL of PBS and transferred to 5-mL plastic tubes (Falcon, BD Biosciences, Woburn, MA).

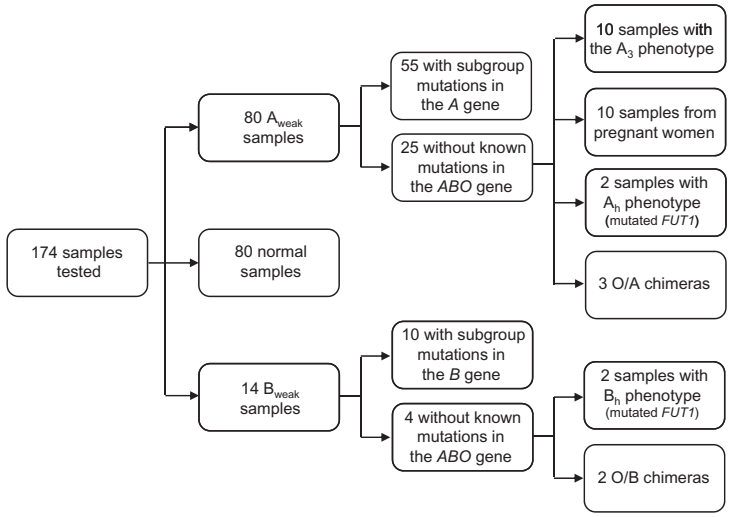

Fig. 1. Flow chart of all samples included in the study.

Analysis was performed on a flow cytometer (FACScan, Becton Dickinson) using the sheath fluid recommended by the manufacturer (FACSflow, BD Biosciences, Erembodegem-Aalst, Belgium). From a sample volume of 300 μL, 10,000 events were collected at a flow rate of 60 μL/minute. Log fluorescence data were gated on a linear forward scatter vs. linear side scatter dot plot. The analysis gate was set to include the viable part of the RBC population (typically at least 90% of all cells), excluding deformed and aggregated cells, that is, cells that gave high values on side and/or forward scatter. Control RBCs of known common (A, B, and O) and relevant rare (e.g., A_x and B_w) ABO phenotypes were included in each run but the individual donors may differ between experiments. The signal from the PE-conjugated secondary antibody used is detected by the FL2 detector. To avoid the risk that autofluorescence (e.g., developing in RBCs transported under suboptimal conditions) would be interpreted as a weakly positive signal, FL1 detection was included in all dot plots. By varying the voltage applied to the FL1 and FL2 photomultiplier tubes the defined negative control RBCs were ensured to fall within the set FL1 and FL2 markers (routinely set at value 10).

Serologic ABO typing

The extensive serologic testing normally performed when weak A or B phenotypes are suspected was performed by the referring laboratories in most cases. In some samples sent to us with scarce serologic information, we performed additional serologic testing including plasma typing, adsorption-elution studies, and a panel of three polyclonal and three monoclonal anti-A, anti-B, and anti-A,B with traditional blood group agglutination methods in tube or gel cards (DiaMed AG) according to standard blood banking practice.

Genomic *ABO* typing

A few samples were received as prepared DNA. Otherwise DNA was extracted from buffy coat with a modified salting out procedure.[22] Genomic typing of the *ABO* locus was performed with established and validated methods used at the Nordic Reference Laboratory for Genomic Blood Group Typing, including polymerase chain reaction (PCR)-restriction fragment length polymorphism,[23,24] PCR with allele-specific primers,[25] and DNA sequencing methods.[13] In some samples additional testing of the enhancer region was performed.[26]

Nomenclature of *ABO* alleles

ABO allele nomenclature poses significant problems that are still under consideration by the International Society of Blood Transfusion. The first time an allele is mentioned here, a reference [in square brackets] to the unofficial but often used terminology found in the Blood Group Antigen Gene Mutation Database (dbRBC)[14] is made whenever possible.

TABLE 1. Characteristics of the subgroup samples included in this study

dbRBC*	Subgroup allele†	Number	Characteristic mutation	Allele in *trans*	Reference	Figures
Ax01	A^x-1	3	646T > A	O^1 *B*	Yamamoto et al.[27]	4A and 4B
Ax03	A^x-2	3	646T > A, 829G > A	O^1 O^2	Olsson and Chester[7]	4C and 4D
Ax02	A^x-3	1	646T> A, 829G > A	O^2	Olsson and Chester[7]	4E
Ax04	A^x-4	3	646T > A, 829G > A	O^1 O^2 *B*	Olsson et al.[13]	4F-4H
Aw02	A^w-2	2	350G > C	O^1	Olsson et al.[13]	5C
Aw03	A^w-3	4	203G > C	O^1 O^{1v}	Olsson et al.[13]	5H
Aw04	A^w-4	1	721C > T	O^1	Olsson et al.[13]	5B
Aw06		2	502C > G	O^{1v}	Seltsam et al.[28]	5F
Aw09	O^{1v}-A^2	5	46G > A	*B*	Hosseini-Maaf et al.[29]	5E
Aw13		3	2T > C	O^1 O^{1v}	Seltsam et al.[30]	5J
Abantu01	A^{bantu}	3	203delG	O^1	Hosseini-Maaf et al.[31]	5G
Ael01	A^{el}-1	12	798-804insG	O^1	Olsson et al.[32]	5A
Afinn01	A^{finn}	11	374 +4A > G‡	O^1 O^{1v} *B*	Olsson et al.[33]	3A-3F
A301	A^3	1	871G > A	O^{1v}	Yamamoto et al.[34]	5D
A304		1	539G > A	O^1	Svensson et al.[35]	5I
Bw03	B^w-3	2	721C > T	O^1 A^1	Olsson et al.[13]	6D
Bw04	B^w-4	2	548A > G	A^1	Olsson et al.[13]	6E
Bw06	B^w-6	1	1036A > G	O^{1v}	Olsson et al.[13]	6B
Bw10	B^w-10	1	556A > G	A^2	Estalote et al.[36]	6F
Bw11	B^w-11	1	695T > C	O^1	Deng et al.;[37] Hosseini-Maaf et al.[38]	6A
Bw14	B^w-14	2	523G > A	A^1	Chen et al.;[39] Hosseini-Maaf et al.[38]	6G

* According to Blumenfeld and Patnaik.[14]
† According to literature if allele name stated in publication.
‡ Previous annotation IVS6 + 4a > g; current annotation according to guidelines from the Human Genome Variation Society at http://www.hgvs.org/mutnomen/recs-DNA.html#DNA

RESULTS

The sample cohort

In total, 94 RBC samples from individuals with atypical A or B antigen expression were tested with monoclonal anti-A and/or anti-B by flow cytometry (Fig. 1). The protocol had been developed and optimized for the purpose of detecting very low residual A and B antigen expression on RBCs after glycosidase treatment.[20] In this study, the high sensitivity of the protocol was employed to examine samples with different degrees of weak or atypical A (n = 80) or B (n = 14) antigen expression. In 65 of the 94 test samples, we identified 15 and seven different known subgroup alleles represented among the A_{weak} (A_w) and B_{weak} (B_w) samples, respectively (Table 1).[7,13,14,27-39]

TABLE 2. Characteristics of the samples with weak ABH expression not caused by mutations in the *ABO* gene

ABO phenotype	*ABO* genotype	Number	Condition	Figures
A_3	A^1 O^1	8	Apparently healthy*	5K
	A^1 O^{1v}	2		
A_w(B)	A^2 *B*	7	Pregnancy	7F-7H
	A^1 *B*	1		
	A^1 O^1	1		
	A^1 O^{1v}	1		
A_w	O^1 O^1 (+A^1)	3	Chimera	7A
A_h	A^1 O^{1v}	1	Para-Bombay†	7C
	A^2 O^1	1		
B_w	O^1 O^1 (+*B*)	2	Chimera	7B
B_h	*B* O^1	2	Para-Bombay†	7D and 7E

* Four of 10 were healthy blood donors. The remaining samples came from patients who were in contact with the health services for reasons other than hematologic malignancies and pregnancy.
† *FUT1* = nonfunctional; *FUT2* = functional.

To our knowledge no additional factors associated with down regulation of ABO expression, for example, pregnancy[13] or hematologic disease,[10,11] were present among these samples. Some samples did not contain *ABO* gene mutations known to cause weak A or B expression but were referred to our laboratory due to ABO forward typing discrepancies and suspicion of ABO subgroup status (n = 29; Table 2). They were included in the study to highlight some characteristic flow cytometric patterns related to the following four categories of samples: A_3, chimerism, para-Bombay, and pregnancy. Ten individuals showed a serologic mixed-field reaction with anti-A, typical for the inherited A_3 subgroup phenotype. We have previously sequenced the A^1 [*A101*]-like allele present in heterozygous form in all A_3 samples encountered by our laboratory[13] but no genetic explanation has been revealed to explain the uniform phenotype noted in all of these patients or donors. In nine other samples the genetic explanation to the weak ABO phenotype was not to be found in the *ABO* gene but elsewhere. Five of these nine samples showed signs compatible with chimerism and four examples were para-Bombay phenotypes, A_h (n = 2) and B_h (n = 2), in which

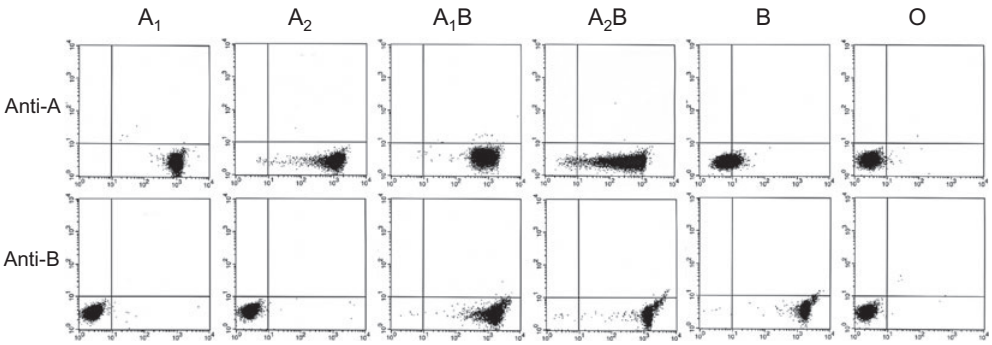

Fig. 2. Dot plots of control RBCs expressing normal levels of ABO antigens as tested with anti-A (clone ES-15) and anti-B (clone 9621A8) and PE-labeled secondary antibodies. The x and y axes represent PE and FL1-derived fluorescence, respectively, on logarithmic scales.

FUT1 mutations were identified. Finally, 10 samples from pregnant women with an apparently weak A phenotype were included.

Common ABO expression with flow cytometry

To establish the flow cytometric pattern of A and B antigen expression in common ABO phenotypes with this system and to confirm specificity of the monoclonal antibodies (MoAbs) selected, RBCs from 80 blood donors with known common phenotypes were examined. The pattern within each ABO group was consistent throughout this study. Dot plots of the common phenotypes are shown in Fig. 2. As expected when tested with anti-A, group A_1, A_2, A_1B, and A_2B RBCs were clearly positive and group O RBCs negative. Group B RBCs were very weakly but still distinctly positive when tested with anti-A due to the small amount of A antigen naturally present on B RBCs.[21] When testing with anti-B, positive reactions were seen with B, A_1B, and A_2B RBCs while negative reactions were obtained with A_1, A_2, and O RBCs. A minor tendency toward agglutination with anti-B of a small percentage of RBCs in B antigen positive phenotypes was noted but did not affect any of the weak/variant phenotypes investigated. A corresponding trend was not observed with anti-A.

Weak A and B expression patterns detected by flow cytometry

Distinguishable flow cytometric patterns based on the A/B expression level and its degree of homogeneity within the RBC population were found for many of the studied ABO subgroups.

The A_{finn} subgroup shows a distinct pattern

All the phenotypically[40] and genetically[33] defined A_{finn} and $A_{finn}B$ subgroup samples displayed a specific flow cytometry pattern with anti-A compared to all other subgroups tested. DNA analysis showed heterozygosity for the Intron 6 splice-site mutation that is characteristic of the A^{finn} [*Afinn01*] allele in 11 individuals of which several were healthy donors. In all cases, the main homogeneous population of cells expressed no or very little A antigen while a small percentage (approx. 3% on average) of the RBCs exhibited A antigen levels that varied from very low to levels comparable to those seen on common group A RBCs. This pattern was consistent and reproducible in RBC suspensions stored for up to at least 4 months, tested fresh versus frozen-thawed and in repeat samples from the same individual with the A^{finn} O^{1v} [*O02*] genotype (Figs. 3A-3D). Tests were also performed on RBCs from an individual with the genetically defined $A_{finn}B$ phenotype (Fig. 3F). The typical flow cytometric pattern of A_{finn} was still obvious but the main population of RBCs is shifted compared to group O controls and the main population of RBCs from A^{finn} O^{1} [O01] donor (Fig. 3E) consistent with the weak A expression normally seen on group B RBC.

The A_x subgroup visualizes the allelic enhancement phenomenon

Four different allelic variants of the A_x subgroup, A^x-1, -2, -3, and -4 [*Ax01, Ax03, Ax02,* and *Ax04,* respectively], were tested (Fig. 4) with a clear tendency for A^x-1 to be the highest expresser of A antigen. While all these variant A^x alleles encode the Phe216Ile substitution at the glycosyltransferase level, the three so-called hybrid A^x alleles (A^x-2, -3, and -4) also add Val277Met. The A_x phenotype RBCs encoded by A^x-1 displayed A antigen expression ranging from very low to almost normal, the majority expressing A

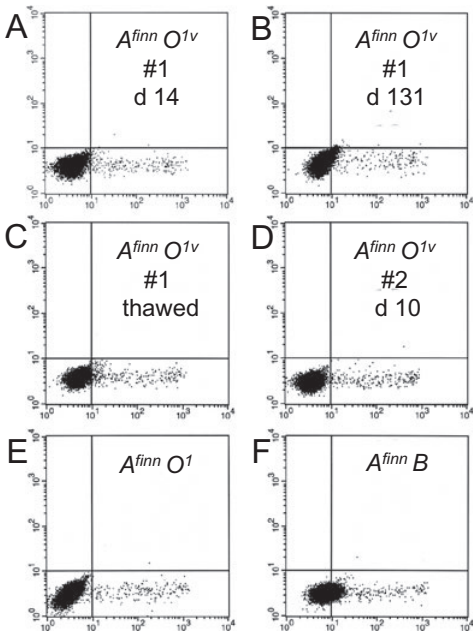

Fig. 3. Dot plots of six different flow cytometric runs with RBCs from individuals with the A_{finn} subgroup and tested with anti-A. The x and y axes represent PE and FL1-derived fluorescence, respectively, on logarithmic scales. (A) Initial testing of the first sample (#1) from an individual with the A^{finn} O^{1v} genotype. (B) Repeat testing of Sample 1 after storage in CellStab solution at 4°C for 4 months. A minor tendency for the RBCs in the main population to become autofluorescent can be noted. (C) Sample 1 tested after having been frozen and thawed. (D) Analysis of a repeat sample (#2) from the same donor as in A through C drawn 6 weeks after the initial sample. (E) Analysis of a sample from an individual with the A^{finn} O^{1} genotype. Sample was tested on Day 3. (F) Analysis of a frozen and thawed sample from an individual with the A^{finn} B genotype.

antigen at medium level with a mean fluorescence intensity (MFI) of approximately 75 (Figs. 4A and 4B). No significant difference was seen whether the second allele was a deletional *O* allele (i.e., with 261delG) or a *B* [*B01*] allele even if, when accompanied by a *B* allele, the staining of the cells seemed to be a bit more homogeneous. In sharp contrast to A^x-1, the hybrid A^x alleles A^x-2 and A^x-4 (Figs. 4C-4D and 4F-4H) did not appear to express any A antigen when evaluated with a deletional *O* allele in *trans*. However, when a nondeletional (i.e., with 261G) allele like O^2 [O03] or *B* allele was present in *trans*, a clear enhancement of the A expression was observed, which is consistent with the concept of allelic enhancement.[9,10] The only example of A^x-3 had an O^2 allele in trans and displayed the typical A_x pattern even if the MFI was somewhat lower than the MFI for A^x-1 O^1 and A^x-2 O^2. Since the A_x hybrids A^x-2, -3, and -4 differ from each other mainly in intronic sequences, it is not unexpected that they behave similarly and in fact, the results are quite similar for these three recombinant alleles when O^2 is present in trans.

A panel of genetically defined A subgroups shows different flow cytometric patterns

Dot plots and histograms of 11 other genetically defined A_w subgroups are displayed in Fig. 5. The samples all depict different flow cytometric patterns and when more than one example of a certain genetically defined subgroup allele was available, the dot plot and histogram patterns between the samples were noticeably consistent. We were able to detect A antigen on A_{el} RBCs, although very weakly, a finding we consider a borderline significant staining compared to the group O control. The majority of the other genetically defined A subgroup samples showed a rather compact cell population in the histograms. This indicates a relatively even distribution of A antigen whereas the histogram curves in two of the samples, A^w-6 [*Aw06*] and A^w-13 [*Aw13*], are more stretched out and heterogeneous, suggesting a more varied A antigen distribution among the cells (Figs. 5F and 5J). *Aw09* is based on an A^2 [*A201*] allelic backbone and is a hybrid O^{1v}-A^2 allele with multiple alterations compared to consensus and displayed a distinct flow cytometric wedge-shaped histogram pattern (Fig. 5E).

A_3 subgroup samples show uniform histograms with dual populations

Ten samples referred to us with classical serologic signs of the A_3 phenotype were all genotyped as A^1 O^1 or A^1 O^{1v} as expected according to previous studies.[13] Here we also obtained the full *ABO* gene sequences from 6 of these 10 samples, Exons 1 through 7 including intronic splice-site regions and the enhancer region. At no point did the A_3-associated *A* alleles deviate from the consensus A^1 sequence. In contrast, all samples displayed a very typical flow cytometric pattern where the majority of the cells were divided into two main cell populations, one of which expressed A antigen at a normal level (for common group A RBCs) and the other expressed A antigen at a level equivalent to the A_x-1 subgroup. In addition, there was also a small amount of cells that expressed A antigen ranging between the two populations (Fig. 5K). The latter finding made this subgroup very different from both naturally occurring chimeras and artificial mixtures of group A cells with other blood groups (data not shown).

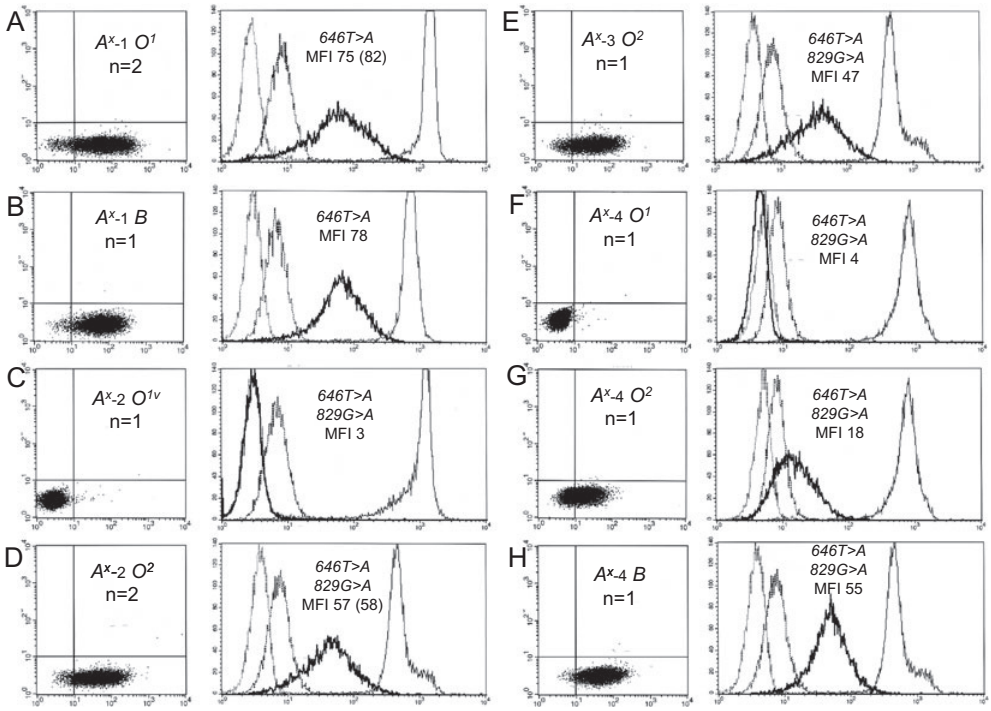

Fig. 4. Dot plots and histograms of eight A_x samples with four different allelic variants of A^x tested with anti-A. In the dot plots, the x and y axes represent PE and FL1-derived fluorescence, respectively, on logarithmic scales. In the histograms PE-derived fluorescence is displayed on the x axis on a logarithmic scale and the number of cells on the y axis. Control RBCs analyzed at the same time as the A_x samples (bold black line) are included in each histogram as follows: group O (dotted thin line to the left), group B (dashed black line to the right of the O control), and group A_2 (solid black line to the far right). The genotypes and the numbers of samples are shown in the dot plots while the implicated mutation(s) are shown in the histogram. In addition, the MFI values for the displayed samples are given in the histograms and when more than one sample was tested the mean MFI is given in parentheses.

B_w subgroups display a more uniform flow cytometric pattern

In the seven genetically diverse variants of naturally occurring B_w subgroups tested in this study (Fig. 6), a few displayed distinct flow cytometric patterns. The majority had a uniform pattern independent of their genetic background after staining with anti-B, reminiscent of the histograms found when A^x-1 cells are labeled with anti-A. Two samples, B^w-11 and B^w-4 [*Bw11* and *Bw04*], displayed a wedge-shaped pattern but were individually different in that B^w-11 had the lowest MFI (17) of all B_w samples tested compared to B^w-4, which had an MFI of 92 (Figs. 6A and 6E). The B_w subgroup with the highest MFI, B^w-14 [*Bw14*], differed the least from normal group B control RBCs and also displayed a wedge-shaped histogram curve but mirroring the wedge direction of B^w-11 and B^w-4 (Fig. 6G). For one allele, B^w-3 [*Bw03*], two samples were available with either A^1 or O^1 allele in *trans* (Fig. 6D). No apparent effect, for instance competition or the allelic enhancement observed for A^x, could be noted. When more than one sample with a defined genetic background was available the pattern was very consistent, as was also the case with genetically defined A_w samples.

Weak ABO antigen expression apparently beyond the control of the ABO *locus*

In the five chimeric samples the main population of cells was group O and a small percentage of the cells were group A or B. While *ABO* genotyping is typically less than ideal to define chimeric states, flow cytometry is known to be well suited for quantification of the two cell populations in a chimera. Figure 7 shows two such samples in

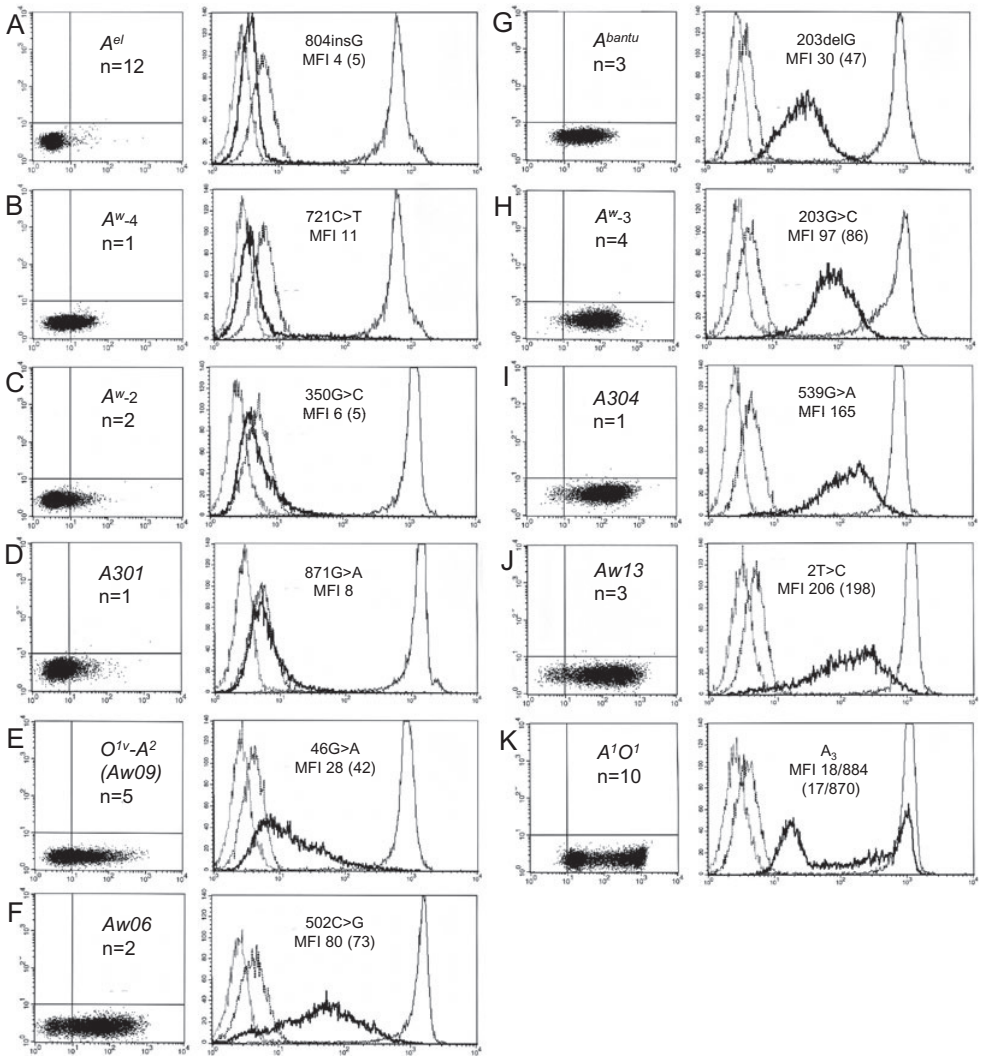

Fig. 5. Dot plots and histograms of 11 samples with different naturally occurring A subgroups tested with anti-A from A_{el} (A) being the weakest expresser of A antigen to Aw13 (J) as the strongest, although A_3 (K) displays dual populations. In the dot plots, the x and y axes represent PE and FL1-derived fluorescence, respectively, on logarithmic scales. In the histograms PE-derived fluorescence is displayed on the x axis on a logarithmic scale and the number of cells on the y axis. Control RBCs analyzed at the same time as the subgroup samples (bold black line) are included in each histogram as follows: group O (dotted thin line to the left), group B (dashed black line to the right of the O control), and group A_2 (solid black line to the far right). The subgroup alleles involved and the number of samples are shown in the dot plots whilst the implicated mutations (or in the case of *Aw09* one of them) are given in the histogram. In addition, the MFI values for the displayed samples are given in the histograms and when more than one sample was tested the mean MFI is given in parentheses. For the A_3 subgroup in K the MFI and mean MFI of the two main populations of cells are given separately in the histogram.

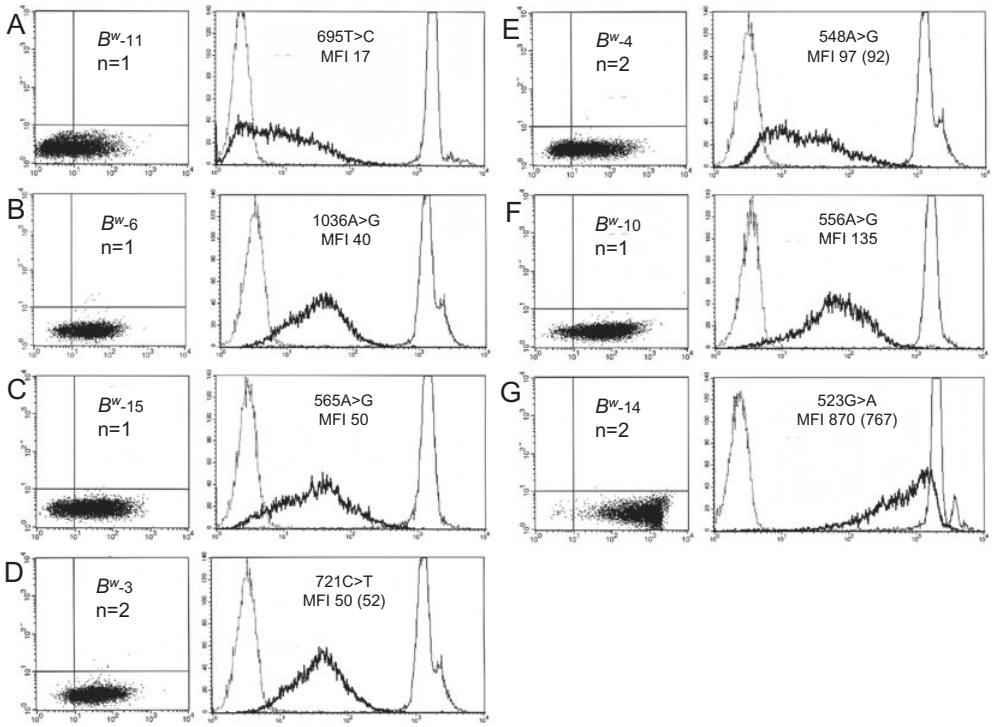

Fig. 6. Dot plots and histograms of seven B_w samples tested with anti-B where the *B^w-11* (A) allele is the weakest expresser of B antigen and *B^w-14* (G) the strongest. In the dot plots, the x and y axes represent PE and FL1-derived fluorescence, respectively, on logarithmic scales. In the histograms, PE-derived fluorescence is displayed on the *x* axis on a logarithmic scale and the number of cells on the y axis. Control RBCs analyzed at the same time as the subgroup samples (bold black line) are included in each histogram as follows: group O (dotted thin line to the left) and group B (solid black line to the right). The subgroup alleles involved and the number of samples are shown in the dot plots while the implicated mutations are given in the histogram. In addition, the MFI values for the displayed samples are given in the histograms and when more than one sample was tested the mean MFI is given in parentheses.

which 3 and 2% of the cell populations were calculated to be of A and B origin, respectively (Figs. 7A and 7B). In the histogram of the O/A sample there was an interesting shift to the right for the main population of group O cells, equivalent to the amount of A antigen normally present on B RBCs. This may be consistent with an uptake of glycolipids with A specificity present in the plasma and derived from the minor group A population. However, this phenomenon was not observed for the O/B samples.

The four para-Bombay samples included in this study had functional *A* or *B* and *FUT2* genes but homozygosity or compound heterozygosity for crippling mutations at the *FUT1* locus.[41-43] In the histograms and dot plots of three of these samples (Figs. 7C-7E) there was a characteristic homogeneous shift to the right for the A_h sample but not as distinct or not at all for the B_h samples. For one of the B_h samples there was a slight shift to the right but for the other no B antigen expression could be detected, which was in concordance with the serology report from the referring blood center where they were not able to detect any B antigen on the RBCs.

Among the samples from 10 pregnant women with an A_w phenotype, the flow cytometric pattern varied and differed with a tendency for the A antigen expression to become weaker toward the end of the gestation period (Figs. 7F-7H). Seven of the 10 samples were genotyped as A^2B and in five cases Exons 1 through 7 including splice sites were sequenced without any deviations from consensus A^2. The upstream polymorphic CCAAT binding factor/nuclear factor Y (CBF/NF-Y)-binding enhancer

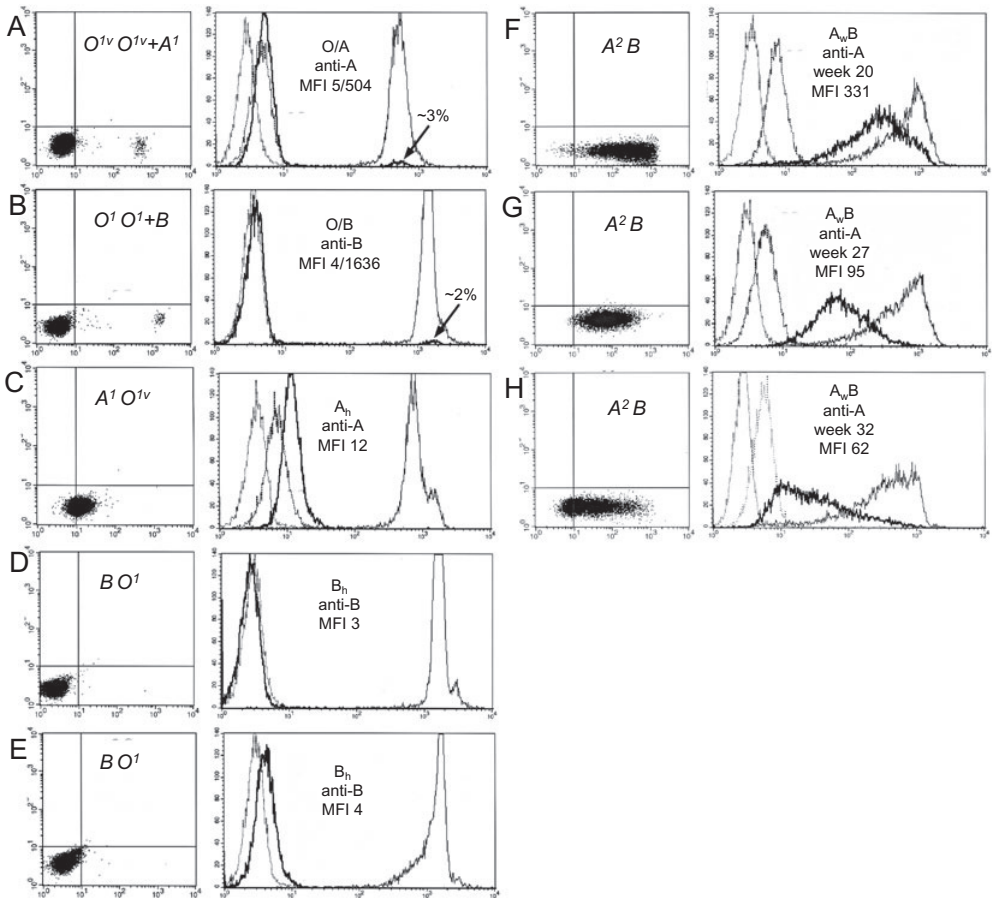

Fig. 7. Dot plots and histograms of RBCs from individuals displaying weak expression of A or B antigen but where no mutations in the *ABO* gene were detected. In the dot plots, the x and y axes represent PE and FL1-derived fluorescence, respectively, on logarithmic scales. In the histograms PE-derived fluorescence is displayed on the x axis on a logarithmic scale and the number of cells on the y axis. *ABO* genotypes are shown in the dot plots while the serologically concluded phenotypes and the MoAb used for detection are given in the histogram. In addition, the MFI values for the displayed samples are given in the histograms. Control RBCs analyzed at the same time as the subgroup samples (bold black line) are included in each histogram as follows: (A and C) group O (dotted thin line to the left), group B (dashed black line to the right of the O control), and group A_2 (solid black line to the far right). (B, D, and E) Group O (dotted thin line to the left) and group B (solid black line to the right). (F-H) Group O (dotted thin line to the left), group B (dashed black line to the right of the O control), and group A_2B (solid black line to the far right). In these latter histograms the positive control (A_2B) was not tested in the same run but added for comparison during the analysis of data given that all the samples were genotyped as A^2B. (A and B) Chimeric samples, the MFI values of which are given separately for the two populations of cells in the histograms as well as the percentage of A or B antigen positive RBCs. (C-E) Para-Bombay samples. (F-H) Samples from three pregnant women with weak expression of A antigen on RBCs probably due to temporary down regulation. The week of gestation is shown in each histogram.

region of these five samples was also tested and the expected number of repeats was found.[26] In addition, four of these women had previously been phenotyped for ABO in a nonpregnant state without any reports of discrepancies. No samples from pregnant women with a B_w phenotype were encountered.

DISCUSSION

A flow cytometric method developed for analysis of very low levels of A and B antigens was used in this study to ask the question if naturally occurring ABO subgroups can be detected and distinguished. As a major reference laboratory we receive numerous samples for in-depth investigation of ABO discrepancies from around the world. In addition to reference serology and DNA-based typing, we have been able to analyze a wide variety of rare and genetically defined samples by flow cytometry, and in many cases accumulated experience from more than one sample of each phenotype/genotype. The A_3 subgroup (n = 10) and phenotypes based on the A^{finn} (n = 12), A^{el} (n = 11), and A^x (n = 10) subgroup alleles appear to be less rare among referrals and accordingly well represented in this study. For many others, only single or few examples of each type have been received. This is a rather expected weakness in a study focusing on rare subgroups. We also included samples causing serologic ABO discrepancies despite the apparent absence of anomalies in the seven *ABO* exons and CBF/NF-Y minisatellite enhancer region.

Samples were evaluated based on a combination of visual inspection of histograms and dot plots to assess antigen density by MFI values and the percentage of antigen-positive cells, with the aim to establish the overall flow cytometric characteristics of each subgroup. Specificity of the antibodies selected for testing was verified by analysis of a large cohort of samples from blood donors with the common ABO phenotypes and no unexpected results were encountered. Sensitivity of the anti-A assay was assessed by the detection of small amounts of naturally occurring A antigen on group B RBCs, and this finding confirms the remarkably high sensitivity of the anti-A clone selected (ES-15). Due to the weak reactions seen with B RBCs, which can also be observed in tube assays or the column agglutination test (gel cards), ES-15 was previously deemed to be an anti-A,B monoclonal reagent.[44] However, the specificity of this reactivity was confirmed by treatment of group B RBCs with exoglycosidase specific for A antigen after which these cells typed and stained negatively with ES-15.[20,21] We were unable to reproduce the weak B reactivity once reported[45] in group A_1 RBCs with the anti-B clone BS-85. The original report used papain-treated A_1 RBCs but neither the anti-B clone selected by us as the most sensitive one (9621A8) nor a fresh batch of BS-85 (a kind gift from Biotest, Dreieich, Germany) was able to stain group A_1 cells by flow cytometry. By immediate-spin agglutination we could only detect very weak microscopic reactions with BS-85 and papain-treated A_1 cells (data not shown), in contrast to the 2+/3+ reaction reported by Voak and colleagues.[45] It is therefore possible that the B antigen sensitivity in this study is lower than that for A antigen.

The A_{finn} subgroup displayed a unique pattern by flow cytometry: the majority of the RBCs expressed no detectable A antigen while a small percentage (2%-4%) of the cells displayed variable amounts of A (Fig. 3). DNA analysis of the A_{finn} samples confirmed heterozygosity for the previously reported splice-site mutation of the fourth nucleotide in Intron 6, from adenosine to guanosine[33] which is predicted to result in skipping of exon 6 in the A^{finn} mRNA. As suggested from identical mutations in other genes,[46,47] this would lead to a premature stop codon due to the fusion of Exons 5 and 7 and is expected to result in the expression of a protein without enzyme activity, de facto making A^{finn} an *O* allele. Since A_{finn} is the only A_w subgroup (in this study) caused by a splice-site mutation, it is especially interesting to note that its almost diagnostic dot plots are reminiscent of those found in chimeras. In fact, we have previously proposed that other rare nondeletional *O* alleles show weak A antigen expression because of a mechanism supposed to involve mitotic recombination and termed "autologous chimerism."[8] A_{finn} is the most common weak ABO subgroup in Finland,[48] which explains why we were able to include 11 A_{finn} individuals in the study. This gave us the opportunity to evaluate the consistency of flow cytometric results between samples from several different individuals but also in the same sample stored over time, in repeat specimens from the same donor, and in frozen-thawed RBCs. The A_{finn}-specific flow cytometric pattern was encountered every time although with a tendency for the cells to become slightly autofluorescent with age of the sample. Since ABO discrepancy cases are often sent to us from other reference laboratories the RBCs are sometimes several weeks old upon arrival. For this reason, the A^x-1, *A304*, B^w-10, and B^w-3 subgroups were all tested systematically for reproducibility over time in a similar way to A_{finn}, although we maximized the testing schedule to 4 weeks (data not shown). These results and other characteristic and similarly consistent data on other subgroups encouraged us to include this assay as part of our ABO investigation arsenal.

The 10 samples from individuals suspected to display the A_3 phenotype all showed an apparently unique flow cytometric pattern with dual populations of RBCs. The classical definition of A_3 serologically is a mixed-field reaction with one or a few large agglutinates among a large number of free unagglutinated RBCs, a picture that may be misinterpreted as the acquired status post–group O-to-A transfusion. The flow cytometric pattern confirms the presence of these expected populations but also reveals two additional findings of interest: 1) the unagglu-

tinated population stains positively with anti-A at a level corresponding to that found in the A_x subgroup (this mirrors previously reported serologic studies);[49] and 2) there is also a continuous range of RBCs with intermediate and varying levels of A expression ranging between those of the two main populations. The latter finding rules out a chimeric state as well as transfusion as the cause of ABO discrepancy in these cases. Even if there have been reports describing mutations associated with A_3,[34,50] we consider the genetic basis of the original A_3 phenotype to be unresolved. Certainly, in the Nordic countries, where the frequency in Denmark has been reported at 1 in 1000,[51] our findings contradict that of the first reported A_3 sample for which the molecular background was determined.[34] In contrast, we have always found samples with the A_3 phenotype to be heterozygous for the consensus A^1 allele.[13,24] In that first case, Yamamoto and colleagues found heterozygosity for an A^1-like allele with the 871G > A mutation.[34] Nevertheless, when the flow cytometric pattern of a sample with the latter allele is compared to all our A_3 samples, the difference is remarkable (Fig. 5D vs. Fig. 5K). Actually, it appears quite likely that they represent different subgroup phenotypes and it is clear that the 871G > A-related profile does not feature the A_3-characteristic dual populations. Thus, in the absence of a genetic marker for the original A_3 subgroup, the flow cytometric approach presented here appears to be an appealing substitute diagnostic tool for the confirmation of A_3 among other inherited subgroups.

Flow cytometric analysis of the A_x samples, which included four allelic variants, displayed interesting variations in their flow cytometric patterns (Fig. 4). When samples containing various A^x alleles with deletional *O* alleles in *trans* were compared, A^x-1 with only one single amino acid substitution (Phe216Ile) gave a positive reaction while two hybrid A^x-alleles (A^x-2 and A^x-4) with two amino acid substitutions (Phe216Ile and Val277Met) were comparable to the group O control. This is in accordance with a previous description of similar hybrids as nondeletional *O* alleles (now renamed *O19* and *O20* in dbRBC[14]) in a Japanese study.[52] However, when other alleles were present in trans together with hybrid A^x alleles (A^x-2, -3, or -4) the flow cytometric pattern resembles that of A^x-1. Even if it has been suggested for O^2[28,53] or proven for B^{54} that non-*A* alleles can contribute toward synthesis of A antigen on RBCs, we have shown that the flow cytometric approach used here does not detect A antigen in group O donors heterozygous for the O^2 allele.[55] The A levels on group B RBCs are also very minor compared to the A expression seen in samples with A^x hybrids combined with O^2 or *B* in *trans*. Thus, we interpret these findings as examples of allelic enhancement.[9,10] To our knowledge, this is the clearest depiction of this enigmatic phenomenon and perhaps also the most comprehensive collection of informative samples for this purpose. The mechanism of action is still unclear though. A possible explanation recently put forward is heterodimer formation by which the function of a defective glycosyltransferase can be rescued by an active enzyme. For instance, it has been experimentally proven that enzymatic turnover is significantly increased when a dimer consisting of two full-length glycosyltransferases is formed.[56,57] This appears to be compatible with the sharp increase in antigen expression when the mutated A_x glycosyltransferase is accompanied by another full-length enzyme as O_2 or B, as opposed to when a deletional O^1 allele was present in *trans*.

In the majority of the other subgroup samples a single mutation was detected as the cause of the weakened A or B antigen expression. For the A_w samples a wide range of A antigen levels was measured and the expression varied from barely detectable (A_{el}) to a more moderate decrease of A antigen (e.g., Aw13). This probably reflects the great variation seen in glycosyltransferase activity present among the numerous different A_w subgroups in this cohort. The pattern observed for A_{el} was only borderline different from group O controls but the presence of a low percentage of positively stained RBCs may be compatible with the heterogeneous immunogold staining results obtained by others previously.[58] Many A subgroups presented diverse flow cytometric patterns but in some cases the differences are very subtle. For instance, *Aw02* and *A301* would be difficult to distinguish and categorize without the genetic data while A_{finn} and A_3 display characteristic and potentially diagnostic patterns as discussed above. The wedge-shaped histogram curves and MFI values noted for *Aw09* and *Aw13* strongly hinted toward their specific genetic backgrounds but may not be diagnostic. As a general limitation to our conclusions, it cannot be ruled out that a subgroup not included in the sample cohort may exhibit a pattern similar to one already defined here.

Genetically defined B_w samples are very rare, at least in the non-Asian part of the world and typically serologically difficult to classify. Of the different variants of B_w samples tested in this study, a few displayed a distinct pattern but the wide range of antigen levels seen in the A_w cohort was not mirrored among the restricted selection of B_w samples available to us. Even if we only had access to samples from more than one donor of a few B_w subgroups, the consistency in flow cytometric patterns between samples with the same genetic background was striking. The very low amounts of A antigen detected in several of the A_w samples had no equivalent in the B_w cohort but this may be due to lack of samples known to express B antigen very weakly, for example B_{el}. It has previously been suggested that the different phenotypically weak B variant names, for example, B_3, B_x, B_v, and so forth, should not be used since the B subgroups are not as distinct serologically as A subgroups.[13] Instead, a terminology based on subgroup alleles or mutations seems more appropriate, a

statement that this flow cytometric evaluation of a limited number of different B_w alleles seems to corroborate.

Chimeras can be serologically difficult to distinguish from A_w or B_w subgroup phenotypes and may also raise the suspicion of a mixed-field reaction due to transfusion of ABO-compatible but nonidentical RBCs. The five cases included in this study were confirmed to be nontransfused patients and they all displayed a small population of A or B RBCs in the main population of group O RBCs. By flow cytometry the percentage of the minor cell population that expressed A or B antigen at normal levels was easily quantified and the interpretation of the serologic results made clear. Artificial in vitro mixes of group A and O or B and O RBCs from 50% down to 1% of A/B cells were used to test the precision of the method (data not shown). The small amount of A antigen seen on the main population of RBCs in the O/A chimera is comparable to the A antigen levels found on group B RBCs (Fig. 7A). We hypothesize that this is due to uptake of glycolipids in plasma derived from the small population of cells expressing A antigen in vivo, although the same phenomenon was not seen for the O/B chimeras (Fig. 7B). It is quite likely that this reflects the same phenomenon described by other investigators using blood group serologic techniques in chimeric patients and samples after transfusion or after bone marrow transplantation in non–group O patients with group O donors.[59-61] The adsorption of glycolipids with A or B specificity from plasma is also the likely cause of the weak A or B antigen expression seen on RBCs from para-Bombay individuals carrying *A* or *B* alleles, respectively (Figs. 7C-7E). The A_h sample seemed to express A antigen at a higher level than B_h expressed B antigen but it is difficult to compare results between MoAbs of different specificities. Nevertheless, three of four para-Bombay samples displayed a distinct cell population where A or B antigen levels appeared to be homogeneous, indicating an even uptake of A or B glycolipids from plasma. In analogy with the O/B chimeras, no B antigen could be detected in one of the B_h samples. The variable levels of A or B antigen on the O-like population in these samples may be due to secretor status or A_1/A_2 status of the patients as previously reported.[60-62] Unfortunately, this was not within the scope of the present study to investigate.

Weakening of ABO antigen levels during pregnancy is known to occur[13] but the underlying cause has not been elucidated. A previous study was not able to correlate weakening of A antigen to a specific *A* allele[13] but in this study 7 of the 10 samples from pregnant women with an apparent weak A phenotype were genotyped as A^2B. In these seven samples no weakening of the B antigen was noted. Since the A_2B phenotype is known to express the lowest amount of A antigen of the common phenotypes it is not surprising if this phenotype would be the most prone to cause discrepancies in serologic testing. It would be interesting to test if there is a general weakening of A antigen on RBCs in pregnant women, phenotyped as A or AB, discrete enough often not to cause discrepancies in routine ABO testing. When semiquantified by flow cytometry, the A antigen levels varied between pregnant women but with a tendency that the lowest A antigen levels were seen in samples from the last trimester. The three samples from women genotyped as A^1 O^1 or A^1 B all expressed higher levels of A antigen than the A_2B women irrespective of the week of gestation. This indicates that the A_1/A_1B phenotypes are weakened to a lesser extent than A_2B.

With a highly sensitive flow cytometric assay developed in our laboratory it was possible to perform an extensive and thorough investigation of A/B antigen expression levels and patterns in different ABO subgroups. To our knowledge, such a study has not been conducted before, probably due to low sensitivity in the flow cytometric assays,[11,17] and too few and insufficiently characterized subgroup samples available for testing. When testing genetically defined subgroups we found a striking reproducibility within subgroups even if the samples were tested fresh/old/frozen and thawed or in repeat samples from the same or different donors. For comparison, we also investigated the patterns of non–ABO subgroup–associated typing discrepancies including chimeras, para-Bombay, and pregnancy-induced acquired weakening of A. The presented approach requires a flow cytometer and trained staff but is rapid, reproducible, and robust. Furthermore, it allows for more objective assessment of unusual ABO phenotypes and results can be easily visualized and quantified as MFI or percentage of positive cells for reporting and comparative purposes. Thus, we found this flow cytometric assay to be a valuable tool and an objective complement to traditional serology and genetic typing in the reference laboratory investigation of clinical ABO discrepancies.

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CONFLICT OF INTEREST

None.

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Paper III

Weak A phenotypes associated with novel *ABO* alleles carrying the A^2-related 1061C deletion and various missense substitutions

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BACKGROUND: The 1061delC single-nucleotide polymorphism (SNP) has been reported mostly in the context of the common A^2 [*A201*] allele and typically produces an A_2 phenotype. This study evaluated new A^{weak} alleles, each containing 1061delC.

STUDY DESIGN AND METHODS: Twenty samples were referred to our laboratory for analysis due to suspected A_{weak} phenotypes originally detected at the referring centers. *ABO* Exons 1 through 7 and flanking intronic regions were sequenced. A antigen expression on red blood cells was analyzed by flow cytometry. Plasma enzyme activity was studied in one case. Molecular three-dimensional modeling techniques studied the potential effects of amino acid changes on the resulting glycosyltransferases (GTs).

RESULTS: Thirteen alleles were discovered, each featuring 1061delC with at least 1 of 12 additional SNPs in the coding region. One of these SNPs disrupts the translation initiation codon. Another constitutes the first reported change in the DVD motif. One SNP found in three alleles causes a substitution of one of the four amino acids that differentiates the wild-type A and B enzymes but plasma enzyme analysis by two methods showed only slightly decreased or normal A_2 activity. Flow cytometric analysis semiquantified the A antigen levels in 16 cases featuring 10 of the alleles and ranged from very weak to nearly A_2 levels. However, the majority of the samples displayed A_x-like patterns. Molecular modeling of some of the GT variants indicated conformational changes that may explain the diminished A expression observed.

CONCLUSION: Missense SNPs were identified in 13 novel A^2-like alleles, which produced a variety of A subgroup phenotypes.

ABO is the most clinically relevant blood group system from both transfusion and transplantation perspectives. The *ABO* coding region consists of 1065 bases and is located on Chromosome 9, and is composed of seven exons.[1-3] Initially, there were three principal alleles described in the ABO system: A^1 [*A101*], which is considered to be the consensus allele, *B* [*B101*], and O^1 [*O01*]; the *A* allele differs from *B* by seven in-frame single nucleotide polymorphisms (SNPs) that cause four-amino-acid substitutions

ABBREVIATIONS: ALL = acute lymphoblastic leukemia; CBF/NF-Y = CCAAT-binding factor/nuclear factor Y; GT(s) = glycosyltransferase(s); GTA = A glycosyltransferase; GTB = B glycosyltransferase; SNP(s) = single-nucleotide polymorphism(s).

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in the resulting glycosyltransferase (GT), while the most common *O* allele only differs by a single-nucleotide deletion from the consensus *A101* allele.[1] However, ABO is now considered one of the most genetically complex blood group systems with approximately 200 alleles described.[4,5] However, most people demonstrate straightforward A, B, O, or AB phenotypes while only few individuals have red blood cells (RBCs) that are weakly agglutinated by anti-A/B. Such patterns can be acquired as a result of transfusion, transplantation, pregnancy, or disease but can also be inherited characteristics based on changes at the *ABO* locus.[6] This latter category of people often has subgroup phenotypes where the common *A* or *B* alleles have been altered by one or more SNPs that negatively affect the GT specificity and/or activity.

One of the most common A subgroup phenotypes is that of A_2. Approximately 20% of Caucasian group A or AB individuals are A_2 or A_2B.[7] Typically, the A_2 phenotype is manifested by strong agglutination with polyclonal or monoclonal anti-A and -H antibodies and *Ulex europeus* lectin, along with no agglutination with lectins produced from either *Dolichus biflorus* or *Helix pomatia*. Anti-A1 is sometimes present in the reverse type, that is, antibodies reacting selectively with A_1 RBCs.[8] The true specificity of the A1 antigen is still under debate but many differences in both A antigen quantity and quality have been reported including the presence of A Type 3 and 4 on A_1 RBCs while these structures are either expressed in much lower amounts or possibly absent in the A_2 phenotype.[9-12]

Among Caucasians, the most common allele that gives rise to the A_2 phenotype (A^2-1 [*A201*]) features two SNPs relative to the *A101* allele: 467C>T (P156L) and 1061delC.[13] The A_2 phenotype is rare in Asia; however, significant allelic heterogeneity among A_2 and A_2B donors in this geographical area has been reported.[14-17] The 1061delC SNP abolishes the normal stop codon such that an additional 21 amino acids are added to the enzyme before translation is finally halted. As the 467C>T SNP has also been identified in *A* alleles [*A102*] producing the common A1 phenotype in the majority of group A individuals in Asia,[18] the reduction of activity of the *A201*-encoded enzyme is caused mainly by the presence of the additional amino acids, possibly due to altered acceptor preference. The *A206* allele features the 1061delC SNP but not 467C>T.[19] The *A201*-encoded enzyme, and probably also that encoded by *A206*, differs from that encoded by *A101* in terms of its optimum pH, cation requirements, isoelectric point, and K_m, and the *A201*-derived plasma-borne enzyme demonstrates considerably less activity than an *A101* plasma enzyme.[13,20,21] While the crystal structure has been solved for A_1 and B enzyme, the A_2 enzyme with its C-terminal extension remains unresolved.[22]

Not all alleles that have been designated as A^2 feature the 1061delC SNP[14,23,24] (see also the Blood Group Antigen Gene Mutation Database[5]), and conversely, 1061delC has been identified in weaker (non-A_2) subtype phenotypes (Table 1). For instance, the weak A_{bantu} phenotype is present in as much as 8% of some populations in southern Africa and was found to be due to a hybrid allele formed near Exon 5 between a previously undescribed *O* allele, O^{1bantu}, and the regular A^2 allele featuring 1061delC.[25] Similarly, the hybrid *Aw09* allele (O^{1v}-A^2 with the crossover region in Intron 4) also includes 1061delC and produces an A_{weak} phenotype in individuals of African descent.[26] Other rare alleles with 1061delC such as *Aw01*, *Aw02*, *Aw03*, and *Aw07* have also been reported to result in weak A phenotypes.[6,26,27] The purpose of this study was to elucidate the genetic basis of the weakened A phenotypes in 20 clinical samples featuring heterozygosity for 1061delC. We discovered 13 novel alleles, each of which contained at least one additional SNP, and characterized their corresponding phenotypes. These new alleles can now be added to the eight previously published weak subgroups based on A^2 backbones.[5]

MATERIALS AND METHODS

Samples

Samples from blood donors and patients exhibiting A_{weak} phenotypes were referred to the Nordic Reference Laboratory for Genomic Blood Group Typing at the University Hospital Blood Centre in Lund, Sweden, for in-depth analysis of their ABO typing discrepancy. On arrival, the RBCs were washed three times and suspended in CellStab preserving solution (DiaMed AG, Cressier, Switzerland) for long-term storage if available. The buffy coat was harvested for DNA preparation.

Flow cytometric detection of A antigens

Flow cytometry was performed as outlined previously,[28] using the anti-A immunoglobulin M clone ES-15 primary antibody (gift from Zymequest, Inc., Beverly, MA) and phycoerythrin (PE)-conjugated rat anti-mouse immunoglobulin kappa light chain secondary antibody (Becton Dickinson, San Jose, CA). Control RBCs of known phenotypes (A_2, A_x, B, and O) were included in each run along with the weak A subgroup sample, 10,000 events were collected, and log fluorescence data were gated on a linear forward scatter versus linear side scatter dot plot.

Serologic ABO typing

The serologic testing normally performed when weak A or B phenotypes are suspected had been performed by the referring laboratories in most cases but the extent of these investigations varied. If samples were sent to us with minimal serologic information, we performed additional serologic testing according to standard blood banking

TABLE 1. Summary of the previously reported alleles featuring the 1061delC SNP

Allele name*	Reference	Exonic SNP	Amino acid change†	Definitive serologic finding	Ethnic/geographical origin of propositus	GenBank Accession Number
A201	Yamamoto et al.[13]	467C>T	P156L	Not reported in the original paper	American	AF134421
		1061delC	FS +21 aa	Confirmed to be A_2 by several groups		
A206	Chen et al.[17]	1061delC	FS +21 aa	Not reported Confirmed by our group to type as A_2	Chinese	Not submitted
A209	Yip et al.,[16] Chen et al.[17]	467C>T	P156L	No agglutination with *D. biflorus*	Chinese	AY20743
		527G>A	R176H	Not reported but listed as A_2	Kuwaiti	
		1061delC	FS +21 aa			
Aw01	Olsson et al.[6]	407C>T	T136M	Weak A expression	UK	AF324010
		467C>T	P156L			
		1061delC	FS +21 aa			
Aw02	Olsson et al.[6]	350G>C	G117A	Very weak A expression	Caucasians from Australia, New Zealand, UK, USA	AF324009
		467C>T	P156L			
		1061delC	FS +21 aa			
Aw03	Olsson et al.[6]	203G>C	R68T	Weak A expression	Scandinavian	AF324008
		467C>T	P156L			
		1061delC	FS +21 aa			
Aw07	Seltsam et al.[27]	467C>T	P156L	Very weak A expression	German	AJ536130
		592C>T	R198W			
		1061delC	FS +21 aa			
Aw09	Hosseini-Maaf et al.[26]	46G>A	A16T	A_{weak} in setting of $A_{weak}B$	Blacks of African origin from Portugal, Sweden, Switzerland, and USA	AY377124
		106C>T	V36F	A_2-like in setting of *AO* genotype		AY377125
		188G>A	R63H			
		220C>T	P74S			
		467C>T	P156L			
		1061delC	FS +21 aa			
A_{bantu}*01*	Hosseini-Maaf et al.[25]	IVS4+1delG	Skipping of Exon 4?	A_{bantu}	Black Africans	AY805750
		467C>T	P156L			
		1061delC	FS +21 aa			
A302‡	Barjas-Castro et al.[37]	829G>A	V227M	Small agglutinates amongst free cells	Likely Brazil	Not submitted
		1061delC	FS +21 aa			
A304	Svensson et al.[11]	467C>T	P156L	Mixed field, no anti-A in reverse typing	Swedish	GU452500
		539G>A	R180H			
		1061delC	FS +21 aa			

* Terminology according to dbRBC.[5]
† The 1061delC creates a frameshift (FS) mutation which abolishes the normal stop codon such that an additional 21 amino acids are translated.
‡ Although listed as A_3 the phenotype is probably best described as A_{weak}.

practice if RBCs were available. Requests for fresh samples were made to continue the serologic investigation and to perform flow cytometry if only DNA had been received. However, in several cases no further sample was obtained.

Genomic *ABO* and *FUT2* typing

DNA was extracted from the buffy coat with a modified salting out procedure,[29] but in a few cases DNA was received directly from the referring laboratory. Genomic typing of the *ABO* locus was performed with the established and validated methods used at the Nordic Reference Laboratory for Genomic Blood Group Typing, including polymerase chain reaction (PCR)-restriction fragment length polymorphism,[30,31] PCR–allele-specific priming (ASP),[32] and DNA sequencing strategies.[6] In addition, PCR testing of the upstream polymorphic CCAAT-binding factor/nuclear factor Y (CBF/NF-Y)-binding enhancer region was performed in all samples as previously described.[33] For the *A212* allele, genetic testing was performed by the Molecular Blood Group and Platelet Testing Laboratory of the American Red Cross (Philadelphia, PA), but testing for the CBF/NF-Y–binding enhancer region was not performed. To determine secretor status, *FUT2* genotyping was performed with a validated, in-house PCR-ASP method that tests for the two most important polymorphic sites in Caucasians (428G/A) and Asians (385A/T) causing nonsecretor status (428A and 385T, respectively).

Conversion of group O RBCs by ABO GT in plasma

A 10% suspension of washed group O RBCs in allogeneic plasma from donors of different blood groups was incubated at 37°C with 0.35 mmol/L final concentration of UDP-GalNAc donor substrate (Sigma-Aldrich, Stockholm, Sweden) and 10 mmol/L $MnCl_2$ (Sigma-Aldrich) for 4, 24, and 48 hours in a total volume of 250 µL (method adapted from Schenkel-Brunner and Tuppy[34]). The treated cells were washed after incubation and subjected to flow cytometric analysis of A antigen expression as described above. Ethylenediaminetetraacetate plasma was used as a source of ABO GT and controls included A_1 and A_2 plasma (positive) and O plasma (negative). The donors of the A_1 and A_2 control plasma samples were genotyped and verified to be heterozygous for the A^1 and A^2 alleles, respectively, while the group O donor was homozygous for 261delG.

Serum enzyme activity assay

The A glycosyltransferase (GTA) activity was analyzed by monitoring the transfer of radioactivity from UDP-[^{3}H]GalNAc to Fucα1,2Galβ-O-$(CH_2)_8CH_3$ acceptor.[35] Reactions were carried out in 0.5-mL microfuge tubes containing 5 µL of serum in 50 mmol/L MOPS buffer, pH 7.0, containing 20 mmol/L $MnCl_2$, 1 mg/mL bovine serum albumin, 50 µmol/L unlabeled UDP-GalNAc, 72,000 to 140,000 dpm radiolabeled donor (American Radiolabeled Chemicals, St Louis, MO), and 200 µmol/L acceptor in a total volume of 10 µL. The mixture was incubated at 37°C for 4 to 5 hours after which the reaction mixture was diluted with 500 µL of water and applied to a reverse-phase cartridge (Sep-Pak C18, Waters, Mississauga, Ontario, Canada).[35] The cartridge was rinsed with 200 mL of water to remove unreacted donor, and then radiolabeled products were eluted from the cartridges into scintillation vials with 3.5 mL of methanol. Radioactivity was measured in a scintillation counter (LS 6500, Beckman, Fullerton, CA) after the addition of 10 mL of liquid scintillation cocktail (EcoLite, ICN, Costa Mesa, CA). Serum samples were assayed four times with different amounts of radiolabeled donor added to the assay tubes. Thus the mean and standard deviation (SD) of the percentage conversion of substrate to product were calculated from the dpm transferred to product/dpm added to the assay. Control reactions were carried out in the absence of acceptor.

Modeling of novel enzymes

In silico mutations were based on the crystal structure of GTA (PDB entry 1LZI) and modeled using the program COOT[36] where the rotamer clashing the least with nearby residues was chosen from the coot rotamer library. H Type 1 and 2 acceptors from the B glycosyltransferase (GTB) structures PDB entry 1ZJ2 and 1ZJ3, respectively, were superimposed onto the GTA structures using the SSM tool in COOT. The structural figures were generated in PyMol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA; http://www.pymol.org).

Nomenclature of *ABO* alleles

ABO allele nomenclature poses significant problems that are still under consideration by the International Society of Blood Transfusion (ISBT). Whenever appropriate (including the first time an allele is mentioned here), a reference [in brackets] to the unofficial but often used terminology found in the Blood Group Antigen Gene Mutation Database is made.[5]

RESULTS

Twenty samples were investigated due either to an ABO discrepancy or a suspected weak A subgroup phenotype. In 18 of these cases, there were no clinical indications of conditions associated with acquired weakening of A antigen expression, while one sample was from a patient

with acute lymphoblastic leukemia (ALL) who had been recently transfused and another came from an otherwise apparently healthy 37-week pregnant female.

Blood group serology

The serologic characterization of most of the samples investigated in this study is summarized in Table 2. In principle, the RBC phenotypes were classified A_{el}, A_x, or A_{weak}. The latter designation was used if weak reactions were obtained with anti-A and no comparison could be made with anti-A,B reactivity. Table 3 lists three samples that shared the same mutation (527G>A; see below) in their A^2-like alleles. Due to underlying conditions (leukemia and/or transfusion and pregnancy), the weakened serologic results with anti-A were difficult to interpret in two of these cases. The third sample in this group (*A212*) came from a potential solid organ donor who demonstrated an A_2-like phenotype, insofar as that his RBCs demonstrated strong agglutination with monoclonal anti-A reagents and was strongly reactive with anti-H lectin but curiously demonstrated very weak agglutination with some anti-A_1 lectins.

Genetic analysis

In all cases, the *A* subtype allele was inherited together with either a deletional *O* allele or a normal *B* allele in trans. The upstream polymorphic CBF/NF-Y–binding enhancer region of all the tested subgroup alleles presented here was found to contain four repetitive motifs as expected for A^2 alleles as well as *B* or deletional *O* alleles.[33]

Most of these 13 new alleles were based on an *A201* backbone (467C>T along with 1061delC) and contained one additional SNP, nine of which are new and one of which had been previously reported (Table 2).[37] In addition, there were three cases in which the alleles contained 1061delC along with the previously reported 527G>A SNP that changes one of the four amino acids that discriminate a wild-type GTA from GTB.[16,17] The details of these three alleles are as follows: 1) The novel *A212* allele is based on *A201* but also had 190G>A (V64I) in Exon 4; [38] 2) the *Aw26* allele is in fact a hybrid allele based on Exons 1 through 5 from the O^1 allele and Exons 6 and 7 from *A201*. The crossover region was defined according to intronic SNPs specific for or related to the common *ABO* alleles (Fig. 1);[26,27] and 3) finally, *Aw27* was based on the *A206* backbone, that is, lacked 467C>T. These findings are summarized in Table 3.

The novel SNPs compounding the A^2 (or hybrid A^2) sequences discovered in this study were scattered throughout the *ABO* gene (Exons 1, 4, 5, 6, and 7), although the majority were located in Exon 7. These missense mutations include several nucleotide substitutions of special interest, for example, a SNP (1A>G in *Aw16*) that is

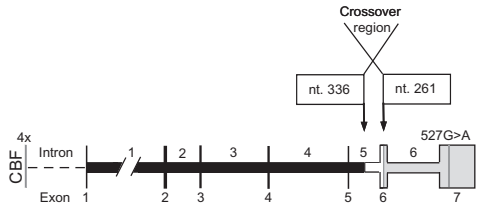

Fig. 1. Overview of the hybrid allele carrying the 527G>A SNP, *Aw26*. Exons are shown as numbered vertical bars of varying width and the intervening segments are introns. Displayed in black is the O^1-derived part of the allele and in gray the A^2-derived portion. The crossover region (white) is located between Nucleotide (nt.) 336 in Intron 5 and nt. 261 in Exon 6 according to 12 allele-specific or allele-related markers.[26,27] Gray thin vertical bars represent exonic SNPs relevant for determination of the crossover region in the hybrid. To the left, the 5′-upstream CBF/NF-Y–binding enhancer region with the expected four repeats is shown. The *ABO* gene is drawn approximately to scale except for the large Intron 1 and 5′-upstream sequences.

predicted to abolish the translation-initiation start codon and one (634G>A in *Aw22*) that is the first described to disrupt the Mn^{2+}-binding DVD motif in the catalytic domain of the GTA (see below and Table 2).

Characterization by flow cytometry

A recently published highly sensitive method for ABO subgroup detection was used to further characterize the phenotypes of the samples.[28] Flow cytometric analysis of A antigen expression was performed on 16 samples including 10 different A_{weak} alleles to provide a semiquantitative visualization of the serologic data (Fig. 2). RBCs were not available for flow cytometry testing for the three remaining alleles. In general, there were three main patterns of A antigen expression: The RBCs encoded by the *Aw19* (Fig. 2E) and by the *Aw24* (Fig. 2H) alleles demonstrated A antigen expression only slightly above that observed on group O RBCs and almost equivalent to the amount of A antigen present on group B RBCs (Fig. 2B). The *Aw17, Aw18, Aw23*, and *Aw25* alleles (Figs. 2C, 2D, 2G, and 2I) showed levels of A antigen expression similar to those displayed by the A_x (A^x-1/O^1) control RBCs that are included for comparison (Fig. 2A). *Aw22* (Fig. 2F) presented a somewhat different flow cytometric pattern compared to *Aw17*/*Aw18*/*Aw23*/*Aw25*, expressing A antigens at a slightly lower level.

The sample with the *A212* allele (Fig. 3A) demonstrated A antigen levels nearly equivalent to the control A_2 [*A201*] RBCs as implied by its name, while the remaining two samples from individuals with the 527G>A SNP

TABLE 2. Summary of the genetic and phenotypic findings for the new alleles described in this study

Proposed allele name	Additional SNP*	Exon	Amino acid substitution†	Figure number	I/C‡	RBC phenotype§	Number¶	Forward typing		Reverse typing		Country of referring center	GenBank Accession Number
								–A	–A,B	A_1	A_2		
Aw16	1A>G	1	M1V	—	—	$A_{el/weak}$?	1	–	–	–	–	Switzerland	GU169166
Aw17	236C>T	5	P79L	2C, 4, 6E	—	A_x	6	–/+^{mf}	+/2+^{mf}	–/2+	–/+	Denmark, Sweden, France**	GU169169
Aw18	347T>C	6	I116T	2D, 4, 6A	C	A_{weak}	2	+^{mf}	NT	2+	—	Sweden	GU169170
Aw19	434A>G	7	**H**145**R**	2E, 4, 6A	C	A_{weak}	1	–	+	+	+	Switzerland	GU169167
Aw20	607G>A	7	**E**203**K**	4	I	A_{weak}	1	+^{mf}	NT	+	NT	Canada	GU169171
Aw21	607G>C	7	**E**203Q	4	I	A_{weak}	1	+/2+^{mf}	4+	2+	NT	Portugal	GU169172
Aw22	634G>A	7	V212M	2F, 4, 5B	I	A_x	1	–/+	+/4+††	+	–	Switzerland	GU169168
Aw23	722G>A	7	**R**241Q	2G, 4, 6C, E	—	A_x	1	+	2+	–	–	France	GU592505
Aw24	742C>T	7	**R**248C	2H, 4, 7	I	$A_{el/weak}$?	1	–	–/+	–‡‡	–	USA	GU169173
Aw25	829G>A	7	V277M	2I, 4, 5D	C	A_{weak}	2	–/+	4+	+	–	Portugal,** Switzerland	GU199605

* The *Aw16-Aw25* alleles were all based on an *A201* backbone (467C>T and 1061delC) and each allele had one additional exonic SNP as indicated.
† Charged residues involved in the amino acid change are indicated in bold.
‡ Invariant (I), conserved (C), or nonconserved (–) residue according to published data on evolutionary conservation in the homologous *ABO* gene family in different species.[55]
§ As reported by the referring center and in accordance with serologic findings.
¶ The allele in trans was a deletional *O* allele in all cases except for *Aw17* (1 of 6), *Aw21*, and *Aw25* (2 of 2) in which it was *B*. In all cases, the expected plasma typing results (i.e., – or 4+) were obtained with group B test RBCs.
** These two donors were of African descent (Northern Africa, and Angola, respectively).
†† The weaker reaction was obtained with polyclonal anti-A,B in DiaMed gel cards and the stronger reaction with monoclonal reagent in the same gel cards.
‡‡ Negative at room temperature but 3+ at 4°C. Also, adsorption onto this individual's RBC with three different anti-A,B reagents (one human and two monoclonal) yielded eluates that reacted positively with A_1 test RBC (3+, 1+, and 4+).
mf = mixed-field agglutination pattern; NT = not tested.

TABLE 3. Genetic and phenotypic descriptions of the three novel alleles featuring the 527G>A SNP

Proposed allele name	Genetic backbone	Additional SNP	Exon	Amino acid substitution*	Figure number	Health status	RBC phenotype†	Number‡	Forward typing		Reverse typing		Country of referring center (ethnic origin if known)	GenBank Accession Number
									–A	–A,B	A_1	A_2		
A212	A^2 [A201]	190G>A	4	V64I	3A, 4	Healthy	A_2?	1	4+§	NT	0	NT	USA	GU592507
		527G>A	7	**R**176H										
Aw26	O^1-A^2 hybrid¶	527G>A	7	**R**176H	3B, 4	Pregnant	A_{weak}?	1	–/+	4+	3+	–	Sweden (Turkish)	GU592506
Aw27	A^2 [A206]	527G>A	7	**R**176H	3C, 4	ALL	A_{weak}?	1	2+	NT	2+	NT	Sweden (Syrian)	GU199604

* Charged residues involved in the amino acid change are indicated in bold.
† As reported by the referring center and in accordance with serologic findings.
‡ The allele in trans was a *B* allele in two cases (*Aw26* and *Aw27*) and a deletional *O* allele [O^{1v} or *O02*] in one case (*A212*). In all cases, the expected plasma typing results (i.e., – or 4+) were obtained with group B test RBCs.
§ Lectin typing results: *U. europeus* 4+ while *D. biflorus* gave very weakly positive reactions using some reagent preparations.
¶ Hybrid breakpoint was defined to be located between Intron 5 Nucleotide 336 and Exon 6 Nucleotide 261.
NT = not tested.

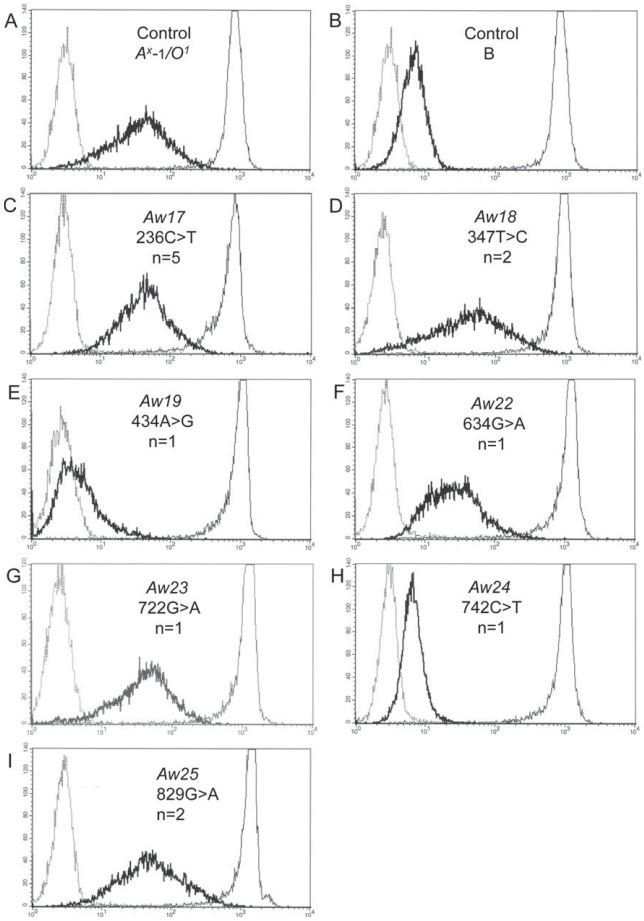

Fig. 2. Histograms of flow cytometric analysis with anti-A (Clone ES-15). PE-derived fluorescence is displayed on the *x*-axis on a logarithmic scale in the histograms and the number of cells on the *y*-axis. Control RBCs analyzed at the same time as the subgroup samples (bold black line) are included in each histogram as follows: group O (dotted thin line to the left) and A_2 (solid black line to the far right). The subgroup alleles involved, the implicated mutations, and the number of samples tested with flow cytometry are given in the histogram. Samples with the same weak *A* alleles gave very similar results and if more than one sample was available for analysis, one representative example is presented here. (A, B) For comparison and to verify the high sensitivity of the assay, a weak control (A_x phenotype with genotype A^x-1/O^1) and a very weak control (group B) are displayed. (C-I) Histograms of seven samples with different novel naturally occurring A subgroups. A antigen expression varied from very low for *Aw19* and *Aw24*, that display antigen levels equivalent to the small amounts of naturally occurring A antigen present on group B RBCs, to levels comparable to the A^x-1/O^1 control RBCs for *Aw17*, *Aw18*, *Aw23*, and *Aw25*.

displayed very different flow cytometric patterns. The *Aw26* allele (Fig. 3B) was discovered during routine testing of a pregnant woman (37 weeks of gestation) and the A antigen level ranged from very low to almost normal with approximately 70% of the cells expressing low amounts of A antigen. The sample containing the *Aw27* allele (Fig. 3C) found in a patient with ALL demonstrated more than one population of RBCs, probably due to recent transfusion, the ABO type of which was not known or available to us. One fraction of RBCs in this patient presented about the same amount of A antigens as that encoded by *Aw19* (Fig. 2E), which is likely to represent some of the transfused group O RBCs (see Discussion), while the other population showed a broad range of A antigen levels that overlapped with the control A_2 RBCs.

Plasma enzyme activity

Plasma was available from the *A212* donor. The activity of the plasma enzyme was slightly lower than that of two healthy A^2/O^1 individuals and clearly higher than two healthy group O individuals (Table 4).

To determine if the *A212*-encoded GTA has the same activity as the regular A_2 enzyme as indicated by phenotyping, flow cytometry was also used to semi-quantify the amount of A antigen detected on group O RBCs after enzymatic conversion using plasma from donors of different blood groups (Figs. 3D-3F). After incubation with group O plasma, the expected negative result was seen after 4, 24, and 48 hr when tested with anti-A. The amount of A antigen present on the O RBCs after incubation with A_1 plasma was clearly detectable after 4 hours and after 48 hours the mean fluorescence intensity (MFI) was equivalent to that of native A_1 RBCs. When comparing the amount of A antigen created by the *A212* plasma to the A_2 control plasma, the cell population incubated with the former did not shift as far to the right as the population incubated with the latter (Figs. 3D-3F),

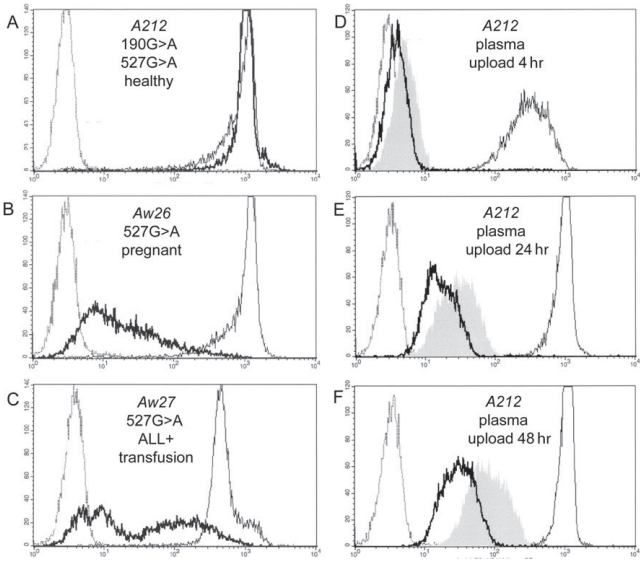

Fig. 3. (A-C) Histograms of flow cytometric analysis with anti-A (clone ES-15) of RBCs from individuals carrying the novel *A212*, *Aw26*, and *Aw27* alleles all featuring the 527G>A SNP. In the histograms PE-derived fluorescence is displayed on the *x*-axis on a logarithmic scale and the number of cells on the *y*-axis. The subgroup alleles involved and important additional information are given in the histogram. Control RBCs analyzed at the same time as the subgroup sample (bold black line) are included in each histogram as follows: group O (dotted thin line to the left) and A_2 (solid black line to the far right). (D-F) Conversion of group O RBCs by ABO GT in A_1, A_2, O, and *A212* plasma tested with anti-A after 4, 24, and 48 hours. O RBCs and O plasma (dotted thin line to the left), O RBCs and A_2 plasma (light gray, filled), O RBCs and A_1 plasma (solid black line to the far right), and O RBCs and *A212* plasma (bold black line).

indicating that the GTA present in the *A212* plasma may be slightly less efficient compared to the enzyme found in A_2 plasma.

Structural modeling of the amino acid substitutions in the novel enzymes

All the amino acid residues encoded by the A^{weak} variant alleles presented in this study were modeled onto a GTA structure (Fig. 4). Residue 79 is located in the N-terminal stem region, which in the crystal structure of the wild-type GTA has been suggested to be involved in forming a homodimer.[39] The C_α-atom of the Pro79 in the wild-type GTA is situated 34 Å away from the active site (measured to the Mn^{2+} ion); replacing the rigid proline with the hydrophobic leucine residue (Pro79Leu), as in the *Aw17*-encoded enzyme, may perturb dimerization and enzyme function.

Residue 116 is located in β3, which is a part of a major β-sheet involved in forming a Rossmann fold responsible for nucleotide donor binding. The C_α-atom of residue Ile116 in the wild-type GTA is located 21 Å from the active site (Fig. 6A). The consequences of the Ile116Thr substitution in the *Aw18*-encoded enzyme are perhaps stereochemically minimal, but introducing a more hydrophilic residue may have an effect on the whole β-sheet.

Residue 145 is located at the N-terminal end of the β5 sheet, approximately 25 Å away from the active site (Fig. 6A). The side chain of His145 in wild-type GTA is in very close proximity to that of Asn106, Val222, and Ile116. Replacing the His residue with Arg and its bulkier side chain, as in the *Aw19*-encoded enzyme, undoubtedly disturbs the configuration of the surrounding amino acids.

Residue 203 is found in the C-terminal end of the α-helix α3 in the *Aw20*-encoded enzyme, near the internal disordered loop of amino acids, which also forms a part of the nucleotide-binding Rossmann fold, approximately 26 Å away from the active site. As with the *Aw18* enzyme, there is ample space surrounding the side chain of Glu203 in the wild-type GTA to accommodate either a Lys or a Gln residue in the *Aw20*- and *Aw21*-encoded enzymes, respectively, without necessarily affecting any surrounding amino acids.

Val212 is placed in between the two Asp residues forming the highly conserved DXD (also known as the DVD) motif known to precisely coordinate the Mn^{2+} ion as well as interact with the donor substrate. The side chain of the Val fits snugly into a hydrophobic pocket formed by Val120, Leu122, Phe133, and Leu134. Replacing the Val with the considerably bulkier Met in the Aw22 enzyme would likely cause clashing with residues in the hydrophobic pocket thus potentially displacing the DXD motif and disturbing the precise positioning of the Mn^{2+} ion. Modeling of the two different residues at Position 212 is shown in Figs. 5A and 5B.

Arg241 is in the same loop as Arg248 but is located far away from the active site. However, in the crystal structure the enzyme forms a dimer with an extensive network of interactions. Although this dimer has not been proven to

TABLE 4. Plasma enzyme activity of the *A212* allele encoded enzyme

Plasma used	Phenotype of plasma donor	Genotype	Percentage conversion of substrate to product (mean ± SD)*
A212	A_2-like	*A212/O*^{1v}	5.8 ± 1.1
Positive controls	A_2	A^2/O^{1v}	6.4 ± 1.1
	A_2	NT	6.7 ± 1.4
Negative controls	O	O^1/O^{1v}	0.2 ± 0.1
	O	NT	0.3 ± 0.3
	Water	NA	0.1 ± 0.05

* Percentage conversion = disintegrations per minute (dpm) isolated in product per total dpm added to assay. The mean percentage conversion and SD from four determinations is reported.
NA = not applicable; NT = not tested.

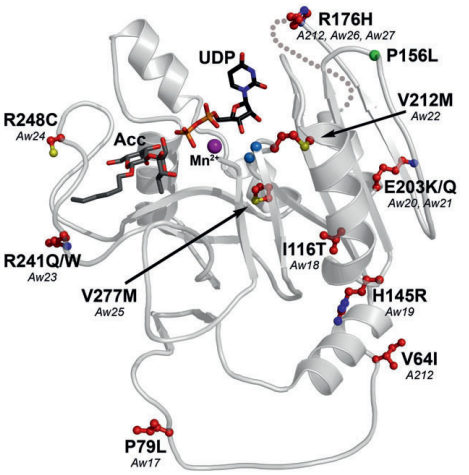

Fig. 4. Ribbon drawing of the overall GTA structure (PDB entry 1LZI) showing the sites of amino acid mutations. The mutated residues are illustrated in red balls and sticks whereas a green sphere indicates the site of the Pro156Leu mutation. The UDP and acceptor are shown in black and gray sticks, respectively, with the Mn^{2+} shown in a purple sphere. The position of the aspartic acids in the DXD motif is shown in light blue spheres. The disordered internal loop is indicated by a gray dotted line.

exist in vivo, it is worth noting that the N-terminal transmembrane regions of both molecules point in the same direction in the dimers formed in vitro and the active site is solvent exposed on each side of the dimer.[39] Thus, if the Arg241 is replaced with a Gln as in *Aw23* several hydrogen bonds to the other monomer will be broken. This may affect enzyme activity and is graphically depicted in Figs. 6B, 6C, and 6E.

Residue 248 is positioned in the loop between β-strands β10 and β11 approximately 23 Å away from the active site (Fig. 4). Despite being remote to the active site, the guanidinium group of the side chain of Arg248 in the wild-type GTA seems to have a role in stabilizing the backbone of residue Thr245, which is known to form a hydrogen bond to the Gal O6-hydroxyl group of the acceptor. The GlcNAc part of both the Type 1 and the Type 2 acceptors does not seem to interact directly with any nearby residues and is primarily solvent exposed. In the presence of Cys248, however, the destabilization or increased flexibility of the loop containing Thr-245 may restrict the *Aw24*-encoded mutant enzyme to allow only or mainly the Type 1 acceptor as substrate, thereby resulting in its para-Bombay-like phenotype (Figs. 2H and 7).

Residue 277 is located in helix α4 approximately 19 Å away from the active site. In the wild-type GTA (Fig. 5C), the side chain faces a hydrophobic pocket formed by Phe270 and Leu280 and there are only approximately 13 Å from the C_α-atom of Val277 to the C_α-atom of Gly268, which is known to be in direct contact with and thus important for determining the specificity of the donor sugar.[22] Val277 and Gly268 are only separated by the side chain of Phe270 in the wild-type GTA; thus the Val277Met mutation found in *Aw25* (Fig. 5D) will almost certainly cause a displacement of the Phe270 side chain, which in turn might displace Gly268.

Residue 176 is placed at the N-terminal edge of the internal disordered loop and is one of four residues which differentiate wild-type GTA from GTB enzymes. The internal disordered loop is highly flexible and is known to fold over the UDP upon donor binding. The Arg176His mutation, as in *A212*, *Aw26*, and *Aw27*, may interfere with the flexibility of the internal loop and also the enzyme's turnover rate.

DISCUSSION

After analysis of 20 samples with weak or unusual A antigen expression, 13 novel alleles each featuring the 1061delC SNP along with at least one additional SNP surfaced. These can now be added to the eight A^2-like alleles previously associated with weak A expression of varying strength, as characterized by traditional serology and a newly developed flow cytometric semiquantification.[28] It is interesting to note that although the amount of A antigen on the RBCs in our study varied considerably, the serologic phenotype produced by most (7 of 12) of these alleles had been classified as A_{weak}, which is an umbrella term that encompasses basically all subtype phenotypes that do not readily fit into another, more specific classification. Flow cytometric characterization was helpful in verifying and extending the categorization based on

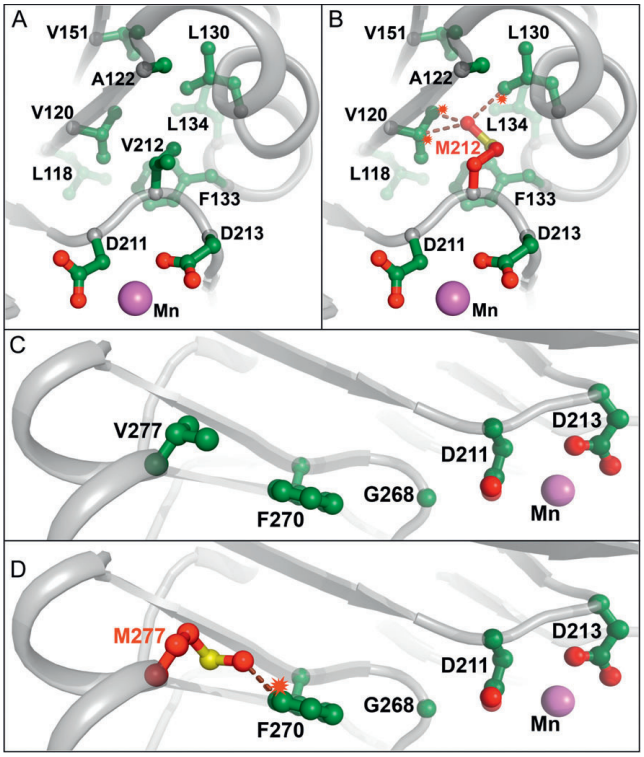

Fig. 5. Three-dimensional modeling of selected A_2 GT mutants. (A) Close-up of the DXD motif in the wt-GTA structure with residues surrounding Val212 shown in green balls and sticks and the Mn^{2+} shown in a purple sphere. (B) The Val212Met mutation modeled into the hydrophobic pocket formed on the backside of the DXD motif. The rotamer (from the rotamer library in COOT) clashing the least with surrounding residues.[36] Dashed lines and red starbursts indicate clashes where the distance is less than 2.8 Å. (C) Close-up of the area around the Gly268 essential for specificity in the wt-GTA structure with surrounding residues. Colored as in A. (D) The Val277Met mutation modeled into enzyme.

serology alone. With one exception only, the RBCs in this study demonstrated clearly less A antigen than regular A_2 RBCs, which indicates that the reduced GTA activity inherent in the *A201*-encoded enzyme relative to that encoded by *A101* was further compromised by the presence of the additional amino acid substitutions. The exception was the *A212*-encoded enzyme where a phenotype similar to A_2 was produced, although lectin studies suggested A_{int}-like features with some reactivity with *D. biflorus* while two independent plasma enzyme assays, including a direct assay of the enzyme's function (Table 4) and an uploading assay using flow cytometry (Figs. 3D-3F), suggested that it had slightly decreased activity.

The *Aw24* (742C>T, R248C) encoded enzyme produced extremely low levels of A antigen. The substitution of a large positively charged arginine with cysteine introduces the potential to form new disulfide bridges that might have a major influence on the overall three-dimensional structure of the protein in addition to perhaps affording less stability to Thr245 and its interaction with the acceptor moiety.[22] This amino acid substitution can also affect many other factors including the acceptor preference of the enzyme. Knowing that this *Aw24* individual is a secretor, it is enticing to speculate that the Arg248Cys substitution virtually eliminated the enzyme's interactions with H Type 2 acceptor chains while not reducing the enzyme's ability to transfer GalNAc onto H Type 1 chains. Although such an effect is possible based on the proximity to the acceptor location, it could not be unequivocally demonstrated by three-dimensional modeling (Fig. 7). Thus, altered acceptor specificity cannot be excluded at this point. As has been previously reported, the RBCs from para-Bombay individuals display small quantities of either A or B antigens synthesized on H Type 1 chains and adsorbed to the cells. In our flow cytometry experiments the A antigen peak in this *Aw24* individual is virtually identical to that seen with para-Bombay samples;[28] this suggests that the low A antigen density on the *Aw24* RBCs might be entirely due to adsorbed A Type 1 glycolipids. If so, the A_{el}/A_{weak} phenotype reported for this sample may in fact be more like A_m, that is, low A expression on RBCs but high levels of A substance in the plasma and secretions (provided that the subject is a secretor). The donor is indeed a secretor according to *FUT2* genotyping (data not shown) but unfortunately, no saliva investigation had been performed. Detailed kinetics of the enzyme encoded by the *Aw24* allele are required to more fully elucidate the impact of the Arg248Cys mutation on enzymatic activity and substrate preference. Other substitutions in the proximity of Residue 248 in the setting of a normal-size GTA include Arg241Trp alone (*Aw04*; 721C>T on an A^1 background),[6] or in combination with Val175Met (*Aw11*),[40] which both produced weak

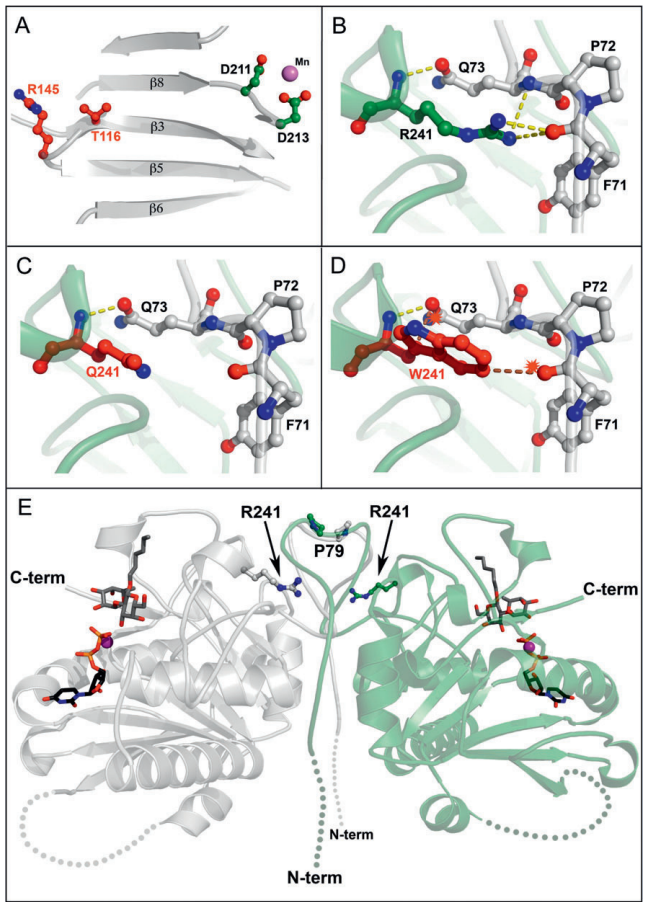

Fig. 6. Three-dimensional modeling of selected A_2 GT mutants and an ABO-GT dimer. (A) The Ile116Thr and His145Arg mutations modeled into the enzyme. The two residues are connected to the active site through a β-sheet, which forms a part of the Rossmann fold responsible for nucleotide-donor binding. (B) Hydrogen bonds (yellow dashed lines) formed between the consensus Arg241 in one monomer and Residues 71 to 73 in the other monomer. (C) The Arg241Gln mutation modeled into the enzyme. Several bonds with residues 71 and 73 are potentially broken compared to the wild type, thus supporting the hypothesis that the Arg241Gln mutation causes weak A antigen expression due to ineffective GT dimer formation. (D) The Arg241Trp mutation modeled into the enzyme. As for Arg241Gln, several hydrogen bonds are potentially broken compared to the wild type. In addition, the chi angles of the tryptophan residue side chain had to be rotated to facilitate its fit into the limited space available with only two minor clashes where the distance is less than 3.1 Å, indicated by reddish-brown dashed lines and red starbursts. (E) Crystal structure of GTA (gray) and the symmetry related monomer (green) together forming a homodimer in the crystal. The potential direction of the N-terminus as well as the disordered internal loop is indicated by dotted lines. The location of amino acid Pro79 and Arg241 involved in dimer formation is shown in sticks. The UDP and acceptor are shown in black and gray sticks, respectively, with the Mn^{2+} shown in a purple sphere.

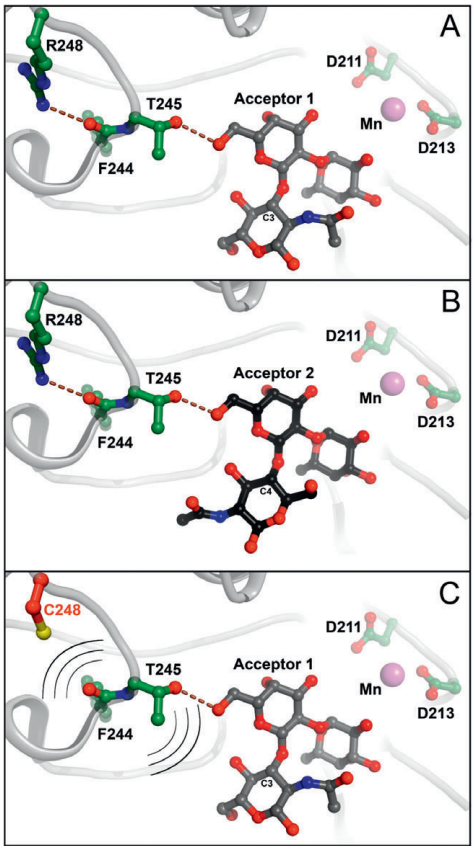

Fig. 7. Modeling of the Arg248Cys mutant. (A) Arg248 forms a hydrogen bond to the carbonyl of Phe244, thereby stabilizing the interaction between Thr245 and the Gal O6 hydroxyl group of the H Type 1 acceptor. (B) The same as A but with H Type 2 acceptor bound. (C) When Arg248 is replaced with a Cys, the hydrogen bond to Phe244 is disrupted and likely a vacant space is left behind Thr245, which may result in a destabilization of the area which could in turn affect acceptor preference of the enzyme.

agglutination on forward typing. In addition, Arg241Trp (721C>T) has also been found in a B^{weak} allele (B^{w}-3).[6] The importance of Residue 241 is further demonstrated by *Aw23*, which results in an A_x-like phenotype due to Arg241Gln. Thus, the same codon is altered in four different ABO subgroup alleles. A flow cytometric comparison between these would be an excellent opportunity to evaluate whether there is synergy between alteration of residue 241 and the A^2-related changes. Surprisingly, the A^2-based *Aw23* sample analyzed in this study displayed higher A antigen levels than those previously found for the A^1-based *Aw04*.[28] However, the comparison between *Aw23* and *Aw04* also involves the change from Arg to either Gln or Trp, respectively, and is therefore suboptimal. At the same time, three-dimensional modeling suggests that Residue 241 is involved in dimerization of ABO GTs (Fig. 6E), a phenomenon not yet confirmed in vivo but observed in in vitro crystallization experiments as well as polyacrylamide gel electrophoresis under nondenaturating conditions (unpublished observations and Lee et al.[39]). In addition, similar phenomena have been described for other GTs.[41,42] In the presence of Gln241 as in the Aw23 enzyme, several hydrogen bonds between the two dimerized enzyme partners are potentially disrupted (Figs. 6C-6E) and it is reasonable to speculate that the dimers become less stable or perhaps are not effectively formed in the first place. The same is true about the Aw04-related Arg241Trp variant. The conformation of the Trp as modeled in Fig. 6D is energetically unfavorable but not necessarily unlikely. Replacing Arg241 with Trp is indeed plausible to interfere with dimer formation to an even greater extent than Gln, consistent with the flow cytometric findings.

There are at least two principally distinct ways to obtain N-truncated ABO GT enzymes in vivo: those found in the plasma encoded by an allele in which translation commenced from the normal start codon but where proteolytic cleavage intracellularly released a N-terminally shortened form into plasma and other extracellular fluids, and those which were encoded by a subtype allele whereby translation started from an alternative downstream start codon. While there is definitive proof of the former type of N-truncated enzymes,[43,44] the existence of enzymes which are N-truncated due to translation from an alternate start codon can so far only be inferred from in vitro experiments. One such study used a human cancer cell line to demonstrate that when translation of the *A101* transcript began from an alternate start codon, a mixed field or weak A phenotype by flow cytometric analysis was produced depending on which alternate start codon initiated translation.[45] In another in vitro expression study using HeLa cells, the amount of A antigen created by N-truncated enzymes was approximately 60% to 65% of that produced by the *A101* enzyme.[46] This indicates that in theory, translation commencing from an alternate start codon should produce GTA capable of synthesizing a measureable amount of A antigen, at least in vitro. One of the novel SNPs reported here, *Aw16* (1A>G, Met1Val in the setting of an *A201* allele), is predicted to abolish the original translation initiation codon. A recent report of a blood donor with a start codon mutation (*Aw13*) demonstrated an A_{weak} phenotype, which was the same serologic result reported for the *Aw16* individual in the current study.[46]

Further enzymologic studies of enzymes translated from alternate start codons are warranted to elucidate their functionality but the finding of naturally occurring start codon mutants the phenotypes of which are not O but A_{weak} lends support to the presence of the idea that genetically N-truncated enzymes exist in vivo.

Some of the SNPs described here have been reported in other contexts. The 607G>A (Glu203Lys) SNP has been deposited in dbRBC as part of an *A101* allelic backbone.[5] In that sample, the RBC serology was listed as A_x. In this study, the *Aw20* allele featuring 607G>A in the context of an *A201* allele produced an A_{weak} phenotype. Unfortunately, an RBC sample was not available for flow cytometric comparison between our *Aw20* sample with the A_x control, but one may speculate that the same SNP on A^2 background should produce an even weaker phenotype than that reported on the A^1 background. That the *Aw21* allele (607G>C, Glu203Gln) also features a mutation at residue 203 speaks to its importance in catalysis; however no apparent explanation for the enzyme's reduced activity was found by the three-dimensional modeling experiments.

The 829G>A SNP is very common in the population due to its presence in the O^{1v} [*O02*] allele.[31] It has also been reported in a series of *A* and *B* alleles underlying various subgroup phenotypes including A_2,[23] A_3,[5] A_{el},[47] A_x,[19,24,25,48] and B_{el}.[49] In a functional allele, this SNP produces a Val277Met change; in both the primary structure and in a three-dimensional surface model of GTA amino acid Residue 277 is located close to Residues 266 and 268 in the enzyme's active center[50] and is predicted to interact with Residue 270, which in turn might impact on the configuration Residue 268 (Fig. 5D). The former two amino acid residues are involved in determining the donor sugar specificity of the enzyme, although none of the reported enzymes that featured Val277Met had altered donor specificity or a cis-AB phenotype. The *A302* allele contains both the 829G>A SNP and the 1061delC SNP;[37] thus it is the most similar of the previously reported alleles to our *Aw25* allele, which is compounded with an additional 467C>T SNP. As indicated in Table 1, the *A302* allele caused mixed-field agglutination while *Aw25* produced a weak A phenotype with an A_x-like pattern of A antigen expression by flow cytometry (Fig. 2I). Even if both our *Aw25* samples carried *B* alleles in trans, which may have increased the A antigen levels, their phenotypes were A_x compared to A_3 for *A302*. As only Exons 6 and 7 were sequenced in the original report of the *A302* allele, it is possible that it contains SNPs affecting other exons elsewhere in the gene that would further change the activity of the resulting GTA.

Although only Exons 6 and 7 were studied in the initial reports,[16,17] the *A209* allele included 527G>A in the setting of an *A201* allele and the serologic phenotype was consistent with A_2.[17] In this study, all seven exons of the *A212* allele were sequenced and the phenotype was A_2-like in the sample from the healthy organ donor but further weakened both in the sample from a pregnant woman and in the patient with ALL. The 527G>A SNP was common to all three individuals but the allelic backbone on which it occurred was different as described above.

The 527G>A SNP present in the *A212*, *Aw26*, and *Aw27* alleles is predicted to cause the amino acid change Arg176His, which affects one of the four amino acids that distinguish a wild-type GTA from a normal GTB. However, the amino acid at Residue 176 does not appear to be involved in donor substrate preference but is more likely to be involved in determining the enzyme's turnover rate due to its proximity to the 16-amino-acid internal disordered loop.[22,51,52] The fact that the flow cytometry experiments on *A212*-encoded RBCs demonstrated A_2 levels of A antigen was thus unexpected given the presence of weak agglutination with anti-A_1 lectins. On the other hand, this highly sensitive flow cytometry method was originally designed to detect the remaining A or B antigens on enzyme-treated RBCs[53] and to differentiate A and B subgroup phenotypes with weak expression levels.[28] Thus, the phenotype produced by *A212* appears to be A_2-like according to flow cytometry, slightly weaker than A_2 by plasma enzyme levels but may possess A_1-like features as suggested by the weak agglutination with *Dolichos/Helix* lectins. The crystal structure of the *A212* enzyme might elucidate whether this enzyme with His176 can work better with Type 3 and/or 4 acceptor chains than the regular A_2 enzyme.

Passive adsorption of A antigen might also explain the small dual peak observed in the ALL patient who had been recently transfused (*Aw27*, Fig. 3C). Although the ABO group of the transfused RBCs is unknown to us, it is reasonable to conclude that they were not A_1 because cells with A_1-antigen expression intensity were not observed in this sample. Plus, as the patient had an ABO discrepancy, it is likely that the transfusion service issued group O RBCs. A small peak with only background levels of A antigen expression (effectively group O) can be seen in this sample along with another small peak immediately adjacent to it on the right. This second peak is at about the same MFI as the major peak in the *Aw24* sample (Fig. 2H) and the A and B peaks in the para-Bombay samples previously reported. As this person was also a secretor according to *FUT2* genotyping, we can thus infer that the patient had been transfused with group O RBCs on two separate occasions as the most recently transfused RBCs had not been exposed to soluble A antigen in the plasma for a long enough period of time to have acquired soluble A antigen.

The RBCs from the pregnant patient with the 527G>A SNP (*Aw26*, Fig. 3B) also demonstrated a significant spread of A antigen expression, although the majority of her RBCs demonstrated low levels of A antigens

approximating that on normal B cells. Due to her co-inheritance of a normal *B* allele, competition for the acceptor substance between the *Aw26*-encoded enzyme and the normal GTB is probably at least partially responsible for the reduced A antigen expression, and furthermore pregnancy itself has been implicated in causing transient subtype phenotypes.[6] In a recent study, it was also shown that 7 of 10 samples from pregnant women with acquired weak A antigen expression were genotyped as A^2B.[28] Interestingly though, this individual had been tested six times during 15 years and every time found to express A antigens weakly and with anti-A1 in plasma.

The mechanism by which the amino acid substitutions encoded by the *Aw18* and *Aw19* alleles effect a reduction in enzyme function is unknown. These two alleles create I116T and H145R mutations, respectively, to an A_2 enzyme encoded by *A201*, and the affected amino acid residues are located in close proximity to Phe121, Ile123, and Tyr126 which all make contact with the donor nucleotide in a model of GTB,[22] and stereochemical effects on other residues cannot be excluded. Even less is known about how the Pro79Leu mutation encoded by the *Aw17* allele reduces enzyme function although it can be postulated that GT dimerization in Golgi may be affected. Interference with heterodimerization has also been speculated to constitute the mechanism behind the enigmatic phenomenon of allelic enhancement.[54] Thus, it is important to keep in mind that the weak *A* alleles accompanied by *B* alleles may give rise to higher A expression levels than would have been the case with deletional O alleles in trans as recently highlighted.[28]

In conclusion, 13 novel *A* subtype alleles based on the A^2 backbone were identified and their resulting phenotypes and enzyme structures in silico were characterized in this study. Although many of these new alleles produced relatively similar phenotypes by conventional serologic techniques, the diversity of A antigen expression by flow cytometry was notable. ABO serology and molecular genetics continue to provide structural insight into GT function.

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CONFLICT OF INTEREST

The authors have no conflicts to disclose.

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Paper IV

Flow cytometry evaluation of red blood cells mimicking naturally occurring ABO subgroups after modification with variable amounts of function-spacer-lipid A and B constructs

Annika K. Hult, Tom Frame, Scott Chesla, Stephen Henry, and Martin L. Olsson

BACKGROUND: Kodecytes bearing synthetic blood group A and B antigens are increasingly being used in transfusion laboratories as serologic mimics of red blood cell (RBC) A_{weak} and B_{weak} subtypes. The aim of this study was to compare the flow cytometry profile of kodecytes with native ABO subgroups.

STUDY DESIGN AND METHODS: A series of A/B kodecytes, each with decreasing A or B antigen expression, were prepared from group O RBCs that were modified with dilutions of function-spacer-lipid KODE technology (FSL) constructs representing a wide serologic range. Using an established flow cytometry method designed for the detection of low levels of A/B antigens, kodecyte profiles were compared with those of native subgroup cells.

RESULTS: Kodecytes with positive tube serology from 4+ to 1+ were created with 15 to 2 μg/mL FSL-A or 78 to 10 μg/mL FSL-B transformation solutions. The kodecytes created with higher concentrations of FSL constructs revealed a uniform and/or even distribution of antigens as seen by a single flow cytometry peak more narrow than the broader peaks produced with lower FSL concentrations similar to those found in native A_x and most B_{weak} subgroups.

CONCLUSIONS: Although kodecytes are created artificially, they can be designed to mimic the serologic and flow cytometric profiles of native ABO subgroup RBCs.

According to UK national[1] guidelines or European directives,[2] monoclonal ABO reagents are required to detect A_x and B_{weak} subgroup red blood cells (RBCs). However, many routine laboratories do not have access to naturally occurring ABO subgroups, and those who do seldom characterize them genetically to confirm their subgroup status. Group O RBCs can be controllably modified with synthetic blood group A and/or B function-spacer-lipid (FSL) constructs,[3] and thus have the potential to be ABO subgroups mimics. These so-called kodecytes[4] (KODE construct modified cells) have been used in quality control (QC) systems[4] and more recently to determine in vivo RBC survival, mimic incompatible transfusion reactions, and neutralize anti-A in an animal model.[5,6] The aim of this study was to examine if kodecytes can be created to mimic the distinct flow cytometric patterns recently established for native ABO subgroups[7] and the optimal concentration of FSLs required to achieve this.

MATERIALS AND METHODS

FSL constructs

Two FSL constructs were obtained from KODE Biotech Materials Ltd (Auckland, New Zealand;

ABBREVIATION: FSL = function-spacer-lipid KODE Technology construct.

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TABLE 1. A_{weak}, B_{weak} kodecytes created by modification of O cells with different concentrations of FSL transformation solutions

FSL-A–modified O cells			FSL-B–modified O cells		
Transformation solution	Tube serology	Flow cytometry*	Transformation solution	Tube serology	Flow cytometry*
FSL-A (μg/mL)	AM201-1	AM201-1	FSL-B (μg/mL)	BM154-1	BM154-1
15	4+	Positive	78	4+	Positive
8	4+	Positive	39	3+	Positive
4	2+	Positive	20	2+	Positive
2	1+	Positive	10	1+	Positive
1	–	Positive	5	–	Positive
0.5	–	–	3	–	–
0.2	–	–	1	–	–

* Positive MFI in relation to the group O control cell (for details see Figs. 1 and 2).

http://www.kodebiotech.com): FSL-A with a blood group A trisaccharide epitope GalNAcα3(Fucα2)Galβ (FSL-A(GALNa3[Fa2]GALb)-SA1-L1, Cat. No. 421604) and FSL-B with a blood group B trisaccharide epitope Galα3(Fucα2)Galβ (FSL-B(GALa3[Fa2]GALb)-SA1-L1, Cat. No. 199283). These constructs are also available from Sigma-Aldrich (St Louis, MO).

Kodecytes

Kodecytes were prepared in two independent batches at different times according to a previously described procedure.[3] Group O RBCs were modified (transformed) with different concentrations of FSLs. The first batch contained concentrations of FSLs down to 2 and 9 μg/mL for FSL-A and FSL-B, respectively, whereas the second batch contained extended dilution steps (Table 1), ranging from 15 to 0 μg/mL for FSL-A and 78 to 0 μg/mL for FSL-B (as serial twofold dilutions). Native RBCs of different ABO phenotypes were used as controls. Manual tube serology was performed by the immediate-spin method (15 sec, 900 × *g*) with 3% to 4% kodecyte suspensions in phosphate-buffered saline (PBS) against monoclonal anti-A (AM201-1) and anti-B (BM154-1; ImmucorGamma, Inc., Atlanta, GA). Kodecytes are defined as cells that can be shown to have acquired the FSL construct[5,6] and can be described by the concentration and type of construct inserted, for example, 4 μg/mL A kodecytes.

Flow cytometry

Kodecytes and control RBCs were washed three times in PBS and 25 μL of RBCs were suspended in 900 μL of PBS. Flow cytometry was as previously described using a highly sensitive method developed to detect A and B antigens in ABO subgroup phenotypes using ABO reagents; anti-A, ES-15 (Serologicals Limited, West Lothian, UK); anti-B, 9621A8 (Diagast, Loos, France) and phycoerythrin-labeled rat anti-mouse Ig kappa light chain (Becton Dickinson, San Jose, CA) as secondary antibody.[7] Each batch was tested three times during a 3-week period. Additional ABO reagents used were anti-A, AM201-1, anti-B, BM154-1, anti-AB, and ABM201-1 (ImmucorGamma, Inc.). Both batches of kodecytes were tested with the additional ABO reagents, the first batch tested once and the second tested twice, 3 weeks apart. Relevant control RBCs A_1, A_2, A_x (A^x-1/O^1 genotype), B, B_{weak} (B^w-3/O^1 genotype), and group O were included in each run, but for simplicity the histograms reported show only the most relevant, selected controls.

RESULTS

A range of A and B kodecytes with decreasing antigen expression was prepared twice from the same group O RBC, starting from the FSL transformation solution concentration that gave a maximum 4+ serologic result by the standard tube method. The group O RBC used for the preparation of the A and B kodecytes were also tested by serology and flow cytometry against anti-A, -B, and -H and behaved like a normal group O control (results not shown).

Tube serology

FSL-A transformation solutions over a four-tube dilution range (15-2 μg/mL) created A kodecytes that gave positive tube serology from 4+ to 1+ (Table 1). The 4 and 2 μg/mL A kodecytes gave serologic reactions in the range expected of A_{weak} subgroups. Similarly FSL-B was also able to create positive tube serology from 4+ to 1 + over a four-tube dilution, but in the range of 78 to 10 μg/mL. The 20 and 10 μg/mL B kodecytes gave serologic reactions in the range expected of B_{weak} subgroups.

Flow cytometry

Flow cytometric testing of kodecytes prepared with higher FSL construct solutions revealed a uniform, reproducible, and even distribution of antigens in the cell population as seen by a single, quite narrow peak in the flow cytometry histograms (Fig. 1). When kodecytes were

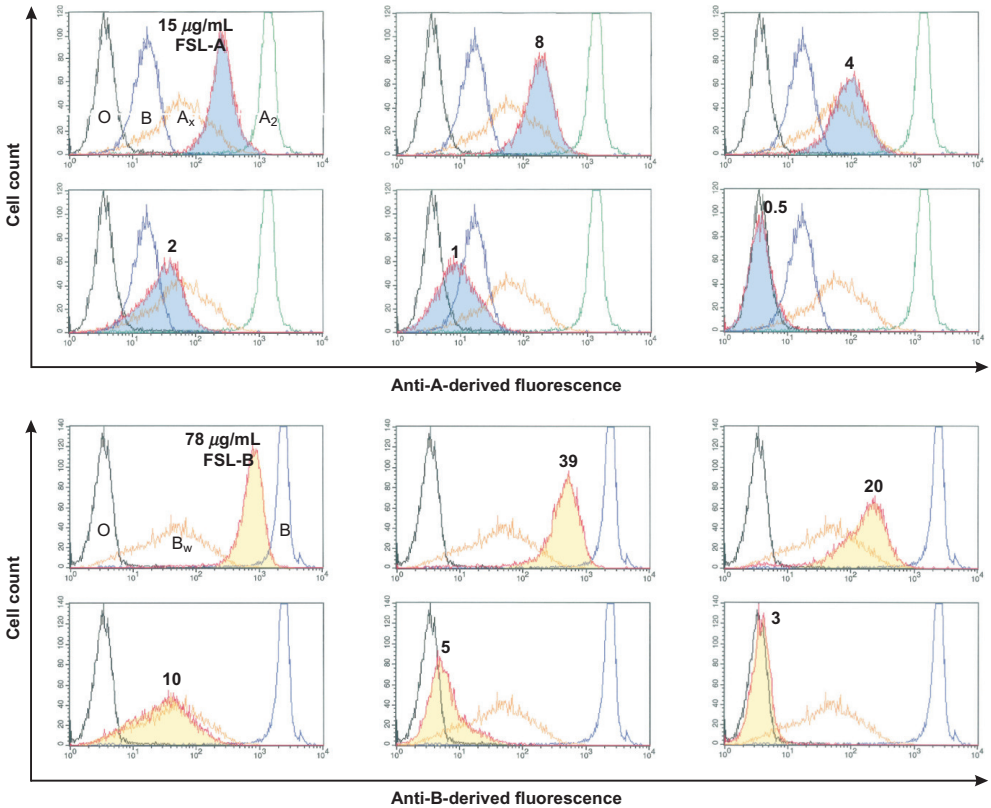

Fig. 1. Representative histograms of A_{weak} kodecytes (top histograms, blue fill), B_{weak} kodecytes (bottom histograms, yellow fill), and natural RBCs against monoclonal reagents (anti-A clone ES-15 and anti-B clone 9621A8). Kodecytes (red lines) of decreasing antigen expression are indicated by a number that represents the μg/mL FSL construct concentration in the transformation solution used to create them (Table 1). Control RBCs analyzed at the same time as the kodecytes are included in each histogram (and labeled in the first histogram) and are as follows: group O (black line), group B (blue line), and group A_2 (green line) plus natural subgroups A_x with the A^x-1/O^1 genotype and B_w with the B^w-3/O^1 genotype are shown as orange lines.

prepared with lower FSL concentrations, peaks tended to broaden to resemble a pattern found in A_x and most B_{weak} subgroups,[7] indicating a more variable antigen site density on the cells in the population, although this is partially explained by the logarithmic scale used to display the results. The concentrations of FSLs that produced kodecytes that resembled the naturally occurring subgroup control RBCs used in this study are approximately 2 to 4 μg/mL for FSL-A and approximately 10 μg/mL for FSL-B. Repeat testing demonstrated good correlation between flow cytometric runs and kodecyte batches (data not shown).

DISCUSSION

Kodecytes are becoming more evident in some transfusion laboratories where they are being routinely used as QC cells expressing low levels of A and B blood group antigens.[4] However, as A/B kodecytes are created by inserting simple glycolipid-like FSL constructs into group O RBCs the question of whether they are representative of native ABO subgroups,[8] which bear a range of simple and complex ABO antigens on both glycolipids[9] and glycoproteins, requires addressing. We used the same methodology in our recent report characterizing low levels of A/B

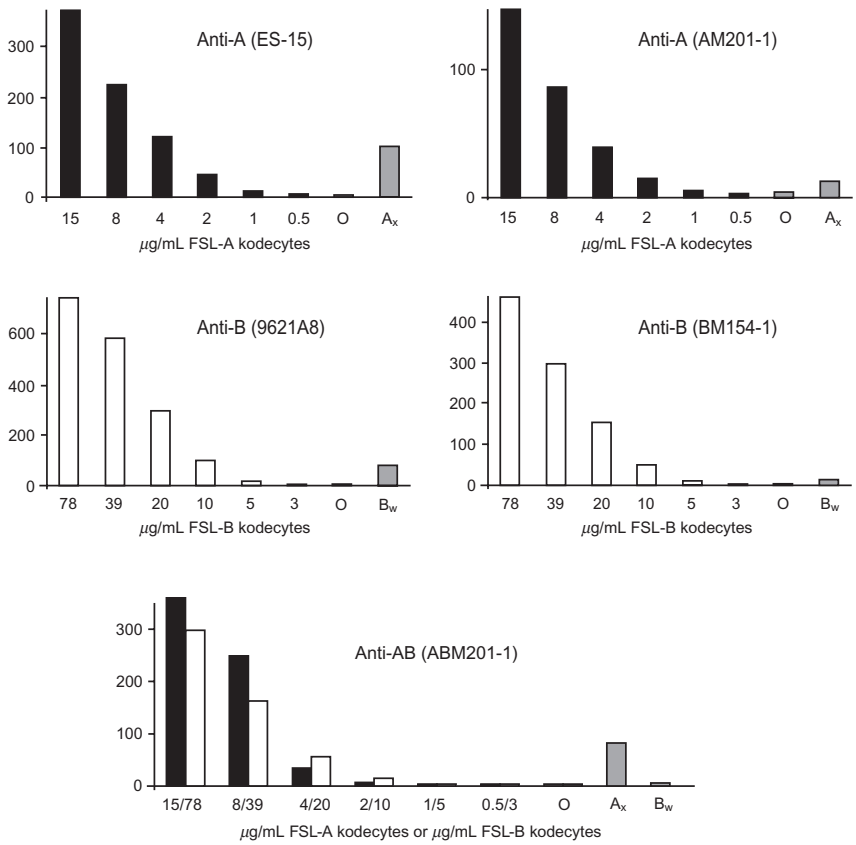

Fig. 2. Mean fluorescence intensity (MFI) comparison of a representative flow cytometric run of A_{weak} and B_{weak} kodecytes against different monoclonal reagents. Natural O, A_x, and B_{weak} samples (gray bars) are included for comparison. Black bars compare A_{weak} kodecytes labeled with two monoclonal anti-A reagents (ES-15 and AM201-1). White bars show B_{weak} kodecytes labeled with monoclonal anti-B (9621A8 and BM154-1). The lower bar graph shows the reactivity with the same kodecytes as above but tested against monoclonal anti-A,B (ABM201-1), with the A kodecytes in black and B kodecytes in white. Note: the scale range for the *y*-axis is different between images.

antigen on a large variety of ABO subgroups,[7] to investigate A and B kodecytes. A similar approach has also been applied as QC of group A, B, and AB RBCs converted by bacterially derived exoglycosidases to type as group O with CE-marked and FDA-approved monoclonal anti-A and -B reagents.[10] Using appropriate concentrations of FSL-A and -B constructs, kodecytes could be easily created that gave manual tube serology and flow cytometry profiles identical to those observed for ABO subgroups expressing low levels of A and B antigens. It was interesting to note that flow cytometry was only slightly more sensitive than manual tube serology when the same antibody was used (Table 1), and although different antibodies did show differences in mean fluorescence with kodecytes (Fig. 2), the endpoints were essentially the same. However, in this article the monoclonal antibodies used were specifically selected for their high sensitivity with some shown to detect native ABO subgroups[7] and remaining A and B antigens after exoglycosidase treatment.[10] It would be expected that different clones would show different sensitivity profiles, as has been shown by serology.[3] Recently, we have also found a few quality monoclonal ABO reagents that do not react with FSL-A/B constructs and others which react with FSL-A/B con-

structs but do not react with natural glycolipid antigens of the same specificity (unpublished). It is clear that further knowledge of the specificity of synthetic antibodies (monoclonal reagents) and synthetic antigens (FSL constructs) is still necessary.

Again[3] the requirement for more blood group B than blood group A antigen was evident, with five times more FSL-B transformation required to be serologically equivalent to FSL-A transformation. This phenomenon is consistent when either monoclonal or human polyclonal antibodies are used and against different variants of both natural and synthetic B antigen (unpublished). It is now believed to be due to the structure of the B antigen, which has an inherently lower affinity for specific antibody, in contrast to its antithetical partner the A antigen, which has the N-acetyl protrusion on its terminal sugar. This concept is also supported by clinical observations that anti-B less frequently causes hemolytic disease of the fetus or newborn.[11]

In agreement with earlier reports[3,4] on the agglutination serology of kodecytes, by simply altering the concentration of FSL construct in the transformation solution "antigen dilutions" were introduced to the kodecytes. By flow cytometry and with higher concentrations of FSLs, the kodecytes created demonstrated a relatively uniform and even distribution of antigens among the cells. Using very low amounts of FSL-A and FSL-B, kodecytes could be made which mimicked the broad antigen density flow cytometry profiles of some natural A_x and B_{weak} subgroup RBCs. However, not all naturally occurring ABO subgroups are equal and some may have either a narrower or wider range of antigen distribution profiles[7] than are seen with kodecytes. The reason for this interesting phenomenon is unknown. It was somewhat surprising that the lower concentrations of FSL solutions produced broader antigen distributions on kodecytes. Even if unlikely, it cannot be excluded that this may be an artifact of preparation, potentially due to transformation equilibration or contact times or possibly a preference of FSLs for some RBCs (perhaps due to lipid differences as a consequence of age). Interestingly, para-Bombay samples, whose weak A phenotype is dependent on acquiring A glycolipids from plasma (and therefore the natural analog to kodecytes), showed a more uniform and even antigen distribution.[7] The same narrow histogram peaks have also been shown to occur after human in vivo plasma incubation, where group O RBCs transfused to group A individuals with the secretor phenotype, will acquire A glycolipids from the plasma of the recipient.[12,13]

This study indicates that kodecytes with low expression of A and/or B antigen have characteristics compatible with use as QCs for monoclonal ABO reagents and could be a valuable addition in the serologic laboratory.

CONFLICT OF INTEREST

TF and SC are employees of ImmucorGamma, a licensee of KODE technology. SH is the CEO/CSO and a stockholder of KODE Biotech Ltd, the patent owner of KODE cell surface engineering technology. AH and MLO have no conflicts of interest regarding this study.

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Paper V

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Regular Article

TRANSFUSION MEDICINE

Forssman expression on human erythrocytes: biochemical and genetic evidence of a new histo-blood group system

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Key Points

- A new histo-blood group system was discovered, based on the identification of Forssman glycolipid antigen on human red blood cells.
- A newly described polymorphism in the *GBGT1* gene activates the encoded enzyme to synthesize Forssman antigen.

In analogy with histo-blood group A antigen, Forssman (Fs) antigen terminates with α3-*N*-acetylgalactosamine and can be used by pathogens as a host receptor in many mammals. However, primates including humans lack Fs synthase activity and have naturally occurring Fs antibodies in plasma. We investigated individuals with the enigmatic ABO subgroup A_{pae} and found them to be homozygous for common *O* alleles. Their erythrocytes had no A antigens but instead expressed Fs glycolipids. The unexpected Fs antigen was confirmed in structural, serologic, and flow-cytometric studies. The Fs synthase gene, *GBGT1*, in A_{pae} individuals encoded an arginine to glutamine change at residue 296. Gln296 is present in lower mammals, whereas Arg296 was found in 6 other primates, > 250 blood donors and A_{pae} family relatives without the A_{pae} phenotype. Transfection experiments and molecular modeling showed that Agr296Gln reactivates the human Fs synthase. Uropathogenic *E coli* containing prsG-adhesin–encoding plasmids agglutinated A_{pae} but not group O cells, suggesting biologic implications. Predictive tests for intravascular hemolysis with crossmatch-incompatible sera indicated complement-mediated destruction of Fs-positive erythrocytes. Taken together, we provide the first conclusive description of Fs expression in normal human hematopoietic tissue and the basis of a new histo-blood group system in man, FORS. (*Blood*. 2013;121(8):1459-1468)

Introduction

Carbohydrate histo-blood group antigens, first recognized on red blood cells (RBCs) in 1900,[1] have been suggested to be part of our innate immune response.[2] Major carbohydrate histo-blood groups in man include the ABO, P1PK, H, Lewis, I, and GLOB systems in which glycoproteins and glycolipids carry immunodominant terminal sugars,[3] defining polymorphic antigens. Other mammals also express carbohydrate histo-blood groups, such as ABO,[4] fucoseless B antigen (Galili),[5] and Forssman (Fs)[6,7] but their expression on RBCs varies among species. Although the biologic function of polymorphic carbohydrates on RBCs is unresolved, these antigens can be used as receptors by pathogens[8-11] and their expression in tissues and bodily secretions are thus believed to reflect microbial selection.[8] In response to blood-group-mimicking glycans on bacterial surfaces, naturally occurring antibodies with the capacity to neutralize various microorganisms are formed. However, these antibodies also constitute substantial transfusion and transplantation barriers.[3,12]

In 1987, 3 families with a supposed ABO subgroup named A_{pae} were reported.[13] Although *Helix pomatia* lectin reacted strongly and polyclonal anti-A weakly with RBCs from some family members, monoclonal (MAb) anti-A reagents were later shown to be nonreactive, thus presenting an apparent paradox. The biochemical and genetic background of this enigmatic phenotype has remained unknown, as has its biologic consequences. We hypothesized that an explanation may be found by studying the glycolipids of this phenotype.[14] Here we report the identification of Fs glycolipids, normally found only on RBCs of selected nonprimate mammals, are strongly expressed on human A_{pae} RBCs. In nonprimates, Fs antigen is synthesized by Fs synthase (globoside 3-α-*N*-acetyl-D-galactosaminyltransferase, EC2.4.1.88),[7] an enzyme homologous to the ABO transferase. We also reveal a genetic polymorphism in the human *Fs* gene (*GBGT1*) that alters the enzymatically inactive human protein[15] to its active nonprimate

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counterpart. Overall, these findings qualify Fs as a new histo-blood group system with potential implications for both transfusion/transplantation medicine and pathogen susceptibility.

Methods

Samples

Five and 3 RBC units were collected from each of 2 unrelated A_{pae} individuals (A_{pae}#1 and A_{pae}#2, respectively) from 2 of the originally reported families.[13] Saliva samples were provided by 14 relatives. Informed consent was obtained according to the Helsinki Declaration after approval from the North Sheffield Local Research Ethics Committee (2008) or Sheffield Research Ethics Committee, National Research Ethics Service (2011), United Kingdom. Blood units were leukocyte-depleted, washed free of plasma with phosphate-buffered saline and frozen at −20°C until processed. Aliquots of plasma, RBCs, and leukocytes were kept for analysis. Approvals from the Regional Ethics Review Board at Lund University were obtained for bone marrow collection from healthy volunteers and genetic blood group analysis on samples from blood donors with known ABO phenotypes. Blood from anonymized Swedish donor samples was used for screening and control purposes.

Glycolipids

Glycolipid preparation. Lysed blood units were thawed and total neutral glycolipids with < 20 sugar residues were isolated (see supplemental Methods, where control glycolipid preparations are also described; available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Open-column chromatography. Total glycolipids (~ 110 mg) from each of A_{pae}#1 and #2 were fractionated by silica chromatography column (5g silica/100 mg lipid; Silica 60, Merck) in a system of chloroform (C) methanol (M) solvent mixes (supplemental Methods).

Thin-layer chromatography-enzyme immunoassay (TLC-EIA). The selected method[16] was recently reported to be sensitive for the characterization of weak A subgroups (supplemental Methods).[17] A wide range of monoclonal and polyclonal antibodies and lectins were used for immunostaining (supplemental Table 1).

Structural analysis

Capillary liquid chromatography: electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS). Endoglycoceramidase II from *Rhodococcus* spp. (TakaraBio Europe) was used to release saccharides from ceramide for analysis by LC-ESI/MS-MS as described.[18]

Proton NMR spectroscopy. ^{1}H-NMR spectra were acquired on a Varian 600-MHz spectrometer at 30°C. Samples were dissolved in dimethylsulfoxide/D_2O (98:2 vol/vol) after deuterium exchange. Two-dimensional double quantum-filtered correlated spectroscopy (2D-DQF-COSY) spectra were recorded using the standard pulse sequence.[19]

Genetic analysis of *ABO* and *GBGT1*

ABO genotyping was performed.[20] *GBGT1* exons 1-7 were sequenced in 11 humans, including 2 A_{pae} propositi, and compared with Ensembl entry ENST00000372040. Exon 7 was sequenced in 6 DNA samples from different nonhuman primates. Two allele-specific primer (ASP) PCR assays were designed to detect 887G > A and 363C > A. RNA extraction, cDNA preparation, and transcript analysis were performed (for details see supplemental Methods and supplemental Table 2).

Transfection

A *GBGT1* (EMBL database; HE583599) wild-type transcript was used as template and 887G > A introduced by site-directed mutagenesis. Wild-type and mutant fragments were cloned into pEF1α-IRES+ZsGreen1 vectors (Clontech Laboratories). A *B3GALNT1* wild-type fragment (GenBank; AB050855.1) cloned into the same vector was cotransfected to enhance the expression of acceptor substrate (P antigen) for Fs synthase. Primers are listed in supplemental Table 2. All constructs were verified by DNA sequencing.

MEG-01 cells were grown in RPMI1640 supplemented with 10% fetal bovine serum (FBS). Transient transfection of 3×10^6 MEG-01 cells with 10 μg of *GBGT1*-allele–containing vector and 10 μg of *B3GALNT1* vector, or 20 μg of empty vector as a negative control, was performed by electroporation at 280 V and 960 μF with GenePulser (Bio-Rad). The MEG-01 cells were resuspended in RPMI1640 supplemented with 10% FBS and grown for 48 hours before flow cytometric analysis of Fs expression. Experiments were run in triplicate (duplicate for mock-treated cells) and repeated 3 times.

Flow cytometry and serology

Flow cytometry was performed with MAb anti-Fs, donor plasma, and eluate made from A_{pae}-RBCs adsorbed with group B plasma. Strongly A_{pae}-hemagglutinating samples were selected by crossmatching. Fresh sera from these donors were used for hemolysin tests[12] against native and papainised RBC (A_{pae}/A_1/B/O). For details, see supplemental Table 1 and supplemental Methods.

Three-dimensional modeling

A molecular model of human Fs synthase was derived by threading onto a human ABO glycosyltransferase crystal structure, PDB entry 2RJ7 (supplemental Methods).

Escherichia coli hemagglutination

A common K12 *E coli* strain, HB101, unmodified or containing either papG (HB101.AD110) or prsG (HB101.JFK102) operons was used for hemagglutination experiments (supplemental Methods). RBCs of various ABO/A_{pae} phenotypes were used, including group O RBCs modified with synthetic glycolipid function-spacer-lipid (FSL) constructs, so-called kodecytes.[21]

Results

ABO testing of A_{pae} propositi

The original serologic work[13] was confirmed and extended with additional ABO reagents. Of 15 anti-A reagents, 3 of 4 polyclonal but only 1 MAb reagent (clone A003) reacted with A_{pae}-RBCs, albeit with weak reactivity. Of 8 anti-A,B reagents, all 3 polyclonal and 1 MAb blend reagent were weakly positive. By *ABO* genotyping, both A_{pae} individuals showed homozygosity for 261delG, the common group O polymorphism.[22,23] These data argued strongly against A_{pae} as a conventional A subgroup in the ABO system.

Glycolipid analysis

Based on the hypothesis that another glycolipid than A antigen may be responsible for the A_{pae} phenotype, total neutral glycolipids from A_{pae}#1 and A_{pae}#2 RBC unit lysates were analyzed. Open-column fractionation and pooling resulted in 5 heterogeneous fractions containing glycolipids with > 4 sugars from each donor.

Thin layer chromatography-enzyme immunoassay. Total glycolipids (Figure 1) and open column fractions (Figure 2) were analyzed by TLC and immunostaining with a variety of reagents. Staining of the heterogeneous open-column fractions of the 2 A_{pae} individuals was very similar (Figure 2), and expected enhancement in reactivity of minor bands compared with the total glycolipid samples was seen. Total A_{pae} glycolipids reacted strongly with *Helix pomatia* in the 5-sugar region consistent with Fs glycolipids (Figures 1B and 2B), with the CM65:35 fraction being of primary interest (Figure 2B lanes 5-14). Staining of total glycolipids with

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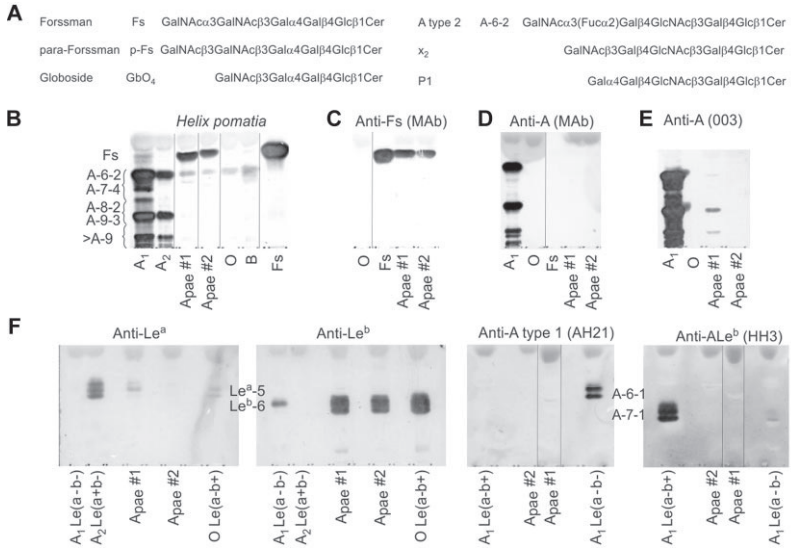

Figure 1. Thin layer chromatography staining of total A_{pae} glycolipids. (A) Nomenclature and structures of selected glycolipids discussed in this paper. (B) *Helix pomatia* lectin staining of the A_{pae} total glycolipid samples and blood group A_1, A_2, O, and B controls plus Fs glycolipid control. TLC migratory positions of known blood group glycolipids are identified in the margin. (C) Staining with MAb anti-Fs. (D) Staining with a representative broadly reactive MAb anti-A (clone 11H5). (E) Unidentified bands recognized by staining with MAb anti-A (003). (F) Staining with Lewis (anti-Le^a, anti-Le^b) and secretory A antigen glycolipid profiles (anti-A type 1, anti-ALe^b). In all panels A_1, A_2, O, and B control samples are total erythrocyte glycolipids whereas Fs is a purified glycolipid fraction. Lewis phenotypes are expressed as red cell phenotypes [where low level Le^a glycolipid is expected in the Le(a−b+) phenotype]. Because of minor migration differences between assays, direct comparison of absolute band positions should be viewed with caution. Black lines indicate where migration controls and unloaded lanes have been removed from the image. All lanes are from the same TLC plate and have not been repositioned.

anti-A and anti-Fs MAbs (Figure 1C-D), and column fractions (Figure 2C) were concordant with the identity of Fs.

Present on the *H pomatia* plate (Figure 1B) with a migration similar to A-6-2, were also faint bands in both A_{pae} samples and B and O controls. The migration and staining of these bands were consistent with p-Fs[24] and/or x_2[25] (Figure 2D-E) or A-6-2. Staining with an anti-A (MAb 2-22, Figure 2D) with known activity against p-Fs[14] found activity in fractions CM65:35, CM60:40 and CM25:75 of the A_{pae}#2 and fractions CM60:40 and CM25:75 of the A_{pae}#1. Staining with another p-Fs-reactive MAb (ES-15) was also consistent with p-Fs in both A_{pae} samples (Figure 2F). Staining with an x_2-reactive MAb found only trace activity from x_2 in fraction CM65:35 of A_{pae}#2, although it was clearly present in all other later eluting fractions of both A_{pae} samples (Figure 2E).

The nonstaining of A_{pae} total glycolipids and concentrated fractions with broadly reactive anti-A reagents (Figure 1D, supplemental Figure 1) was indicative of the absence of A glycolipids. The bands seen with anti-A (A003, Figure 1E) in A_{pae}#1 were not because of either Fs or A and remain unresolved yet consistent with the weak hemagglutination results observed with A003. Staining with 7 other anti-A MAbs (supplemental Table 1, supplemental Figure 1) was unable to detect any bands in the A_{pae} samples. These results strongly suggest that the aberrant bands seen with some anti-A reagents are not because of A antigens. Finally, staining with Lewis MAbs established that both individuals are Le(b+) (Figure 1F), and, therefore, if blood group A they would be expected to have A-7-1 (ALe^b) glycolipids,[26,27] which were not found.

Overall both A_{pae} samples could be demonstrated to have staining consistent with the presence of Fs, p-Fs, x_2 but absence of A structures.

Structural analysis. The LC-ESI/MS-MS and ^{1}H-NMR results of both A_{pae} samples were essentially the same. Only A_{pae}#2 results are shown as it had less globoside content (Figure 2A). Released oligosaccharides from fraction CM65:35 were analyzed by LC-ESI/MS-MS.[18] Despite the identical molecular masses of p-Fs and Fs (*m/z* 909 corresponding to an oligosaccharide with 2 HexNAc and 3 Hex) the reference pentasaccharides of Fs eluted as 2 peaks at 10.1-10.4 minutes retention time (Figure 3Aiii), whereas those of p-Fs eluted at 9.8-10.2 minutes (Figure 3Aiv). These elution time differences were used for differentiating Fs from p-Fs by LC-ESI/MS-MS. Fraction CM65:35 of both A_{pae} samples had *m/z* 909 peaks at 10.0 and 10.4 minutes, corresponding with Fs (eg A_{pae}#2, Figure 3Aii), whereas no peaks at 9.8-10.2 minutes were present. The MS-MS spectrum of the ion at *m/z* 909 (Figure 3Av) produced a series of C-type fragment ions (C_2 at *m/z* 423, C_3 at *m/z* 585, and C_4 at *m/z* 747) consistent with a pentasaccharide with HexNAc-HexNAc-Hex-Hex-Hex sequence (Figure 3B). The fragmentation pattern obtained was identical to both reference Fs/p-Fs pentaglycosylceramides (Figure 3Avii-viii) but could be differentiated on the basis of retention time (Figure 3Aiii-iv). In addition, the A_{pae}#2 CM65:35 fraction had a *m/z* 909 oligosaccharide eluting at 14.0-14.2 minutes (Figure 3Aii), with MS-MS spectrum (Figure 3Avi), consistent with a HexNAc-Hex-HexNAc-Hex-Hex sequence as in x_2 (Figure 3B). Dominant ions in the base sample could be assigned to globoside (P/GbO_4), neolactotetraosylceramide (nLc_4), and the H-5-2 pentaglycosylceramide, respectively (Figure 3Ai).

The 1D-^{1}H-NMR spectra (results not shown) were complex and 2D-DQF-COSY spectra was required to determine the presence of Fs and differentiate it from p-Fs pentaglycosylceramides. The most abundant glycolipids present in A_{pae}#2 fraction CM65:35 (Figure

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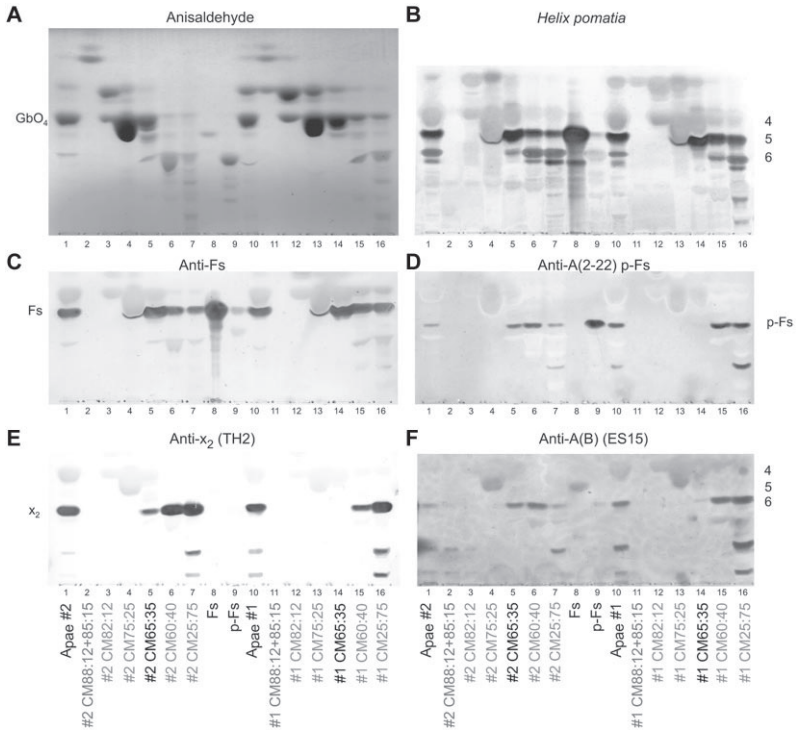

Figure 2. Thin layer chromatography staining of open column fractions. (A) Anisaldehyde semiquantitative chemical staining. (B) *Helix pomatia* lectin staining (with approximate sugar sizes indicated on the right of the plate). (C) MAb anti-Fs staining. (D) p-Fs crossreactive MAb anti-A 2-22 (clone AY209) staining. (E) Anti-x_2 (clone TH2) staining. (F) p-Fs and Fs crossreactive MAb anti-A(B) (ES-15) staining. In all panels, total glycolipids are indicated as A_{pae}#1, A_{pae}#2, whereas open column fractions are identified by their elution solvent (with the fraction used for structural analysis indicated in black). Fs and p-Fs are purified reference glycolipids. Because of minor migration differences between assays, direct comparison of absolute band positions should be viewed with caution.

3C) were GbO_4, followed by nLc_4, H-5-2, P1, and GbO_5. The COSY spectrum (Figure 3C) identified Fs pentaglycosylceramide (as indicated by the H1/H2 connectivities outlined by red ellipses in Figure 3C) as a minor glycolipid. With respect to connectivities and reference glycolipids, identification of Fs was unambiguous. Equally clear in this fraction was the absence of p-Fs pentaglycosylceramide (essentially excluded by fractionation) and indicated by blue circles in Figure 3C. The same structures were also identified in fraction CM65:35 of A_{pae}#1. Importantly, there was no evidence for any structures carrying blood group A determinants. Sugar sequences and NMR resonances are given in supplemental Table 3.

Extended serologic analysis

Positive reactions were obtained by flow cytometry using MAb anti-Fs against ovine, canine and A_{pae}-RBCs, whereas other human RBCs were negative (Figure 4A). Approximately 6% of 278 random blood donor plasmas agglutinated A_{pae}-RBCs by the indirect antiglobulin test (anti-IgG/-C3d) and ~ 23% caused direct IgM agglutination. Selected plasmas reacting strongly with A_{pae}-RBCs were further investigated (Figure 4B). By adsorption of one such plasma onto A_{pae}#2-RBCs and testing the eluted antibodies against various RBCs, reactivity against both A_{pae} and nonprimate mammal RBCs but not other ABO groups was noted (Figure 4C-D), thus strongly suggesting that the antibody eluted is not crossreactive anti-A but indeed anti-Fs.

Freshly drawn group B and O sera from normal (non-A_{pae}) donors were incubated with native and papainised A_{pae} and control RBCs. Hemolysis was observed with A_{pae} and ABO-incompatible RBCs (Figure 4E-F) and quantified. These in vitro results indicate that anti-Fs can activate complement and may have the potential to cause intravascular lysis of Fs-positive RBCs.

Genetic analysis

***The sequence of* GBGT1.** Based on the finding that A_{pae} RBCs carry Fs antigens, exons 1-7 of the *GBGT1* gene were sequenced in 11 individuals because this supposed human pseudogene is homologous to mammal Fs synthase genes.[28] No deviations from the GenBank consensus sequence were detected in 9 non-A_{pae} individuals, whereas in both A_{pae}#1 and A_{pae}#2, heterozygosity for a previously unreported single nucleotide polymorphism (SNP), 887G > A, that results in Arg296Gln was identified (supplemental Figure 2). In addition, A_{pae}#2 was homozygous for a previously noted SNP, 58C > T (Leu20Phe), which shows that A_{pae} has arisen from heterozygosity for different alleles in the 2 families, with or without 58C > T but including 887G > A. A_{pae}#1 also had another

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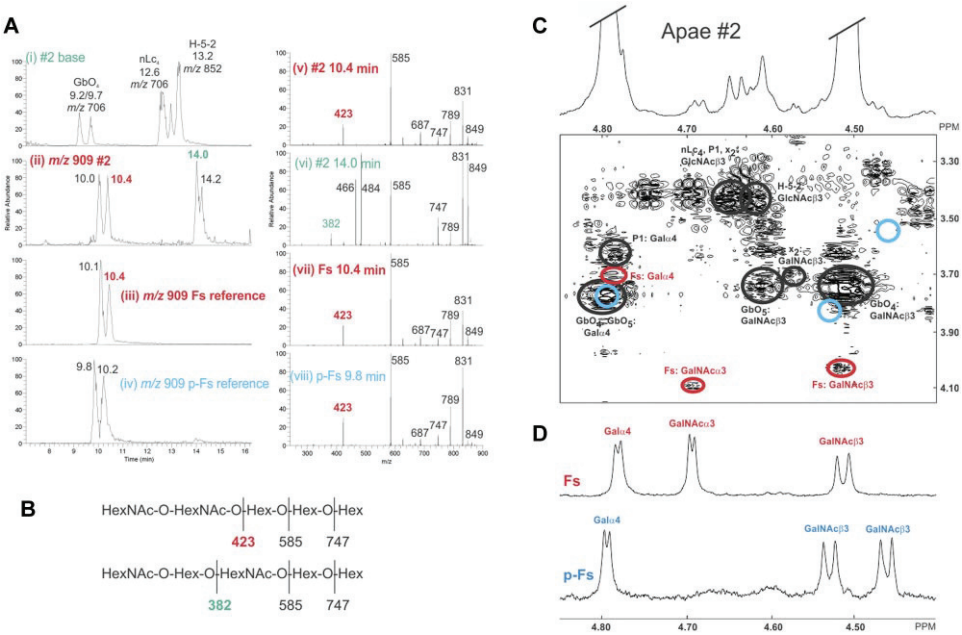

Figure 3. Structural analysis of A_{pae} glycolipids. Structural signatures indicative for the identification of Fs are indicated in red font. (A) LC-ESI/MS and MS-MS analysis of the oligosaccharides derived from fraction CM65:35 of A_{pae}#2 glycolipids (#2) and Fs and p-Fs references. (Ai) Base peak chromatogram from LC-ESI/MS, representing the main oligosaccharides in fraction CM65:35 of A_{pae}#2. (Aii) Mass chromatogram of oligosaccharides with *m/z* 909 (indicative for Fs, p-Fs, and x_2) in the same fraction as in panel Ai shows peaks at retention time 10.1 and10.4, corresponding to Fs. (Aiii) Reference Fs *m/z* 909 mass chromatogram, showing the diagnostic retention time at 10.1 and10.4. (Aiv) Reference p-Fs *m/z* 909 mass chromatogram with retention time 9.8 and 10.2. (Av) MS-MS spectrum of the ion at *m/z* 909; see (Aii) retention time 10.4 minutes. (Avi) MS-MS spectrum of *m/z* 909 (retention time 14.0 minutes) indicating a x_2 oligosaccharide. (Avii) Reference Fs MS-MS spectrum of *m/z* 909 (retention time 10.4 minutes). (Aviii) Reference p-Fs MS-MS spectrum of *m/z* 909 (retention time 9.8 minutes). (B) Interpretation MS-MS fragmentation formulas. (C) Low-field part of the 600-MHz proton NMR spectrum shown on top of the DQF-COSY spectrum, corresponding to the H1/H2 connectivities of the signature resonances for the structures present in the A_{pae}#2 CM65:35 fraction. The anomeric resonances from the 2 terminal residues of the dominating globoside structure (GbO_4) have been truncated at a height corresponding to approximately 20% of the total amplitude. In the COSY spectrum, connectivities belonging to expected structures (nLc_4, P1, H-5-2, GbO_5) have been outlined by black ellipses. H1/H2 connectivities of the 3 terminal residues (GalNAcα3GalNAcβ3Galα4−) of Fs are indicated with red ellipses. Also shown by blue circles is the absence in this fraction of the 3 terminal residues of p-Fs (GalNAcβ3GalNAcβ3Galα4−). (D) Reference NMR spectra of Fs and p-Fs glycolipids isolated from chicken and human erythrocytes, respectively.

SNP in exon 7, 363C > A, predicting a premature stop codon following residue 120 (supplemental Figure 2). Cloning and allele-specific amplification with subsequent sequencing verified that 363C > A and 887G > A are located on different alleles.

Nine non-A_{pae} individuals with different ABO phenotypes conformed to consensus, although 58C > T and 363C > A were detected in 5 and 1, respectively, of the 18 examined alleles. The prevalences of *GBGT1*-SNPs are given in supplemental Table 4.

Exon 7 of *GBGT1* in 6 different non-human primates was also sequenced, verifying that all had the human consensus 887G, as opposed to non-primates.[15,28,29]

PCR-ASP assays were designed to screen saliva DNA from members of the 2 A_{pae} families, and random blood donors for 363C > A and 887G > A (supplemental Figure 2, supplemental Table 4). The A_{pae} versus non-A_{pae} phenotypes defined in the 1987 paper[13] were concordant with the presence of 887G > A (supplemental Figures 3-4).

Transcript analysis. It is unknown whether Fs synthase is expressed during hematopoiesis, especially in erythroid cells, although *GBGT1*-mRNA has been detected in other tissues.[15]

We measured transcript levels in buffy coats from 4 random blood samples of different ABO groups (A_1/A_2/B/O) and the 2 A_{pae} blood samples. Transcripts were readily detected in mRNA preparations from all samples but no apparent quantitative differences were noted, suggesting that lack of Fs is not because of absence of mRNA. Transcript levels were ~ 4 × higher in erythroid cultured cells from bone marrow (supplemental Figure 5).

Transfection of MEG-01. Based on the hypothesis that 887G > A in *GBGT1* induces Fs synthesis in A_{pae} individuals, a Fs-negative megakaryoblastic cell line, MEG-01, was cotransfected with the genes encoding P synthase (*B3GALNT1*) and 2 allelic forms of Fs synthase (*GBGT1*). Mutant (887A) but not wild-type (887G) *GBGT1* resulted in a statistically significant increase in Fs-positive cells (Figure 5). This indicates that 887G > A (Arg296Gln) changes the inactive human Fs synthase to become enzymatically active.

Human Fs synthase model

The close homology (45% amino-acid identity) between *ABO* and *GBGT1* permitted creation of a 3-dimensional model of the Fs-synthesizing enzyme based on a crystal structure of ABO transferase.[30] The modeled structure of the putative human Fs synthase was generated by threading onto a human group B

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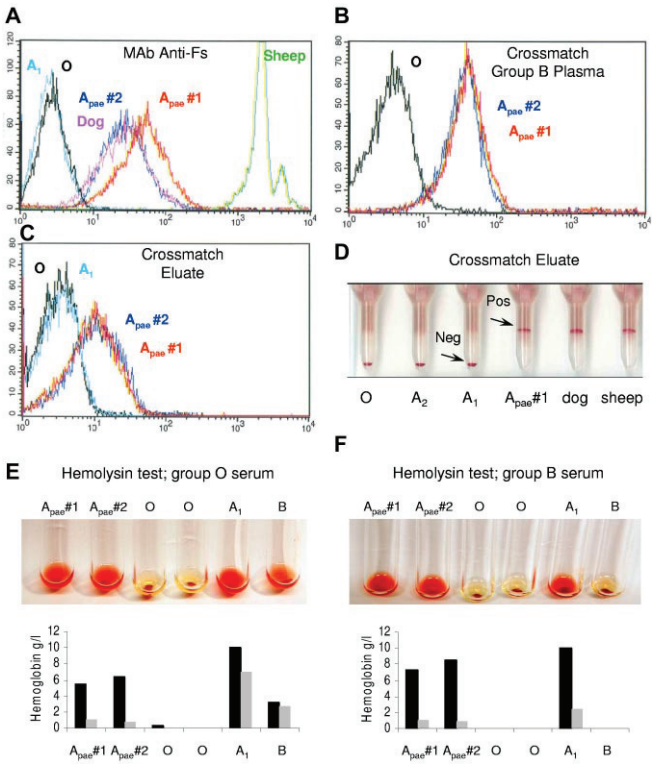

Figure 4. Flow cytometric and serologic testing of the 2 A_{pae} individuals. The y-axis displays the number of cells on a linear scale whereas the x-axis shows the fluorescence intensity on a logarithmic scale. Flow cytometry line color codes: Sheep RBCs (green), dog RBCs (purple), A_{pae} #1 (red), A_{pae} #2 (blue) group A_1 (light blue), and group O RBCs (black). (A) MAb anti-Fs with goat anti-rat IgM labeled with phycoerythrin as secondary antibody. Sheep RBCs were used as a strong positive control and human RBCs of various ABO groups as negative controls (only A_1 and O are shown for clarity). (B) Reactivity of plasma antibodies from a group B donor against A_{pae} RBCs and visualized with rabbit anti-human IgM labeled with FITC as secondary antibody. Group O RBCs were used as a negative control. Similar reactions could be obtained with O plasma (not shown). Because of the expected, strong agglutination of group A RBCs, that is the positive control, it was not possible to run the A RBCs on the flow cytometer hence they are not included in the histogram. (C) Reactivity of an eluate prepared from group B plasma antibodies adsorbed to and eluted from A_{pae}#2 RBCs and visualized with rabbit anti-human IgM FITC secondary antibody. Group A_1 and O RBCs were included as negative controls. (D) Gel column hemagglutination test between the eluate [prepared from A_{pae}#2 cells, also used in (C)] against RBCs of various phenotypes. Negative reactions were observed with the common human blood groups (A_1, A_2 and O), whereas positive reactions were seen with RBCs from the A_{pae}#1 individual and both dog and sheep RBCs known to express Fs antigen. (E-F) The hemolysin test was performed with 2 strongly A_{pae}-reactive sera. Lysis patterns for group O and B serum are displayed in panels E and F, respectively. Results for RBCs of A_{pae} and 3 common ABO phenotypes (A_1, B, and O) are shown. ABO-compatible and ABO-incompatible combinations serve as negative and positive controls, respectively. Incubations were performed with both native (gray) and papain-treated (black) RBCs. Diagrams show hemoglobin levels (g/L) in all the tubes tested, whereas the photos show the results with papainised cells.

glycosyltransferase (GTB) variant (PDB entry 2RJ7). The model also shows an overall similarity to the human group A glycosyltransferase (GTA) structure (PDB entry 1LZI), a 3-α-*N*-acetylgalactosaminyltransferase similar to Fs synthase (Figure 6A). Moreover, the catalytically important His301 in GTA maps to the same position as Arg296 in Fs synthase. UDP-Galactose (Gal) co-crystallized with the GTB structure can be easily superimposed into the binding pocket of modeled Fs synthase. In sharp contrast to His301 in GTA, none of the preferred side chain rotamers of Arg296 in the Fs synthase model seems well suited to create the critical hydrogen bond to the O6 of the donor-sugar moiety. Furthermore, assuming that UDP-*N*-acetylgalactosamine (GalNAc) binds in a similar way to Fs synthase as UDP-Gal binds to GTB, the GalNAc can be easily accommodated in the binding pocket (Figure 6B). One reason for this is that the corresponding position of Met-266 in GTB (important for UDP-Gal specificity by sterically preventing binding of UDP-GalNAc)[30] is glycine in Fs synthase (Gly261), which leaves ample space for GalNAc. The A_{pae}-associated Gln296 mutant is more probable to form a hydrogen bond with O6 of the donor substrate (Figure 6C), which could explain why the Gln296 enzyme gets activated.

E coli hemagglutination

P-fimbriae are well-known virulence factors of uropathogenic *E coli*.[31] The papG adhesin recognizes the terminal Galα4Gal

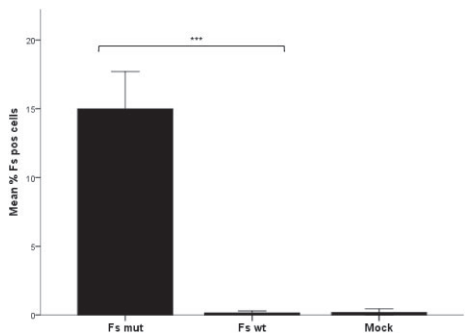

Figure 5. Expression of Fs antigen in transfected MEG-01 measured by flow cytometry. The bar graph shows the mean percentage of Fs-positive cells after 3 independent transfection experiments, each performed in triplicate with the open reading frame of *B3GALNT1* and either *GBGT1* 887A (Fs mut) or 887G (Fs WT). As a negative (background) control included in all 3 experiments, cells were transfected with the empty vector without insert (mock) and run in duplicate. The mean percentage of Fs-positive cells among the viable transfected (GFP-positive) cells is shown. Non-viable cells were disqualified by exclusion gating of cells positive for 7AAD. Error bars depict the standard error of the mean (SEM). The difference in Fs antigen expression after transfection was calculated to be statistically significant for the Fs mutant compared with the Fs wild-type cells and mock-treated cells according to the independent Kruskal-Wallis test. The significance level for the difference between wild-type and mutant is shown by the asterisks above the bars. Data were considered statistically significant with respect to the following criteria: $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

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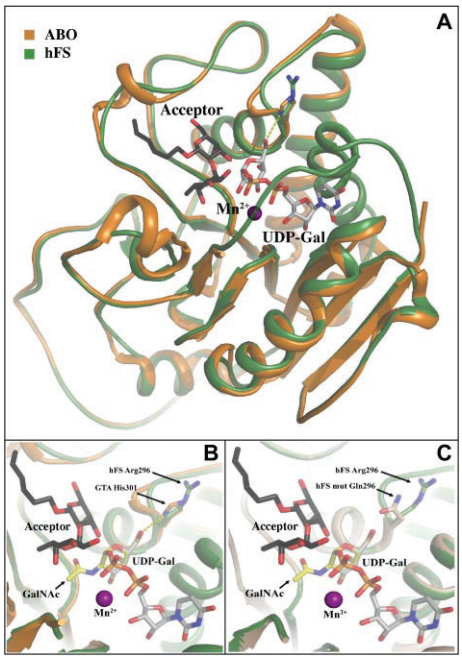

Figure 6. Superimposition of a model human Fs synthase threaded onto human ABO glycosyltransferase. This GTB (a GTB variant, PDB entry 2RJ7) contains UDP-Gal and an antigen acceptor derivative in the binding site. Furthermore, this enzyme structure has adopted a closed conformation where an internal loop (residue 180-200) and the C-terminus (residue 345-354) have folded over the active site. The threading and minimization was done using Moe and resulted in a model highly similar to the GTB structure. (A) The GTA structure (orange; PDB entry 1LZI) has been superimposed onto the human Fs synthase model (green), although this GTA structure has a disordered internal loop and C-terminus. UDP-Gal (gray) from the GTB mutant (PDB entry 2RJ7) was also superimposed onto the figure to highlight the probable location of the donor substrate. The catalytically important His301 in GTA/GTB is shown as sticks and potentially forms a hydrogen bond to the O6 position on the Gal of the UDP-Gal (yellow dashed line). In the human Fs synthase model the Arg296, which corresponds to and overlaps His301 in ABO transferase, is shown as sticks and has adopted a rotamer side chain conformation away from the Gal. In almost any other conformation, the Arg296 side chain would be clashing with either nearby residues in the enzyme itself or with the donor. The H-antigen acceptor from the GTB structure is shown as black sticks and the manganese ion as a purple sphere. (B) Close-up of the active site from panel A. A GalNAc molecule from the HIC-Up database (yellow) is superimposed onto the Gal without introducing any clashes to the molecule. Assuming the UDP-GalNAc binds in a similar way to the Fs synthase as UDP-Gal does to GTB, the acetamido group can be easily accommodated. One reason for this is that at the corresponding position of Met-266 in GTB (known to be important for UDP-Gal specificity by sterically preventing binding of UDP-GalNAc) there is a glycine in Fs synthase which leaves ample space for the acetamido group. Color scheme as described. (C) The threaded structure of the modeled human Fs synthase. Close-up of the active site showing the position of the Arg296 (green) versus the Gln296 mutant (light-brown) as it was modeled in Moe with the residue replacement introduced. It is evident that residue Gln296 in the mutant is more probable to form a hydrogen bond with O6 of the Gal which could explain why the enzyme goes from inactive to active when the mutant is introduced.

moiety of P^k and P1 blood group glycolipids but also the internal Galα4Gal of P (globoside) antigen. The prsG adhesin recognizes Galα4Gal-containing glycolipids less well but preferentially binds to the Fs structure terminating in GalNAcα3GalNAc.[32] The papG adhesin readily agglutinates all common human RBCs, although the prsG adhesin may crossreact weakly with A_1 (but not A_2/B/O) RBCs but binds strongly to sheep and dog RBCs which are Fs-positive. We tested the ability of *E coli* strain HB101, unmodified or expressing either the papG (HB101.AD110) or prsG (HB101.JFK102) adhesin to agglutinate RBCs. Table 1 shows that neither the parent (HB101) nor the prsG-expressing (HB101.JFK102) strain agglutinates A_1/A_2/O RBCs under these conditions, whereas the papG-expressing (HB101.AD110) strain agglutinates all tested RBCs as expected. Only A_{pae}-RBCs, sheep RBCs, and human group O RBCs containing synthetic FSL-Forssman constructs (Fs kodecytes), but not FSL-B (B kodecytes), were readily agglutinated by prsG-expressing (HB101.JFK102) bacteria.

Discussion

We report biochemical, serologic, and genetic data that show conclusively for the first time the expression of Fs antigen on human RBCs. Fs is better known for its expression on animal RBCs,[33] and has been implicated as a target for a variety of microorganisms and toxins.[34-37] The discovery that humans can also express Fs on their RBCs was a consequence of the immunochemical evaluation of glycolipids from individuals with the so-called ABO subgroup, A_{pae},[13] the terminology of which now needs to be revised.

Before 2012, there were 30 blood-group systems.[38] Six of these are carbohydrate-based and their genes code for glycosyltransferases. To define a new blood-group antigen, the International Society of Blood Transfusion (ISBT) requires that it be shown independent of all other blood-group antigens, be expressed on RBCs, and is inheritable. Furthermore, at least 1 individual with the corresponding antibody because of lack of the antigen, must be known (in this case naturally occurring anti-Fs is already known to exist).[37,39,40] We have proven the unambiguous existence of structurally defined Fs glycolipids on the surface of RBCs from 2 unrelated A_{pae} individuals and have excluded Fs in RBCs from others. Related and potentially crossreactive glycolipids, such as p-Fs, x_2 and blood-group A antigens, were excluded as causative. In addition to the phenotypic characterization, we linked this phenotype to a SNP in *GBGT1*, the human Fs synthase gene. The inheritance of Arg296Gln was associated with the Fs-positive phenotype in A_{pae} families. As our results satisfy the ISBT criteria, we proposed to designate this new system FORS, in honor of Prof John Forssman who a century ago discovered antibodies to this heterophile antigen by injecting extracts from guinea pig tissues into rabbits whose immune sera hemolyzed sheep RBCs.[6] In July 2012, Fs was recognized as a blood-group antigen by ISBT in the newly formed 31st blood-group system given the name FORS.

Previous studies have deemed the Fs synthase inactive because of mutations in its catalytic domain encoded by exon 7 of *GBGT1*.[15] The human protein differs from the canine enzyme by 58 amino-acids but can be reactivated by adding the canine C-terminus to the human N-terminus.[15] The consensus non-primate sequence corresponding to human residue 296 is Gln, whereas all primate and human (excluding A_{pae}) sequences code for Arg. *GBGT1* has been considered a human pseudogene, together with other homologous genes except *ABO* in the GT6 superfamily.[28] Interestingly, a recent study noted that *GBGT1* has not decayed as much as other related pseudogenes, and was therefore predicted to have retained some unknown function.[29] Although speculative, the apparently inactive human consensus form of *GBGT1*-encoded enzyme (with Arg296) may have as yet unrecognized enzymatic

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Table 1. Hemagglutinating ability of *E coli* displaying different fimbriae

	Reactions against RBCs								
Strain	A_1	A_2	O	O	$A_{pae\#1}$	$A_{pae\#2}$	Sheep	Fs kodecyte*	B kodecyte*
HB101†	0	0	0	0	0	0	0	0	0
HB101.AD110‡	++	++	++	++	++	++	+	++	++
HB101.JFK102§	0	0	0	0	++	++	+++	++	0

Grading according to standard blood bank practice, where ++++ is the strongest, +++, ++, and + are gradually weaker but positive reactions, and 0 is negative.
*Group O human RBCs modified with synthetic Fs and B FSL glycolipid-like KODE constructs.
†Parental strain.
‡Expressing papG adhesin.
§Expressing prsG adhesin.

activity. In contrast, the Gln296 variant described here has become effective at synthesizing Fs, in analogy with the enzyme in many non-primate mammals. The A_{pae}-associated substitution was not found in 256 blood donors but its frequency in other populations around the world remains to be established. In addition to the 3 originally reported families (2 of which were studied here and surprisingly found to have 2 different 887G > A-containing alleles), we noted another 887G > A-positive individual (phenotype unknown) in a database (supplemental Table 4).

To establish if Arg296Gln directly causes Fs synthesis, we expressed the wild-type and mutant forms in a hematopoietic cell line. After optimization of the experiment, including cotransfection of *B3GALNT1* (P synthase) to increase the number of precursors/acceptors for Fs synthase, a striking increase in Fs-positive cells (from background level) was noted with the A_{pae} construct. A possible mechanism behind the reactivation of the enzyme was suggested by the Fs synthase model based on the crystal structure of the homologous GTA/GTB.[30]

GBGT1 is transcribed in several human tissues but does not appear to result in Fs antigen expression.[15] However, Fs antigen may be expressed in carcinomas[41,42] and has been suggested to constitute the so-called incompatible A antigen found in non–group-A individuals with cancer.[42] We demonstrated here for the first time that *GBGT1*-mRNA is readily detectable in human blood and erythroid bone marrow cultures at levels similar to, if not higher than, *ABO*-mRNA.[43] This indicates that Fs synthase is probably expressed in erythroid progenitors and thus able to make Fs on RBCs if mutated Arg296Gln.

The carbohydrate histo-blood group systems in man are ancient and the biologic pressures that created and maintained them are still largely unknown.[44] The generally accepted belief is that these polymorphisms arose as a consequence of selective pressure exerted by microorganisms.[8,45] We investigated the possible biologic consequences of Fs expression on human cells and found that uropathogenic *E coli* containing a PrsG-encoding plasmid bound only to A_{pae} (and animal) cells. Thus, by acquiring an animal-associated *GBGT1* polymorphism, A_{pae} individuals express an antigen which protects against shiga toxin 1 (Stx1)[34] but risks making them more susceptible to Stx2e, a common variant of Stx2 in porcine feces, recently reported to bind Fs.[37] *E coli* strains capable of infecting the urinary tracts of canine and other Fs-expressing mammals adhere to uroepithelial cells via Fs-binding PrsG adhesins.[32,35] Expression of canine Fs synthase induces binding of canine *E coli* strains to human and monkey epithelial cell lines.[15] As suggested by hemagglutination, A_{pae} individuals could potentially be more prone to certain toxins and pathogens. However, it remains to be established whether Fs is also present in non-erythroid tissues and secretions of A_{pae} individuals, where it may constitute a biologically more relevant target. From these observations, we predict that Fs glycolipids will be expressed outside the hematopoietic compartment when Fs synthase is activated by Arg296Gln, on the basis that other globoseries glycolipids, such as P^k and P (the precursor of Fs), are expressed in uroepithelial cells. Furthermore, *GBGT1* is transcribed in most tissues investigated.[15]

This report provides an explanation for the enigmatic A_{pae} phenotype. Because A_{pae} is unrelated to the ABO system and its gene, the ISBT accepted that this old terminology should be abolished and replaced by FORS, a new system based on our findings. Accordingly, the (former) A_{pae} phenotype was redefined as FORS1-antigen positive and independent of ABO group, that is, not comparable with weak A subgroups. We identified a missense SNP that according to transfection data offers a plausible mechanism of reactivation of Fs-synthase activity, and allows for genetic screening of the phenotype. Even if our hemolysin results are limited in scope and mainly show an effect on papainized RBCs, it can be questioned if FORS1-positive individuals should be accepted as blood/organ donors. It cannot be excluded that anti-Fs, such as anti-A or anti-P, can be hemolytic in certain donor-recipient combinations and thereby constitute a threat to patient safety. If FORS1-positive units were to be transfused, we recommend that they are not given after electronic (type-and-screen) crossmatch but require a negative reaction between patient plasma and donor RBCs. However, it should be emphasized that the clinical consequences of naturally occurring anti-Fs are still unknown and require further studies. Taken together, these findings provide the genetic and structural bases for the new histo-blood group system FORS with possible implications for both transfusion and transplantation medicine as well as trans-species microbial susceptibility.

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Authorship

Contribution: A.H. and L.S. performed most experiments; R.S. provided and characterized samples; J.Å. and S.T. obtained and analyzed the structural data; J.R.S. designed and performed the bacterial binding experiments; R.J. made the molecular modeling experiments and figures; L.S., A.H., S.M.H., L.R., and M.L.O. designed the study and interpreted data; and L.S., A.H., S.M.H., and M.L.O. wrote the paper and the remaining coauthors reviewed, revised, and approved the paper.

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A. The rise in MFI is clearly seen for the O RBC/ A_1 secretor plasma mix. **b**. O RBC mixed with either O, B secretor or B non-secretor plasma incubated at 37°C and tested with anti-B. There was no obvious rise in MFI in comparison to the O plasma control. **c**. O RBC mixed with either O, A_1 secretor or A_1 non-secretor plasma incubated at 37°C tested on day five with anti-H. A small decrease in MFI was noticed for O RBC/A_1 secretor and A_1 non-secretor plasma mixes in comparison to the O plasma control.

Figure 3. Histograms and dot plots from flow cytometric testing of cell-cell mixes after 24 hour incubation at 37°C under constant mixing. A clear difference in MFI is seen between cell-cell mixes including secretor RBC versus non-secretor RBC. **a**. Group O RBC mixed with either A_1 secretor (solid red line) or A_1 non-secretor (dashed red line) RBC and tested with anti-A. **b**. Group O RBC mixed with either B secretor (solid red line) or B non-secretor (dashed red line) RBC and tested with anti-B. **c**. Flow cytometric testing of cell-cell mixes with either PBS or A non-secretor plasma added. Control RBC are included in each run. Different levels of A antigen were detected with anti-A in the different mixes.