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"Towards universal donor blood: enzymatic conversion of A and B to O type"

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ABSTRACT

Transfusion of blood, or more commonly red blood cells (RBCs), is integral to healthcare systems worldwide, but requires careful matching of blood types to avoid serious adverse consequences. Of the four main blood types, A, B, AB, and O, only O can be given to any patient. This universal donor O-type blood is crucial for emergency situations where time or resources for typing are limited, so it is often in short supply. A and B blood differ from the O type in the presence of an additional sugar antigen (GalNAc and Gal, respectively) on the core H antigen found on O-type RBCs. Thus, conversion of A, B, and AB RBCs to O-type RBCs should be achievable by removal of that sugar with an appropriate glycosidase. The first demonstration of a B-to-O conversion by Goldstein in 1982 required massive amounts of enzyme, but enabled proof-of-principle transfusions without adverse effects in humans. New α-galactosidases and α-*N*-acetylgalactosaminidases were identified by screening bacterial libraries in 2007, allowing improved conversion of B and the first useful conversions of A-type RBCs, although under constrained conditions. In 2019, screening of a metagenomic library derived from the feces of an AB donor enabled discovery of a significantly more efficient two-enzyme system, involving a GalNAc deacetylase and a galactosaminidase, for A conversion. This promising system works well both in standard conditions and in whole blood. We discuss remaining challenges and opportunities for use of such enzymes in blood conversion and organ transplantation.

Blood transfusion is an indispensable part of the health care system, saving many thousands of lives annually. To make this possible approximately 85 million units of packed red blood cells (RBCs) are obtained annually around the world[1], largely via donation. This requires a massive organization to secure supplies and yet, according to the World Health Organization, demand for RBCs is expected to grow further.[2] This growth is fueled in part by the demographically aging population and consequent rise in blood-intensive procedures such as solid organ transplants, hematopoietic

stem cell transplants, hemorrhage resuscitation, and aggressive chemotherapy against cancer. To counter this demand significant improvements have been made in the collection and management of blood-component supply, including reassessment of policies concerning when blood is needed and in what quantities. This has helped to reduce the usage of blood products, somewhat stabilizing supply vs demand. However, problems remain, especially given the challenge of correctly matching blood types on all occasions.

The human blood group system is complex: 30 discrete blood groups are known, defined by 270 antigens plus 38 that have not been assigned to a particular group [3]. These blood group antigens are based either on oligosaccharide epitopes (ABO, P and Lewis antigens) or on specific amino acid sequences of proteins (Rh, Kell and Duffy antigens). The majority of the antigens are integrated into the cell membrane but some, like the Lewis system, are plasma antigens that are adsorbed onto the red cell surface [4]. The A, B and H (O-type) carbohydrate antigens of the ABO blood group system are the most important clinically [3,5], with about 1 million antigens present on the surface of each red blood cell (RBC) [6]. Careful matching of the host and donor ABO blood types is essential in order to avoid transfusion incompatibility events, which are fatal in 10% of all cases [7]. This applies to transfusion of whole blood, RBCs or platelets as well as tissue or organ transplants, since ABO antigens are not only present on RBCs but also on most other tissues in the human body. The only exceptions are the so called non-secretors for whom a mutation in one of the fucosyl transferases leads to the absence of ABH antigens on secretory epithelial cells in the salivary glands, gastrointestinal tract and respiratory cavities [8].

Under unhurried circumstances transfusion is therefore performed with donor blood for which the antigens match those of the recipient as closely as possible. To confirm compatibility, cross-matching studies are performed in which small samples of donor and recipient blood are mixed and monitored for possible agglutination reactions that would indicate antigen/antibody interaction. In emergency situations "universal" O-type blood (preferably O negative) is employed since, as explained below, it is compatible with A, B, AB and of course O-type blood. Minor incompatibilities due to other antigen mismatches are not typically life-threatening. This review provides a brief background to blood antigens then describes attempts to convert A, B and AB blood to O type blood by enzymatic removal of their differentiating glycan antigens. Technologies developed must be compatible with current procedures for collection, storage and handling of blood. Typically, after collection in sterile bags blood is stored at room temperature for 24 hours then cooled to 4°C and can be stored for up to 42 days. Most blood is separated into its plasma, red blood cell and platelet components early in the process, and most commonly transfusion is performed with red blood cells rather than whole blood.

In this report we review methods that have been employed to identify enzymes that work efficiently at the neutral pH conditions required by RBCs, culminating in the recent identification of a highly efficient enzyme pair from the human gut microbiome. For excellent reviews providing more detail on the earlier stages of this path of discovery we refer the reader to the following publications: Olsson 2004 [9], Olsson and Clausen 2008 [10] and Garratty 2008 [11].

The ABO blood group system defined

The base structure of the ABO blood group system is the fucosyl galactose H antigen of O-type blood (Figure 1a), which is attached to glycoproteins and lipids on the surface of RBCs and other tissues. The A- and B- antigens differ from this H-antigen through the addition of an extra sugar moiety, this being α-N-acetylgalactosamine for A-type or α-galactose for B-type RBCs and both in the case of AB-type (Figure 1a). Individuals with A-type blood harbor Anti-B antibodies, with the result that transfusion

of an A individual with B-type RBCs would lead to agglutination of the RBCs and subsequent hemolysis. Likewise, B type blood contains A antibodies thus the complementary transfusion may not be performed. People with O type blood harbor both anti-A and anti-B antibodies, thus may receive only O type blood.

O-type RBCs have a special status, since the H antigens on their surface are not recognized by the Anti-A or Anti-B antibodies and no specific H (O) antibody is formed, likely because A and B antigens are synthesized via the H-antigen, which is thus recognized as "self". Consequently, O type RBCs can be used as universal donor for any other ABO blood type, thus are in great demand (Figure 1b). As can be seen in Table 1 the distribution of blood types varies with race demographic. O type is the most common in all cases, followed by A, then B, with only small numbers of AB. The second most clinically important antigen is the Rh (D) factor, which is a transmembrane protein and renders the individual Rh positive (Rh+)[3,13,14]. The vast majority of people are Rh Positive, with only 18 % of Caucasians being Rh negative, and other racial groups even less, as shown in Table 1[15]. Rh negative (Rh-) patients may produce antibodies to the Rh protein, in which case they cannot receive blood from Rh positive individuals. This is a particular concern during childbirth if the fetus is Rh positive and the mother Rh negative since, should fetal blood enter the maternal bloodstream it would induce anti Rh antibodies. While these are not a problem at that time, this could be an issue during a subsequent pregnancy if the fetus is Rh+. In that case maternal antibodies could cross the placenta and destroy fetal RBCs. Second pregnancies after an initial mismatch are thus especially closely watched by measurement of anti-Rhesus antibody levels. If problems are suspected an injection of Rh immune globulin is provided at 28 weeks to suppress Rh antibody formation. Some 1-8% (O-) of the population in the U.S. are thus "true" universal donors while 37-53% (O+) are universal donors within the (very large) Rh (D) positive group. Consequently, O type blood is always in high demand, especially in emergency situations where there is no time for blood group typing or just no possibility to perform it. Notably, in emergency settings in Canada at least, Rhesus positive units are transfused to all recipients except women of childbearing age, irrespective of the Rhesus status of the recipient. Thus, even though it is the largest of the blood groups within the worldwide population there is a constant shortage of O-type blood.

Subtypes of ABH antigens and their biosynthesis

The ABH antigens of the ABO blood group are composed of carbohydrate chains bound either to glycolipids (approximately 10%) or glycoproteins (approximately 90%). These antigens exist as a number of sub-types that differ in internal linkages within the linking oligosaccharide, as shown in Figure 2. Interestingly expression of these antigens on tissues can be organ-specific, with important implications for organ transplantation[16,17].

These glycans are built up from two main groups of carbohydrates. Lactotriaosylceramide (GlcNAc-β-3-Gal-β-4-Glc-β-Cer) forms the core structure of the Type 1, 2 and 3 antigen linkage types [18–21]. Transfer to this of galactose in a β-3 linkage or β-4 linkage followed by fucose in an α-2 linkage to the galactose yields the Type 1 and Type 2 H antigens respectively. From these the A and B antigens are derived through the transfer of N-acetylgalactosamine or galactose by the A-transferase (GTA) or B-transferase (GTB) respectively[22]. Further extension of the Type 2 A antigen by transfer of galactose and fucose produces the Type 3 H antigen, which can be decorated again with N-acetylgalactosamine, yielding the Type 3 A antigen, which is also called repetitive A, due to the repetition of the trisaccharide structure[21]. Around 50% of the A Type 2 antigens synthesized on glycolipids are modified further to these Type 3 Antigens [21].

The other core structure is that of globotriaosylceramide (Gal-α-1,4-Gal-β-1,4-Glc-β -Cer, Gb3 or Pk), from which the Type 4 antigen linkage is built [20]. Initial addition of β-3-GalNAc yields the P antigen; further addition of galactose and fucose produces the Type 4 H

antigen, from which the Type 4 A antigen is derived by transfer of N-acetylgalactosamine [23] (Figure 2).

The 2-α-fucosyltransferases (FUT) play key roles in defining which tissues within the body present ABO blood group antigens. FUT1, which produces the Type 2 and Type 4 H antigens, is the most important in terms of RBC-based antigen production [24,25] while FUT2 is specifically required for formation of the Type 1 and Type 3 H antigens. This latter enzyme is inactive in 20% of the Caucasian population, leading to the previously mentioned non-secretor phenotype, in which most non-secretors no longer present the ABH antigens[8]. Loss of FUT1 and FUT2 leads to the rare Bombay phenotype, which is characterized by an absence of H antigens on RBCs. Bombay patients can only receive autologous transfusions or blood from another Bombay blood group individual. FUT3 to FUT7 are responsible for the production of Lewis antigens by addition of α-1,4- or α-1,3- fucose to N-acetylglucosamine/galactosamine residues of the appropriate precursor. The presence of the ABO blood antigens and their different linkage types will vary between different tissues in the body [26].

A Blood types are further classified as A_1 or A_2 based on the density of the antigens on the RBC surface and specifically upon the presence of the Type 3A repetitive structure on A_2 RBCs. This difference is rooted in GTA enzymes that have higher activity in A_1 RBCs than in A_2. As a consequence, in the more prevalent A_1 RBCs more H-antigens are modified and each of the Type 1, 2, 3 and 4 subtypes is formed. By contrast in Type A_2 RBCs most of the H antigens are unmodified, and only Type 2 antigens are formed at significant levels, with very little formation of Type 1 and 4 linkages and no formation of Type 3. Indeed, experimental distinction of A_1 from A_2 RBCs is based on antibodies to the Type 3 structure. Other A and B sub-groups are known but are not as common as A_2.

Enzymatic blood type conversion – first generation

The finding that the only apparent difference between A, B and O RBCs resides in the presence or absence of either GalNAc or Gal on the terminal H-antigen raised the possibility that A and B RBCs could be converted to O RBCs if that sugar could be selectively removed (Figure 3). Such enzyme- converted O RBCs (ECO-RBCs) might then be usable as universal donor blood in place of normal O type blood, though this procedure would clearly not affect the RBC Rh status. Accordingly, conversion of A+ and B+ RBCs would yield O+ while A- and B- RBCs would yield O-.

The first full reports demonstrating the feasibility of such enzymatic conversion were published by Goldstein and colleagues[27,28] in the early 1980's, based upon preliminary findings by Sharon and his group[29]. Goldstein's group focused upon the conversion of B RBCs rather than A, primarily because at that time the only commercially available enzyme that might be applicable to such a task was the α-galactosidase from green coffee beans. The low pH optimum of this enzyme required that conversion be performed under sub-optimal conditions for RBCs (pH 5.7) and using large amounts of enzyme. Nonetheless full conversion to the H-antigen was confirmed and RBC structure and viability were established. After demonstrating that converted B-erythrocytes from gibbons could be safely transfused back into the donor gibbons and that the transfused erythrocytes enjoyed normal circulation times, the team moved into a small human trial with three patients of blood type A, B and O. RBCs from the B type donor were converted then, after washing to remove enzyme, 5 mL samples of packed ECO-RBCs were injected into volunteers. Again, the ECO-RBCs were shown to exhibit a normal half-life and to be well tolerated. This same group followed up on these studies in the early 1990s, showing that, initially, a full unit (200 mL) of such ECO-RBCs and ultimately 2 or 3 units could be transfused into A and O type patients with no ill effects and normal RBC circulatory survival times. It was however noted that higher galactosidase levels (2 g per packed RBC bag (6 mg/ml for 80%

hematocrit)) were needed for samples transfused into O than A volunteers in order to avoid a small increase in anti-B titre, though the reason why was not clear[30–32].

A larger, Phase 2 clinical trial carried out in 2000 by a second group came to similar conclusions [33]. Using a recombinant form of the coffee bean enzyme to generate their ECO-RBCs 21 patients were given ECO-RBCs and no adverse events were recorded, even though small increases in B-titre were again seen with some patients. Cross-matching studies, wherein ECO-RBCs were mixed with sera from group A and group O patients, resulted in some level of agglutination in 20% of serum samples from A patients and 40% from O, even though the ECO-RBCs could not be agglutinated by murine anti-A and anti-B. While somewhat perplexing, this would appear not to be clinically significant given the lack of adverse effects upon transfusion. The study concluded that, pending the development of suitable enzymes for conversion of A type blood, enzymatic conversion could indeed be used to create a universal (Group O) donor blood supply. However, it was evident that enzymes of improved pH optimum and activity were needed. Further research was done in an attempt to improve the efficiency of the enzymes. Here a key player was ZymeQuest (now Velico Medical) who developed a kinetically superior soy bean alpha-galactosidase[34]. This enzyme was able to work at a higher pH of 5.8 and the amount used per packed RBC bag was reduced to 0.5 g [9]. However, this was still not suitable for serious usage. Further, given the relatively small percentage of the North American and European population of B blood type it was clear that such conversion approaches would not be viable until efficient A-cleaving enzymes were identified.

Towards A-cleaving glycosidases and better B-antigen cleavers

Success in the production of B ECO RBCs, inspired the search for enzymes that can convert the A antigen to O. In considering other glycosidases that might be useful in blood type conversion it is useful to review the broader classification of such enzymes. Glycoside hydrolases have been grouped into over 160 sequence-related families within the Carbohydrate-Active enZYmes (CAZy) database[35]. Since sequence dictates structure, mechanism and ultimately substrate specificity this database, along with the sister CAZYPEDIA site (www.cazypedia.org) has provided an extremely valuable organising principle to glycoenzymology. The coffee bean α-galactosidase belongs to CAZy family GH27, a grouping of bacterial and eukaryotic α-galactosidases and α-N-acetylgalactosaminidases, including the human lysosomal enzymes whose deficiency leads to the Fabry and Schindler lysosomal storage disorders. The low pH optimum of the coffee bean enzymes is perhaps thus unsurprising. Another family of closely related α-galactosaminidases and α-galactosidases is that of GH36: enzymes from these two families have in common a catalytic domain with an $(\alpha/\beta)_8$ fold, along with an associated beta sheet domain.

Early attempts to identify suitable alpha-N-acetylgalactosaminidases focused on the bacterium *Clostridium perfringens*, which had earlier been identified as an "A-destroying" organism [36–39]. The enzyme responsible for this activity was identified (and later shown to be a member of CAZy family GH36), but while it was able to convert the A antigens to H antigens on A_2 RBC membrane fragments, it was not active on intact RBCs so was not useful. Around the same time the company ZymeQuest started to use a GH27 α-N-acetylgalactosaminidase from chicken liver to convert the A antigens [34]. However, it was only able to fully convert A_2 RBCs; conversion of A_1 RBCs, with their much higher antigen content, was incomplete. Further its optimal pH range was not really compatible with RBCs (3.8-5.7), meaning that up to 3 g enzyme per packed RBC unit was needed [40]. Other α-N-acetylgalactosaminidases from different sources were found in the next few

years[41–43], but unfortunately none showed full conversion activity on A_1 RBCs [41–43].

Enzymatic blood type conversion – 2nd generation

A key publication describing the discovery of new enzymes for blood type conversion was that of Liu et al. in 2007 [44]. TLC-based screening of a library of some 2500 fungal and bacterial lysates using fluorescently tagged tetrasaccharides that represent the A and B antigens identified new groups of α-N-acetylgalactosaminidases and α-galactosidases that function at pH 7. The α-N-acetylgalactosaminidase activity was similar to that of the *Elizabethkingia meningosepticum* α-N-acetylgalactosaminidase that had been identified by similar means previously[45]. BLAST analysis then identified a family of enzymes that were subsequently assigned to GH109 and shown to hydrolyse their substrates with net retention of anomeric configuration. Members of this family use an NAD cofactor and cleave the glycosidic bond through an unusual mechanism involving transient redox-assisted elimination and addition steps, as had first been shown for members of GH4 [46]. A three-dimensional structure of the enzyme confirmed the location of the NAD cofactor near H3 of the substrate, and the absence of any metal ion requirement. It also provided structural insight into the strict specificity of the enzyme for A-antigens and its broad specificity therein for all A subtypes. They also discovered a new family of inverting α-galactosidases that cleave the B-antigen efficiently at pH 7, and assigned these enzymes to GH110. A sub-family (GH110B) that also cleaves the linear B-antigen (missing the fucose) was reported in the following year[47], but at present no three dimensional structures are available to explain these findings.

The *E. meningosepticum* enzyme was indeed shown to cleave A-antigens from red blood cells, but only if buffers of low ionic strength were employed. This behavior was attributed to the need for the enzyme, which is predominantly cationic around the active site, to interact with the net negatively charged red blood cell surface[48]. Under such conditions they were able to use as little as 300 ug mL^{-1} of enzyme to fully convert A red blood cells to O as measured using standard typing agents. This corresponds to approximately 60 mg enzyme per unit of RBCs. Conversion of B type RBCs by the alpha-galactosidases was considerably more efficient. Using the GH110A enzyme from *B. fragilis* in the same low ionic strength buffer a full unit of B type RBCs could be converted using only 2 mg of enzyme. Indeed, by combining the two enzymes they were also able to convert Type AB RBCs. The conditions employed were further studied by other groups, who showed that a standard low ionic strength glucose-containing buffer was equally good for use in conversion of A, B and AB RBCs, but that the enzymes were not stable to storage at low ionic strength [49,50]. Considerably improved cleavage performance at higher ionic strengths could be attained by inclusion of molecular crowders such as dextrans in the reaction mixtures [51]. These polymers effectively increase the enzyme concentration by decreasing the reaction volume available to them, thereby bringing them closer to the RBC surface. Given their long history as blood expanders that are used to maintain plasma volume in emergency situations, dextrans can probably be used safely in this manner.

Inspired by these studies our laboratory explored the potential of a class of bacterial endo-□-galactosidase that cleaves the entire A or B trisaccharide off red blood cells in the hope that a single enzyme could be used for conversion of both blood types. These GH98 enzymes, discovered by the Li group, had been shown to cleave the dominant β-1,4-linked Type 2 antigens effectively, but to have relatively low activities on the β-1,3-linkages of other subtypes [52]. By use of iterative steps of directed evolution, guided by available crystal structures, we were able to increase the activity of a GH98 enzyme from *Streptococcus pneumonia* some 170 fold in cleaving Type 1 linkages without significant loss

of Type 2 cleavage activity [53]. Crucial to our success was our development of an efficient coupled assay in which the methylumbelliferyl glycoside of the Type A1 pentasaccharide, synthesized chemoenzymatically, was employed. Cleavage of the terminal α-GalNAc residue exposed the oligosaccharide to sequential degradation by the α-fucosidase, exo-β-galactosidase and β-hexosaminidase that were included in the assay mixture, resulting in release of the fluorescent methylumbelliferone. Overall this provided an important demonstration of the potential for improving the activities of these enzymes [53,54]. However, the reality that this process would have to be repeated to create good enzymes for Type 3 and 4 linkages, and the concern that the presence of a terminal GlcNAc residue could lead to RBC clearance led us to reconsider the wisdom of this specific approach. This was especially true in the light of new opportunities presented by our development of high-throughput screens suitable for metagenomic analyses.

Enzymatic blood type conversion – 3rd generation

The advent of functional metagenomic analysis has opened up new opportunities for discovery of novel enzymes since it allows the interrogation of the genomic repertoire of potentially all microorganisms within an environmental sample, not just those that can be cultured [55]. By extracting all the DNA from the sample then expressing fragments thereof within a host organism such as *Escherichia coli* a library is generated that can be assayed for the desired activity. Crucial to this approach, which can be performed with either small (3 - 10 kB) or large (30 - 40 kB) DNA inserts, is the availability of a suitable high throughput screen.

So armed, we first considered what environments might harbour organisms that degrade A and B antigens. After rejecting ideas based upon parasites such as mosquitoes or leeches that feed on human blood (since only primates have the ABO system) we settled on the human gut microbiome. The mucins that line the gut wall display many sugar structures on their termini, including the ABH antigens[56]. Since gut bacteria are known to forage upon these mucin glycans it seemed probable that some of the bacteria therein would produce glycosidases that could cleave the A and B antigens. Based on this, and upon a study showing a correlation of human microbiome content with blood type [57] we generated a large insert metagenomic library from a donor of AB blood type. Screening of these 20,000 clones identified several new GH109 enzymes as well as a GH36 α-N-acetylgalactosaminidase, though none of these exhibited superior properties to those of the *Em*GH109 previously found [44] . However, one other particularly active clone contained a pair of genes encoding two enzymes that work in concert to cleave the A antigen. The first of these is a metallo-GalNAc deacetylase (DeAc) that is highly specific for the A-antigen, but works on all subtypes of A, generating a galactosamine (GalN) at the terminus in place of GalNAc. Crystallographic analysis of a product analogue complex revealed the metal-containing active site as well as a set of hydrogen bonding interactions with the fucose moiety that explain the specificity for A antigen (Figure 4). Its C-terminal carbohydrate binding module binds tightly to LacNAc structures, consistent with its docking to the RBC cell wall while acting on the A antigen. The second enzyme is a GH36 α-galactosaminidase that efficiently cleaves the monosaccharide from the terminus, generating the H-antigen [58] (Figure 3 and Figure 4). This latter enzyme is much less specific, but since no other GalN is likely present on the cell surface this is not likely to be a problem. Indeed, testing of this enzyme on O and B RBCs revealed no release of GalN. These two enzymes, working together, cleave the A-antigen very efficiently, requiring some 15-30 fold less enzyme than the previous best (EmGH109) and doing so in standard buffers without the need for molecular crowders, though their addition further enhances activity. Indeed, they work particularly well in whole blood, both at room temperature or at 4°C

and can be removed from RBCs by simple washing during the centrifugation steps that are employed in normal RBC processing. As little as 1 mg per unit of RBCs may be needed for conversion. These properties should facilitate the use of these enzymes within the current structure for blood processing with minimal disruption. Interestingly good evidence for the probable existence of such GalNAc deacetylases that work on A type RBCs was already present in the medical and forensic science literature. Some sepsis patients had been observed to undergo a transient change in their blood type from A to B until the infection cleared up, when their A blood type returned. Kabat had in fact suggested that this process, known as the Acquired B phenomenon [59], might be caused by a bacterial deacetylase generating a galactosamine moiety that was recognised as galactose by the polyclonal antibodies in use at that time, though no enzyme was purified. Similar phenomena of variable blood type had also been seen during attempts to type body parts recovered from the River Thames, again presumably due to river-borne bacteria [60].

Current status and future prospects for enzymatically converted RBCs

Despite the early success of converting B to O type RBCs and their subsequent transfusion into A and O type patients [33] without adverse effects the technology has not yet moved into clinical practice. One hold-up has been the efficiency and efficacy of the A-cleaving enzymes available. This was in part alleviated by ZymeQuest's development of the GH109 enzymes, although 15-60 mg of enzyme per unit of RBCs was still required. The other problem, noted earlier, is the low level agglutination observed when ECO converted RBCs are cross-matched with serum from Type A (in 20% of cases) or Type O (in 40% of cases) serum from other patients. The cause of this has not been determined, but it could arise from low levels of residual B-antigen caused by incomplete cleavage. Similar problems have also been seen after cleavage of the A-antigen [9] and, interestingly, agglutination was more frequent when using serum drawn from patients with previous infections[61]. Again, the cause of this cross-matching agglutination, which does not seem to result in hemolysis, is not clear. Importantly, however, the presence of small amounts of residual A antigen does not appear to be an issue for transfusion since it is known that some Type B individuals also express a low level of A antigen due to their B-galactosyl transferase having a loose specificity. Blood from such individuals has been used for transfusions to other B patients for years with no issue. As a consequence blood typing reagent manufacturers now adjust antibody concentrations to avoid detecting this phenomenon and unnecessarily rejecting blood [33]. Importantly the residual A antigen site density of A-ECO-RBCs that were converted by GH109 enzymes is lower than that present on these B-type RBCs, and indeed the low levels of A antigen on these B type RBCs can be removed by treatment with GH109 enzymes [10]. Consequently, it is likely that the ECO A type RBCs are indeed safe for transfusion.

Another suggested explanation revolves around the way in which Type 3 chain A antigens are cleaved by an exo-GalNAcase since only the terminal GalNAc would be cleaved, not the internal ones (Figure 2). This generates an H-antigen capping an internal A-antigen. If the internal GalNAc residue is recognised by anti-A or other antibodies, this could result in the agglutination observed [11,61–64]. A further possibility is that removal of the A or B antigens from the surface might lead to a rearrangement of other clustered antigens in the vicinity, exposing antigens that were previously hidden. While there is no direct evidence for this, changes in glycan clustering around the ABH antigens upon their enzymatic removal was shown to differentially affect the recognition of cell surface sialic acids by cognate receptors, most likely through altered clustering [65]. The only clinical trials reported on A-ECO RBCs are Phase 1 trials conducted by ZymeQuest in 2005 wherein small volumes of A-ECO RBCs were re-infused into the original

donor with no ill effects (https://www.clinicaltrials.gov #NCT00261274) [10]. Clearly further trials are needed.

As we move forward a primary concern is clearly to understand the basis of these agglutinations during cross matching and to evaluate whether these are of clinical significance. This most likely requires identification of the antibodies causing such agglutination and their specificity while also investigating the possible causes suggested above. Some clarity on these issues would greatly improve prospects of moving into further clinical trials given the high activity of the newest enzymes and consequent low enzyme loadings needed. Prospects of removal of the Rh antigen are more limited, given that it is associated with a transmembrane protein. However, the much more invasive antigen masking approach using cell surface polymers is one method that has been proposed[66].

Meanwhile another possible application of these enzymes is in the area of organ transplantation. Of great importance among the antigens that need to be matched in identifying suitable organs for transplantation to an individual patient are the ABO antigens. Removing the A/B antigen enzymatically may seem impractical at first since the organ will regenerate the cleaved antigen. However, the greatest challenge with incompatible transplants is during the first few days after transplantation. If the immune response during this time can be reduced substantially then success rates are expected to improve. Indeed, when wait times have become dangerously long kidney transplantations across the ABO barrier are already performed at some centres. Typically plasmapheresis is performed on the recipient to remove antibodies, along with immunosuppression and possibly splenectomy [67,68]. Removal of the A/B antigens from the donor organ prior to transplant would greatly reduce the need for immunosuppression and possibly plasmaphoresis. Several papers relate the use of glycosidases to remove tissue surface antigens from whole organs during perfusion [67,69,70], but studies have not progressed beyond that point. The advent of improved enzymes and improved perfusion procedures give new life to this field.

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CONFLICTS OF INTEREST

The authors are named inventors on a patent submission by the University of British Columbia regarding enzymes that may be used for removal of A antigens from red blood cells. They have no other conflicts of interest.

REFERENCES

1. American Red Cross Blood Facts and Statistics. 18–20 (2012). Available at: https://www.redcrossblood.org/learnabout-blood/blood-facts-and-statistics.
2. Laxmi, V. The Global Blood Industry. *BCC Res. Rep.* (2017).
3. Daniels, G. & Reid, M. E. Blood groups: the past 50 years. *Transfusion* **50**, 281–289 (2010).

4. Quraishy, N. & Sapatnekar, S. Advances in Blood Typing. *Adv. Clin. Chem.* **77**, 221–269 (2016).
5. Durand, J. K. & Willis, M. S. Karl Landsteiner, MD. *Lab. Med.* **41**, 53–55 (2010).
6. Economidou, J., Hughes-Jones, N. C. & Gardner, B. Quantitative measurements concerning A and B antigen sites. *Vox Sang.* **12**, 321–328 (1967).
7. Simmons, D. P. & Savage, W. J. Hemolysis from ABO Incompatibility. *Hematol. Oncol. Clin. North Am.* **29**, 429–443 (2015).
8. Kelly, R. J., Rouquier, S., Giorgi, D., Lennon, G. G. & Lowe, J. B. Sequence and expression of a candidate for the human Secretor blood group alpha(1,2)fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. *J. Biol. Chem.* **270**, 4640–9 (1995).
9. Olsson, M. L. *et al.* Universal red blood cells—enzymatic conversion of blood group A and B antigens. *Transfus. Clin. Biol.* **11**, 33–39 (2004).
10. Olsson, M. L. & Clausen, H. Modifying the red cell surface: towards an ABO-universal blood supply. *Br. J. Haematol.* **140**, 3–12 (2008).
11. Garratty, G. Modulating the red cell membrane to produce universal/stealth donor red cells suitable for transfusion. *Vox Sang.* **94**, 87–95 (2008).
12. Varki, A. *et al.* Symbol Nomenclature for Graphical Representations of Glycans. *Glycobiology* **25**, 1323–1324 (2015).
13. Landsteiner, K. & Wiener, A. S. An Agglutinable Factor in Human Blood Recognized by Immune Sera for Rhesus Blood. *Exp. Biol. Med.* **43**, 223–223 (1940).
14. Rosenfield, R. E., Allen, F. H. & Rubinstein, P. Genetic Model for the Rh Blood-Group System. *Proc. Natl. Acad. Sci. U. S. A.* **70**, 1303–1307 (1973).
15. Weinstock, C. It is worthwhile filling in the remaining blank spots for blood group antigen frequencies. *Blood Transfus.* **12**, 3–6 (2014).
16. Jeyakanthan, M. *et al.* Chemical Basis for Qualitative and Quantitative Differences Between ABO Blood Groups and Subgroups: Implications for Organ Transplantation. *Am. J. Transplant.* **15**, 2602–2615 (2015).
17. Jeyakanthan, M. *et al.* ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation. *Am. J. Transplant.* **16**, 1548–1558 (2016).
18. Rege, V. P., Painter, T. J., Watkins, W. M. & Morgan, W. T. Three new trisaccharides obtained from human blood-group A, B, H and Lea substances: Possible sugar sequences in the carbohydrate chains. *Nature* **200**, 532–4 (1963).
19. Donald, A. S. R. A-Active Trisaccharides Isolated from A1 and A2 Blood-Group-Specific Glycoproteins. *Eur. J. Biochem.* **120**, 243–249 (1981).
20. Bremer, E. G. *et al.* Characterization of a glycosphingolipid antigen defined by the monoclonal antibody MBr1 expressed in normal and neoplastic epithelial cells of human mammary gland. *J. Biol. Chem.* **259**, 14773–14777 (1984).
21. Clausen, H., Levery, S. B., Nudelman, E., Tsuchiya, S. & Hakomori, S. Repetitive A epitope (type 3 chain A) defined by blood group A1-specific monoclonal antibody TH-1: chemical basis of qualitative A1 and A2 distinction. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1199–203 (1985).
22. Hakomori, S., Stellner, K. & Watanabe, K. Four antigenic variants of blood group A glycolipid: Examples of highly complex, branched chain glycolipid of animal cell membrane. *Biochem. Biophys. Res. Commun.* **49**, 1061–1068 (1972).
23. Clausen, H. *et al.* Blood group a glycolipid (AX) with globo-series structure which is specific for blood group A1 erythrocytes: One of the chemical bases for A1 and A2 distinction. *Biochem. Biophys. Res. Commun.* **124**, 523–529 (1984).
24. Marionneau, S. *et al.* ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. *Biochimie* **83**, 565–573 (2001).
25. Mollicone, R., Cailleau, A. & Oriol, R. Molecular genetics of H, Se, Lewis and other fucosyltransferase genes. *Transfus. Clin. Biol.* **2**, 235–242 (1995).

26. Rydberg, L., Skogsberg, U. & Mölne, J. ABO antigen expression in graft tissue: is titration against donor erythrocytes relevant? *Transplantation* **84**, S10-2 (2007).
27. Lenny, L, Goldstein, J. Enzymatic removal of blood group-B antigen from gibbon erythrocytes. *Transfus. (Philadelphia, Pa.)* **20**, 5 (1980).
28. Goldstein, J., Siviglia, G., Hurst, R., Lenny, L. & Reich, L. Group-B Erythrocytes Enzymatically Converted to Group-O Survive Normally in A, B, and O Individuals. *Science* **215**, 168–170 (1982).
29. Harpaz, N., Flowers, H. M. & Sharon, N. Studies on B-antigenic sites of human erythrocytes by use of coffee bean α-galactosidase. *Arch. Biochem. Biophys.* **170**, 676–683 (1975).
30. Lenny, L. L., Hurst, R., Goldstein, J., Benjamin, L. J. & Jones, R. L. Single-unit transfusions of RBC enzymatically converted from group B to group O to A and O normal volunteers. *Blood* **77**, 1383–8 (1991).
31. Lenny, L., Hurst, R., Goldstein, J. & Galbraith, R. Transfusions to group O subjects of 2 units of red cells enzymatically converted from group B to group O. *Transfusion* **34**, 209–214 (1994).
32. Lenny, L., Hurst, R., Zhu, A., Goldstein, J. & Galbraith, R. Multiple-unit and second transfusions of red cells enzymatically converted from group B to group O: report on the end of phase 1 trials. *Transfusion* **35**, 899–902 (1995).
33. Kruskall, M. S. *et al.* Transfusion to blood group A and O patients of group B RBCs that have been enzymatically converted to group O. *Transfusion* **40**, 1290–1298 (2000).
34. Clausen, H., De, L. V. H., Hill, C. & Liu, Q. P. Enzymatic conversion of blood group A, B, and AB red blood cells using alpha-N-acetylgalactosaminidases and alpha-galactosidases with unique substrate specificities and kinetic properties. European Patent 1436387A2. (2004).
35. Lombard, V., Ramulu, H. G., Drula, E., Coutinho, P. M. & Henrissat, B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* **42**, D490–D495 (2014).
36. Levy, G. N. & Aminoff, D. Purification and properties of alpha-N-acetylgalactosaminidase from Clostridium perfringens. *J. Biol. Chem.* **255**, 11737–42 (1980).
37. Hsieh, H.-Y., Mitra, M., Wells, D. C. & Smith, D. Purification and Characterization of a-N-Acetylgalactosaminidase from Clostridium perfringens. *IUBMB Life* **50**, 91–97 (2000).
38. Calcutt, M. J., Hsieh, H. Y., Chapman, L. F. & Smith, D. S. Identification, molecular cloning and expression of an α-N-acetylgalactosaminidase gene from Clostridium perfringens. *FEMS Microbiol. Lett.* **214**, 77–80 (2002).
39. Hsieh, H.-Y., Calcutt, M. J., Chapman, L. F., Mitra, M. & Smith, D. S. Purification and characterization of a recombinant α-N-acetylgalactosaminidase from Clostridium perfringens. *Protein Expr. Purif.* **32**, 309–316 (2003).
40. Zhu, A., Monahan, C., Wang, Z.-K. & Goldstein, J. Expression, Purification, and Characterization of Recombinant α-N-Acetylgalactosaminidase Produced in the Yeast Pichia pastoris. *Protein Expr. Purif.* **8**, 456–462 (1996).
41. Hata, J. *et al.* Purification and characterization of N-acetyl-alpha-D-galactosaminidase from Gallus domesticus. *Biochem. Int.* **28**, 77–86 (1992).
42. Izumi, K., Yamamoto, K., Tochikura, T. & Hirabayashi, Y. Serological study using α-N-acetylgalactosaminidase from Acremonium sp. *Biochim. Biophys. Acta - Gen. Subj.* **1116**, 72–74 (1992).
43. Bakunina, I. Y. *et al.* α-N-acetylgalactosaminidase from marine bacterium Arenibacter latericius KMM 426T removing blood type specificity of A-erythrocytes. *Biochem.* **67**, 689–695 (2002).
44. Liu, Q. Y. P. *et al.* Bacterial glycosidases for the production of universal red blood cells. *Nat. Biotechnol.* **25**, 454–464 (2007).
45. Landry, D. Isolation and composition of a novel glycosidase from chryseobacterium. US Patent US20020072104A1.
46. Yip, V. L. Y. *et al.* An unusual mechanism of glycoside hydrolysis involving redox and elimination steps by a family 4 beta-glycosidase from Thermotoga maritima. *J. Am. Chem. Soc.* **126**, 8354–8355 (2004).

47. Liu, Q. Y. P. *et al.* Identification of a GH110 subfamily of alpha 1,3-galactosidases - Novel enzymes for removal of the alpha 3Gal xenotransplantation antigen. *J. Biol. Chem.* **283**, 8545–8554 (2008).
48. Fernandes, H. P., Cesar, C. L. & Barjas-Castro Mde, L. Electrical properties of the red blood cell membrane and immunohematological investigation. *Rev Bras Hematol Hemoter* **33**, 297–301 (2011).
49. Gao, H.-W. *et al.* Glucose buffer is suitable for blood group conversion with α-N acetylgalactosaminidase and α-galactosidase. *Blood Transfus.* **12**, 61–6 (2014).
50. Gao, H. *et al.* Application of α-N-acetylgalactosaminidase and α-galactosidase in AB to O Red Blood Cells Conversion. *Artif. Cells, Nanomedicine, Biotechnol.* **41**, 32–36 (2013).
51. Chapanian, R. *et al.* Enhancement of biological reactions on cell surfaces via macromolecular crowding. *Nat. Commun.* **5**, 4683 (2014).
52. Anderson, K. M. *et al.* A clostridial endo-β-galactosidase that cleaves both blood group A and B glycotopes: The first member of a new glycoside hydrolase family, GH98. *J. Biol. Chem.* **280**, 7720–7728 (2005).
53. Kwan, D. H. *et al.* Toward Efficient Enzymes for the Generation of Universal Blood through Structure-Guided Directed Evolution. *J. Am. Chem. Soc.* **137**, 5695–5705 (2015).
54. Kwan, D. H., Ernst, S., Kötzler, M. P. & Withers, S. G. Chemoenzymatic Synthesis of a Type 2 Blood Group A Tetrasaccharide and Development of High-throughput Assays Enables a Platform for Screening Blood Group Antigen-cleaving Enzymes. *Glycobiology* **25**, 806–811 (2015).
55. Handelsman, J. Metagenomics: Application of genomics to uncultured microorganisms (vol 68, pg 669, 2004). *Microbiol. Mol. Biol. Rev.* **69**, 195 (2005).
56. Tailford, L. E., Crost, E. H., Kavanaugh, D. & Juge, N. Mucin glycan foraging in the human gut microbiome. *Front. Genet.* **5**, (2015).
57. Mäkivuokko, H. *et al.* Association between the ABO blood group and the human intestinal microbiota composition. *BMC Microbiol.* **12**, 94 (2012).
58. Rahfeld, P. *et al.* An enzymatic pathway in the human gut microbiome that converts A to universal O type blood. *Nat. Microbiol.* 4, 1475-1485 (2019).
59. Gerbal, A., Maslet, C. & Salmon, C. Immunological aspects of the acquired B antigen. *Vox Sang.* **28**, 398–403 (1975).
60. Judd, W. J. & Annesley, T. M. The acquired-B phenomenon. *Transfus. Med. Rev.* **10**, 111–117 (1996).
61. Gao, H.-W. *et al.* Evaluation of group A1B erythrocytes converted to type as group O: studies of markers of function and compatibility. *Blood Transfus.* **14**, 168–74 (2016).
62. Goldstein, J. Conversion of ABO blood groups. *Transfus. Med. Rev.* **3**, 206–12 (1989).
63. Hoskins, L. C. & Boulding, E. T. Changes in immunologic properties of group A RBCs during treatment with an A-degrading exo-alpha-N-acetylgalactosaminidase. *Transfusion* **41**, 908–916 (2001).
64. Liu, Q. P. *et al.* Bacterial glycosidases for the production of universal red blood cells. *Nat Biotech* **25**, 454–464 (2007).
65. Cohen, M., Hurtado-Ziola, N. & Varki, A. ABO blood group glycans modulate sialic acid recognition on erythrocytes. *Blood* **114**, 3668–3676 (2009).
66. Li, L., Noumsi, G. T., Kwok, Y. Y. E., Moulds, J. M. & Scott, M. D. Inhibition of phagocytic recognition of anti-D opsonized Rh D+ RBC by polymer-mediated immunocamouflage. *Am. J. Hematol.* **90**, 1165–70 (2015).
67. Yee, J. Increasing Access to Kidney Transplantation: Easy as A-B-O. *Adv. Chronic Kidney Dis.* **23**, 277–279 (2016).
68. Senior, M. Beating the organ clock. *Nat. Biotechnol.* **36**, 488–492 (2018).
69. Kobayashi, T. *et al.* Removal of blood group A/B antigen in organs by ex vivo and in vivo administration of endo-ß-galactosidase (ABase) for ABO-incompatible transplantation. *Transpl. Immunol.* **20**, 132–138 (2009).

70. Kobayashi, T. *et al.* Alternative strategy for overcoming ABO incompatibility [5]. *Transplantation* **83**, 1284–1286 (2007).

TABLES

Table 1: Distribution of ABO and Rh (D) groups
(https://www.redcrossblood.org/donate-blood/blood-types.html)

	Rh (D) Positive				Rh (D) Negative			
Race demographic*	**O+**	**A+**	**B+**	**AB+**	**O-**	**A-**	**B-**	**AB-**
Caucasian	37	33	9	3	8	7	2	1
African-American	47	24	18	4	4	2	1	0.3
Asian	39	27	25	7	1	0.5	0.4	0.1
Latino-American	53	29	9	2	4	2	1	0.2

* data based on U.S. population

FIGURES

Figure 1

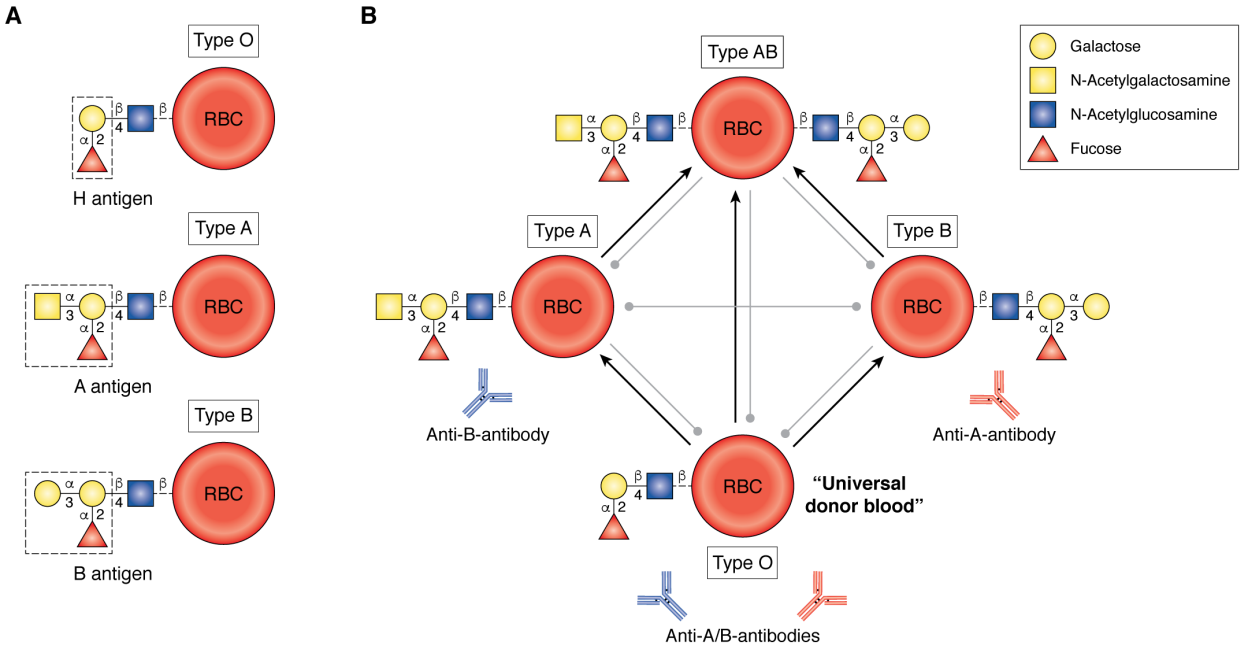

Figure 1: a. Overview of basic A, B and H antigens on the surface of Type A, B and O RBCs. **b.** representation of blood transfusion compatibility (black arrow =can be transfused; gray = no transfusion). O RBCs are the only ones lacking a recognized blood group antigen and can therefore be universally donated. Sugars are shown using the Consortium for Functional Glycomics notation [12].

Figure 2

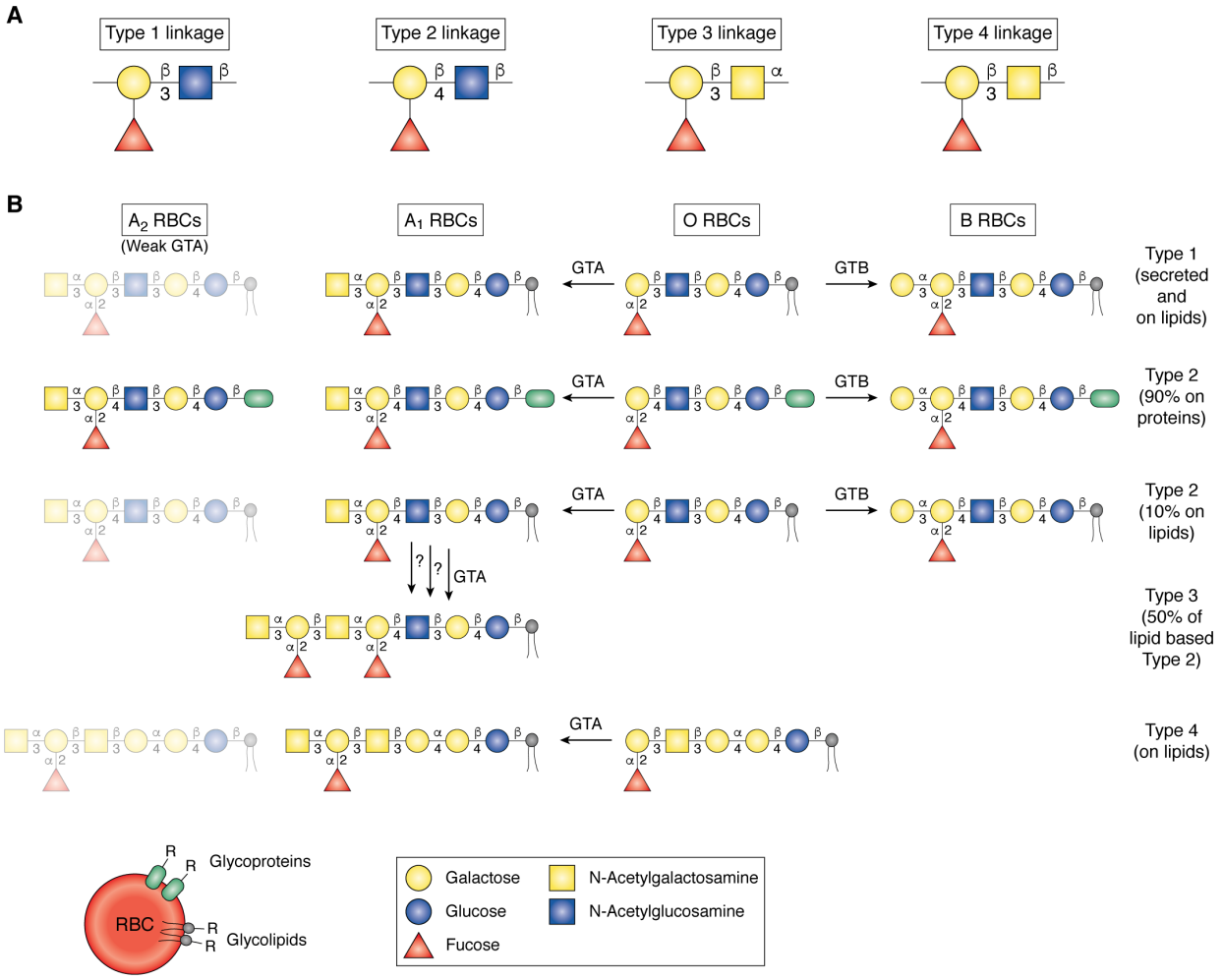

Figure 2. Presentation of the blood antigen linkage types present on the RBCs of different blood types. **a)** Presentation of the difference of the 4 linkage types on RBCs. **b)** The main groups are O, B, A_1 and A_2. O type blood presenting the different H antigens is the starting point for the synthesis of A and B antigens, depending on whether a GTA or GTB is present. Sub-group A_2 only possesses a low activity (weak) GTA, which leads to only minor amounts of Type 1 and 4 A antigen production (This is symbolised by the faded antigens) and no Type 3 production. Sugars are shown using the Consortium for Functional Glycomics notation [12].

Figure 3

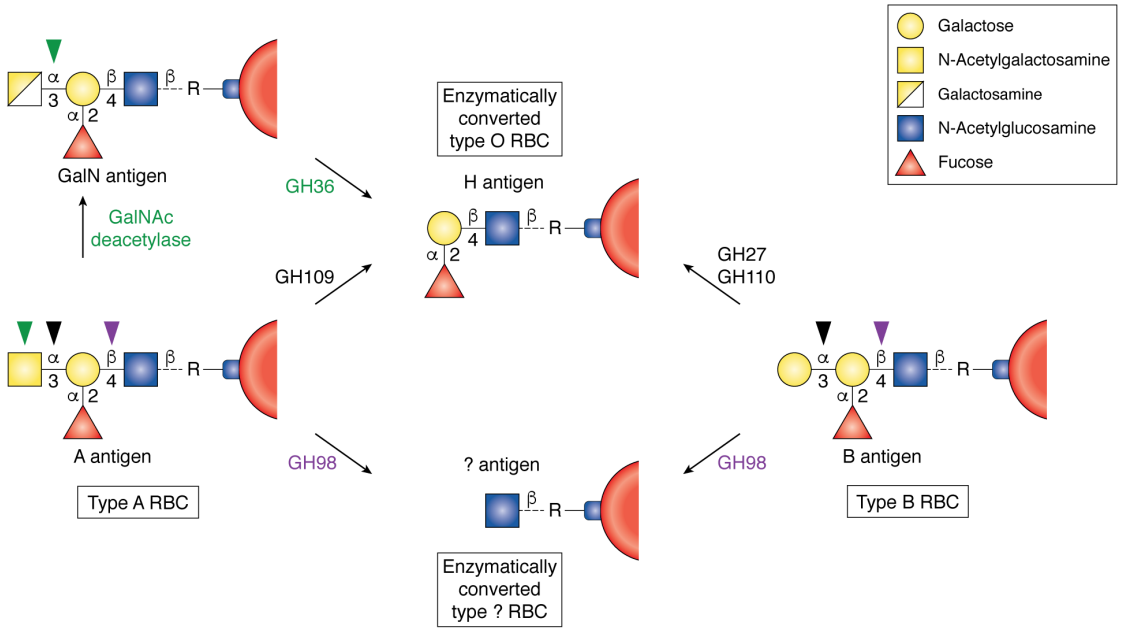

Figure 3. Overview of enzymatic conversion of A and B antigens to H antigens as routes to production of enzymatically converted (ECO) O RBCs. Presented are the known enzymatic pathways for the conversion of A or B to H antigens. The enzymes with conventional exo-cleavage activities are presented in black, enzymes with endo-cleavage activity in purple (producing ECO ? RBCs, with unknown antigenicity) and the two-step pathway in green. Sugars are shown using the Consortium for Functional Glycomics notation [12].

Figure 4

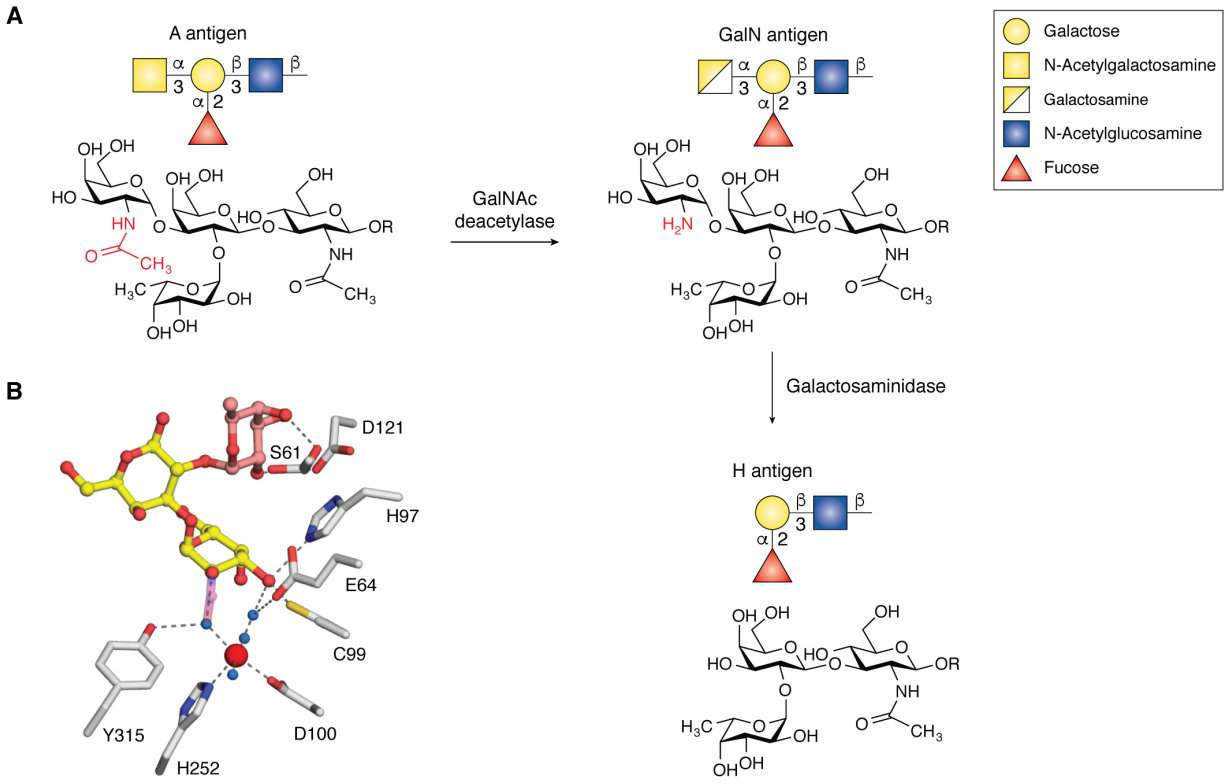

Figure 4. Deacetylation pathway for A antigen cleavage. **a)** The A antigen is deacetylated by the GalNAc deacetylase (DeAc), followed by cleavage of galactosamine by the galactosaminidase (GH36), yielding the H antigen. Sugars are presented as chemical structures (red labelled part of the chemical structure is the functional group being converted by the deacetylase) and symbols using the Consortium for Functional Glycomics notation [12]. **b)** Presented are the active site residues of the GalNAc deacetylase, involved in interactions with the non-reducing end galactosyl group. Polar interactions are shown as dashed grey lines and water molecules are shown as blue spheres. The N-acetyl group of the GalNAc in an A antigen trisaccharide modelled onto the structure of the complex with B-antigen is shown in semi-transparent magenta stick. Electron density ($2F_o$-F_c) map of the B antigen trisaccharide ligand is shown in cyan mesh. The H252 and D100 residues coordinate the divalent metal ion (red sphere). Galactosyl residues are shown in yellow sticks and the fucosyl residue in red.

Towards universal donor blood: enzymatic conversion of A and B to O type

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