

REVIEW ARTICLE

ABO blood group and related antigens, natural antibodies and transplantation

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Abstract

The current success rate of transplant surgery and immunosuppression has led to a demand for organs that has outstripped the supply. This has required investigation of alternate strategies. Therefore, allotransplantation across the ABO blood group barrier has commenced, and pig-to-human xenotransplantation is under consideration. The first immunological barrier to both these types of transplantation is the prevention of the antibody-mediated rejection. This rejection is a result of natural preformed antibodies circulating in the serum of the recipient binding to either ABO (for allo) or α -galactose (α -Gal) (for xeno) antigens expressed on the donor tissue. These antibodies recognise antigens that are, in both cases, carbohydrate molecules with the characteristic feature that the nonreducing terminal carbohydrate is either a Gal or N-acetylgalactosamine residue in an α 1,3 linkage. These epitopes are synthesised by closely related members of a single family of glycosyltransferases. This review discusses the carbohydrate antigens, the enzymes involved in their synthesis and the consequences of natural antibodies binding these antigens.

Introduction

It is 100 years since Karl Landsteiner discovered the ABO blood groups that became the basis for matching blood from individuals prior to blood transfusion [for review see Landsteiner (1)]. The ABO family of antigens remains highly relevant to medicine today, especially in the field of transplantation. In the early days, it was realised that donation of blood, organs or tissues between individuals who express incompatible blood groups would result in rejection. This process of rejection is mediated by natural antibodies (Nabs) and is known as hyperacute rejection (HAR). In recent times, the issue of ABO incompatibility has again come to the fore. A shortage of donor organs has led to a reversal of the clinical practice of avoiding transplantation across ABO incompatibilities, and allotransplantation of these organs has recommenced in several countries (2, 3). Another potential solution to the organ shortage explored by researchers in the last decade is xenotransplantation (pig to human), where an antigen related to the ABO blood group antigens also leads to rejection mediated by Nabs (4, 5). Thus, two potential

scenarios for alleviating the organ shortage problem are characterised by related antigens that are bound by Nabs, leading to an immediate rejection process called HAR.

The ABO and related antigens are carbohydrates**ABO blood group antigens**

In contrast to major histocompatibility complex, A, B and O (H) blood group antigens are oligosaccharide moieties formed on precursor backbones by glycosyltransferases, glycoprotein enzymes that reside in the Golgi apparatus and sequentially add carbohydrate units to growing oligosaccharide chains on proteins and lipids (6, 7). It is the specificity of the catalytic domains of these enzymes for donor sugar and acceptor structures, as well as the tissue distribution of expression, that determines the form of the ABO blood group expressed. The glycosyltransferases that form A and B blood group antigens and the related Gal α (1,3)Gal, isogloboside 3 (iGb3) and Forssman antigens are members of the same glycosyltransferase family

(discussed below). The fucosyltransferases that form H substance are from another gene family; however, they are discussed here, as they are important to the synthesis of A and B blood groups.

The disaccharide precursors that form the backbone of ABO blood group antigens are known as Type I, II, III, IV and V. Type I precursor of the fucosyltransferases is a galactose (Gal) in a β 1,3 linkage to N-acetylglucosamine (GlcNAc); Type II, a Gal in a β 1,4 linkage to GlcNAc; Type III, a Gal in a β 1,3 linkage to N-acetylgalactosamine (GalNAc) on serine or threonine; Type IV, a Gal in a β 1,3 linkage to GalNAc, and then, via other carbohydrate residues, linked to ceramide; Type V is Gal in a β 1,4 linkage to glucose (Glc) linked directly to ceramide (lactosylceramide) or Gal in a β 1,4 linkage to Glc (free lactose) (Figure 1).

To form A and B blood groups, initially, H substance (O blood group) is formed by the addition of a fucose to the precursor by either the H-type- α 1,2fucosyltransferase or Se-

type- α 1,2fucosyltransferase. Although the fucosyltransferases have high amino acid identity (8–10), they differ in tissue distribution and utilisation of precursor structures. The H-type- α 1,2fucosyltransferase is mainly expressed on erythrocyte precursors and red blood cells (RBCs) and utilises Type II and IV precursors, and, in contrast, the Se-type- α 1,2fucosyltransferase is mainly expressed in epithelial cells and utilises Type I and III precursors.

The A and B transferases transfer a terminal carbohydrate residue and specifically utilise the fucosylated precursors [for review see Yamamoto (11)]. They transfer, in an α 1,3 linkage, either a Gal residue (B transferase) or a GalNAc residue (A transferase) (Figure 2). The tissue expression and specificity of the fucosyltransferases, therefore, affect the expression of A and B blood groups. The ABO blood group antigens are expressed on either membrane proteins or on glycolipids and can be found on RBCs, epithelial cells and (if on precursors formed by the Se-type- α 1,2fucosyltransferase) various soluble glycans.

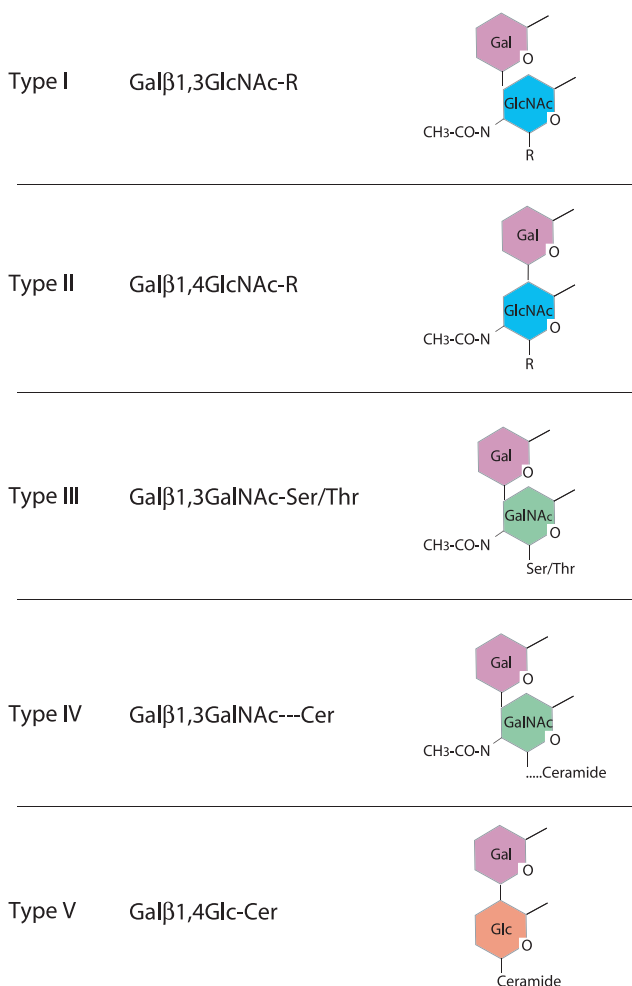


Figure 1 The disaccharide precursors utilised by glycosyltransferases to form the ABO and related α -galactose (α -Gal) and α -N-acetylgalactosamine (α -GalNAc) antigens.

Related antigens

Unlike the ABO blood group antigens, the related α -Gal and α -GalNAc antigens do not require fucosylation of the precursor structure (Figure 2), and in fact, the enzymes that synthesise these epitopes are unable to utilise the fucosylated precursor.

The Gal α (1,3)Gal xenoepitope was also originally described by Lansteiner as 'B-like' antigen in animals (1). This epitope can be synthesised by the α 1,3galactosyltransferase (α 1,3GT) that adds a Gal in an α 1,3 linkage to N-acetyllactosamine (NAcLac) on either protein or lipid (12, 13). Structurally, Gal α (1,3)Gal synthesised by α 1,3GT is very similar to the B blood group antigen on Type II precursor except that it lacks the Fuc in an α 1,2 linkage on the penultimate Gal (Figure 2).

A second enzyme also synthesises Gal α (1,3)Gal; however, in contrast to α 1,3GT, the epitope is exclusively produced on lipid to form iGb3. In this case, the iGb3 synthase (iGb3S) synthesises Gal α (1,3)Gal on lactose rather than on NAcLac: the precursor is Gal in a β 1,4 linkage on Glc and this in turn is attached to ceramide, or the Type V precursor (14, 15). The difference between Gal α (1,3)Gal on Type II (synthesised by α 1,3GT) and on Type V (synthesised by iGb3S) begins at the third carbohydrate residue, which is a GlcNAc and Glc, respectively. The presence of an N-acetyl group in this third position may influence the binding of anti-Gal antibodies (J. Milland and M. S. Sandrin, unpublished data).

Finally, the A blood group-related Forssman epitope, GalNAc α (1,3)GalNAc (Figure 2), is formed by Forssman synthetase utilising the globo and isoglobo series pathways. The globoside 4 (Gb4) synthase that adds GalNAc in a β 1,3 linkage to a Gal can utilise either Gb3 (P blood group) or iGb3 to produce Gb4 (globoside) or iGb4 (isogloboside),

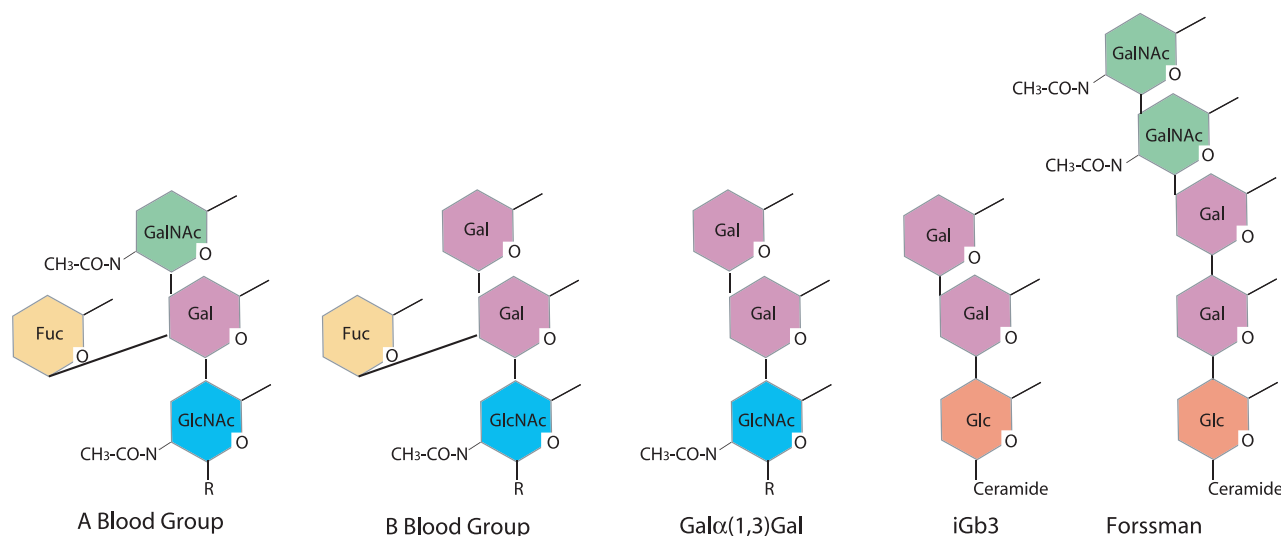


Figure 2 The terminal saccharide structures of the ABO and related α -galactose (α -Gal) and α GalNAc antigens. The A and B blood groups and Gal α (1,3)Gal terminal saccharides are shown on Type II and the isogloboside 3 (iGb3) and Forssman are shown on Type V.

respectively. Forssman synthetase then adds a terminal GalNAc in an α 1,3 linkage to form the epitope GalNAc α (1,3)GalNAc (16). When this epitope is produced on the iGb4 precursor, it is known as isoForssman, whereas when it is formed on Gb4, it is called Forssman.

The biological function of all these related antigens remains obscure although ABO blood group antigens have been linked to infectivity and cancer [for review see Greenwell (17)]. iGb3 has been suggested as the natural ligand presented by CD1d to natural killer T cells (18) [for review and comment see Godfrey et al. (19) and Savage et al. (20)]. This appears to be true in mice (21, 22), whereas, in humans, there is no direct biochemical evidence as yet (18).

It is the ABO blood group glycans on any precursor and Gal α (1,3)Gal on Type II and Type V precursors that are important to transplantation, as binding of Nabs to these epitopes may cause rejection of ABO-incompatible allo-transplants and xenotransplants, respectively.

Human natural anticarbohydrate antibodies recognise these antigens and can lead to HAR

Nabs to these carbohydrate epitopes are present in all humans with intact immune systems. Traditionally, it was believed that A blood group individuals have anti-B Nabs, B blood group have anti-A Nabs, O blood group have anti-A and anti-B Nabs and AB blood group have neither anti-A nor anti-B Nabs as they express both antigens. This is due to the fact that an A blood group individual can develop an immune response to B-like antigens in the bacterial environment (e.g. in the intestine) but not to A-like (or self) antigens, and so on for B blood group and O blood group individuals. However, there is some evidence that normal

human serum does contain antibodies that react with self-antigens (e.g. anti-A antibodies in the serum of A blood group individuals) without any evidence of pathology (23). This may not be surprising as many other Nabs are directed to self-antigens such as intracellular and plasma proteins (24, 25) but most are not pathological.

In contrast, anti-Gal antibodies are present in all individuals due to the complete lack of Gal α (1,3)Gal expression in humans. Again, these antibodies probably arise as an antibody response to microorganisms and environmental antigens (e.g. plant) that contain the epitope. These antibodies, like most Nabs, are encoded by germline genes and are mainly of the immunoglobulin (Ig) M and IgG isotypes and represent 1%–2% of all Ig (26). In contrast, anti-ABO antibody levels are estimated as one quarter of those of anti-Gal as measured by the number of circulating B cell clones that synthesise the antibody (27).

As already suggested, Nabs to self-antigens do not always cause pathology. Examples of this relevant to transplantation are the existence of antibodies to autologous ABO blood group antigens (23) or the existence of anti- α -Gal antibodies in α 1,3GT knockout mice (–/–) that contain α -Gal synthesised by iGb3S (15). On the other hand, circulating Nabs to carbohydrate antigens can lead to rapid rejection of an allotransplant or a xenotransplant.

Naturally occurring anti-ABO and anti- α -Gal antibodies play a significant role in transplantation and indeed represent the first obstacle to be overcome for successful engraftment [for review see Sandrin and McKenzie (4)]. The binding of the cognate antigen by the antibodies, in conjunction with complement fixation, leads to immediate graft destruction that occurs within minutes of the antibody recognising antigen. For the simplest form of transplantation, blood

transfusion, this represents intravascular haemolysis of RBCs, whereas for a vascularised organ the phenomenon is known as HAR. The hallmarks of HAR include lysis/destruction of the vascular endothelium by complement components, which, as a result of a loss of vascular integrity, leads to oedema and haemorrhage into the underlying tissue. Furthermore, the coagulation pathway is also activated leading to deposition of fibrin, formation of microthrombi and eventually intravascular coagulation leading to infarction of the organ. The pathological features of HAR resemble the Schwartzman phenomenon, and the severity of the reaction is dependent on the level of antibody and antigen expression and the complement system. Elimination or inhibition of any one of these factors can attenuate HAR. The effect of complement inhibition in HAR has been reviewed (28), and this will not be discussed further.

In the allogeneic setting, it is possible to match blood groups of the donor and recipient, thus preventing antibody-mediated graft rejection. Due to the chronic shortage of donors, strategies are being developed to transplant across the ABO barrier. First, it is possible to choose an ABO-incompatible donor/recipient combination in which the level of antigen expression in the donor is low and the transplant is not rejected by HAR, e.g. A2 to O graft (29). Another strategy is to remove the preformed antibodies by immunophoresis and then use appropriate short-term immunosuppressive therapy to target both T and B cells. It appears that if the anti-ABO antibodies can be kept at low or undetectable levels for 2–3 weeks posttransplant, long-term graft survival is possible even with the later return of antibodies. The mechanism for the survival is unclear but may involve the phenomenon of 'accommodation' (30). In contrast, if the antibodies return within the first 2 weeks posttransplant, the graft is destroyed by antibody-mediated mechanisms (29, 31).

The results from many studies show that in the xenogeneic setting, either complement inhibition or depletion/inhibition of antibodies in humans is not clinically practical and that the only alternative is the production of antigen-negative pigs. Currently, $\alpha 1,3\text{GT}^{-/-}$ pigs have been developed by several groups, and these appear to be Gal-ve (32, 33, P. Cowan and A. d'Apice, unpublished data). However, we have recently reported remaining $\text{Gal}\alpha(1,3)\text{Gal}$ on $\text{Gal}^{-/-}$ mice (15), and preliminary unpublished data suggest that $\text{Gal}^{-/-}$ pigs may also have residual $\text{Gal}\alpha(1,3)\text{Gal}$. This residual antigen is most likely synthesised by iGb3S [see above and for review see Milland et al. (34)]. The implication of this iGb3 for clinical xenotransplantation is currently unclear.

ABO and related α -Gal antigens are synthesised by glycosyltransferases from the α -Gal/GalNAc family

Carbohydrate antigens on proteins and lipids have a profound influence on many aspects of the immune system.

Understanding the ABO and related carbohydrate antigens, in particular, has become crucial to future efforts of transplantation. An examination of the cell biology and biochemistry of the enzymes that produce these epitopes is critical to the development of strategies to overcome the carbohydrate rejection barrier.

Structure and localisation

The glycosyltransferases are Type II integral membrane proteins. This protein orientation has an N-terminal cytoplasmic tail followed by a stem region, transmembrane domain and a C-terminal catalytic domain residing in the lumen of the endoplasmic reticulum (ER), Golgi or *trans*-Golgi network (TGN).

The enzymes are localised in subcompartments of the secretory pathway, where they perform their enzymatic function. That is, glycosyltransferases that add saccharide units to the glycan core reside in the ER and *cis*-Golgi, whereas those that add the terminal saccharides reside in the *trans*-Golgi or TGN. A number of mechanisms were proposed for the localisation of glycosyltransferases. The lipid bilayer theory suggested that they are localised by the length of their transmembrane domain. In glycosyltransferases, this is typically ~17 amino acids long. The glycosyltransferases would be partitioned into the Golgi/TGN, as to transit to the plasma membrane would require a longer transmembrane domain of ~22 amino acids, typical of plasma membrane proteins (35). The other mechanism proposed was kin recognition (36), where similar or like proteins aggregate and are retained in the secretory pathway. Finally, there is increasing evidence that the cytoplasmic domain is involved in localisation either via specific sequences (37–40) or length (41). It is likely that a combination of mechanisms localise glycosyltransferases. However, understanding these mechanisms and manipulating the location of enzymes is one way we can alter the carbohydrate profile of a cell to give improved clinical outcomes in settings where disease is linked to carbohydrate antigens.

Family 6 glycosyltransferases: the $\alpha 1,3\text{Gal}/\text{GalNAc}$ family

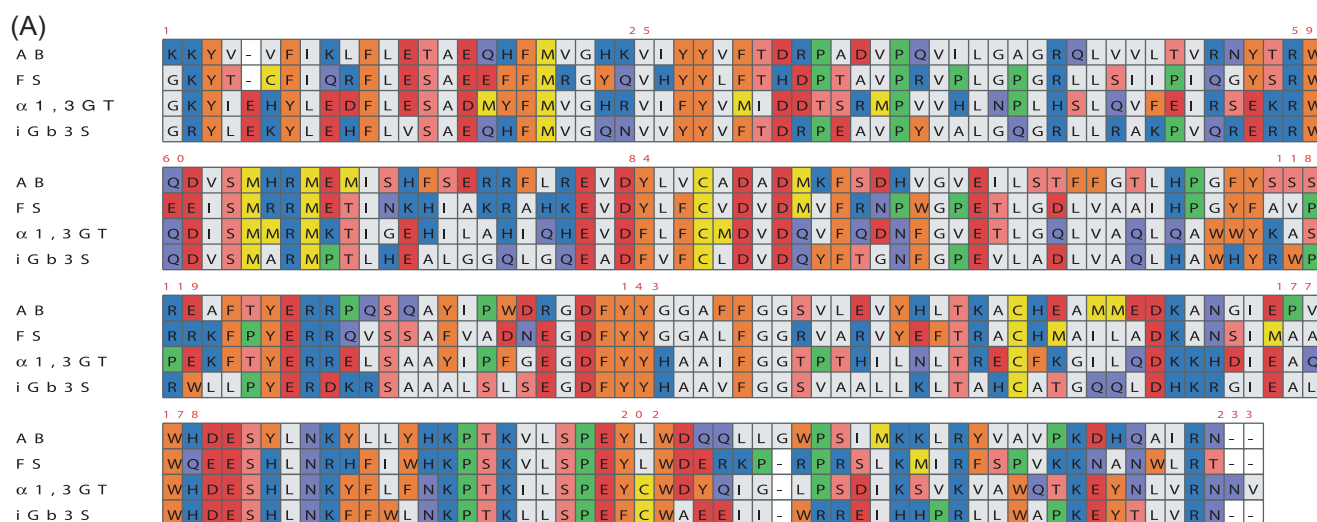
The ABO and related enzymes are all members of the Family 6 of glycosyltransferases [as defined by the Glycosyltransferase Family Server of the CAZy (Carbohydrate-Active enzymes) database]. They transfer either an α -Gal or αGalNAc to their respective acceptor molecules (see above). They are all retaining enzymes, which means they retain their anomeric configuration i.e. during catalysis, they retain the α configuration that existed between the nucleoside and the sugar.

The Family 6 glycosyltransferases also exhibit high sequence conservation (Figure 3). Using the mouse as a representative species (chosen as all members of this family are catalytically active), the glycosyltransferases have an overall amino acid identity of 45.2% and if conservative amino acid substitutions are taken into account, there is an overall homology of 60.1% (Figure 3A,B). The genes encoding the individual family members share an overall common structure: the cytoplasmic tail and transmembrane domain are encoded in one exon, followed by a variable number of exons that encode the stem region; the catalytic domain is encoded over two exons, a small one towards the N-terminus and a larger one encoding the majority of the domain.

Understanding the molecular mechanisms of A and B blood group synthesis began when the complementary

deoxyribonucleic acid (cDNA) clones for A and B transferases were isolated (42). This was a landmark event not only for tissue typing and transplantation but also for the field of glycobiology, as these were among the first mammalian glycosyltransferases to be cloned. The remarkable feature is that the predicted amino acid sequences of the A and B transferases differed at only four residues and that O blood group is a result of a single nucleotide deletion that leads to a shift in the reading frame resulting in a truncated nonfunctional A transferase. Subsequent *in vitro* mutagenesis of these four amino acid residues demonstrated that the side chain of residue 268 determines the donor substrate specificity by recognition of the carbohydrate moiety of the nucleotide sugar (43, 44).

The isolation of cDNAs encoding glycosyltransferases allows direct rapid demonstration by transfection that



(B) % Sequence identity

	α1,3GT	iGb3S	FS
AB	42.5 (57.6)	41.6 (54.4)	46.1 (63.9)
α1,3GT		49.6 (62.5)	42.7 (62.9)
iGb3S			43.2 (59.1)

Figure 3 Family 6 glycosyltransferases in mouse. (A) Alignment of the predicted amino acid sequences of the catalytic domains of AB transferases (AB, exon 5), Forssman synthetase (FS, exon 5), α1,3galactosyltransferase (α1,3GT, exon 9) and iGb3 synthase (iGb3S, exon 5). (B) Percent sequence identity of catalytic domains of Family 6 members in mouse. The figure in parentheses is an adjusted percentage that takes conservative amino acid substitutions into account.

a particular gene is indeed responsible for phenotype. This is in contrast to the complex genetic analysis and serology used in the past to define blood groups and related antigens. Transfection allows the confirmation of the dogma one gene → one protein → one epitope. Thus, several studies have used transfection to examine the effects of alteration of individual amino acid residues on catalytic function and both substrate and donor specificity of α 1,3GT (45, 46) and A and B transferases (43, 44).

The next advance in the field was the crystallisation and determination of the tertiary structure of the catalytic domain of Family 6 glycosyltransferases: bovine α 1,3GT (47, 48) and human A and B transferases (49). The Family 6 glycosyltransferase catalytic domain of bovine α 1,3GT [Figure 4, based on 1G93.pdb (48) and modelled using VMD software (50)] is a unique form of α/β protein domains, comprising two central β -sheets, each composed of four β -strands, surrounded by four long α -helices. The molecule can be further subdivided into two distinct subdomains: (1) the amino-terminal subdomain involved in nucleotide-sugar binding consists of four parallel β -strands, surrounded by two of the α -helices (yellow in Figure 4) and (2) the carboxyl-terminal subdomain involved in acceptor substrate binding made up of two parallel β -strands flanked by two anti-parallel β -strands and surrounded by the two other α -helices (green in Figure 4). There is an additional small β -sheet domain composed of two β -strands (purple in Figure 4) that is preceded by the conserved DVD motif (orange in Figure 4) that lies at the base of the catalytic pocket formed by portions of the two subdomains.

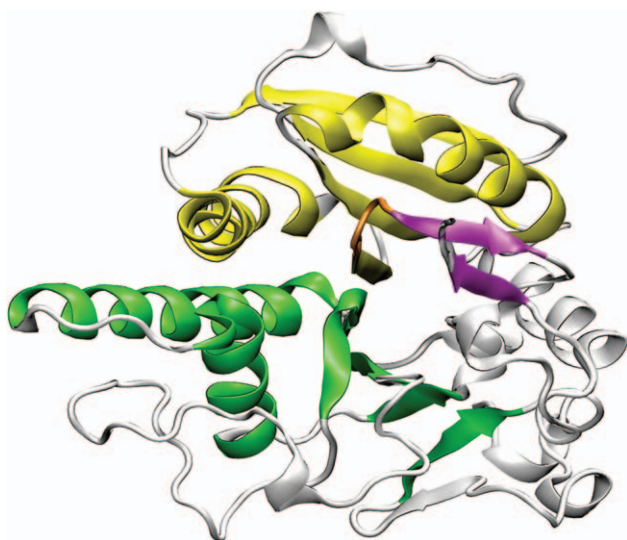


Figure 4 Looking down into the catalytic pocket of bovine α 1,3galactosyltransferase. The subdomain that supports the interaction with (1) the acceptor substrate is shown in green, (2) the donor sugar is yellow, (3) the small sheet flanking the DVD motif in purple. The DVD motif is shown in orange. The remaining catalytic domain is represented in white.

These structural determinations allow the understanding at the atomic level of the interactions of individual amino acid residues with donor and acceptor carbohydrate and direct observation of the effect of the individual mutations described above and below. Mutational/functional studies can be used to explain variation at the molecular level of AB and related antigens, and more importantly, they may be used to examine the effect of modification of the carbohydrate profile of cells to improve transplantation outcomes.

Natural mutations in the Family 6 glycosyltransferases in humans have led to a number of consequences for transplantation. As mentioned, a deletion in the A transferase gene has resulted in the O phenotype, and amino acid differences between A and B form the blood group phenotypes that have resulted in ABO incompatibilities (42). These differences have been partially overcome by plasmapheresis, leading to accommodation (see above). Furthermore, humans are the only organisms (along with Old World monkeys) that do not express Gal α (1,3)Gal due to inactivation of the α 1,3GT gene, the consequence of which is HAR in pig-to-human xenotransplantation. Transplantation outcome will hopefully be improved by the current work involving genetic inactivation of the glycosyltransferases forming this epitope and the expression of molecules to protect from complement and other coagulation-promoting molecules.

The actual biological reason for the evolution and phenotypic variations of the ABO and related carbohydrate epitopes remains obscure, but it is known that they can affect infection by viruses and bacteria (17). The challenge is for us to manipulate these epitopes to enable successful transplantation.

Conclusions

For the last decade or so, the concentrated study of the biology of carbohydrates important to transplantation has led to a marked increase in knowledge, especially for xenotransplantation. The reality is that we will not be able to genetically alter organs in humans to improve allotransplantation across the ABO blood group barrier. Therefore, workers in this field will need to study tolerance induction and the phenomenon of accommodation to improve transplantation of ABO-mismatched vascularised organs.

Nonetheless, even if ABO-incompatible donor organs could be successfully transplanted, the problem still remains that there are not enough organs available to meet the demand. Xenotransplantation may fill this shortfall. By using our knowledge of carbohydrate biology, we were able to alter the carbohydrate profile of cells in transgenic animals by overexpressing the H-type-fucosyltransferase to reduce Gal α (1,3)Gal expression (51). The α 1,3GT knockout pigs (32, 33) provided the best solution to this problem by

completely eliminating Gal α (1,3)Gal on Type II precursor (but perhaps not Gal α (1,3)Gal on Type V precursor).

Xenotransplants of organs from α 1,3GT $^{-/-}$ pigs into immunosuppressed baboons have extended the life of a transplant from minutes to months (52, 53). Interestingly, similar transplants into nonimmunosuppressed baboons have extended the life of a transplant from minutes to days (P. Cowan and A. d'Apice, unpublished data). These experiments definitively show that the problem of HAR has been solved as the true reaction of the baboon's immune system was measured without interference from immunosuppressive treatments.

Thus, by understanding carbohydrate and anticarbohydrate biology and by manipulating or eliminating Family 6 glycosyltransferases, HAR has been eliminated. The field of xenotransplantation can now concentrate on developing strategies to overcome downstream rejection events.

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