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The Galα1,3Galβ1,4GlcNAc-R (α-Gal) epitope: a carbohydrate of unique evolution and clinical relevance

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Abstract

In 1985, we reported that a naturally occurring human antibody (anti-Gal), produced as the most abundant antibody (1% of immunoglobulins) throughout the life of all individuals, recognizes a carbohydrate epitope Galα1-3Galβ1-4GlcNAc-R (the α-gal epitope). Since that time, an extensive literature has developed on discoveries related to the α-gal epitope and the anti-Gal antibody, including the barrier they form in xenotransplantation and their reciprocity in mammalian evolution. This review covers these topics and new avenues of clinical importance related to this (α-gal epitope/anti-Gal) unique antigen/antibody system in improving the efficacy of viral vaccines and in immunotherapy against cancer.

Keywords

Anti-Gal; α-1,3galactosyltransferase; α-gal epitopes; xenotransplantation; cancer immunotherapy; cancer vaccine; viral vaccine

1. Introduction

The distribution of the α-gal epitope (Galα1-3Galβ1-4GlcNAc-R) is unique in mammals, being abundantly expressed on glycoconjugates of non-primate mammals, prosimians and New World monkeys. In contrast, the α-gal epitope is not expressed on glycoconjugates of Old World monkeys, apes and humans; instead, they produce in very large amounts the natural anti-Gal antibody that specifically binds the α-epitope. This absence of the α-gal epitope is the result of an evolutionary event in ancestral Old World primates which led to the inactivation of the glycosyltransferase UDP-Gal: 3Galβ1-4GlcNAc α1-3-galactosyltransferase, EC 2.4.1.151 (α1,3GT) that is estimated to have occurred less than 28 million years ago. The 3D structure of α1,3GT is known and the crystal structures has revealed that the enzyme undergoes a conformational rearrangement of the C-terminal region, creating a highly ordered structure that appears to be part of the active site. Anti-Gal is the most abundant natural antibody in humans, apes and Old World monkeys constituting ~1% of circulating immunoglobulins. Anti-Gal mediates the rejection of pig xenograft organs in humans and monkeys by binding α-gal

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epitopes on the pig cells and inducing complement mediated destruction and antibody dependent cell mediated destruction. This barrier to xenotransplantation has been eliminated recently by producing α1,3GT knockout pigs. Since anti-Gal is ubiquitous in humans, the α-gal epitope has clinical potential in the production of vaccines expressing α-epitopes that can be targeted to antigen presenting cells (APC), thereby increasing the immunogenicity of viral and other microbial vaccines. Expression of α-gal epitopes on tumor cells by transduction with gene therapy vectors such as adenovirus or retrovirus vectors containing the α1,3GT gene, can also result in anti-Gal mediated targeting of vaccinating tumor cells to APC. A similar targeting can be achieved via the biosynthesis of α-gal epitopes on tumor cells with recombinant α1,3GT. In addition, intratumroal injection of glycolipids carrying α-gal epitopes results in the destruction of these lesions by mechanisms similar to xenograft rejection, which in turn, converts the lesions into an endogenous anti-tumor vaccine. A summary of findings related to each of the topics described in this introduction is presented in this review.

2. Galα1-3Galβ1-4GlcNAc-R glycoconjugates

2.1 Glycolipids with α-gal epitopes

Yamakawa and coworkers first reported a glycolipid, ceramide pentahexoside (CPH), from rabbit red blood cells containing the non-reducing terminal sequence Galα1-3Galβ1-4GlcNAc-R in 1968 [1]. The structure of the rabbit red blood cell CPH was further characterized by Hakomori and coworkers in 1972 [2]. Subsequent reports by Dabrowski, Egge, Hanfland and coworkers demonstrated that rabbit red blood cells contain a range of glycolipids of varying lengths that terminate with the Galα1-3Galβ1-4GlcNAc-R structure. These glycolipids, with the exception of ceramide heptahexoside (CHH), vary in size by increments of 5 monosaccharides, and each increment forms an additional branch [3-5]. Honma et al. reported that these glycolipids may be multi-branched, reaching a size of 35 and 40 carbohydrate per glycolipid [6]. Glycolipids with α-gal epitopes were also reported to be present on bovine neutral glycolipids and gangliosides [7-9].

The identification of a naturally occurring human antibody, anti-Gal [10-12], and development of a mouse monoclonal antibody, Gal-13 that bind to compounds with a non-reducing terminal sequence Galα1-3Galβ1-4GlcNAc-R on glycolipids [13] provided a tool to evaluate the species distribution of these types of glycoconjugates. Hendricks et al. evaluated the distribution of Galα1-3Galβ1-4GlcNAc-R glycolipids in kidney tissue from sheep, pig, rabbit, cow and rat [14]. Among these species, only rat did not contain Galα1-3Galβ1-4GlcNAc-R neutral glycolipids (although α-gal epitopes are found on some rat cells). Two of the species (pig and sheep) also contained sialic acid-containing glycolipids (gangliosides) with the Galα1-3Galβ1-4GlcNAc-R structure, most likely the same branched, ganglioside identified by Watanabe et al. in bovine red blood cells [9].

Thymus tissue from sheep, pig and rabbit were also evaluated for Galα1-3Galβ1-4GlcNAc-R glycolipids and shown to contain a range (compounds with 5-11 sugars) of neutral glycolipids terminating with Galα1-3Galβ1-4GlcNAc- [15]. In addition, sheep thymus was found to contain a Galα1-3Galβ1-4GlcNAc-R ganglioside [15]. Interestingly, although, kidney and thymus from sheep, pig, and rabbit express Galα1-3Galβ1-4GlcNAc-R glycolipids, these glycolipids were not detected in brain tissue demonstrating a tissue specific expression of these compounds.

2.2 Glycoproteins with α-gal epitopes

Spiro and Bhoyroo [16] demonstrated that thyroglobulin isolated from various mammals contain Galα1-3Galβ1-4GlcNAc terminal structures, and Vliegenthart and coworkers [17] used NMR to further characterize the structure on bovine thyroglobulin N-linked glycans.

Galα1-3Galβ1-4GlcNAc terminal structures on N-linked glycans also have been isolated from secreted glycoproteins such as mouse and bovine laminin [18-20]. Thall and Galili [20] have shown that fibrinogen and immunoglobulins, as well as thyroglobulin, from various species contain varying numbers of Galα1-3Galβ1-4GlcNAc terminal structures [20]. Kobata and coworkers [21,22] reported that recombinant proteins produced in some mammalian cells also carry Galα1-3Galβ1-4GlcNAc terminal structures.

Studies on freshly isolated cells or cell lines from mouse [23-26], bovine [27] and porcine origin [28] further demonstrated the presence of α-gal epitopes in large amounts on cell surface glycoconjugates. More recently, Kim et al. carried out a detailed analysis of N-linked glycans from pig kidney [29]. Using a preparation enriched in plasma membranes, they released N-glycans from glycopeptides (produced by protease treatment) using PNGase F and structurally characterized the products. Structures were assigned using a combination of glycosidase treatment and mass spectrometry (MALDI-TOF and ESI-QTOF). The results of their analyses demonstrated that Galα1-3Galβ1-4GlcNAc-R termini were found on a variety of complex bi-, tri- and tetra-antennary N-glycans. Accordingly, immunohistochemistry studies by Oriol et al. [28], demonstrated that Galα1-3Galβ1-4GlcNAc-R containing glycans can be detected via anti-Gal staining on pig renal proximal convoluted tubules, respiratory epithelium, pancreatic ducts, epidermis, as well as, on vascular endothelium.

3. Anti-Gal

In 1984, Galili et al. reported that one percent of circulating IgG in human sera has a specificity for α-linked galactose. This antibody, anti-Gal, is found in high titer in sera from all humans who are not immuno-compromised [10]. It is produced throughout life as a result of continuous antigenic stimulation by carbohydrate antigens on gastrointestinal bacteria of the normal flora [30]. Our initial collaborative studies, which started in 1984, aimed to characterize the specificity of anti-Gal. The main method we used for our characterization of anti-Gal was immunostaining of glycolipids of known structures separated by thin layer chromatography (TLC); a method described by Magnani et al. [31] in 1981. Using this method, we demonstrated that anti-Gal recognizes glycosphingolipids with non-reducing terminal Galα1-3Galβ1-4GlcNAc-R structures, whereas it does not bind to a range of closely related glycosphingolipids (Table I). Initially, we demonstrated that anti-Gal binds to a series of Galα1-3Galβ1-4GlcNAc-R glycosphingolipids isolated from rabbit red blood cells, but not to glycosphingolipids with Galα1-4Galβ1-4Glc(NAc)-R structures (i.e., CTH and P_1 antigen), establishing that anti-Gal distinguishes between structures that have the same carbohydrate sequence and anomerity, but which differ in the linkage positions of the terminal Gal residues [11]. Further analyses demonstrated that anti-Gal does not bind to glycosphingolipids with a non-reducing terminal GalNAcα1-3GalNAc disaccharide (Forssman) or the A blood group structure GalNAcα1-3(Fucα1-2)Gal-R. When anti-Gal is purified from the sera of A and O blood group individuals, it also binds to blood group B antigen. Our analyses demonstrated that approximately 85% of purified anti-B antibodies are anti-Gal antibodies [12]. In contrast, anti-Gal purified from sera of blood group B or AB, bound to α-gal epitope, but not to blood group B antigen [12]. As much as 1% of the human B cell population in an individual is capable of producing anti-Gal [32]. The studies demonstrating the differences in the fine specificity of natural anti-Gal in individuals of various blood types implied that the multiple B cell clones produce anti-Gal antibodies which have specificities that differ slightly from each other, and thus recognize various “facets” of the α-gal epitope in its 3 dimensional form. The range of this specificity is limited by self antigens (i.e. immune tolerance that prevents production of autoantibodies to self carbohydrate antigens). Thus, blood group A and O individuals produce a variety of anti-Gal clones. Some of these clones can bind only to the α-gal epitope, whereas others are capable of binding the α-gal epitopes to which a fucose is linked α1,2 to the penultimate galactose (i.e. blood group B antigen). In A and O individuals, this fucose does

not prevent many clones of anti-Gal from binding to the α-gal epitope. In contrast, in blood group B and AB individuals (in whom blood group B antigen is a self antigen), the immune tolerance mechanism prevents the development of B cell clones producing anti-Gal antibodies that are capable of binding to α-gal epitopes which also have a fucose linked to the penultimate galactose [11,12]. Similar fine specificity variation may also be found in natural antibodies to other carbohydrate antigens.

4. Evolutionary relationship between Anti-Gal and the α-Gal epitope

The observations that human serum contains anti-Gal, and that Galα1-3Galβ1-4GlcNAc-R glycoconjugates are found in a range of mammalian species, suggested that there might be an interesting evolutionary relationship between the expression of anti-Gal and the α-gal epitope. To investigate this, we isolated anti-Gal from normal human blood type AB serum by affinity column chromatography and tested its binding red blood cells and nucleated cells from various species. Parallel analysis was performed with the α-gal-specific, lectin *Bandeiraea (Griffonia) simplicifolia* IB4 [33]. Our studies demonstrated that the α-gal epitope is abundantly expressed on red blood cells and nucleated cells of marsupials as well as non-primate placental mammals, but is absent on cells from nonmammalian vertebrates including fish, amphibians, reptiles and birds (Table 2) [34,35]. The α-gal epitope was also found on cells from several groups of primates. It is abundant on cells of lemurs (prosimians) and of New World monkeys (i.e. monkeys of South and Central America-*platirhines*) such as marmoset, squirrel monkey and spider monkey [34,35]. TLC immunostaining of glycolipids from red blood cells of these species demonstrated that CPH with α-gal epitopes, which is abundant in rabbit and bovine red blood cells, is also present in New World monkey (squirrel monkey) red blood cells [34]. In contrast, no α-gal epitopes are present on red blood cells, and nucleated cells obtained from primates including Old World monkeys (monkeys of Asia and Africa-*catarhine*), apes and humans, all of which produce large amounts of the natural anti-Gal antibody (Table 2) [34, 35]. This striking differential expression of α-gal epitopes could be further demonstrated by Western blot immunostaining of thyroid glycoproteins using the natural anti-Gal antibody [36]. In these studies, the α-gal epitope was found to be most abundantly expressed on glycoproteins from cow and pig, where it was detected on a wide range of glycoproteins, whereas other nonprimate mammals and New World monkeys had lower amounts of such glycoproteins on thyroid cell membranes [36]. Analysis of α-gal epitopes on thyroglobulin as a representative secreted glycoprotein, demonstrated the same distribution as that summarized in Table 2 [16,20]. The observation that the α-gal epitope is absent in fish, amphibians, reptiles and birds indicates that the expression of the genes involved in the formation of this carbohydrate structure is a relatively recent event in the evolution of vertebrates. This event must have occurred early in the evolution of mammals, before placental and marsupial mammals diverged from each other (estimated to have occurred ~125 million years ago [mya]), since the epitope is present in both groups of mammals.

5. α1,3GT Expression and Evolution

The synthesis of the α-gal epitope is catalyzed by the enzyme UDP-Gal: 3Galβ1-4GlcNAc α1-3-galactosyltransferase (α1,3GT). α1,3GT utilizes UDP-Gal (nucleotide sugar donor) and either a glycosphingolipid or glycoprotein carrying Galβ1-4GlcNAc-R or Galβ1-3GlcNAc-R (acceptor substrate) as substrates and catalyzes the following reaction:

$$\underset{\text{Acceptor substrate}}{\text{Gal}\beta 1\text{-}4\text{GlcNAc-R}} + \underset{\text{uridine diphosphate-galactose}}{\text{UDP-Gal}} \xrightarrow{\alpha 1,3\text{GT}} \underset{\alpha\text{-gal epitope}}{\text{Gal}\alpha 1\text{-}3\text{Gal}\beta 1\text{-}4\text{GlcNAc-R}} + \text{UDP}$$

α1,3GT activity was first detected in rabbit bone marrow cells by Basu and Basu [37] and subsequently in rabbit intestinal submucosa by Betteridge and Watkins [38], in mouse

plasmacytoma cells by Blake and Goldstein [39] and in bovine thymocytes by Blanken and van den Eijnden [40]. Approximately 15 years after its first characterization, the enzyme was cloned by Lowe and coworkers [41] from mouse and by Joziasse et al. [42] from bovine cells. Transfection studies by Smith et al. [43] with the cloned α1,3GT demonstrated that the enzyme functions in the Golgi apparatus and competes with sialyltransferase for the same *N*-acetyllactosamine non-reducing terminal residues on glycolipids and glycoproteins. In general, the species distribution of α-gal epitopes expressed on cell membranes or on secreted glycoproteins parallels the distribution of α1,3GT activity. Thus, α1,3GT was found in cells and tissues of non-primate mammals and New World monkeys, but not in Old World monkeys and humans [35,36].

The α1,3GT is a membrane bound type 2 protein (i.e. the N-terminus is part of the cytoplasmic domain) with ~370 amino acids in length. The availability of the α1,3GT gene sequence provided the opportunity to gain an understanding of the molecular basis for the evolutionary distribution of α-gal epitopes in various species. Coding sequences for α1,3GT were determined for several species (bat, mink, dog, sheep and dolphin), all of which were shown to contain Galα1-3Galβ1-4GlcNAc-R glycoconjugates [44]. Although some variation in amino acid sequence is found in the N-terminal region (amino acids 1-99) of the α1,3GT sequences from this wide range of species, there is a remarkably high degree of sequence homology (~90%) among the sequences from amino acid 100 to the C-terminus, a region of the protein known to be critical for enzyme activity (see below).

Southern blots of human DNA probed with the cow α1,3GT sequence demonstrated that humans contained a homologous pseudogene [42]. The human α1,3GT pseudogene was found on chromosome 9 and it was shown to contain the same exon/intron organization as the active gene in mouse [45]. The longest exon (exon 9) which encodes most of the catalytic domain of the α1,3GT gene, was cloned by Lowe and coworkers [46] from human DNA, and its sequence was found to contain two point mutations (deletions) which results in a frameshift and a premature stop codon. Sequencing of this exon in chimpanzee showed the same two mutations, whereas the sequence from gorilla and orangutan only include the first frameshift mutation that produces a premature stop codon [47]. Analysis by Koike et al. [48] and Lanteri et al. [49] of the α1,3GT gene expression by reverse-transcriptase PCR assays demonstrated that mRNA from this gene can be detected in humans, orangutan and Old World monkey cells (48,49). Additional frameshift mutations in exons 7 and 8 were found, indicating that structural mutations (i.e. deletions in the coding regions of the gene), rather than regulatory mutations have been the primary cause for evolutionary inactivation of the α1,3GT gene. The α1,3GT sequences of exon 9 from humans and apes α1,3GT pseudogene have similar mutations which differ from those found in Old World monkey α1,3GT sequences, whereas common mutations are found among the Old World monkey α1,3GT sequences [47]. These findings may suggest that the α1,3GT gene was inactivated in ancestral Old World primates sometime after the divergence of apes and monkeys [47,50,51]. This divergence is estimated by paleontologists to have occurred ~28 million years ago [52]. Finally, another intronless α1,3GT pseudogene in chromosome 12 was reported by Joziasse et al. [45], and was observed in other primates as well [52]. This pseudogene contains several deletions and appears to be the result of the conversion of α1,3GT mRNA into cDNA with subsequent insertion into human chromosome 12. Based on detailed analysis of the two pseudogenes in Old World monkeys vs. that of the active gene in New World monkeys and prosimians, Koike et al. [52] suggested that the inactivation of the α1,3GT gene occurred shortly before the divergence of monkeys and apes, i.e. ~28 mya.

6. Possible causes for the evolutionary inactivation of α1,3GT

Since α1,3GT is only found in mammals and not in other vertebrates, it must have appeared at a relatively recent stage of vertebrate evolution. The function of the α1,3GT product, the α-gal epitope, is unknown. The fact that it was inactivated in ancestral Old World primates (monkeys and apes) after they diverged from New World monkeys implies that it was not essential for survival. However, some biological significance for this epitope may be inferred from the observation that mice with a disrupted α1,3GT gene (i.e. knockout mice for the α1,3GT gene) have cataracts [53,54]. Thus, in some mammalian species the α-gal epitope may have certain biological roles, possibly in cell-cell or cell-matrix interaction. Based on a detailed comparative analysis of the α1,3GT gene in primates that express an active α1,3GT (i.e. prosimians and New World monkeys) with the pseudogene in Old World monkeys and apes, Koike et al. [52] suggested that the inactivation of the α1,3GT gene likely coincided with the appearance of other glycosyltransferases which produced products that replace the biological functions of the α-gal epitope.

The fact that α1,3GT and α-gal epitopes have been conserved in New World monkeys (evolving on the South American continent which was separated from the African continent for >35 million years) and in lemurs (prosimians residing in the Madagascar, a land mass isolated from the African continent for > 60 million years) suggests that the inactivation of the α1,3GT gene in ancestral primates occurred along distinct geographical boundaries, i.e. in primates that resided on the “Old World” land mass comprised of Africa, Asia and Europe [34,35,50,51]. It is possible that the inactivation of the α1,3GT gene, which resulted in the disappearance of α-gal epitope expression in ancestral Old World primates and the subsequent appearance of the anti-Gal antibody, was associated with a strong selective pressure on primates, which was confined to the Old World continents. Because of the geographical separation of the New and Old World continents, this selective pressure would not have affected the evolution of primates in the South American continent (i.e. New World monkeys) or in Madagascar (i.e. lemurs). The observed inactivation of primate α1,3GT along distinct geographical boundaries, suggests that it was exerted by an infectious agent that was endemic to the connected continents of the Old World. This infectious agent may have been a virus, bacteria, or protozoan that was detrimental to monkeys and apes and that expressed α-gal epitopes, or an immunologically cross-reactive carbohydrate structure. Inactivation of the α1,3GT gene, resulting in suppression of α-gal expression in ancestral Old World primates would have eliminated immune tolerance to this epitope and thus enable production of the anti-Gal antibody as means of protection against the pathogen(s) expressing α-gal epitopes. This possibility is supported by the following observations: i) enveloped viruses propagated in cells with active α1,3GT express α-gal epitopes on their glycoproteins, whereas the same virus propagated in cells lacking α1,3GT, do not express α-gal epitopes [55-57]; ii) bacteria and protozoa have been found to express carbohydrate epitopes with structures similar to the α-gal epitopes, as indicated by their ability to bind anti-Gal [30,58-60]; and iii) anti-Gal has been shown to destroy viruses and protozoa expressing α-gal epitopes [61-63].

An alternative cause for inactivation of the α1,3GT gene could have been the use of the α-gal epitope as a cellular receptor by a pathogen. (e.g. a docking receptor for a virus, or a receptor for a bacterial toxin). A current example for such an activity is the enterotoxin A of *Clostridium difficile*. The primary ligand for this toxin on intestinal cells is the α-gal epitope [64]. Thus, endemic infection of Old World primates with a bacterial strain producing an α-gal binding toxin could have induced a selective pressure for the evolution of primates which lacked α-gal epitopes and thus, were not susceptible to the toxin's effect. Individuals successfully suppressing α-gal epitope expression would have lost immune tolerance to the α-gal epitope and would have produced the natural anti-Gal antibody in response to antigenic stimulation by gastrointestinal bacteria expressing carbohydrate epitopes with structures similar to that of the

α-gal epitope. Currently, such antigenic stimulation in humans is mediated by a number of bacterial strains in the intestinal flora [30,58].

There is an intriguing parallelism between the selective evolutionary event of α1,3GT inactivation in ancestral Old World primates and the fossil record. Based on the large number of ape fossils and the variety of ape species inferred by these fossils, Andrews suggested that apes were a very successful group of primates in the late Miocene (~26 million years ago [mya]) [65]. However, their numbers and the variety of their species started declining ~20 mya, to an extent that no ape fossils have been recovered from the period of 5-10 mya. Subsequently, apes fossils dated at <5 million years old reappeared as the various hominoids and hominids. These observations raise the possibility that the strong selective pressure for suppression of α-gal epitope expression may have been associated with the observed decline of ape species in the period between 5-20 mya [51]. A parallel analysis is impossible with Old World monkeys since their fossil record in periods earlier than 12 mya is very poor, and is limited to only one or two species [51,65].

An interesting example for a possible recent inadvertent “microevolution” for elimination of α-gal epitope expression is that of Chinese hamster ovary (CHO) cells. These cells originate from hamster, which like other nonprimate mammals, has active α1,3GT. However, the α1,3GT gene is not expressed in CHO cells and thus, they lack α-gal epitopes [43,66]. It is possible that the processes associated with culturing these cells in culture medium supplemented with human serum during the 1950s (before fetal calf serum was introduced into culture media) resulted in the selection of CHO cells lacking α-gal epitopes. This is because the natural anti-Gal antibody present in the human serum bound to cells expressing α-gal epitopes (i.e. CHO cells with active α1,3GT) and thus, prevented their growth.

7. Structure, mechanism and acceptor substrate specificity of α1,3GT

Although glycosyltransferases as a protein family have little amino acid sequence homology, they do share a common protein domain structure composed of a short cytoplasmic domain, a transmembrane domain that anchors glycosyltransferases in the Golgi membrane, and a stem domain that can be cleaved to release a soluble, catalytically active fragment which is located in the lumen of the Golgi. Truncation studies of α1,3GT demonstrated that the first 90 amino acids of the marmoset enzyme were unnecessary for enzyme activity, whereas removal of additional amino acids resulted in proteins with lower enzyme activity [67]. Minimal activity was observed for constructs containing amino acids 94 to the C-terminus (amino acid 376). Similar results were obtained with murine α1,3GT. Interestingly, removal of 3 amino acids at the C-terminus of the primate or murine protein resulted in the complete elimination of the catalytic activity [67]. Thus, nucleotide deletions in the α1,3GT gene sequence in human and ape DNA which produce a stop codon at the amino acid equivalent to 284 in the marmoset α1,3GT sequence would produce an inactive protein [67]. As described below, structural studies of α1,3GT have demonstrated that the C-terminal region of α1,3GT is critical for the formation of the active site of the enzyme.

Gastinel and co-workers published the first X-ray crystal structure of the catalytic domain (amino acids 80-368) of bovine α1,3GT at the 2.5Å level [68]. The structure (Form I) was obtained from crystals formed in the absence of substrates, and analyzed both in absence and presence of UDP-Gal and Mn^{2+} after soaking the crystals in this substrate. Both structures were of globular shape with folding of an α/β protein having central β-sheet and α-helical elements. The electron density of the side chain carboxyl group of E317 was extended in the substrate-bound enzyme, and this observation led Gastinel and co-workers to propose that galactose was covalently bound to E317 [68]. Thus, they proposed that the reaction mechanism occurred by a double-displacement process.

Boix et al. further refined the structure of the bovine α1,3GT construct to a level of 1.53Å [69]. At this higher resolution, a clearer picture of the structure of the catalytic site of the enzyme was obtained. In the substrate bound form (Form II), the C-terminal segment (amino acids 358-368 of the bovine sequence) was found to adopt a highly structured conformation that was part of the catalytic site, presumably due to a donor substrate induced conformational change upon binding. As described above, we demonstrated that truncation of the C-terminal region by as few as three amino acids of α1,3GT eliminates the enzyme's activity[67].

In another study by Boix et al. [70], the structural and mechanistic properties of α1,3GT were further clarified. Crystal structures of α1,3GT containing the donor UDP-Gal or UDP-Glc and acceptor lactose or N-acetyllactosamine substrates were obtained [70]. Structural and calorimetric binding studies were consistent with an ordered binding mechanism in which the donor substrate binds first causing a conformational change followed by acceptor binding with the direct participation of UDP. The results demonstrated that catalysis proceeded by cleavage of the galactose-UDP bond producing non-covalent complexes containing buried galactose. The location of the buried monosaccharide and results from molecular modeling studies also suggested that E317 was an important residue in UDP-Gal binding, but for a different reason than acting as the catalytic nucleophile. Boix et al. proposed that the mechanistic significance of E317 maybe to induce a distorted conformation of UDP-Gal to properly place it in the enzyme catalytic site [70]. In contrast to the covalent intermediate double-displacement mechanism proposed by Gastinel and co-workers [68], this mechanism is more consistent with an S_Ni mechanism involving nucleophilic attack by the acceptor simultaneously with UDP release on the same side of the galactose ring [71].

We [72] recently prepared a series of E317 mutants (Glu to Asp, Ala, Cys and His) to evaluate the importance of this residue. Under a double-displacement mechanism, site-directed mutagenesis of E317 to Asp would be expected to retain perhaps most enzyme activity, however substitution of either Ala or Cys would be expected to result in inactive proteins or ones with very low activity. Our results demonstrated that changes of E317 to either Asp, His, Ala, or Cys resulted in mutant enzymes that had substantially reduced, but measurable activity compared to the wild-type enzyme under standard reaction conditions. Substrate saturation studies demonstrate that a minor change in the functional group at amino acid 317 of bovine α1,3GT resulted in a substantial increase in the apparent Km for both substrates, and may reflect a change in the enzyme's affinity for the two substrates. Our results indicate that either a residue in the active site other than E317 is involved in a nucleophilic double-displacement mechanism or, that the reaction proceeds via a reaction mechanism not requiring a covalent intermediate such as an S_Ni mechanism [72]. Clearly, further studies are needed to identify the actual mechanism for α1,3GT.

Zhang et al. [73] evaluated the function of four tryptophan residues (Trp residues 245, 250, 314 and 356) that are near the active site of α1,3GT in substrate binding and catalysis. Substitution of a Gly for Trp^{249} reduced the affinity of the enzyme for lactose, and Trp^{250} (Tyr) reduces the k_{cat} for transfer of galactose to lactose. The mutant has significantly reduced transferase activity, but its UDP-Gal hydrolysis activity was similar to that of the wild type enzyme. Structural analysis of Trp^{249} (Gly) and Trp^{314} (Tyr) showed that the mutations had little effect on the overall structure of the enzyme, but the amino acid substitution of Trp^{249} (Gly) altered local amino acid interactions, whereas the local structure of the Trp^{314} (Tyr) mutant was very similar to that of the wild type enzyme. Finally, the mutation of Trp^{356} (Thr) produced a protein had lower transferase and hydrolase activity, and a significantly higher apparent Km for the acceptor substrate.

More recently, Brew and coworkers [74] have described the crystal structure of a mutant form of α1,3GT (Arg365Lys) bound to a UDP-Gal inhibitory analogue, UDP-2F-Gal. The inhibitor

is bound in a bent configuration to the mutant α1,3GT and conformational changes in the protein are observed when comparing the apo protein vs. the protein with UDP-2F-Gal bound. Based on these results, Brew and colleagues propose that there is a role for ground state destabilization in the catalytic process of α1,3GT. They also observed that UDP-2F-Gal binding results in a reduction in the flexibility of two loops, one centered around Trp195 and the other containing residues near the C-terminus of the enzyme. They conclude that the structural changes observed are connected to the formation of the binding site for the acceptor, cleavage of the nucleotide sugar bond and UDP release. Disordered loops with highly conserved amino acids have been observed in other glycosyltransferases, including the blood group A and B transferases as reported by Palcic and coworkers [75]. In mutants of the A and B transferases that contain single amino acid substitutions for one of the highly conserved loop amino acids, Yazer and Palcic [75] have demonstrated that these mutation affect enzyme turnover.

We [76] probed the acceptor substrate specificity of the α1,3GT using structural variants of Galβ,4GlcNAc (i.e. azido, hydroxyl, formamido, propionamido, succinimido and N-acrylamido) of various sizes and hydrophobicity, and showed that it tolerates modifications at C2 and C3 of the N-acetylglucosamine residue, and at C3 of the galactose residue, and produces a family of compounds with a nonreducing α1,3 linked galactose. From these studies it appears that a portion of the active site involved in acceptor substrate binding is hydrophobic and does not tolerate disaccharides with charged substituents at some sites around the sugar rings (an exception is the C3 of galactose). Interestingly, a 2-amino substitution at the C2 position of GlcNAc in Galβ,4GlcNAc was not tolerated by the α1,3GT. Thus, groups larger or smaller than the naturally occurring N-acetyl do not eliminate binding to the enzyme, but the presence of a positively charged group at this position does. Although α1,3GT does not bind the type 1 (Galβ1,3GlcNAc) structural analogue of Galβ1,4GlcNAc, the type 1 disaccharide is bound by the enzyme and acts as an effective competitive inhibitor [76].

8. Clinical significance of anti-Gal and the α-gal epitope in xenotransplantation

The inactivation of the α1,3GT gene in ancestral Old World primates and the subsequent production of the natural anti-Gal antibody has resulted in the formation of a unique immunological barrier that presently prevents transplantation of pig organs in humans (referred to as xenotransplantation). Xenotransplantation is a research area of considerable clinical significance since the number of organ donors (e.g. heart and kidney donors) in humans is insufficient. Thus, many patients in need of a heart or kidney transplant die without receiving a graft to replace their failing organ. Extensive research as been performed on the possible use of pig organs (xenografts) as grafts instead of human organs (allografts). Pig is considered a suitable organ donor because its organs are of similarity in size to many human organs. However, the binding of human natural anti-Gal to the millions of α-gal epitopes expressed on each pig cell was expected to cause xenograft rejection [77]. This rejection is the result of cell lysis following complement activation by bound anti-Gal, and antibody dependent cell mediated cytolysis (ADCC) in which anti-Gal IgG molecules binding to α-gal epitopes on the pig cells direct the subsequent binding of cytolytic effector cells such as macrophages and NK cells [77-81]. Indeed, pig kidneys and hearts transplanted into Old World monkey such as rhesus, cynomolgus and baboon, were found to be rejected within 30 min to several hours by a process designated as hyperacute rejection [82,83]. In addition to complement activation, binding of anti-Gal antibodies to $>10^7$ α-gal epitopes expressed per endothelial cell of the xenograft blood vessel walls was shown by Platt and colleagues [82] to induce platelet activation and aggregation resulting in collapse of the xenograft vascular bed, ischemia and rapid rejection of the graft.

That anti-Gal is the major cause for hyperacute rejection was demonstrated by Cooper and coworkers [84] in studies on transient neutralization of this antibody *in vivo* by infusion of the disaccharide Galα1-3Gal. This infusion process delayed hyperacute rejection by several hours [80]. Moreover, removal of anti-Gal from the blood of monkeys using an affinity column with α-gal epitopes, was shown by Sachs and coworkers [85] to delay of the rejection for up to 10 days. However, reappearance of anti-Gal in the circulation due to its continuous production by the immune system caused rejection of xenograft in these monkeys [85].

Because α1,3GT competes with other “capping” glycosyltransferases within the Golgi apparatus for the same acceptor substrates, several research groups attempted to decrease expression of α-gal epitopes by the introduction of α1,2fucosyltransferase [86,87], or sialyltransferase [88] into cells to reduce the level of α-gal epitopes. A decreased expression of α-gal epitope was also achieved in transgenic pigs expressing *N*-acetylglucosaminetransferase III [89]. This enzyme can potentially compete with other branching enzymes, producing N- glycans with bisecting GlcNAc residues. Although the exact mechanism of suppressing the level of α-gal epitopes in these animals is unknown, it may be caused by the inhibition of N-link glycan branching or a reduction in the processing of the glycan chains [89]. These various approaches, were effective in decreasing the number of α-gal epitopes/cell, however, they did not completely eliminate this epitope. Transplantation of xenograft expressing even small numbers of α-gal epitopes is detrimental to the graft because as many as 1% of human B lymphocytes are quiescent, but are capable of producing anti-Gal when activated [32]. These anti-Gal B cells are readily activated by even a small number of α-gal epitopes on a xenograft, resulting in an increased amount of high affinity anti-Gal antibodies that are potent enough to mediate destruction of the xenografts [90-92].

Complete elimination of α-gal epitopes was achieved in pigs by targeted disruption (i.e. knockout) of the α1,3GT gene. The feasibility of cloning an α1,3GT knockout (KO) in nonprimate mammals was first demonstrated in mice by Thall, Lowe and coworkers [53,93] and by d'Apice and coworkers [54,94]. Subsequent success of Ayares and coworkers [95,96] and of Prather and coworkers [97,98] in cloning of α1,3GT KO pigs by nuclear transfer, brought xenotransplantation closer to clinical reality. Transplantation of kidneys or hearts from these pigs into monkeys, performed by Sachs, Cooper and coworkers [99-101] and by Zhong and coworkers [102] did not result in hyperacute rejection since anti-Gal does not bind to the grafted cells and xenografts functioned for few weeks to several months.

However, anti-Gal is not the only obstacle to successful xenotransplantation. Most pig proteins are likely to be immunogenic in humans since they differ from the homologous proteins in humans in some of their amino acid sequences. Thus, when the human immune system is exposed to the various pig proteins, antibodies to the multiple foreign peptide epitopes are likely to be produced. The activity of these antibodies in humans (referred to as anti-non gal antibodies) has been demonstrated in patients who received pig ligament transplants for replacement of torn anterior cruciate ligament (ACL) [103]. The pig ligaments were treated with recombinant α-galactosidase [104] to eliminate α-gal epitopes, then underwent mild cross linking of collagen fibers by glutaraldehyde. The latter step was performed to prevent rapid destruction of the ligament graft due to immune rejection. Patients transplanted with these pig ligaments did not display a significant increase in anti-Gal activity since the ligaments were devoid of α-gal epitopes. However, there was a continuous production of anti-non gal antibodies against a very large number of pig ligament proteins [103]. These antibodies gradually destroyed the implanted xenograft. This destruction is very slow because of the mild cross linking of the ligament fibers by glutaraldehyde. Based on the activity of anti-non gal antibodies, it was estimated that the pig ligament is destroyed within 2 years of implantation. However, this slow rate of rejection enables the patient's fibroblasts to infiltrate into the destroyed regions, align themselves with the scaffold of pig collagen fibers and secrete their

own collagen. Therefore, the pig tissue is gradually replaced by the recipient's autologous ligament tissue (in a process called “ligamentization”) without losing the biomechanical function of the ligament. The continuous production of anti-non gal antibodies against the xenograft suggests that a similar response is likely to occur in recipients of organs. The appearance of these anti-non gal antibodies was reported in monkeys transplanted with pig kidneys devoid of α-gal epitopes (i.e. from α1,3GT KO pigs) [102]. Since live xenograft organs must maintain their viability, they cannot be cross linked by glutaraldehyde. Therefore, extensive research is currently conducted in controlling the production of anti-non gal antibodies (e.g. by immune suppression, or by induction of specific immune tolerance) while preserving the ability of the immune system to protect the recipient against opportunistic infections.

9. Exploitation of anti-Gal and the α-gal epitope for increasing immunogenicity of influenza (flu) and HIV vaccines

Since anti-Gal is produced in large amounts in all humans (unless they are severely immunocompromized) and since its ligand, the α-gal epitope can be readily synthesized by a variety of chemical, biochemical and genetic engineering methods, anti-Gal has potential for a variety of clinical uses, particularly in the areas of viral and cancer vaccines. The immunogenicity of some viral vaccines, including flu vaccine for use in the elderly, and HIV vaccine, is viewed as suboptimal. A major reason for the insufficient efficacy of vaccines is associated with poor uptake of the vaccine by antigen presenting cells (APC) such as dendritic cells and macrophages. Flu vaccine comprised mainly of the viral envelope glycoprotein “hemagglutinin” (HA) may serve as a useful example for the use of anti-Gal for vaccine targeting to APC. When a flu vaccine is administered by injection intramuscularly in the arm, no immune response occurs at the vaccination site, except for the uptake (internalization) of the vaccinating HA glycoproteins by APC. The internalized vaccine is transported by the APC to adjacent lymph nodes. The APC further process the vaccinating glycoproteins into peptides that are presented on the APC in association with class I or class II major histocompatibility complex (MHC) molecules. T cells can recognize the vaccine and be activated by it only if the T cell receptors can bind to the corresponding specific peptides presented on MHC molecules of APC. An effective T cell activation by the vaccine peptides is a prerequisite for eliciting a protective anti-flu antibody response (which is dependent on helper T cell activation) and activating cytotoxic T cells (CTL) that destroy flu virus infected cells. If the vaccine is delivered in large enough amounts, its uptake by APC will suffice to induce an effective immune response. However, due to the paucity of the vaccinating HA glycoproteins within annual vaccine (15μg HA of each of the 3 vaccinating strains in “subunit” or “split” vaccine), and since these vaccinating glycoproteins have no markers that identify them for uptake by APC at the vaccination site, uptake of the vaccine by APC is minimal since it is only mediated by random endocytosis. This results in suboptimal immunogenicity.

Immunogenicity of flu vaccine may be greatly improved if it is effectively targeted for uptake by APC. This can be achieved by processing the vaccinating HA to carry α-gal epitopes on its multiple carbohydrate chains. Injection of α-gal carrying HA results in an *in situ* binding of anti-Gal to these epitopes and formation of immune complexes. APC, including macrophages, dendritic cells and Langerhans cells of the skin, all express Fcγ receptors (FcγR) that effectively bind the Fc portion of IgG molecules once these IgG molecules are bound to their corresponding antigens by their combining sites [105-109]. The APC targeting activity of any IgG antibody molecule bound to its corresponding antigen is referred to as opsonization and is the most effective mechanism by which APC identify and internalize vaccinating antigens [105-109].

In most strains of flu virus, HA has 6-8 N-linked carbohydrate chains, most of which are of the complex type [110-112]. Flu virus is usually propagated in embryonated chicken eggs.

Since, chickens, like other birds, lack α-gal epitopes, and since sialic acid (SA) is removed from viral glycoproteins by viral neuraminidase, the carbohydrate chains on HA terminate with N-acetyllactosamine (Galβ1-4GlcNAc-R) [110,111]. Thus, α-gal epitopes could be added to all carbohydrate chains of HA using recombinant α1,3GT [113,114]. To illustrated that this is possible, we cloned the α1,3GT gene from a New World monkey cell line [67], produced the recombinant enzyme in the yeast *Pichia pastoris* expression system [115], and used it to glycosylate inactivated flu virus. Using the recombinant α1,3GT and UDP-Gal in a 2h incubation at 37°C at least 3000 α-gal epitopes/virion were added, mostly on HA [113].

To determine whether inactivated flu virus processed to express α-gal epitopes is more immunogenic than the original virus lacking these epitopes, we used the α1,3GT knockout (KO) mouse generated by Thall, Maly and Lowe [53] as an experimental model. Wild type (WT) mice lack the ability to produce anti-Gal, since the α-gal epitope is a self antigen in mice and thus, they are immunotolerant to it. This immune tolerance is similar to that found in humans who have blood group A and thus, they cannot produce anti-A antibodies. The α1,3GT KO mice (referred to as KO mice) lack α-gal epitopes and thus, have lost immune tolerance to it. After being immunized with xenograft tissue such as pig kidney membrane homogenate, KO mice produce anti-Gal in titers comparable to those found in human serum. Immunization of KO mice with 1μg of inactivated flu virus vaccine expressing α-gal epitopes, administered twice in 2 weeks interval, resulted in ~100 fold higher anti-flu virus antibody and T cell response in comparison to the immune response in KO mice immunized with the original inactivated flu virus lacking α-gal epitopes [114]. Moreover, intranasal infection (challenge) of the immunized KO mice with live virus and monitoring their survival for one month, demonstrated 11% mortality in mice immunized with the vaccine expressing α-gal epitopes, vs. 89% mortality in KO mice immunized with vaccine lacking this epitope [114]. These findings imply that the *in situ* formation of immune complexes between the flu vaccine and anti-Gal of the vaccinated mice resulted in effective targeting of the vaccine to APC, thereby increasing the efficacy of the vaccine many fold. It is probable that immunization with HA expressing α-gal epitopes, rather than with the intact inactivated virus, will also result in increased immunogenicity due to anti-Gal mediated targeting. This is suggested from our studies on immunization with gp120 of HIV processed to express α-gal epitopes [116].

The major glycoprotein in the envelope of HIV is gp120 which has 24 N-linked glycosylation sites of which 13-16 carbohydrate chains are of the complex type. When produced as a recombinant protein in CHO cells, the gp120 carbohydrate chains have the terminal structure SA-Galβ1-4GlcNAc-R [117]. We could convert these structures to α-gal epitopes by incubation of the glycoprotein with a mixture of neuraminidase (for removal of SA), α1,3GT and UDP-Gal [116]. Two immunization of KO mice with 5μg gp120 processed to express α-gal epitopes resulted in ~100 fold higher anti-gp120 antibody response and a similar higher T cell response against gp120 peptides, in comparison to the immune response in KO mice receiving similar immunizations with gp120 lacking α-gal epitopes [116].

This principle of increasing vaccine immunogenicity by expression of α-gal epitopes, followed by anti-Gal binding to these epitopes, and resulting in targeting of the immune complexes to APC, is theoretically applicable to any microbial, soluble or particulate vaccine. If the vaccine has carbohydrate chains of the complex type, α-gal epitopes may be readily synthesized on the vaccine by the use of recombinant α1,3GT. Alternatively, vaccinating recombinant glycoproteins may be produced in cells that have active α1,3GT (e.g. CHO cells transfected with this gene [118]). If, however, the vaccine lacks these types of carbohydrate chains, synthetic α-gal epitopes may be coupled to the vaccine. If such a process is used, it is important to determine that the coupling process does not affect the immunogenic epitopes of the vaccine. For example, if the immunogenic regions of the vaccine include lysine residues, coupling α-gal epitopes to these lysines may affect the immunogenicity of those regions.

10. α-gal epitopes on autologous tumor vaccines

Anti-Gal may also be exploited for eliciting an immune response against vaccinating tumor antigens, thereby inducing the destruction of tumor cells by the immune system. Numerous studies, both in experimental animal models and in humans have indicated that tumor cells express a variety of tumor specific antigens. Some of the tumor antigens which have been characterized (e.g. carcinoembryonic antigen and MUC 1) are common to many types of tumors. The immune response to these common tumor antigens is usually very low because these antigens may also be expressed on normal cells. However, a large proportion of the tumor antigens are specific to the tumor type and to the patient. They appear in tumor cells as a result of the multiple mutations occurring in the course of the development of the malignant tissue. Therefore, the autologous tumor could be considered a suitable source for vaccine material.

As with viral vaccines, induction of an immune response against tumor antigens requires the effective uptake of the vaccinating tumor cells and cell membranes containing these antigens by APC. Tumors, however, evade the immune system by eliminating markers identifying them for uptake by APC. APC are “oblivious” to tumor cells, to the extent that such tumor cells can reside even within lymph nodes without being internalized by APC. Exploiting anti-Gal as an antibody targeting vaccines to APC can overcome the inefficient tumor vaccine uptake by APC, provided that such vaccines express α-gal epitopes.

10.1 Tumor vaccines engineered in vitro to express α-gal epitopes

We hypothesized that tumor cells or cell membranes can be subjected *in vitro* to synthesis of α-gal epitopes [119,120]. Tumor cells obtained from patients with hematological malignancies (leukemia, lymphoma and myeloma), or tumor cell membranes obtained from resected solid tumors that are homogenized, could be modified *in vitro* to express α-gal epitopes by incubation in a solution containing neuraminidase, recombinant α1,3GT and UDP-Gal [119,120]. After washing, and irradiation to kill any remaining live tumor, this autologous vaccine could be injected into the patient. Anti-Gal binding to the α-gal epitopes should induce effective targeting of the vaccinating cells and cell membranes to APC at the vaccination site, thereby eliciting a protective immune response against tumor antigens that are expressed on tumor cells remaining in the body. Treating tumor cells with neuraminidase, recombinant α1,3GT and UDP-Gal was found to produced ~10^6 α-gal epitopes/cell on a variety of human tumors [120]. The efficacy of these vaccines in eliciting a protective anti-tumor immune response was demonstrated in the α1,3GT KO mice experimental model. Mouse B16 melanoma was used as the tumor model since it lacks α-gal epitopes, and thus it mimics human tumor cells in this manner [121]. B16 cells expressing α-gal epitopes were generated by stable transfection with a plasmid containing the α1,3GT gene [121,122], transduction with an adenovirus vector [123], or with a retrovirus vector containing this gene [124,125]. Immunization of anti-Gal producing KO mice with irradiated B16 cells expressing α-gal epitopes resulted in the induction of an immune response that protected the mice against challenge with live B16 tumor cells [122-125]. This treatment did not induce any immune response to normal antigens on the vaccinating tumor cells and thus, did not result in an autoimmune response [125,126].

10.2 In situ conversion of tumor lesions into endogenous xenograft-like anti-tumor vaccines by expression of α-gal epitopes

In advanced states of cancer, metastatic tumor cells released from the primary tumor, develop into lesions that if they are refractory to chemotherapy, they are lethal. Even if these lesions are destroyed by various methods of ablation (e.g. irradiation, injection of ethanol, or thermal ablation), tumor cells within invisible micrometastases that are usually present in the patients, develop within weeks or months into lethal metastases. We have developed a method to use anti-Gal for the destruction of visible lesions and their conversion into endogenous vaccines

[127]. These endogenous vaccines elicit an immune response that can target micrometastatic tumor cells and destroy them. The treatment involves the intratumoral injection of glycolipids carrying α-gal epitopes (α-gal glycolipids). These glycolipids are isolated in relatively large amounts from rabbit red blood cell membranes [1-6] [128]. The red cell membranes are incubated with a mixture of chloroform, methanol and water for the extraction of glycolipids, phospholipids and cholesterol. By performing Folch partition [129], the α-gal glycolipids with carbohydrate chains of 5-25 carbohydrate units (e.g. CPH and CHH in Table 1 and multiantennary glycolipids with 10, 15, 20 and 25 carbohydrates) [1-6], are extracted in the aqueous phase. Phospholipids and cholesterol remain in the organic phase of the partition. When the α-gal glycolipids are dissolved in water, they form micelles. When injected into tumor lesions, the micellar glycolipids insert spontaneously into the tumor cell membranes. The α-gal glycolipids within the injected lesions interact with anti-Gal and induce activation of complement, generation of complement chemotactic factors C5a and C3a, and ultimately induce a local inflammation within the treated lesion. This inflammatory response recruits APC, as well as other inflammatory cells into the lesion. Tumor cells in the treated lesion expressing α-gal epitopes (i.e., the α-gal glycolipids) are destroyed by anti-Gal in a manner similar to xenograft rejection. Anti-Gal further opsonizes the tumor cells and targets them for effective uptake by APC, which transport the internalized tumor antigens to lymph nodes for the activation of tumor specific T cells.

We demonstrated the efficacy of this treatment in studies in the experimental model of KO mice and cutaneous B16 melanoma lesions. Intratumoral injection of 1mg α-gal glycolipids into 5mm melanoma lesions resulted in the destruction of most of the treated lesions, as well as in the induction of a systemic protective anti-tumor immune response. This protective immune response could be transferred by lymphocytes to naïve recipients, thus protecting them against challenge with B16 cells [127]. This treatment with α-gal glycolipids and the treatment above using autologous tumor vaccines engineered *in vitro* to express α-gal epitopes, are currently in Phase I clinical trials for evaluating the maximum tolerated dose and determining whether they cause any toxicity in patients. It should be stressed that, if successful, these anti-Gal mediated immunotherapies may be considered for most types of human tumors since cancer patients usually display anti-Gal activities in titers that are similar to those observed in healthy individuals (unpublished observations).

11. Conclusions

The α-gal epitope displays a distribution that is unparalleled by any other known carbohydrate structure. Its synthesis is catalyzed by the glycosyltransferase, α1,3galactosyltransferase (α1,3GT), only in a limited group of mammals including nonprimate mammals, prosimians and New World monkeys (monkeys of South America). The α-gal epitope is absent, however, in Old World monkeys (monkeys of Asia and Africa), apes and humans, all of which produce large amounts of the natural anti-Gal antibody that binds specifically to α-gal epitopes. The absence of α-gal epitopes in Old World monkeys, apes and humans is the result of evolutionary inactivation of the α1,3GT gene in ancestral primates due to deletion mutations that prevent the synthesis of a catalytically active α1,3GT. Anti-Gal is continuously produced in humans due to constant antigenic stimulation by bacteria of the normal intestinal flora. This antibody functions as a barrier to pig xenografts since anti-Gal binding to α-gal epitopes on xenograft cells results in immune mediated rejection. This barrier has been overcome by the generation of α1,3GT knockout pigs lacking α-gal epitopes. Since anti-Gal in naturally present in large amounts in all humans that are not severely immuno-compromised, it can be exploited in a clinical setting for increasing the immunogenicity of viral vaccines and autologous tumor vaccines processed to express α-gal epitopes, by targeting such vaccines to antigen presenting cells (APC) at the vaccination site. In the experimental animal model of α1,3GT KO mice, anti-Gal binding to α-gal epitopes on flu or gp120 HIV vaccines results in their opsonization

and effective targeting to APC which transport the vaccine to lymph nodes. The APC process and present the immunogenic peptides for effective activation of the immune system against the vaccinating antigens, resulting in a marked increase in immune protection against live virus. Immunization with tumor cells engineered to express α-gal epitopes also resulted in increased protection of KO mice against challenge with tumor cells lacking this epitope. In addition, intratumoral injection of α-gal glycolipids results in insertion of these molecules into tumor cell membranes, anti-Gal mediated destruction of the tumor lesion in a manner similar to xenograft rejection and effective targeting of the tumor cell membranes to APC. This results in conversion of the treated lesions into endogenous anti-tumor vaccines. Research on the α-gal epitope and the natural anti-Gal antibody may yield additional novel methods for clinical exploitation of this unique natural antibody that is present in large amounts in all humans.

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Table 1

Glycosphingolipid binding by anti-Gal

Glycosphingolipid	Anti-Gal reactivity
Glcβ1-1Cer (glucosylceramide)	-
Galβ1-4Glcβ1-1Cer (lactosylceramide)	-
Galα1-4Galβ1-4Glcβ1-1Cer (CTH)	-
GalNAcβ1-3Galα1-Galβ1-4Glcβ1-1Cer (Globoside)	-
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (Paragloboside)	-
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (CPH)	+
GalNAcα1-3GalNAcβ1-3Galα1-Galβ1-4Glcβ1-1Cer (Forssman antigen)	-
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (CHH)	+
Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (P_1 antigen)	-
Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (B antigen)	-,+
GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (A antigen)	-

CTH- ceramide trihexoside, CPH- ceramide pentahexoside. CHH- ceramide heptahexoside

Table 2

Expression of α-gal epitopes, α1,3galactosyltransferase (α1,3GT) and anti-Gal in mammals.

Mammals	α-gal epitope expression	α1,3GT activity	Anti-Gal production
Marsupials	+	+	-
Non-primate mammals	+	+	-
Prosimians	+	+	-
New World monkeys	+	+	-
Old World monkeys	-	-	+
Apes	-	-	+
Humans	-	-	+