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Invited Commentary

Potential impact of the non-human sialic acid *N*-glycolylneuraminic acid on transplant rejection risk

A major obstacle to clinical applications of xenotransplantation is the expression of immunogenic xenoantigens that provide targets for immune recognition of xenografts, leading to activation of host immunity and consequent rejection or poor engraftment. Among the best-known xenoantigens is the ''αGal'' epitope (Galα1-3Galβ1-(3)4GlcNAc-R, where R is an underlying glycoconjugate) characterized by Galili and colleagues. This epitope is widely expressed by most mammals other than old-world primates and recognized by abundant circulating human anti-α-Gal antibodies [1]. Such antibodies are universally induced after birth in humans via exposure to gut bacteria bearing similar epitopes [2], and the resulting difficulties in xenotransplantation [3] have even encouraged the production of alpha1,3-galactosyltransferase gene-knockout pigs [4] as a potential solution.

However, a second class of anti-carbohydrate xenoantibody recognizes glycans carrying the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) [5–9]. Unlike the case with αGal, the potential impact of Neu5Gc-glycans as xenoantigens is less well recognized. Here, we address the similarities and differences between these two xenoantigens (see Table 1 for a summary and comparison), concluding that anti-Neu5Gc antibodies are of potential relevance not only to xenotransplantation but also to allotransplantation and even to autotransplantation if methods are incorporated that require an ex vivo culturing phase using animal materials, including stem-cell-based therapies.

Sialic acids (Sias) are 9-carbon backbone acidic sugars terminating the glycan chains of various glycoproteins and glycolipids at vertebrate cell surfaces [10]. *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form Neu5Gc are the two major Sias in mammals, with the activated form CMP-Neu5Ac serving as the precursor for synthesis of CMP-Neu5Gc, catalyzed by the enzyme CMP-Neu5Ac hydroxylase (CMAH). This enzyme is specifically inactivated in humans, in contrast to other mammals studied to date, including old-world primates and pigs [10].

Despite inactive CMAH in humans and lack of an alternative pathway for Neu5Gc synthesis [11], Neu5Gc is found at low levels on human epithelia and endothelia and is especially enriched in tumors, apparently originating from dietary

Table 1. Similarities and differences between α-Gal and Neu5Gc xenoantigens in humans

Feature	α-Gal	Neu5Gc
Enzyme		
Biosynthetic enzyme	*GGTA1* gene encoding α1-3-galactosyltransferase (α1-3GT)[a]	*CMAH* gene encoding CMP-Neu5Ac hydroxylase (CMAH)
Inactive	α1-3GT inactive in old-world monkeys, apes, and humans	*CMAH* specifically inactive in humans, after the common ancestor with chimpanzees
Active	α1-3GT active in non-primate mammals and new-world monkeys	*CMAH* active in primate and non-primate mammals studied to date, except humans
Epitope(s)		
Presence	Abundant in non-primate mammals, prosimians, and new-world monkeys	Abundant in all primate and non-primate mammals studied to date
Absence	Absent in humans, apes, and old-world monkeys	In addition to humans, absent in non-mammalian vertebrates of the sauropsid lineage, including reptiles and birds
Metabolic incorporation from extrinsic sources	Not possible	Can be incorporated into humans, and into cultured cells in vitro, from Neu5Gc-rich mammalian sources
Presence in human tissues	Not possible	Incorporated into normal human epithelial and endothelial cells, enriched in human tumors
Target epitopes	Defined (Galα1-3Galβ1-(3)4GlcNAc-R) on glycoproteins, or on some glycolipids[a]	Many possible Neu5Gc-glycan epitopes on glycoproteins or glycolipids
Antibodies		
Levels	High in all individuals	Highly variable levels against different Neu5Gc-glycan epitopes
Isotypes	Anti-α-Gal IgA, IgG, IgM	Anti-Neu5Gc IgA, IgG, IgM

[a]In addition to α1-3GT, the glycosyltransferase iGb3 synthase (iGb3S) generates the α1-3Gal-containing glycosphingolipid called isoglobotrihexosylceramide (isogloboside 3, or iGb3) [42]. This enzyme is also inactive in humans [43].

Neu5Gc-rich foods, e.g., red meat and milk products (Neu5Gc is low or undetected in fish and poultry) [12,13]. In this regard, Neu5Gc can be taken up by cultured human cells in vitro from Neu5Gc-containing media supplements like fetal calf serum (FCS), and, being compatible with intrinsic human biochemical pathways, it is metabolically incorporated, resulting in its cell surface expression [13]. However, unlike the case with these biochemical pathways, the human immune system recognizes Neu5Gc as foreign, resulting in a humoral response involving a polyclonal highly diverse antibody profile in all humans [14,15]. This unique combination of metabolically incorporated Neu5Gc and circulating anti-Neu5Gc antibodies has a likely impact on human health issues related to consumption of Neu5Gc-rich foods, e.g., chronic inflammation–mediated tumor growth stimulation and exacerbation of vascular disease [16,17]. Importantly, unlike Neu5Gc, dietary α-Gal cannot undergo metabolic incorporation, as it would simply be converted into free galactose in the digestive tract or in cellular lysosomes. Thus, Neu5Gc is the first example of a ''xeno-autoantigen'', a non-human molecule that becomes part of ''self'', even while inducing an antibody response [15].

Persisting controversies about the importance of Neu5Gc in biological therapies

Despite all the previous facts, there are persisting questions about the significance of Neu5Gc as a target for immune reactions against biotherapeutic agents or cellular and tissue transplants [5–9]. The roots of this confusion can be traced back to two early misconceptions: the first that there might be alternate pathways for intrinsic production of Neu5Gc in humans [18] and second that anti-Neu5Gc antibodies are only present at insignificant levels in healthy humans [19]. The first issue has been effectively laid to rest by multiple studies showing that Neu5Gc found in human tissues or human stem cells is not of intrinsic origin [11,20]. The second misconception can be explained by the erroneous view that Neu5Gc is a single antigen, against which antibodies can be detected with a single ELISA assay [19]. Instead, Neu5Gc is a key component of a complex ensemble of Neu5Gc-glycan antigens that were not previously recognized.

Complexities of Neu5Gc-containing glycoconjugates and anti-Neu5Gc antibodies

The antigenic complexity of Neu5Gc-glycans arises at multiple levels: (i) modification of Neu5Gc by *O*-acetyl esters at positions C4, C7, C8, and/or C9, (ii) various linkages to underlying glycans (α2-3 Gal, α2-6Gal(NAc), or α2-8Sia), (iii) underlying glycans that can vary in length and/or structure, (iv) and their attachment to various scaffolds, e.g., glycolipids, or O-linked or N-linked glycans on glycoproteins [15]. Cell surface organization and density adds yet another level of complexity to the potential ''Neu5Gc sialome'' [21].

As the molecular weight of Neu5Gc is only 325.3 daltons, the binding pocket of an antibody should fit several additional underlying structures [22,23], either by fitting the full epitope into an extended groove or in shallow cavities with anchoring of the terminal monosaccharide [9]. Indeed, there are multiple potential epitopes that can be recognized by various corresponding ''anti-Neu5Gc'' antibodies, and the human anti-Neu5Gc antibody spectrum is broad and variable [15]. Such antibodies can be present at quite high levels, and yet be easily missed in ELISA assays, depending on the targets and protocols used for study. For example, using just α-linked Neu5Gc as a target allows the detection of only low levels of human anti-Neu5Gc antibodies [12]. However, this changes dramatically when using more extended glycan epitopes with terminal Neu5Gc – and even then, any one epitope picks up only a fraction of the antibodies [15]. As an analogy, using just alpha-linked Gal to test for the α-Gal epitope (Galα1-3Galβ1-(3)4GlcNAcR) would result in a much lower/inaccurate detection of the relevant antibodies.

Another major confounding factor in the detection of human anti-Neu5Gc antibodies is the presence of Neu5Gc in the animal-derived reagents commonly used in immune assays. For example, bovine serum albumin (BSA) is a common blocking reagent in many applications, including ELISAs. However, although BSA is not itself sialylated, even highly purified preparations are contaminated with other bovine serum glycoproteins that contain Neu5Gc glycans (V. Padler-Karavani & A. Varki, unpublished observations). These can absorb out anti-Neu5Gc antibodies, resulting in a markedly lower observed signal for anti-Neu5Gc antibodies. Many other commonly used blocking agents (e.g., milk or bovine/porcine gelatin) suffer from the same problem.

To overcome this issue, we have been using chicken ovalbumin [15] or fish gelatin [24] as blocking reagents, as they both lack Neu5Gc and thus do not interfere with detection assays. Using such methods, we detected a broad and variable spectrum of anti-Neu5Gc antibodies of IgM, IgG, and IgA constituting ~0.1% of total Igs, ranging at 0.1–23 μg/ml against several potential targets,

some at levels similar to anti-α-Gal antibodies [15]. We are currently studying this matter further using a sialoglycan array presenting multiple Neu5Gc-glycans, which further confirms and extends the previous findings (V. Padler-Karavani & A. Varki, unpublished data). However, to date, there is no precise way to measure total anti-Neu5Gc antibodies, and we suspect that even our array approach is underestimating amounts. Finally, given the high degree of variability of antibody levels and types in individual humans, studies that use just a few uncharacterized sera [25] can be quite misleading [26,27].

Potential significance of Neu5Gc and anti-Neu5Gc antibodies in transplant rejection

Given the above-mentioned considerations, it is time to revisit the many misconceptions about the significance of anti-Neu5Gc antibodies. As with the xenoantigen α-Gal, xenografts from other mammalian sources would potentially carry Neu5Gc-containing epitopes. Considering that all human sera have some level of anti-Neu5Gc antibodies, this may result in rejection of the xenograft. Indeed, this was recently demonstrated in a ''human-like'' mouse model, where Neu5Gc-deficient $Cmah^{-/-}$ mice induced to have pre-existing anti-Neu5Gc antibodies showed rejection of allotransplanted syngeneic Neu5Gc-positive $Cmah^{+/+}$ islets [6]. In the days when such approaches were considered ethical, attempts were also made to transplant organs from chimpanzees into human patients. Despite their close similarity (including lack of the α-Gal epitope), most of these chimpanzee heterografts failed within 2 months or less [28,29]. While serum samples from these experiments were not saved (K. Reemtsma, personal communication), it is reasonable to suggest that anti-Neu5Gc antibodies contributed to rejection. The same might be true of failed attempts at baboon heart transplants into humans [30].

In contrast, porcine cardiac valve transplants can have long-term success. However, in this approach, the valve is cleaned of all pig cells, leaving only a connective tissue matrix, which is repopulated by human cells in vivo [31]. It would be interesting to know whether there is any Neu5Gc remaining in such valve matrices after preparation for transplant and whether this alters efficacy. The same might be asked of biologic scaffold materials composed of mammalian extracellular matrix that are commonly used in regenerative medicine and surgical procedures for reconstruction of various tissues and organs [32].

Unlike the case with α-Gal, Neu5Gc can also be metabolically incorporated into human cells through consumption of Neu5Gc-rich foods, such as red meat, and it is mostly found in normal human epithelial and endothelial cells, likely reflecting the individual's diet. Hence, even allotransplantation from another human individual could potentially result in graft reaction or rejection, dependent on the actual situation for Neu5Gc accumulation and anti-Neu5Gc antibodies in the donor and recipient, respectively.

Given the almost ubiquitous presence of Neu5Gc in animal-derived materials used in the in vitro culture of cells in biotechnology [33], this potential problem even extends to human embryonic stem cells cultured in vitro [20,34–38]. Indeed, even the recent advances in induced pluripotent stem cell technology could result in return of autologous cells into humans that are contaminated with Neu5Gc glycans from the culture materials used in vitro.

On a more general note, it should also be recognized that lack of clear-cut in vitro complement-mediated killing [25] is no guarantee of safety, as deposition of complement and anti-Neu5Gc antibodies on transplanted cells in vivo could mobilize other immune processes, such as macrophage recognition and antibody-dependent cellular cytotoxicity (V. Padler-Karavani & A. Varki, unpublished). Likewise, model systems, such as Neu5Gc-deficient mice, could create a false sense of security about transplant success [6] unless human-like levels and patterns of anti-Neu5Gc antibodies are assured (something that is difficult to do in such mice, C. Gregg and A. Varki, unpublished observations).

Approaches to prevent potential anti-Neu5Gc antibody-dependent rejection

Based on all of the above considerations, it is reasonable to take a cautionary approach toward transplants where Neu5Gc of intrinsic or extrinsic origin could be present and where the recipient has anti-Neu5Gc antibodies against the specific Neu5Gc-glycan epitopes present in the graft. With regard to the anti-α-Gal xenoantibody barrier of hyperacute rejection, several approaches to depletion have been pursued, including ex vivo organ perfusion, plasmapheresis, immunoadsorption, complement inhibition, and, finally, the generation of an α-Gal-deficient pig by eliminating the α1-3-galactosyltransferase (α1-3GT) encoded by the *GGTA1* gene [4,29,39]. Analogous approaches could be explored to address the potential adverse effects of Neu5Gc and anti-Neu5Gc xeno-autoantibodies,

and eventually, to generate an engineered pig that is both Neu5Gc-deficient and Gal-deficient (*Cmah*$^{-/-}$ *Ggta1*$^{-/-}$ DKO). In this regard, thymocytes from *Cmah*$^{-/-}$*Ggta1*$^{-/-}$ DKO mice were recently tested and shown to have attenuated complement-dependent cytotoxicity of human serum antibodies, when compared with cells from wild-type mice and single-deficient mice [40].

In addition to the previous approaches bearing similarity to α-Gal, the unique capability of Neu5Gc to be metabolically incorporated into cells intended for transplant can now be addressed. We recently showed that the Neu5Gc content of cultured human and non-human cell lines can be markedly reduced by simply adding an excess of Neu5Ac (the precursor non-immunogenic human sialic acid) to the culture medium [33]. This approach could also be exploited to "chase-out" Neu5Gc from cells and/or organs before xenotransplantation [26]. The recent successes in large-scale production of Neu5Ac [41] also render such an approach economically feasible. Indeed, while methods are being developed to predict the true relevance of anti-Neu5Gc antibodies in a given transplant situation and recipient, it seems logical to try this simple, non-toxic, and relatively inexpensive approach to minimize any risks involved. As we stated in an earlier publication on this issue: "If any of us were unfortunate enough to require (such) treatment…..we would strongly prefer that the non-human Neu5Gc molecule not be present on grafted cells." [26].

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