In vivo Neutralization of Naturally Existing Antibodies against Linear α(1,3)-Galactosidic Carbohydrate Epitopes by Multivalent Antigen Presentation: A Solution for the First Hurdle of Pig-to-Human Xenotransplantation

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Dedicated to Professor Daniel Belluš on the occasion of his 70th birthday

Abstract: Pig-to-human xenotransplantation of islet cells or of vascularized organs would offer a welcome treatment alternative for the ever-increasing number of patients with end-stage organ failure who are waiting for a suitable allograft. The main hurdle are preexisting antibodies, most of which are specific for ‘Linear-B’, carbohydrate epitopes terminated by the unbranched Gal-α(1,3)Gal disaccharide. These antibodies are responsible for the ‘hyper-acute rejection’ of the xenograft by complement mediated hemorrhage. For depletion of such antibodies we have developed an artificial injectable antigen, a glycopolymer (GAS914) with a charge neutral poly-lysine backbone (degree of polymerization n = 1000) and 25% of its side chains coupled to Linear-B-trisaccharide. With an average molecular weight of 400 to 500 kD, presenting 250 trisaccharide epitopes per molecule, this multivalent array binds anti-αGal antibodies with at least three orders of magnitude higher avidity on a per-saccharide basis than the monomeric epitope. In vivo experiments with non-human primates documented that rather low doses – 1 to 5 mg/kg of GAS914 injected i.v. – efficiently reduce the load of anti-Linear-B antibodies quickly by at least 80%. This treatment can be repeated without any sensitization to GAS914. Interestingly, although the antibody levels start raising 12 h after injection, they do not reach pretreatment levels. The polymer is degraded and excreted within hours, with a minute fraction remaining in lymphoid tissue of anti-αGal producing animals only, probably binding to and inhibiting antibody-producing B-cells. The results of pig-to-non-human primate xenotransplantations established GAS914 as a relevant therapeutic option for pig-to-human transplantations as well. The synthesis of GAS914 was successfully scaled up to kg amounts needed for first clinical studies. Key was the use of galactosyl transferases and UDP-galactose for the synthesis of the trisaccharide.

Keywords: Carbohydrate antigens · Enzymatic glycosylation · GAS914 · Glycopolymer · Xenotransplantation

The availability of human organs for transplantation to treat end-stage organ failure is limited to about 25% of patients on waiting lists. This has led to a great interest in the possibilities of xenotransplantation with the main focus on the pig as donor. Although organs from the larger non-human primates would pose the least immunogenic barrier, ethical considerations as well as the high risk of cross-species virus transmission preclude such an option.[1] On the other hand, the immune reaction towards a pig-to-human xenograft includes all branches of the immune system, resulting in hyperacute rejection, acute vascular rejection, and – after a period of accommodation – cellular rejection as well as chronic rejection. The first hurdle – the hyper-acute rejection – is initiated within minutes upon contact with monoreactive naturally existing antibodies directed to carbohydrate epitopes, leading to complement activation and destruction of the organ by hemorrhage and thrombosis within hours. These preexisting antibodies originate from cross-immunization by bacterial flora upon colonization of the intestine after birth. The predominant antigen is related to a carbohydrate epitope found on proteins and lipids of all mammals except primates.[2] This epitope – also called ‘Linear B’ (1) – is terminated with an α(1,3)-galactosidic linkage on an unbranched galactose, i.e. the α(1,2)-linked fucose of human blood group epitopes (2, 3) is missing (Fig. 1). Such variations in glycosylation patterns are the result of species-specific differences in glycosyl transferase genes. The fraction of preformed antibodies directed to these antigens widely differs between individuals, and can reach 3% of all preexisting antibodies.
The hyper-acute rejection and to a large part also the acute vascular rejection are caused by anti-Linear-B antibodies and the connected complement activation. One way to overcome this hurdle is to breed transgenic animals expressing factors regulating complement activation such as 'hDAF', human decay accelerating factor,[1a,3] or by depleting the xenoreactive antibodies using extracorporeal plasmapheresis with affinity columns.[4]

Another strategy is to 'inhibit' these naturally existing antibodies by injection of carbohydrate antigen.[5] Such monovalent epitopes have, however, to compete with a multivalent presentation on cell surfaces, e.g. of endothelial cells on blood vessels. High concentrations of at least 1 mM have to be reached to compete for the xenoantibodies, which are divalent receptors in the case of the IgG subclass, and decavalent for the pentameric IgM subclass, which plays a major role in hyper-acute rejection. The well-established principle of multivalent potentiation of weak carbohydrate–protein interactions[6] should also be operative for anti-carbohydrate antibodies. For this purpose attempts with high molecular weight oligosaccharides from pig-stomach mucins,[7] with serum albumin conjugates,[8] with multivalent arrays on oligo-ethylene glycol scaffolds,[9] and with poly-acryl amide conjugates[10] were made.

Our poly-lysine based system, which had already been successfully applied for potent E-selectin ligands,[11] would be well-suited for a multivalent presentation of Linear-B. This scaffold has several advantages over many of the other approaches for multivalency. Poly-lysine is commercially available with different degrees of polymerization. After derivatization a charge-neutral and hydrophilic amide linked polymer results, which has a low propensity for immunogenicity and is degradable, as opposed to poly-acrylates or poly-ethylene glycols. On the other hand, albumin conjugates[8] and other proteins may be immunogenic, the ethylene glycol oligomer carries only eight carbohydrate epitopes,[9] and poly-acryl amide with a lipophilic backbone is not degradable, and the molecular weights have upper limits.

To prepare poly-lysine conjugates a Linear-B oligosaccharide with a 3-aminopropyl aglycon had to be synthesized. A purely chemical synthesis using for the most part conventional transformations is

Scheme 1. Chemical synthesis of Linear-B type II trisaccharide 4 with an amino-propyl aglycon.
depicted in Scheme 1. The per-acetylated tetrachloro-phthalimide-protected glucosamine derivative 5 \(^{[12]}\) is converted to the anomeric bromide 6. Helferich-glycosylation of N-benzoyloxycarbonyl-protected propanolamine 7, deacetylation and transacetalization affords the 4,6-benzal derivative 8, which is reduced to the 6-O-benzyl protected glucosamine 9. By virtue of the bulky tetrachloro-phthalimido residue the 3-hydroxy group need not be protected for the regioselective glycosylation of the 4-hydroxy group with the Gal-α(1,3) Gal disaccharide 10. This intermediate is obtained by glycosylation of the selectively protected S-ethyl-1-thiogalactose derivative 11 \(^{[13]}\) with trichloro-acetimide 12. \(^{[14]}\) The crucial glycosylation of 9 with glycos donor 10 is mediated by activation with dimethyl-thiomethyl-sulfonium trifluoromethyl-sulfonate (DMTST) affording the protected trisaccharide 13 in 79% yield. Cleavage of the tetrachloro-phthalimide with ethylene diamine in ethanol, acetylation, and deprotection finally gives the trisaccharide 4. The conjugation of Linear-B-propanolamine 4 to polylysine is described in Scheme 2. Reaction of 4 with thiobutyrolactone gives thiol 16. Commercially available poly-(l)-lysine hydrobromide batches 14 with average degree of polymerization \(n\) around 40, 250, or 1000 are then converted to the DMF-soluble per-chloroacetamides 15 which in turn are coupled with different equivalent amounts \(x\) of thiolated trisaccharide 16 as described previously. \(^{[11]}\) The final Linear-B conjugates 17 are obtained by capping the remaining chloroacetamide groups with racemic thioglycolic acid \(^{[13]}\). The composition of these polymeric materials is given by the specification of the starting batch of 14 (vendor), and by \(^1\)H-NMR measurements at elevated temperatures. Assessment by integration of selected signals, assigned to either backbone, thioglycolic, or trisaccharide, closely matched the stoichiometry of reactants (cf. ref. [11]).

These polymers were then tested in vitro for binding avidity with anti-\(\alpha\)Gal antibodies, and in a functional assay for inhibiting complement dependent hemolysis of pig erythrocytes by human serum. \(^{[16]}\) In binding assays competition for anti-Linear-B antibodies (pooled human AB-serum) between antigenic carbohydrate coated on well plates and the soluble antigen test compounds is measured with an ELISA (enzyme-linked immunosorbent assay) format, selective either for the divalent IgG or the decavalent IgM subtypes. The results shown in Table 1 list inhibitory IC\(_{50}\) concentrations based on equivalent weight, i.e. trisaccharide concentration, rather than on molecular weight of the polymer. Thus, these equivalent weights are only dependent on the fraction \(x\) of lysine residues coupled to saccharide, determined by NMR, and are independent of the degree of polymerization \(n\), determined by the vendor (SIGMA) by physical methods (size exclusion chromatography coupled to low angle laser light scattering). Polydispersities also specified by the vendor vary between 1.1 and 1.2 (cf. also refs [11b,c]). It is evident that multivalent arrays 17 inhibit anti-Linear-B antibodies better than monovalent trisaccharide 4 (entry 1), and for most cases also better than the human serum albumin (HSA) conjugate tested (entry 7). For IgM-binding and inhibition of cytotoxicity the potency increases with the degree of polymerization \(n\): 36 (entry 2), 250 (entry 4), and 1050 (entry 6). Also 25% loading \(x\) is better than either 10% or 60% (entries 3, 4, 5). For the IgG subtype a less distinct but similar trend can be seen. For the best composition of 17 (entry 6) a potency gain of several orders of magnitude (2 to 6), compared to monovalent 4, is observed. Further experiments led to the con-
clusion that the configuration of lysine and thioglycerol, as well as the spacer length between carbohydrate and backbone play a less significant role (data not shown).

First in vivo experiments confirmed the superiority of high molecular weight poly-lysine conjugates 17. Therefore the polymer with 25% carbohydrate loading (ε: 0.25) and a degree of polymerization n between 900 to 1200 entered development with the code GAS914. A major technical hurdle turned out to be the chemical synthesis of trisaccharide 4 (Scheme 1). In a Prep Lab-setting the 24-step sequence with six chromatographic separations could be scaled up to 20 g of 4. Still, some toxic, smelly, or touchy reagents, such as DMTST (see above), and the amount of waste estimated to 10 tonnes per kg of trisaccharide would make this route a daunting task. After some attempts at optimization[17] it was decided to resort to enzymatic glycosylations with glycosyl transferases and sugar nucleotides as glycosyl donors. The advantages of total stereo- and regioselectivity without the need of protecting groups has to be balanced against cost and availability of enzymes and activated sugars, difficulties of reaction control, reproducibility, and process scale up. A research procedure starting with Z-protected 1(3-aminopropyl)-N-acetyl-glucosamine 18[18] had been elaborated,[19] but needed to be optimized for robustness, and toxic buffer (Na-cacodylate) should be replaced. Also a risk assessment concerning proteins of ruminant origin – bovine serum albumin, β(1,4)-Gal-transferase from cow milk, calf intestine alkaline phosphatase – was needed. As shown in Scheme 3, a first galactosylation of 18 with commercially available β(1,4)-Gal-transferase from cow milk gave N-acetyl-lactosamine 19 in 61–89% yield. The pH 7.5 cacodylate buffer was successfully replaced by acetate buffering at pH 5.5, a measure which at the same time alleviated substrate inhibition. Addition of Mg²⁺ solved the issue of excess cofactor Mn⁴⁺, which otherwise was depleted by precipitation of its phosphate. Equally successful was the optimization and scale up of the second galactosylation with recombimant α(1,3)-Gal-transferase giving the Z-protected trisaccharide 20 in 89% yield. Crucial here was the quality of the commercial enzyme in respect of minimized β-galactosidase activity, which leads back to monosaccharide 18. Again the arsenic buffer could be replaced by a pH 6.5 acetate buffer. In this case addition of Mg²⁺ was avoided, as it appeared to be connected with the unwanted glycosidase activity. Besides inorganic phosphates uridine 21 is the only byproduct of these enzymatic glycosylations. It is formed by phosphate cleavage with CIAP (calf intestine alkaline phosphatase) from uridine diphosphate, an inhibitor of galactosyl transferases. The intermediates 19 and 20 were purified by reversed phase chromatography, and a crystallization from ethanol/water yielding products of ≥98% purity. A membrane filtration before hydrogenolytic cleavage to the target Linear-B propanolamine 4 ensured removal of any larger entities such as endotoxins, prions, and viral particles. The deprotected 3-aminopropyl-trisaccharide 4 was also amenable to purification by recrystallization from ethanol/water. The remaining steps to the glycopolymer 17 (GAS914) followed the research procedure (Scheme 2) with some adjustments. The thiolated oligosaccharide 16 could be isolated by crystallization from the concentrated reaction mixture, thereby alleviating the separation from high boiling thiouylactone. This thiol was protected from oxidation to disulfide by the addition of antioxidant. Isolation of chloro-acetylated poly-(l)-lysine 15 and the final glycopolymer 17 by precipitation was a major hurdle. Precipitates were finally obtained in reproducible quality by direct precipitation in a stirrable pressure filter under high dilution, a re-dissolving of the product cake directly on the filter. For the final purification by tangential flow ultra filtration compatible filter material had to be evaluated. Characterization of the product after lyophilization was done with NMR, gel permeation chromatography, and in vitro biochemical assays for biological properties. The optimized procedure thus allowed the preparation of kg-quantities of drug substance of sufficient quality to prepare injectable solutions for first clinical studies.

In vivo experiments were first done with non-human primates – Cynomol-
With the aid of monoclonal antibodies raised against the poly-(-1)-lysine backbone of GAS914 it could be shown with immuno-histochemical methods that the radioactivity remaining in lymphoid tissue was associated with intact GAS914 and also co-staining with B-cell regions. [16] It might therefore be speculated that such collocation indicates binding of GAS914 to B-cells, and suppression of anti-Gal antibody production, resulting in the observed long term lowering of antibody levels. [16] During these treatments no adverse side effects such as immune complex mediated glomerulonephritis were observed, and complement activation remained very low. To our knowledge, GAS914 is the most potent inhibitor of α-Galactosidic carbohydrate antibodies reported. In the case of oligo-ethylene glycol conjugates 50 mg/kg doses have to be injected for similar effects, [9] and poly-acryl amide conjugates appear to be even less efficient, especially for the poly-ethylene glycol conjugates 50 mg/kg doses having xeno-antibodies was discontinued. It should, however, be noted that the poly-lysine backbone of GAS914 offers an excellent base for other antigen-specific therapies in antibody mediated diseases, be it as injectables or as ligands for immuno-apheresis.

In the past years interest in pig-to-human xenotransplantation has to some extent diminished mainly because of the risk of transmitting infections by the Porcine endogenous retrovirus (PERV). [23] Then the successful production of α(1,3)-galactosyl transferase doubly knockout pigs [24] by nuclear transfer cloning [24] was another device currently in use. From recent pig-to-primate transplantations with lactosyl transferase doubly knockout pigs it became evident that further genetic modifications would be necessary to prolong survival of xenografts beyond the six months currently achieved. Along these lines anti-coagulant genes should prevent thrombotic microangiopathy, causing the majority of organ losses. [25] Yet another complication might be inflammation and loss of pig-xenografts by infection with Human cytomegalovirus. [26] Other options are tolerance induction through chimeric bone marrow cells, so far restricted to transplantations in early infancy, before establishment of full immune competency, or organogenesis from developing animal organ primordials rather than from Human embryonic stem cells. [27] Under these circumstances the clinical development of GAS914 for eliminating xeno-antibodies was discontinued. It should, however, be noted that the poly-lysine backbone of GAS914 offers an excellent base for other antigen-specific therapies in antibody mediated diseases, be it as injectables or as ligands for immuno-apheresis.

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