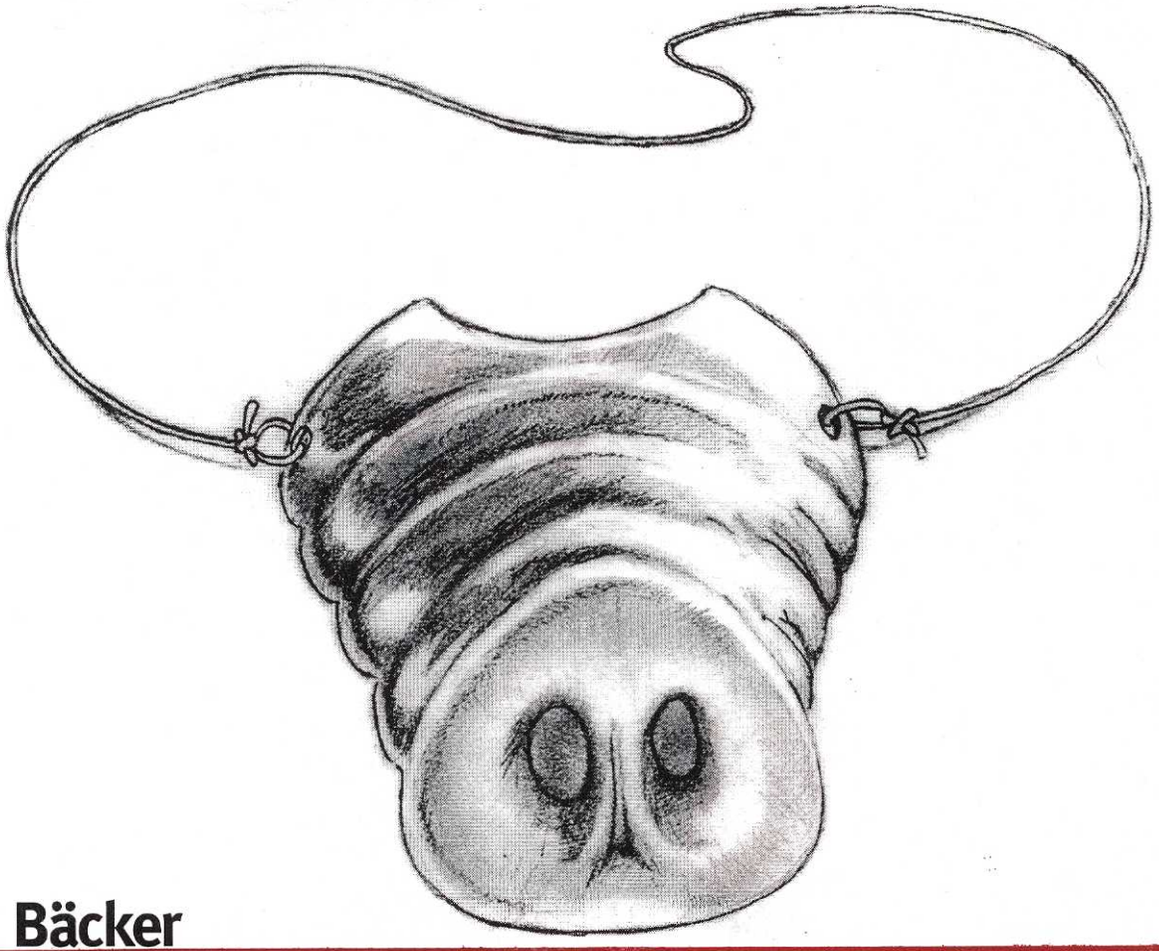
Carbohydrate antigens in pig with special relevance to human xenotransplantation

– Aspects on structural characterisation and organ distribution.



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Annika E. Bäcker

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2. **Bäcker, A.E.**, Holgersson, J., Samuelsson, B.E. and Karlsson, H. (1998) Rapid and sensitive GC/MS characterisation of glycolipid released Galα1,3Gal-terminated oligosaccharides from small organ specimens of a single pig. *Glycobiology*, 8(6): 533-545.

3. Olling, A., Sandberg, P., **Bäcker, A.E.**, Hallberg, E.C., Larson, G., Samuelsson, B.E., and Soussi, B. (1999) Continuous flow LC-high field NMR spectroscopy of glycolipid mixtures. *Journal of Magnetic Resonance Analysis*, In press.

4. **Bäcker, A.E.**, Thorbert, S., Rakotonirainy, O., Hallberg, E.C., Olling, A., Gustavsson, M., Samuelsson, B.E., and Soussi, B. (1999) Liquid Chromatography "on-flow"^{1}H Nuclear Magnetic Resonance on native glycosphingolipid mixtures together with Gas Chromatography/Mass spectrometry on the released oligosaccharides for screening and characterisation of carbohydrate-based antigens from pig lungs. *Glycoconjugate Journal*, In press.

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Abstract

Transplantation between humans is today an accepted treatment for several diseases. The lack of human donors is however the major obstacle against widening the indications for organ transplantation. Transplantation of tissue between different species e.g. xenotransplantation, may be one solution to the problem. Different animal species have been suggested, but the pig is today considered as the most suitable donor. The primary barrier to pass when transplanting between species is the hyperacute rejection which appears when the organ is connected to the recipient's blood stream. This is caused by preformed natural antibodies in the recipient, which react with carbohydrate antigens exposed on the endothelial cells of blood vessels in the transplanted organ. A galactose linked in an α1,3 linkage to another galactose (Galα1,3Gal) as a terminal carbohydrate sequence is the major target for the human antibodies.

We have studied and characterised the expression of carbohydrate structures in different porcine organs. The structural elucidation of the cell surface carbohydrates was made with antibodies and different mass spectrometric and/or nuclear magnetic resonance spectroscopy methods.

The development of improved, more sensitive, methods for carbohydrate analysis have made it possible to analyse carbohydrates in small amounts of tissue. By using the GC/MS technique we were allowed to look for differences in carbohydrate expression in small tissue specimens from pig small intestine, heart, spleen, liver, salivary gland, kidney and lung. The advantages of the technique is the small sample amount needed, the sensitivity and the speed of analysis, and the screening pattern obtained, showing both qualitative and quantitative differences in the analysed mixtures of oligosaccharides. By using the LC"on-flow"NMR technique, we got the possibility to separate, and at the same time analyse single carbohydrates, e.g. glycolipids, in a mixture from pig lung. This is the first time this method has been used for native glycolipids.

The enzymes involved in carbohydrate chain biosynthesis in pig small intestine were also studied. The biosynthetic products from *"in vitro"* experimental studies with prepared enzymes were compared to the carbohydrate expression *"in vivo"*, produced by the same enzymes. The enzymes were shown to accept a variety of precursor carbohydrate chains in the *"in vitro"* situation compared to the *"in vivo"* situation where one precursor chain type was dominant.

It is still a long way to go before the mysteries of xenotransplantation are solved. By characterising the carbohydrates on the cell surfaces in organs of interest, we have taken a small step towards a complete understanding of the mechanisms of xenotransplant rejection.

Key words: ABH blood group, glycosphingolipids, tissue distribution, carbohydrate antigen, xenotransplantation, pig, gas chromatography, mass spectrometry, NMR spectroscopy.

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List of publications

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We have studied and characterised the expression of carbohydrate structures in different porcine organs. The structural elucidation of the cell surface carbohydrates was made with antibodies and different mass spectrometric and/or nuclear magnetic resonance spectroscopy methods.

The development of improved, more sensitive, methods for carbohydrate analysis have made it possible to analyse carbohydrates in small amounts of tissue. By using the GC/MS technique we were allowed to look for differences in carbohydrate expression in small tissue specimens from pig small intestine, heart, spleen, liver, salivary gland, kidney and lung. The advantages of the technique is the small sample amount needed, the sensitivity and the speed of analysis, and the screening pattern obtained, showing both qualitative and quantitative differences in the analysed mixtures of oligosaccharides. By using the LC"on-flow"NMR technique, we got the possibility to separate, and at the same time analyse single carbohydrates, e.g. glycolipids, in a mixture from pig lung. This is the first time this method has been used for native glycolipids.

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"Skriv en bok som alla läser,
Ge pengar till ett institut
Gör en sång som barnen sjunger
när terminen tagit slut...

Korsa apor med kaniner
och få ett pris ur kungens hand
Rita hus som står för evigt
eller sätt en värld i brand

Vi kommer alltid att leva,
Vi kommer aldrig att dö"

Ur "Vi kommer aldrig att dö"
Bo Kasper och David Shutrick, 1996

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1. Blood group ABH cell surface carbohydrates

Blood group ABH active oligosaccharides are expressed in cells, tissues and body fluids. At the cell surface they are bound to the cell membranes, linked by either lipids or proteins as glycolipids or glycoproteins. Carbohydrates can also exist as free molecules in the body fluids.

Glycosphingolipids

Glycosphingolipids (GSL) are composed of a carbohydrate part and a lipid part, which anchors the molecule into cell membranes. The carbohydrate part can range in size from a single residue to more than 60 sugar residues and can consist of a variety of different sugars, each of which can be attached to each other in a variety of ways (linkage and anomerity). The complex oligosaccharide chain of GSLs can be either linear or branched and carry blood group determinants. The lipid part, consisting of a long chain base, sphingosine, and an amide bonded fatty acid, is also known as ceramide, and shows significant heterogeneity due to variation in carbon chain length, number of double bonds, methyl branching and hydroxyl groups [1, 2]. Although the lipid tail is not directly involved in determining the blood group epitope carried by the carbohydrate chain, it can influence the steric presentation of the epitope at the cell surface [3]. The GSL can be neutral or acidic, the latter ones containing sulphate (e.g. sulphatides) or sialic acid (e.g. gangliosides).

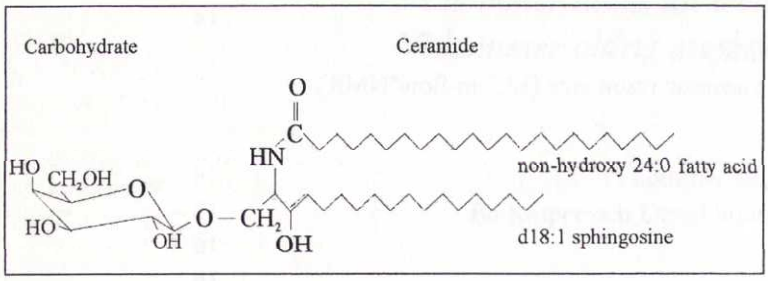

Fig. 1 A schematic formula of a simple GSL monoglycosylceramide. The GSL consists of a carbohydrate and a ceramide part. The ceramide part in this example contains a non-hydroxy 24:0 fatty acid and a dihydroxy d18: 1 long chain base (sphingosine).

Glycoproteins

Carbohydrates bound to proteins, e.g. glycoproteins, can be linked to the sugar chain moiety through an N-acetylglucosamine to an asparagine residue (N-linked) [4] or through an N-acetylgalactosamine to a serine or a threonine residue (O-linked) [5-7]. The complexity of the carbohydrate chain is like that of the ceramide bound carbohydrate [8]. Although, unlike ceramide bound glycoconjugates, more than one species of oligosaccharide chain may exist on the same protein.

The N-linked glycoproteins contain a common pentasaccharide core structure (Manα1,6(Manα1,3)Man β1,4GlcNAcβ1,4GlcNAc-Asn) which is extended to form three different forms of oligosaccharides, one complex type which is often terminally sialylated and therefore gives the structure a net negative charge, a hybrid type and a high mannose type [4]. Important N-liked glycoproteins are e.g. the interleucin 1β(IL-1β) [9] and interleucin-6 (IL-6) [10].

The O-linked oligosaccharides show four major core structures. The O-linked glycoproteins consists either of simple disaccharide chains bound to the hydroxyl oxygen of serine or threonine (Galβ1,3GalNAcα1-O-Ser/Thr) or are more complex chains with an extension or branching of lactosamine structures [6, 7]. These oligosaccharides are expressed in e.g. the gastrointestinal tract, lung and glandular tissue [5]. Blood group reactivity has been found on O-linked glycoprotein [11]. O-liked N-acetylglucosamines have also been found in cytoplasmic and nucleoplasmic glycoproteins involved in the regulation of phosphorylation [12, 13].

The proteoglycans are glycoproteins with a dominating glycan part, compared to the glycoproteins, which have a dominant protein part. The proteoglycans consist of a repeating unit of disaccharides, which also can be sulphated. The carbohydrate part can be linked to the serine via xylose [14].

Phenotypes

The blood group phenotype of an individual is determined by the structures of the carbohydrates present at the terminus of the oligosaccharide chain. A variety of different chain types can be found on glycoconjugates and they can be grouped/named according to their precursor chains (Table 1). This nomenclature recognises the types of sugars, anomerity and linkage position of the terminal saccharides in the chain. ABH blood group determinants can reside on a variety of these different chain types with each representing a different ABH structure, which may show a species- [15-18], tissue- [19] and cell- [20] specific distribution. For example, type 1 ABH antigens are only found in individuals who are blood group secretors, while the type 3 A antigen is generally only found on the red cells of individuals of the A_1 phenotype, and the A type 4 antigen is strongly expressed in kidney. The structure of the chain bearing the blood group determinant also influences the steric presentation of the epitope at the cell surface, which thus can be recognised by specific antibodies [21].

In the gastrointestinal tract, GSLs bearing blood group determinants are predominantly short (4 to 7 sugars in length), while those on the red cell, bearing similar blood group determinants (albeit on different chain types) are predominantly large branched structures (30-60 sugars) [22,23].

Type 1	Galβ1,3GlcNAcβ1-R
Type 2	Galβ1,4GlcNAcβ1-R
Type 3	Galα1,3GalNAcβ1-R
Type 4	Galβ1,3GalNAcβ1-R
Type 5	Galβ1,3Galβ1-R*
Type 6	Galβ1,4Glcβ1-R

Table 1. Structures of possible disaccharide precursors [24]. (* not described in humans).

Histo-blood group expression

At the beginning of this century, Landsteiner discovered the ABO blood group system [25]. He observed that erythrocytes from one individual could be agglutinated by serum from certain people but not from others. Much later, the H blood group system was defined, thereby defining the structure of the "O antigen" as the precursor of A and/or B. More recently, several studies have shown that the expression of the blood group ABH antigens is not limited to red blood cells and the antigens can be found in most tissues and fluids of the body. Today the term histo-blood group system, first used by Clausen and Hakomori [23] is used to better describe the expression of the carbohydrate blood group system antigens. The histo-blood group antigens also show a tissue specific expression [2] and can be secreted in the body fluids [26-28]. Histo-blood group expression often shows similarities between different species and closer related species shows more similarities, e.g. humans and monkeys [29].

Carbohydrate expression in different tissues

A specific pattern of the carbohydrate expression is found in different organs and tissues, reflecting the cell-specific carbohydrate phenotypes of the cell types constituting a particular tissue [2, 23, 30]. Glycosyltransferases act on the carbohydrate chain, adding different monosaccharides to elongate the chain. Some of the transferases are shown to act on both GSL and glycoproteins [31].

The type 1 chain

The type 1 GSL chain (Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ1,1Cer) is synthesised in ectodermal tissues as in the case of small intestinal mucosa and glandular epithelium [2]. This chain is the most common carrier of the Lewis and ABO antigens in body fluids and secretions. The type 1 chain is not synthesised in endodermal tissues with exception of the non-keratinised oral epithelium, nor in mesodermal derived tissues. Type 1 chain based antigens can however be present on erythrocytes and lymphocytes but as a consequence of absorbing freely circulating GSLs from the plasma.

The secretor gene (Se) encodes an α1,2 fucosyltransferase, which can bind a fucose residue in a α1,2 position to the terminal galactose of the type 1 chain e.g. lactotetra, to form H type 1 [32]. The product of the secretor gene is found in bodily secretions and seminal fluids. The gene is generally expressed in tissues related to exocrine secretion of type 1 chains.

The Lewis blood group determinants (Le) are also based on the type 1 chain and are present on both proteins and GSLs. The Lewis gene encoded α3/4 fucosyltransferase adds a fucose residue in an α1,4 position to the subterminal N-acetyl-glucosamine of the type 1 precursor which gives rise to the Le^a antigen (Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer) [32]. If the secretor fucosyltransferase had previously modified the type 1 precursor into H type 1 before the Lewis transferase added the subterminal fucose, the Le^b antigen ((Fucα1,2)Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer) results. The secretor transferase cannot make Le^b from Le^a. The Le^a and Le^b antigens predominantly exist as 5 and 6 sugar GSL's, but much larger GSLs bearing these epitope are also common [32]. Lewis antigens bearing ABO determinants exist. If the H type 1 antigen is converted into A type 1 the Lewis transferase is still able to add its fucose and a difucosylated compound antigen ALe^b is formed. Likewise modification of B type 1 by the Lewis transferase results in the BLe^b antigen. The Le^b antigen is, however, not a suitable substrate for the A or B glycosyltransferases.

Red cell phenotype	Glycosyltransferases			Major product in secretions		
ABO	Lewis	Le	Se	A	B	
A, B, AB, O	$Le^{(a-b-)}$	-	-	+/-	+/-	type 1 precursor
O	$Le^{(a-b-)}$	-	+	-	-	H type 1
A, B, AB	$Le^{(a-b-)}$	-	+	+/-	+/-	A type 1 and/or B type 1
A, B, AB, O	$Le^{(a+b-)}$	+	-	+/-	+/-	Le^a
O	$Le^{(a-b+)}$	+	+	-	-	Le^b
A, B, AB	$Le^{(a-b+)}$	+	+	+/-	+/-	ALe^b and/or BLe^b

Table 1. The major blood group antigens present in saliva in individuals with different ABO, Lewis and Secretor phenotype. For simplicity, A, B and AB have been grouped together, but only the A products are present when the A glycosyltransferase is present and the same for the B products.

The type 2 chain

The type 2 GSL chain (Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer) is expressed in tissues of ectodermal and mesodermal origin, i.e. skin and erythrocytes. Type 2 chain based ABH antigens are the predominant ABH antigens present on the red cells. The chain can also be found in some endodermal tissues together with the type 1 chain. The type 2 precursor can be fucosylated by either the H or the Se fucosyltransferases to form H type 2. The type 2 chain based ABH antigens in saliva are not formed by the H gene encoded enzyme (because it is not expressed in the secretory compartments), but instead by the action of the secretor fucosyltransferase. In the gastrointestinal tract, the expression of type 2 chain based antigens is generally weak [33].

In addition, the Lewis α3/4 fucosyltransferase can modify the type 2 precursor to form Le^x (X), H type 2 to form Le^y (Y) and A and B type 2 to form ALe^y (AY) and BLe^y (BY) respectively. The Lewis transferase is not the only transferase capable of α3 fucosylation, and the above structures can also be formed by other α3 fucosyltransferases such as FUT4, FUT5, FUT6 and FUT7 [34].

The type 3 chain

The type 3 chain (Galβ1,3GalNAcα1) exists in two major forms. One is an O-linked chain of mucin type [35]. The disaccharide is a part of the T/Tn (Galβ1,3GalNAcα1-O-Ser/Thr/GalNAcα1-O-Ser/Thr) blood group system (Thomsen-Friedenreich antigen and related antigen). The chain is common in many cells and tissues, including erythrocytes, but is almost always substituted with a sialic acid. The substituted structure is often present in ovarian cysts and gastric mucosa. It is also associated with human gastric intestinal metaplasia and carcinomas for example in breast, colon, lung, kidney, ovary, and rectum [36-38].

The type 3 chain also exists as an extension of the blood group A antigen (GalNAcα1,3(Fucα1,2)Gal

β1,3GalNAcα1,3(Fucβ1,2)Galβ1,4GlcNAcβ1,3Galβ 1,4Glcβ1,1Cer). This repetitive blood group A structure was first described in 1985 by Clausen *et al* [39]. This extended A GSL structure, also known as A-9-3, represents an elongation of the A type 2 chain, first by the addition of a galactose, to produce the type 3 precursor, then modification by the H transferase to form H type 3, then glycosylation by the A_1 transferase to produce A type 3. The A_2 transferase is unable to affect the transfer and the H type 3 precursor remains unsubstituted in the blood group A_2 individual.

The type 4 chain

The type 4 chain GSL (GalNAcβ1,3Galα1,3Gal β1,4Glcβ1,1Cer) is also referred to as globoside and belongs to the globo-series of GSLs. Globoside is a major glycolipid on erythrocyte membranes but can, together with other globo-series glycolipids, also be found in kidney, small intestine, spleen, salivary gland, liver and heart in different species [29].

Blood group P related structures

In the early days, Landsteiner and Levine identified an antigen in the blood which they named P. The antigen was later renamed to the blood group P_1 antigen (Gal α1,4Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer) and is, together with the two other serological related antigens P (GalNAcβ1,3Galα1,4Galβ1,4Glcβ1,1Cer) i.e. globoside, and P^k (Galα1,4Galβ1,4Glcβ1,1Cer) i.e. globotriaosylceramide, named the blood group P system. Other structures related to the system are the Globo-5 (Galβ1,3GalNAcβ1,3Galα1,4Galβ1,4Glcβ1,1Cer), globo-H (Fucα1,2Galβ1,3GalNAcβ1,3Galα1,4Gal β1,4Glcβ1,1Cer), globo-A, also called A type 4 (Gal NAcα1,3(Fucα1,2)Galβ1,3GalNAcβ1,3Galα1,4Galβ 1,4Glcβ1,1Cer) and the Forssman antigen (GalNAcα 1,3GalNAcβ1,3Galα1,4Galβ1,4Glcβ1,1Cer).

The ganglio-series

The ganglio-series of GSLs can express ABO epitopes. The ganglio chains can be substituted with sialic acid and are in these cases called acidic GSLs or gangliosides. The GSL gangliotetraosylceramide (Galβ1,3Gal NAcβ1,4Galβ1,4Glcβ1,1Cer) is commonly found in cells with neural origin [40].

Other glycosphingolipid chains

The glycosphingolipid chains mentioned above are the most commonly found chains in human and pigs. Other glycosphingolipid chains exist but are not discussed here.

Biological functions and implications

Variations of an oligosaccharide chain can be accomplished by different monomers (i.e. fucose, glucose, galactose, N-acteylgalactosamine), different carbohydrate sequences, changes in binding positions, branching/non-branching or binding configurations [2]. All these variations of the carbohydrate part may potentially have an impact on the function of the GSL. Fifteen years ago, glycosylation of glycoproteins and GSLs was not believed to play any significant functional role. Today functions are still largely unknown, but a number of proteins bind to the oligosaccharide portions of GSLs and glycoproteins which indicate involvement in cell-cell recognition [41-43], leucocyte adhesion and recruitment (E-selectin and sialyl-Le^x) [44]. GSLs are also shown to interact in cell surface recognition events [45]. Glycosylation may furthermore affect the function of the cell [46]. Both the extent and type of glycosylation can play a role in the glycoprotein activity. Oligosaccharides are believed to be involved in neural differentiation [40], oncogenesis [47-49] and metastasis [50]. Many hormones are also glycosylated e.g. gonadotropins, LH and FSH. [51]. GSLs have been shown to be involved in the adhesion of bacteria, viruses and toxins to cellular surfaces (microbial ligands) [52, 53]. Carbohydrate chains can also function as therapeutic targets in i.e. allergic inflammatory disorders [54], auto-immune rheumatic diseases [55] and in the hyperacute rejection during xenotransplantation, see below [56].

Variation of ABO expression during development and cell differentiation

Expression of the ABO glycolipids have been shown to be closely related to cell differentiation phenomena [57].

Branching of linear type 2 chains can be accomplished by a Galα1,6 glycosyltransferase regulated by the I gene. Before birth, there is very little expression of branched chains and thus the blood group ABH determinants of the red cells are found on linear chains. The late onset of the I gene, and thus sparse expression of branched ABH determinants at birth, has been suggested to be an evolutionary phenomenon to reduce the occurrence of serious cases of ABH haemolytic diseases in new-borns. The binding of an IgG anti-A antibody on to both epitopes of a branched antigen has been called monogamous bivalency and shown to activate complement more efficiently.

Transfusion between individuals and species

Blood transfusions between individuals became a relatively safe procedure after Landsteiners discovery of the blood group ABH system. Before that, transfusions between individuals often resulted in acute haemolysis and a mortality of about 30%.

A transfusion of blood from animals like sheep, calves and pigs does not necessarily lead to haemolysis at the first transfusion. A second transfusion, after boosting the sparsely occurring natural antibodies, will though elicit a life treating condition.

Transplantation between individuals and species

Transplantation between individuals has been made more or less regularly since the 60's, and has increased in number as a consequence of successive improvements in immunosuppressive regimens and graft survival. Primary donor-recipient selections in organ transplantation are routinely blood group ABO matched. Blood group ABO incompatible organ transplantation is, however, feasible under special circumstances or actions [58]. Blood group A_2 to O kidney transplantation has been shown possible due to the low blood group A expression in kidneys in these individuals. Major ABO incompatibilities are also possible provided that plasmapheresis or specific absorption lowers isoaggutinin titres. The spleen also needs to be removed. In all cases, long term survival and tolerance e.g. accommodation [59], can be induced.

Xenotransplantation (transplantation between species) has been performed through history with limited success [60-62].

In recent years the realism of success for xenotransplantation has gradually increased. The identification of the Galα1,3Galβ1,3 iso antigen/antibody system (e.g. similar to blood group ABO incompatibility in allotransplantation) has provided a handle for surpassing the initial hyperacute barrier. Pig kidneys have been connected to the blood stream outside the body of human dialysis patients [63, 64]. Human anti-pig antibodies were heavily depleted through extensive plasmapheresis and protein A absorption. In one case, urine was produced for 6 hours.

2. Methods

GSL isolation and fractionation

Over the years several different protocols have been described for isolation and purification of GSLs [65-67]. The GSL preparation and purification method described by Karlsson [66] was used in this thesis.

The preparation procedure used can be summarised as follows;

The chosen tissue sample was cut into small pieces and lyophilised. Lipids were extracted from the tissue in a Soxhlet apparatus using different mixtures of chloroform and methanol. The extracted lipids were subjected to alkaline methanolysis to remove alkali-labile phospholipids, and silicic acid chromatography to remove non-polar lipids (i.e. mainly methyl esters of fatty acids). The DEAE-cellulose columns separated the acidic glycolipids from the non-acidic glycolipid components. In order to remove sphingomyelin the sample was acetylated and subjected to silicic acid chromatography. Glycolipids having multiple O-acetylation sites were first eluted, while sphingomyelin, having only one O-acetylation site, was retarded and eluted later. The isolated glycolipids were O-deacetylated and further purified by additional DEAE-cellulose and silicic acid chromatography.

The resultant total non-acid GSL mixture was further purified and fractionated on a silicic acid HPLC column [68] using chloroform/methanol/water solvent mixtures in linear gradients and a constant flow rate [68](Paper1-4). Fractions were collected and monitored by high performance thin layer chromatography (HPTLC). The pooling of fractions was based on information from HPTLC (Paper 1 and 2). In paper 4 the pooling procedure was in some cases performed on the basis of LC"on-flow"NMR identification of components.

A substantial amount of GSL appeared to be lost during the final stages of the GSL preparation in the pig lung preparation (Paper 4). The silicic acid columns were found to irreversible bind (preferentially longer) GSLs, which normally should be eluted by the polar mixture of chloroform/methanol/water in proportions 40:40:12 (by volume). This have been found by others [69] and losses can be diminished to some extent if the slowly moving fraction eluted with chloroform/methanol/water (40:40:12) from the initial silicic acid column is not further processed. This will result in a less pure fraction of extended GSLs (often in small amounts) which can be derivatised and used for mass spectrometric analysis.

Ceramide glycanase-cleavage of GSLs

The polar GSL mixtures used for ceramide glycanase cleavage were incubated with sodium cholate, sodium acetate buffer and ceramide glycanase [70]. After incubation, the product was passed through C18 reverse phase cartridges in order to remove ceramides and potential traces of non-cleaved GSLs [71]. The ceramide fraction was used to determine the digestion yield. The non-adsorbed fraction was analysed by TLC and non-digested GSL could be visualised by anisaldehyde staining. The glycanase digestions was estimated to have an efficiency of more than 90 % (Paper 2,4).

Thin layer immunostaining

By using thin layer immunostaining with antibodies against carbohydrates, the achieved binding information can be used for the assignment of i.e. blood group specificity to different GSLs. The chromatographic separation on the HPTLC plate also gives information related to carbohydrate chain length, +/- one sugar component. Both sugar chain length and ceramide differences contribute to the chromatographic mobility of the GSL. The achieved data are empirical and based on prior experience, e.g. references.

The immunostaining analyses were performed either by the method of Magnani *et al* [72, 73] (Paper 1 and 2) or by the method of Hynsjö *et al.* [74] (Paper 2 and 4). Depending on the complexity of the mixtures, 5-10 μg of the GSL mixture were applied to HPTLC plates. Chromatography was performed using a solvent mixture of chloroform/methanol/water in proportions 60:35:8 (by volume). The anisaldehyde reagent was used for chemical detection of the GSLs on the HPTLC plates [66]. Monoclonal antibodies (MAb) and immunostaining of thin layer plates were used to identify blood group related epitopes. The primary anti-carbohydrate MAb was detected by labelled secondary antibodies, either ^{125}I and visualised by autoradiography using a γ-sensitive film (Paper 1 and 2) or by alkaline phosphatase labelled antibodies, made visible by the catalysis of a colour reaction (Paper 2 and 4).

Glycolipid biosynthesis.

In Paper 1, a biosynthetic study was performed with enzymes prepared from pig small intestine epithelial cells. The study was made to evaluate the potential of prepared enzymes to produce GSL products *in vitro* from the available precursor GSLs and radioactive labelled sugar residues (e.g. fucose, galactose and N-acetylgalactosamine) and to compare the result with the *in vivo* expression of GSL structures.

A crude microsomal fraction was prepared from mechanically or enzymatically released epithelial cells [75, 76] and was used as the enzyme source. Defined precursor glycolipids were incubated with the enzyme preparation and radio-labelled substrates (e.g. UDP-[U-^{14}C]galactose, UDP-N-acetyl-D-[1-^{14}C]galactosamine or GDP-[U-^{14}C]fucose). Tris-HCl, ATP, $MnCl_2$ (or $MgCl_2$ depending on assay), NaN_3, and Triton X-100 was added prior to the incubation [75, 76]. The products were passed through prewashed C18 cartridges to trap the glycolipids and separate them from the radiolabelled substrates and enzymes [76]. The glycolipids (including those, which had been radiolabelled by the enzyme reaction) were eluted with methanol. HPTLC and autoradiography identified the products.

Glycolipid and oligosaccharide preparation for MS analysis

Permethylation

Native GSLs and oligosaccharides can be analysed by fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS), liquid secondary ion mass spectrometry (LSIMS)

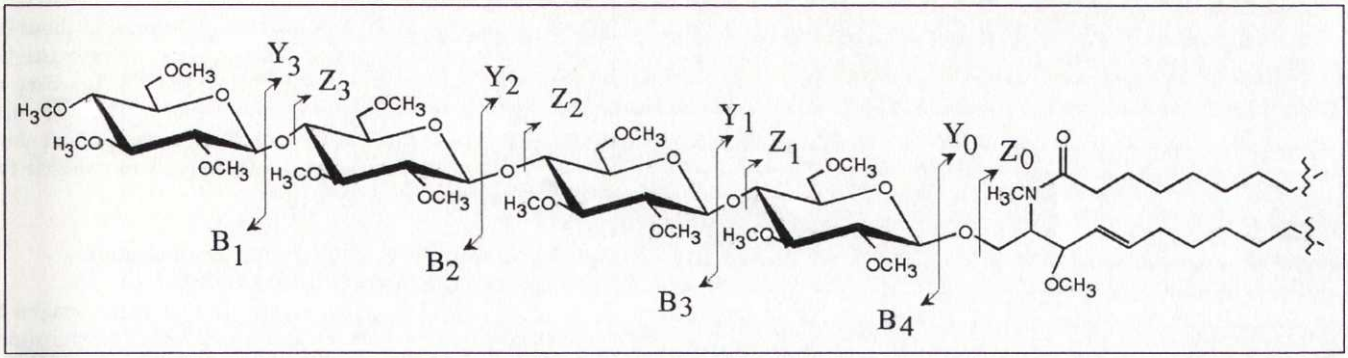

Figure 2. Fragment ions in EI^+ mass spectra from permethylated GSLs and oligosaccharides. The nomenclature is according to Domon and Costello [80].

and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). However, the sensitivity of these methods on native GSL structures is low, but can be 20-50 fold increased by derivatization [77].

In order to analyse glycoconjugates and oligosaccharides by EI-MS these molecules have to be derivatized. The derivatization increases the volatility and thermal stability of the molecules. This is a prerequisite for EI-MS analysis. Permethylation was performed using solid NaOH in dimethyl sulfoxide and iodomethane according to Ciucanu and Kerek [78] as modified by Larson *et al* [79]. The procedure results in a methylation of all carbohydrate hydroxyl and amide groups. Permethylated GSLs and oligosaccharides analysed by EI-MS give sequence information due to the formation of oxonium ions (B_i) along the carbohydrate chain. (See figure 2.)

Reduction

The reduction procedure was performed in diethyl ether using $LiAlH_4$ [81]. The reduction of GSLs converts amides to amines, which in electron ionisation (EI-MS) strongly favours the formation of stable immonium ions $[imm]^+$. The ionization occurs at the nitrogen of the ceramide and the immonium ions are formed by a α-cleavage of the sphingosine base and contain the carbohydrate chain together with the fatty acid part. The advantage with permethylated and reduced GSLs in EI-MS is that the immonium ions give the size of the molecules. This is often not the case with just permethylated GSLs. (See figure 3.)

Glycolipid preparation for NMR analyses

In Paper 1 and 4, conventional proton NMR was used for the characterisation of the GSLs. The native glycolipid fractions were dried and later deuterium exchanged in excess of $CDCl_3/CD_3OD$, dried again and dissolved in a mixture of dimethyl sulfoxide-d_6 containing 2% of D_2O.

Glycolipid preparation for LC-NMR analyses

In Paper 3 and 4, the LC"on-flow"NMR technique was used for the GSL analysis. The GSL fractions were dried and deuterium exchanged in excess of $CDCl_3/CD_3OD$, dried again and dissolved in a mixture of $CDCl_3/CD_3OD/D_2O$.

Mass spectrometry

Direct inlet EI^+ mass spectrometry

Permethylated and permethylated-reduced GSLs can be analysed by EI-MS using the fractionated evaporation technique (Paper 1). This "in-beam" sample technique uses a cuvette for sample loading, which is brought in close proximity (1-2mm) to the electron beam. The ion source temperature is then programmed from 180 to 360° C to allow the GSL mixture to evaporate and be ionized [82].

High-Temperature Capillary Gas Chromatography (GC)

High-temperature capillary gas chromatography was used to analyse oligosaccharides (Paper 2, 4) due to the high resolution and sensitivity achieved. The retention and resolution of sample components (solutes) in capillary GC result from the differential distribution (partition) of the solutes between the stationary liquid and the mobile gas phases. As a result of the solution-dissolution process of the solute molecules into and out of the stationary phase, solute retention and resolution in the column are obtained. The magnitude of retention depends on the partition coefficient (K), an equilibrium constant, which is defined as the ratio of the concentrations of solutes in the stationary and

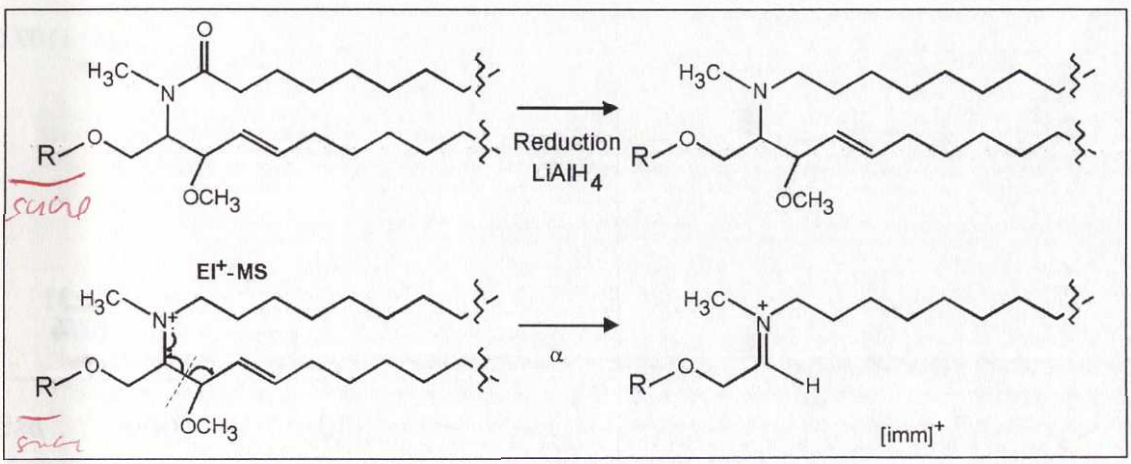

Figure 3. Reduction of amides to amines and formation of immonium ions by a α-cleavage of the sphingosine base in EI-MS of permethylated-reduced GSLs. The "fish-hooks" half-arrows indicate transfer of a single electron.

mobile phases. The larger the value of the partition coefficient for a sample component, the higher solubility and longer retention in the stationary phase. The partition coefficient is related to the column phase ratio (β) and the retention factor (k) by K=βk. The retention factor k is a measure of the retention time for a sample component relative an unretarded component. The column phase ratio can be expressed as $\beta=V_g/V_l=r/2d_f$, where V_g is the gas phase volume, V_l is the stationary liquid phase volume, r is the column radius and d_f is the film thickness. In order to decrease k, β has to be increased since the product (K) is a constant. β can be increased either by increasing the column inner diameter (a wider column) or by decreasing the film thickness (d_f) or both. A standard capillary column with a diameter of 0.25 mm and a film thickness of 0.25μm has a β value of 250. A column with the same diameter, but with a film thickness of 0.02 μm has a β-value of 3125. β is increased by a factor of 12.5, which results in a much shorter retention time. In our case ultra-thin films were necessary in order to chromatograph the large permethylated oligosaccharides.

For fast analysis at high linear gas velocity the zone spreading is decreased by using a carrier gas with low viscosity and high diffusivity such as hydrogen. The permethylated oligosaccharides were dissolved in ethyl acetate, injected on-column into a gas chromatograph equipped with a flame ionisation detector. A linear temperature program from 70 °C to 400 °C was used [83].

High-Temperature Gas Chromatography/ Mass Spectrometry (GC/MS)

High-resolution chromatography and structural information can be achieved by using high-temperature gas chromatography in combination with mass spectrometry (GC/MS)(Paper 2,4). The mass spectra of permethylated oligosaccharides are simple to interpret, as they are mainly composed by sequence oxonium ions (B_i) and in the presence of HexNAcs inductive ions (Z_i) (Figure 4). In some cases, information about binding position can be achieved (e.g. differentiate between type 1 and type 2 chains) [84, 85, Teneberg and Karlsson, unpublished observation]. The chromatographic conditions were identical to those given above, except helium acted as carrier gas and constant flow mode were used [86].

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

MALDI-MS (Paper 2 and 4) is used to analyse the dynamic mass range of oligosaccharide mixtures prior to gas chromatography analysis, as the MALDI mass spectrum gives molecular ions of the oligosaccharides. This gives an indication of the size of the molecules in the mixture and the feasibility for GC/MS analysis. A thin-film matrix surface was prepared using the fast evaporation technique from 2,5-dihydroxybensoic acid in acetone doped with 10mM LiCl. Permethylated oligosaccharides were dissolved in ethyl acetate and applied to the matrix surface. Lithium adducts of the molecular ions $[M+Li]^+$ were produced.

Nuclear Magnetic Resonance

Proton NMR spectroscopy (^{1}H NMR)

NMR of native glycolipids was performed in Paper 1 and 4. Spectra were recorded at 500 MHz at a temperature of 30 °C (Paper 1) or RT (Paper 4). The chemical shifts were given relative to tetramethylsilane [87].

Liquid Chromatography-Nuclear Magnetic Resonance (LC"on-flow"NMR)

Papers 3 and 4 concern the LC"on-flow"NMR technique for rapid screening and pooling of native GSLs. The method is still under development. A LC pump with a straight phase silica column was connected on-line to the NMR probe and GSLs were separated prior to 500 MHz NMR analysis using a constant flow gradient in a solvent of $CDCl_3/CD_3OD/D_2O$. An UV-

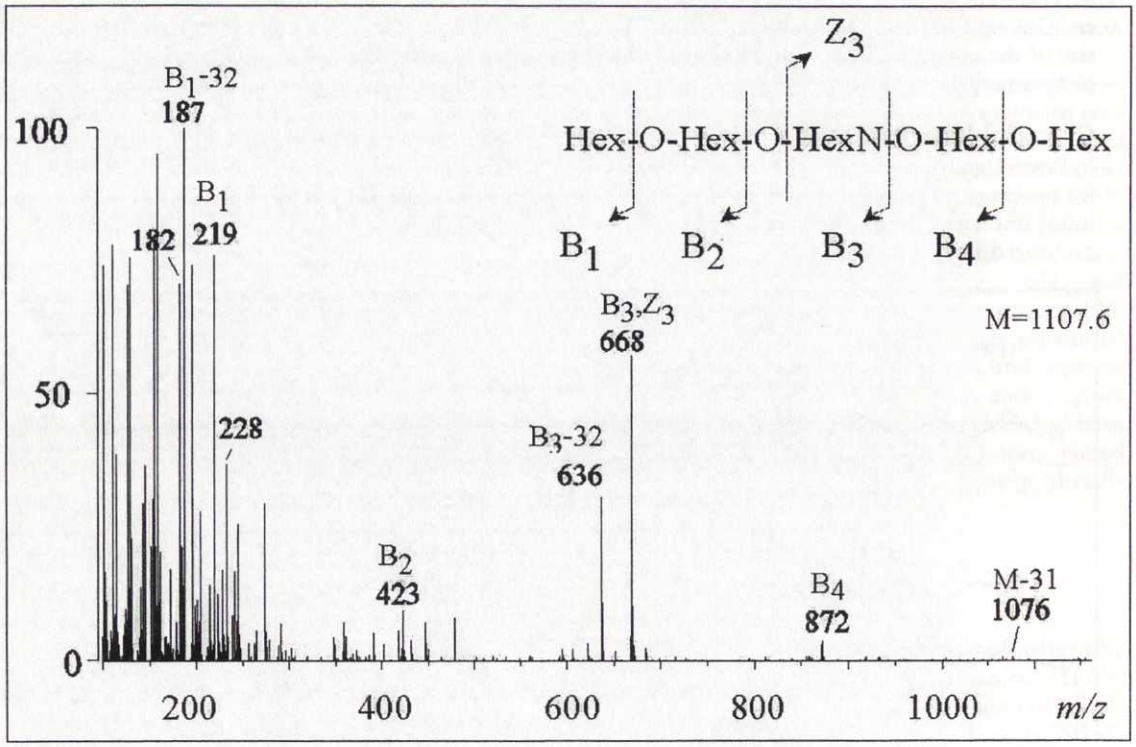

Figure 4. Mass spectrum of the Hex-O-Hex-O-HexN-O-Hex-O-Hex component from pig kidney [85].

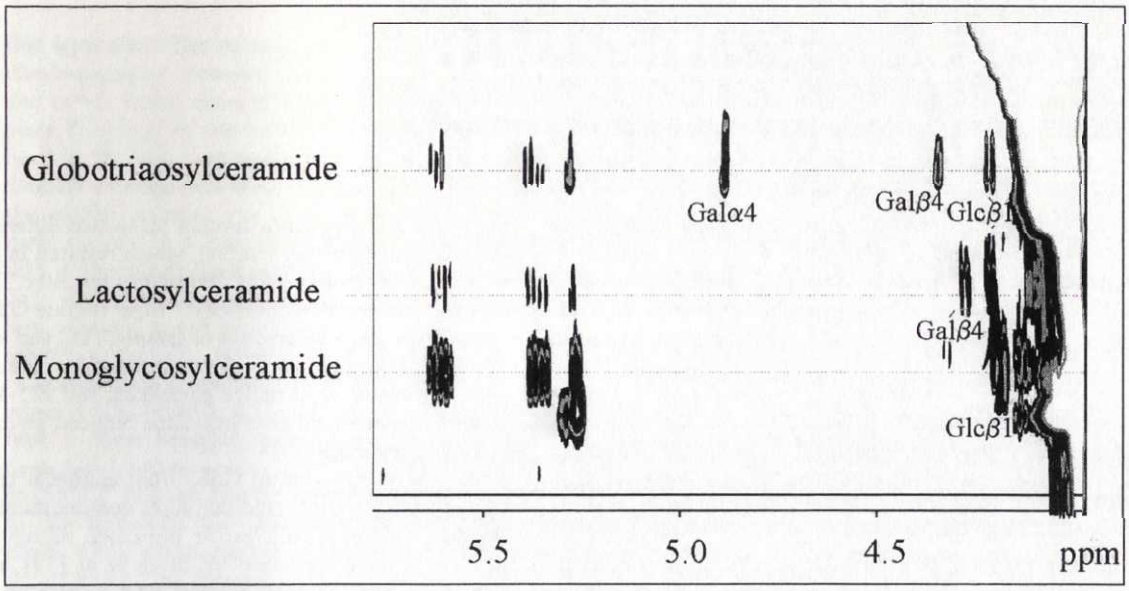

Figure 5. 2D spectra of the slowly moving fraction from pig lung (Paper 4).

detector for fractionation observation was connected in the interface between the LC and the NMR probe head. Continuous flow ^{1}H spectra were obtained using a one-dimensional NOESY pulse sequence providing improved solvent suppression. Pseudo-2D spectra were obtained using a sine-squared window multiplication prior to Fourier Transformation in the f2-dimension. The 2D spectra consisted of all obtained 1D spectra added in a single plot, with time on the y-axis and the chemical shift region on the x-axis. Smaller GSLs, which eluted early from the column, were found in the lower regions of the 2D spectra. Longer and hence more polar GSLs were found in the higher regions of the plot. The anomeric proton region (5.5-4.0 ppm) was used for monitoring the fractionation or elution. The 2D spectra showed the dynamics of the gradient elution with increasing or decreasing signals from significant anomeric protons appearing in the plot. (Figure 5).

The different anomeric protons from the sugar chains are indicated in the spectra to facilitate the interpretation.

3. A presentation of present work

The four papers presented in this thesis are part of a wider interest in xenogenic antigens involved in human xenotransplantation.

Paper one is aimed towards understanding carbohydrate expression at the level of biosynthesis regulation in the pig small intestine. The paper also deals with structural characterisation of pig small intestine GSLs and *in vitro* biosynthesis of GSLs with enzymes isolated from the same intestine.

Paper two shows oligosaccharide expression in small tissue specimens from different pig organs of a single semi-inbred pig. It demonstrates the quantitative differences in expression of carbohydrates in the organs and discusses their impact as antigens.

Paper three is a methodology paper describing for the first time LC"on-flow"NMR of native GSLs. The GSL fractions are separated and identified from the obtained spectra and TLC analysis.

Paper four is a development and an application of the LC"on-flow"NMR method described in paper three. It is concerned with an uncharacterised GSL mixture derived from pig lungs. The LC"on-flow"NMR method was used together with GC and GC/MS for the characterisation of the GSLs. The NMR method was also shown to be useful for pooling of GSL fractions after chromatography.

Paper 1

This paper describes the expression of blood group and histo-blood group GSLs in the epithelial cells of porcine small intestine, both from a structural and a biosynthetic perspective. We initiated this descriptive study to see if the expression of blood group ABH GSLs in the pig intestine, similarly as in humans, confined to type 1 chains [2, 18].

Through biosynthetic studies using crude enzyme preparations from pig intestinal epithelial cells and known characterised precursor glycolipids, we also aimed at some understanding of the glycolipid antigen expression on the level of precursor and enzyme availability and specificity. The paper also contains structural characterisation of GSLs based on thin layer immunostaining, EI^+-MS and ^1H NMR.

Non-acidic GSLs were isolated from porcine small intestinal epithelial cells, separated by column chromatography and pooled into nine non-polar fractions (NP1-9) and five polar fractions (P1-5)(Paper 1, Figure 1). Due to the small amount of sample, pooled GSL fractions were allowed to contain up to two different sugar chain lengths per fraction, which resulted in up to four different GSLs being present in a test tube. The last polar fraction P5 contained ≥7 sugar residue GSLs due to the very small amount of sample. TLC and immunostaining monitored GSL separation. Proton NMR spectroscopy of native glycolipids and EI^+-MS of permethylated and permethylated-reduced glycolipids were performed.

The characterisation of GSLs from epithelial cells with EI^+-MS, ^1H NMR and thin layer immunostaining showed similarities with earlier published results on the human small intestine by Björk *et al* [18] and Holgersson *et al* [2]. Like humans, type 1 chain blood group ABH GSL and type 1 and 4 as precursor chain molecules dominate the small intestine GSL expression. This expression is similar to the human intestine expression, except for the lack of A-7-4 in man [2]. Because only A type 1 structures were found, the dominating *in vivo* precursor used/available must be Lc_4Cer, which is supported by the *in vitro* studies.

The biosynthetic studies were performed with crude enzyme preparations from pig intestinal epithelial cells to evaluate the potential of the enzyme mix to produce GSLs *in vitro* with the added available precursor chains and radiolabelled sugars in the test tube. The result was compared with the *in vivo* expression of GSL from the same pig small intestine (Table 2).

The identification of the product of Lc_4Cer + fucose (Paper 1, Figure 5, Lane 1) was performed by TLC migration following acetylation. Since both H-5-1 and Le^a-5 are possible products in the Lc_4Cer + fucose experiment, (the fucose is bound to the terminal Gal in an α1,2 position for H-5-1, compared to a α1,4 linkage to the subterminal GalNAc for Le^a-5), an acetylated pure fraction of each of these references was also tested by TLC. The difference in chromatographic mobility allows product identification on the TLC plate. The product chromatographed like acetylated H-5-1 (Paper 1, Figure 6a-b). The same experiment was performed with nLc_4Cer + fucose (Paper 1, Figure 5, Lane 2) which could form as a product either H-5-2 or Le^x-5 (Fucα1,2 to terminal Gal for H-5-2, and Fucα1,3 to subterminal GalNAc for Le^x-5). The acetylated product migrated similar to acetylated H-5-2 (Paper 1, Figure 6c-d).

The product formed by Lc_3Cer + galactose (Paper1, Figure 7a) migrated as a triplet band on the

Precursors	GDP-[U-^{14}C] fucose	Precursors	UDP-N-acetyl-D[1-^{14}C] galactosamine	Precursors	UDP-[U-^{14}C]galactose
Lc_4Cer	+	H-5-1	+	Lc_3Cer	+
nLc_4Cer	+	H-5-2	+	Gg_3Cer	-
Gg_4Cer	+	H-5 on Gg_4	+		
Gb_5Cer	+	H-6-4	+		

Table 2. The result of the *in vitro* biosynthetic studies with microsomal enzyme fractions, radioactive labelled sugars and precursor chains.

thin layer plate. The references used created different chromatographic patterns, as in the cases for H-5-1 and Le^a-5, which allowed identification on the TLC plate. Pure Lc_4Cer was found to migrate as a doublet band in the same region. β-galactosidase specific for Galβ1,4 cleavage was used to treat the product, and the purified mixture was applied to TLC. The treatment resulted in a loss of the lower band and about 50% of the middle band of the triplet (Paper 1, Figure 7b). The experiment was repeated and the product now counted in a β-scintillator. The enzyme cleaved off 40% of the radioactivity.

Immunological reactivity was found to the blood group A GSLs based on all four chain types with the type 1 chain hexaglycosylceramide being predominant. A-6-1 was however clearly dominating, as assessed by TLC and was also the only component detected in that molecular size interval by EI^+-MS and ^1H NMR. Thus, the dominating *in vivo* precursor used should be Le_4Cer. This was also in agreement with our identification of the precursor Lc_4Cer, and the apparent absence of the nLc_4Cer precursor. Furthermore, this was also supported by the *in vitro* studies, which showed that all precursor chains (Lc_4Cer, nLc_4Cer, Gg_4Cer, Gb_5Cer) could serve as precursors for fucose with the enzymes prepared from the pig small intestine. The *in vitro* biosynthetic studies with blood group H GSLs based on the four precursor chains used (H-5-1, H-5-2, H-5 on Gg_4Cer and H-6-4), showed that all could serve as acceptors of GalNAc to produce blood group A structures with the enzymes prepared.

Lc_3Cer can, in our biosynthetic studies, using enzymes isolated from the pig, form both Lc_4Cer and nLc_4Cer. However, only Lc_4Cer could be identified in the pig small intestine epithelial cells by immunostaining, MS and ^1H NMR as mentioned earlier. The apparent absent of nLc_4Cer was established by probing TLC plates with anti-Galβ1,4GlcNAc MAbs (Paper 1, Figure 2a-b). These results are in contradiction with our *in vitro* studies where we could see a conversion of Lc_3Cer to nLc_4Cer. The *in vitro* situation may however be explained by a different localisation in the Golgi apparatus.

The β1,3galactosyltransferase in turn could loose its *in vivo* specificity when it is extracted from its environment within the intact cell and be able to add galactose also in a β1,4 position *in vitro*. This could also account for the lack of specificity seen for the α1,2fucosyltransferase and α1,3fucosyltransferases as well. A non-specific β-galactosidase specificity is less possible, because the high specificity for the Galβ1,4 linkage of the β-galactosidase was established during the development of the method.

Paper 2

GC/MS was used in the second article for rapid screening of GSL structures in small samples from kidney, spleen, small intestine, liver, salivary gland and heart from a single pig of a semi-inbred pig strain. The carbohydrate based blood group antigen distribution with special reference to GSLs with terminal Galα expression was characterised by MALDI-MS, GC, GC/MS and thin layer immunostaining.

Terminal Galα carrying GSLs in the pig is of particular interest for human xenotransplantation (transplantation between different species, *see chapter 4 Xenotransplantation*), where these antigenic structures have been shown to be involved in hyper acute rejection (HAR) when attempting to transplant across the species barrier i.e. pig organs to humans. The Galα1,3Gal epitope is not present in humans and higher primates but is present in most other species. Humans have also preformed circulating antibodies to these structures. This antigen and the preformed antibodies are responsible for the immediate HAR of xenotransplanted organs.

Characterisation of GSLs was performed by MALDI-MS, GS and GC/MS. GC/MS is a very sensitive method, which can be used for small samples and can be contrasted with full-scale preparation and conventional GSL analysis. Picomoles of components in the GSL mixture were found to be sufficient for analysis and gave clear and interpretative mass spectra. Due to the small sample amounts, no ^1H NMR was used for structural identification. Instead, thin layer immunostaining, GC and MALDI-MS were used to determine basic structural identities. We were able to interpret structural identity for the oligosaccharides present by combining the acquired information from our experiments with studies by others.

Total GSL fractions and the slower moving fractions ≥ 4 sugar GSLs) from kidney, spleen, small intestine, liver, salivary gland and heart were used for thin layer immunoanalysis. Staining with antibodies specific for the Galα1,3 epitope detected five-sugar compounds in kidney, salivary gland and heart, and a six-sugar compound in kidney and heart. Additional staining of eight- and ten-sugar Galα– components could also be seen in the organs. The findings were confirmed by lectins reactive against Galα- epitopes that bound to compounds in the five/seven sugar region on the TLC for the kidney and heart. An anti-A reagent bound to six- and seven sugar compounds in the salivary gland and heart.

Oligosaccharides cleaved off from the GSLs in the slowly moving fractions were characterised by MALDI-MS, GC and GC/MS. MALDI-MS gave information about the dynamic mass range of the fractions, GC was used to screen the number of components in the mixture and GC-MS gave sequence information of the oligosaccharide components.

The characterisation revealed high concentrations of a five-sugar oligosaccharide with terminal hexose, probably galactose, in all organs, earlier indicated by thin layer immunostaining. The structures were identified as Galα1,3nLc_4Cer which is in accordance with others [88]. The kidney also revealed a six sugar terminal Hex (Gal) component consistent with Galα 1,3Le^xCer [89].

Limited linkage information can be achieved using high temperature GC/MS in situations where conventional linkage analysis techniques, such as ^1H NMR and degradation can not be used. The high resolving power obtained by GC using ultra thin films was used for demonstration of the partly resolved oligosaccharides derived from H-5-2 and H-5-1. The oligosaccharide isomers could be differentiated on basis of their mass spectra due to relative intensity differences of the fragment ions at *m/z* 182 and *m/z* 228 since the isomers were chromatographically separated. This information also gave us the possibility to identify the blood group A hexasaccharide based on the type 1 chain in the salivary gland and heart together with its characteristic sequence fragment ions (B_1, B_2 and B_3, *see chap-*

ter 2) and the indication by anti-A MAbs.

Conventional GSL preparation and analysis can be compared with the small scale preparation and analysis involving thin layer immunostaining, MALDI-MS, GC and GC/MS used in this paper. The small-scale preparation and analysis appear to have an advantage when organs from a single animal are to be analysed, e.g. in intra-individual studies. The methods allow different parts from an organ to be separately analysed. Small tissue specimens (10-140 g) can be used instead of kilograms. GC separation makes it possible to analyse individual components of a mixture when coupled to MS. Another advantage is that acidic and non-acidic components could be analysed simultaniously with the above techniques.

The kidney is rich in GSLs containing terminal Gal(residues such as Galα1,3nLc$_4$Cer and Galα1,3Le^{x}Cer [88, 89]. The high Galα1,3 content in the pig kidney may indicate problems of HAR because of pre-existing antibodies which react well against this epitope, and thus make it a less suitable organ for pilot xenotransplantation experiments. There are indications of extended Galα1,3nLc$_4$Cer structures in the pig heart, which probably will give the same immune reaction as the shorter ones. The complex Galα– GSL expression of the heart suggests similar problems as are seen with the kidney, or even worse, since they are more accessible for antibodies. The liver, small intestine and salivary gland show the lowest amount of Galα– expression on GSLs.

Paper 3

The third and fourth papers evaluate LC"on-flow"NMR separation and analysis of native GSLs. The aim of Paper 3 was to evaluate the possibility of using the LC"on-flow"NMR technique to separate native GSL mixtures and also obtain interpretative spectra.

Three pure GSL components from human A$_1$ erythrocytes were pooled together. The criteria for suitable GSLs for the experiments were 1) well characterised different components, 2) easy GSLs to resolve by column chromatography, 3) present in sufficient amounts and 4) a presence of both α- and β-anomeric protons. LcCer (Galβ1,4Glcβ1,1Cer), Gb$_3$Cer (Galα 1,4Galβ1,4Glcβ1,1Cer), and Gb$_4$Cer (GalNAcβ 1,3Galα1,4Galβ1,4Glcβ1,1Cer) fulfilled these criteria.

A straight-phase silica column was interfaced to the NMR instrument. The sample mixture was injected on the column, and a gradient system of deuterated solvents (e.g. CDCl$_3$/CD$_3$OD/D$_2$O) with an increasing polarity was used. Provided optimal conditions, a separation of the GSLs were achieved. The NMR probe recorded continuous "on-flow" spectra during the experiment. NMR spectra were recorded for each component during the period the GSL was inside the probe. The fractions were collected and monitored by TLC after the LC"on-flow"NMR analysis.

The results were presented in a 2D contour plot, showing the time and dynamics of the separation on the y-axis and the chemical shifts on the x-axis (Paper 3, Figure 2). The coarse of separation could be followed on the y-axis, starting with the earliest eluted GSL, lactosylceramide, in the lower region of the plot. As the solvent became more polar, the longer, more polar globotriaosylceramide and later globotetraosylceramide were eluted and entered the NMR probe. These extended GSLs are found in the top region of the 2D plot.

We found the LC"on-flow"NMR technique to be useful for native GSL separation and also resulted in interpretative spectra if large amounts of sample (about 300µg of each component) were present and the GSL were separable on the column. The chemical shifts tended to drift when a more polar solvent was introduced into the system, which complicated the interpretation of more complex GSLs.

Paper 4

In the last paper, the LC"on-flow"NMR technique was used for separation, screening and characterisation of an unknown mixture of native GSLs. The information obtained was intended to assist pooling of the generated GSL fractions. We also wanted to evaluate the limitations of the method regarding GSL separation, analysis and detection. The paper includes thin layer immunostaining, MALDI-MS, GC and GC/MS of released oligosaccharides, used to assist in the structural interpretation as described in chapter 2. As part of the analysis, the LC"on-flow"NMR spectra were compared with conventional ^{1}H NMR spectra from the GSL fractions obtained.

GSLs from 4 pig lungs were prepared [66]. The fraction contained GSLs in different amounts and of different chain length (e.g. up to 5 sugar GSLs). The mixture was injected onto the column, which was interfaced with the NMR probe. A solvent gradient of CDCl$_3$/CD$_3$OD/D$_2$O with an increasing polarity was used for the silicic acid column separation. Smaller, less polar GSLs were the earliest eluted and entered the NMR probe first. With increasing polarity, longer and more complex GSLs entered the probe. NMR spectra were recorded "on-flow" during the run and were presented in a pseudo 2D plot (see Figure 5).

Since the obtained spectra contained solvent information and in some cases showed signal drift, conventional ^{1}H NMR was prepared on the separated fractions. At this early state of LC"on-flow"NMR development the ^{1}H NMR was used for reproduction and confirmation of the LC-NMR spectra findings. These spectra were interpreted and compared to the LC-NMR screening and also compared with reference spectra to give structural confirmation. The spectra were also used to evaluate signal drift and exclude false signal interpretation due to solvent interference. MALDI-MS gave the dynamic mass range for the prepared oligosaccharides deriving from the GSLs. The gas chromatogram gave an appreciation of the number and amount of components in the mixture. The different retention times also gave an indication about the relative size of the molecules. GC/MS gave sequence information by the oxonium ions (B$_i$) detected from the oligosaccharides. The thin layer immune staining with different MAbs recognised GSLs with antibody defined epitopes.

Since the techniques used complement each other, the generated information was combined and used together for structural characterisation. Some thin layer immunostaining results are shown in Table 3 (*for a total summary, see Paper 4*, Table 1a-b).

The characterised oligosaccharides from GSLs in the slowly moving fraction are presented in Table 4. For the first time, the Galα1,3nLc$_4$Cer structure was identified and reported in pig lung. A quantitative

Antibody specificity	Binding	Binding region on the TLC. Estimated number of sugars in the carbohydrate chain
anti-H-2	+	5,7
anti-Le^{b}H (H type 1)	+	5
anti-Galα1,3	+	5,6 7/8 10/12
anti-B	+	5

Table 3. Results from thin layer immunostainings of pig lung fractions with MAbs by the method of Hynsjö *et al* [74].

TIC peak	Interpreted GSL structure	Structure	M (Da):
1	Gb_3	Hex-O-Hex-O-Hex	658.4
2	nLc_3/Lc_3	HexN-O-Hex-O-Hex	699.4
3	Gb_4	HexN-O-Hex-O-Hex-O-Hex	903.5
4	nLc_4	Hex-O-HexN-O-Hex-O-Hex	903.5
5	H-5-2/ H-5-1	dHex-O-Hex-O-HexN-O-Hex-O-Hex	1077.6
6	Galα1,3nLc_4	Hex-O-Hex-O-HexN-O-Hex-O-Hex	1107.6

Table 4. The oligosaccharide structures released from GSLs in the slowly moving fraction from pig lungs as identified by ^{1}H NMR, GC/MS and MALDI-MS.

dominating expression of GSLs of the globo-series was also found, which is in accord with other studies of EC rich pig tissues e.g. aorta [90].

Since the GSL composition required a more polar gradient of the deuterated solvents (cp. Paper 3), a pronounced signal drift of the GSL protons and solvent signals were recorded. Another drawback was the discovery of a loss of longer GSLs (the longest GSL contained five sugars).

We also evaluated the possibility to use the LC"on-flow"NMR technique for guidance when pooling fractions after chromatography.

TLC analysis is based on the chromatographic mobility on the silicic acid on the plate, and can be empirically compared to known references. Sugar- and ceramide differences can increase or decrease the mobility of the GSLs, which can complicate identification. Continuously recorded NMR spectra give instead signals from i.e. anomeric protons, the information being absolute and both qualitative and quantitative. In this way, using the α– and β– signals that are easily identified together with their quantitative dynamics during separation, even gives us a possibility to discriminate between different components that are not totally separated over the column. An advantage with TLC is that small sample quantities are required compared with the large amounts required by LC-NMR. However, the small amount of GSL applied on the TLC is consumed, compared to no loss or destruction of the sample after LC-NMR analysis. When the LC-NMR analysis is completed, the separation is also complete and spectra are obtained. In contrast TLC analysis is made after the chromatographic separation, and MAbs may take several hours (depending on the technique used) to return a result on the epitopes present. Furthermore, TLC immunostaining only produces results related to the specificity of antiserum or lectin used. TLC is still a very valuable and cost effective technique. Regarding LC-NMR, the time consuming steps during a preparation procedure may be minimised by LC-NMR and in this way saving money. Also, LC-NMR accessories can now be considered standard NMR instrumentation and can be complemented with a final on-line MS detector.

The LC"on-flow"NMR technique can be summarised as follows. The technique can be used for pooling and characterisation of GSLs. The sensitivity of the technique is low compared with GC and GC/MS, but compared to conventional ^{1}H NMR the sensitivity is reasonable. For example, GC and GC/MS could easily detect nLc_3Cer but not conventional NMR. Validation by ^{1}H NMR is important for the development of the LC"on-flow"NMR technique. MALDI-MS, GC, GC/MS and thin layer immunostaining together allow structural characterisation of unknown GSLs in a mixture. In addition, the structural information showed the presence of a Galα1,3nLc_4Cer GSL not previously shown in pig lung.

4. Xenotransplantation

Introduction

A transplantation where the species barrier is crossed is usually called xenotransplantation. Two types of xenotransplantations have been defined in the literature, the concordant and the discordant transplantation. Usually the two types are referred to as a closely related species transplantation for the concordant model (e.g. baboon to human) and transplantation between distant related species for the discordant (e.g. pig to human) [91]. The terms are used to distinguish between two clinical states of xenograft rejection. In the discordant model there is an immediate hyper acute rejection (HAR) due to the presence of pre-existing antibodies in the host, while in the concordant model the graft is initially accepted but will probably be rejected over time by host immune responses. There are several factors that can contribute to an organ transplant rejection, for example mismatched blood groups or the susceptibility of specific organs to acute rejection. Kidneys are usually easily hyperacutely rejected while the liver seems to be relatively resistant.

Hyperacute rejection

HAR leads to typical clinical signs of a vascular rejection with oedema, haemorrhage and infarction with thrombosis in the organ vessels [92]. The expression of the Galα– antigen is heterogeneous within different organs, with preferential, non-homogenous expression in small vessels [93]. HAR is considered to be caused by preformed, xenoreactive natural antibodies (XNA) in the recipient species reacting with antigens exposed on the endothelium in the donor's organ vessels.

Microscopically, the event is characterised by complement activation (via the classical pathway), endothelial cell activation, platelet aggregation [94, 95], extravasation of white blood cells [96] and a perturbed endothelial layer [97].

Antibodies and antigens

XNAs are found in all animals. In the pig to human xenotransplantation situation, human XNAs, reactive with xenogenic pig antigens are directed against antigens of carbohydrate origin. The major antigens involved express the carbohydrate Galα1,3Gal epitope [98] that is due to expression of the gene encoding the α1,3 galactosyltranferase (α1,3 GT). In humans and Old World monkeys, apes and chimpanzees the Galα1,3 antigens are not expressed due to inactivation of the (α1,3 GT) glycosyltransferase. The Galα– carbohydrate epitope is expressed on both lipids and proteins. GSL expression shows great variation in different organs of the pig, with e.g. an increased expression in the heart and the kidney and decreased expression in the small intestine and the liver [85].

Endothelial cell (EC) activation by the immune system is at the centre of HAR, in part because of the ECs location in the blood vessels. The ECs actively contribute to the rejection process by promoting pathological changes, which lead to the acute graft failure [99]. EC activation is thought to be initiated by the complement factors (e.g. C5a) and the membrane attack complex (MAC).

Different approaches to avoid HAR

Reduction of the Galα1,3Gal expression

How can HAR be avoided? No one knows for sure that the reaction can be avoided entirely, but many results show a decrease and a slower rejection when modifying the donor organs or the host's immune response. Donor organ animals can be modified in several ways; genetic modification of the biosynthetic pathway responsible for Galα1,3Gal expression by introducing competing transferases e.g. an α1,2FT [100] or by introducing human complement regulatory proteins [101]. Knockout techniques which turn-off a specific gene [102] and hydrolase treatment of the pig organs which destroys the undesired antigen are other ways tested to decrease HAR. These experiments do not show a 100% reduction of HAR, but in some cases, up to 70% in reduction has been achieved [100]. The "ideal situation" would be non-HAR animals whose organs are suitable for grafting to humans and which do not express the Galα– epitopes. These animals do not exist naturally and are very difficult to produce.

With regards to the pig, which is a mammal residing high up in the evolutionary tree, probably has a range of α1,3GT in the cells, the knockout technique will be very difficult. If the knockout technique succeeds in eliminating one Galα glycosyltransferase, potentially another α1,3GT could still Galα– glycosylate the organ. No one knows how many knockouts would be required to eliminate the Galα1,3Gal epitope from the pig. Hydrolase treatment is thought to reduce the level of Galα1,3Gal epitopes expressed on the EC, but the treatment is only temporary as the antigens are known to reappear on the surfaces after a shorter time span.

Manipulation of the immune response

Manipulation of the host is an alternative approach to reduce HAR. This can be done by immunosuppressive drugs, or by absorption of the antibodies involved in the rejection. Immunosuppressive drugs are commonly used after allotransplantation and combinations of the drugs have been used in the xenotransplantation situation. Xenotransplanted patients probably need more specific other types and perhaps more intensive immunosuppression than allotransplanted patients do, due to the fact that the immune response to xenotransplantation is probably stronger than in allotransplantation [97]. HAR leads directly to T cell activation by the porcine presenting cells which expresses the pig antigens in significant amounts on the endothelium. Human MHC class II restricted T cells also recognise this pig antigen exposure, and participate in HAR [99]. The matching of pig organs to the recipient is primitive compared to allotransplants, which show good host-matching and low expression of foreign epitopes. A problem in xenotransplantation is that the heavily immunosuppressed host also is exposed to microbial agents which include those of pig origin, such as bacteria and endogenous retrovirus agents or possibly even prions. The concern that xenotransplantation may carry the potential for a new epidemic has been highlighted by recent experiences with both bovine spongiform encephalopathy and human immunodeficiency diseases [103].

Reduction of antibodies

The reduction of human anti-pig antibodies before transplantation is another way of trying to prevent HAR. This could be done more or less specifically and can be accomplished by pre-transplant organ perfusion, plasmapheresis [63, 104], or by circulating the blood from the recipient extracorporally through affinity columns, prior to transplantation, which bind human immunoglobulins [105, 106]. Different absorbents can be used, but protein A or immunoglobulin-binding antibodies are most commonly used in such columns [105, 106].

A more specific absorption of xenoreactive pig antibodies can be achieved by perfusing the blood through columns expressing the Galα1,3Gal disaccharide [107]. However, as shown in Paper 4 [85] and by others [89], Galα1,3-terminated structures can be attached to core chains other than those of the common Galili antigen, e.g. Galα1,3nLc4, in the porcine tissues. The porcine α1,3 GT is probably responsible for the biosynthesis of a spectrum of Galα1,3 containing immuno dominant epitopes. It is also possible that the human antibodies directed against the repertoire of pig antigens can distinguish between these different epitopes, just as there are antibodies that can distinguish between blood group A determinants carried by different core saccharide chains [108]. An efficient absorber of human anti-pig xenoreactive antibodies should therefore contain a variety of Galα1,3-terminated structures in order to absorb as much human anti-pig α1,3 determined antibodies as possible [109].

Function of Galα1,3Gal?

The function of the Galα1,3Gal epitope in the pig is not yet known. Humans can do without it, but can the pig? Reports on mice with an inactivated α1,3GT gene, found that these mice are viable and have normal organs but develop cataracts [102]. If this also is true for the pig is yet to be determined.

Delayed Xenograft Rejection

When HAR is avoided, delayed xenograft rejection (DXR) can still occur. DXR can be averted or delayed either by removing and suppressing XNAs and other humoral factors, or by depleting inhibiting complement components. DXR is characterised by abnormal thrombo regulation, donor organ EC activation, and infiltration of host monocytes and natural killer cells in the graft [97]. The cell infiltration (with cytokine production), together with EC activation (with an up-regulation of adhesion molecules, pro-coagulant proteins and production of cytokines), promotes intragraft inflammation and thrombosis which are believed to be the major mechanisms behind the DXR [110].

DXR in xenotransplanted laboratory animals [59] shows some similarities with the chronic rejection [110] seen after allotransplantation in humans.

The role of accommodation

The role of accommodation of the transplanted organs despite circulating antibodies in the bloodstream, have been discussed [92]. Accommodation is defined as the survival of an organ graft in the presence of anti-graft antibodies and complement. Allotransplantations of ABO incompatible kidneys have shown that removal of donor antibodies from the recipient could increase graft survival, even if the antibodies returned. The same phenomenon can be seen in xenografting, though not as frequent [92, 111].

Other considerations

Many questions still needs to be answered before xenotransplantation is an alternative to allotransplantation. One is if the xenotranplanted organs will function in humans. When the pig was chosen as a model animal donor, it was chosen because of its physiological and biochemical similarities to human. There are similarities, but the differences between the two species are in majority [112]. Just to examplify, how will organs adapt when transplanted to humans considering the much shorter life span of the pig (8-10 years)? Or, if a pig kidney or liver is transplanted to a human, will the enzymes from the pig organ function in the human body, and in turn, can or will the human control mechanisms and hormones affect the pig organ? Some enzymes are found to function, but others do not as in the case of human anti-diuretic hormone (hADH) [112]. Without ADH controlling the urine production of the pig kidney it will produce excessive amounts of urine which may result in physiological problems or a dependency on drug control. Other questions regarding pig physiology like organ size, horizontal organ position and blood pressure will also need to be considered [112]. Also the risk for bacterial and/or virus transmission [113] and foreign protein antigens have to be considered.

5. Final comments and future prospects

As mentioned earlier, the ideal theoretical solution to HAR in the pig to human xenotransplantation situation is a pig donor with α1,3 GT knocked out. This animal does not yet exist due to difficulties to grow proper pluri-potent stem cells. However, the knock-out technique have earlier produced transgenic mice and should, in principle, be useful on pigs too. It might just be a matter of time before a Galα1,3Gal deficient pig exist. Though, *is* this the solution of HAR?

Serious doubts have recently been put forward from a population risk perspective. It has been postulated that the loss of Galα1,3 expression in man might be an evolutionary advantage for protection against retrovirus transfer. When a virus particle is produced in e. g. the pig, the Galα- antigen is incorporated in its membranes. If the virus particle tries to enter a human, it will immediately be destroyed, since humans have naturally occurring circulating anti-Gal antibodies.

In this perspective, it might be advantage to think in terms of accommodation, a phenomenon earlier mentioned in chapter 4. This reaction has been studied in ABO incompatible organ transplantation. The phenomenon refers to a state of tolerance, where both antigen and antibodies are present, though without eliciting HAR. Several conditions contribute to induce accomodation. In the allotransplantation situation there is a low concentration of cell surface antigens present (as in the case of blood group A_2 to O kidney transplantation). A low concentration of specific antibodies in serum, achieved through plasmapheresis or specific/non-specific immunoabsorption, is also a prerequisite.

In the xenotransplantation situation, as well as in allotransplantation, there is a need for screening of low antigenic expressing organs. Screening of different organs may select for individuals showing a natural or genetically manipulated weak Galα- expression. If pig organs, transgenic for complement regulatory proteins (e.g. DAF), will be used for transplantation there is still a need to control differences in carbohydrate antigen expression in order to avoid HAR.

The methods used and/or developed in this thesis (e.g. GC/MS and LC"on-flow"NMR), might be the methods of choice for these comparative screening experiments. GC/MS will then be chosen for its high resolving and rapid screening which gives both qualitative and quantitative information and LC"on-flow"NMR for its quick and reproducible preparation of relevant antigen fractions. The qualitative aspect is important due to the varying expression of Galα- carrier chains/molecules. Both organ- as well as individual differences exist. The quantitative aspects of expression are important both in xenotransplantation and in allotransplantation. Different individual/organ antigen profiles may require specially tailored immunoabsorption columns with a very specific and appropriate solid phase to ensure a complete and specific removal of cytotoxic antibodies.

There is still a long way to go before the mysteries of xenotransplantation are solved. By the characterisation of carbohydrates on cell surfaces in organs of interest, we take a small, but important step in furthering our understanding of the mechanisms of xenotransplant rejection.

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