Transpl Int (1994) 7: 405–413
© Springer-Verlag 1994

ORIGINAL ARTICLE

Rafael Oriol
Frédérique Barthod
Anne-Marie Bergemer
Yong Ye
Eugen Koren
David K. C. Cooper

Monomorphic and polymorphic carbohydrate antigens on pig tissues: implications for organ xenotransplantation in the pig-to-human model

Received: 9 December 1993
Accepted: 16 February 1994

R. Oriol (✉) · F. Barthod
INSERM U-178,
16 Av. Paul Vaillant-Couturier,
F-94807 Villejuif Cedex, France

A.-M. Bergemer
Department of Pathology,
Ambroise Pare Hospital,
Boulogne, France

Y. Ye · D. K. C. Cooper
Oklahoma Transplantation Institute,
Baptist Medical Center,
Oklahoma City, USA

E. Koren
Oklahoma Medical Research Foundation,
Oklahoma City, OK 73112, USA

Abstract The existence of the αGal epitope in 137 pigs belonging to 23 different breeds suggests that this antigen is either monomorphic or occurs at a high incidence in the porcine species. Its histological location at the surface of pig vascular endothelial cells makes it a target for human natural anti-αGal antibodies and complement, which may be responsible for the hyperacute vascular rejection of transplanted pig organs. The precursor carbohydrate chain (*N*-acetyllactosamine) and NeuAc-substituted epitopes are also exposed at the surface of pig vascular endothelium and were found in all pigs in this study. However, humans also have these two epitopes on vascular endothelium and, consequently, have not made natural antibodies against these carbohydrate antigens. Therefore, these two pig epitopes cannot be the main target of the hyperacute vascular rejection process. Three pig phenotpyes – A + (51 %), A: H + (38 %), and A – H – I + (11 %) were identified among 37 Large-white pigs by the presence of polymorphic A, H, and I carbohydrate antigens on the brush border of the surface epithelium of small intestine. These antigens were also present in other exocrine secretions but were not detected on vascular endothelium of the same pigs, suggesting that they are not involved in the hyperacute vascular rejection, although the pig A tissue antigen can induce an immune response in 0 or B blood group recipients. Once the problem of the initial hyperacute vascular rejection directed against the αGal epitope is overcome, typing donor pigs for A, H, and I, as well as for the protein swine leukocyte antigens (SLA) and other pig antigens, may help in elucidating antigens involved in acute or chronic xenograft rejection.

Key words Xenotransplantation, pig, natural antibodies · Pig, carbohydrate antigens, xenotransplantation · Antigens, xenotransplantation, pig

Introduction

Two groups have explored the role that human anti-αGal antibodies, originally described by Galili et al. [12], might play in the hyperacute vascular rejection of pig organ xenotransplants.

The Oklahoma group reported at the first International Congress on Xenotransplantation (Minneapolis, 1991) that purified human natural anti-pig antibodies (obtained after perfusion of normal plasma through the vascular bed of pig kidneys or hearts) demonstrated strong reactivity with synthetic carbohydrate antigens containing a terminal, nonreducing αGal epitope: (i) αGal1 → 3β Gal → R, (ii) αGal1 → 3β Gal1 → 4β Glc → R, and (iii) αGal1 → 3β Gal1 → 4β GlcNAc → R (Chembiomed, Alberta Research Council, Edmonton, Canada). "Neutralization" of these antibodies by binding to added αGal oligosaccharides, or depletion of the

antibodies by passage of the serum through an immunoabsorbent column of an αGal oligosaccharide, resulted in a significant reduction in the cytotoxicity of human serum on pig cells [6, 7, 16].

The Göteborg group identified a terminal non reducing αGal1 → 3βGal1 → 4GlcNAc epitope on glycolipids extracted from porcine aorta, and the epitope was found on the pig aortic endothelium [19]. Furthermore, they later confirmed the structure of the epitope and determined the complete pentasaccharide structure of the major kidney αGal neutral glycosphingolipid to be αGal1 → 3βGal1 → 4β GlcNAc1 → 3β Gal1 → 4β Glc1 → 1 Ceramide, by gas chromatography/mass spectrometry [2, 30].

Human natural anti-αGal antibodies have been purified by affinity chromatography on immunoadsorbents containing synthetic oligosaccarides with the αGal epitope and have been used to define the tissue distribution of the pig αGal epitopes by immunofluorescence. The αGal epitope was found on all vascular endothelial cells [13], including capillaries of islets of Langerhans [32, 34], epithelial cells of renal proximal tubules, hepatocytes, lung epithelium, pancreatic ducts, and deep layers of the epidermis, as described elsewhere [26]. A similar tissue distribution of this epitope has recently been confirmed by two independent Australian groups who detected, in addition, αGal epitope on the apical pole of epithelial cells of biliary ducts [10, 35]. The αGal epitope has also been reported at the surface of pig red cells [12], leukocytes [10, 31], and platelets [33]. It has been independently identified in both glycolipids [2, 4, 19] and glycoproteins [35]. Finally, digestion of tissues [26] or cells [3] with α-galactosidase, which removes the terminal nonreducing αGal, completely abolished the positive reactions obtained with the purified natural anti-αGal antibodies and the *Griffonia simplicifolia* 1 B4 isolectin, which are both specific for the αGal1 → 3β Gal → R structure.

Since the αGal epitope on pig vascular endothelium is believed to be a major target for the natural antibodies present in human plasma, leading to hyperacute vascular rejection, it is important to know if all pigs express the same amounts of αGal epitope, and whether the porcine species is, therefore, monomorphic for expression of this antigen, or if there are individual variations from one pig to another, as has been recently proposed for a triad of pig glycoprotein antigens (gp 115, gp 125, and gp 135) [15]. It is evident that if there are differences in the expression of αGal epitopes, the selection of pigs with genetically no or low antigen expression could help to diminish the hyperacute vascular rejection of pig organ xenografts in humans.

A second point addressed in the present work is the identification of other pig carbohydrate antigens on vascular endothelium or in exocrine epithelium. Our previous study demonstrated that, in addition to the αGal epitope, there are sialylated antigens on pig vascular endothelium and polymorphic A and H histo-blood group-related antigens in exocrine secretions [26]. In the present work these observations are extended to 39 pigs from three different strains; new monomorphic and polymorphic carbohydrate antigens on pig tissues are described.

Immunofluorescent staining of (i) a Yucatan pig cryostat section labeled with human natural antibodies affinity-purified on αGal1 → 3βGal1 → 4βGlcNAc → R (anti-αGal; Fig. 1), (ii) paraffin sections of Poland China pig tissues stained with the lectin of *Solanum tuberosum* (*STL;* Fig. 2–6), and with the lectin of *Lycopersicon esculentum* (*LEL;* Fig. 7), and (iii) on a paraffin section of a Large-white pig stained with the lectin of *Ulex europaeus 1* (*UEA;* Fig. 8)

Fig. 1 Liver stained with human anti-αGal showing strong positive staining of all vascular endothelial cells and the apical pole of the epithelial cells of biliary ducts. Weak staining of connective tissue of portal tracks is also seen, as previously reported on cryostat sections [26] (× 100)

Fig. 2 Heart section stained with *STL*. All vascular endothelial cells are positive (× 250)

Fig. 3 Surface mucosa of the stomach stained with *STL*. All epithelial cells are strongly stained. The vascular endothelial cells in the stroma are also stained, but the brightness of the epithelial cells gives the false impression that endothelium is poorly stained (× 250)

Fig. 4 Mucosa of the small intestine stained with *STL*. Only the vascular endothelium in the stroma and irregular areas of the brush border are positive. Goblet cells and the body of absorptive epithelial cells are negative. Although the vascular endothelium staining of Figs. 3 and 4 is identical, the proximity of strongly positive epithelial cells gives the impression that vascular endothelium is weaker in the stomach (× 250)

Fig. 5 Cortex of the kidney stained with *STL*. Vascular endothelium of intertubular capillaries and glomeruli are positive. Apical poles of epithelial cells of distal convoluted tubules are also positive, but proximal convoluted tubules are negative. The same proximal tubules were strongly positive with the human anti-αGal and *Griffonia simplicifolia* 1 B4 lectin, the two reagents specific for αGal1 → 3Gal epitope. As in Fig. 3, the proximity of strong staining of proximal tubules made the positive vascular endothelium reaction (obtained with the anti-αGal reagents) less evident than the reaction with *LEL* or with *STL* in this figure (× 250)

Fig. 6 Pancreas stained with *STL*. Vascular endothelium of small and large vessels and the apical pole of epithelial cells of pancreatic ducts are positive (× 250)

Fig. 7 Lung stained with *LEL*. The apical pole of epithelial cells and bronchiolar secretion are strongly positive. Vascular endothelium of large and small vessels is also positive. All pulmonary alveolar parenchyma is positive and it is difficult to differentiate alveolar epithelial cells from vascular endothelial cells, as previously observed with anti-αGal reagents [26] (× 100)

Fig. 8 Mucosa of small intestine of an A– H+ Large-white pig stained with *UEA* 1. Both brush border of surface epithelium and goblet cells are strongly positive. The vascular endothelium is negative (× 250)

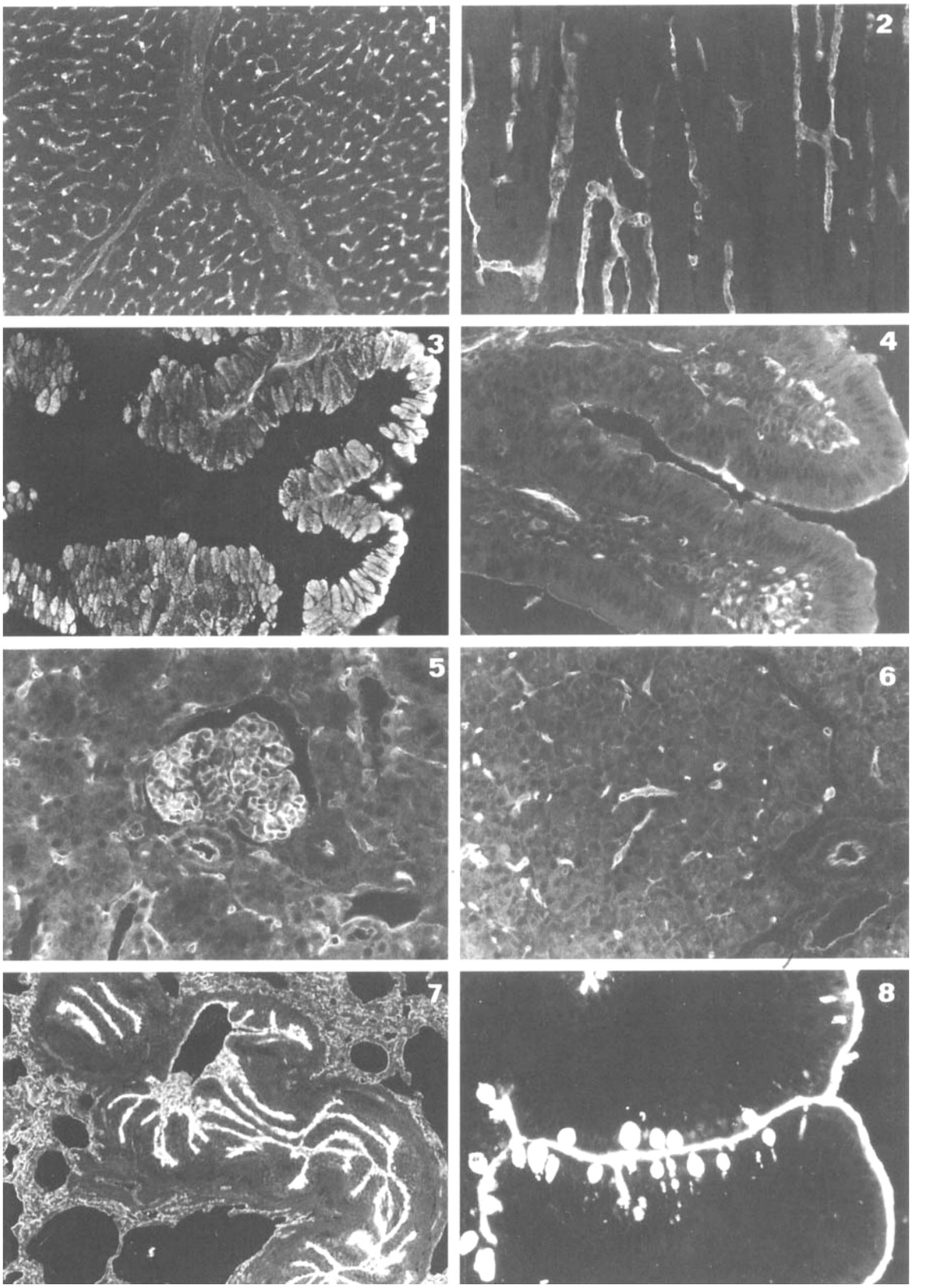
1
2
3
4
5
6
7
8

Table 1 List of pig strains or breeds reported by different teams to have αGal epitope in tissues

Pig strain or breed	Number tested	City	Reference
Local breed	> 1	San Francisco	[12]
Pig cell lines	> 1	San Francisco	[13]
Poland China	2	Oklahoma City	[7, 16]
Yorkshire	9	Oklahoma City	[7, 16, 26]
Local breed	> 1	Göteborg	[4, 19]
Local breed	> 1	Oxford	[3]
Inbreed (R. Binns)	1	Cambridge	[2]
German Landrace	4	Kiel	[34]
Belgium Landrace	4	Kiel	[34]
Duroc	4	Kiel	[34]
Hampshire	4	Kiel	[34]
Pietrain	4	Kiel	[34]
Göttingen minipig	4	Kiel	[34]
Inbred miniature	3 lines	Boston	D. H. Sachs (personal communication)
White Landrace	10	Stockholm	[32]
White Landrace	10	Victoria	[10]
Large-white	7	Victoria	[31]
Large-white	20	Nantes	[33]
Large-white	3	Cambridge	D. White (personal communication)
Large-white	5	Paris	[37]
Large-white	37	Paris	This article
Yucatan	1	Mineapolis	This article
Poland China	1	Oklahoma City	This article
Total 23	137	13	16

Materials and methods

Pig tissues

Tissue samples of myocardium, aorta, kidney, liver, lung, stomach, small intestine, thymus lymph node, spleen, and brain were fixed in formalin 10 % and embedded in paraffin wax by routine histological techniques.

Lectins

Lectins labeled with fluorescein (FITC): *Griffonia simplicifolia* 1 isolectins B4 (GSIB4) and A4 (GSIA4), *Triticum vulgare* (WGA), *Limax flavus* (LFA), *Anguilla anguilla* (AAA), *Limulus polyphemus* (LPA), *Helix pomatia* (HPA) and labeled with rhodamin (TRITC): *Maakia amurensis* (MAA), *Sambucus nigra* (SNA), *Vigna radiata* (VRA), *Artocarpus intergrifolia* (Jacalin), *Cytisus scoparius* (CSA), *Sophora japonica* (SJA), *Ricinus communis* (RCA) were obtained from E-Y (San Mateo, Calif., USA) and lectins labeled with FITC: *Euonymus europaeus* (EEL), *Ulex europaeus* 1 (UEAI), *Arachis hypogaea* (PNA), *Solanum tuberosum* (STL), *Licopersicon esculentum* (LEL), *Erythrina cristagalli* (ECL) were obtained from Vector Laboratories (Burlingame, Calif., USA).

Antibodies

Monoclonal anti-A (018), anti-A type 3 (009), anti-B (063), and anti-H type 2 (063) were obtained from the panel of the Second International Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens, Lund, Sweden (1990) [5, 25]. Anti-Le^y (77/180) was obtained from F. Real (IMIM, Barcelona, Spain), anti-Le^x (82H5) from Chembiomed (Alberta Research Council, Edmonton, Canada), and anti-I (18.3) from P. Edwards (Cambridge, UK).

Affinity-purified FITC-labeled sheep anti-mouse immunoglobulins (H + L) were obtained from Pasteur Diagnostic (Marnes la Coquette, France).

Immunofluorescence

Both unfixed cryostat sections and formalin-fixed, paraffin-embedded sections gave similar results with the pig carbohydrate epitopes and the reagents used. Therefore, the majority of this study was performed on routine fixed, paraffin-embedded tissues.

Fresh cryostat or fixed deparaffinated slides were incubated for 30 min in a wet chamber with affinity-purified FITC or TRITC-labeled lectins at 20 μg/ml. Indirect fluorescence was performed with a first 30-min incubation with monoclonal antibody and a second 30-min incubation with FITC-labeled anti-mouse immunoglobulin antibody. In both cases, coverslips were mounted with one drop of Vectashield (Vector Laboratories, Burlingame, Calif., USA) and fluorescence was observed on a Leitz SM-Lux microscope equipped with a lamp source of 200 W HBO, and a Ploemopak illuminator. Pictures were taken with a Leitz Photoautomat MPS 50 on 400 ASA Kodak TMAX black and white 24 × 36 mm film.

Results

The αGal epitope – is it monomorphic or polymorphic?

The αGal epitope was previously found on all vascular endothelial cells from all organs tested with affinity-purified human anti-αGal and the GSIB4 lectin in four American Yorkshire pigs [26]. In the present study we obtained the same results on vascular endothelium of all organs of a Yucatan pig (Fig. 1) and a Poland China pig. In addition, small intestine mucosa of 37 French Large-white pigs was screened for the presence of αGal epitope on vascular endothelium of the stroma of intestinal villi with the lectin GSIB4, and they were all also found to be positive. We therefore concluded that the αGal epitope of pig vascular endothelium is either monomorphic or has a high incidence in the porcine species since we have not found any pig negative for this antigen.

Furthermore, we sent a questionnaire to all the research groups who had mentioned the αGal epitope on pig tissues at the second International Congress on Xe-

Table 2 Structures of the oligosaccharides known to bind to the lectins employed in this study and the results obtained by immunofluorescence of labeled lectins on vascular endothelium of sections of pig heart and brain (*IF* results of the direct immunofluorescent test at 20 μg/ml, *R* glycolipid or glycoprotein carrier molecules)

Taxonomic name	Trivial name	Abbreviation	Inhibitor oligosaccharides	IF[a]
Griffonia simplicifolia 1 B4	bandeiraea	GS1B4	αGal1 → 3βGal → R	+ + +
Griffonia simplicifolia 1 A4	bandeiraea	GS1A4	αGal1 → 3βGal → R < αGalNAc → R	+
Maackia amurensis		MAA	αNeuAc2 → 3βGal1 → 4βGlcNAc/βGlc → R	+ + +
Sambucus nigra	elderberry	SNA	αNeuAc2 → 6βGal → R	+
Lycopersicon esculentum	tomato	LEL	$(\beta Gal1 \rightarrow 4\beta GlcNAc)_n$, $(\beta GlcNAc1 \rightarrow 4\beta GlcNAc)_n$	+ + +
Solanum tuberosum	potato	STL	$(\beta Gal1 \rightarrow 4\beta GlcNAc)_n$, $(\beta GlcNAc1 \rightarrow 4\beta GlcNAc)_n$	+ +
Euonymus europaeus	spindle tree	EEL	βGal1 → 4βGlcNAc < αFuc1 → 2βGal1 → R	+ +
Erythrina cristagalli	coral tree	ECA	βGal1 → 4βGlcNAc > βGal1 → 4Glc > GalNAc > Gal	+
Vigna radiata	mung bean	VRA	αGal → R	–
Artocarpus intergrifolia	breadfruit	Jacalin	αGal → R	–
Cytisus scoparius	scotch broom	CSA	GalNAc > βGal1 → 4Glc > αGal1 → 6Glc > Gal	–
Sophora japonica	pagoda tree	SJA	GalNAc > Gal	–
Helix pomatia	snail	HPA	GlcNAc, αGalNAc → R	–
Arachis hypogaea	peanut	PNA	βGal1 → 4Glc > βGal → R	–
Ricinus comunis	castor bean	RCA	βGal1 → 4Glc	–
Ulex europaeus 1	gorse	UEA1	αFuc1 → 2βGal1 → 4βGlcNAc → R (H type 2)	–
Anguilla anguilla	fresh water eel	AAA	αFuc → R	–
Triticum vulgare	wheat germ	WGA	$(\beta GlcNAc)_3 > (\beta GlcNAc)_2 > \beta GlcNAc$ > NeuAc	–
Limax flavus	garden slug	LFA	NeuAc, N-glycolylneuraminic acid	–
Limulus polyphemus[b]	horseshoe crab	LPA	NeuAc, N-glycolylneuraminic acid	–

[a] + + + strong staining, + + intermediate staining, + weak staining, – no staining

[b] Tested in 0.05 M TRIS, 0.15 M NaCl, 0.01 M $CaCl_2$

notransplantation (Cambridge, 1993). We received positive replies from 16 different groups, spread over three continents, with information on 137 pigs belonging to 23 different breeds (Table 1). These results illustrate the interest that has now arisen in this antigen and strongly suggest that the αGal epitope may, indeed, be monomorphic in the porcine species.

Other carbohydrate antigens on pig vascular endothelium

In addition to the αGal epitopes, pig vascular endothelium also reacted with the lectin *Maackia amurensis* (MAA), suggesting the presence of terminal nonreducing NeuAc linked in the α2 → 3 position to the subterminal Gal residue [26].

In an effort to identify other carbohydrate antigens exposed at the surface of endothelial cells, twenty lectins were tested on serial sections of the heart and brain of two pigs (Table 2). Heart (Fig. 2) and brain were selected for this screening because they are devoid of secretory mucins, which can mask the staining of vascular endothelium in other tissues as the stomach (Fig. 3). Strong positive results with GSIB4 and MAA confirmed the presence of αGal and αNeuAc epitopes, respectively, on pig vascular endothelium. A weak reaction with the lectin *Sambucus nigra* (SNA) suggested that either there are some αNeuAc epitopes linked in α2 → 6 or that this last lectin crossreacts slightly with the α2 → 3-linked NeuAc.

Four new lectins with similar specificity were found to be positive on pig vascualr endothelium: *Lycopersicon esculentum* (LEL), *Solanum tuberosum* (STL), *Erythrina cristagalli* (ECA), and *Euonymus europaeus* (EEL). The first three are known to react with a common carbohydrate structure, the type 2 precursor chain βGal1 → 4βGlcNAc → R (*N*-acetyllactosamine), either in its single terminal nonreducing form (ECA) or as extended poly-*N*-acetyllactosamine chains (LEL and STL). The relative intensity of the fluorescence was: LEL > STL > EEL > ECA, suggesting that pig vascular endothelium may have poly-*N*-acetyllactosamine epitopes, which more closely configurate to the lectins of LEL and STL than do single *N*-acetyllactosamine units. Finally, the fourth lectin, EEL, is known to react with some H epitopes but also crossreacts with the type 2 precursor chain. This last lectin had previously been reported to be positive on vascular endothelium of humans [27] and mammals, including the pig [28]. We can conclude from these results that there are also some *N*-acetyllactosamine-ended chains at the surface of pig vascular endothelium. This is not surprising since *N*-acetyllactosamine is the most abundant precursor chain in the biosynthetic pathway for the formation of αGal and αNeuAc epitopes. If not all of the type 2 precursor chains are transformed into sialylated or αGal epitopes, *N*-acetyllactosamine-ended chains can remain accessible at the surface of pig vascular endothelial cells.

The new pig strains tested in this study – Yucatan (Figs. 1–2), Poland China (Figs. 3–7), and Large-white

Table 3 Immunofluorescent staining of A, H, Le^y, Le^x and precursor chain epitopes on the brush border of surface epithelial cells of the small intestinal mucosa from 37 Large-white pigs (+ + + strong staining, + + intermediate staining, + weak fluorescent staining, – no staining)

Identification	A	A type 3	H	UEA	Le^y	Le^x	PNA	I	Phenotype
D57BJ15	+ +	+	–	–	–	–	–	–	A+
D58AJ21	+ +	+	–	–	–	–	–	–	
D60EJ4	+ + +	+	+	+	–	–	–	–	19/37 = 51 %
D3N3Aa	+ + +	+	–	–	–	–	–	–	
D5N5Aa	+ + +	–	+	–	–	–	–	–	
D6N6Aa	+ + +	+	–	–	–	–	+	–	
D30N14Y	+ + +	+	–	–	–	–	–	–	
D36N17Y	+ + +	+ +	–	–	–	–	–	–	
D38N19Y	+ + +	+	–	–	–	–	–	–	
D49N24Y	+ + +	–	–	–	–	–	–	–	
D57N28Y	+ + +	+ +	–	+	–	–	–	–	
D34N15bisY	+ + +	+	–	–	+	–	–	–	
D37N18Y	+ + +	+	+	+ +	–	–	–	–	
D16N10Fa	+ + +	–	–	–	–	+ +	–	+ +	
D11N7	+ + +	–	–	–	–	–	–	+ +	
D17N11	+ + +	+ +	–	–	–	–	–	+ + +	
D4N4Aa	+ + +	+	–	–	–	–	–	+ + +	
D31N15Y	+ + +	+	–	–	–	–	–	+ + +	
D55N25bisY	+ + +	+	–	–	–	–	–	+ + +	
D48N23bisY	–	–	+ + +	+ + +	+ + +	+	–	–	A– H+
D58N29Y	–	–	+ + +	+ + +	+ + +	–	–	–	
D59N30Y	–	–	+ + +	+ + +	+ + +	–	–	–	14/37 = 38 %
D60N31Y	–	–	+ + +	+ + +	+ + +	–	–	–	
D12N8	–	–	+ + +	+ + +	+ +	–	–	–	
D13N9	–	–	+ + +	+ + +	+ +	–	–		
D61CJ7	–	–	+ + +	+ + +	+ +	–	–	–	
D2N2Aa	–	–	+ + +	+ + +	+ +	–	+	–	
D44N22Y	–	–	+ + +	+ + +	+ +	–	–	–	
D47N23Y	–	–	+ + +	+ + +	+ +	–	–	–	
D54N26Y	–	–	+ + +	+ + +	+ +	–	–	–	
D42N21Y	–	–	+ + +	+ + +	+ +	+ +	–	+	
D53N25Y	–	–	+ + +	+ + +	+ + +	–	–	+ +	
D35N16Y	–	–	+ +	+ + +	+	–	–	+ + +	
D28N12Y	–	–	–	–	+	+ + +	+ + +	+ + +	A– H– I+
D41N20Y	–	–	–	–	–	+ + +	+ + +	+ +	
D29N13Y	–	–	–	–	–	+	+ + +	+ + +	4/37 = 11 %
D56N27Y	–	–	–	–	–	–	–	+ + +	

(Fig. 8) – gave similar fluorescent staining on vascular endothelial cells of all the organs tested. The lectins reacting positively with NeuAc and *N*-acetyllactosamine-ended chains were also tested on the vascular endothelium of sections of small intestine of the 37 Large-white pigs. They all showed similar positive reactions, suggesting that the expression of αNeuAc and *N*-acetyllactosamine epitopes might also be a monomorphic trait in pigs, as seems to be the case for the αGal epitope.

Polymorphic A, H, Le^y, Le^x, and I epitopes on the mucosa of small intestine

The epithelium of the intestinal mucosa of 37 Large-white pigs was tested with reagents specific for histo-blood group-related antibodies and lectins (Table 3). Histo-blood group A and H antigens have been previously described in pig glycolipids [18, 29], and the expression of these antigens is genetically polymorphic [1, 24].

Nineteen of the 37 pigs were strongly stained with anti-A on goblet cells and brush border of the surface epithelium (A+ phenotype, 51 %). Most of these A+ pigs were also positive on the same structures with anti-A type 3.

The brush border of surface epithelial cells was completely negative with the anti-A reagents in 18 of the 37 animals (A– phenotype, 49 %). The majority of these A– animals (14/18) demonstrated positive staining of the brush border (i) with reagents specific for the precursor of the A antigen, the H type 2 epitope, and (ii)

Table 4 Expression of carbohydrate antigen specificities at the surface of pig and human vascular endothelium. The structures in *bold type* and *underlined* are the only antigenic differences detected by histochemistry between the two species (*R* glycolipid or glycoprotein carrier molecules)

Pig	Human
βGal1 → 4βGlcNAc → R	βGal1 → 4βGlcNAc → R
αGal1 → 3βGal1 → 4βGlcNAc → R	**A or B1** → 3βGal1 → 4βGlcNAc → R 1 ↑ 2 **αFuc**
αNeuAc2 → 3βGal1 → 4βGlcNAc → R	αNeuAc2 → 3βGal1 → 4βGlcNAc → R

with anti Ley and *Ulex europaeus* lectin 1 (UEA) (Fig. 8); these last two reagents also react with H type 2. This particular A– H+ phenotype represented 38 % of the population.

The remaining four A – pigs were negative with all the anti-A and anti-H reagents but had positive staining of the brush border of surface epithelial cells with reagents specific for precursors of the H structure, such as anti-I or peanut agglutinin (PNA) and with anti-Lex, which reacts with the type 2 precursor chain substituted with fucose linked in α1 → 3 to the penultimate βGlcNAc residue (βGal1 → 4(αFuc1 → 3)βGlcNac → R). These pigs with A – H – I + phenotype represented 11 % of the overall population (Table 3).

Since, in the biosynthetic pathway, *N*-acetyllactosamine (βGal1 → 4βGlcNAc → R) is the precursor of H (αFuc1 → 2βGal1 → 4βGlcNAc → R) and H is, in turn, the precursor of A (αGalNAc1 → 3(αFuc1 → 2)βGal1 → 4βGlcNAc → R), it is not surprising that some of the A+ pigs also had spurious positive reactions with incomplete molecules reacting with anti-H or anti-precursor chains and that some of the H+ pigs also reacted with antiprecursor reagents.

The three main phenotypes (A+, A– H+, and A– H– I+) shown in Table 3, were clearly identified at the level of the brush border of the small intestinal surface epithelium but were more difficult to see on goblet cells. Indeed, the mucins of goblet cells of all pigs were positive with anti-H reagents (Fig. 8), irrespective of their phenotype on the brush border, suggesting that the genetic control of the expression of histo-blood group-related antigens in goblet cells is not identical to that of absorptive epithelial cells. A similar phenomenon has been recently observed between goblet and epithelial cells in human conjunctiva [14].

Discussion

All the pigs tested expressed the αGal epitope on vascular endothelium, suggesting that this antigen might be monomorphic in the porcine species (Table 1). However, it is worth continuing to look for αGal-negative pigs because such animals could exist at low frequency, as does the Bombay phenotype in the human species (estimated to be < 1 : 10,000). Breeding of such αGal-negative pigs, if they are ever found, would be easier than producing αGal-negative pigs by genetic manipulation.

We believe that this antigen is the main target involved in the hyperacute vascular rejection of pig organs [7, 16, 26] induced by the natural αGal antibodies (and complement) that are present in all higher primates [11]. However, some of the teams quoted in Table 1 [33, 37] think that, in addition to the αGal epitopes, other carbohydrate antigens may play a role in the rejection of pig xenotransplants.

Histochemistry studies indicate the cellular location of positive reactions between tissue epitopes and lectins or antibodies, but do not give hard information on the structure of the epitope itself. More precise technology, such as mass spectrometry and nuclear magnetic resonance [2, 18], will be needed to define the precise structure of the epitopes involved in these reactions.

The importance of the αGal epitope has received further support from experiments showing that human and baboon natural αGal antibodies are able to kill pig vascular endothelial cells and the pig kidney cell line PK-15 in the presence of complement [21]. The specificity of this cytotoxic reaction was further confirmed by specific inhibition of the reaction by the addition of synthetic oligosaccharides containing the terminal nonreducing αGal1 → 3Gal epitope [23].

Both neutral precursor and sialylated carbohydrate epitopes were also detected by immunofluorescence with specific lectins on vascular endothelial cells of all pigs in the present work. However, these antigens are also present in human vascular endothelium (Table 4), and humans have not developed natural antibodies against these epitopes, suggesting that they are not involved in the hyperacute vascular rejection of pig xenotransplants.

Three main pig phenotypes: A+, A– H+, and A– H– I+ were identified with reagents specific for A, H, Ley, Lex, and I epitopes (Table 3). However, these antigens were not found on vascular endothelium, suggesting that they also are not involved in the hyperacute vascular rejection, even if natural anti-A antibodies are found at

high titers as in humans with histo-blood groups 0 and B. Nevertheless, these polymorphic pig carbohydrate tissue antigens may be reached and attacked by human antibodies once the vascular endothelial barrier has been disrupted. They would also contribute to an immune response in transplant recipients who lacked the corresponding antigens. Indeed, A-O incompatibility has been previously reported to accelerate rejection in a pig allograft skin model in the same way and with similar efficacy to that of incompatibility of the major swine leukocyte antigen (SLA) system [22].

The recent cloning of the gene encoding for the αGal transferase enzyme of the pig [9] suggests that in the near future it may be possible to "knock out" the expression of this gene in pigs and create a breed of pigs devoid of αGal epitopes on vascular endothelium [8]. This would certainly be expected to help in preventing hyperacute vascular rejection and might also allow for the detection of other targets that may be masked by the violence of the reaction of the anti-αGal antibodies.

A transgenic pig that combines regulators of complement receptors [17, 20, 36] with lack of expression of αGal epitope on vascular endothelium would seem to be the most promising donor of organs for xenotransplantation in humans.

Acknowledgement This study was partially supported by the Centre National de la Recherche Scientifique (CNRS).

References

1. Andresen E (1962) Blood groups in pigs. Ann N Y Acad Sci 97: 205–225
2. Bäcker AE, Holgersson J, Karlsson H, Binns RM, Samuelsson BE (1993) Structural characterization of glycosphingolipids in different organs of a semi-inbred pig strain using GC/MS of ceramide glycanase-released oligosaccharides (abstract). Second International Congress of Xenotransplantation. September 1993, Cambridge
3. Cairns T, Hammelmann W, Gray D, Welsh K, Larsen G (1993) Enzymatic removal from various tissues of the galactose α1,3galactose target antigens of human anti-species antibodies (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
4. Cairns T, Karlsson E, Holgersson J, Welsh K, Samuelsson BE (1993) Confirmation of a major target epitope of human natural IgG and IgM anti-pig antibodies: terminal galactose α1,3galactose (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
5. Chester MA, Johnson U, Lundblad A, Löw B, Messeter L, Samuelsson BE (1990) Monoclonal antibodies against human red blood cells and related antigens. Lund. pp 208–209
6. Cooper DKC (1992) Depletion of natural antibodies in non-human primates – a step towards successful discordant xenografting in man. Clin Transplant 6: 178–183
7. Cooper DKC, Good AH, Koren E, Oriol R, Malcolm AJ, Ippolito RM, Neethling FA, Ye Y, Romano E, Zuhdi N (1993) Identification of α galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transplant Immunol 1: 198–205
8. Cooper DKC, Koren E, Oriol R (1993) Genetically engineered pigs. Lancet 342: 682–683
9. Dabrowski PL, Vaughan HA, McKenzie IFC, Sandrin M (1993) Characterization of cDNA clones encoding pig α(1,3)galactose transferase (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
10. Fournier AM, Birchall IE, Kyriazis AG, Pearse MJ, d'Apice AJF (1993) A human naturally occurring antibody, anti-Gal, recognises epitopes in pig kidney, heart, and liver and is cytotoxic to endothelial cells in the presence of rabbit complement (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
11. Galili U (1993) Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: a major obstacle for xenotransplantation. Immunol Today 14: 480–482
12. Galili U, Clark MR, Shohet SB, Buehler J, Macher B (1987) Evolutionary relationship between the natural anti-Gal antibody and the Galα1 → 3Gal epitope in primates. Proc Natl Acad Sci USA 84: 1369–1373
13. Galili U, Shohet SB, Kobrin E, Stults CLM, Macher BA (1988) Man, apes, and Old World monkey differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J Biol Chem 263: 17755–17762
14. Garcher C, Bara J, Bron A, Oriol R (1994) Expression of mucin peptide and blood-group ABH and Lewis related carbohydrate antigens in normal human conjunctiva. Invest Ophthalmol Vis Sci 35: 1184–1191
15. Geller RL, Rubinstein P, Platt JL (1993) Variation in expression of gp115/135 on porcine platelets (abstract). Second International Congress of Xenotransplantation, September 1993, Cambridge
16. Good AH, Cooper DKC, Malcolm AJ, Ippolito E, Koren E, Neethling FA, Ye Y Zuhdi N, Lamontagne LR (1992) Identification of carbohydrate structures that bind human anti-porcine antibodies: implications for discordant xenografting in humans. Transplant Proc 24: 559–562
17. Harland RC, Logan JS, Kooyman D, Byrne GW, Platt JL (1993) Ex-vivo perfusion of mouse hearts expressing the human complement regulatory protein CD59 (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
18. Holgersson J, Jovall PA, Samuelsson BE, Breimer ME (1990) Structural characterization of non-acid glycosphingolipids in kidneys of single blood group 0 and A pigs. J Biochem 108: 767–777

19. Karlsson E, Samuelsson BE, Holgersson J (1992) Blood group glycosphingolipids of porcine aorta (abstract). Carbohydrate International Meeting. July 1992, Paris
20. Kooyman D, Byrne GW, McClellan S, Nielsen DL, Kagan DT, Coffman T, Walsh LA, Tone M, Waldmann H, Platt JL, Logan JS (1993) Erythroid specific expression of human CD59 and transfer to vascular endothelial cells (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
21. Koren E, Neethling FA, Koscec M, Kujundzic M, Richards SV, Ye Y, Oriol R, Cooper DKC (1994) In vitro model for hyperacute rejection of xenogeneic cell. Transplant Proc 26: 1166
22. Leight GS, Kirkman R, Benjamin A, Rasmusen BA, Rosenberg SA, Sachs DH, Terrill R, Williams GM (1978) Transplantation in miniature swine. III. Effects of MSLA and A-0 blood group matching on skin allograft survival. Tissue Antigens 12: 65–74
23. Neethling FA, Koren E, Ye Y, Richards SV, Kujundzic M, Oriol R, Cooper DKC (1994) Protection of pig kidney (PK15) cells from the cytotoxic effect of anti-pig antibodies by α-galactosyl oligosaccharides. Transplantation 57: 959–963
24. Oriol R (1987) Tissular expression of ABH and Lewis antigens in humans and animals: expected value of different animal models in the study of AB0-incompatible organ transplants. Transplant Proc 19: 4416–4420
25. Oriol R, Samuelsson BE, Messeter L (1990) Workshop 1. ABO antibodies. Serological behaviour and immunochemical characterization. J Immunogenet 17: 279–299
26. Oriol R, Ye Y, Koren E, Cooper DKC (1993) Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 56: 1433–1442
27. Roussel F, Tayot J (1987) *Euonymus europaeus* agglutinin as a marker of endothelial cells in the human. Acta Anat 129: 92–95
28. Roussel F, Dalion J (1988) Lectins as markers of endothelial cells: comparative study between human and animal cells. Lab Anim 22: 135–140
29. Sako F, Gasa S, Makita A, Hayashi A, Nozawa S (1990) Human blood group glycosphingolipids of porcine erythrocytes. Arch Biochem Biophys 278: 228–237
30. Samuelsson BE, Cairns T (1994) Carbohydrate antigens as targets for human allo- and xeno-antibodies: pig-to-primate xenotransplantation. Alfred Benzon Symposium 36. Munksgaard, Copenhagen
31. Sandrin MS, Vaughan HA, Dabrowski PL, McKenzie IFC (1993) Anti-pig antibodies in human serum react predominantly with Galα(1,3)Gal epitopes. Proc Natl Acad Sci USA 90: 11391–11395
32. Satake M, Kawagishi N, Kumagai-Braesch M, Samuelsson BE, Rydberg L, Tibell A, Andersson A, Korsgren O, Groth CG, Möller E (1994) Specificity of human xenoantibodies formed in response to fetal porcine islet-like cell clusters. Transplant Proc 26: 1122
33. Thibaudeau K, Anegon I, Lemauff B, Soulillou JP, Blanchard D (1994) Human natural antibodies to porcine platelets. Transplantation 57: 1110–1115
34. Ulrichs K, Eckstein V, Korsgren O, Tibell A, Groth C-G, Müller-Ruchholtz W (1993) Characterization of natural and induced human xenophile antibodies before and after transplantation of the fetal porcine endocrine pancreas (abstract). Second International Congress on Xenotransplantation. September 1993, Cambridge
35. Vaughan HA, McKenzie IFC, Sandrin M (1994) Biochemical analysis of pig xenoantigens detected by naturally occurring human antibodies. Transplantation 00: 000–000 (in press)
36. White DJG (1993) Complement regulation. Second International Congress on Xenotransplantation. Cambridge, September 1993, Main lecture
37. Zhao Z, Michalski JC, Chéreau C, Calmus Y, Houssin D, Weill B (1993) An approach to the structure of carbohydrate epitopes recognized by human natural antibodies on porcine endothelial cells (abstract). Second International Congress on Xenotransplantation, September 1993, Cambridge