The Sda/GM2-glycan is a carbohydrate marker of porcine primordial germ cells and of a subpopulation of spermatogonia in cattle, pigs, horses and llama

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Abstract

Spermatogonia are a potential source of adult pluripotent stem cells and can be used for testis germ cell transplantation. Markers for the isolation of these cells are of great importance for biomedical applications. Primordial germ cells and prepubertal spermatogonia in many species can be identified by their binding of *Dolichos biflorus* agglutinin (DBA). This lectin binds to two different types of glycans, which are α -linked N-acetylgalactosamine (GalNAc) and β -linked GalNAc, if this is part of the Sda or GM2 glycotopes. We used the MAB CT1, which is specific for the trisaccharides motif NeuAc α 2–3(GalNAc β 1–4)Gal β 1-, which is common to both Sda and GM2 glycotopes, to further define the glycosylation of DBA binding germ cells. In porcine embryos, CT1 bound to migratory germ cells and gonocytes. CT1/DBA double staining showed that the mesonephros was CT1 negative but contained DBA-positive cells. Gonocytes in the female gonad became CT1 negative, while male gonocytes remained CT1 positive. In immunohistological double staining of cattle, pig, horse and llama testis, DBA and CT1 staining was generally colocalised in a subpopulation of spermatogonia. These spermatogonia were mainly single, sometimes paired or formed chains of up to four cells. Our data show that the Sda/GM2 glycotope is present in developing germ cells and spermatogonia in several species. Owing to the narrower specificity of the CT1 antibody, compared with DBA, the former is likely to be a useful tool for labelling and isolation of these cells.

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Introduction

The search for new markers for spermatogonial stem cells has been fostered by the development of two new biotechnological applications for this cell type. First, the development of testis germ cell transplantation in rodents (Brinster & Zimmermann 1994) and domestic livestock species offers new potentials for the generation of genetically modified offspring and also alternatives to artificial insemination (Hill & Dobrinski 2006). Secondly, there is evidence that pluripotent stem cells can be derived from spermatogonial stem cells of rodents and humans without genetic transformation (Ko *et al.* 2009, Kossack *et al.* 2009). Such cells would be an alternative to embryonic stem cells (Dym *et al.* 2009).

In many species, primordial germ cells (PGCs) and spermatogonia show a very specific surface glycosylation pattern, which differs from that of the surrounding cell populations. The biological significance of this glycosylation pattern is still largely unknown, but it seems likely that the glycans play a role in cell migration and adhesion. Lectins and carbohydrate-specific

antibodies are used to identify the specific glycosylation pattern of these cells. These reagents can be used to monitor cell purification procedures or for positive cell sorting (Herrid *et al.* 2009). A widely used marker for early stages in germ cell development in domestic mammals is the plant lectin *Dolichos biflorus* agglutinin (DBA), which binds to terminal N-acetylgalactosamine (GalNAc) residues (Piller *et al.* 1990). DBA is often regarded as specific for α -GalNAc, although it has been shown to bind to β -GalNAc, if this is part of the Sda (NeuAc α 2–3(GalNAc β 1–4)Gal β 1–4GlcNAc-R) or GM2 (NeuAc α 2–3(GalNAc β 1–4)Gal β 1–4Glu-R) glycotopes (Piller *et al.* 1990, Wu *et al.* 1998, Klisch *et al.* 2008).

Several studies showed that the binding profile of DBA to germ cells changes during development. In cattle, DBA binds to prespermatogonia in early postnatal stages and also to the Golgi region in some basal spermatogonia in postpubertal bulls (Ertl & Wrobel 1992). In the pig, DBA binds to PGCs and gonocytes, but to very few spermatogonia in the adult testis (Goel *et al.* 2007). Binding of DBA to bovine and porcine migratory PGCs has been demonstrated by Wrobel & Suss (1998) and

Takagi *et al.* (1997) respectively. DBA has been used to label spermatogonia in cattle (Izadyar *et al.* 2002, Herrid *et al.* 2007, Aponte *et al.* 2008, Fujihara *et al.* 2011), pigs (Takagi *et al.* 1997, Kim *et al.* 2010) and horses (Verini-Supplizi *et al.* 2000, Ha *et al.* 2003). It has also been used for the isolation of spermatogonia from bovine testis (Herrid *et al.* 2009, Anglin *et al.* 2010).

The use of DBA as a marker for isolating germ cells has the disadvantage that it also binds other cell types in neighbouring areas such as the mesonephros, as shown in the pig (Takagi *et al.* 1997). This lack of specificity restricts the application of this lectin for labelling cells prior to sorting by fluorescence-activated cell sorting (FACS) or magnetic beads.

In this study, we use a monoclonal mouse antibody CT1, which binds to the Sda/GM2 glycotope, to elucidate the configuration of GalNAc residues in spermatogonia of cattle, pigs, horses and llama, as well as porcine migratory PGCs and gonocytes.

Results

CT1 binds only to the Sda and GM2 glycotopes

On the Consortium of Functional Glycomics (CFG) glycan array, the CT1 antibody bound to three structures with very high specificity (Fig. 1). These were the Sda glycotope (Neu5Ac α 2-3(GalNAc β 1-4)Gal β 1-4GlcNAc β -), which is present on the array twice (linked with two different spacers) and to the GM2 glycotope (Neu5Ac α 2-3(GalNAc β 1-4)Gal β 1-4Glc β -; data are available on the CFG website: <http://www.functionalglycomics.org/>). A schematic drawing of these carbohydrate structures is given in Fig. 2. The binding specificity of DBA has been determined on an earlier

Figure 1 Binding data of CT1 and DBA to Consortium of Functional Glycomics (CFG) glycan arrays. The 13 carbohydrate structures with the strongest binding are shown. Complete data are available on the CFG website (<http://www.functionalglycomics.org/>). RFU, relative fluorescence unit. CT1 strongly binds to only two different glycans antibody, which are the Sda glycotope Neu5Ac α 2-3(GalNAc β 1-4)Gal β 1-4GlcNAc β - (linked to the array by two different spacers (Sp0 and Sp8)) and the GM2 glycotope (Neu5Ac α 2-3(GalNAc β 1-4)Gal β 1-4Glc β -). All other, glycans show affinities that are about three orders of magnitude lower. DBA also binds strongly to the two glycans, which are recognised by CT1, but shows additional binding to several structures that mainly contain terminal α 1-3GalNAc.

Figure 2 The Sda and GM2 glycotopes are branched carbohydrates, which have a terminal structure in common, which consists of sialic acid (NeuAc), N-acetylgalactosamine (GalNAc) and galactose (Gal). In the Sda glycotope, R stands for underlying carbohydrate chain, and in the GM2 ganglioside, R is a ceramide.

version of the CFG glycan array. These data show that DBA binds to the Sda and GM2 glycotopes, but also to other structures, which mainly contain terminal α 1-3-linked GalNAc (Fig. 1).

CT1 is expressed in early migratory pig PGCs

We determined whether CT1 was expressed in early migratory pig PGCs. A clear CT1 signal was detected in cells located in the hindgut of E15 embryos (Fig. 3). These CT1-positive cells can be clearly distinguished from their somatic neighbours because they are large and show prominent round nuclei, which are typical features of PGCs. The CT1 cells also stained positive for DBA lectin (Fig. 3A). No other embryonic cells, except epidermal cells (Fig. 3A) and some rare gut epithelial cells, were stained with this antibody. The CT1-positive cells were also co-immunostained with POU5F1 (also known as OCT-4; Fig. 3B), which identifies migratory PGC at this stage of development (Hyldig *et al.* 2011).

CT1 is expressed in gonocytes colonising the fetal gonad

Then, we investigated the expression of CT1 binding sites during the development of the fetal gonad in the pig. CT1 staining was detected in late migratory PGC reaching the gonadal primordium of E22 embryos (Fig. 4). These cells also showed weak DBA staining. Later, by E28, PGCs colonising the gonads were abundant, and colocalisation of CT1 and DBA staining was detected in almost all PGCs, although some DBA-positive cells were CT1 negative (Fig. 4, E28). The mesonephros of these embryos showed no CT1 immunoreactivity; however, many cells were stained with DBA. In the gonads of E28, E32, E42 and E51 embryos, CT1 and DBA were colocalised, although a small proportion of DBA+/CT1- cells were always detected.

In ovaries from E75 embryos, CT1- and DBA-stained groups of gonocytes located in close proximity. Ovaries containing small primordial follicles showed a reduction

Figure 3 CT1 marks early migratory pig PGCs. Sections of E15 embryos co-stained with CT1 antibody (green), and either DBA (red) and anti-POU5F1 antibody (red). DAPI (blue) was used as counterstaining. The lower row of images for each panel shows a closer view demarcated by the square in the merge images. (A) Arrows point to the PGCs in the hindgut, which are CT1 and DBA positive. The epidermis is CT1 positive (arrowhead) but shows only weak DBA staining. Some CT1/DBA staining was also detected in the extraembryonic membranes localised at the bottom of the picture. (B) The CT1-positive cells (arrows) in the hindgut are also POU5F1 positive. The higher magnification shows that the CT1 label is mainly cytoplasmic, while POU5F1 is located in the nuclei. Scale bar: 50 μ m for lower magnification and 20 μ m for higher magnification images.

in CT1 but maintained DBA staining. In the testis of E75 embryos, bright CT1 and DBA staining was detected in gonocytes located in the testicular cords.

To confirm that CT1-positive cells were PGCs/gonocytes, we performed double immunostaining with POU5F1 and UCHL1 (Fig. 5). Sections of E32 embryos show clear nuclear POU5F1 staining and CT1 in the cytoplasm and membrane of PGCs. CT1 and UCHL1 staining was colocalised in E70 gonocytes.

CT1 identifies a subpopulation of spermatogonia in postpubertal testes

In postpubertal testis of cattle, pig, llama and horse, the CT1 antibody binds to a subpopulation of cells located at the basal membrane of the seminiferous tubules (Figs 6 and 7). The frequency of these cells was relatively high in cattle and lower in the llama and horse. In the sections of postpubertal pig testis, only very few CT1 immunoreactive cells were detected. In cattle and horse, the CT1 binding was mainly restricted to the perinuclear region of the cells, but membrane binding was also visible. The pig and llama showed a more diffuse cytoplasmic staining and also labelling of the cell membrane. The CT1-positive cells were generally single cells, but also pairs (see llama in Fig. 6) or 'chains' of up to four cells could be observed (not shown). This occurrence of such groups of CT1-immunopositive cells was most obvious in the llama specimens. In the llama, some cells, which were more apically located, showed immunostaining of a dense structure adjacent to the cell nucleus (data not shown).

To further elucidate the identity of the CT1-positive cells, we used a marker for spermatogonia (UCHL1,

previously known as PGP9.5) in double staining with CT1 (Fig. 6). The UCHL1 antibody labelled spermatogonia in cattle, pig and llama, but not in horses. In cattle, the CT1 and UCHL1 staining was generally colocalised. In pig and llama, only a small fraction of the UCHL1-positive cells were also CT1 positive (Fig. 6). In prepubertal porcine testis, CT1-positive cells were more frequent than in postpubertal testis. They were located either at the periphery of the seminiferous tubules (Fig. 6) or centrally (Fig. 7).

The CT1/DBA double staining (Fig. 7) revealed that CT1-positive cells in the seminiferous tubules were also DBA positive. DBA bound also to other CT1-negative cells. In cattle, there was DBA staining of spermatids. In sections of prepubertal pig testis, many cells inside the tubules were DBA positive, but CT1 negative. In horse and llama, CT1 and DBA labelled the same population of cells.

In controls, the omission of the primary reagent (DBA/CT1/anti-UCHL1) generally reduced the staining to a diffuse background, without any prominent labelling of a specific structure or cell type (see [Supplementary Figures 1 and 2](#), see section on [supplementary data](#) given at the end of this article). One exception was the pre- and postpubertal pig testis, which showed a strong green signal in intertubular regions (probably Leydig cells) in the control sections.

Discussion

The lectin DBA binds to glycoepitopes with terminal α -linked GalNAc (e.g. blood group A, Forssman and Tn antigens; [Piller *et al.* 1990](#)) and is therefore often regarded as specific for GalNAc in α -linkage. But it also

Figure 4 CT1 marks gonadal pig PGCs. Sections of embryos between E22–E75 stained with CT1 antibody (green) and DBA lectin (red). Arrows point to a migratory PGC reaching the gonadal primordium (dashed area) in an E22 embryo. CT1 staining was detected in individual gonadal PGCs (E28–E32), but did not mark cells in the E28 mesonephros, as is typical for DBA. A few DBA-positive cells (arrowheads in E28) were CT1 negative. The inset in E32 shows the colocalisation of DBA/CT1 in higher magnification. In later stages (E75), testicular cords and ovarian nests (E75N) showed prominent CT1/DBA staining; however, some areas of E75 ovaries with primordial follicles (E75P) showed a marked reduction in CT1. Scale bar: 100 µm.

shows high affinity to β -linked GalNAc, if it is part of the Sda (GalNAc β 1–4(NeuAc α 2–3)Gal β 1–4GlcNAc-R) or the GM2 (GalNAc β 1–4(NeuAc α 2–3)Gal β 1–4Glc-R) glycotopes (see Figs 1 and 2; Kamada *et al.* 1991, Klisch *et al.* 2008). Sda and GM2 glycotopes share the common terminal trisaccharide (GalNAc β 1–4(NeuAc α 2–3)Gal β 1–), but differ only in the underlying sugar, which is GlcNAc in Sda and Glc in GM2. The binding of DBA to α -GalNAc as well as β -GalNAc is possibly due to an unusual configuration into which the β -GalNAc is forced by the adjacent α 2–3-linked NeuAc (Harris & Thornton 1978, Piller *et al.* 1990). The CT1 antibody shows a narrower binding specificity, since it only recognises the Sda and GM2 glycotopes.

Our results of the DBA staining in pigs and cattle are consistent with most previously published data. In our

study, we found very few DBA-positive cells in porcine testis, which is in agreement with Goel *et al.* (2007), who also found a small number of DBA-positive cells in testis of a 5-month-old pig. Pinart *et al.* (2001) reported no DBA affinity to cells of the seminiferous epithelium in 9-month-old postpubertal porcine testes, indicating that in older boars the epitope bound by DBA is no longer present in spermatogonia. Very few cells bound DBA in the horse testis (Ha *et al.* 2003). Verini-Supplizi *et al.* (2000) found no DBA-positive cells in the postpubertal equine testis, but a strong reaction with degenerating spermatogonia in the prepubertal testis. Ertl & Wrobel (1992) described DBA binding to the Golgi complex of bovine prespermatogonia and spermatogonia. Weak binding of DBA to bovine spermatogonia, acrosomes of spermatids and Leydig cells in the postpubertal tissue was described by Abd-Elmaksoud (2005).

Combined UCHL1 (PGP9.5) and DBA immunostainings were done in cattle (Herrid *et al.* 2007) and pig testis (Goel *et al.* 2007). Results of both studies are consistent with our findings. In pre- and postpubertal cattle, UCHL1 and DBA stain the same cells (Herrid *et al.* 2007). In pigs, the frequency of DBA-positive cells rapidly decreases after parturition, while UCHL1 staining remains constant (Goel *et al.* 2007). Our findings show that in the llama and in the pig, only a small proportion of the UCHL1-positive spermatogonia is CT1 positive. Since CT1 marks PGCs and gonocytes in earlier stages of development, the spermatogonia, which remain CT1 positive in the postpubertal testis, might be relatively undifferentiated. Whether this is linked to potential stem cell properties of these CT1-positive cells will need further elucidation.

The colocalisation of DBA and CT1 staining in the tissues studied suggests that the Sda glycotope is present in migrating porcine PGCs and a subpopulation of spermatogonia. Since GM2 ganglioside, which is also recognised by CT1, is a glycosphingolipid, the use of organic solvents during the histological preparation of our specimen should have removed this structure (Debarbieux *et al.* 2009). We would expect that only the Sda antigen, as part of glycoproteins, is responsible for the CT1 and DBA binding. The Sda glycotope is formed by the addition of GalNAc in a β 1–4 linkage to terminal 3'-sialyllactosamine residues. This final step of the synthesis is catalysed by the β -4-N-acetylgalactosaminyltransferase-2. The Sda glycotope has only been demonstrated in a limited number of glycoproteins. It was detected on N-glycans in bovine pregnancy-associated glycoprotein (Klisch *et al.* 2008) and on Tamm-Horsfall protein (Easton *et al.* 2000a). The Sda antigen was also found on human and mouse zona pellucida protein-3 (Easton *et al.* 2000b) and on murine CD45 in activated cytotoxic T-cells (LeFrancois & Bevan 1985). The functions of the Sda glycotope still remain unknown. It might be involved in the regulation of the half-life of glycoproteins in plasma (Klisch *et al.* 2008).

Figure 5 Anti-POU5F1 and anti-UCHL1 antibodies (red) were used to identify spermatogonial cells. The insets in both merged images show the colocalisation of these markers with CT1 (green) in gonocytes of E32 and E70 stages. Scale bar: 100 µm.

and is possibly involved in protecting the human urinary tract from pathogenic *Escherichia coli* (Serafini-Cessi *et al.* 2005).

Surface markers are ideally suited for labelling germ cells prior to sorting using FACS or magnetic beads, and DBA has frequently been used for these purposes. DBA, however, binds to carbohydrates that are present in

multiple cell types; thus, efficient sorting of cells based on the binding properties of this molecule depends on the location of the germ cells. For instance, in testis samples, DBA has been used for isolating type A spermatogonia using FACS (Izadyar *et al.* 2002) and magnetic separation (Herrid *et al.* 2009). For isolating PGCs from migratory stages, however, the use of DBA

Figure 6 CT1 and UCHL1 staining in postnatal testis. CT1 (green) and UCHL1 (red) double immunostaining of cattle (pre- and postpubertal), pig and llama testis. In prepubertal cattle, CT1 staining is found in all UCHL1-positive spermatogonia. The CT1 label is concentrated mainly in the perinuclear region (arrowheads) of the cells. The postpubertal cattle shows a similar pattern. At a higher magnification, the membrane staining with CT1 is clearly visible (arrow). In prepubertal pig testis, only a small proportion of UCHL1-labelled cells were CT1 positive. These were located either in the periphery of the tubules (arrowhead) or centrally. In the postpubertal pig testis, CT1-positive cells were very rare (arrowhead) and were a subpopulation of the UCHL1-positive cells (arrows). The strong green staining of intertubular cells in the pig specimens was unspecific, since it was also present in controls, in which the primary antibody (CT1) was omitted. In llama, CT1-positive cells were often seen in pairs (arrowheads), but single CT1-positive cells or chains of four cells were also present (not shown). The CT1 cells were a subpopulation of the UCHL1-positive cells (arrows). Scale bars: 50 µm.

Figure 7 CT1 and DBA staining in postnatal testis. CT1 (green) and DBA (red) double immunostaining of postpubertal cattle, prepubertal pig, llama and horse testis. CT1-positive cells are highlighted by arrowheads. In cattle testis, CT1 and DBA are colocalised in spermatogonia. The staining is mainly in the perinuclear region of the cells, but also at the cell membrane. In cattle, some cells, which are located more apically in the seminiferous epithelium, show a dense perinuclear DBA staining (arrows). In the porcine prepubertal testis, only a few intratubular cells are CT1 positive (arrowheads), while many more are DBA positive (arrows). In llama and horse testis, CT1 and DBA staining is localised in cells (arrowheads) located basally in the seminiferous tubules. Scale bars: 50 μ m.

may be more problematic because cells in the mesonephros, a neighbouring structure to the migratory path of PGCs and to the gonadal ridges, also bind this molecule (our study; Takagi *et al.* (1997)). Our results with CT1 show that this antibody specifically marks PGCs in their migratory path and cells in the mesonephros are not labelled, making this antibody potentially suitable for cell-sorting applications. The strong background produced by the secondary antibody in our study could be overcome using a direct labelling approach for the CT1 antibody prior to FACS sorting. Further experiments with the CT1 antibody will be necessary to show whether this antibody is superior to the DBA lectin for the isolation of PGCs and spermatogonia in domestic animals.

In conclusion, this study established that Sda/GM2 epitopes are present on germ cells during early stages of development and become restricted to a small population of cells in the postpubertal male gonad. The identity of these rare cells in the postpubertal gonad is currently unknown, and future studies using CT1 antibody will determine whether these spermatogonia represent a unique population of germ cell progenitors.

Materials and Methods

Materials

All the procedures involving animals have been approved by the School of Biosciences Ethics Review Committee (University of Nottingham, UK). Porcine embryos were collected from artificially inseminated British Landrace sows or Yorkshire X Landrace gilts. Embryos were recovered from the pregnant uteri within between 30 min and 2 h of slaughter. Embryos of each of the ages (15, 22, 28, 32, 42, 51, 70 and 75 days) were fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Tissue was hereafter dehydrated through increasing ethanol concentrations to xylene and embedded in paraffin. Transversal sections of 5 μ m thickness containing the PGC were collected on SuperFrost Plus microscope slides (Menzel, Braunschweig, Germany).

Testicular tissue of cattle ($n=2$), pigs ($n=3$), horses ($n=2$) and llamas ($n=2$) was fixed in Bouin's fixative for 24 h and embedded in paraffin. The porcine and bovine testes were collected at a commercial abattoir. Llama and horse testes were obtained from castrations. Except for the llamas, which were aged 2–10 years, no information about the age of the animals was available. The testes of two animals (one cattle and one pig) were classified as prepubertal due to the absence of spermatozoa in the seminiferous ducts.

The CT1-hybridoma was a gift from Leo Lefrancois, University of Connecticut, Farmington, CT, USA. The hybridoma cells were grown in medium (DMEM Ham's F12, 10% FCS, 2 mM L-glutamine, 1× penicillin/streptomycin and 1 mM sodium pyruvate) until the cells died. The cells were removed by centrifugation and the supernatant was concentrated ~20-fold by ultrafiltration. The antibody concentration was estimated by dot-blot in comparison to an IgM solution of known concentration.

Determination of CT1 binding specificity

An aliquot of the CT1 antibody (0.1 mg IgM, freeze dried in culture medium) was sent to the protein-carbohydrate interaction core (H) of the CFG for determination of its carbohydrate binding specificity. The antibody (10 µg/ml) was incubated on the version 4.1 of the printed Glycan Array, which consists of 465 glycans in replicates of six. Concentrated culture medium without antibodies was used as negative control.

Immunohistochemistry

Paraffin sections (5 µm) were dewaxed in histoclear, rinsed in three changes of ethanol, rehydrated in descending concentrations of ethanol and rinsed in distilled water. Antigen retrieval was performed by boiling the slides for 10 min in citrate buffer (0.01 M sodium citrate, 0.5% Tween 20 and pH 6.0) in a microwave oven, followed by 30 min cool down and 10 min wash in distilled water. For double staining, the sections were blocked in 10% normal goat serum or 10% normal donkey serum (for all POU5F1 staining) in PBS for 1 h and incubated with the primary antibodies/lectin for 1 h at room temperature or overnight at 4 °C in blocking solution. The CT1 antibody (2 µg/ml) was used in combination with one of the following: biotinylated DBA (10 µg/ml, Sigma), anti-POU5F1 (2 µg/ml, Santa Cruz Biotechnology Santa Cruz, CA, USA) or anti-UCHL1 (rabbit antiserum, 1:1000, Enzo Life Science, Exeter, UK). Slides were washed twice for 5 min in PBS. The antibody/lectin binding was visualised by incubation with fluorochrome-labelled secondary antibodies in 10% blocking serum in PBS for 90 min. CT1 was detected with FITC-labelled donkey anti-mouse (0.5 µg/ml, Vector Laboratories, Burlingame, CA, USA) and the biotinylated DBA with streptavidin-Cy3 (0.25 µg/ml, Invitrogen). A Cy3-labelled donkey anti-goat antibody (4 µg/ml, Vector Laboratories) was used to visualise POU5F1 and an Alexa488-labelled goat anti-rabbit (2 µg/ml, Invitrogen) was used to visualise the UCHL1. Slides were washed three times for 10 min in PBS, counterstained with DAPI (1:10 000) and mounted in Vectashield (Vector Laboratories). In controls, the incubation was carried out under identical conditions, but without the primary reagents (CT1, POU5F1, UCHL1 antibodies or DBA).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0007>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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