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Long-term IgG response to porcine Neu5Gc-antigens without transmission of PERV in burn patients treated with porcine skin xenografts

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Abstract

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Acellular materials of xenogenic origin are used worldwide as xenografts and Phase I trials of viable pig pancreatic islets are currently being performed. However, limited information is available on transmission of porcine endogenous retrovirus (PERV) after xenotransplantation and on the long-term immune response of recipients to xenoantigens. We analyzed the blood of burn patients who had received living pig skin dressings for up to 8 weeks for the presence of PERV as well as for the level and nature of their long term (maximum 34 years) immune response against pig antigens. Whilst no evidence of PERV genomic material or anti PERV antibody response was found, we observed a moderate increase in anti Gal antibodies and a high and sustained anti non-Gal IgG response in those patients. Antibodies against the non-human sialic acid Neu5Gc constituted the anti non- Gal response with the recognition pattern on a sialogly can array differing from that of burn patients treated without pig skin. These data suggest that anti-Neu5Gc antibodies may represent a barrier for long-term acceptance of porcine xenografts. As anti-Neu5Gc antibodies can promote chronic inflammation, the long-term safety of living and acellular pig tissue implants in recipients warrants further evaluation.

Keywords

xenotransplantation; pig; skin graft; xenoantibody; PERV

Introduction

The prospect of clinical xenotransplantation could result in a medical revolution in the near future (1). Clinical xenotransplantation trials involving pig cells and tissues are imminent and range from cornea, to islet cells for diabetes to brain cells for neuronal diseases including Parkinson's disease. For example, a clinical trial of islet xenotransplantation is ongoing (2, 3). Transient xenograft transplantation for fulminant organ failure (e.g., heart or liver) has been used as a bridge to allotransplantation (4). In addition, devitalized animal tissues including heart valves, skin and tendons, are currently widely implanted to patients (5–7). Likewise, vital pig skin (PS) has been widely used as a dressing for burn patients (8, 9). In these clinical settings, where patients are exposed to xenogeneic antigens and pathogens in unnatural fashions, microbial safety (10) and immunological effects (11, 12) are of critical importance and require extensive assessments.

The porcine endogenous retroviruses (PERV) has been regarded as the major potential risk of zoonoses in xenotransplantation (10). A limited number of studies on patients who had received xenotransplantation found no evidence of PERV infection in humans (13–19). However, retroviruses in other species often establish latent infection and cause chronic immunological or neurological diseases as well as cancer. Therefore, although the relevance of PERV is debated (20), it is considered necessary to monitor for PERV infection regardless (21). Thus further testing of xenograft recipients for chronic PERV infection is warranted.

Another risk in xenotransplantation stems from the well-known xeno-reactive antibodies that can cause rejection of xenografts (22–25). Humans, apes and old world monkeys are defective in the *GGTA1* gene encoding the alpha1-3-galactosyl-transferase enzyme (1-3GT) and produce high levels of anti- Gal antibodies (25–27), largely due to continuous exposure to Gal-expressing bacteria in the gastrointestinal normal flora. These antibodies can cause hyperacute rejection (HAR) of porcine organ xenografts (25, 28, 29). To prevent HAR, pigs with knocked-out 1-3GT have been generated and are currently being investigated (30–35). However, pig grafts express many non- Gal antigens (36, 37) and induction of other xeno-reactive antibodies has also been observed (11, 38–40). *N*-Glycolylneuraminic acid, Neu5Gc, is a dietary non-human sialic acid that incorporates into

diverse human glycoconjugates. Such modified glycoconjugates then become immunogenic and lead to the generation of anti-Neu5Gc xeno-autoantibodies (37, 41–44). However the major difference between anti- Gal and anti-Neu5Gc antibodies lies in the fact that Neu5Gc-antigens can metabolically incorporate into normal human tissues, in contrast to the Gal antigen, which cannot (43). Furthermore, it was shown that persistent presence of anti-Neu5Gc antibodies may result in chronic inflammation due to the concomitant presence of Neu5Gc-antigens, likely leading to exacerbation of vascular diseases and cancer (42, 44–46).

Here we sought to investigate these two major long-term risk factors in xenotransplantation, especially in light of the anticipated future increase in recipients of xenografts (or animal-derived medical devices). For this purpose, we took a retrospective approach by using clinical samples of xenograft-treated patients. The use of skin harvested from commercial pigs (vital PS) was demonstrated to be effective in treating patients with severe burns by limiting wound secretions and facilitating granulation and epithelialization of the affected areas (47, 48). We tested long-term immunological effects of exposure to PS in blood and serum samples collected from burn patients up to 34 years post-treatment with PS compared with age-matched control burn patients without PS treatment. We present compelling evidence for lack of PERV infection in PS recipients but persistent and high anti-Neu5Gc IgG response to porcine Neu5Gc antigens.

Materials and Methods

Burn patients and control groups

This study was approved by Ethical Committee of The University Hospital Kralovske Vinohrady, Prague, No. EK/25/2007. The study was also conducted in compliance with the requirement of Ethics Committee of the European Commission that supported the study.

A total of 220 burn patients among more than 15,000 patients who received PS treatment (BP-PS) at BMC-Prague between 1973 and 2005 were invited to participate in this study. These patients were selected for their burn injury severity and recent hospital contact records. 54 of them responded positively and provided their informed consent and blood samples. Blood samples were collected, de-identified, and serum and peripheral blood mononuclear cells (PBMC) prepared. No specific chronic and/or unexplained illness potentially related to PS treatment was noted in the patient record and their interview by the clinician prior to sampling. One individual was still undergoing treatment for her injuries at the time of sampling (one month post xenograft). Samples from control burn patients not treated with PS (BP-CTRL) were also collected at Burn Medicine Clinic, Faculty Hospital of Královské Vinohrady, Prague (BMC-Prague) (n=7) or Centre Hospitalo Universitaire, Nantes (n=7); average duration between injury and sampling was 113.7 months for BP-PS and 45.8 months for BP-CTRLs. Burn wounds of these BP-CTRL patients were tailored by removing necrotic tissues and healed by autografting. Healthy controls (HC, n=27) were mainly voluntary laboratory workers at Institute of Molecular Genetics, Prague.

Clinical characteristics

PS-treated burn patients (BP-PS) were admitted with full-thickness and deep dermal burns and 10% to 80% total body surface area (TBSA) burns equating to 2nd (II) and 3rd (III) degree burns between year 1976 and 2005. Patients age ranged from 18 months to 71 years at time of treatment. This included 4 children aged 18 months, 8, 15 and 17 years. Prior to use the PS, strips were immersed in an antibiotic solution containing streptomycin, chloramphenicol, and furantione for a period of 20 minutes and then assessed for microbial contamination. Viability of skin was determined by assessing glucose metabolism and then

either snap frozen in cryoprotective media containing 15% glycerol for storage in liquid nitrogen or used fresh (8). Patients were exposed to porcine xenografts up to 8 weeks and a maximum number of 18 xenograft changes ranging from 2–5 days apart. At the time of accident, the use of fresh porcine skin xenografts at BMC-Prague was considered the best life-saving treatment. All cases were historical and blood collected at an average of 4 years post accident with the exception of patient number 27 who had a more recent burn injury and was sampled 1 month after treatment.

Burn patients not treated with PS dressing (PS-CTRL) were age matched and presented similar burn lesion grading. Burns were here treated by removal of necrotic tissue and auto grafts.

In vitro transmission of PERV

Blood samples were taken from the commercial herd (Large White x Landrace x Goland Poland) used for collection of skin for xenografts (n=10). Peripheral blood mononuclear cells were isolated as described in (49, 50), co-cultured with porcine fibroblasts (ST-Iowa) and human epithelial (293) cells and assayed for PERV production as described in (13, 51).

Determination of PERV in patient samples

Both total RNA and vRNA was extracted from fresh whole blood (RNeasy kit, Qiagen, Surrey, UK) and serum (Qiagen viral RNA kit, Surrey, UK) respectively according to the manufacturer's instructions. The presence/absence of PERV RNA was confirmed by RT-qPCR using the following primers and probe P218F 5 -CCGGCTCTCATCCTGATCA-3 and P315R 5 -TCTTGGTTTATTTAGCCATGGTTTAA-3 and probe P241T 5 -FAM-CCCTATATCCTTACGTGGCAAGATTTGGCA-TAMRA-3 targeting the pol region. Reactions were carried out according to the manufactures instructions using TaqMan one step RT-PCR master mix (Applied Biosystems, Warrington, UK), $0.5\mu M$ of each primer, $1\mu M$ of probe and $3\mu l$ of RNA sample. The cycling conditions were 48°C for 30mins, 95°C for 10mins, then 40 cycles of 95°C for 15secs, 53°c for 30secs and 60°C for 30secs (ABI 7500 fast, Applied Biosystems, Warrington, UK). For vRNA, each reaction was spiked with Taqman exogenous internal positive control (Applied Biosystems, UK) and for total RNA, reactions included 18S RNA as a reference using primers and probe from the 18S rRNA control kit (Eurogentec, Hampshire, UK) to avoid false negatives due to the absence of PERV RNA and/or RT-PCR failures.

DNA was prepared from white blood cells collected from the patients using the Nucleon Bacc2 kit (GE healthcare, Buckinghamshire, UK) according to manufacturer's instructions. Patient DNA was tested for the presence of PERV DNA with the same conditions as above using $1\mu g$ of DNA and Gene Expression Master Mix (Applied Biosystems, UK). Reactions were carried out according to manufacturer's instructions with $0.5\mu M$ of each primer and $1\mu M$ of probe. Absolute quantification was used to define results and samples were compared against a plasmid standard expressing the gag-pol region of PERV (52). Patient DNA was also tested for the presence of pig centromeric DNA to determine if patients displayed microchimerism according to the method and sensitivity described previously by Paradis *et al* (13).

Validation of these assays showed that the detection limit of this qPCR assay for PERV DNA was one copy of PERV per $1\mu g$ of DNA (300,000 cells) (Supplementary Fig S1C), which gave a confidence level of >99.9% of detecting = copies, and therefore <0.01% chance of a false negative. The sensitivity of the vRNA PCR was 5 copies per $3\mu l$ of vRNA preparation and validation of this assay showed that we could consistently detect 475 viral particles per ml of serum.

Neutralizing antibodies to PERV

A total of 11 xenograft recipients and 4 control samples were tested for seroneutralisation of PERV. The recombinant PERVA/C virus 14/220 (49) was replicated in 293T cells, cell free supernatant containing virus was recovered, divided into aliquots and stored at −80°C. The stock virus was titrated by immunostaining on 293T cells. The human sera were inactivated for 30 min at 56°C. Thirty µl of the serum were incubated with 30 µl of the virus dilution containing 120 focus-forming units of PERVA/C virus for 1 h at 37C. Then 50 µl of the mixture was added in duplicate to 293T monolayers in 96-well plate and incubated for 1 h at 37°C. Viral inocula were replaced with culture medium and the cells were incubated for 48 h and then fixed with methanol-acetone. Viral antigens were detected by immunostaining using a rabbit anti-capsid serum and counting foci as previously described (50).

Anti-αGal IgG ELISA

The ELISA for detection of human IgG-antibodies specific for the Gal 1-3Gal disaccharide epitope was adapted from a previously described method (53). In brief, polystyrene microtiter plates (NUNC Maxisorp, NUNC AB, Roskilde, Denmark) were coated with 100 µl per well of 5 µg/ml Gal 1-3Gal-polyacrylamide conjugate (PAA-Bdi, Syntesome GmbH, Munich, Germany) in 0.1 M (0.1 mol/l) carbonate buffer, pH 9.6, overnight at 4°C. The plates were then washed and saturated with a 0.5% solution of fish gelatin (Sigma) diluted in PBS. Sera diluted in PBS-Tween 0.1% were incubated for 2 h at 37°C, in triplicates. Instead of serum, PBS-Tween was used as a blank. Goat anti-human IgG antibodies (diluted 1: 1000, Jackson) and a TMB substrate was used to reveal bound antibodies. After a 5-min revelation and addition of H_2SO_4, optical density (OD) values were read at 405 nm.

Cell binding

The level of xeno-reactive antibodies was assessed in 53 burn patients who had received PS xenografts (BP-PS), 14 burn patients not treated with PS (BP-CTRL) and 27 healthy control (HC) samples. FACS antibody binding assays were performed on porcine aortic endothelial cells (PAEC) isolated from wild type (WT) or galactosyl-transferase knock-out (GalKO) pigs (with no expression Gal epitope). Cells were isolated, phenotypically characterized and grown as we previously described (54). An aliquot of a pool of normal male AB blood group sera (n=250) was used as an internal control in every experiment. IgM xenoreactivity was assessed using undiluted sera on both WT and GalKO PAEC. In contrast, for the measurement of IgG xenoreactivity, sera were assessed on WT PAEC at a 1:32 dilution and on GT-KO PAEC at a 1:4 dilution. Briefly, heat inactivated sera were incubated for 30 min at 4°C with 1 to $2×10^5$ PAEC, cells were washed twice in cold FACS buffer, then PAEC were incubated separately for 30 min at 4°C with FITC-labeled anti-human IgM goat antibodies (Jackson ImmunoResearch, West Grove, PA, USA) or FITC-labeled anti-human IgG goat antibodies (Beckman Coulter, Indianapolis, IN, USA). After staining and washing steps, cells were harvested in Phosphate Buffer Saline/paraformaldehyde 2% and analyzed using a Canto-II Flow cytometer (Becton Dickinson, San Diego, CA, USA) with DIVA (Becton Dickinson) and FlowJo software (Tree stars, Ashland, OR, USA). The difference of the Median of Fluorescence Intensity (MFI) between test samples and negative staining controls (average of quadrupled experiments with secondary FITC-labeled antibodies only) were presented as MFI shifts. Statistical analyses were performed with the Mann Whitney non parametric test and the Kruskal Wallis test.

In order to assess the proportion of anti-Neu5Gc IgG among IgG binding to GalKO PAEC, selected serum samples were incubated for 2h at 4°C with Neu5Ac or Neu5Gc at various concentrations: 0, 2.5, 5, and 7.5 mM in FACS buffer. Then, for each selected sera, these different conditions were tested by the FACS binding assay on GalKO PAEC for the IgG as described above.

Detection of anti-Neu5Gc antibodies by EIA and ELISA

Anti-Neu5Gc antibodies were detected in 1:100 diluted human serum samples by ELISA inhibition assay (EIA method) on wild-type mouse sera, as previously described (55). To demonstrate Neu5Gc-specific binding, diluted human sera were pre-incubated for 2 hours on ice in EIA buffer (55) supplemented with 5 mM Neu5Gc or control 5 mM Neu5Ac prior to testing binding to coated wells. To demonstrate sialic acid specific binding, the coated ELISA plates were either pre-treated with mild periodate or mock-treated (55).

Detection of anti-Neu5Gc antibodies by sialoglycan microarray

Arrays were fabricated by KAMTEK Inc. (Gaithersburg, MD) on Epoxide-derivatized slides (Corning, Thermo Fisher Scientific) with 8 full sub-arrays per slide as described (Array 1) (56). Anti-Neu5Gc antibodies were detected in 1:250 diluted human serum by sialoglycan microarray version 13 at 200 μl/sub-array and developed as described (56).

Results

Burn patients and control samples cohort

Blood samples from 55 burn patients treated with vital pig skin (BP-PS), 14 control burn patients without pig skin treatment (BP-CTRL) and 27 healthy controls (HC) were obtained. Table 1 shows demography of patients and controls.

In vitro transmission of PERV from porcine peripheral blood monouclear cells

To assess potential risk of infectious PERV in pigs used for burn patient treatments, samples from the animals of commercial herd that had been used for PS treatment at Burn Medicine Clinic, Faculty Hospital of Královské Vinohrady were tested for PERV transmission *in vitro* by co-culture. Of the 10 animals tested, 4 were found to transmit PERV to porcine cells and none were found to transmit PERV to human 293 cells (Supplementary Fig S1 A and B).

Analysis of PERV in PS-treated patient and control samples

Sensitive qPCR-based PERV assays were validated according to previous xenotransplantation studies (13). Of the 55 patient samples tested, one produced a false positive result, yielding an overall false positive rate of 0.83%. However, RNA was re-extracted from this sample and confirmed to be negative in a duplicate. We also received a new sample from the patient and confirmed it is negative. Tests for micro-chimerism (i.e., the presence of circulating pig cells) were also negative.

Antibody testing against PERV elements was also carried out in these patients. Consistent with previous studies (13), most of the reactivity observed was due to background cross reactivity and were considered to be negative for antibody to PERV in comparison to normal healthy control individuals. In addition, no significant neutralization of PERV infection was detected (Supplementary Fig S1D).

Altogether, these results provided no evidence of PERV transmission or presence of specific anti-PERV antibodies in these patients, consistent with previous studies, one with 15 BP-PS recipients (13) and additional other studies (13, 15, 16, 19).

Xenoantibodies binding to porcine cells

There is limited information on long-term antibody responses and nature of non- Gal antibodies in recipients (11). To investigate this, we first examined the anti- Gal antibody level using a previously established ELISA-based antibody binding assay (53) (Fig 1 A and B). Significantly, albeit modestly, higher anti- Gal IgG levels were observed for BP-PS

samples at low serum dilutions compared to BP-CTRL samples (both 1/40 and 1/160 dilutions) and HC samples at 1/40 dilution (not significant at higher dilutions of 1/640 and 1/2580, Supplementary Fig S2). Next, we set up cell binding assays to detect anti-pig cell antibodies by FACS analyses (See Supplementary Fig S3A–C for typical FACS profiles). Wild type porcine aorta endothelial cells (PAEC) were used to detect both anti- Gal and anti-non- Gal Ig (Fig 1C), while PAEC derived from Gal-deficient pigs (1-3GT knock out; GalKO) were used to specifically detect anti-non Gal Ig (Fig 1D). PS recipients (BP-PS) had significantly higher levels of IgG that bound to both WT and GalKO PAEC compared to HC and BP-CTRL and their levels are on average 2–3 fold (WT PAEC) and 4–6 fold (GalKO PAEC) higher than the controls (Fig 1). This increased response is evidenced in PS-treated patients at an average of 9.4 years (range 1 to 408 months; Table 1) following exposure to PS. Although several BP-PS recipients had strikingly high levels of anti-non- Gal antibodies (5 to 30 fold higher than the average of BP-CTRL), about one third of BP-PS recipients remained in the control range (Fig 1D). We have also examined IgM binding to these porcine cells (Supplementary Fig S3D and E). No significant difference was observed between BP-PS and BP-CTRL in IgM binding to both WT and GalKO PAEC. These results raised a question of the nature of these non- Gal antigens that are recognized by IgG in BP-PS recipients. Neu5Gc might be an important xenoantigen as recent data on Gal/CMAH-double-KO pigs (57) suggests that it is a major target for preformed human anti-pig antibodies.

Blocking of IgG binding to Gal KO PAEC by Neu5Gc

To estimate the proportion of anti-Neu5Gc IgG binding to GalKO PAEC in sera of BP-PS, cell binding FACS assays were carried out in the presence of Neu5Gc or Neu5Ac at various concentrations (Fig 2). At high concentration of Neu5Gc, but not Neu5Ac, IgG binding reached a plateau and was reduced from 10% to 50%, especially for the samples that had higher binding to GalKO PAEC in the absence of Neu5Gc. These results suggest that Neu5Gc glycans are major non- Gal antigens that induced long-term IgG responses in BP-PS recipients, although other non- Gal antigens may also be involved.

High levels of anti-Neu5Gc IgG in sera of BP-PS recipients long after PS treatment

Humans can produce xeno-autoantibodies against various Neu5Gc-epitopes and pig tissues and cells bear such Neu5Gc antigens (58). We therefore characterized the anti-Neu5Gc response in 10 representative BP-PS recipients that showed significantly higher levels of IgG binding to GalKO PAEC, as well as in 14 BP-CTRL patients and 10 HC. To detect anti-Neu5Gc antibodies we used a newly developed sensitive ELISA that allows detection of overall anti-Neu5Gc response in human sera to multiple Neu5Gc-antigens in a single assay (55). Compared to HC or BP-CTRL patients, serum samples of BP-PS recipients had significantly higher levels of anti-Neu5Gc IgG or IgM, but not IgA (Fig 3A). Pre-treatment of coated sialoglycan-antigens with mild periodate (that truncates the sialic acid side chain) abrogated Sia-specific binding of antibodies (46, 59) both in BP-PS or BP-CTRL suggesting Sia-specific binding in both groups, albeit higher in BP-PS (Fig 3B). Furthermore, we examined several representative samples by inhibition with increasing concentrations of either Neu5Gc or with the human sialic acid *N*-acetylneuraminic acid (Neu5Ac), a control monosaccharide which differs by only one oxygen atom from Neu5Gc. This is also relevant to the *in vivo* state where many circulating proteins in the serum are glycosylated and most carry Neu5Ac-glycoconjugates (rarely Neu5Gc). So *in vivo*, anti-Neu5Gc antibodies must be able to pass this pool of high concentration of Neu5Ac. No inhibition was observed with Neu5Ac. In contrast we saw inhibition with Neu5Gc that was maximal at 5 mM (Fig 3C). Subsequently, Neu5Gc-specificity was tested in all samples in the presence of 5mM Neu5Gc or Neu5Ac revealing specific inhibition of anti-Neu5Gc IgG binding with Neu5Gc but not with Neu5Ac (Fig 3D). These results indicate greater anti-Neu5Gc response in BP-PS (Fig

3C). To address this in more detail, we quantified anti-Neu5Gc antibody response in all these samples, relative to an IgG standard curve (Fig 3E). These results collectively indicate that BP-PS recipients can have high and persistent levels of anti-Neu5Gc IgG, which likely contributes to the high IgG binding to GalKO PAEC (Fig 1B).

Analysis of anti-Neu5Gc IgG profile on a sialoglycan microarray

Neu5Gc can be attached to a wide variety of naturally occurring glycans, resulting in the generation of a wide variety of epitopes. To reveal a more detailed Neu5Gc-antigen binding profile, anti-Neu5Gc IgGs were further characterized in BP-PS recipients and in BP-CTRL using a sialoglycan microarray (46, 56, 60). The arrays were printed with matched pairs of Neu5Gc- or Neu5Ac-glycans and serum IgG binding was analyzed (Fig 4). Consistent with the above ELISA assay, BP-PS recipients showed significantly higher IgG binding to Neu5Gc-glycans compared to the BP-CTRL, and both groups did not show significant binding to Neu5Ac glycans (Fig 4A–B). To further delineate the binding preferences of anti-Neu5Gc IgG in BP-PS recipients, we performed receiver operating characteristic (ROC) analyses for each of the Neu5Gc-glycans (Fig 4C). Overall this array analysis suggests that both N-linked (Neu5Gc 2-6LacNAc/Lac) and O-linked (Neu5Gc 2-3Core1) glycans contribute to anti-Neu5Gc immune response in BP-PS recipients (Fig 4C).

Discussion

Commercially available vital porcine skin has been shown to be effective in treating patients with severe burns as the grafts are viable for up to 5 days after transfer to the wound and facilitate swift healing (8). This treatment has been practiced less frequently since the year of 2005, mainly because of the concern of potential risk of zoonosis (50). Here we examined transmission of PERV, the major safety issue in xenotransplantation, and long-term status of anti-pig antibodies in pig skin recipients (BP-PS), which may bring unique information on expected long term humoral response to a xenotransplantation in the clinic.

In this study we did not find any evidence for PERV transmission in patients up to 408 months after xenotransplantation, the longest term analysed to date. This supports previous negative results in patients who had received various types of xenotransplantation treatment (13–19). These results support the use of closely monitored xenotransplantation clinical trials, especially those that do not require the use of systemic immunosuppression, such as alginate embedded pancreatic islet cell transplantation (1, 61). However, our analysis of the humoral response in BP-PS revealed that PS treatment can induce persistent anti-pig cell IgG responses (including the common anti- Gal): a major component of which is of anti-Neu5Gc IgG that can be detected even 34 years after PS removal from their burn injuries.

Our study shows that BP-PS recipients possess increased xeno-reactivity long after the treatment despite the short exposure to PS. Because humans have lost the functional gene *CMAH*, encoding a hydroxylase that converts the acetylated form of neuraminic acid (Neu5Ac) to its glycolylated form (Neu5Gc) (62, 63), Neu5Gc epitopes are recognized as foreign by the human immune system, similar to the Gal epitopes (37, 39–41, 43). Using a recently developed method designed to detect overall anti-Neu5Gc reactivity to multiple Neu5Gc-glycans in a single assay (55), we showed that the sera of BP-PS had a significant increase in anti-Neu5Gc IgG and IgM compared to BP-CTRL even years following the burn PS dressing. The specificity of antibody binding for Neu5Gc was confirmed by several means including competitive inhibitor experiments. Moreover, using a sialoglycan microarray displaying the various underlying molecular scaffolds of Neu5Gc, we showed that the distribution pattern of the antibodies for these various determinants was strongly affected by the PS treatment (Fig 4C). In BP-PS anti-Neu5Gc IgG showed strong preference to Neu5Gc 2-6LacNAc/Lac (Fig 4C). We also detected elevated anti-Neu5Gc to

Neu5Gc 2-3-linked to Core-1 *O*-glycan that was rare in BP-CTRL. Furthermore, using specific competitive inhibition of anti-Neu5Gc antibodies in the GalKO PAEC cell binding assay, we show that a substantial IgG fraction of anti non- Gal antibodies (ranging from 10% to 50%; average 30%) in the tested sera were anti-Neu5Gc. This highlights the possibility that induction of anti-Neu5Gc antibodies represents a major late immune response in clinical xenotransplantation, although antibodies against other uncharacterized pig cell antigens were likely to be induced in BP-PS as well.

High anti-Neu5Gc antibody responses (up to 30 fold higher than controls) in BP-PS were observed for a strikingly long time (up to 34 years; average 9.5 years) after the PS treatment. This occurred even though the PS was applied only temporarily (average for 3.9 weeks and up to 8 weeks for the maximum application time, with applications/changes up to a maximum of 18 times). It is likely that despite short term exposure to pig tissues in BP-PS, pig antigens were presented in the strong and persisting inflammatory context of a severe burn (64). This may have provided a vigorous stimulus resulting in the increased basal level of immunity against Neu5Gc-containing epitopes followed by class switch to IgG as a long term immune response. The immune response against some carbohydrate antigens (e.g. bacterial capsular polysaccharide) can elicit strong and long lasting production of antibodies if the BCR cross linking by the repetitive antigenic motif is also contemporary to strong T or non T cell help (65). Understanding the long lasting and some time lifelong response following vaccination or virus infection has generated several models including continuous stimulation (boost of vaccines, repeated infections, cross reactivity driven memory etc). However, convincing experiments suggest that the initial "imprinting" resulting from a vigorous BCR cross linking and T helper cell and/or TLR driven innate immunity help is determinant for establishing long term memory B cells and plasma cells (66). Possibly, high density of Neu5Gc and/or its protein/lipid scaffolds on xenogeneic cells synergize to major innate and cognate immunity helper signals contemporary to a severe burn itself and to the frequent surrounding bacterial complications. Furthermore, co-existence of Neu5Gc antigens, antibodies and complement in a normal individual may promote long lasting persistence of Neu5Gc on follicular dendritic cells in germinal centers of lymph nodes (67), all factors possibly contributing to the significant elevation of the antibodies almost a decade after the burns.

The concomitant presence of the xenoautoantigen Neu5Gc and related anti-Neu5Gc xeno-autoantibodies at significant titers in humans promoted several studies and hypotheses suggesting that longterm exposure to the resulting inflammation may facilitate inflammatory diseases (44–46). In a preclinical study, alginate embedded pig islets implantation also generated a vigorous humoral anti-pig response (61), indicating that the alginate protection does not prevent immunization. However, non-human primates (NHP) retain the intact *CMAH* gene (62). Thus the human host response to Neu5Gc epitopes cannot be studied in preclinical xenotransplantation experiments using NHP. Instead, it is possible that patients currently treated with alginate embedded (3) neonatal pig islets also develop persisting anti-Neu5Gc antibodies showing that this technique does not prevent their possible deleterious effects on recipients. Furthermore, our data raise the possibility that anti-Neu5Gc response may also occur in other xenotransplant regimes involving acellular pig tissues such as heart valves, or skin that is routinely utilized in the clinic. Of note, however, is that the current study of BP-PS may have only limited implication to the use of acellular pig skin which are expected to be less immunogenic than vital pig skin, vascularized xenografts or injected porcine cells (47). Indeed, the analysis of the xeno-antibody response in acellular pig skin recipients is of interest for future studies. However unlike this study, there is theoretically no risk of PERV transmission. In any case pigs doubly null for GT and CMAH may represent desirable or even required source animals for clinical xenotransplantation. It is also likely that there are additional antigens requiring some control measure and such antigens are yet

to be identified. This may require further studies on patients' sera after exposure to xenografts, like the current study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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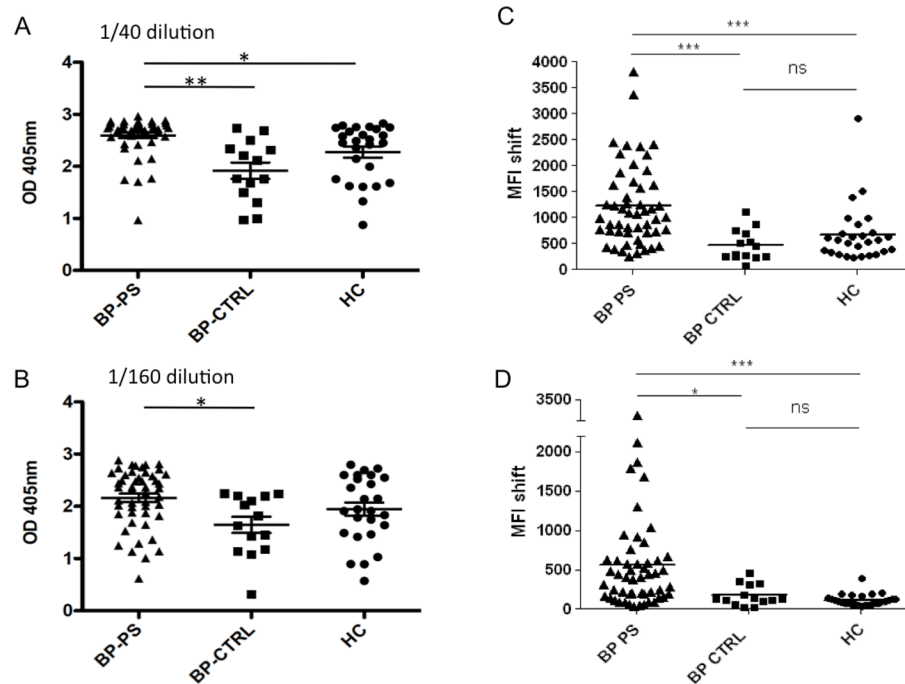
Fig. 1. Assessment of xeno-reactive IgG antibodies
Dilutions at 1/40 (A) and 1/160 (B) of serum from healthy volunteers (, HC; n=26), burn patients treated conventionally   , BP-CTRL; n=14) or who received a pig skin plaster (BP-PS; n=49) were reacted against Gal 1-3Gal-polyacrylamide conjugate and revealed with anti-human IgG antibodies by ELISA. P-values are based on One-Way ANOVA: **, p<0.001; *, p<0.05. IgG binding to WT and GalKO PAEC IgG patient serum xeno-reactivity to WT and GalKO PAEC. Serum xenoreactivity of healthy controls (, HC; n=27), control burn patients (, BP-CTRL; n=14) and pig skin treated burn patients (, BP-PS; n=53) was assessed by flow cytometry: IgG serum reactivity was assessed against WT PAECs at a 1/32 dilution (C) and against GalKO PAECs at a 1/4 dilution (D), representing respectively the anti- Gal and non- Gal IgG Ab (C) and the anti-non- Gal IgG Ab (D).
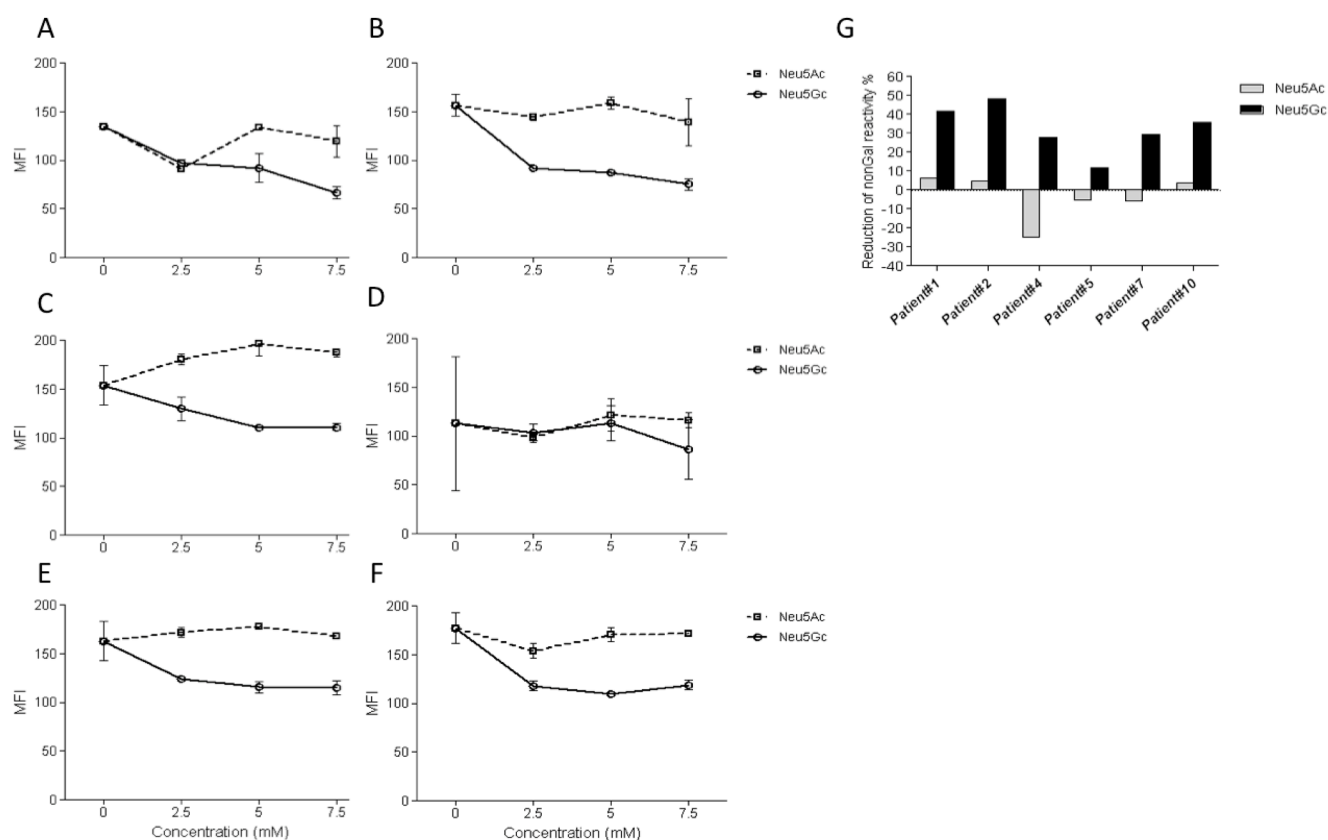
Fig. 2. Blocking of IgG binding to GalKO PAEC by Neu5Gc

Anti-non- Gal IgG reactivity of serum with or without Neu5Ac (cir;, dashed line) or Neu5Gc (, plain line) respectively at 0; 2.5; 5 and 7.5 mM: sera from patient #1 (A), patient#2 (B), patient#4(C), patient#5 (D), patient#7 (E) and patient#10 (F) diluted in FACS buffer. Percentage of reduction of anti-non- Gal IgG reactivity after serum absorption with Neu5Ac (grey bars) or NeuGc (black bars) in the 6 patients (#1, 2, 4, 5, 7 and 10) (G).
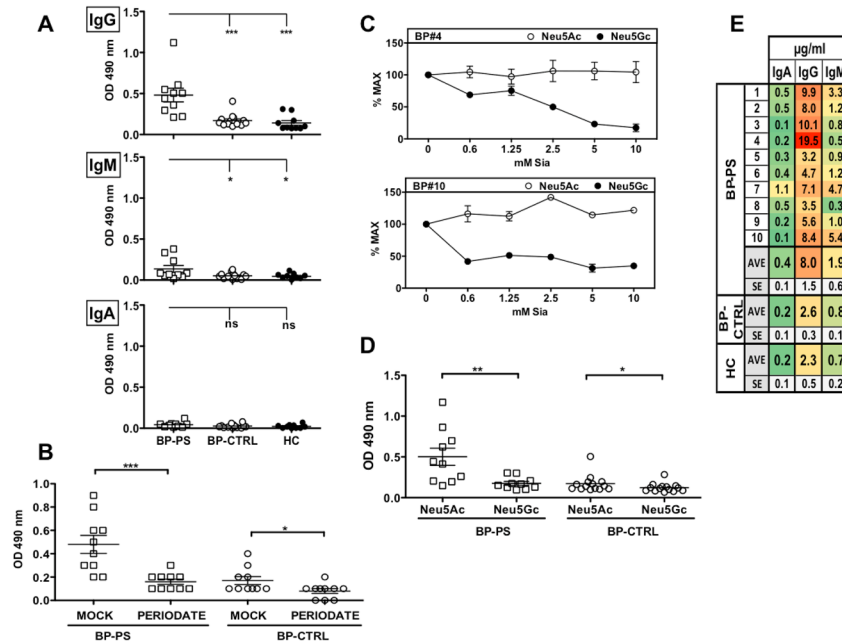
Fig. 3. Characterizing the anti-Neu5Gc antibody response
A. Anti-Neu5Gc response was tested in PB-PS (burn patients treated with PS), BP-CTRL (burn patients not treated with PS) and HC (healthy controls) at 1:100 diluted serum by EIA method revealing higher anti-Neu5Gc IgM or IgG (but not IgA) in BP-PS compared to BP-CTRL or HC (two independent experiments; One-Way ANOVA, ns P=0.1502, * P=0.0177, *** P< 0.0001). **B.** Binding is specific to Sialic acids (Sia) because pre-treatment of coated glycans with mild-periodate (that removes Sia side chain), but not control mock pre-treatment, eliminates binding (representative of two experiments; paired t-test, *** P=0.0004, * P=0.0187). **C.** EIA IgG binding (of two sera with high anti-Neu5Gc reactivity) is inhibited by increasing concentrations of Neu5Gc, but not with Neu5Ac (two independent experiments showing mean and SEM). **D.** IgG binding is specific to Neu5Gc because serum EIA binding is inhibited by 5 mM Neu5Gc but not 5mM Neu5Ac (representative of two experiments; paired t-test, ** P=0.0083, * P=0.0213). **E.** Serum anti-Neu5Gc antibodies (detected by EIA) were quantified by standard curves of human Ig (for each Ig standard curve, all sera samples were tested in duplicates on the same plate; two independent experiments showing mean and SEM). This analysis confirmed that BP-PS have 3–4 fold higher anti-Neu5Gc antibodies (IgG: 8±1.5 μg/ml; IgM: 1.9±0.6 μg/ml) compared with BP-CTRL (IgG: 2.6±0.3 μg/ml; IgM: 0.8±0.1 μg/ml) or HC (IgG: 2.3±0.7 μg/ml; IgM: 0.7±0.2 μg/ml).
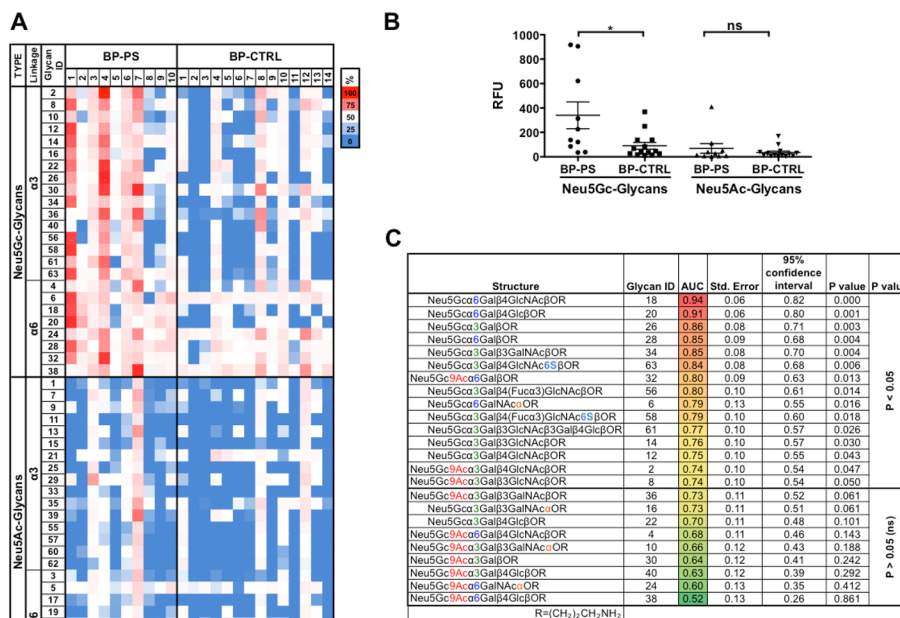
Fig. 4. Analysis of serum binding to sialoglycan microarray
A. Binding pattern of BP-PS versus BP-CTRLs (at 1:250 serum dilution) on sialoglycan microarray, including various matched pairs of Neu5Ac-glycans and Neu5Gc-glycans (Array 1 full glycan list is detailed in ref. (Padler-Karavani et al., 2012, J Biol Chem)). Digital array data are presented in Supplementary Table S1. **B.** Statistical analysis of serum binding to the various glycans reveals that only binding to Neu5Gc-glycans is significantly higher in BP-PS compared to the BP-CTRL (Mann Whitney test; Neu5Gc-glycans, P=0.0207; Neu5Ac-glycans, P=0.5775). **C.** ROC curve analysis on binding to Neu5Gc-glycans in BP-PS versus BP-CTRL. The area under the ROC Curve (AUC) revealed a significantly (P<0.05) elevated IgG response to some Neu5Gc-glycans in BP-PS compared to the BP-CTRL. In general, the least difference is observed for antibodies to Neu5Gc-glycans that are modified with *O*-Acetylation at position C-9 (Neu5Gc9Ac), suggesting those antigens do not contribute to anti-Neu5Gc immune response to PS (P<0.05, glycans 36, 4, 10, 30, 40, 24, 38). However, there is a strong preference to Neu5Gc 2-6LacNAc/Lac over Neu5Gc 2-6GalNAc or Neu5Gc 2-3LacNAc (glycans 18, 20 > 6, 12) with no binding to Neu5Gc 2-3Lac (glycan 22). In addition, Neu5Gc 2-3Core1 is preferred over Neu5Gc 2-3Type1, Le^X or 6S-Le^X (glycans 34 >14, 56, 58).

Table 1

Summary of patients' history

55 BP-PS				
Sex Male/Female	**48/7**			
	Average	**Median**	**Min**	**Max**
Age at time of sampling	47.6	45	10	92
Age at time of accident	38.2	35	1.5	71
Months since burn	113.7	88	1	408
% burn	32.3	30	5	87

14 BP-CTRL recipients				
Sex Male/Female	**8/6**			
	Average	**Median**	**Min**	**Max**
Age at time of sampling	44.2	46	23	66
Age at time of accident	40.4	42.5	7	65
Months since burn	45.8	24.5	2	240
% burn	31.6	24.5	7	90

27 HC				
Sex Male/Female	**14/13**			
	Average	**Median**	**Min**	**Max**
Age at time of sampling	34.1	29	23	79