

Xenoantibodies to porcine non-galactose α 1,3 galactose antigens in non-human primates cross-react and cause apoptosis to human endothelial cells

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Abstract

Xenoantibodies show a particular reactivity to endothelial cells of multiple species. This cross-reactivity is considered negligible in most of the cases, and xenoantibodies are disregarded in assays determining anti-endothelial cell antibodies. However, there is also evidence that xenoantibodies may cause apoptosis to endothelial cells of several species. In this study we examined in non-human primates the characteristics of anti-porcine xenoantibodies targeting non-galactose α 1,3-galactose (Gal) antigens, boosted by exposure to porcine red blood cells (PRBC) and the depletion of anti-Gal antibodies with GAS914. Production of anti-non-Gal antibodies correlated on day 10 with an augmented IgM and IgG antibody reactivity to L35 (porcine lymphoblastic cells). On days 20 and 30 there was an increased binding of IgG to AOC-40 (porcine endothelial cells), which paralleled an IgG antibody binding to HMEC-1 (human microvascular endothelial cells). These antibodies caused the apoptosis of AOC-40 and HMEC-1 cells through two different pathways, with and without DNA fragmentation, respectively. Western blotting of anti-non-Gal antibodies showed the increased intensity of several protein bands in AOC-40 and HMEC-1 lysates, and the transient detection of a few new bands, compared to samples before PRBC injection. Treatment with cyclophosphamide in one animal led to the virtual disappearance of anti-non-Gal antibody binding to AOC-40 and HMEC-1 proteins without modifying the cell surface antibody reactivity or apoptosis of these cells. Therefore, exposure of baboons to PRBC increases xenoantibody binding to porcine lymphoblastic cells and to porcine and human endothelial cells. The xenoantibodies caused apoptosis of porcine and human endothelial cells by apparently targeting non-protein antigens. This study substantiates a cross-reactivity of xenoantibodies to endothelial cells from different species, which may be particularly relevant if there is a xenogenic exposure. In this setting, disregarding these antibodies may impair the proper assessment of the prevalence and role of anti-endothelial cell antibodies in human and animal disorders.

Introduction

Xenotransplantation of pig organs is not a current clinical practice because the tools available to date fail to control the humoral immune response triggered by the xenograft in humans and non-human primates. Initially, antibodies directed against the oligosaccharide galactose α 1,3-galactose (Gal) were found to be responsible for the hyperacute rejection of pig grafts transplanted into humans and non-human primates and major contributors to acute humoral xenograft rejection (AHXR) [1-3]. However, the continuous depletion of these antibodies by polymers containing multiple Gal epitopes and the use of grafts from pigs lacking this epitope (α 1,3 galactosyltransferase knockout; Gal-KO) evidenced that antibodies to non-Gal antigens play a major role in AHXR of pig organs in non-human primates [4-6]. This rejection is characterized by a thrombotic microangiopathy that results from the activation, proliferation and apoptosis of endothelial cells [7], suggesting a particular reactivity of non-Gal antibodies to these cells [8].

Immunization of mice and rabbits with human and murine endothelial cells, respectively, generates IgG xenoantibodies cross-reactive with endothelial cells of several species [9,10]. These antibodies cause apoptosis of endothelial cells leading to anti-angiogenic and anti-tumour effects [9,10]. Thus, xenoantibodies produced by the

injection of human endothelial cells impacted likewise over human and mice endothelial cells and tumours. Identical response occurs after immunization with mice endothelial cells. The cross-reactivity of xenoantibodies generated by the exposure to xenogenic endothelial cells was attributed to protein antigens shared by endothelial cells of different species. However, xenoantibody responses are species-restricted not cell-specific and exposure to xenoantigens on other cells aside endothelial also produce cross-reactive antibodies to endothelial cells of different species [11].

Endothelial cells include a wide variety of antigens and anti-endothelial cell antibodies (AECA) have been reported in connective tissue diseases, vasculitis and other inflammatory diseases both in humans and animals [12,13]. AECA may cause different

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pathophysiological effects, including direct or indirect cytotoxicity, endothelial cell activation and apoptosis [14]. However, AECA also includes xenoantibodies that are considered negligible and cause of interference in immunoassays used for the detection of AECA and other antibodies, leading to false-positive results [15,16]. In consequence, an antibody absorption step using other species animal proteins to remove xenoantibodies is recommended before assaying AECA [16].

In the present study we induced anti-porcine xenoantibodies to non-Gal antigens in non-human primates by exposure to porcine red blood cells (PRBC) and compared the reactivity and effects of these antibodies on porcine and human endothelial or lymphocytic cells. A better characterization of the reactivity of xenoantibodies to endothelial cells of different species may be relevant for xenotransplantation. However, it may be even more important to understand the potential role of xenoantibodies in assays evaluating AECA, both in human and animal disorders. We selected PRBC instead of porcine endothelial cells because contact to xenogeneic endothelial cells is exceptional both in humans and animals. In contrast, exposure to xenogeneic antigens in other type of cells such as those present in the food may occur much more often.

Material and methods

Animals

A total of 5 baboons (*Papio anubis*; Consort Bioservices, Steyming, UK) were used in this study. The animals weighing 10 to 15 kg were housed in a facility approved and accredited by the Spanish Environment Minister and the Galicia Department of Agriculture. Two hDAF transgenic pigs (Novartis Pharma A.G. Basel, Switzerland) were used as source of PRBC.

Ethics

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication n° 85-23 revised 1996) and the *European Agreement of Vertebrate Animal Protection for Experimental Use* (86/609), and was approved by the Research Committee of INIBIC-Complejo Hospitalario Universitario A Coruña.

PRBC injections, baboon blood sampling and side effects

Baboons received intravenous injections of PRBC ($6-6.5 \times 10^6$ cells/ml) with the goal of achieving sustained levels of anti-non-Gal hemolytic anti-porcine antibodies (HAPA) higher than those present with anti-Gal antibodies. Three baboons (C79, D29 and D33) received a single injection of 50 ml of PRBC. D29 took in an additional injection of 5 ml 60 days after de first inoculation. Two more baboons were

immunized with either 5 daily injections of 10 ml (C54) or 10 daily injections of 5 ml (C63) (Table 1). Baboon blood was obtained on days 5, 7, 10, 15, 20 and 30 after initial PRBC injection. Animal D29 has additional blood drawn on days 65, 67, 70, 75, 80, 90 and 120) (Table 1). PRBC injections were associated with side effects in only one animal (D29), which showed mild respiratory insufficiency during the initial injection of 50 ml. No signs of unwarranted autoimmunity were observed in any animal up to 9 months of follow-up.

GAS914 and Cyclophosphamide (CyP) treatment

All the animals were treated with 1 mg/kg/day of GAS914 (Novartis Pharma, A.G. Basel, Switzerland) subcutaneously from day -5, relative to the first PRBC injection, to 10 days after the last injection [4]. After that, they were treated with 1 mg/kg of GAS914 every other day until day 30 of the first injection. Animal D29, which received two injections of pig blood separated by 60 days, was treated with GAS914 during 120 days with daily injections from days -5 to 10 and 55 to 70 and every other day the remaining days (Table 1).

Baboon C63 was treated at day 20 after the initial PRBC injection with 200 mg/kg of CyP over 4 days. The dose of CyP was chosen because it largely eliminates the mature immune system, leading to a major reduction in antibody and autoantibody levels while leaving hematopoietic precursors intact (Table 1) [17].

Anti-Gal and HAPA assays

The anti-Gal and HAPA were measured as previously described [18], using an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-Gal antibodies and porcine red blood cells and rabbit complement for the hemolytic assay.

Cell lines

L35 (porcine lymphoblastic cell line), HMEC-1 (human microvascular endothelial cell line), and Jurkat E6.1 (human leukemic T cell lymphoblast line) were obtained from American Type Culture Collection (Rockville, MA, USA). AOC-40 (porcine endothelial cell line) was a kind gift from Dr. Rodriguez-Barbosa, Leon, Spain [19].

Determination of cell-surface antibody reactivity by flow cytometry

Cell lines (AOC-40, HMEC-1, L-35 and Jurkat E6.1) were placed in a 96-well V-bottom plates (NUNC, Denmark) at $2.5 \times 10^5-3.5 \times 10^5$ cells/well and washed twice with FACS buffer (BD Biosciences, Franklin Lakes, NJ, USA). Then, they were incubated at 4°C for 40 minutes with 50 μ l of testing sera (1:5 dilution in FACS buffer), followed by three washes with FACS buffer. Anti-human IgM (μ -chain specific) or IgG

Table 1. Animal identification, blood groups, and injection, sampling and treatment protocols.

Baboon	Baboon Blood Group	Pig	Pig Blood Group	PRBC injections (Volume and days)	Baboon blood sampling (days)	GAS914 Administration (days)	Cyclophosphamide
C54	AB	A-751	0	10cc days 0 to 5	-5,0,5,7,10, 15,20,30	Daily days -5 to 15 QOD days 16 to 30	No
C63	B	A-750	0	5cc days 0 to 10	-5,0,5,7,10, 15,20,30	Daily days -5 to 20 QOD days 21 to 30	50 mg/kg days 20,21,22 and 23
C79	B	A-750	0	50cc day 0	-5,0,5,7,10, 15,20,30	Daily days -5 to 10 QOD days 11 to 30	No
D33	A	A-751	0	50cc day 0	-5,0,5,7,10, 15,20,30	Daily days -5 to 10 QOD days 11 to 30	No
D29	AB	A-751	0	50cc day 0 5cc day 60	-5,0,5,7,10, 15,20,30, 40, 50, 60, 65, 67, 70, 75, 80, 90, 120	Daily days -5 to 10 and 55 to 70 QOD days 11 to 54 and 71 to 120	No

fluorescein isothiocyanate (FITC)-conjugated (DAKO, Denmark) (1:20 dilution) were used as secondary antibody by incubating the cells in the dark at 4°C for 30 minutes. Following three washes with FACS buffer, 5000 cells/tube were analyzed in FACScan cytometer with CellQuest program (BD Biosciences) in a histogram plot representing FL1. Cells incubated with secondary antibody only were used as negative control.

Annexin V (AnV)/ Propidium iodide (PI) assay

AOC-40 and HMEC-1 cells at 5×10^4 cells/well were cultured in 24-well plates with 100 μ l of baboon serum (diluted 1:2 in serum-free media) for 24 hours. Then, cells were detached using trypsin-EDTA (Life Technologies, CA, USA) and stained with An V-Fluos labelling reagent (Roche Applied Science, Germany) (1:50) and PI solution (1:1000) (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol.

Determination of DNA fragmentation

DNA fragmentation was assessed in AOC-40 and HMEC-1 cells with the APO BrdU Kit Assay (BD Biosciences). To this end, 5×10^4 cells/well were cultured in 24 well plates with 100 μ l of baboon serum (diluted 1:2 in serum-free media) for 24 hours. Then, cells were detached using trypsin-EDTA (Gibco) and stained according to the manufacturer's protocol.

Western blot analysis

AOC-40 and HMEC lysates were prepared using lysis buffer, containing 50 mM Tris-base (Bio-Rad, Hercules, CA, USA), 155 mM NaCl (Sigma), 2 mM EDTA (Sigma), 0.2% Triton X-100 (Sigma), 0.3% NP-40 (Sigma), 0.1 mM PMSF (Sigma), for 1 hour at 4°C. Lysates were then clarified by centrifugation at 10,000 g for 15 minutes, and supernatant protein concentrations were determined with a Bio-Rad assay kit (Bio-Rad). The samples were then mixed with 5 \times sample buffer and 1% β -mercaptoethanol (Bio-Rad) and heated at 100°C for 5 minutes, 30 μ g/ μ l of lysates were loaded onto 10% SDS-polyacrylamide gels (Bio-Rad) and transferred to immune-blot polyvinylidene difluoride membrane, Immobilon-P S-Q (Millipore, Billerica, MA, USA). After transfer the membranes were blocked for 1 h in 5% non-fat dried milk, washed, incubated with the appropriate dilution of baboon sample sera (1:100) overnight at 4°C, washed, incubated with secondary antibodies (diluted 1/8000–1/6000) anti-human IgG (γ -chain specific) or anti-human IgM (μ -chain specific) peroxidase-conjugated (Sigma) for 1 h at room temperature, and washed. Proteins were visualized using ECL system (GE Healthcare Biosciences, Little Chalfont, UK) in a digital camera CCD LAS-3000 (Fuji-Film, Tokyo, Japan). Membrane incubated only with secondary antibody (without baboon serum) was used as negative control.

Results

Induction of anti-non-Gal HAPA

Before the injection of PRBC, removal of anti-Gal antibodies by GAS914 led to the complete disappearance of HAPA in all the baboons (Figure 1). PRBC injection along with the administration of GAS914 was associated with the generation of anti-non-Gal HAPA from day 5 that peaked between days 7 and 10 of the initial inoculation (Figure 1). An identical pattern of response was also observed after the second injection in baboon D29 and in baboons C54 and C63 that received 5 daily injections of 10 ml or 10 daily injections of 5 ml, respectively. The highest levels of HAPA attained were between 2 to 4-fold the levels present with anti-Gal antibodies, which were observed after the

second injection in baboon D29 (Figure 1C) and in animals C54 or C63 (Figures 1D and 1E). Baboon C63 was also treated at day 20 after the initial PRBC injection with 200 mg/kg of CyP over 4 days. The animal died on day 32 as a result of CyP treatment toxicity, without changes in the level of anti-non-Gal HAPA on day 30 (Figure 1E).

Reactivity of anti-non-Gal HAP

Removal of anti-Gal antibodies with GAS914 was associated with a notable reduction in the binding of IgM and IgG antibodies to AOC-40 and L35 (Figure 2A and 2B), whilst only minor effects were observed in binding to human cell lines HMEC-1 and Jurkat E6.1. After injection of PRBC and induction of anti-non-Gal antibodies, all the animals had at day 10 an IgM and IgG antibody binding to AOC-40 similar to that observed before GAS914 treatment, whilst against L35 was notably higher (Figure 2A and 2B). On days, 20 and 30, reactivity of IgM and IgG to L35 and IgM against AOC-40 declined, whereas binding of IgG to AOC-40 augmented. The highest reactivities were observed in animals C63 and D29, in the latter case after the second injection of PRBC, with increases between 2 and 9-fold compared to the binding

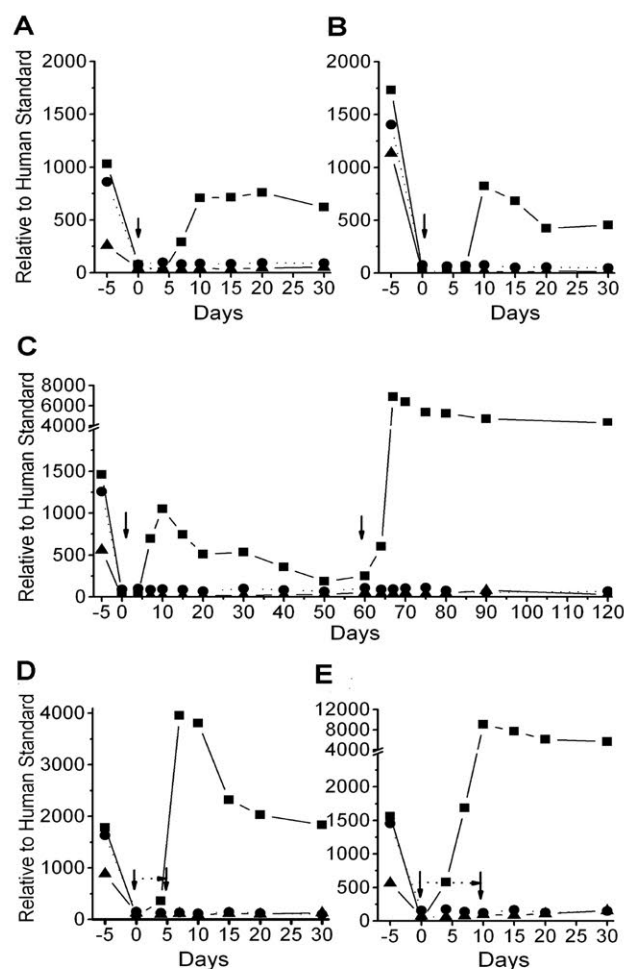


Figure 1. Levels of anti-Gal IgM (circles), IgG (triangles) and HAPA (squares) in baboons immunized with pig blood. The immunization protocols are described in Materials and Methods. The arrows indicate the days of pig blood injection. A) baboon C79; B) D33; C) D29; D) C54; E) C63.

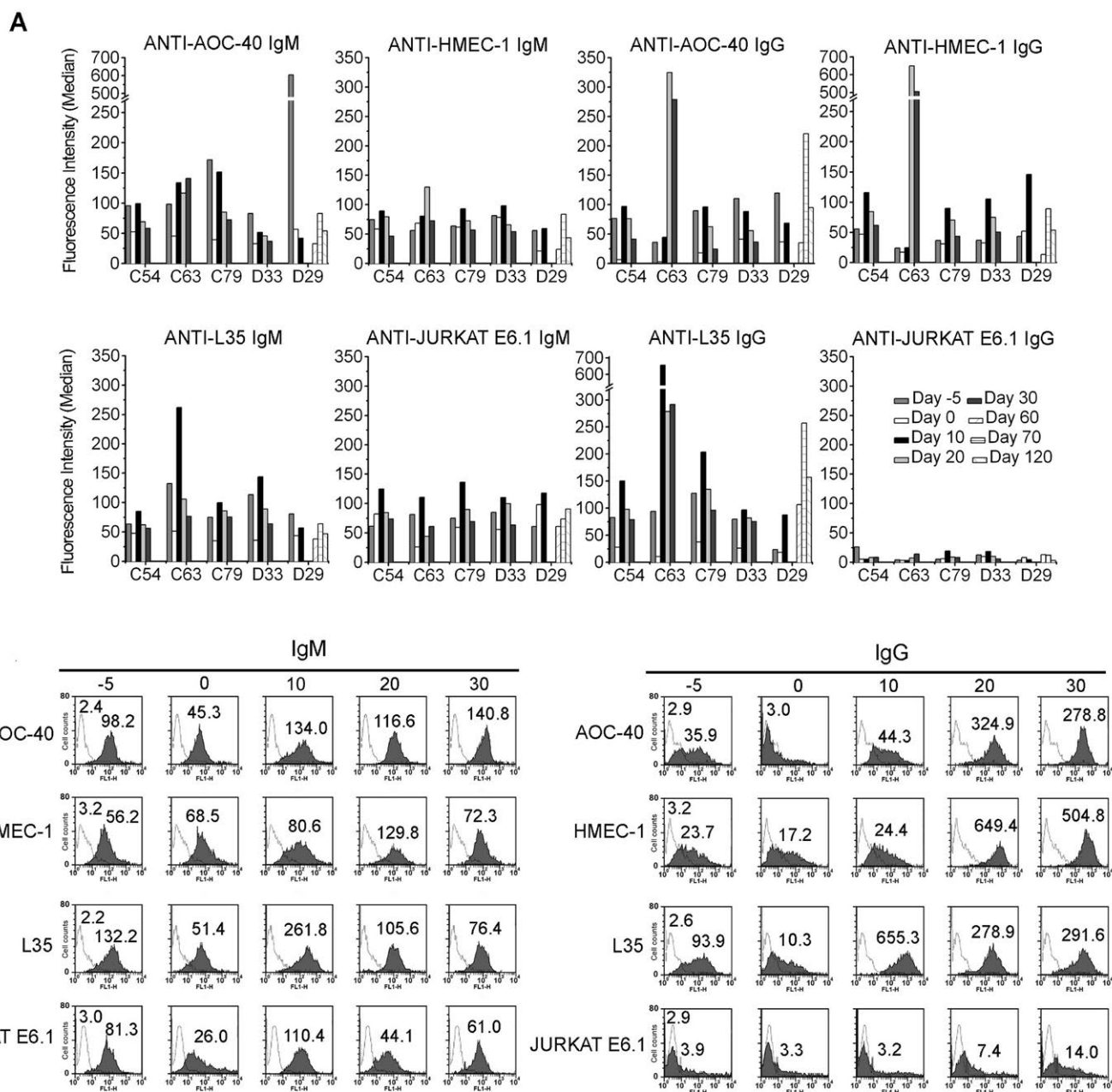


Figure 2. Sequential measurement by flow cytometry of baboon antibody binding to AOC-40, HMEC-1, L-35 and Jurkat E6.1. A) Pattern of IgM and IgG antibody binding on days -5, 0, 10, 20 and 30, in baboons C54, C63, C79 and D33, and on days -5, 0, 10, 60, 70 and 120 in baboon D29. Data are representative of two independent experiments. B) Pattern of IgM and IgG antibody binding in baboon C63. Values represent median fluorescence intensity on day -5 (before GAS914 administration); day 0 (first day of pig blood injection); day 10 (last pig blood injection); day 20 (first day of CyP treatment); day 30 (10 days after CyP initiation and two days before animal death). Data are representative of two independent experiments.

detected on day 0 after the removal of anti-Gal antibodies and before the injection of PRBC.

Boost of baboon IgG anti-AOC-40 reactivity on days 20 and 30 was paralleled by an IgG antibody binding to HMEC-1, which reached 27-fold and 3-fold augments in C63 and D29, respectively (Figure 2A and 3B). No change was detected with anti-non-Gal antibodies in the reactivity of IgM to HMEC cells. Also, no IgG antibody binding to Jurkat E6.1 was evidenced, although some IgM reactivity was present (Figure 2A and 3B). Treatment with CyP in baboon C63 had a minimal impact on IgM and IgG antibody binding to porcine and human cell lines.

Effect of anti-non-Gal HAPA in AOC-40 and HMEC-1 cells

AOC-40 and HMEC-1 were exposed to sera from baboons and stained with AnV and PI to investigate whether the xenoantibodies could induce apoptosis of these cells. AOC-40 cells incubated with baboon sera collected before the removal of anti-Gal antibodies showed an AnV-/PI+ (necrosis) pattern, which was also observed 10 days after the initial PRBC injection with baboon sera containing anti-non-Gal antibodies (Figure 3A and 3B). This pattern changed to an AnV+/PI+ (apoptosis) profile on days 20 and 30 after the first PRBC exposure (Figure 3A and 3B). Incubation of HMEC-1 cells with baboon sera containing anti-Gal antibodies showed minimal AnV+/PI+ labelling,

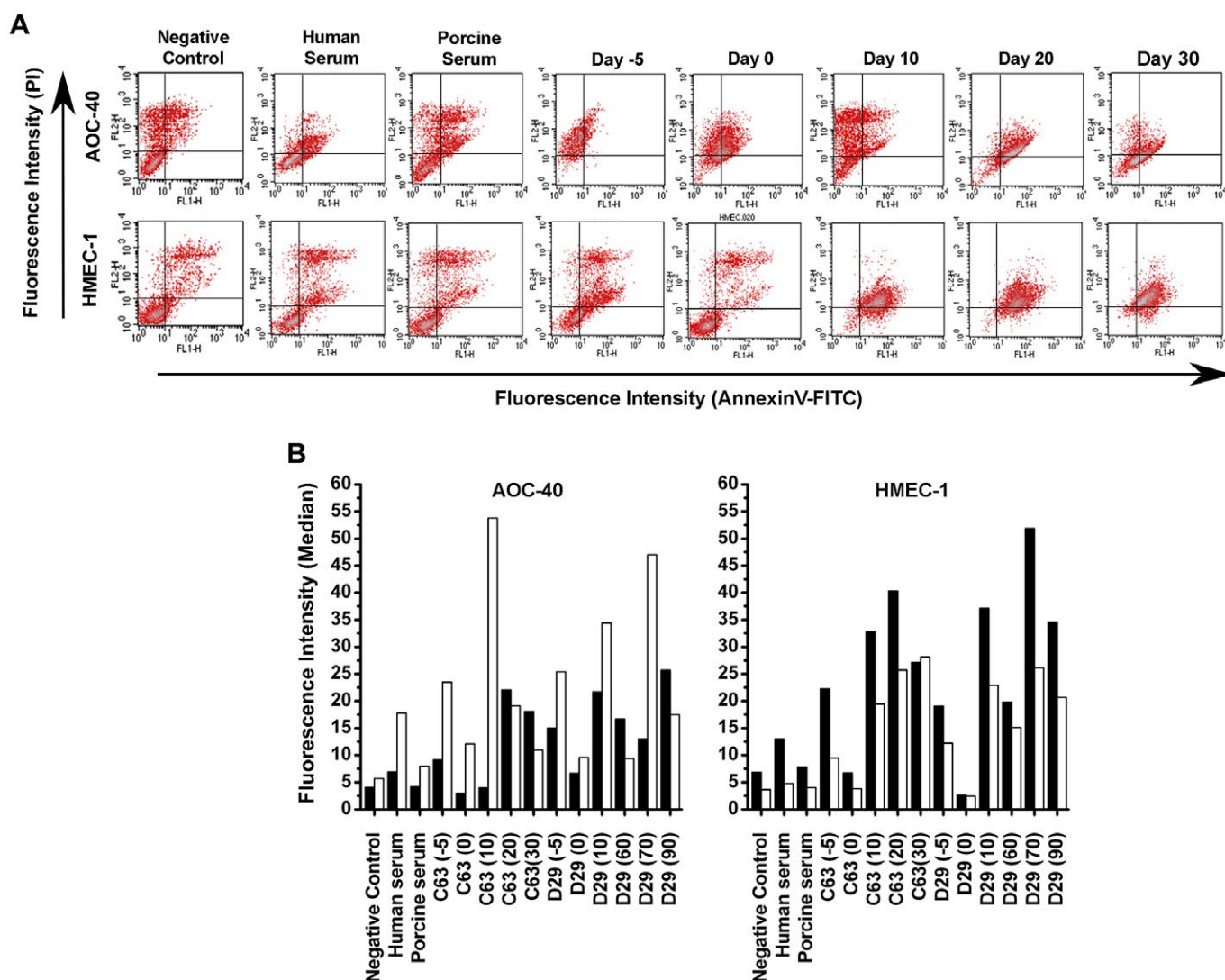


Figure 3. Annexin V and PI staining of AOC-40 and HMEC-1 cells after incubation with baboon sera. Sample identification is the same described in Figure 2. A) AOC-40 and HMEC-1 cells cultured with baboon sera from animal C63 for 24 hours were stained with FITC-annexin V and PI and analyzed by flow cytometry. B) Median fluorescence intensity of AV (black column) and PI (white column) after incubation of AOC-40 and HMEC-1 with baboons C63 and D29 sera samples. Data are representative of two independent experiments.

which increased significantly after exposure to baboon sera containing anti-non-Gal antibodies (Figure 3A and 3B). The highest levels of AnV-/PI+ in AOC-40 and AnV+/PI+ labelling in both AOC-40 and HMEC-1 were observed with sera from animals C63 and D29 (Figure 3A and 3B).

To further characterize the cause of cell death, we also investigated DNA fragmentation, a specific indicator of apoptosis. AOC-40 cells incubated with baboon sera containing anti-Gal antibodies showed minimal DNA fragmentation. However, this phenomenon was present in more than 50% of cells exposed to anti-non-Gal antibodies. Exposure of HMEC-1 cells to baboon serum with either anti-Gal or anti-non-Gal antibodies did not lead to DNA fragmentation in these cells. The highest levels of DNA fragmentation in AOC-40 cells were detected with sera from animals C63 and D29, in the latter case after the second injection of PRBC (Figure 4A and 4B). Treatment with CyP did not modify the pattern of AnV/PI labelling to AOC-40 and HMEC-1 or

DNA fragmentation to AOC-40 caused by anti-non-Gal antibodies in baboon C63.

Protein recognition of anti-non-Gal HAPA in AOC-40 and HMEC-1 cells

To investigate whether anti-non-Gal antibodies target specific proteins in pig and human endothelial cells, we performed Western blots with lysates of these two cell types and the sera of baboons C63 and D29. The lack of enough serum samples did not allow assessing other animals. Incubation of blots with baboon serum containing anti-Gal antibodies produced multiple bands in both AOC-40 and HMEC-1 lysates (Figures 5A and 5B). Removal of anti-Gal antibodies decreased the intensity of reactivity for most AOC-40 proteins while this effect was not observed for HMEC-1 (Figure 5A and 5B).

A few new bands were detected when assessing samples obtained after exposure to PRBC compared to results from preimmune sera

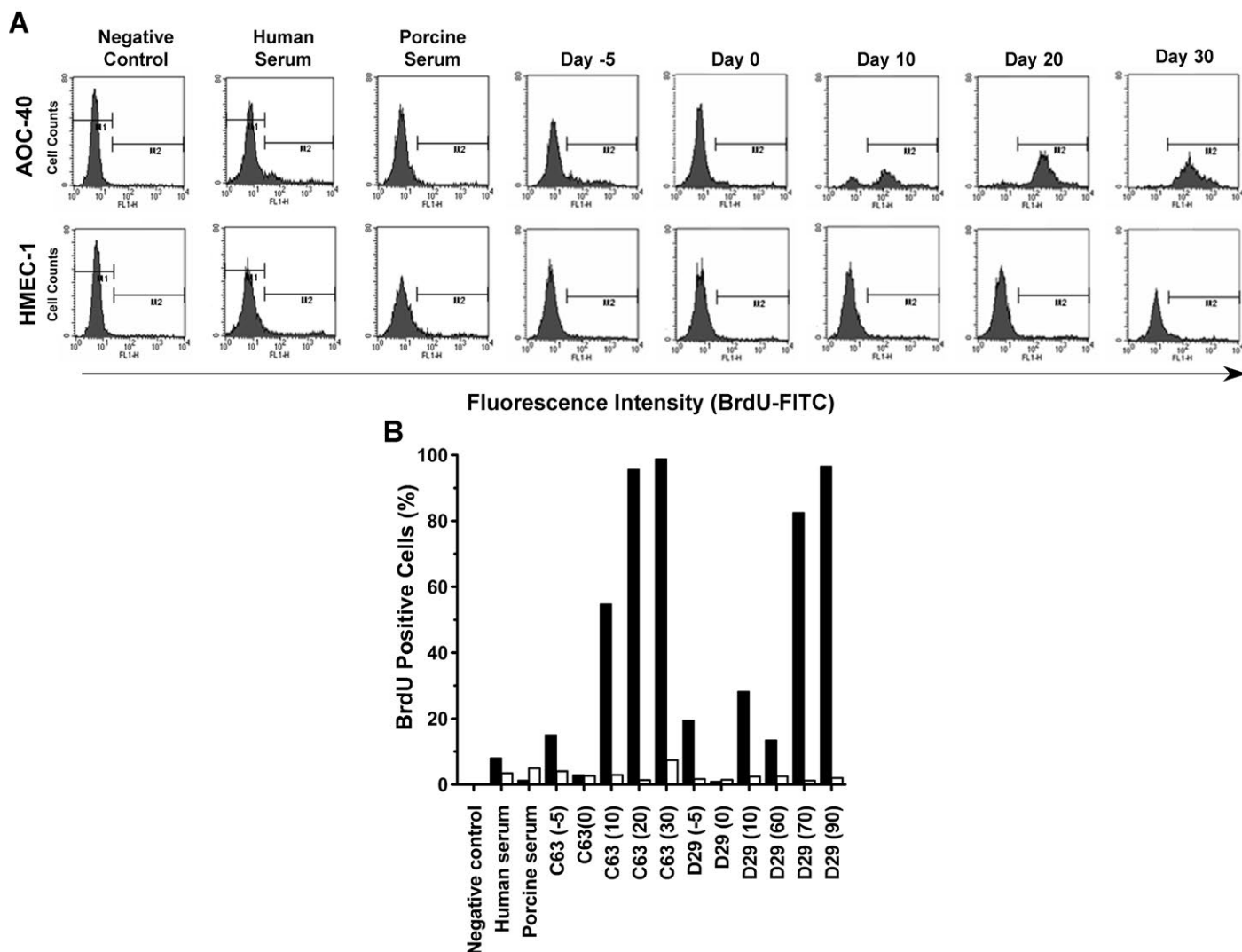


Figure 4. BrdU staining of AOC-40 and HMEC-1 cells after incubation with baboon sera. Sample identification is the same described in Figure 2. A) AOC-40 and HMEC-1 cells cultured with baboon serum from animal C63 for 24 hours were stained with FITC-labelled anti-BrdU and analyzed by flow cytometry. M1 corresponds to the negative control and M2 to the stained cells. B) Percentage of BrdU positive cells after exposure of AOC-40 (black column) and HMEC-1 (white column) cells to C63 and D29 sera samples. Data are representative of two independent experiments.

though they differed in each baboon (Figure 5A and 5B). Thus, D29 serum IgG corresponding to days 70 and 90 showed reactivity to a new band of 32.0 kDa in AOC-40 and HMEC-1 lysates. Also, a new band of 106.9 kDa was transiently detected by C63 IgG on day 10 (only in HMEC-1 lysates). IgM reactivity was observed on new bands of 57.7, 120.7 and 127.6 kDa in AOC-40 and HMEC-1 lysates when incubated with D29 sera from days 70 and 90, whilst the 57.7 kDa band was also detected for C63 on day 10 only in AOC-40 lysate.

Treatment with CyP in animal C63 was associated with a remarkable reduction in the intensity of all bands detected by IgG antibodies and almost the disappearance of bands reacting with IgM antibodies, as observed in western blots of both AOC-40 and HMEC-1 cell lysates (Figures 5A and 5B). This contrasted with the prominent detection of cell-surface antibody reactivity and cytotoxicity after CyP treatment determined by flow cytometry using the same sera samples and target cells. Together, these results suggest that anti-non-Gal HAPA cross-

reactive to AOC-40 and HMEC-1 include antibodies that do not target protein antigens on these cells.

Discussion

In the present study we generated anti-non-Gal IgG xenoantibodies in baboons by exposure to PRBC, which reacted to AOC-40 and L35 porcine cell lines and to HMEC-1 human cell line, causing apoptosis of both porcine and human endothelial cells. This confirms the particular reactivity of xenoantibodies to endothelial cells of different species, which is, at least in part, related to xenogeneic antigens and not only to endothelial cell antigens as it was proposed [9]. Whether the immunogenic capacity is confined to xenogeneic antigen/s present on endothelial cells and blood cells, which are intimately linked to development [20], or may be extended to other cell types such as epithelial cells cannot be answered by the present study. However, xenotransplantation studies in rodents suggest that skin xenografts

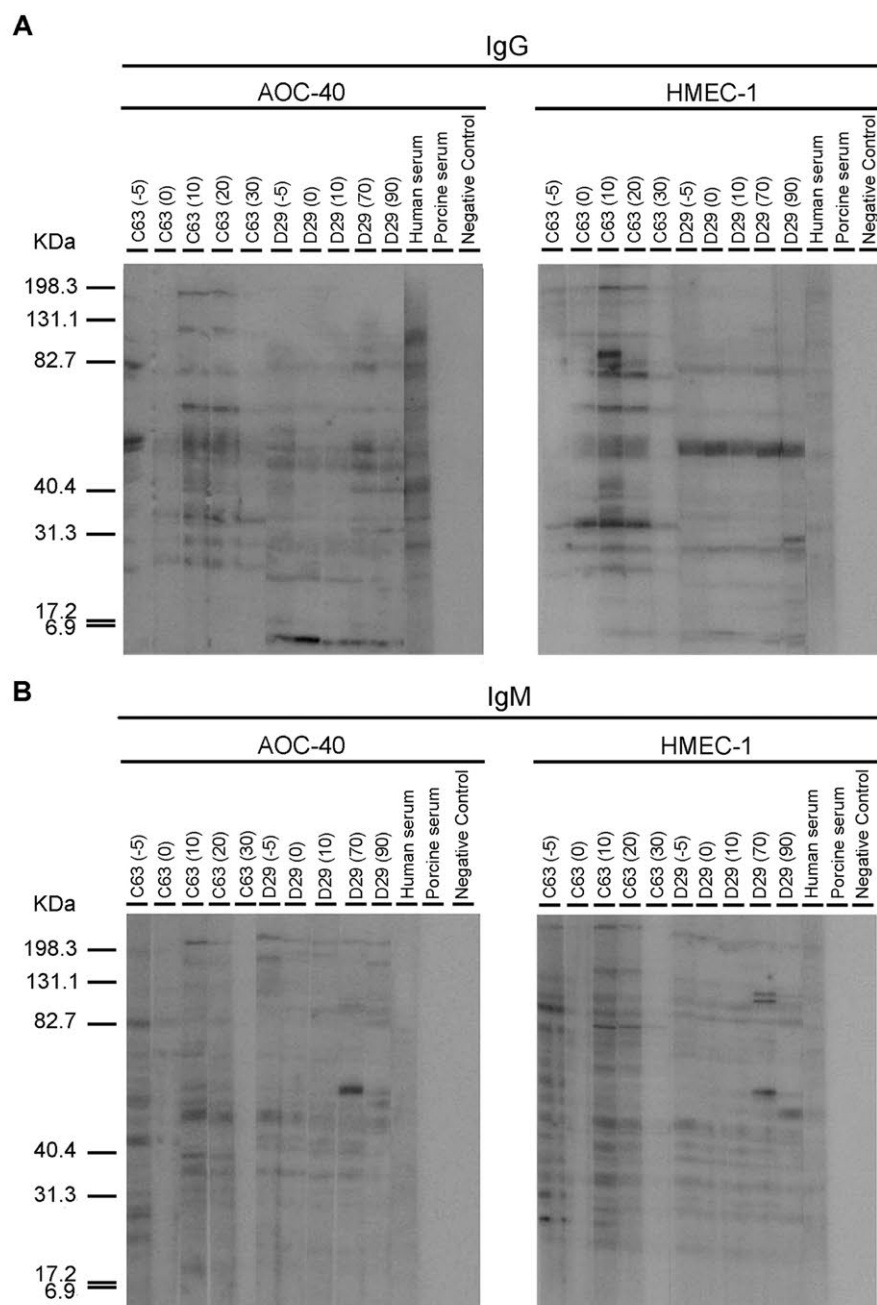


Figure 5. Reactivity of C63 and D29 baboon serum samples against AOC-40 and HMEC-1 cell lysates in western blots. Sample identification is the same described in Figure 2. A) A new band of 32.0 kDa was detected in AOC-40 and HMEC-1 lysates by D29 serum IgG from days 70 and 90. Also, IgG from C63 at day 10 transiently reacted to a new band of 106.9 kDa only in HMEC-1 lysates. Treatment with CyP (C63 day 30) was associated with a noteworthy reduction of IgG reactivity to AOC-40 and HMEC-1 lysates. B) IgM reactivity was detected on a new band of 57.7 kDa in AOC-40 and HMEC-1 lysates when testing D29 sera from days 70 and 90. This band was also observed for C63 on day 10 in AOC-40 lysate. There were also for D29 on days 70 and 90 two new reactive bands of 120.7 and 127.6 kDa detected in AOC-40 and HMEC-1 lysates. Treatment with CyP (C63 day 30) implied the complete disappearance of IgM binding to AOC-40 and HMEC-1 lysates.

may also enhance or reduce the reactivity to endothelial cells from other species [11].

AOC-40 cells exposed to baboon anti-non-Gal antibodies present after the injection of PRBC, underwent apoptotic cell death determined by AnV/PI staining and DNA fragmentation. Apoptosis of endothelial cells has been also described with xenoantibodies present at AHXR of

Gal-KO pig hearts transplanted into baboons [7]. In contrast, apoptosis was not observed in AHXR of wild and pig organs transgenic for human complement proteins transplanted in non-human primates that were mainly mediated by IgM antibodies [21]. In our study, anti-Gal antibodies, mostly IgM, caused the minimal apoptosis of AOC-40 cells, being necrosis the predominant death pathway triggered by these antibodies even without adding an exogenous complement

to the assay. After GAS914 treatment and injection of PRBC anti-non-Gal IgG antibodies prevailed in baboon serum samples, which produced the apoptosis of AOC-40 cells. In addition, these antibodies caused apoptosis of human endothelial cells without including DNA fragmentation, indicating a different pattern of apoptosis than the observed in porcine endothelial cells. Apoptotic cell death can occur even when DNA fragmentation is prevented [22]. Also, with the An V/PI binding assay that is the analysis of apoptosis mostly used [23], a maximum extent of apoptosis has been detected 8 hours earlier than it was detected by measuring of DNA fragmentation [24]. This reflects the highly dynamic process of apoptosis during which characteristic morphological and biochemical markers may be observed in cells for only a limited period. The durations of these periods vary depending on the cell type, the cell-cycle status, and the type and concentration of apoptosis inducer. Moreover, cells of the same population are not uniform in their susceptibility to an apoptosis inducer and may initiate apoptosis at different times of the exposure [24]. In our study is unclear whether the apoptosis of human endothelial cells induced by baboon anti-porcine antibodies included a lack or delay of DNA fragmentation.

After PRBC exposure, new proteins from AOC-40 and HMEC-1 cells were targeted simultaneously though the pattern was different in the two animals studied. These results are similar to those observed after transplantation of organs from pigs transgenic for human complement proteins or Gal-KO in non-human primates. They included the lack of new reactivity after transplantation compared to pre-transplant samples, or the detection of several new bands with a pattern different from each transplantation and without an immunodominant target common to distinct transplants [6,25]. Some of the new bands observed in our study, such as 32.0 kDa, were similar to those detected after immunization with xenogeneic endothelial cells in mice and in the anti-non-Gal antibody response of baboons transplanted with Gal-KO pig hearts [6,9].

CyP treatment was associated 10 days later with the virtual disappearance of IgM anti-non-Gal antibody binding to both AOC-40 and HMEC-1 proteins and a notable reduction in IgG reactivity. The lack of protein binding by anti-non-Gal antibody contrasted to the substantial detection of antibodies on both AOC-40 and HMEC-1 cells by FACS analysis. Although having only one animal treated with CyP is a shortcoming and does not allow drawing general conclusions, the results suggest that part of the anti-non-Gal antibodies cross-reactive to porcine and human endothelial cells target other antigens rather than proteins. Carbohydrates have been considered potential antigens for anti-non-Gal antibodies after porcine xenograft transplantation in non-human primates, although it has not been possible to associate any with AHXR [26]. In spite of this, the lack of usefulness of CyP over anti-non-Gal IgG cross-reactive xenoantibodies appears similar to that we previously demonstrated with antibodies targeting the Gal antigen [18]. Carbohydrates are considered T-cell independent antigens and antibodies against them are produced by long-lived plasma cells much more resistant to irradiation or immunosuppression than short-lived plasma cells. Neu5Gc sialic acid has been proposed as potential target of xenoantibodies in humans exposed to animal cells, tissues and foods [27-29]. However, baboon's express Neu5Gc and lack antibodies against this antigen [26], indicating that other antigens are responsible for the reactivity of anti-non-Gal IgG antibodies with human endothelial cells observed in our study.

Humans have been exceptionally transplanted with organs, tissues or cells from other animal species (xenografts) [30,31]. However, devitalized animal tissues including heart valves, skin, and tendons are

commonly implanted in patients [32,33]. Also, vital pig skin has been used as dressing for burns [29,34]. Patients exposed to animal antigens under these conditions showed a moderate increase of xenoantibodies such as anti-Gal and a high and sustained anti-non-Gal IgG response, similar to the findings we are describing here in non-human primates [29]. In addition, animals and humans are continuously exposed to other animal antigens through the food. Thus, red meats may deliver immunogenic molecules such as Neu5Gc to the human endothelium, leading to antibody and complement-dependent activation, and potentially contributing to vascular pathologies such as atherosclerosis [28]. The binding of human xenoantibodies to endothelial cells observed in this setting was abrogated if serum is previously absorbed with animal serum, similarly to what happens with antibody absorption before assaying AECA antibodies. Thus, considering xenoantibodies as interference and absorbing them in AECA assays may be inaccurate if there is a previous exposure to xenogeneic antigens.

Conclusion

Our studies demonstrate that PRBC immunization along with the depletion of anti-Gal antibodies in baboons induces anti-non-Gal IgG xenoantibodies reactive to porcine and human endothelial cells. These xenoantibodies cause apoptosis of porcine and human endothelial cells through two different pathways, apparently reacting to non-protein antigens. The findings of this study confirm the particular reactivity of xenoantibodies to endothelial cells of different species and challenge the idea that xenoantibodies are false-positive results in AECA assays. After xenogeneic antigen exposure, which may occur much more often than we think, disregarding these antibodies may impair the proper assessment of the prevalence and role of AECA in different human and animal disorders.

Conflict of interest statement

The authors declare that they have no competing interests.

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