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Prevention of hyperacute xenograft rejection in orthotopic xenotransplantation of pig hearts into baboons using immunoadsorption of antibodies and complement factors

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Abstract To prevent hyperacute xenograft rejection (HXR) caused by preformed natural antibodies (XNAb) after orthotopic heart xenotransplantation (oXHTx) of landrace pig hearts into baboons, we used immunoadsorption of immunoglobulins IgG, IgM and IgA and complement with the reusable Ig-Therasorb column. In addition to functional data, tissue was sampled for histological, immunohistochemical and electron microscopical analysis. We performed three oXHTx of landrace pig hearts to baboons using extracorporeal circulation (ECC) connected to the immunoadsorption unit. Intraoperative treatment consisted of four cycles of immunoabsorption (IA). One oXHTx of a baboon without IA served as a control. A mismatch of donor and recipient heart size was prevented by selecting a 30–40% lower body weight of donor pigs than recipients. Four cycles of IA removed more than 80% of IgG, IgM and IgA, 86% of anti-pig antibodies and 66% of complement factors C3 and C4 from plasma. The graft of the control animal failed after 29 min. Orthotopic xenotransplantation with IA was selectively terminated after 100 min, 11 h and 21 h, respectively without any histological signs of HXR in light and electron microscopy. After weaning off from ECC these donor xenografts showed sufficient function with normal ECG and excellent cardiac output in echocardiography and invasive measurement (1.93 ± 0.035 l/min). The myocardium of the control xenograft demonstrated more deposits of Ig and complement components (C3, C4) than in the IA group. Baboons survive HXR after orthotopic pig heart xenotransplantation due to antibody depletion by reusable Ig-Therasorb column treatment. Long-term survival in an orthotopic baboon xenotransplantation model after IA, especially in combination with transgenic pig organs, could be a reliable preclinical trial for future clinical xenotransplantation programs.

Key words Immunoadsorption · Ig-Therasorb · Hyperacute xenograft rejection · Baboon · Orthotopic xenotransplantation · Xenoreactive antibodies

Introduction

Currently only 15% of patients awaiting cardiac transplantation undergo the procedure in a given year [11]. If established criteria for cardiac transplantation were to be extended to all potential recipients younger than 65 years of age, the current donor pool would provide only 5–6% of the necessary organs. Bridging therapy with mechanical left ventricular assist devices has recently provided relative success in carefully selected adult patients with left ventricular failure [22]. The increasing shortage of donors for allotransplantation has

generated interest in the potential use of animal organs for clinical use (xenotransplantation). The first attempt of clinical xenotransplantation was performed in 1906 by Jaboulay [18], who achieved survival of a kidney xenograft for 72 h. The first cardiac xenotransplantation was performed by Hardy et al. in 1964 with a chimpanzee heart into a 68-year-old man in cardiogenic shock. The patient died after 1 h [15]. All subsequent clinical trials of xenotransplantation with pig, sheep, baboon and chimpanzee donor hearts failed after a maximum of 24 h, a disappointing result with respect to clinical application. The only exception was the case of baby Fae: in 1984 Bailey transplanted an ABO-incompatible baboon heart into a human neonate with hypoplastic left heart syndrome, and obtained adequate hemodynamic function and graft survival for 20 days [4]. The most recent clinical orthotopic cardiac xenotransplantation was performed by Czaplicki, who in 1994 transplanted a pig heart into a patient with Marfan's syndrome after extracorporeal depletion of anti-pig antibodies using an additional pig heart and application of embryonal and fetal calf serum. This graft survived for 24 h, a disappointing result in view of further clinical trials. It was reported that no evidence of rejection despite immunohistochemical deposition of complement in myocardial tissue was detected [10].

The first hurdle in a xenotransplantation between widely divergent species is hyperacute xenograft rejection (HXR) [2], which differs markedly from the more progressive vascular and cellular rejection process which can be seen with transplants between different primate species [25]. Species combinations that are subject to the HXR of vascularized xenografts are termed "discordant", whereas species combinations not subject to this type of rejection are termed "concordant" [9]. A pig xenograft transplanted into primates undergoes HXR within minutes to hours. The histological signs of this process are the rapid thrombotic occlusion of graft vessels, platelet aggregation and infiltration with polymorphonuclear leucocytes. Pre-existing natural xenoreactive antibodies (XNAb) mostly of the IgM isotype [21] are present in adult primates [only in humans, apes and Old World monkeys (the platyrrhines)] with an inactivated α1–3-galactosyltransferase gene [12, 13] and primarily initiate HXR [30]. These XNAb are thought to develop early in life after exposure to gut microorganisms expressing the α1–3Gal-epitope [26]. In HXR, binding of XNAb to the α1–3Gal-epitopes of glycoproteins and glycolipids of the vascular endothelium of the xenograft [14] activates complement [23], leading to endothelial cell activation followed by cell injury, thrombosis and ischemia of the graft [6]. This endothelial cell activation leads to a procoagulant state due to the loss of thrombomodulin and heparansulfate/antithrombin III and increase of plasminogen activator inhibitor which causes fibrin clot formation, platelet activation and vascular thrombosis [17]. Adhesion molecules are expressed, which enables inflammatory cells like macrophages and neutrophil cells to migrate into the xenograft. HXR, which is analogous to ABO-incompatible rejection after allograft transplantation, is only secondarily triggered by direct endothelial cell activation via monocytes and natural killer cells [16].

The major therapeutic approaches to overcome HXR are depletion or inhibition of anti-αGal antibodies and elimination or inhibition of complement and the use of organs from genetically engineered pigs transgenic for human complement regulartory proteins.

Many strategies for removal of XNAb and complement depletion [28] were investigated, including plasma exchange, plasmapheresis, xenogeneic organ perfusion and the use of haptens like αGal1–3Gal-fragments and penicillamine [3]. Plasma exchange and organ perfusion result in a loss of coagulatory and plasma proteins and are therefore clinically unattractive. In recent times, particularly columns using antibody conjugates as immunosorbents represent a novel approach for the selective removal of plasma immunoglobulins. Contrary to plasmapheresis or organ perfusion, antibody-based immunoadsorption (immunoapheresis) provides a highly specific depletion technique, using immobilized polyclonal antibodies generated against human immunoglobulins. The purpose and aim of our study was to investigate selective IgG-, IgM- and IgA-antibody removal from baboon blood by immunoadsorption (IA). Therefore we used an Ig-Therasorb column. The reusable Ig-Therasorb column contains polyclonal sheep antihuman IgG antibodies conjugated to sepharose beads, which remove specifically IgG, IgM and IgA from the baboon plasma. In our recent experiments for ex-vivo testing of IA in a working heart model, perfusing pig hearts with human blood Ig-Therasorb column treatment prevented HXR [7, 8, 19] not only by sufficient antibody elimination, but also by a removal of more than 50% of complement components C3 and C4. To investigate analogously Ig-Therasorb immunoadsorption in vivo in primates, we selected orthotopic xenotransplantation model of landrace pig hearts into baboons to prevent HXR by IA. Only orthotopic heart transplantation as a reliable life-supporting model represents the clinical xenotransplantation situation.

Materials and methods

Animals and surgical procedure

Donor animals for orthotopic xenotransplantation were non-transgenic landrace pigs (body weight: 13–14 kg). Pigs were anesthetized with azaperon, ketamine hydrochloride and xylacine. A tracheotomy was performed and the animals were ventilated mechanically. Anesthesia was maintained with N_2O/O_2 (2 l/4 l per min), intravenous pancuronium and fentanyl citrate. After thoracotomy

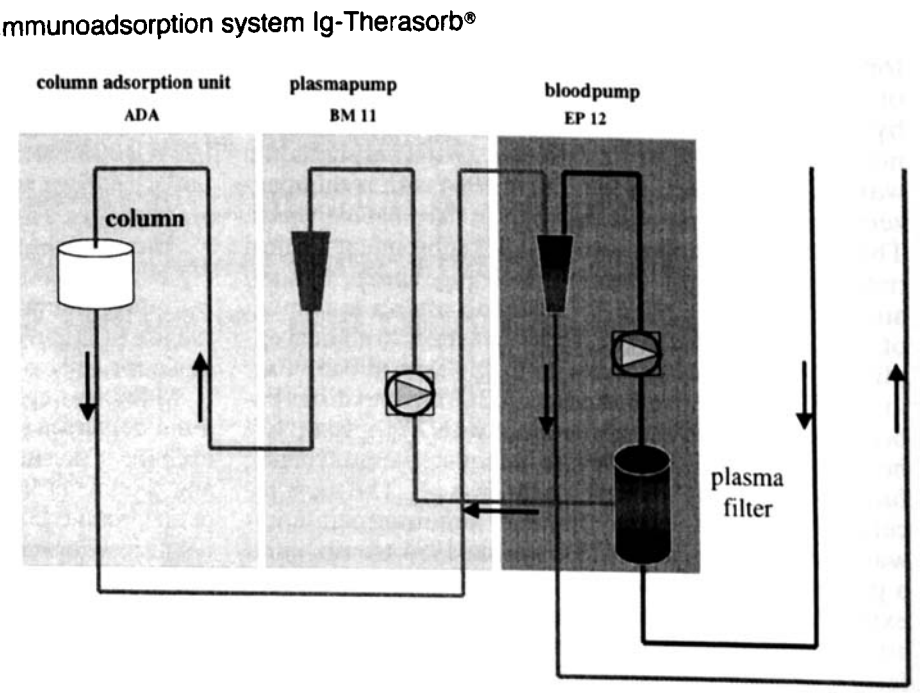

Fig. 1 The Ig-Therasorb® unit consists of a pair of reuseable columns. In a first step, baboon whole blood is separated by plasmapheresis and the erythrocyte fraction returned to the blood sample. Blood flow at the separation device should reach a rate of approximately 20–50 ml/min to ensure a constant plasma flow. The plasma flow is directed to the first column (300 ml) and then switched to the second column. In the meantime, bound immunoglobulins are lysed from Sepharose by glycine and PBS buffer solution and the column is regenerated

Table 1 Immunoadsorption group G1 and control group G2. There was no difference of body and heart weight between both groups. We selected a 30–40% smaller body weight of donor pigs than recipients to prevent a heart weight mismatch. Anti-pig antibody levels were higher in the control baboon. Lower levels in the IA group would still have been sufficient to induce HXR

Groups	Immunoadsorption group ($n = 3$)	Control group ($n = 1$)
Body weight of donor pigs (male landrace pigs)	13.7 ± 0.25 kg	13 kg
Body weight of recipient baboons (male, *Papio anubis*)	20.3 ± 2.12 kg	16.9 kg
Heart weight of donor pig	73.4 ± 5.3 g	76.2 kg
Heart weight of recipient baboon	89.4 ± 10.6 g	80.0 g
Ischemic time	3.26 ± 0.47 h	3.97 h
Anti-pig-antibody level before immunoadsorption (1/*n*)	298 ± 82.2	1024
Anti-pig-antibody level after immunoadsorption (1/*n*)	42.7 ± 8.2	1024

the pig heart was explanted after induction of cardioplegic arrest with 4 °C cold Celsior-solution (Imtix, Pasteur Merieux Serums & Vaccines, Lyon, France).

As recipients we used adult baboons (body weight: 17–26 kg) of the *Papio anubis* type (Table 1). The surgical technique used for xenotransplantation is well known from allogenic heart transplantation according to Lower and Shumway. Anatomical differences between a human and a porcine heart include a short ascending aorta with a thick wall in pigs, a hemizygos vein that is attached to the upper left atrium and a heart axis and left anterior descending coronary artery deviation to the right side if compared to humans. We selected a 30–40% smaller body weight of donor pigs than recipient baboons to prevent a mismatch of donor and recipient heart size.

After thoracotomy during extracorporeal circulation (ECC) the recipient heart was excised and the anastomoses between donor and recipient left atrium, right atrium, pulmonary artery and finally the ascending aorta were made. Before releasing the aortic clamp steroids were given IV. After a reperfusion time of 45 min, weaning from extracorporeal circulation was started with initial use of catecholamines if necessary.

We used IA in three oXHTx of landrace pig hearts into baboons (B1, B2, B3) in group 1 (G1) by connecting the ECC to the IA unit. Treatment consisted of four cycles of IA. A blood pump (BM11) separated the recipient's blood into plasma and the cellular fraction by a plasma filter. In a second pump-driven circuit the plasma flow was directed to the Ig-Therasorb column. One oXHTx of a baboon (B4; body weight: 16.9 kg) without IA treatment served as a control (G2).

Immunoadsorption

The IA unit (ADA) consists of a pair of sterile and pyrogen-free Ig-Therasorb glass columns, which contain polyclonal sheep anti-human IgG antibodies (heavy chain- and light chain-specific) conjugated to cyanogen bromide-activated Sepharose beads. The anti-Ig column, with a total volume of 300 ml, removes specifically IgG (subclasses 1–4), IgM, IgA, circulating immune complexes and fragments of immunoglobulins with an average Ig reduction of 60–70% per cycle. The column is loaded with a storage buffer containing phosphate-buffered saline (PBS) and 0.01% sodium azide (pH 7.2 at 4 °C) until use. A second circuit ensures a constant plasma flow directed to the first Therasorb column (Fig. 1). Following passage through the first column, the bound immunoglobulins are released from the sepharose beads by glycine (pH: 2.8) and PBS buffer solution (pH: 7.2). The column is regenerated by glycine

and PBS solution, while the second column is loaded. Thus the Therasorb columns become re-usable.

Hemodynamic parameters

Arterial, central venous pressure, heart rate, ECG and esophageal and rectal temperature was registered by a Sirecust 960 monitor (Siemens, Erlangen, Germany). For cardiac monitoring after oX-HTx, we used echocardiography (Sonoline 2000, Siemens, Erlangen, Germany) for the measurement of ejection fraction (EF in %) and fraction shortening (FS in %). Invasive measurement of cardiac output before and after xenotransplantation was performed with a Swan-Ganz catheter positioned in the pulmonary artery.

Serology

Blood samples were taken from baboons at fixed intervals before, during and after IA and xenotransplantation. Plasma samples were stored at -70 °C after centrifugation of blood at 4 °C. As serological parameters for evaluation of the efficiency of IA levels of IgA, IgG, IgM, complement components C3 and C4 and anti-pig antibodies were measured. For measuring anti-pig-antibody levels baboon plasma (0.5 ml) was serially diluted (Fig. 4 a). Washed pig RBC were incubated with the plasma for 30 min and hemagglutination was titrated under a light microscope. The last detection step where agglutination was still present was determined the anti-pig-antibody titer. Creatine kinase (CK and CK-MB), lactate dehydrogenase (LDH) and glutamic ocalacetic transaminase (GOT) were determined by clinical standard methods as parameters for myocardial damage.

Histology

For light/electron microscopy and immunohistochemical examination myocardial tissue from both atria and ventricles of the xenograft were sampled at the end of the experiment.

Light microcopy

Frozen tissue sections of 4–6 μm were stained using hematoxylin and eosin and examined under a light microscope (LM).

Transmission electron microscopy (EM)

Tissue sections were embedded in Tissue Tek (Miles, USA), snap-frozen in liquid nitrogen, and stored at -70 °C until use. Other tissue samples were fixed in glutaraldehyde 6.25% and stored until further saccharose (0.2 mol/l) processing. Hemithin 0.5 μm sections prepared with epon resin were first coloured with toluidine-methylene blue in order to gain an overview by light microscopy. Ultrathin tissue sections of interest (100 nm) were laid on copper grids and stained with uranylacetate and lead. The examination with transmission electron microscopy (Philips 300) was performed at two magnifications (× 10000 and × 16000).

Immunohistochemistry

Cryostat-prepared tissue specimens were stained with FITC-conjugated goat antibodies specific for C3, C4, C5b-9. Monoclonal antibodies were obtained from Dako (Hamburg, Germany) and Immunotech Diagnostics (Marseille, France). Deposition of IgA, IgG and IgM in myocardial tissue were stained according to the avidin-biotin method.

Results

Duration of ECC was 2.36 ± 0.17 h (G1: 2.32 ± 0.16 h; G2: 2.5 h), aortic cross clamp time was 1.16 ± 0.09 h (G1: 1.10 ± 0.13 h; G2: 1.33 h). Cold ischemic time of the xenograft was 3.43 ± 0.35 h (G1: 3.26 ± 0.47 h; G2 3.97 h, Table 1). Selection of a 31% smaller body weight of donor pigs (13.5 ± 0.29 kg) than baboon recipients (19.5 ± 2.13 kg) resulted in a 15% smaller heart weight of pig hearts (74 ± 4.9 g) compared to baboon hearts (87.1 ± 11.5 g).

Xenograft survival and hemodynamic parameters

Our study was not authorized as a long-term survival experiment. As the animals were not allowed to recover from anesthesia, the experiments had to by terminated within 24 h. The xenograft of the control animal failed after 29 min with a dilated fibrillating ventricle and signs of HXR. After cardiac arrest, this experiment was terminated after 1 h of reperfusion during extracorporeal circulation. In the immunoadsorption group ($n = 3$), acute graft failure caused by HXR did not occur. Our first oXHTx (B1) with IA was terminated after weaning off from ECC and stabilization of the circulation 100 min after reperfusion. The second oXHTx (B2) was killed after 11 h and the third oXHTx (B3) after 21 h. All animals of G1 demonstrated stable circulation after weaning from ECC. The donor xenografts of the IA group displayed sufficient ventricular function in echocardiography (EF: 65 ± 13%, FS: 32 ± 6%). In ECG sinus rhythm without arrhythmia and without significant ST-segment elevation as a sign of ischemia was determined. The cardiac output was 1.93 ± 0.035 l/min in Swan-Ganz catheter measurement.

Serology

Immunoglobulins

According to Fig. 2, IA (four cycles) eliminated 94% of IgM (from 0.6 ± 0.19 g/l to 0.1 ± 0.01 g/l), 85.5% of immunoglobulins IgG (from 9.43 ± 0.76 g/l to 1.37 ± 0.23 g/l), and 83.5% of IgA (from 0.97 ± 0.39 g/l to 0.17 ± 0.08 g/l) from baboon serum.

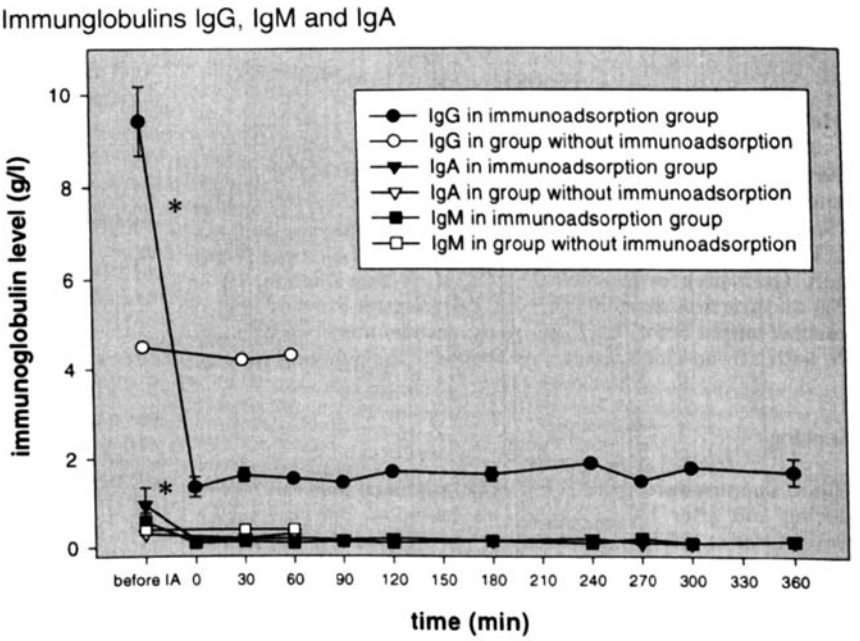

Fig. 2 Four cycles of IA significantly removed IgM by 94%, IgG by 85.5% and IgA by 83.5% in baboon serum

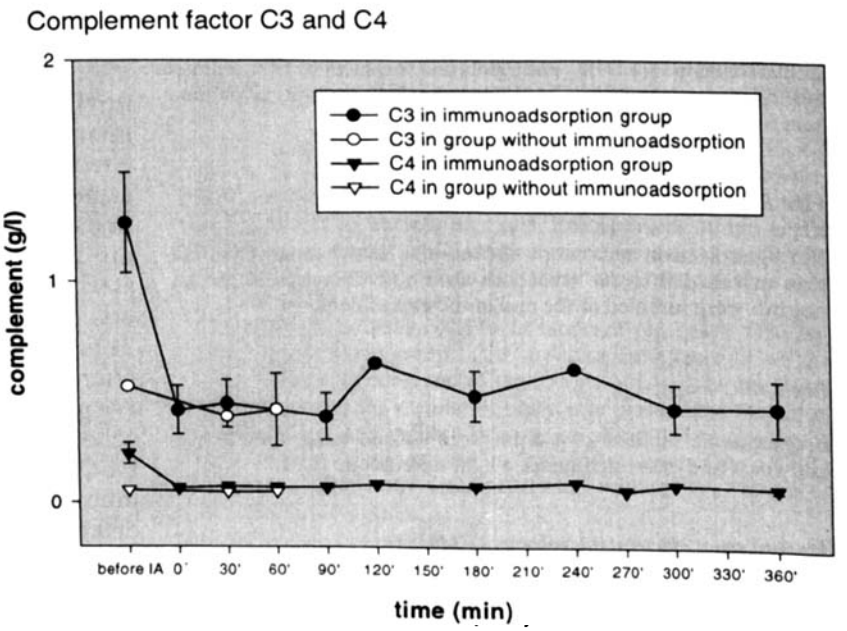

Fig. 3 Complement component C3 was eliminated by 63% and C4 by 62.5% after four cycles of IA. In control animal levels of C3 and C4 remained constant

Anti-pig-antibodies

Anti-pig antibody levels (Table 1) were higher in the control animal (B4: 1:1024) than in the IA group (mean: 298 ± 82; B1: 1:512, B2: 1:128, B3: 1:256), but in both groups levels were sufficient to induce HXR. Immunoadsorption removed XNAb in G1 by 86% to 1:64, 1:32 and 1:32, respectively (Fig. 4b). In the control animal, anti-porcine antibodies were markedly decreased during perfusion of the xenograft presumably caused by adsorption of the antibodies onto the graft.

Complement

Circulating complement components C3 and C4 were additionally removed by IA. A marked elimination of C3 by 63% and of C4 by 62.5% could be demonstrated in G1 (Fig. 3: C3: group 1: from 1.27 ± 0.22 g/l to

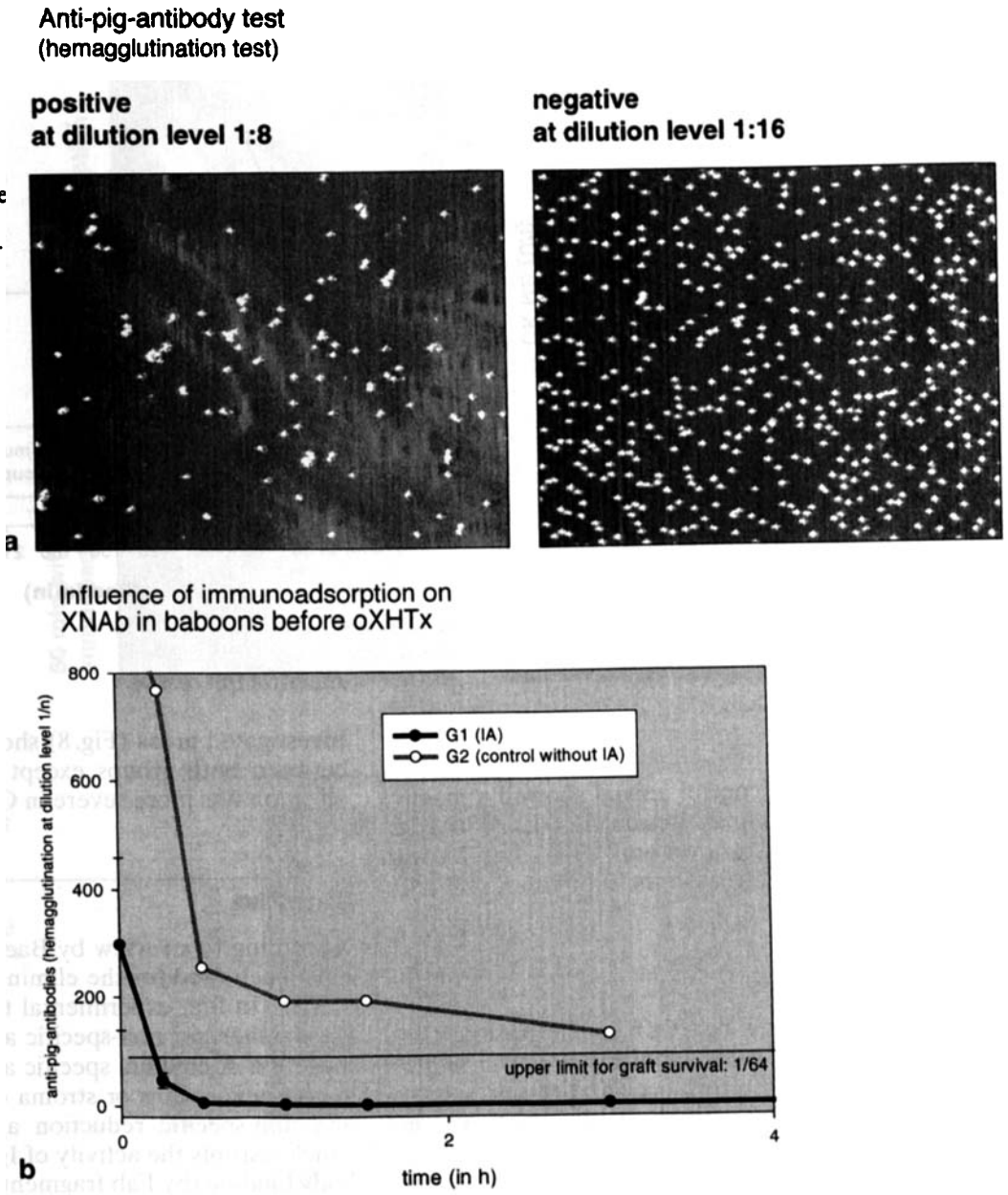

Fig. 4 **a** For anti-pig-antibody monitoring baboon plasma (0.5 ml) was serially diluted. Washed pig red blood cells were incubated for 30 min and hemagglutination was titrated under a light microscope. **b** Anti-porcine antibodies were eliminated after four cycles of IA by 86%. In the control animal XNAb were removed by 87.5% caused by adsorption onto the xenograft

0.47 ± 0.19 g/l after IA; group 2: from 0.52 g/l to 0.41 g/l; C4: group 1: from 0.2 ± 0.037 g/l to 0.075 ± 0.02 g/l after IA; group 2: from 0.058 g/l to 0.049 g/l). In G2 levels of C3 and C4 remained constant during the experiment.

Serological parameters of myocardial damage

Serological parameters like CK, CK-MB (Fig. 5), GOT, GPT and LDH indicating cardiac damage increased in both groups.

Hematology

Hemodilution turned out to be a major problem during immunoadsorption and ECC. Hemoglobin levels were decreased to a minimum of 6 mg/dl. In our recent experiments, this problem was solved by homologous and autologous blood transfusion. Platelet count was markedly reduced by 50% to critical levels below 100 G/l. White blood cell count increased 4 h after xenotransplantation to 20–30 G/l.

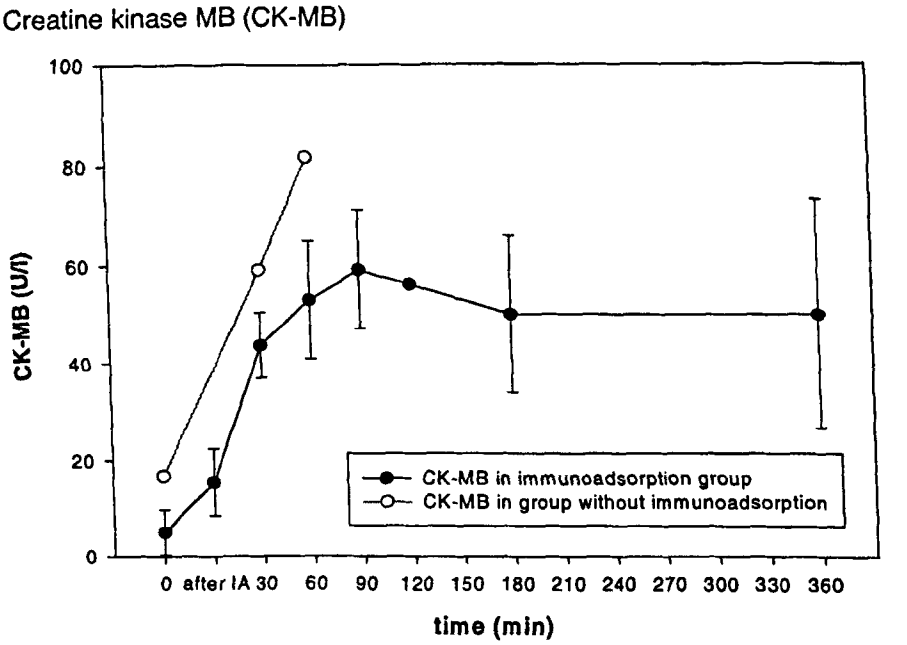

Fig. 5 As a serological parameter CK-MB, a specific cardiac enzyme, increased in both groups

Histology

Macroscopy

The xenograft of the control animal showed a massive hemorrhage and transmural necrosis (Fig. 6) if compared with the inconspicuous graft 20 h after IA with only minimal necrotic areas in the transversal section.

Light microscopy

Hematoxylin/eosin staining (Fig. 6, magnification 1:100) of the control graft tissue demonstrated typical signs of HXR like single cell necrosis, occluded vessels, interstitial hemorrage and interstitial edema which was not found in the tissue of the IA group xenografts.

Immunohistochemistry

IgM staining of the myocardium of the IA group demonstrated patchy small deposits of IgM within the myocardium and on the vascular endothelium (Fig. 7). A marked IgM staining of the vascular endothelium as well as of interstitium was found along the destroyed myocytes of the control graft. Immunohistochemical results with IgG and IgA staining (data not shown) were similar. Detection of complement components C3, C4 (not shown) and C5b-9 was only possible on vascular endothelium of the control graft.

Electron microscopy

Investigated areas (Fig. 8) showed no major difference between both groups except that mitochondria vacuolization was more severe in G2.

Discussion

According to a review by Bach [3], numerous methods have been used for the elimination or neutralisation of XNAb. In first experimental trials plasma exchange or plasmapheresis, non-specific antibody sorbents such as a protein A column, specific antibody sorbents such as xenogeneic organs or stroma cells, injectable antigens, and non-specific reduction agents like penicillamine which disrupts the activity of IgM and blockade of antibody binding (by Fab fragment application) were investigated. In experimental models predominantly plasmapheresis or organ perfusion was performed. Immunoadsorption columns (immunoapheresis) entered the clinical arena initially using staphylococcal proteins A and G. After preclinical experiments in a pig-to-dog renal transplant model [1], Ig-Therasorb columns were used for renal transplant patients with anti HLA antibodies [24].

Stoffel et al. [29] performed the first animal experiment with antibody-based IA for depletion of LDL-cholesterol in pig plasma using sheep antibodies. While the first clinical trial (1983) was performed for LDL-cholesterol elimination [27], a second generation of columns using polyclonal antibodies directed against human immunoglobulins (Ig) was extremely effective for remov-

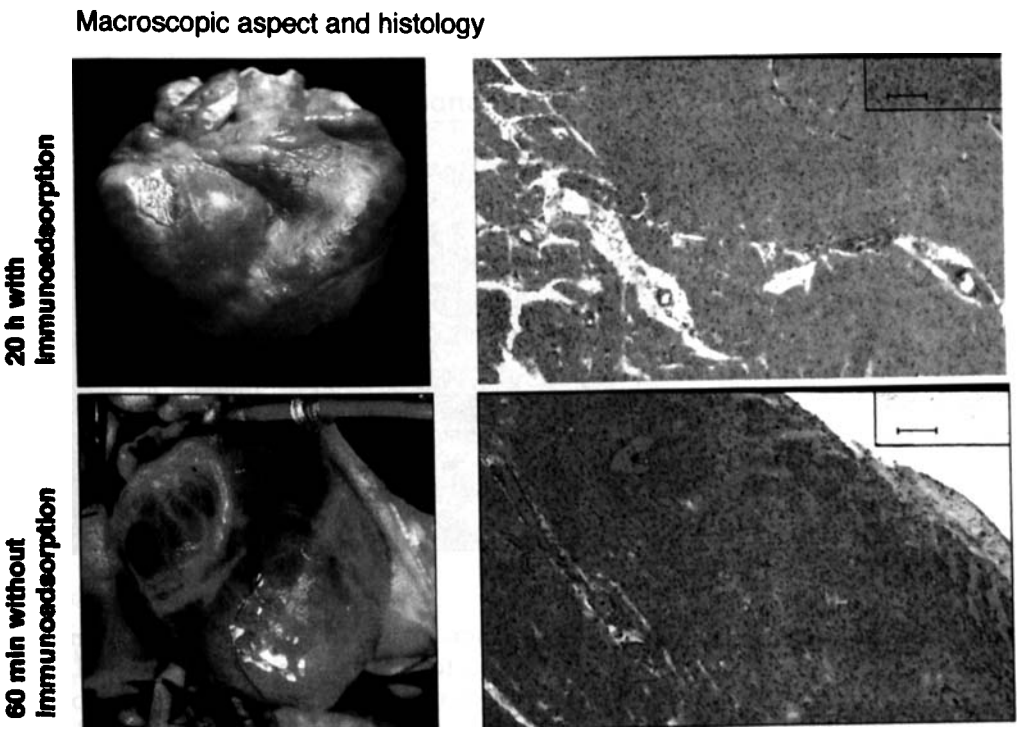

Fig. 6 *Left side*: after HXR the xenograft of the control animal showed massive hemorrhage if compared to the inconspicuous graft of the antibody-adsorbed animal after 20 h. *Right side*: histological examination with light microscope (hematoxylin/eosin staining, magnification: 1:100) demonstrated open vessels in an inconspicuous tissue of the IA group (*upper side*) in contrast to typical signs of HXR like cell necrosis, occluded vessels and interstitial hemorrage in the control animal

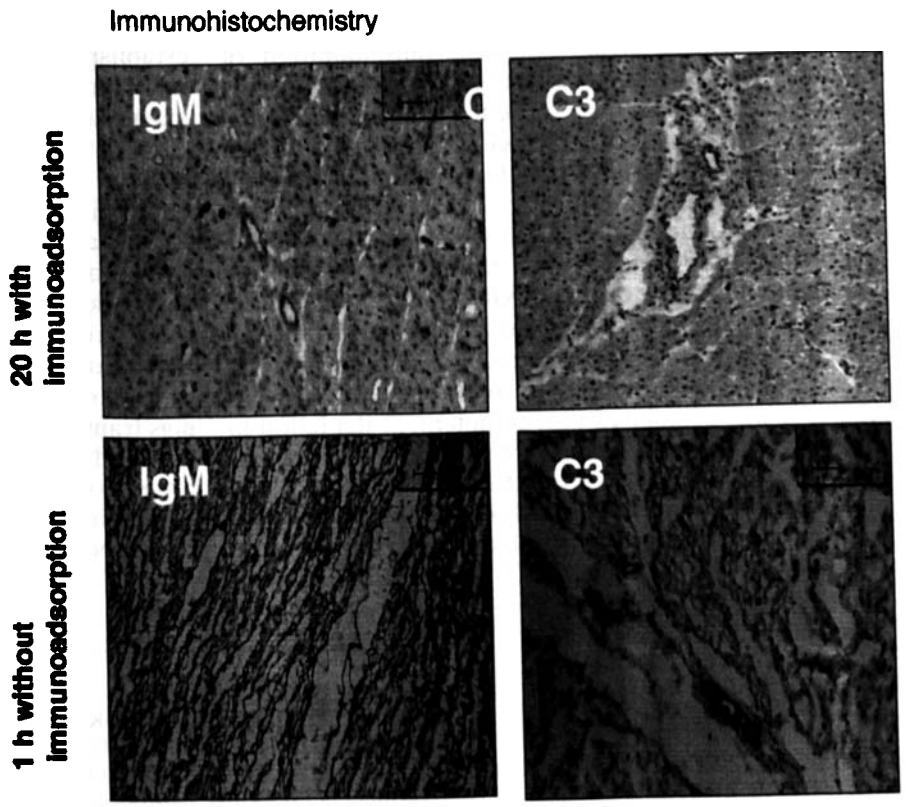

Fig. 7 Immunohistochemical staining showed slight deposition of IgM after four cycles of IA in contrast to marked IgM staining of the destroyed control myocardium (*left*). We detected complement component C3 on the vascular endothelium of the control graft (*right*)

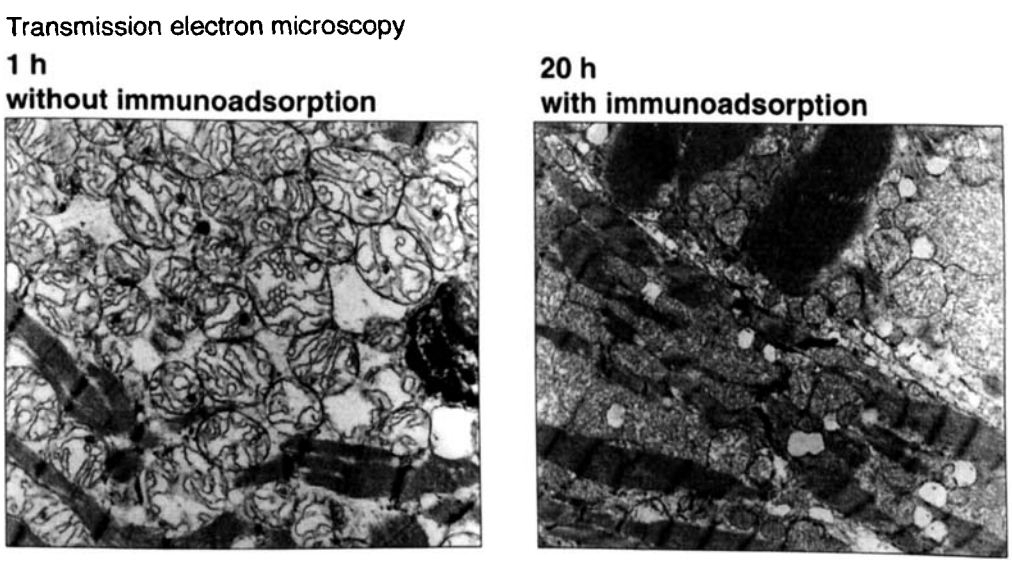

Fig. 8 Electron microscopy investigation showed mitochondria vacuolization in G2 (left, magnification: × 16000) in contrast to G1 (*right*, magnification: × 10000), where interstitial edema was detected

ing human IgG and IgM XNAbs from plasma of patients with autoimmune diseases. According to these clinical applications no significant impact on coagulatory and plasmatic proteins was found [20].

The aim of our study was application of IA using a reusable antihuman Ig-Therasorb column (Therasorb, Baxter Corp., Unterschleissheim, Gemany) for removal of XNAb before orthotopic xenotransplantation of landrace pig hearts into baboons.

Our experiments give evidence that baboons can overcome the period of hyperacute xenograft rejection after xenotransplantation if antibody depletion by Ig-Therasorb column is performed. Plasma levels of complement components C3 and C4 were reduced which could clearly have an influence on the activation of the complement cascade during HXR. We suppose that C3 and C4 may be fixed to the antibodies which are absorbed to the column or by direct consumption within the extracorporeal immunoadorption system. In recent studies it was postulated that complement depletion in addition to antibody removal would be beneficial for prolonging xenograft survival [5]. IA obviously has the potential to improve xenograft function to sustain circulation in a life-supporting primate model. In the future even more than four cycles of IA will be used for more effective elimination of XNAb before oXHTx of baboons, because IgM deposition could still be detected in xenograft tissue of G1 (Fig. 7). IA reduced myocardial damage in histology, immunohistochemistry and serological parameters. Measurement of agglutinating anti-pig-antibody-levels was a reliable method for monitoring the efficiency of IA. In further experiments we established a critical "deadline" for anti-pig antibodies necessary to induce HXR at a 1:64 dilution level, the maximum antibody level, which a xenograft can survive. IA with the Ig-Therasorb system can be handled easily during extracorporeal circulation or by taking recipient blood via a central venous catheter. Current problems include a decrease in platelet count, which was probably caused by the plasma filter of the IA unit, and a hemoglobin decrease to critical levels.

In order to overcome delayed xenograft rejection and chronic humoral rejection mechanisms after xenotransplantation a combination of IA, the use of donor pigs transgenic for hDAF and other complement inhibitors and an ideal combination of immunosuppressive drugs possibly including soluble complement receptor 1, may improve long-term survival of xenografts in future experiments.

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