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EX VIVO LUNG MODEL OF PIG-TO-HUMAN HYPERACUTE XENOGRAFT REJECTION

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Objective: Our objective was to study lung hyperacute rejection in the pig-to-human xenotransplantation combination. **Methods:** Pig lungs were harvested and continuously ventilated and perfused ex vivo, using a neonatal oxygenating system, with either xenogeneic unmodified human blood ($n = 6$) or autogeneic pig blood ($n = 6$). **Results:** Autoperfused lungs displayed normal hemodynamics, oxygen extraction (arteriovenous oxygen difference), and histologic characteristics throughout the 3-hour study period. By contrast, xenoperfused lungs displayed, within 30 minutes, severe pulmonary hypertension and abolishment of arteriovenous oxygen difference culminating in massive pulmonary edema, hemorrhage, and lung failure after 115 ± 44.2 minutes of reperfusion. Within 30 minutes, the human blood showed a significant drop of anti- α Gal immunoglobulin M and G xenoreactive antibodies (enzyme-linked immunosorbent assay) and complement activity, consumption of clotting factors, and hemolysis; total circulating human immunoglobulins remained substantially normal. Histologically, lungs perfused with human blood were congestive and showed alveolar edema and hemorrhage and multiple fibrin and platelet thrombi obstructing the small pulmonary vessels (arterioles, capillaries, and venules) but not large (segmental or lobar) pulmonary vessels. On immunohistologic examination, deposits of human immunoglobulin M and

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complement (C1q and C3) proteins were observed on the alveolar capillaries. Conclusions: This pig-to-human xenograft model suggests that the pig lung perfused with human blood has an early and violent hyperacute rejection that results in irreversible pulmonary dysfunction and failure within approximately 150 minutes of reperfusion. (*J Thorac Cardiovasc Surg* 1997;114:315-25)

The ongoing shortage of human organs for allotransplantation, coupled with scientific¹ and biotechnologic² advances, has catalyzed new attempts to use animal organs in human beings, a field known as xenotransplantation. The source of such animals is, however, controversial. Although nonhuman primates, like chimpanzees and baboons, offer the advantages of phylogenetic proximity (concordant xenotransplants), they are unlikely to bridge the organ gap because they are limited in number and size, can harbor pathogens lethal to human beings,³ and present ethical and social dilemmas that are difficult to overcome.⁴

Conversely, nonprimate donors, most notably pigs, are more likely to be favored because they are easily bred, physiologically similar, and accepted for human consumption. Their clinical application is, however, limited by hyperacute rejection (HAR). This reaction, observed when organs are engrafted between phylogenetically distant species such as the pig-to-primate (discordant xenotransplants) combination,^{4,5} is triggered by binding of human xenoreactive natural antibodies to endothelial xenograft cells with subsequent complement activation. It leads to irreversible xenograft damage and loss within minutes or 1 to 2 hours in untreated recipients.⁶⁻¹¹

However, recent experimental^{2,12,13} and clinical^{14,15} cardiac xenotransplantation¹⁶ studies have certainly shown that the seemingly insuperable barrier of HAR in the pig-to-primate model is crumbling.¹ Unfortunately, similar advances in the domain of lung xenotransplantation are yet not available.^{17,18} This work was done to depict the HAR in the pig-to-human lung combination and the functional, immunologic, and histologic events contributing to its onset.

Materials and methods

Animals and study design. Outbred pigs of the race Large White weighing approximately 20 to 30 kg served as lung donors. All animals received care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals"

formulated by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). Human blood was obtained from informed patients (Claude Huriet Law, No. 88-1138, December 20, 1988) affected by hemochromatosis undergoing therapeutic phlebotomies; blood units (two AB+, three O+, and one A+) were collected at the Blood Banks of the West Ile-de-France Public Interest Group (GIP) and screened for virally transmitted diseases. Each blood unit (400 ml) was anticoagulated with 1.2 units of heparin per milliliter and used within 30 days.

Pig left lungs were harvested and then ex vivo perfused with pig blood for 30 minutes to stabilize the perfusion and ventilation system without the oxygenating system. A neonatal oxygenating system was then interposed in the perfusion circuit, and lungs were perfused either with autogeneic pig blood for a 3-hour study period ($n = 6$) or xenogeneic unmodified human blood until the onset of HAR ($n = 6$). To study the functional, immunologic, and coagulation effects of autogeneic and xenogeneic lung perfusion, we took blood samples at baseline and after 10, 20, 30, 60, 90, 120, 150, and 180 minutes of reperfusion and plasma was subsequently stored in aliquots at -80°C until use.

Harvest technique. Animals were premedicated with intramuscular ketamine hydrochloride (25 mg/kg) and atropine sulfate (1 mg/kg) and anesthetized with intravenous sodium pentobarbital (25 mg/kg). After orotracheal intubation, anesthesia was maintained with inhaled halothane and the animals' lungs were ventilated (Labaz Inc. Ventilator, Chemin Cami-Saliè, Pau, France) at an equal gas mixture of oxygen and protoxide. Ear venous catheters were placed for infusion of crystalloid solutions and adequacy of ventilation and oxygenation was assessed by arterial blood gas analysis and pulse oximetry. After completion of a median sternotomy and upper laparotomy, the abdominal viscera were lifted away from the diaphragm to avoid compression of the lower lung lobes and the anterior pericardium was opened and suspended. After systemic heparinization (3 mg/kg), a 4-0 polypropylene (Prolene, Ethicon, Inc., Somerville, N.J.) purse-string stitch was placed on the anterior surface of the ascending aorta, and an aortic root cannula (DLP, Inc., Grand Rapids, Mich.) was inserted for collection of approximately 400 ml of autogeneic blood. The left lung was then harvested after topical electrically induced cardiac arrest. Care was taken to have the maximum length of the left pulmonary artery and main bronchus and a large cuff of left atrium incorporating the two left pulmonary veins and the venous return from the infracardiac lobe of the right lung. Once harvested, a 4-0 Prolene purse-string suture was placed on the free cuff walls of the left atrium.

Fig. 1. The isolated perfused and ventilated ex vivo lung model. Human blood (1) fills the oxygenator cardiotomy reservoir, where it is continuously deoxygenated (93% nitrogen, 5% carbon dioxide, and 2% oxygen) by the neonatal oxygenating system (2), enters through an arterial line into the pulmonary artery, perfuses the lung, and returns to the cardiotomy reservoir through the venous line. A heater (3) warms the blood and lung to 37° C. This last, continuously ventilated by a mechanical ventilator (4) (75% nitrogen, 20% oxygen, and 5% carbon dioxide gas mixture), is suspended in a humidified chamber (5).

Then, the left lung was instrumented by placing a 3/8-inch straight tube connector (Dideco Inc., Mirandola, Italy) in the left pulmonary artery for pulmonary artery inflow, one in the left main bronchus for mechanical ventilation, and one in the cuff of the left atrium to passively drain pulmonary venous blood outflow. Ligatures were then tied around the cannulas.

Ex vivo pig-to-human xenograft model. An ex vivo perfusion and ventilation model for the study of isolated, working pig lungs perfused with either autogeneic pig blood or xenogeneic human blood was developed as shown in Fig. 1. The left lungs were suspended by the left main bronchus connectors in a thermostated humidified chamber, continuously ventilated with room air (inspired oxygen fraction = 0.21) by a mechanical ventilator (Siemens-Elema, Solna, Sweden) through noncompressible tubing (tidal volume of 200 ml at 20 breaths/min with 5 cm H₂O positive end-expiratory pressure), and perfused with autogeneic undiluted pig blood for the first 30 minutes in both groups to obtain adequate stabilization. Hemolysis of the residual pig blood was avoided by flushing the pigs' lungs with 0.5 L saline solution before reperfusion with human blood. Thereafter, lungs were continuously ventilated with a 75% nitrogen, 20% oxygen, and 5%

carbon dioxide gas mixture and perfused through the inflow and outflow connectors attached to the blood-primed perfusion circuit through silicone tubing (Bentley ByPass 70 tubing; Baxter Healthcare Corp., Bentley Div., Irvine, Calif.), with care taken to avoid introduction of air. Venous blood return was collected through the outflow cannula by gravity into the oxygenator cardiotomy reservoir, continuously deoxygenated (93% nitrogen, 5% carbon dioxide, and 2% oxygen) with the use of a neonatal oxygenating system (Polystan Safe Micro, Copenhagen, Denmark), and returned to the left pulmonary artery through a roller pump (Ismatec mod, Bioblock, Strasbourg, France) at a flow rate of 100 ml/min. The entire perfusion circuit was limited to an internal volume of 200 ml of heparinized blood (0.15 ml/kg). A heater (HAAKE FK/F4391, Berlin, Germany) warmed the humidified chamber and blood to 37° C. An additional 200 ml of donor autogeneic or xenogeneic blood was maintained in reserve to replace circuit volume loss resulting from blood sampling. Perfusion pressure was continuously monitored with a pressure transducer (Gould Electronics BV, Bilthoven, The Netherlands) placed in series with the perfusion inflow line. The perfusion flow rate was manually adjusted to maintain a constant perfusion pressure of

Fig. 2. Hemodynamics (pulmonary vascular resistance, PVR) at baseline (time 0) and at specific time intervals after perfusion of pig lungs with pig or human blood. Data are expressed as mean $\pm$ standard deviation (error bars) of the experiments. The difference was statistically significant ($p < 0.0001$). During the time interval between -30 and 0 minutes, the pig lungs were perfused with autogeneic blood to stabilize the perfusion system.

Fig. 3. Oxygen extraction (AVO₂) of pig lungs at specific time intervals after perfusion of pig lungs with pig or human blood. Data are expressed as mean $\pm$ standard deviation (error bars) of the experiments; hemoglobin concentrations to calculate the AVO₂ were normalized for the observed hematocrit value. The difference was statistically significant ($p < 0.0001$). First 30 minutes as in Fig. 1.

Fig. 4. Histologic studies of pig lungs perfused with human blood. A, Intraarteriolar thrombus (big arrow) of fibrin and platelet; the respiratory epithelium of a small bronchus is indicated by the small arrow. (Original magnification $\times 360$.)

10 mm Hg. During reperfusion, pH and Na⁺, K⁺, Mg²⁺, and Ca²⁺ ions were monitored and eventually adjusted to maintain physiologic values.

Functional measurements. Arterial and venous blood samples were collected for blood gas analysis (ABL3, Radiometer A/S, Copenhagen, Denmark) and hematocrit evaluation (Cellokritt, AB Lar Lyunberg, Stockholm, Sweden). Blood electrolytes and pH adjustments were made as necessary during the study period to maintain physiologic arterial blood pH (7.3 to 7.4). Arterial samples were taken from the left atrial outflow cannula and venous samples from blood directly exiting the oxygenator. The arteriovenous oxygen difference (AVO₂) was calculated from the following formula:

$$\text{AVO}_2 (\text{ml O}_2/100 \text{ ml blood}) = ([1.34 \times \text{Hb}] \times [\text{S}_{\text{art}}]) - ([1.34 \times \text{Hb}] \times [\text{S}_{\text{ven}}])$$

where S is the arterial (S_{art}) or venous (S_{ven}) oxygen saturation and Hb is hemoglobin concentration (grams per deciliter).

The pulmonary vascular resistance (PVR) was calculated from measurements of the blood flow with a flow probe (Statham SP2202, Biomedical Div., Oxnard, Calif.)

Fig. 4, cont'd. B, Venular thrombus of fibrin and platelets in an interalveolar septum. C, Diffuse capillary congestion and alveolar edema and hemorrhage. (Original magnifications $\times 180$.)

and the pulmonary artery pressure (PAP) (Kipp et Zonen BD112, Gagny, France) and calculated as follows:

$$\text{PVR} = \frac{\text{PAP}}{\text{Blood flow}}$$

where pulmonary vascular resistance was expressed in millimeters of mercury per milliliter per minute, pulmonary artery pressure in millimeters of mercury, and blood flow in milliliters per minute.

Complement activity. A quantitative assay was used to detect complement activity of the human blood, (Kallestad Quantiplate, Sanofi Diagnostics Pasteur S.A., Marnes-la-Coquette, France). In brief, human serum is placed in wells and diffuses through an agarose gel medium containing standardized sheep erythrocytes sen-

sitized with hemolysin. Total complement activity (CH_{100}) is subsequently estimated by comparing the extent of the lysis of the sheep erythrocytes caused by the human serum with that caused by a standard reference serum. By definition, one unit of complement (CH_{100}) at pH 7.4, 37°C in veronal buffer for 60 minutes will lyse 2×10^8 sheep erythrocytes sensitized with two units of hemolysin. Pathologic CH_{100} values fall less than 70 CH_{100} units per milliliter.

Immunoglobulin levels, hemolysis, and coagulations in human blood. The levels of total circulating human immunoglobulins (Ig G, A, and M (grams per liter) were evaluated with the use of class-specific antibodies (Roche Diagnostics, Nevilly-sur-Seine, France) and measured by end-point turbidity at 340 nm. Lysis of red blood cells was determined by measuring the absorbance of a diluted plasma at 547 nm with reference to a standard curve of

Fig. 5. Wet/dry weight ratio of pig lungs perfused with pig (autogeneic) or human (xenogeneic) blood. Data are expressed as mean $\pm$ standard deviation (error bars) of the experiments. The difference was statistically significant ($p < 0.01$).

known hemoglobin values and expressed as milligrams of hemoglobin per 100 ml. Prothrombin time and activated partial thromboplastin time were measured on a fibrin timer 10-semiautomatic device (Behring, Rueil Malmaison, France) by in vitro activation (37°C) of plasma with human placental thromboplastin (Thromborel S, Behring), and micronized silica (automated APTT, Organon Teknika, Fresnes, France), respectively. Chronometric determination of fibrinogen level (grams per liter) was made according to the Clauss method,¹⁹ on ST 888 semiautomatic device (Diagnostica-Stago, Asnieres, France) by in vitro activation (37°C) of plasma with bovine thrombin (Fibrinomat, BioMérieux, Marcy-l'Estoule, France). The effects of heparin on the activated partial thromboplastin times were evaluated by the anti-Xa activity, which was kept less than 0.5 IU/ml.

Measurement of natural αGal antibodies. The enzyme-linked immunosorbent assay for isotype-specific detection of human natural xenoreactive antibodies to the αGal disaccharide epitope was performed as previously described by one of us.²⁰ Microtiter plates (NUNC maxisorp, NUNC AB, Roskilde, Denmark) were coated overnight at 4°C with synthetic $\text{Gal}\alpha 1-3\text{Gal}$ disaccharide coupled to polyacrylamide (PAA-Bdi, Syntesome, Munich, Germany) in 0.1 mol/L carbonate buffer, pH 9.6. The plates were then washed with phosphate-buffered saline solution, pH 7.4, containing 0.02% Tween 20 (PBS-Tween). The sera were diluted 1:40 in phosphate-buffered saline solution, pH 7.4, containing 1% bovine serum albumin and 5% Tween (PBS-BSA-Tween) and incubated (100 μl /well) for 2 hours at 37°C . After being washed, bound anti- αGal antibodies were detected by means of monoclonal antibodies to human IgG (clone HB43), IgM

(clone HB57), or IgA (clone 4E8). These secondary monoclonal antibodies are mouse IgG1 and were in turn revealed by biotinylated goat antimouse IgG1 (SBA, Birmingham, Ala.), streptavidin-alkaline phosphatase conjugate (Amersham International, Amersham, United Kingdom), and 4-nitrophenyl-phosphate substrate. The optical density at 405 nm was measured with the use of a microplate reader.

Histology. In each case, the whole left lung was macroscopically assessed regarding its edematous and hemorrhagic appearance. At least four samples of representative areas including the hilum with large vessels, pleura, interlobular septa, and all abnormal lesions were taken. The tissues were inflated in 10% formaldehyde and routinely processed into paraffin wax. Sections were cut at 5 μm and stained with hematoxylin and eosin. Sections from paraffin blocks were also used for immunohistochemical studies performed with the LSAB Kit (Dako SA, Trappes Cedex, France). Rabbit antibodies were used: antihuman IgM and IgG (both at 1:1000) and anti-C3 at 1 to 30 (Biogenex-Menarini, Chevilly-Larue, Paris, France).

Fresh tissue was also snap-frozen in liquid nitrogen and stored at -70°C . Immunostaining was carried out on cryostat sections by direct fluorescence. Fluorescein isothiocyanate-conjugated rabbit antibodies (Dako) were used diluted 1 to 10: antihuman IgM, C1q, C3, and antifibrinogen or anti-IgG were diluted 1 to 20.

Wet/dry weight ratio. Wet lung weight was estimated by measuring the weight of the lungs at the beginning and at the end of the experiment. Lungs excised at the end of the experiments were dried in an oven at 60°C for 30 days and weighed again to allow determination of wet/dry weight ratio.

Statistical analysis. All quantitative sera or plasma data measured at baseline and after the ex vivo reperfusion period were normalized for hemodilution by correcting them for changes in hematocrit value. Data are expressed as mean $\pm$ standard deviation of the number of observations and analyzed by one-way analysis of variance (repeated measures) with Fisher's protected least significance difference. Data were analyzed by means of a software package (STATVIEW 4.02, Abacus Concepts, Inc., Berkeley, Calif.). The a priori level of significance was set at $p < 0.05$.

Results

Pulmonary function. In the lungs perfused with autologous pig blood, normal pulmonary vascular resistance and AVO_2 persisted for the entire 3-hour study period. Conversely, xenoperfused lungs never displayed normal function. Pulmonary vascular resistance rose profoundly in the first 30 minutes of reperfusion, declined thereafter, and remained at values triple the ones observed in the autoperfused lungs (Fig. 2). Likewise, the AVO_2 was always below its physiologic values, with levels diminishing to zero or near zero by 48.3 ± 26.3 minutes of initiation of reperfusion (Fig. 3). Graft failure occurred in all lungs perfused with human

Fig. 6. A, Immunofluorescence with antihuman fibrinogen showing multiple thrombosis in the capillary lumen of the alveolar septa. A thrombus seen longitudinally is indicated by the arrow. B, Immunofluorescence with antihuman C3 showing diffuse deposition of the C3 protein along the alveolar capillaries. (Original magnifications $\times 200$.)

Fig. 7. Total complement activity (CH_{100}) in human plasma at baseline (time 0) and at specific time intervals after xenogeneic perfusion of pig lungs with human blood. Data are expressed as mean $\pm$ standard deviation (error bars) of the experiments and normalized for the observed hematocrit value. Values below the dotted line are pathologic ($< 70 CH_{100}$ units/ml). The CH_{100} activity dropped from 274.4 ± 43.2 to 47.7 ± 6.4 units/ml over the study period ($p < 0.0001$). First 30 minutes as in Fig. 1.

blood after 60 minutes ($n = 2$), 120 minutes ($n = 1$), and 150 minutes ($n = 3$) (mean survival time, 115 ± 44.2 minutes) of reperfusion and was associated with no gas exchange, severe pulmonary hypertension, and gross evidence of pulmonary hemorrhagic edema.

Fig. 8. Hemolysis at baseline (time 0) and at specific time intervals after perfusion of pig lungs with pig or human blood. Data are expressed as mean $\pm$ standard deviation (error bars) of the experiments and normalized for the observed hematocrit value. The dotted line represents values normally observed during human cardiopulmonary bypass procedures at our institution (< 150 mg/100 ml). The difference was statistically significant ($p < 0.0001$). First 30 minutes as in Fig. 1.

Histopathology and immunopathology. All autoperfused lungs were macroscopically and histologically intact. By contrast, xenoperfused lungs appeared macroscopically congestive, hemorrhagic, and mottled with red and yellows areas. Microscopically, the lungs perfused with human blood showed

Fig. 9. Hematocrit value at baseline (time 0) and at specific time intervals after perfusion of pig lungs with autogeneic or untreated human blood. Data are expressed as mean $\pm$ standard deviation (error bars) of the experiments. The difference was statistically significant ($p < 0.0001$). First 30 minutes as in Fig. 1.

severe congestion, alveolar edema and hemorrhage, and several fibrin and platelet thrombi localized in and obstructing small pulmonary vessels (arterioles, capillaries, and venules) but not large pulmonary vessels (segmental or lobar), a finding consistent with HAR (Fig. 4). No inflammatory cells were attached to the endothelial surfaces or infiltrating the lung parenchyma. The wet/dry weight ratio was 4.1 ± 0.3 in the lungs perfused with human blood and 2.55 ± 0.2 in those perfused with autologous pig blood (Fig. 5).

By immunohistologic examination, the lungs perfused with human blood showed diffuse staining of C1q, C3, fibrinogen, and human IgM within the alveolar capillary (Fig. 6); we were unable to find positive IgG and IgA stainings. The autoperfused lungs were always negative for pig IgG, IgM, C1q, and C3.

Immunologic functions. Xenogeneic blood showed a 69% reduction in total complement activity, as measured by CH_{100} , after 20 minutes of reperfusion (Fig. 7). Hemolysis increased gradually over the study period and was significantly higher ($p < 0.0001$) in the xenogeneic than autogeneic blood (Fig. 8). Similarly, the hematocrit level remained

Table I. Changes of coagulation factors in the human blood used for ex vivo perfusion of pig lungs

Lung perfusion time (min)	PT (sec)	APTT (sec)	Fibrinogen (gm/L)
Baseline	19.2 ± 6.1	35.5 ± 7.8	8.8 ± 1.6
10	13.8 ± 4.3	56.3 ± 36.3	6.3 ± 1.2
20	13.1 ± 3.7	65.3 ± 36.3	6.8 ± 1.5
30	12.3 ± 2.8	68.8 ± 33.5	6.5 ± 1.7
60	12 ± 2.8	61.8 ± 48.2	5.5 ± 2
90	11 ± 0.9	69.6 ± 26.1	5.7 ± 1.4
120	10.3 ± 0.9	79 ± 28.6	5.6 ± 0.4
150	11 ± 1.3	88.8 ± 26.8	4.7 ± 0.5

PT, Prothrombin time; APTT, activated partial thromboplastin time. Fibrinogen levels were normalized for the hematocrit value. Data are expressed as mean $\pm$ standard deviation. PT and fibrinogen levels were significantly reduced over the study period ($p = 0.03$ and $p = 0.04$, respectively); the prolongation of APTT did not reach statistical significance ($p = 0.08$).

stable in the autogeneic blood whereas it increased gradually, reaching values 1.5 times that of baseline levels, at the end of the xenogeneic experiments (Fig. 9). Xenogeneic blood showed a significant activation of the coagulation system, as demonstrated by reduction in prothrombin time ($p = 0.03$) and plasmatic fibrinogen ($p = 0.04$) and the twofold prolongation of the activated partial thromboplastin time ($p = 0.08$) (Table I).

Human blood units had a substantial concentration variability of the baseline levels of anti- α Gal IgM (614.7 ± 367.1), IgG (764.9 ± 433.8), and IgA (2837.3 ± 3190.5) xenoreactive natural antibodies; no anti- α Gal antibodies were detectable in the pig blood. On reperfusion, a significant early (10 minutes) drop was noted in human anti- α Gal IgM (85%) and anti- α Gal IgG (63%), and this diminution became only slightly more pronounced over time; the decrease in anti- α Gal IgA was not significant over time (Fig. 10). Despite this significant reduction of natural anti- α Gal xenoantibodies, total human immunoglobulins remained relatively unchanged throughout the reperfusion time (Table II).

Discussion

We have developed an ex vivo system that allows the study of isolated, working, human blood-perfused pig lung. Owing to the closed nature of this model by virtue of the continuously ventilated and perfused lungs, we were able to accurately depict and reproduce the HAR in a species combination with potential clinical relevance. The results demonstrate that on reperfusion of pig lungs with human blood, lung HAR and failure occur within 60 to 150 minutes. This time span is longer than that observed

Table II. Changes of total immunoglobulin concentration in the human blood used for ex vivo perfusion of pig lungs

Lung perfusion time (min)	IgG (gm/L)	IgM (gm/L)	IgA (gm/L)
Baseline	29.1 ± 5.8	2.8 ± 1	6.2 ± 2.9
10	22.5 ± 6.5	2 ± 0.5	4.2 ± 1.9
20	22.3 ± 6.8	2 ± 0.5	4.4 ± 1.8
30	21.5 ± 8.9	2.1 ± 0.6	4.6 ± 2.1
60	18.9 ± 10.6	2 ± 0.6	4 ± 2.4
90	21.3 ± 11.1	1.9 ± 0.8	4.6 ± 2.4
120	18.1 ± 9.9	2.1 ± 0.8	4.1 ± 2.1
150	20.1 ± 10.2	2.1 ± 1.1	4.2 ± 2.4

Data are expressed as mean ± standard deviation. Immunoglobulin (Ig) levels were normalized for hematocrit value. The changes in the total IgG, IgM, and IgA levels were not significant ($p > 0.2$) over the study period.

when pig hearts are perfused with human blood, for example, 25.2 ± 5.6 minutes,^{11, 21} which could suggest that the heart may be less xenotolerant than the lung. However, pig lungs never showed normal oxygen extraction function after reperfusion with human blood, and early and long-lasting severe pulmonary hypertension developed. These abnormalities were always observed within 30 minutes of xenogeneic reperfusion, probably as a consequence of the obstruction of the small pulmonary vessels (arterioles, capillaries, and venules) by fibrin and platelet thrombi. If this hypothesis is correct, our results provide evidence that the lung is as susceptible to HAR as the heart and kidney. These results challenge recent suggestions made in a pig-to-baboon xenotransplantation model by Kaplon and associates,¹⁷ in which lung xenotransplants were found to be less susceptible to HAR. This discrepancy probably reflects the different species combination used or the criteria used to define HAR.

During the first 30 minutes after lung reperfusion with human blood, the most violent reactions in the human blood included a significant reduction of the human anti- α Gal antibodies and complement activity. It is well established that the two primary immunologic factors determining susceptibility of xenogeneic pig organs to HAR by human beings are (1) the recognition of endothelial cell antigens expressed in the pig by xenoreactive natural antibodies in the circulation of the human recipient and (2) the incompatibility of complement regulatory proteins of the donor with the recipient's complement system.⁸ About 1% of the total normal human immunoglobulins are anti- α Gal natural antibodies directed against Gal α 1,3Gal β 1,4GlcNAc (α Gal epitope).²² The reason is that human beings,

Fig. 10. Plasma IgM, IgG, and IgA anti- α Gal antibodies at baseline (time 0) and at specific time intervals after perfusion of pig lungs with untreated human blood. Data are expressed as mean ± standard deviation (error bars) of the experiments and normalized for the observed hematocrit. IgM ($p = 0.003$) and IgG ($p = 0.03$) anti- α Gal natural antibodies decreased significantly over the study period, whereas IgA did not reach the threshold of significance ($p = 0.08$). However, the three human antibodies had a similar kinetic pattern over time, because their decrease was particularly important in the first 30 minutes ($p < 0.0001$) and remained stable thereafter. First 30 minutes as in Fig. 1. OD, Optical density.

like apes and Old World monkeys, lack the α 1,3-galactosyltransferase^{22, 23} necessary for the synthesis of the α Gal epitope, which is, conversely, densely distributed along the vascular tree of pig heart, lung, liver, kidney, and pancreas.^{24, 25} Interestingly, we observed a significant early reduction of the natural anti- α Gal IgM and IgG but not IgA antibodies, and this is in line with previous observations demonstrating that the anti- α Gal IgM and, to a lesser extent, IgG antibodies bind the α Gal epitope on the endothelial cells^{5, 26, 27} and initiate the HAR of pig-to-human xenotransplants. This is indirectly confirmed in this study, in which the anti- α Gal IgM and IgG human blood concentration dropped significantly in the first 30 minutes after reperfusion whereas the amount of total circulating immunoglobulins remained substantially unchanged. Moreover, human IgM deposits were found on the pig alveolar capillaries, where the α Gal epitope is strongly expressed²⁴; the lack of IgG and IgA

staining does not mean that they are not on the capillaries but rather that the immunofluorescence technique was unable to detect them because the severe alveolar-capillary membrane hemorrhage weakens the contrast between the staining and the background.

Although differing from results reported by Kaplon and colleagues¹⁷ of a pig-to-baboon orthotopic lung transplantation model, in which no antibody-mediated HAR was observed, our results suggest that natural anti- α Gal IgM and IgG antibodies play a key role in inducing xenotransplant injury in the pig lung-to-human combination. Interestingly, the baseline levels of human xenoreactive antibodies varied substantially among individuals, and this is in line with the findings of Platt and coworkers.²⁸ Probably, the different baseline blood concentrations of IgM and IgG anti- α Gal natural antibodies²⁸ or heterogeneous expression of pig antigens on donor organs²⁹ may account for the substantial diversity in survival time we observed.

Development of HAR depends on activation of the complement system, as demonstrated by its prevention when xenograft recipients are treated with complement-inhibitor agents like cobra venom factor, complement receptor type I, or gamma globulin.^{8, 21, 30} In this study, human blood circulating into pig lungs showed an almost 70% reduction in the CH₁₀₀ and a remarkable hemolysis within 30 minutes. These two features were, in principle, not related to the ex vivo circuit by itself, because hemolysis did not occur in pig lungs perfused with pig blood. The reduction of CH₁₀₀ is most probably due to complement consumption because accumulation of C3 complement proteins was observed along the alveolar capillary walls. Thus it is not utopistic to postulate that after binding of xenoreactive human IgM and IgG antibodies to the pig lungs harboring the α Gal epitope, the complement pathway becomes activated, as observed in most pig-to-primate xenotransplants.^{5, 31} This in turn triggers HAR by an increase of vascular permeability and activation of coagulation and, thus, alteration of the endothelial cell function. In favor of these mechanisms is the constant hematocrit increase and onset of edema in xenogeneic but not autogeneic perfused lungs and the significant consumption of the clotting factors over time. Leukocyte infiltrations were absent, suggesting that cellular reactions are not at the origin of lung HAR; this is further confirmed by the fact that a similar time-related and violent HAR still develops in pig lungs perfused with

human blood depleted of its leukocytes (data not shown).

In conclusion, the results generated from our ex vivo pig-to-human xenotransplantation model demonstrate that pig lungs perfused with human blood have an early and violent HAR that results in the loss of gas exchange, severe pulmonary hypertension, and, ultimately, xenograft failure. The fact that this model permits the analysis of several functional parameters is particularly attractive, because it provides a useful tool to investigate how to prevent lung HAR.

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