

Chimeric Pig Hearts Resist Hyperacute Rejection in ex vivo Perfusion Model

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Abstract: With surrogate tolerogenesis, the recipient immune system is engrafted within the donor pig before organ transplant. Chimeric pig hearts may resist hyperacute rejection by inducing accommodation. This hypothesis was tested using an ex vivo isolated piglet heart perfusion model. Processed sheep marrow was infused into fetal pigs at 45 days gestation. Heart explants from chimeric or nonchimeric pigs were suspended in a Langendorff apparatus and perfused with plasma from unsensitized sheep or sensitized sheep. Nonchimeric hearts perfused with plasma from unsensitized functioned for 240 min ($N = 3$). Non-

chimeric hearts perfused with sensitized plasma deteriorated rapidly, functioning at 19 ± 12 min ($N = 6$); Immunohistochemistry of heart graft revealed extensive deposition of IgG, IgM in the microvascular. In contrast, chimeric hearts perfused with sensitized plasma functioned for 183 ± 46 min ($N = 3$) ($p < .001$); Deposition of IgG, IgM had substantially less. Heart grafts procured from chimeric pigs survived in the presence of antidonor IgG, IgM, and complement, demonstrating that chimeric pig hearts resist hyperacute rejection. **Keywords:** hyperacute rejection, surrogate tolerogenesis. *JECT. 2001;33:181-184*

The use of xenografts could provide a solution to the critical shortage of organs available for use in clinical heart transplantation. Pigs are easily bred, physiologically similar to humans, and ethically acceptable, because they are also used for human consumption. However, with current technology, xenografts transplantation have generally failed because of hyperacute rejection (HAR) and vascular rejection (1). In discordant transplant combination, preformed natural antibodies initiate HAR. The ensuing activation of the complement cascade via the classical pathway leads to intravascular coagulation, thrombosis, interstitial edema, and hemorrhage (2). As with HAR, the xenograft is destroyed by intravascular coagulation, thrombosis, interstitial hemorrhage, and edema. If HAR and acute vascular rejection can be prevented for 2 to 4 weeks in the recipient, xenograft develops resistance to cytotoxic antidonor antibodies and antibody-mediated rejection (3). Platt and Bach defined accommodation as a state in which vascularized organ grafts would survive despite the presence of cytotoxic antibodies and circulating complement (4,5). A major goal for xenotransplantation is to achieve long-term survival of xenografts by inducing accommodation of the xenograft.

To prevent xenograft rejection, we have induced chimerism immune tolerance in the donor pig before transplant rather than after transplant (6). Using surrogate tolerogenesis (ST), the recipient's lymphocytes are infused into preimmune fetal pigs. Later, the chimeric lymphocytes and organ graft are transplanted into the recipient. Previous studies of ST have demonstrated a specific effect on the cellular immune response to pig antigens (7,8). In following study, ST also provided protection against HAR and vascular rejection in sheep sensitized to pig cells. This protection was unexpected, because suppressor cells should not have any effect on preformed antibodies. There is the possible protection against HAR and acute vascular rejection to identify in pig-to-sheep transplants.

Therefore, the aim of this study is to investigate if chimerism in the donor animal induces accommodation in addition to immune tolerance before heart transplantation. This hypothesis was tested using an ex vivo isolated pig heart perfusion model based on the Langendorff system.

MATERIALS AND METHODS

The animals in this study were housed at the University of Nebraska Medical Center and its satellite facilities. These facilities are all American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

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Fetal pigs were injected at 45 days gestation. Sheep bone marrow was prepared on the day before injection. Using ultrasound guidance, the fetal pigs were infused with 1×10^7 nucleated cells, partially depleted of CD4⁺ and CD8⁺ cells. At term, the pigs were delivered by Caesarean section. Peripheral blood mononuclear cells were analyzed for chimerism by flow cytometry. Chimerism was confirmed by polymerase chain reaction (PCR) on the blood or spleen cells. The chimeric pigs used in this study had 5–18% chimerism in the peripheral blood as measured by flow cytometry.

Unsensitized blood was obtained via the jugular vein from sheep without detectable cytotoxic antibodies to pig lymphocytes. The blood was placed into tubes containing heparin (14 units/mL final concentration, Gsi ELKINS-SINN, Inc., NJ) and centrifuged at 720 *g* for 30 min. The plasma was collected and stored at -20°C until used (within 24 hours). Immediately before use, the plasma was thawed and filtered through a 0.8-micron filter.

A sheep sensitized with 2×10^7 pig peripheral blood lymphocytes on three to five occasions at 2–4 week-intervals. It received a boost at 2–4 weeks before each study. The sensitized sheep plasma had high levels of lymphocytotoxic antibodies (titers of 32–128) as determined by lymphocytotoxic assay. The plasma was collected and frozen as described for unsensitized plasma. Immediately before use, sensitized plasma was also thawed and filtered.

Heart grafts were isolated from 2–3-week-old chimeric and nonchimeric pigs. Animals were anesthetized (ketamine 20mg/kg, xylazine 2mg/kg, and inhalation of isoflurane). Pigs were then subjected to thoracotomy. The pericardium was incised and heparinized (sodium heparin, 300 units/kg) by means of right atrium injection. The heart was rapidly extracted and placed in 4°C modified Krebs–Henseleit (K–H) buffer. The time from interruption of normal blood flow until the establishment of retrograde perfusion was less than 90 sec. Isolated hearts were attached via the aorta to a Langendorff apparatus, and retrograde perfusion was performed with K–H buffer solution at a constant perfusion pressure of 70 mmHg. The perfusion solution was enriched continuously with a mixture of 95% O₂ and 5% CO₂ to achieve an oxygen partial pressure over 500 mmHg. The temperature of the perfusate was monitored to maintain the heart between $36\text{--}37^{\circ}\text{C}$. The PH was adjusted to 7.35–7.45. The perfusate was filtered through a prefilter before addition to the Langendorff apparatus. Various physiological parameters were measured to assess the function of the heart preparation. Change in heart rate (beats per min) was monitored using a transducer attached to the apex of the heart.

There were three study groups: Group I ($N = 3$) consisted of nonchimeric pig hearts perfused with unsensitized sheep plasma; Group II ($N = 6$) consisted of nonchimeric pigs hearts perfused with sensitized sheep

plasma; Group III ($N = 3$) consisted of chimeric pig hearts perfused with sensitized sheep plasma. The pig hearts were allowed a 30-min stabilization period of normothermic perfusion using the oxygenated K–H buffer solution. Baseline hemodynamic data were taken. After equilibration, the volume of the perfusion medium in system was reduced to 200 mL and recirculated. The plasma from unsensitized sheep or sensitized sheep was added to the recirculating buffer reservoir to achieve a final concentration of 20% (v/v). Hearts were perfused until functional damage caused their failure with the cessation of the heartbeat (<30 beats/min) or until an arbitrary endpoint of 240 min was reached.

Functional Analysis

Following the perfusion of plasma, heart beating time, heart rate, and coronary flow were recorded at predefined timepoints.

Immunohistological Analysis

Myocardial biopsies were taken immediately at the time of cessation of function or an arbitrary endpoint of 240 min. All specimens were embedded in frozen tissue media and snap frozen for immunofluorescence labeling studies.

Statistical Analysis

All values are expressed as the mean values (\pm SEM) of *n* independent observations. Student's *t*-test was used to determine significant differences within each group. Statistical significance was met at the $p < .05$ level.

RESULTS

Nonchimeric Heart Survival versus Chimeric Heart Survival

Each of the nonchimeric pig hearts perfused with plasma from unsensitized sheep (Group I) survived for 240 min when the study was terminated. Nonchimeric pig hearts perfused with plasma from sensitized sheep (Group II) ceased contraction within 40 min (mean time 19 ± 12 min). Chimeric pig hearts perfused with plasma from sensitized sheep (Group III) functioned for 183 ± 46 min. The survival of Group II was significantly prolonged, as compared with Group III ($p < .0001$, Figure 1).

Nonchimeric Heart Function versus Chimeric Heart Function (Table 1)

The heart rate and coronary flow rates were significantly different between chimeric hearts (Group III) and nonchimeric hearts perfused with sensitized plasma (Group II) ($p < .01$). There was no significant difference between Group I and Group II within 60 min. However, there was some deterioration after perfusion 60 min, as compared with the control hearts perfused with unsensitized plasma.

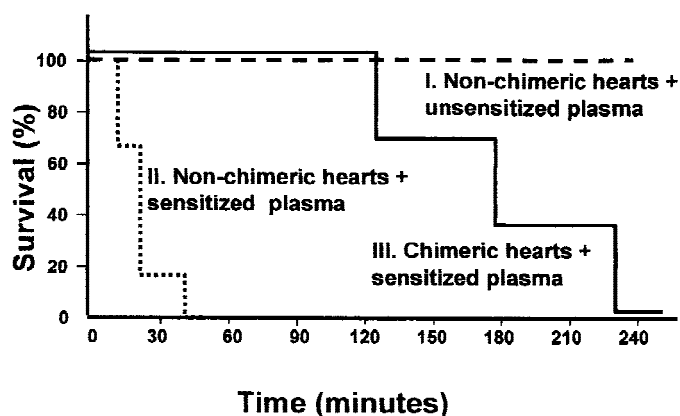


Figure 1. Survival of pig heart grafts. The survival of nonchimeric hearts perfused with sensitized plasma (Group II) was significantly prolonged, as compared with chimeric hearts (Group III) ($p < .0001$).

Immunohistologic Characteristics of Nonchimeric versus Chimeric Perfused Heart

The pattern and intensity of IgG and IgM deposition of three experimental groups were shown in Figure 2. The hearts from Group I revealed minimal deposition of IgG and IgM. The grafts from Group II showed extensive deposition of IgG and IgM in the endothelium of the capillaries and vessels and on the myocardial sarcolemma. In contrast, chimeric hearts had substantially less deposition of IgG and IgM on the microvascular. The distribution and intensity of deposition was significantly different.

DISCUSSION

In this study, the sheep was sensitized with pig lymphocytes several times and received a boost before the experiments. The high levels of anti-pig antibodies in the sensitized sheep plasma were determined by lymphocytotoxicity assay and flow cytometry (FCM) (titers of 32–128). When nonchimeric pig hearts perfused with plasma from sensitized sheep plasma the function of hearts deteriorated rapidly with the cessation of contraction between 8–40 min. Immunohistologic analysis showed diffuse deposition of IgG and IgM in the microvascular and vessels; whereas, nonchimeric pig hearts perfused with plasma from unsensitized sheep plasma survived for 240 min and revealed minimal deposition of IgG and IgM when the

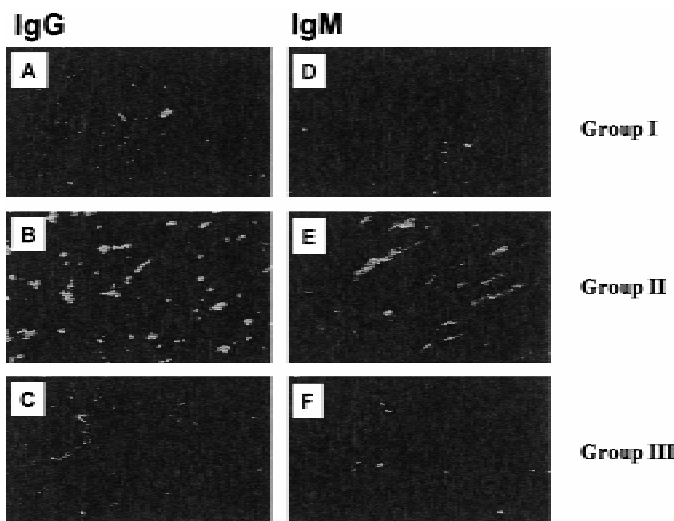


Figure 2. The pattern and intensity of IgG and IgM deposition. Deposition of sheep immunoreactions in perfused pig hearts. (A) IgG was negative at nonchimeric hearts perfused unsensitized plasma, (B) positive at perfused sensitized plasma, but (C) negative at chimeric hearts perfused sensitized plasma for capillaries and other vessels. (D) IgM was negative at nonchimeric hearts perfused unsensitized plasma, (E) positive at perfused sensitized plasma, but (F) negative at chimeric hearts perfused sensitized plasma for most vessels.

study was terminated. The study indicated HAR is associated with deposition of IgG and IgM antidonor antibodies in the vascular endothelium and early complement components (9). The pig-to-primate xenotransplantation model, IgM binding to graft endothelium, was determined by immunofluorescence assay (10). The activation of complement following deposition on the graft endothelium of xenoreactive antibodies was not shown in this study, because reagents for sheep complements were unavailable. Nonchimeric pig hearts perfused with plasma from sensitized sheep plasma simulates hyperacute rejection of pig xenografts in discordant transplants. The heart functional deterioration is similar to that seen when pig heart was used ex vivo with human blood containing nature antibodies (11).

In contrast, the hearts from chimeric pigs perfused with plasma from highly sensitized sheep functioned approximately ten times as long, 137–229 min. Chimeric pig hearts survived in the presence of antidonor antibodies and ac-

Table 1. Heart rate and coronary flow parameters.

Group	Heart Rate (beats/min)			Coronary Flow (mL/min)		
	Baseline	30'	60'	Baseline	30'	60'
I	112 ± 10	126 ± 26	118 ± 15	114.6 ± 6.9	110.7 ± 8.31	13.3 ± 6.1
II	109 ± 12	21 ± 19*	0*	97.2 ± 19.6	12.0 ± 3.1*	0*
III	111 ± 14	105 ± 2*	98 ± 2*	116.0 ± 6.9	90.0 ± 18.0*	84.0 ± 6.0*

The heart rate and coronary flow rates was significantly different between chimeric hearts (Group III) and nonchimeric hearts perfused with sensitized plasma (Group II) ($*p < .01$).

tivated complement. Immunohistopathology demonstrated that vascular deposition of IgG and IgM in the chimeric pig hearts was substantially less. Survival of the chimeric hearts may be attributable, in part, to their resistance to cytotoxic anti-pig antibodies and antibody-mediated rejection by the down-regulation of antigen expression. In the present study, we have established the resistance of chimeric hearts to sheep anti-pig IgG and IgM antibodies and complement that cause HAR of non-chimeric pig hearts. This resistance may also depend on the expression in hearts endothelial cells (EC) and smooth muscle cells of a number of protective genes (5,12), or other changes protecting the graft we have been studying (9). Because the assay used only the hearts from the chimeric pigs, protection against antibodies-mediated rejection was attributable to factors within the graft rather than within circulation. This experiment of Miyatake et al. (5) established that the basis of accommodation was in the organ, not the circulating factors or cells. This observation supports the hypothesis that, in addition to tolerance, vascular organs from chimeric pigs are accommodated when they are removed from the donor.

The mechanism of accommodation is under investigation. It not only seems to be the down-regulation of antigen. The induction of select protective genes contributes to the graft resistance; including heme oxygenase-1, bcl-2, and A-20 (5). These products could inhibit apoptosis of EC. Other changes protecting the graft include decreased adhesion molecules and increased nitric oxide synthetase (12).

In summary, chimerism in the donor animal inducing accommodation in addition to immune tolerance before heart transplantation was identify using an ex vivo isolated pig heart perfusion model. However, the mechanism of chimerism in donor-inducing accommodation must be investigated further.

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REFERENCES

1. Saadi S, Platt JL. Immunology of xenotransplantation. *Life Sci.* 1998; 62:365-87.
2. Platt JL, Fischel RJ, Matas AJ, et al. Immunopathology of hyperacute xenograft rejection in swine-to-primate model. *Transplantation.* 1991;52:214-20.
3. Platt JL, Vercellotti GM, Dalmaso AP, et al. Transplantation of discordant xenografts: A review of progress. *Immuno Today.* 1990; 11:450-6.
4. Cooper DK, Ye Y, Niekrasz M, et al. Specific intravenous carbohydrate therapy. A new concept in inhibiting antibody-mediated rejection—Experience with ABO-incompatible cardiac allografting in the baboon. *Transplantation.* 1993;56:796-77.
5. Miyatake T, Koyamada N, Hancock WW, et al. Survival of accommodated cardiac xenografts upon retransplantation into cyclosporine-treated recipients. *Transplantation.* 1998;65:1563-9.
6. Beschorner WE, Lomis TJ. Xenotransplantation: Potential opportunity versus resistant challenge. In: *Kidney Transplant Rejection: Diagnosis and Treatment*, 3rd ed Racusen LC, Solez K, Burdick JF, eds. New York: Marcel Dekker, 1998:201-38.
7. Beschorner WE, Qian Z, Mattei P, et al. Induction of human chimerism and functional suppressor cells in fetal pigs: Feasibility of surrogate tolerogenesis for xenotransplantation. *Transplant Proc.* 1996;28:648-9.
8. Sudan DL, Radio SJ, Matamoros A, Jr., et al. Effect of surrogate tolerogenesis on the vascular rejection of pig heart xenografts. *Transplantation.* 2000;69:232-5.
9. Forty J, White DJG, Wallwork J, et al. A technique for perfusion of an isolated working heart to investigate hyperacute discordant xenograft rejection. *J Thorac Cardiovasc Surg.* 1993;106:308-16.
10. Soares MP, Lin Y, Sato K, et al. Accommodation. *Immunol Today.* 1999;20: 434-7.
11. Parker W, Holzknicht ZE, Platt JL, et al. Fate of antigen in xenotransplantation: Implication for acute vascular rejection and accommodation. *Am J Pathol.* 1998; 152:829-39.
12. Dorling A, Delikouras A, Nohadani M, et al. In vitro accommodation of porcine endothelial cells by low-dose human anti-pig antibody: Reduces binding of human lymphocytes by accommodated cells associated with increased nitric oxide production. *Xenotransplantation.* 1998;5:84-92.