Correlation of biochemical and hematological changes with graft failure following pig heart and kidney transplantation in baboons

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Abstract
We have explored biochemical and hematologic parameters that might indicate acute humoral xenograft rejection (AHXR) following pig organ transplantation in baboons. Baboons (n = 15) received an immunosuppressive regimen, and underwent a miniature swine or hDAF kidney (Group 1, n = 6) or heart (Group 2, n = 7) transplantation. Control baboons (Group 3, n = 2) received the immunosuppressive regimen without organ transplantation. Blood chemistry and hematologic parameters were measured daily. Baboon and porcine cytomegalovirus were monitored. In Groups 1 and 2, organ grafts survived for up to 29 days. A plasma fibrinogen of 600 U/L and aspartate transaminase of >300 U/L, were associated with the development of AHXR in both heart and kidney grafts. In Group 1, a decrease in platelet count of >150,000/microL within 3 days, or a count of 500 U/L was associated with graft failure. In Group 3, no abnormalities were observed. The possibility that porcine CMV may play a role in [...]
Correlation of Biochemical and Hematological Changes with Graft Failure Following Pig Heart and Kidney Transplantation in Baboons


We have explored biochemical and hematologic parameters that might indicate acute humoral xenograft rejection (AHXR) following pig organ transplantation in baboons.

Baboons (n = 15) received an immunosuppressive regimen, and underwent a miniature swine or hDAF kidney (Group 1, n = 6) or heart (Group 2, n = 7) transplantation. Control baboons (Group 3, n = 2) received the immunosuppressive regimen without organ transplantation. Blood chemistry and hematologic parameters were measured daily. Baboon and porcine cytomegalovirus were monitored.

In Groups 1 and 2, organ grafts survived for up to 29 days. A plasma fibrinogen of <80 mg/dL on 2 consecutive days, and a serum lactate dehydrogenase of >600 U/L and aspartate transaminase of >300 U/L, were associated with the development of AHXR in both heart and kidney grafts. In Group 1, a decrease in platelet count of >150,000/μL within 3 days, or a count of <50,000/μL, were associated with AHXR. In Group 2, a creatine phosphokinase of >500 U/L was associated with graft failure. In Group 3, no abnormalities were observed. The possibility that porcine CMV may play a role in graft injury could not be excluded.

Noninvasive parameters were identified that have predictive potential for AHXR. Monitoring of these might enable therapeutic intervention to reverse rejection.

Key words: Acute humoral xenograft rejection, baboon, consumptive coagulopathy, endothelial cell activation, heart transplantation, kidney transplantation, pig

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Introduction

Acute humoral xenograft rejection (AHXR) is currently the major immunological barrier to successful discordant xenogeneic transplantation (Tx) in pig-to-nonhuman primate models. Although hyperacute rejection can be overcome either by the use of organs from pigs transgenic for one or more regulators of complement activation, e.g. human decay accelerating factor (hDAF) (1,2), or by immunoadsorption of anti-Gal1−3Gal (Gal) antibody (Ab) from the potential recipient before organ Tx (2–4), increasing immunoglobulin deposition on the vascular endothelium of the transplanted organ appears to be related to the development of AHXR (5). Although elicited anti-Gal and antinongal Abs play a significant role in the development of AHXR, we have previously reported evidence indicating that when the elicited Ab response has been successfully prevented by therapy with an anti-CD154 monoclonal antibody (mAb), natural anti-Gal Ab alone can lead to this complication (6).

Our previous studies indicated that pig kidney Tx in baboons may be associated with a fall in the level of plasma fibrinogen (6). We hypothesize that this is a consequence of endothelial cell activation. Consumptive coagulopathy (CC) may develop in these cases (6). We found no definitive correlation between the extent of histopathological features of AHXR and the development of CC, as CC could occur in the presence of mild or moderate histopathologic changes. The development of CC was also associated with a decrease in platelet count, an abrupt terminal increase in prothrombin time, and in activation of porcine cytomegalovirus (PCMv) in some animals (7). However, AHXR could occur in the absence of the development of CC and, in such cases it could only be monitored by biopsy and histological examination of the transplanted kidney. As one native kidney was left in situ in our studies, changes in serum creatinine or blood urea nitrogen could not be followed as indicators of graft dysfunction. Changes in these
two parameters are associated with graft dysfunction and rejection (8).

When heterotopic heart Tx is performed in the pig-to-human primate model, no simple serum marker has been reported as an indicator for AHXR, and so we have monitored the blood for various biochemical and hematological parameters in an effort to assess their association with graft dysfunction. We here report our retrospective observations in animals that underwent an identical immunosuppressive regimen, and show that changes in certain parameters, when taken together, are indicative for kidney or heart graft failure well in advance of significant histopathological changes. The exact cause of the systemic changes remains uncertain. We also asked whether these changes could be associated with the immunosuppressive therapy being administered rather than with the presence of the transplanted pig organ. In baboons receiving the same immunosuppressive regimen, but not receiving a pig organ, none of these biochemical or hematological changes was seen. We have attempted to quantify these changes and believe that they may allow the opportunity for modification of therapy before graft failure develops.

Materials and Methods

Animals
Baboons (Papio anubis, n = 15) of known ABO blood group and of body weight 8–17 kg (Biological Resources, Houston, TX, or Mannheim Foundation, Homestead, FL) were used as recipients. A Massachusetts General Hospital (MGH) MHC-inbred miniature swine (n = 6) of blood group O, 1.5–4 months old, 6.5–40 kg body weight (Charles River Laboratories, Wilmington, MA) or Large White/Landrace crossbreed pigs transgenic for hDAF (n = 7) (Novartis Pharma/Harlan, Madison, WI) of blood group O, 1.5–3.5 months old, weighing 9–35 kg, served as donors of kidneys or hearts. All experiments were conducted in accordance with the NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical procedures
Anesthesia, intravascular line placements in pigs and baboon, kidney or heart excision in pigs, splenectomy, and kidney or heart Tx in baboons have been described in detail previously (4,9). In the baboons that underwent kidney Tx, one kidney was left in situ to allow the potential for survival and follow up in the event of the need for donor organ excision for rejection. At the time of graft excision, cuffs of a pig’s renal artery and vein remained in the wall of the baboon’s aorta and inferior vena cava, respectively, and a remnant of the pig’s ureter remained in the bladder wall. Heterotopic heart Tx was performed through a midline abdominal incision. Graft function was monitored by daily palpation, and recorded by using a four-grade classification system with grade 3, representing excellent graft function, and grade 0, representing cessation of contractions. After excision of a rejected heart graft, remnants of pig aorta and pulmonary artery remained in the baboon.

Extracorporeal immunoadsorption in baboons
Anti-Gal Ab was depleted from the baboon’s circulation by the perfusion of plasma through immunoadsorption columns containing synthetic Galα1–3Galβ1–4GlcX-Y (a Gal type 6 trisaccharide, Alberta Research Council, Edmonton, Alberta, Canada), as described previously (10–12).

Conditioning regimen in baboons
All baboons in Groups 1–3 (see later) underwent splenectomy on day –8, received horse antihuman thymocyte globulin (ATGAM, Upjohn, Kalamazoo, MI) 50 mg/kg/day i.v. on days –3, –2 and –1, underwent extracorporeal immunoadsorption of anti-Gal Ab on days –3, –2 and –1, and received thymic irradiation (700 cGy) on day –1 (4,10). All baboons also received mycophelonolate mofetil by a continuous i.v. infusion (at approximately 110 mg/kg/day) from day –8 to maintain a whole blood level of 3–6 μg/mL (13). Cobra venom factor at approximately 100 units/kg i.v. was given from day –1 until graftectomy to maintain the CH50 at 0% (14). Prostacyclin (PGI2; 20 ng/kg/min by continuous infusion) was given for 14 days and methylprednisolone (2 mg/kg × 2 daily i.v. for 7 days, reducing weekly to 0.25 mg/kg daily at 28 days) were started on day 0. Murine antihuman CD154 mAb (ICB, ATCC, Rockville, MD, and prepared by Immerge BioTherapeutics, Cambridge, MA) (20 mg/kg i.v.) therapy was started on day –1 or 0 (two doses) and administered on alternate days until graft excision or recipient death (6). Anti-CD154 mAb levels were measured and maintained >300 μg/mL. Heparin (10–20 U/kg/h by continuous infusion) was started on day 2 and continued until graftectomy. Cyclophosphamide was administered at a total dose of 60–80 mg/kg i.v. in the peripheropic period between days –6 and 4. After Tx, when the white blood cell count increased to >3000/mm³ or signs of rejection were evident, additional doses of cyclophosphamide (5–20 mg/kg) were administered. Pig kidney Tx (and unilateral native nephrectomy, Group 1) or pig heterotopic heart Tx (Group 2) was performed on day 0. Prophylactic ganciclovir (6 mg/kg/day) was administered to five baboons in Group 2, beginning on day –5 in three animals and on day 4 in two animals. In Group 3, no organ was transplanted. Prophylactic ganciclovir was given in one of these control subjects, starting on day –5.

Monitoring and supportive therapy
Blood chemistry, including troponin T levels, blood cell counts, international normalized ratio and partial thromboplastin time (PTT), fibrinogen, and fibrin degradation products were measured daily by standard methods. Washed irradiated red blood cells from AB-matched baboon donors were administered to maintain the hematocrit >20%. Erythropoietin was administered to some baboons at a dose of 100 units/kg subcutaneously × 1–2 weekly; this did not appear to affect outcome. Thrombocytopenia of <10 000 platelets/mm³ was corrected by the transfusion of fresh-washed irradiated baboon platelets. All baboons received daily prophylactic cefazolin sodium (500 mg/day i.v.) or levofloxacin (100 mg/day i.v.) throughout the period of immunosuppressive therapy. Surveillance blood cultures were drawn twice weekly, and specific antibiotic therapy was initiated when indicated. Quantitative, real-time polymerase chain reaction analyses of blood using primers for cytomegalovirus (CMV) that were baboon- or pig-specific were performed in six baboons × 3 weekly (Group 2, n = 4; Group 3, n = 2), on the organ after graftectomy (n = 9), and on the native baboon tissues at necropsy (n = 9).

Assays for detection of baboon anti-Gal antibody and of antibody directed against porcine nonGal determinants
Details of these methods have been reported previously (10,11). IgM and IgG Ab reactive with Gal type 6 and type 2 were determined by ELISA, and antipig IgM and IgG by flow cytometric analysis.

Histopathology, immunohistopathology, and ultrastructure of tissue biopsies
Tissues were fixed in 10% formalin and paraffin-embedded. Five-micron sections of tissue were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff for light microscopy. Immunohistochemical staining for immunoglobulins (IgM and IgG), complement, and fibrin was performed on frozen sections; details have been reported previously (5). For electron microscopy studies, tissues were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide, and embedded in
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Epon 812. Ultrathin sections were stained with lead citrate and examined with a Phillips 301 electron microscope.

Measurement of porcine and baboon CMV DNA
Total DNA extracted from tissue or peripheral blood mononuclear cells was quantified as described previously (7).

Statistical analyses
Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for biochemical and hematological parameters. Confidence intervals for sensitivity, specificity, and positive and negative predictive values were computed as an exact binomial 95% confidence interval, using SPSS 9.0 for Windows (SPSS Inc., Chicago, IL).

Experimental groups
Group 1 (n = 6) consisted of baboons that underwent either MGH (n = 3) or hDAF kidney (n = 3). Tx. Group 2 (n = 7) underwent heterotopic Tx of either MGH (n = 3) or hDAF (n = 4) hearts. Group 3 (n = 2) were subjected to the same induction and maintenance immunosuppressive therapy as Groups 1 and 2, but did not receive an organ transplant.

Results

Graft survival
Pig kidney (Group 1) and heart (Group 2) grafts survived for up to 29 and 28 days, respectively. Human decay accelerating factor kidneys survived longer than MGH kidneys (median survival 29 vs. 7 days); failure of the MGH kidneys resulted from several different causes (Table 1), whereas failure of the hDAF kidneys uniformly resulted from AHXR. There was little difference in survival of the heart grafts from these two sources (median 27 vs. 24 days) (Table 1). Acute humoral xenograft rejection was the prime reason for termination of the experiment in seven cases (Table 1), in all cases confirmed by histopathology. In six cases, the experiment was terminated through death of the baboon from infection (n = 3) or other complications (Table 1) [pulmonary thrombosis (n = 1), necrosis of the distal ureter (without signs of AHXR in the kidney graft) (n = 1), heart graft failure of uncertain cause (cardiac distension with no definite histopathologic features of AHXR) (n = 1)]. Consumptive coagulopathy developed in four cases (Table 1); it proved fatal in two cases, and in one the baboon was euthanized.

Changes in biochemical and hematological parameters
There was a steady reduction in hematocrit and hemoglobin during the course of all experiments in Groups 1–3, which was considered to be related to frequent blood withdrawal and the effect of immunosuppressive therapy on the bone marrow. Serum bilirubin remained normal in all cases in all groups, suggesting that there was no significant hemolysis. In both Groups 1 and 2, following the initial surgery, there was a temporary increase in lactate dehydrogenase (LDH), aspartate aminotransaminase (AST), and creatine phosphokinase (CPK). Increases in these parameters were more marked in the Group 2 baboons, some of which developed a temporary rise in troponin T as a result of reversible ischemic injury to the transplanted pig heart. Plasma fibrinogen and platelet count fell during the first week postTx in most baboons in all groups, as a result of induction therapy with cyclophosphamide.

Group 1

Biochemical parameters. An increase in LDH to >600 U/L occurred in four baboons, and rose to a mean of 1144 (± 800) before graft failure, which was determined by the development of CC or histological features of AHXR; a sustained increase of >600 U/L was associated with the

Table 1: Causes of graft failure and recipient death in Groups 1 and 2

<table>
<thead>
<tr>
<th>Group (Baboon #)</th>
<th>Donor pig (days)</th>
<th>Graft survival (cause of recipient death)</th>
<th>Cause of graft failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B69-254</td>
<td>MGH</td>
<td>6</td>
<td>Minimal AHXR (infection)</td>
</tr>
<tr>
<td>B129-23</td>
<td>MGH</td>
<td>13</td>
<td>AHXR/CC (bleeding)</td>
</tr>
<tr>
<td>B133-59</td>
<td>MGH</td>
<td>7</td>
<td>Distal ureter necrotic/perforated (euthanized)</td>
</tr>
<tr>
<td>B117-63</td>
<td>hDAF</td>
<td>28</td>
<td>AHXR/CC (bleeding)</td>
</tr>
<tr>
<td>B182-323</td>
<td>hDAF</td>
<td>29</td>
<td>AHXR necrotizing pancreatitis</td>
</tr>
<tr>
<td>B69-169</td>
<td>hDAF</td>
<td>29</td>
<td>AHXR/CC (survived)</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B116-16</td>
<td>MGH</td>
<td>21</td>
<td>No AHXR(infection)</td>
</tr>
<tr>
<td>B69-126</td>
<td>MGH</td>
<td>26</td>
<td>AHXR (euthanized)</td>
</tr>
<tr>
<td>B69-134</td>
<td>MGH</td>
<td>27</td>
<td>AHXR (euthanized)</td>
</tr>
<tr>
<td>B69-210</td>
<td>hDAF</td>
<td>19</td>
<td>No AHXR pulmonary thrombosis</td>
</tr>
<tr>
<td>B69-321</td>
<td>hDAF</td>
<td>28</td>
<td>AHXR/CC (euthanized)</td>
</tr>
<tr>
<td>B69-714</td>
<td>hDAF</td>
<td>15</td>
<td>Graft failure of uncertain cause (euthanized)</td>
</tr>
<tr>
<td>B69-171</td>
<td>hDAF</td>
<td>28</td>
<td>No AHXR (infection)</td>
</tr>
</tbody>
</table>

AHXR = acute humoral xenograft rejection, CC = consumptive coagulopathy.

1Indicates those experiments in which changes were seen in systemic parameters.
development of AHXR on histological examination (Table 2 and Figure 1). The increase to >600 U/L occurred at a mean of 6 (±2) days before final graft failure. Serum creatinine was not predictive, as one native kidney remained in situ (see Methods). Changes in other biochemical parameters, e.g. AST (Figure 2) and CPK, were not associated with graft failure (Table 2).

**Hematological parameters.** Excluding the initial transient reduction (associated with cyclophosphamide therapy), a plasma fibrinogen level of <80 mg/dL on 2 consecutive days, or a decrease >80 mg/dL over 3 days, was associated with AHXR (Table 2 and Figure 3) and occurred 6 (±6) days before the need to excise the graft. The platelet count also fell in five of six baboons initially, but a subsequent reduction of >150 000/µL within a period of 3 days, or a fall to an absolute count of <50 000/µL, was associated with impending AHXR, and occurred 6 (±2) days before graft failure (Table 2 and Figure 4). Partial thromboplastin time and the international normalized ratio remained stable over the entire course, but increased acutely to values of >150 s and 17, respectively, when CC occurred. Fibrinogen degradation products showed a variable pattern, but were high (>160 µg/mL) when CC occurred.

**Group 2**

**Biochemical parameters.** An increase of LDH to >600 U/L occurred in five baboons, beginning 15 days (±8 days) before cessation of contractions, and continued to rise to a maximum mean level of 3471 (±2303) U/L (Table 3 and Figure 1). The maximum LDH was higher in those baboons that received MGH pig hearts than hDAF hearts. In two baboons, an increase in AST (to >300 U/L) was associated with AHXR (4 and 7 days, respectively, before graft failure), and in two it was associated with infection or pulmonary thrombosis (Table 3). The rate of increase of LDH or AST (Figure 2) correlated with the speed of development of graft failure. An increase in CPK to >500 U/L occurred in two cases in association with AHXR, and in one when graft failure occurred for uncertain cause, 10 (±3) days before graft failure (Table 3). Measurement of human CPK MB isoenzymes showed normal parameters in all cases, and appears not to reflect injury in pig hearts, which has been confirmed by other studies in pigs at our center.

Troponin T rose significantly immediately after heart Tx as a result of the ischemic injury experienced during Tx (confirmed by alloTx studies), but returned to baseline level within 3–4 days. It showed no increase during the period when LDH and AST were rising, but only began to rise 2 days before functional graft failure from AHXR. This terminal rise in troponin T correlated with a deterioration in contractility of the transplanted heart as detected by palpation, particularly when AHXR was severe (confirmed by histopathology) when the level rose to >2 mg/dL.

**Hematological parameters.** A steady decrease in fibrinogen to 80–110 mg/dL over the first week occurred in all baboons. A further decrease to <80 mg/dL, sustained for 2 consecutive days [which occurred 11 (±2) days before graft failure], was associated with the development of AHXR (Table 3 and Figure 3). In one baboon, a fall in platelet count of >150 000/µL within a period of 3 days occurred as a result of *Pseudomonas aeruginosa* sepsis, but was not seen when graft rejection occurred. No such decrease occurred in any other baboon (Table 3 and Figure 4). An increase in PTT over time occurred in all animals, but was not associated with the development of AHXR or graft failure (Table 3). Other parameters showed variable levels, but were not associated with the development of AHXR.

In those animals in Groups 1 and 2 that survived after successful excision of the graft for AHXR with CC (n = 2), all biochemical and hematological parameters normalized within 48 h, and remained within normal limits thereafter, with follow up for >150 days.

### Table 2: Sensitivity, specificity, positive predictive value, and negative predictive value of biochemical and hematological parameters predicting graft failure after porcine kidney transplantation in baboons (Group 1)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity (95%CI)</th>
<th>Specificity (95%CI)</th>
<th>PPV (95%CI)</th>
<th>NPV (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH (&gt;600 U/L)</td>
<td>100% (51–100%)</td>
<td>100% (34–100%)</td>
<td>100% (51–100%)</td>
<td>100% (34–100%)</td>
</tr>
<tr>
<td>Fibrinogen (decrease &gt;80 mg/dL over 3 days, or to &lt;80 mg/dL on 2 consecutive days)</td>
<td>100% (51–100%)</td>
<td>100% (34–100%)</td>
<td>100% (51–100%)</td>
<td>100% (34–100%)</td>
</tr>
<tr>
<td>PLT (decrease &gt;150 000/µL over 3 days, or to &lt;50 000/µL)</td>
<td>100% (51–100%)</td>
<td>100% (34–100%)</td>
<td>100% (51–100%)</td>
<td>100% (34–100%)</td>
</tr>
<tr>
<td>AST (&gt;300 U/L)</td>
<td>0% (0–49%)</td>
<td>100% (34–100%)</td>
<td>0% (0–100%)</td>
<td>33% (70–97%)</td>
</tr>
<tr>
<td>CPK (&gt;500 U/L)</td>
<td>0% (0–49%)</td>
<td>100% (34–100%)</td>
<td>0% (0–100%)</td>
<td>33% (70–97%)</td>
</tr>
</tbody>
</table>

CI95% = binomial 95% confidence interval, LDH = lactate dehydrogenase, PLT = platelet count, AST = aspartate aminotransaminase, CPK = creatine phosphokinase.
Figure 1: Changes in lactate dehydrogenase (in U/L) in baboons in which pig organ failure developed; (A) pig kidney recipients (Group 1); (B) pig heart recipients (Group 2); and (C) control baboons: no organ graft (Group 3). Massachusetts General Hospital pig organs (—), and human decay accelerating factor pig organs (–).

Figure 2: Changes in aspartate transaminase (in U/L) in baboons in which pig organ failure developed; (A) pig kidney recipients (Group 1); (B) pig heart recipients (Group 2); and (C) control baboons: no organ graft (Group 3). Massachusetts General Hospital pig organs (—), and human decay accelerating factor pig organs (–).

Group 3

After initial falls in fibrinogen and platelet count (associated with the induction therapy), no abnormalities in
Prediction of Pig Organ Failure in Baboons

biochemical and hematological parameters were observed during the 4- or 6-week period of immunosuppressive therapy (Figures 1 and 3).

Figure 3: Changes in plasma fibrinogen (in mg/dL) in baboons in which pig organ failure developed; (A) pig kidney recipients (Group 1); (B) pig heart recipients (Group 2); and (C) control baboons: no organ graft (Group 3). Massachusetts General Hospital pig organs (—), and human decay accelerating factor pig organs (–).

Figure 4: Changes in platelet count (/µL) in baboons in which pig organ failure developed; (A) pig kidney recipients (Group 1); (B) pig heart recipients (Group 2); and (C) control baboons: no organ graft (Group 3). Massachusetts General Hospital pig organs (—), and human decay accelerating factor pig organs (–).

Correlation of changes in combined parameters with graft failure
The three parameters that most closely correlated with graft failure after kidney Tx (Group 1) were LDH (increase), fibrinogen (decrease), and platelet count (decrease), and those after heart Tx (Group 2) were LDH (increase),
Table 3: Sensitivity, specificity, positive predictive value, and negative predictive value of biochemical and hematological parameters predicting graft failure after porcine heart transplantation in baboons (Group 2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity (CI95%)</th>
<th>Specificity (CI95%)</th>
<th>PPV (CI95%)</th>
<th>NPV (CI95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH (&gt;600 U/L)</td>
<td>100% (44–100%)</td>
<td>50% (15–85%)</td>
<td>60% (23–88%)</td>
<td>100% (34–100%)</td>
</tr>
<tr>
<td>Fibrinogen (decrease &gt;80 mg/dL over 3 days or to &lt;80 mg/dL)</td>
<td>75% (30–95%)</td>
<td>100% (44–100%)</td>
<td>100% (44–100%)</td>
<td>25% (5–70%)</td>
</tr>
<tr>
<td>AST (&gt;300 U/L)</td>
<td>67% (21–94%)</td>
<td>50% (15–85%)</td>
<td>50% (15–85%)</td>
<td>67% (21–94%)</td>
</tr>
<tr>
<td>CPK (&gt;500 U/L)</td>
<td>67% (21–94%)</td>
<td>50% (15–85%)</td>
<td>50% (15–85%)</td>
<td>67% (21–94%)</td>
</tr>
<tr>
<td>PLT (decrease 150 000/lL over 3 days or to &lt;50 000/lL)</td>
<td>0% (0–56%)</td>
<td>100% (51–100%)</td>
<td>0% (0–100%)</td>
<td>50% (25–84%)</td>
</tr>
<tr>
<td>PTT</td>
<td>100% (44–100%)</td>
<td>0% (0–49%)</td>
<td>43% (16–75%)</td>
<td>0% (0%–100%)</td>
</tr>
</tbody>
</table>

PTT = partial thromboplastin time, CI95% = binomial 95% confidence interval, LDH = lactate dehydrogenase, PLT = platelet count, AST = aspartate aminotransaminase, CPK = creatine phosphokinase.

Table 4: Scoring system of biochemical and hematological changes occurring during graft failure

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH &gt;600 U/L</td>
<td>1</td>
</tr>
<tr>
<td>&lt;600 U/L</td>
<td>0</td>
</tr>
<tr>
<td>Fibrinogen Decrease &gt;80 mg/dL over 3 days, or to &lt;80 mg/dL on 2 consecutive days</td>
<td>1</td>
</tr>
<tr>
<td>No decrease &gt;80 mg/dL over 3 days, or to &lt;80 mg/dL on 2 consecutive days</td>
<td>0</td>
</tr>
<tr>
<td>Platelets Decrease &gt;150 000/mL over 3 days, or to &lt;50 000/mL</td>
<td>1</td>
</tr>
<tr>
<td>No fall &gt;150 000/mL over 3 days, or to &lt;50 000/mL</td>
<td>0</td>
</tr>
<tr>
<td>AST &gt;300 U/L</td>
<td>1</td>
</tr>
<tr>
<td>&lt;300 U/L</td>
<td>0</td>
</tr>
</tbody>
</table>

In an attempt to quantify the changes occurring in these three parameters, the changes in each baboon of each group were scored as in Table 4; the mean sum of the scores was correlated with clinical outcome. The minimum score was 0 (indicating no deterioration in any of the three parameters), and the maximum that could be achieved was 3 (indicating a significant change in all three parameters).

Group 1: In baboons that developed AHXR (n = 4), the mean score began increasing approximately 2 weeks before graft failure, with the maximum possible score of 3.0 being reached 3 days before graft failure (Figure 5). In contrast, in baboons that did not develop AHXR (n = 2), the score increased only terminally to a mean of 0.3 (not shown).

Group 2: In baboons that developed graft failure (n = 4) (i.e. cessation of contractions, including three with AHXR and one without conclusive histopathologic evidence of AHXR), the score began increasing approximately 2 weeks before graft failure. The maximum score of 3.0 was reached 4 days before termination of the experiment (Figure 5). The increase in score began several days before any decrease in cardiac contractions, as determined by palpation. In baboons without graft failure (n = 3), the increase in score began later and was far less marked, reaching a maximum score of only 0.5 (not shown).

Group 3: There was no increase in score greater than the baseline of 0 in either baboon (not shown).

Anti-Gal and anti-nonGal antibody responses
In all baboons in Groups 1–3, whenever anti-CD154 mAb therapy was being administered, anti-Gal Ab remained at
low levels. There was no obvious correlation between anti-Gal IgM or IgG levels and the biochemical and hematological changes described earlier. No Ab to nonGal pig determinants developed in any baboon in Groups 1 and 2 until discontinuation of anti-CD154 mAb therapy (which was always after graftectomy).

**Histopathology, immunohistopathology, and ultrastructure of pig organ graft and native baboon tissue biopsies**

Light microscopy on biopsies of functioning grafts taken 14 days after organ Tx indicated minimal to moderate changes of AHXR in Group 1, and no abnormalities in Group 2. Biopsies of the graft taken at necropsy or at the time of graftectomy in both groups indicated a range of histopathological features from normal to severe AHXR.

In the excised kidney grafts of Group 1, immunofluorescence showed IgM deposition in all cases with no or minimal IgG, fibrin, or C3 deposition. C4d staining was positive in all grafts with mild or moderate AHXR. There was no correlation between immunoglobulin deposition and the biochemical and hematological changes observed in the baboons (data not shown). No histopathological changes were seen in any of the baboons’ native organs except where infection or other complication had developed. Although electron microscopic studies indicated endothelial cell activation within the pig organs (data not shown) (6), there was an absence of features suggesting significant immune complex deposition in any of the native baboon organs, in particular in the lungs, liver, and kidneys (not shown).

In Group 2, immunofluorescence showed IgM deposition in three of seven cases, IgG in two of seven, fibrin in one, no C3 deposition, but C4d deposition in cases of moderate to severe AHXR. As in Group 1, there was no correlation between the extent of immunoglobulin deposition and the observed biochemical and hematological changes. No histopathological or ultrastructural changes were seen in native organs except where related to specific complications.

In the Group 3 control baboons, no significant histopathologic changes were seen in any native organ during the period of therapy.

**Monitoring for baboon and porcine CMV**

These changes will be described in detail in a separate report (Mueller et al., in preparation). In summary, in Groups 1–3, increased copy numbers of baboon CMV occurred in 10 of the 12 baboons (83%) in which tissues where available. In Groups 1 and 2, porcine CMV DNA copy numbers were greatly increased in six of the 10 organ grafts (60%) available for testing. The activation of porcine CMV was many times greater than that of baboon CMV (7). The increase in baboon CMV in Group 3 indicates that activation of baboon CMV was unlikely to be playing a role in the systemic changes documented. Studies in our laboratory have suggested that activation of porcine CMV is associated with vascular endothelial cell activation; in the present study, we cannot absolutely exclude a correlation between the increase in porcine CMV and the systemic changes seen in Groups 1 and 2.

**Discussion**

This is the first report of changes in noninvasive biochemical and hematological parameters that indicate that graft dysfunction in the pig-to-nonhuman primate model is occurring (or will soon occur) and that graft failure will result. These biochemical and hematologic abnormalities began to develop over a period of 7–14 days before any graft dysfunction could be detected by palpation of the heart or by an increase in troponin T, and before there were significant changes of AHXR on heart biopsy. No single parameter was a reliable indicator that graft failure was developing. However, when the three parameters that correlated most positively with AHXR or graft failure after kidney and heart Tx, respectively, were considered together, they indicated conclusively that graft failure was occurring (Figure 5).

The possibility that soluble antigen from the graft was being bound by anti-Gal Ab and being metabolized in the liver has been considered as a cause of the increases in LDH, AST, and CPK. However, there were no other indicators of liver dysfunction, such as elevation in alanine aminotransaminase or serum bilirubin. There were also no ultrastructural changes suggestive of immune complex deposition in the liver, native kidney, or other major organ. Impaired hepatic function associated with the metabolism of immune complexes would therefore not appear to be the cause of these changes. Nor did the changes appear to be related to red blood cell dyscrasia, as there was an absence of hemolysis, based on parameters such as serum bilirubin or schistocytosis on blood smears. The exact causes for the changes remain uncertain, although it seems likely that injury in the graft is a major factor in the elevation of LDH, AST, and CPK.

Troponin T is a reliable marker of ischemic injury of the myocardium, although its value as an indicator of allograft rejection is inconclusive (15,16). Troponin T rose significantly after heart Tx, confirming that it is a sensitive marker of pig heart ischemic injury. However, it showed no increase during the period when LDH, AST, and CPK were rising, but only increased 2 days before functional graft failure from AHXR. The much earlier rise in LDH, AST, and CPK therefore would not appear to be associated with ischemic changes taking place in the graft.

In all groups, although fibrinogen and platelet count fell initially in some baboons (associated with induction therapy), they both stabilized in the low/normal range. A subsequent
fall in fibrinogen was predictive that graft failure from AHXR was likely to develop soon. A fall in fibrinogen to <35 mg/dL was always associated with the development of CC (n = 4). The late reduction in fibrinogen was presumably related to abnormal consumption of fibrinogen and/or fibrin deposition in the graft resulting from endothelial activation, possibly accentuated by physiological incompatibilities between baboon and pig (and from reduced liver synthesis as a result of cyclophosphamide treatment). A late fall in platelet count was seen only when a kidney had been transplanted (Group 1) and not after heart Tx (Group 2), and was possibly related to aggregation of platelets within the graft, possibly indicating a greater endothelial procoagulatory state in the kidney than in the heart, although we have no conclusive immunohistological evidence for this hypothesis.

There was little correlation between the development of CC and the extent of AHXR seen on histopathology of the excised organ. However, recent unpublished data by Gollackner et al. (in preparation) indicate that, whenever there is any immunoglobulin deposition, tissue factor expression was up-regulated. From these in vitro and in vivo observations, we suggest that a low level of tissue factor expression by natural anti-Gal Ab deposition is sufficient to cause endothelial cell activation (that may lead to CC), whereas perhaps the massive deposition of elicited Ab causes rapid AHXR. One other factor that may be playing a role in endothelial cell activation is porcine CMV, which showed significant up-regulation in most grafts; we cannot exclude this as a factor in the systemic changes observed.

In recent and ongoing studies, using a different immunosuppressive regimen, a different histopathological picture is being seen in the grafted organs. Fibrin thrombi in the vessels with some ischemic injury have been seen rather than the classical features of AHXR. In these studies, changes in the parameters identified here have continued to indicate graft injury. The scoring system has been demonstrated to be entirely reliable when, in addition to AHXR, the transplanted organ was from a CMV-positive pig. When the organ was from a CMV-negative pig, individual parameters, e.g. LDH, still indicated graft injury, but the overall score did not reach 3.0. In the present study, all transplanted organs were from CMV-positive pigs, and this may be an important additional factor in the validity of the scoring system. Major changes in the immunosuppressive regimen, such as the inclusion or omission of cyclophosphamide, may affect some parameters, although we believe this is less likely in view of the absence of changes seen in the Group 3 baboons in the present study. Our more recent data, which confirm the value of the systemic parameters we identify as indicators of graft injury and pending failure, will be reported fully when these studies have been completed.

We believe it is important to be aware that systemic changes, which can be easily monitored, may indicate graft injury as a result of AHXR, perhaps in the presence of porcine CMV or other factors. With increasing observation and experience, diagnostic criteria will evolve that will prove useful in monitoring recipients following discordant xenotx, hopefully even in the clinical setting.

References


