The Necrotic and Apoptotic Injury of Cardiac Xenotransplants
Caused by Human Serum.

by

Jamie George Lucien

A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy,
Graduate Department of the Institute of Medical Sciences, in the
University of Toronto

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Title: The Necrotic and Apoptotic Injury of Cardiac Xenotransplants Caused by Human Serum.

Written and submitted by Jamie George Lucien in conformity with the requirements for the Degree of Doctor of Philosophy from the Institute of Medical Sciences, University of Toronto in the year 2001.

Abstract

Xenotransplants are injured by preformed immune components found in the serum of the recipient. Human serum mediates the necrosis of a xenotransplant by the activation of the complement cascade by preformed xenoantibody. This form of immunological rejection is called hyperacute rejection (HAR), which has been attributed to cause endothelial cell necrosis. In the absence of HAR there is the onset of delayed xenograft rejection (DXR). The pathological mechanism of DXR remains controversial, however apoptosis has been implicated as a possible mechanism of injury. These studies investigate the role of human serum in mediating both necrotic and apoptotic injury to xenotransplants and the participation of these processes in HAR and DXR.

Monoclonal antibodies directed against human xenogeneic IgM antibodies (anti-XeIgM) significantly reduced xenogeneic IgM (XeIgM) binding to cultured porcine endothelial cells (PAEC) as much as 83.2±2.1%, as measured by enzyme linked immunosorbant assay. There was no significant reduction in IgG binding by any anti-XeIgM. The reduction of XeIgM also reduced complement mediated cell cytotoxicity of PAEC in vitro, and prevented HAR in vivo. The pathological analysis of heart biopsies from mice that received human serum treated with anti-XeIgM revealed no signs of necrosis and no classical signs of HAR.
Heart biopsies from mice transfused with human serum were examined for the presence of apoptosis in situ by terminal dUTP nicked end labeling (TUNEL). Mice in whom HAR was prevented had approximately a ten fold increase in the percentage of apoptotic cells (% apoptosis) 18 hours post injection compared to animals given saline, and a four fold increase over animals that experienced HAR. Administration of cobra venom factor (CVF) decomplemented human serum did not significantly (p>0.05) alter the % apoptosis demonstrating that the classical mediators of HAR, namely antibodies and complement, do not participate in xenograft apoptosis. The addition of 20 mM Gal α 1,3 Gal to human serum significantly (p<0.05) reduced % apoptosis to levels observed in saline treated control animals. Human serum induces apoptosis of isolated pig and mouse rod shaped cardiomyocytes, but not cultured human cardiomyocytes in vitro. Supplementation of culture media with recombinant human TNF-α did not induce apoptosis of xenogeneic cardiomyocytes at concentrations found in normal human serum. These results demonstrate that human serum initiates both a necrosis and an apoptosis via two separate pathways to injure the cardiac xenograft.
Acknowledgements

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Finally, I would like to thank the Physician Services Incorporated, The Hospital for Sick Children, The University of Toronto, and the Medical Research Council of Canada for providing financial support of this project and my graduate stipend.
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<td>ABO</td>
<td>A B O blood group system</td>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>B cell lymphoma protein-2-associated x protein</td>
</tr>
<tr>
<td>Bcl</td>
<td>B cell lymphoma protein</td>
</tr>
<tr>
<td>bcl</td>
<td>B cell lymphoma gene</td>
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<tr>
<td>C1q</td>
<td>Complement component 1 q</td>
</tr>
<tr>
<td>C2aC4b</td>
<td>Complement component 3 convertase</td>
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<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
<tr>
<td>C3c</td>
<td>Complement component 3 fragment c</td>
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<tr>
<td>Ca^{2+}</td>
<td>Soluble calcium ion</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CED</td>
<td><em>Caenorhabditis elegans</em> development protein</td>
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<td>Cl^-</td>
<td>Soluble chloride ion</td>
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<tr>
<td>CVF</td>
<td>Cobra venom factor</td>
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<tr>
<td>CR1</td>
<td>Complement receptor type 1</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Degrees Celsius °C</td>
<td>Degrees Celsius</td>
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<td>DFF</td>
<td>DNA fragmentation factor</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dUTP</td>
<td>Uridine triphosphate</td>
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<td>DXR</td>
<td>Delayed xenograft rejection</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<td>FADD</td>
<td>Fas-associated death domain</td>
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<td>Fc</td>
<td>Immunoglobulin gamma tails</td>
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<td>FLICE</td>
<td>Fas-associated death domain like interleukin -1β-converting enzyme</td>
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<td>g</td>
<td>Gram</td>
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<td>Gal α 1,3 Gal</td>
<td>Galactose alpha 1.3 galactose</td>
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<td>&gt;</td>
<td>Greater than</td>
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<td>h</td>
<td>Hour(s)</td>
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<td>HAR</td>
<td>Hyperacute rejection</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>I.V.</td>
<td>Intravenous</td>
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<td>ICAD</td>
<td>Inhibitory caspase-activated DNase</td>
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<td>Abbreviation</td>
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<tr>
<td>ICE</td>
<td>Interleukin-1β-converting enzyme</td>
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<td>Interferon gamma</td>
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<td>Immunoglobulin alpha</td>
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<td>IgM</td>
<td>Immunoglobulin mu</td>
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<tr>
<td>IKK</td>
<td>Inhibitory kappa B alpha</td>
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<td>IU</td>
<td>International unit of activity</td>
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<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
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<tr>
<td>k</td>
<td>Kilo</td>
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<td>K⁺</td>
<td>Soluble potassium ion</td>
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<tr>
<td>L</td>
<td>Liter</td>
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<td>&lt;</td>
<td>Less than</td>
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<td>Molar</td>
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<td>m</td>
<td>Milli</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>MASP</td>
<td>Mannose-binding lectin-associated serine protease</td>
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<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
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<tr>
<td>MCP</td>
<td>Membrane cofactor protein</td>
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<tr>
<td>min</td>
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<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Nedd</td>
<td>Neuronal precursor cells expression developmentally down-regulated</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NIK</td>
<td>Nuclear factor kappa B inducing kinase</td>
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<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
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<td>PAEC</td>
<td>Porcine aortic endothelial cell(s)</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
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<td>%</td>
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<td>±</td>
<td>Plus/minus</td>
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<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
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<td>rTNF-α</td>
<td>Recombinant tumour necrosis factor alpha</td>
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<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
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<td>SCID</td>
<td>Severe combined immunodeficient</td>
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<td>sCR1</td>
<td>Soluble complement receptor type 1</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium docecyl sulphate</td>
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<td>STSG</td>
<td>Split thickness pig skin graft</td>
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<td>TCR</td>
<td>T cell receptor</td>
</tr>
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<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<td>TNFR-1</td>
<td>Tumour necrosis factor receptor one</td>
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<td>TRADD</td>
<td>Tumour necrosis factor receptor-associated death domain</td>
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<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Tumour necrosis receptor-related apoptosis-mediating protein</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal d uridine triphosphate nicked end labeling</td>
</tr>
<tr>
<td>Xab</td>
<td>Xenoreactive antibody/ xenogeneic antibody/ xenoantibody</td>
</tr>
<tr>
<td>XelgG</td>
<td>Xenogeneic immunoglobulin gamma</td>
</tr>
<tr>
<td>XelgM</td>
<td>Xenogeneic immunoglobulin mu</td>
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I. INTRODUCTION: Xenotransplantation and Xenotransplant Rejection.

A. The Requirement for Xenotransplantation

End-stage organ failure is one of the most important public health issues today. End-stage heart failure alone takes more lives than communicable diseases, such as human immunodeficiency virus (HIV) infection, and cancer. End-stage organ failure is the result of an organ being either unable to repair itself or to properly perform its function. Therapies that supplement or augment the function of an organ have little impact on end-stage diseases. In essence, the only effective therapy for end-stage organ failure remains to be replacing the afflicted organ by transplanting a healthy donor organ in its place.

Transplantation of hearts, kidneys, lungs, livers, pancreas, and intestine have become an accepted modality of treatment with recognized benefits to the patient’s health, quality of life, and burden to the hospital. In the United States of America over 20,884 organ transplants were performed in 272 transplant centers. The majority (81.1%) of the organs for these transplants was from cadaveric donors. In some categories, such as heart transplants, all the organs came from cadaveric donors. The majority of living donors gave kidneys (30.1% of all kidneys donated and 17.7% of all donated organs). Individuals receiving a heart transplant had a 72±0.4% survival rate, and transplanted hearts had a 70.5±0.4% survival rate after four years (October 1987- December 1996) based on Kaplan-Meier graft and patient survival index.

Currently, the limitation of transplantation to be utilized widely therapeutically is the shortage of suitable donor organs. At the end of 1997, a total of 56,716 patients (<1 to >65 years of age) were registered on the National Transplant Waiting list in the United
States of America. Of these, 3897 patients were waiting for a donor heart, 773 additional patients died while waiting for a donor heart, and 2292 additional patients were transplanted. Excluding the individuals who were removed due to recovery or due to the administration of an alternative therapy, the total number of patients waiting for a donor heart was approximately 6962. The total number of donor hearts recovered in 1997 was 2427, which is 34.9% of the total number required to provide a transplant to all registrants (1). Moreover, it is estimated that as many as 45–50 thousand individuals under the age of 65 in the United States of America could benefit from a heart transplant (2).

Utilization of a living donor pool from animals (xenotransplantation) could be used to as an alternative to human donors (allotransplantation). Xenotransplantation utilizing taxonomically closely related donors, such as primates, has been proposed since it reduces the genetic disparity between the donor and the human recipient. This combination in xenotransplantation is commonly referred to as concordant xenotransplantation (3). Concordant transplantation rejection is characterized by activation of T and B lymphocytes and thus is similar to allotransplantation. Although this potential solution appears attractive, many primate species are endangered or are not available in large numbers with only a few hundred in captivity, and thus this is not a feasible means to address the shortage in the donor pool. Xenotransplantation utilizing donors from a taxonomically disparate domesticated species, such as pigs, that are abundant in large numbers (hundred of thousands to millions) has the potential of addressing the donor shortage. This combination of taxonomically disparate species is commonly referred to as discordant xenotransplantation. Discordant xenotransplantation
rejection is characterized by the presence of recipient preformed natural anti-xenograft antibodies and an incompatibility of the donor complement inhibitor membrane proteins with the recipient complement. The presence of preformed xenoreactive factors in the recipient serum leads to rapid rejection of the xenograft commonly referred to as hyperacute rejection (Figure. 1) (4-7). Despite this formidable obstacle, the potential of using animals donors is considered to be the most attractive and viable strategy in light of the overwhelming numbers required to meet the current shortfall in organ donations. Thus, researchers have invested their efforts to studying the mechanisms of discordant xenotransplant rejection.

B. Xenotransplant Rejection

Transplant rejection, be it xenogeneic or allogeneic, is caused by the host immune system targeting and destroying the cells that make up the transplanted tissue or organ. There are many cytotoxic mechanisms at the disposal of the immune system, however they can be described as either necrotic or apoptotic (8, 9). The basis of categorizing the mechanisms cell death is based on the physical, biochemical and physiological characteristics of the dying cell (9). Necrosis is thought to be a form of “accidental” cell death in which the dying cell loses membrane integrity and ceases to perform its physiological functions. Thus, the catastrophic failure of homeostasis is a key characteristic of necrosis (10-12). Typically, necrosis is thought to be mediated by an external effector that causes lysis of the target cell, and not through the activation of cellular biochemical pathways to facilitate its own destruction (13, 14).
Xenoreactive antibody recognizes and binds to Gal α 1.3 Gal (▲) present on the cell surface of the xenograft's endothelium (1.). Deposits of xenoantibody activate serum complement, which leads to the lysis of the endothelial cells (2.). Loss of the barrier properties of the endothelium results in platelet aggregation (3.), fibrin deposition (4.), hemorrhage (5.) and edema of the tissue (6.).
Apoptosis is thought to be a form of "programmed" cell death. In apoptosis, cell death may be triggered by an extracellular or intracellular stimulus that activates specific "death pathways" to mediate the destruction of the cell (9). However, this categorization of cell death into either necrotic or apoptotic does not mean that the two processes are necessarily mutually exclusive. It has been demonstrated that apoptotic cells may undergo secondary necrosis under certain conditions. In contrast, necrotic cells cannot undergo apoptosis, since they have lost the intracellular machinery required to initiate and carry out apoptosis. Thus, it has been proposed that the relationship between apoptosis and necrosis is that of a progression from apoptosis toward necrosis. However, not all necrotic agents are able to induce apoptosis and not all apoptotic agents are able to induce a significant amount of necrosis (15). Therefore an alternate relationship has been proposed in which a cell may be directed to undergo necrosis without first the induction of apoptosis. Furthermore, apoptotic cells may undergo necrosis by virtue of the fact that they have an intact membrane and intracellular environment that may be disrupted (Figure 2.).

C. Necrosis

Necrosis is a form of cytotoxicity that results from a sudden and uncontrollable catastrophic/lethal loss of homeostasis. Necrosis does not require a cell to utilize its energy stores, nor to utilize its biochemical machinery to facilitate cytotoxicity. Typically, necrosis occurs in contiguous cells (i.e. patches or discrete areas of tissue), and is accompanied by inflammation of the viable tissue that surrounds the necrotic area (14).
The proposed linear relationship between apoptosis and necrosis proposes that a cytotoxic insult initiates either apoptosis or necrosis based on the dose of the cytotoxic agent (A.). However, some cytotoxic insults cause only apoptosis or necrosis (B.). Thus, it is not obligatory for a cytotoxic agent to initiate apoptosis at low doses and necrosis at high doses. However, apoptotic cells are still vulnerable to secondary necrosis.
The key characteristics of necrosis are the loss of cell membrane integrity and a
disruption of the cytoskeleton. These features are caused by a loss of selective
permeability of the necrotic cell's membrane, which allows unregulated passage of
extracellular fluid and ions into the dying cell. The result is a morphological change of a
normal cell to a swollen cell. Moreover, the loss of cell membrane selective permeability
results in disruption of the intracellular environment and loss of homeostasis. As a result,
there is damage to the organelle plasma membranes of the mitochondria, endoplasmic
reticulum, and lysosomes, and spontaneous activation of $\text{Ca}^{2+}$-activated enzymes (9, 13).
The loss of the boundaries of the cell results in a release of the intracellular contents into
the interstitial space, which in turn triggers local inflammation. The nucleus of necrotic
cells are thought either to be lost to the intracellular environment like the other
organelles, or disintegrated by the disruption of the nuclear envelope and the liberation of
lysosomal enzymes (16). Currently, there is no evidence that there are specific
biochemical pathways that lead to the degradation of cellular DNA, protein, lipids, or
carbohydrates during necrosis. Instead, it is thought that degradation of these molecules
is mediated by unregulated release of the content of disrupted lysosomes.

C.1. The Role of Necrosis in Hyperacute Rejection

Hyperacute rejection is characterized by interstitial hemorrhage, edema, platelet
aggregation, fibrin deposition, and thrombi formation (17). This histological profile is
the result of a severe and rapid injury to the endothelium of a xenograft. Hyperacute
rejection is mediated by the deposition of preformed xenoreactive antibody directed
against a ubiquitous xenotransplant antigen (Gal $\alpha$ 1,3 Gal) activating serum complement
(18–22).
Xenoreactive antibody is a naturally occurring antibody found in the serum of unimmunized individuals. These antibodies may be of the IgG, IgM, or IgA isotype as demonstrated by the presence of all these isotypes are found in hyperacutely rejected xenografts (23). The xenoreactive antibody found in humans that participate in xenotransplant hyperacute rejection is mainly directed against a carbohydrate disaccharide antigen, Gal α 1.3 Gal, that is not expressed by normal human cells. Gal α 1.3 Gal is ubiquitously expressed in many animal tissues, and thus provides adequate antigen density to form dense antigen-antibody deposits (24-26). Xenoreactive antibody deposition onto the surfaces of a xenograft alone does not induce cytotoxicity. Instead, antibody marks the tissue for immunological clearance and recruits additional immunological factors such as the complement system (27). This is demonstrated in vitro by utilizing models of antibody dependent complement mediated cytotoxicity. In these experiments, the absence of either human xenoreactive antibody or active complement prevents the cytotoxicity of cultured porcine aortic endothelial cells. In vivo, immunopathological analysis of hyperacutely rejected tissue from experimental and clinical xenotransplants demonstrate heavy deposition of antibody and complement (28-31). This has lead to the development of several therapeutic strategies that focus on preventing antibody-antigen binding. The immunoaffinity depletion of xenoreactive antibody from the circulation of xenograft recipients initially prevents hyperacute rejection of the xenograft (32-34). However, this technique requires frequent depletion of xenoreactive antibody from the recipient, and is subject to antibody rebound. Antibody rebound is the recipient’s immune system responding to low circulating xenoreactive antibody levels by producing a large amount xenoreactive antibody that exceeds the
circulating antibody levels prior to antibody depletion. In the absence of sustained depletion of circulating xenoreactive antibody, the alternative therapy is to prevent the expression of Gal α 1,3 Gal by the donor species. The compete prevention of the expression of Gal α 1,3 Gal is a developmentally lethal mutation in relevant species such as pigs. Thus, the approach of expressing a competitive glycotransferase that produces a non-immunostimulating human antigen have been used to prevent the formation of Gal α 1,3 Gal. Fucosyltransferase is responsible in the formation of human blood group H antigen, and has been expressed in mice and pigs in sufficient levels to successfully compete with the galactosyltransferase that is responsible for the formation of the terminal Gal α 1,3 Gal structure found in xenografts(35-37). The reduction in the expression of Gal α 1,3 Gal and the increase in the H antigen has been associated with decreased xenoreactivity and increased resistance to human serum-mediated cytolysis (38).

The complement system is the primary mediator of necrosis in xenograft hyperacute rejection. The complement system is a collection of serum proteins that upon activation react in a specific sequence to form an active enzyme cascade that generates a variety of immunological factors (Figure 3.). The major biological activity of the complement cascade is to opsonize pathogens for phagocytosis and to generate mediators of inflammation, and thus like antibody, complement facilitates cell mediated immune reactions (39, 40). However, the complement cascade is also able to mediate the lysis of pathogens via the assembly of pore forming units (the membrane attack complex) in the membrane of the opsonized cell (41).
Figure 3. The classical, lectin and alternative complement activation pathways.

**Classical Pathway**

- Immune Complex → C1q → C4 → C4b → C2 → C4bC2a

**Lectin Activation Pathway**

- MBL-MASP → C4a → C4bC2aC3b

**Alternative Pathway**

- C3 → C3bBb → C3bBbC3b → C5a

**C5 Convertase**

- C4bC2aC3b → C5a

Terminal Attack Complex

C5b-C9n

The classical pathway is initiated by the binding of C1q to adjacent Fc tails of immune complexes. The lectin activation pathway is initiated by mannose-binding lectin (MBL) that is associated with a novel serine protease (MASP). In the presence of active MASP both C4 and C2 are cleaved to form C4bC2a. The alternative pathway is activated by the hydrolysis of C3 by cell surface molecules, perturbation or spontaneous activation. Both pathways produce a C3 convertase, which cleaves C3 to generate a C5 convertase and C3a. C5 convertase then generates C5a and the terminal attack complex.
The complement cascade may be activated by any one of three distinct pathways. The classical pathway is the primary pathway by which human xenoreactive antibody mediates hyperacute rejection (42). The activation of this pathway is initiated by the binding of C1q to adjacent Fc tails. This leads to the formation of the C3 convertase C2aC4b. The lectin activation pathway is mediated by serum mannose-binding lectin (MBL) and MBL-associated serine protease (MASP) that are able to form a complex that is able to activate the complement cascade in a manner similar to the classical pathway (43). The MBL is a C-type lectin that has a similar bouquet-like structure to C1q. However, MBL functions more like IgM by directly binding to and opsonizing terminal mannose and N-acetylglucosamine structures that are normally not exposed on mammalian cell surfaces but are present on microorganisms such as yeasts and viruses (44). In addition to its function as an opsonin, MBL-MASP complexes are able to activate the complement cascade. MASP resembles activated C1s and can cleave both C4 and C2 to generate active C4bC2a C3 convertase (45). The last complement activation pathway is the alternative pathway, which is the result of the spontaneous activation of C3 to form C3b (46). This results in the formation of C3bBb C3 convertase, which activates more C3 in an amplification loop.

The production of either the C4bC2a or the C3bBb C3 convertase eventually leads to the formation of the membrane attack complex (47) that is essential to the induction of necrosis and xenograft hyperacute rejection. The membrane attack complex causes necrosis by disrupting cell membrane integrity, and thus disrupting cellular homeostasis (47).
Although the xenograft donors have a complement system, their complement proteins are not able to regulate the human complement system. This lack of compatibility prevents the endogenous complement regulatory proteins from protecting the xenograft from complement-mediated damage. Both pharmacological and molecular approaches have been developed to prevent the activation of complement. The pharmacological approaches focus on depleting or uncoupling the complement cascade to prevent the formation of the membrane attack complex. Thus, molecules such as cobra venom factor (CVF), C1-inhibitor, anti-C5 antibodies, soluble complement receptor type 1 (sCR1), or compstatin have been used to prevent the formation of C5 convertase (48-52). This provides an effective therapy to prevent complement activation, however these therapies required periodic administration and prevent all complement activation including the mobilization of complement against bacterial, viral, and parasitic attack. The molecular approach is to express human cell surface complement regulatory proteins such as complement receptor type 1 (CR1), decay accelerating factor (DAF, CD55), CD59, and membrane cofactor protein (MCP, CD46) (53, 54, 55). This has advantage of leaving the recipient’s complement system entact to allow participation against infection or parasitic attack, while providing protection from complement activation specifically directed against the xenograft.

Multiple transgenic animals expressing both complement regulatory proteins and fucosyltransferase are free of hyperacute rejection, however the xenograft is infiltrated by macrophages, T cells and B cells in the absence of immunosuppression. Furthermore, the recipients experienced profound thrombocytopenia and abnormalities in coagulation (56).
C.2. The Role of Necrosis in Acute Rejection

Acute rejection is characterized by inflammation, edema and massive infiltration. Pathological examination of rejected tissues reveals large areas of necrosis associated with areas of heavy infiltration. Cytotoxic lymphocytes are able to produce a pore forming protein called perforin. Perforin is a 65-kDa polypeptide lytic agent that inserts itself into lipid bilayers (57, 58). Its calcium dependent polymerization forms transmembrane channels that expose the cytosol to the extracellular environment (59). Thus, perforin is very much like the complement membrane attack complex, and this similarity lead to the belief that perforin could mediate necrosis (60, 61). However, unlike the complement system, perforin is not produced in sufficient quantity to disrupt the homeostasis of the target cell to induce necrosis. Thus, perforin induced necrosis is only seen in vitro using experimental systems were there are high effector to target ratios. Cell mediated cytotoxicity in vivo is thought to be mainly apoptotic, and the necrosis seen in processes such as acute rejection may be secondary necrosis of apoptotic cells (62).

D. Apoptosis

The distribution of apoptotic cells contrasts with that of necrotic cells in that apoptosis may have a scattered distribution throughout a tissue. This is possible by virtue of the fact that apoptosis is ultimately performed by individual cells and not necessarily mediated by the actions of an exogenous cytotoxic agent. The time course from the initiation of apoptosis to its conclusion ranges from minutes to days and is dependent on the cell type (9, 14). Unlike necrosis, apoptosis is not typically accompanied by inflammation, however induction of overwhelming apoptosis by toxic agents may stimulate inflammation due to the induction of secondary necrosis of apoptotic cells. The
most fundamental difference between necrosis and apoptosis is that apoptosis utilizes cellular suicide pathways to mediate cytotoxicity of the cell. Morphologically, an apoptotic cell undergoes a process of condensation of the cytoplasm and nuclear chromatin in a process of shrinkage. The shrinkage of the apoptotic cell is potentially the result of two processes. First, there is an outward movement of fluid from the cell causing condensation of the cytoplasm (63). This process is potentially mediated by a Na⁺-K⁺-Cl⁻ co-transport system (12). Secondly, the shedding of “boils” or apoptotic bodies, which are small membrane-bound vesicles containing cytoplasm, cellular organelles, and portions of fragmented DNA leads to a reduction of intracellular contents and membrane surface area of the apoptotic cell. The process of shrinkage ultimately causes the apoptotic cell to detach from both neighboring cells and its basement membrane. Achieving an accurate assessment of apoptotic cells using morphological criteria is complicated in tissues in that there is rapid phagocytosis of the apoptotic fragments by neighboring cells or natural immune cells, such as macrophages (64).

D.1. The Biochemical Pathways of Apoptosis

There is increasing evidence that apoptosis is mediated by a set of tightly regulated molecular pathways that include activation of both pro- and anti-apoptotic signals (Figure 4.). Pro-apoptotic pathways may include specific death receptor ligation or the entry of molecules, which leads to activation of intracellular protease cascades. The caspase family of cysteine proteases holds a prominent position, and is thought to be an essential element of apoptosis induction. Anti-apoptotic pathways include the activation of some Bcl-2 family proteins and NF-κB. Thus, the induction of apoptosis requires that the balance of intracellular signals favor the pro-apoptotic.
Apoptosis may be initiated by classical receptor/ligand interactions. The death receptors are a group of membrane proteins of the TNF-receptor superfamily that transduce pro-apoptotic signals (65). These receptors are characterized by an 80 amino acid intracellular death domain that is required to transduce the apoptotic signal. Five distinct groups of death receptors have been identified; they are TNFR-1 (66, 67), Fas (68, 69), TRAMP (70), and TRAIL receptor 1 and 2 (71). Typically, the death receptors require oligomerization in order to initiate the apoptotic signal. All of the death receptors share common signal transduction mechanisms to induce apoptosis.

After receptor oligomerization, there is recruitment of adapter proteins to the death domains of the receptors. The death domain of the receptors exerts its effects via homophilic interaction (72). Therefore, death domains either form self-associated interactions or interact with a second molecule that possess a death domain. This property has implications in oligomerization, as seen with the death receptors and facilitation of cross talk between different death receptor pathways via death domain containing adapter proteins (73). There is some understanding of the structure and function of death domain containing adapter proteins. Overexpression of the death domain containing adapter proteins spontaneously induces apoptosis, and thus demonstrates some independence from receptor/ligand binding. The transient expression of the N-terminus region of FADD is also able to spontaneously induce apoptosis. This is in contrast to transient expression of the C-terminus region, which acts much like a dominant negative mutant to provide protection against apoptosis. Thus, the N-terminal region of FADD has been termed the death effector domain (74). Death domain containing adapter proteins that lack the death effector domain, such as TRADD and RIP,
Figure 4. A schematic representation of the apoptosis signaling pathways.
The binding of the death receptors to its ligand results in clustering of the receptors cytoplasmic death domains and the recruitment of other death domain (DD) bearing cytoplasmic proteins (e.g. TRADD and FADD) to form the death inducing complex (DISC). Formation of the DISC results in recruitment of FLICE/Pro-Caspase-8 via the death effector domain (DED). Liberation of active Caspase-8 initiates an intracellular enzyme cascade that eventually leads to the activation of DNA fragmentation factor (DFF) and the activation of endonucleases. The caspase cascade also stimulates mitochondria (MT) to release cytochrome c (Cyt C) which promotes activation of DFF via caspase-9 and caspase-3. B-cell lymphoma proteins 2 and XL (Bcl-2/XL) inhibit initiation of DFF by Cyt C. However, members of the Bcl-2 family of proteins, such as BAX, stimulate Cyt C release by MT. Death receptor ligation leads to the initiation of stress kinases (SAPK/JNK) which are implicated in initiating apoptosis. In addition to pro-apoptotic signals, there is activation of survival pathways via activation of NF-κB via nuclear factor κ B inducing kinase (NIK) and inhibitory κB kinase (IKK).
are speculated to recruit other death domain containing adapter proteins involved in pro-apoptotic pathways (i.e. FADD), to activate stress activated protein kinase cascades, and to activate anti-apoptotic pathways via activation of NF-κB. Thus, death domain containing adapters form heterogeneous complexes termed death-inducing signaling complexes that activate both pro-apoptotic and anti-apoptotic signals.

A key characteristic of apoptosis pathways is the activation of caspase cascades, which are speculated to be the key effector molecules of apoptosis. Activation of the caspases is facilitated by the cysteine protease of the caspase family, FLICE/caspase-8. FLICE/caspase-8 possesses a N-terminal death effector domain, as characterized in FADD, and a C-terminal region with a typical cysteine protease structure found in interleukin 1β converting enzyme (ICE) (75, 76). FLICE/caspase-8 is thought to interact with the death-inducing signaling complex via the death effector domain interaction with FADD (77). It is speculated that FADD’s interaction with FLICE/caspase-8 leads to a conformational change of FLICE/caspase-8 that results in autoproteolytic activation and release of the C-terminal cysteine protease portion of FLICE/caspase-8 into the cytoplasm (78). Overexpression of inactive FADD and FLICE/caspase-8 block Fas, TNFR-1, and TRAMP apoptosis signaling (79, 80). Moreover, the addition of caspase inhibitors in many experimental systems abrogates the induction of apoptosis. This evidence suggests the existence of a common intracellular apoptosis pathway characterized by a requirement of caspase activation.

The interaction of FLICE/caspase-8 with FADD is an initial step in the activation of apoptosis caspase cascades. The active caspase is a heterotetrameric complex of two large subunits of approximately 20 kDa that make up the active site, and two smaller 10
kDa subunits. Currently, ten members of the mammalian caspase family have been identified, and they are divided into three families based on phylogenetic analysis. The families are the ICE-like protease family, the CED-3 family, and Nedd2 family. Cytoplasmic caspases are found in the form of inactive zymogens, or proenzymes that require proteolytic cleavage for activation (81, 82). Upon activation FLICE/caspase-8 has been characterized to cleave caspases-3, -4, -7, -9, and -10 in vitro (83). The activation of multiple caspase targets is an early branching point in the cascade that may facilitate activation of multiple biological and biochemical cell processes. The exact order of activation of the caspases is unclear, and thus it is unknown if there are additional branching points in the cascade. However, an increasing number of targets vulnerable to caspase cleavage have been identified. They include proteins involved in genome function (e.g. DNA repair enzyme poly(ADP-ribose) polymerase, PARP) (84), regulators of cell-cycle progression (e.g. retinoblastoma protein) (85), proteins that are integral in maintaining nuclear architecture (e.g. lamins) (86, 87), and cell structural proteins (e.g. actin and gelsolin) (88, 89). Perhaps one of the most interesting implicated targets for the apoptosis caspase cascade is murine inhibitory caspase-activated DNase (ICAD). ICAD and its human homologue DNA fragmentation factor (DFF) have been demonstrated to be a substrate for caspase-3, and upon cleavage release an active endonuclease (90, 91). This provides a putative link between apoptosis signal transduction and the activation of endonucleases.

There is increasing evidence that the caspase cascade may activate the stress-activated protein kinase (SAPK) pathway. Cleavage of p21-activated kinase-2 (PAK2) produces a constitutively active protein kinase that is implicated in SAPK/JNK
activation. Dominant-negative PAK2 mutants do not form apoptotic bodies during Fas stimulated apoptosis. However, these mutants have no alteration in the formation of apoptotic nuclear bodies. The activation of the SAPK/JNK pathway by death receptors is typically slow and sustained (92). It is unclear, at this time, if this response represents a collateral apoptosis pathway, or if this is an independent apoptosis pathway.

Bcl-2 was discovered in human B-cell lymphoma arising from a t(14;18) chromosome translocation (93, 94). Expression of Bcl-2 provides a cell with increased resistance to the induction of apoptosis. Additional members of the Bcl-2 family have been identified in mammalian tissues. Some members, such as Bcl-2, Bcl-X_L, and Bcl-w, confer resistance to apoptosis. Other members, such as Bax, Bad, and Bcl-X_S, have been demonstrated to promote apoptosis (95, 96). Studies have demonstrated that members of the Bcl-2 family are able to homo- and heterodimerize with each other, and thus allow facilitate antagonizing or enhancing the function of one another (97, 98). Bcl-2 has been implicated in preventing apoptosis by three mechanisms of action. The first potential mechanism is the regulation of ions, specifically Ca^{2+}, across the mitochondrial membrane. Bcl-2 has been shown to regulate mitochondrial Ca^{2+} levels and the loss of membrane potential produced by the induction of apoptosis (99). Several studies have demonstrated that both Bcl-2 and Bcl-X_L may be pore-forming proteins that are able to insert into the membranes of intracellular constituents. The formation of pores in organelles, such as the mitochondria, has implications on membrane transport functions and the maintenance of membrane potentials (100). However, it is not clear what the implications of the modification of mitochondria membrane physiology is on the induction and progression of apoptosis.
Bcl-2 has been demonstrated to reduce the availability of cytochrome c in the cytoplasm. Cytochrome c participates in the activation of caspase-3, and thus, prevents the progression of apoptosis after early caspase activation events. It has been demonstrated that Bcl-2 is able to prevent the release of cytochrome c from the mitochondria (101, 102), and that both cytoplasmic Bcl-2 and Bcl-X\(_L\) are able to bind to cytochrome c and does so in competition with the pro-apoptotic factor, Bcl-X\(_S\) (103).

NF-κB is a ubiquitous transcription factor that is associated with the \textit{de novo} production of anti-apoptotic survival factors (104). Induction of apoptosis via death receptors, Fas and TNFR-1, is associated with activation of NF-κB. Thus, the induction of apoptosis leads to paradoxical activation of anti-apoptotic factors. There is speculation that this relationship represents a mechanism of cell cycle control. During apoptosis, TNF-receptor-associated factor-2 (TRAF-2) is recruited to the death receptor. TRAF-2 is able to activate the SAPK/JNK pro-apoptotic pathway, and NF-κB-inducing kinase (NIK) (105). NIK activates inhibitory-κB kinase (IKK), which has been demonstrated to phosphorylate IκBα (106). The phosphorylation of IκB triggers proteosome-mediated degradation of IκB and the active translocation of NF-κB to the nucleus (107).

\textbf{D.2. The Detection of Apoptosis in vitro and in vivo}

The identification of apoptotic cells and necrotic cells is crucial to the understanding of the mechanism of xenograft injury and rejection. By understanding how the immune system induces cytotoxicity of xenograft tissues, one would be able to develop strategies to prevent cell death based either on preventing apoptosis or necrosis. This is unlike the prevention of allograft transplant rejection, which relies primarily on immunosuppression, since lymphocyte activation is a necessary event. On the other
hand, xenotransplant rejection may be mediated by innate preformed components of the humoral immune system making immunosuppression impractical, and the prevention of the cytotoxicity by engineering the donor tissues attractive.

D.2.1. **Gross Morphological Changes**

Visible, fluorescent light, and electron microscopy techniques remains to be a valuable tool in determining the nature of cell death *in vivo* and *in vitro*. This is mainly due to the fact that the final manifestations of the initiation of apoptosis are the morphologic changes that ultimately lead to the demise of the cell. Combining visible light and fluorescence light microscopy techniques allow assessment of gross cellular morphology and nuclear morphology *in vitro* (Figure 5.) (108). A combination of nuclear binding dyes may be employed to determine if the cell membrane is intact by utilizing ethidium bromide, which is a cell membrane impermeable dye that has red fluorescence upon entry into membrane damaged cells (i.e. necrotic cells) and binding to DNA (109). The detection of apoptotic cells may be performed by determining if the genomic DNA is condensed or fragmented. Cell membrane permeable dyes, such as acridine orange (green fluorescence) and Hoechst 3342 (blue fluorescence), allow visualization of the nuclear DNA content and its distribution (i.e. intact or fragmented nucleus) (110). The drawback to this technique is that it requires fine judgment and experience to determine if a cell is necrotic or apoptotic, and thus is most useful for rudimentary screening purposes.
The gross cellular morphological and nuclear morphological characteristics of apoptosis include a shrunken cell with an intact cellular membrane producing blebs or boils containing cellular constituents (1.), the condensation of the cytoplasm (2.), and a shrunken nucleus shedding blebs of condensed DNA fragments (3.). Unlike necrotic cells, apoptotic cells typically have intact cell membranes and thus are unstained by membrane impermeable DNA dyes.

**Normal**

**Necrotic**

**Apoptotic**

Membrane Impermeable DNA Dyes
(e.g. ethidium bromide)
Electron microscopy provides a detailed image of a cell, and thus it is able to determine if a cell has many of the gross cellular cytoplasmic characteristics of apoptosis. Thus, a determination of membrane status, nuclear content, mitochondrial status, and cell size can easily be made. As with visible light and fluorescent light microscopy, transmission electron microscopy allows for easy identification of two key characteristics of apoptosis, namely chromatin condensation, and the formation of apoptotic bodies (8, 111). Unfortunately, this technique requires examination of individual cells and is not conducive to screening a large population of cells.

**D.2.2. Nuclear Biochemical Changes**

DNA fragmentation by endonucleases is a primary biochemical event that is characteristic of apoptosis. The activation of endonucleases leads to DNA cleavage of the chromatin between the nucleosomes to form fragments of multiple units of 180-200 kDa in size. This uniform fragmentation of the DNA produces a pattern that may be visualized by separating the fragments based on size utilizing an agarose gel containing ethidium bromide (108). The characteristic DNA-ladder has been used to determine the presence of apoptosis in experimental systems, and remains one of the most popular techniques. A variation of this technique is the “comet assay”, in which cells are embedded in a low gelling temperature agarose and lysed. The released DNA is allowed to migrate in an electric field, and visualized using ethidium bromide or other DNA-binding dye. The assessment of apoptosis is made based on the length of the “comet tail”, as the amount of DNA that is able to migrate is dependent on the number of strand breaks and the size of the DNA fragments (112, 113). Unfortunately, these techniques do
not allow identification of the cell type of an apoptotic cell nor the assessment of the number of apoptotic cells in a heterogeneous population.

Nuclear fragmentation can be detected in situ by first labeling DNA strand breaks and then visualizing the strand breaks using immunohistochemical methods. This technique is commonly referred to as terminal dUTP nicked end labeling (TUNEL) (Figure 6.) (114). The ends of DNA fragments are labeled by repetitive addition of dUTP conjugated to a biotin, digoxigenin or other immunohistochemical label onto exposed 3’ and 5’- hydroxyl ends (115). The intensity of the DNA labeling is parallel to the extent of the degree of DNA fragmentation. The advantage of this technique is that it may be employed to detect apoptotic cells in both tissue sections and single cell suspensions. This provides the flexibility to perform either microscopy or flow cytometry. TUNEL is satisfactory for identifying the initial fragmentation of the chromosomes that occurs in the early stages of apoptosis (116). This technique is particularly powerful if a microscopic technique is employed, as this allows simultaneous evaluation of both the biochemical marker of DNA fragmentation, and the evaluation of cell morphology to determine the presence of necrosis. However, sufficient non-specific degradation of DNA during the process of necrosis may also be labeled by TUNEL. This prohibits the use of TUNEL alone in experimental systems in which the same cytotoxic stimulus induces both necrosis and apoptosis (e.g. ischemic cytotoxicity). In these cases, TUNEL staining must be either be accompanied by a second technique to determine the level of necrosis in a sample by evaluating the overall tissue architecture of a tissue, and the distribution of the TUNEL positive cells. The tissue architecture may be evaluated using
The apoptosis of a cell initiates endonucleases that cleave the genomic DNA into fragments of 180 kDa (A.). The fragmentation of the DNA results in an increase in the abundance of exposed 3' and 5'-OH ends within the margins of the nuclear envelope. A digoxigenin-dUTP conjugate is added to the 3' and 5'-OH ends of the fragmented DNA by terminal deoxynucleotidyl transferase (B.). The digoxigenin-dUTP tails are labeled with an anti-digoxigenin antibody conjugated to fluorescein (C.). The fluorescein tag may be visualized under ultraviolet light (494 nm) to give a green fluorescence (523 nm).
hematoxylin and eosin stained serial sections for tissues, and vital dye staining for cell suspensions.

D.2.3. Membrane Biochemical Changes

The mitochondria of a cell experience some profound changes in its physiology upon initiation of apoptosis. The mitochondrial outer membrane is permeated by the insertion of the pore forming Bcl-2 protein resulting in modification of the mitochondrial membrane function and preventing the release of cytochrome c (101, 102). There is also an increase in the consumption of ATP by mitochondria during apoptosis (117). The net result is a decrease of the mitochondrial transmembrane potential (118). Although the full impact of these events is not well understood, they remain to be indicators of apoptosis. A decrease in the mitochondrial transmembrane potential may be detected by a reduction in the uptake of cationic lipophilic fluorochromes, such as rhodamine 123 (119). This technique should be performed in conjunction with vital dye staining in order to rule out the presence of necrosis in the sample.

During apoptosis there is loss of plasma membrane phospholipid asymmetry with externalization of phosphatidylserine residues (120). Annexin V binds preferentially to negatively charged phospholipids, such as phosphatidylserine, and in conjunction with propidium iodide staining may be used to detect apoptosis (121). Unfortunately, this technique is best used on unfixed cell suspensions, and is not appropriate for fixed cells or tissue sections.

D.3. The Biological Significance of Apoptosis

Unlike necrosis, apoptosis is an essential process in healthy tissue development and maintenance. Thus, apoptosis takes a biological role in providing a counter balance
for cell proliferation, and does so without invoking potentially damaging immunostimulatory and inflammatory processes. Apoptosis has been documented to be essential in embryonic development (122, 123), tissue renewal and repair (124), hormone dependent involution in adults (125), and immune system development, regulation and defense (126). The disturbance of the balance between cell proliferation and cell death may result in a variety of human diseases and disorders. For example, it has been demonstrated that a suppression of apoptosis plays a fundamental role in tumorigenesis (95), autoimmune diseases (127), and AIDS progression (128). In contrast, unregulated induction of apoptosis is thought to be the basis of many neurodegenerative (e.g. Alzheimer's disease) (129) and neurodevelopmental diseases (130).

With the exception of acute rejection, the role of apoptosis in mediating rejection of xenotransplants is currently unknown. However there is increased speculation that it may play a role in mediating hyperacute rejection and may play a role in delayed xenograft rejection. The sets of studies that are outlined in this thesis seek to clarify the role of apoptosis in xenotransplant injury and rejection.

D.4. The Role of Apoptosis in Acute Rejection

The detection of foreign or infected cells is primarily performed by specialized cytotoxic lymphocytes. The massive infiltration of activated cytotoxic lymphocytes is a characteristic of acute rejection (131). The cytotoxic lymphocyte specializes in the destruction of a particular target cell. They induce cytotoxicity by secreting cytotoxic cytokines such as TNF-α or IFN-γ, and by inducing cell death in a contact dependent process. Cell mediated cytotoxicity is mediated primarily by natural killer cells and cytotoxic lymphocytes. Cytotoxic lymphocytes may be CD8+ or CD4+; the difference
between the two being that CD8+ cytotoxic lymphocytes primarily use the perforin/granzyme system and CD4+ cytotoxic lymphocytes rely more on the Fas/FasL system (132). Thus in transplant rejection in which both CD8+ and CD4+ cells may be activated by directly or indirectly via an antigen presenting cell, the induction of apoptosis by cytotoxic lymphocytes could contribute to the rejection of a graft.

The cytotoxic lymphocyte may stimulate its target cell via engagement of a death receptor expressed on the surface of the target cell. It may utilize these pathways by producing and secreting TNF-α or upregulation of membrane bound FasL. Death receptor induced cytotoxicity relies on the aggregation of the receptor death domains for the recruitment of cytoplasmic pro-apoptotic adapter proteins. The recruitment of cytoplasmic adapter proteins leads to the formation of DISC and the activation of the pro-apoptotic caspase cascades via FADD (133).

Alternatively, apoptosis may be induced independently of death domain engagement via the production and secretion of granules containing perforin and granzymes. The constituents of the granules are synthesized 24-48 hours after the initial stimulation of the T lymphocyte via the T-cell receptor (TCR) (134). The activated cytotoxic T lymphocyte then infiltrates the graft, and recognizes its target cell via the TCR and the accessory molecules. The granules stream towards the site of contact, where they fuse with the lymphocyte membrane and release their contents directly to the target cell membrane (135). Perforin polymerizes in the presence of calcium to form channels in the target cell membrane (59). These channels allow the easy passage of the granzymes to diffuse into the cytoplasm of the target cell and activate the pro-apoptotic caspase cascades or to travel to the nucleus and directly induce apoptosis of the target.
Recent evidence has demonstrated that the participation of perforin is not necessary to the induction of apoptosis (136-138). This suggests that the granzymes alone are able to penetrate the target to induce apoptosis. Although this phenomenon is well documented in vitro, its contribution in vivo is unknown.

D.5. The Role of Apoptosis in Hyperacute Rejection

There is some speculation that apoptosis may play a role in hyperacute rejection (139, 140). Beranek, has suggested that apoptosis may mediate the rejection of guinea pig heart transplants by rats (140). This assertion is mainly based on his observations that the myocardial defects around the muscular vessels that are found in hyperacutely rejected hearts are preserved nuclei from cardiomyocytes fragmented into eosinophilic droplets. Furthermore, these defects occur to individual myocytes in the absence of an immune cell infiltrate. Beranek reasons that complement activation and the formation of the membrane attack complex allow entry of apoptotic factors, much like cytotoxic lymphocyte perforin allows the entry of granzymes. The mechanism by which the myocytes undergo apoptosis is by the influx of Ca\(^{2+}\) and the subsequent induction of Ca\(^{2+}\)-sensitive protease calpain, which mediates the digestion of fodrin located in the sarcolemma, intercalated disks, and Z-bands. Beranek suggests that since fodrin digestion by calpain is detected in the apoptosis of hematopoietic cells, fodrin digestion by calpain in myocytes is indicative of apoptosis.

D.6. The Role of Apoptosis in Delayed Xenograft Rejection and Accommodation

Recent studies have implicated a role for apoptosis in mediating delayed xenograft rejection and the accommodation of discordant xenografts. Delayed xenograft rejection is a form of injury to xenograft that is attributed to a host of putative
immunological mechanisms with a focus on endothelial activation. Endothelial activation is a process by which endothelial cells are stimulated to change in their morphology and physiology to provide a pro-coagulant environment and a loss of barrier properties (141, 142). The mechanism by which endothelial cell activation and delayed xenograft rejection are mediated requires further investigation. It is clear that current immunosuppressive protocols are insufficient to prevent this form of xenograft rejection. Accommodation is a physiological state in which a xenograft remains free of injury and rejection in an environment of xenogeneic antibody and active complement. Accommodation was first observed in the transplantation of ABO incompatible kidney allografts. The temporary depletion of either the recipient’s anti-A or anti-B antibodies allowed the engraftment of an ABO mismatched donor kidney even with the return of isohemagglutinins (143). This same phenomenon has been observed in discordant xenotransplant models in which plasmapheresis and immunosuppressive drug therapy were employed. Unfortunately, none of the transplanted animals survived due to complications associated with heavy pharmacological therapy and the requirement for plasmapheresis (144, 145). Currently, the induction of accommodation is the only known avenue to prevent delayed xenograft rejection.

The characterization of accommodated xenogeneic cells has revealed a potential role for apoptosis in mediating delayed xenograft rejection. Bach et al. have recently reported that endothelial cells and smooth muscle cells of accommodated hamster hearts transplanted into rats which were administered cobra venom factor (CVF) and cyclosporine A exhibit upregulated expression of anti-apoptotic genes (“protective genes”) such as A20, bcl-2, and bcl-XL in contrast to hamster hearts that undergo delayed
xenograft rejection (146). In addition, Bach et al. provides evidence that apoptotic endothelial cells may be found rejected xenotransplants that fail to undergo accommodation. These studies provide evidence that apoptosis is associated with xenograft injury and therefore may play a role in a delayed rejection process.
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II. Objectives

One of the major barriers to clinical xenotransplantation is the rapid and potent rejection of xenografts by the human immune system. This is mediated by the activation of the classical complement pathway by preformed, naturally occurring xenoreactive antibody. This leads to rejection within minutes to hours of revascularization of the xenograft, and thus it is referred to as hyperacute rejection. Most of these studies are based on surrogate models of various species combinations that are characterized as models of hyperacute rejection. Unfortunately, the majority of these models potentially lack specific human anti-xenograft immunological reactions. This factor is of major importance, since the major xenoantigen (Gal α 1,3 Gal) is expressed by the majority of potential xenograft animal donors, and not expressed by humans and Old World monkeys. Thus, species combinations that utilize donor and recipient species that both express Gal α 1,3 Gal as self antigens will lack any anti-xenograft responses that rely on the Gal α 1,3 Gal.

The humoral immunological reaction against xenografts has been extensively studied and has been characterized to be mainly a necrotic event directed against the endothelium of the primary vascularized xenograft. Thus, the strategies developed to inhibit human serum xenograft rejection have focused on preventing the necrosis of the endothelium. Currently, there has been little investigation to the contribution of the alternate form of cell death, apoptosis, to xenograft injury. Apoptosis has been implicated to mediate several clinical and experimental models of progressive organ dysfunction. Thus, it becomes imperative to characterize both necrotic and apoptotic
mechanisms of cytotoxicity in xenotransplant rejection in order to devise a coherent strategy to enable clinical xenotransplantation.

It is the focus of this study to investigate the role of both necrotic and apoptotic mechanisms of human serum anti-xenograft reactions. In order to achieve this end, the objectives of this study are as follows:

1. To develop a small animal model of human anti-xenotransplant humoral immunity.

2. To develop and investigate the efficacy of anti-human xenogeneic IgM monoclonal antibody therapy to prevent serum mediated necrosis of xenogeneic tissues in vitro and in vivo.

3. To investigate the contribution of apoptosis as a mechanism of xenotransplant injury in vivo and in vitro.

4. To investigate the contribution of human classical immunological mediators of xenotransplant rejection (i.e. antibodies, and complement) to apoptotic injury of xenogeneic tissues in vivo and in vitro.
Chapter 1: Pig Skin Engraftment onto Severe Combined Immunodeficient Mice

Introduction

The use of pig to human transplants may provide a source of suitable tissue to meet the shortfall of allogeneic tissue donation. Pig to human xenotransplantation is, however, complicated by the presence of human preformed natural antibody directed against pig antigens expressed on the xenograft endothelium cell surface. This form of anti-xenograft immunity is commonly known as hyperacute rejection (HAR) and is characterized by rapid rejection of primary vascularized organs (6,17). In contrast, xenogeneic skin transplants are secondarily vascularized with the circulation re-established by the third day post-transplant (147). It is well established that the cellular immune system plays a central role in the rejection of allogeneic and xenogeneic skin transplants (148-150). However, the contribution of the preformed anti-xenograft antibodies to xenograft skin rejection is unclear. Previous studies have demonstrated that presensitization of the recipient (151) and administration of anti-serum raised in hosts different than the skin graft recipient results in skin graft rejection by targeting the vascular bed (152-154). These studies demonstrate that antibody and complement can induce rejection of the graft, but they do not specifically assess whether pre-formed antibodies can induce rejection.

Severe combine immunodeficient (SCID) mice lack functional T and B cells and have no detectable antibodies in their serum. This allows the animal to be reconstituted with human antibody and immune cells, and thus provides an in vivo model of human immunity. In addition, these mice have been shown to tolerate xenogeneic skin grafts.
from a variety of animals. Immunodeficient mice reconstituted with human peripheral blood mononuclear cells have been successfully utilized to assess human anti-pig cellular immune responses \textit{in vivo} (155). The aim of this study is to characterize the engraftment of pig skin onto SCID mice and to investigate the humoral immune response of preformed human anti-pig xenoantibody against pig skin xenografts \textit{in vivo}.
Methods

A. Severe Combined Immunodeficient Mice

Inbred homozygous Severe Combined Immunodeficient (SCID) Beige mice C.B-17/lcrCrl-scidBR were obtained from Charles River Canada and were bred and maintained in microisolator cages. The mice were fed autoclaved food and water, and all manipulations were done in a sterile laminar flow hood. All experiments were performed on mice 4-6 weeks old.

B. Harvesting of Thin Split Skin Grafts from Pig Donors

Outbred domestic swine served as skin graft donors. Pig donors (n=20) weighing approximately 30-50 kg were anesthetized by intramuscular injection of an atropine (0.1 mg/kg), acepromazine (0.2 mg/kg), and ketamine HCl (10 mg/kg) cocktail, and maintained on inhalation of 2% halothane. Anesthetized animals were intubated and maintained at 15 breaths/min, and given up to 1 L lactated Ringers solution I.V. Two to three rectangular 5 cm² split-thickness pig skin grafts (STSG) were taken from the thorax and abdomen of each donor using a straight razor. The skin grafts ranged from 100 to 150 μm in thickness. Evaluation of the uniformity of graft thickness was based on the graft’s translucency and the bleeding pattern of the exposed dermis of the donor site. Harvested skin grafts were placed in sterile 0.9% saline on ice, washed of any blood by 3 exchanges of ice cold sterile saline, and transplanted within 1 hour.
C. Transplantation of STSG onto SCID Beige Mice

SCID Beige mice (n=57) were anesthetized using 0.2% Avertin anesthetic. The right flank was shaved and swabbed with Betadine surgical scrub solution (Purdue Frederick Inc., Pickering, ON). A full-thickness window 1 cm in diameter was removed using iris scissors. Pig skin of uniform thickness (100-150 μm) was transplanted onto the exposed body wall of the right flank of SCID Beige mice. The STSG were trimmed to size and sutured in place using 4-0 braided polyester nonabsorbable sutures. The engrafted skin was treated with an antibiotic ointment and covered with a bandage. The bandage was left on for at least 3 days to ensure adequate protection of the pig skin graft. All pig skin transplants onto SCID Beige mice were performed in a sterile laminar flow hood using autoclaved instruments.

D. Histological Analysis of Engrafted Pig Skin

On days 0, 3, 7, 14 and 30, 300 μl of a 25% (v/v) Indian ink:0.9% I.V. saline solution was injected I.V. into the lateral tail vein of the SCID mice (n=5/group) and the mice were sacrificed immediately after injection. The grafts and the underlying mouse body wall were harvested and fixed in 10% neutral buffered formalin. Cut sections of the grafts were examined for the presence of ink in intact blood vessels in eosin stained slides. Hematoxylin and eosin stained slides were used for assessment of inflammatory response, cell viability, and dermal architecture.
E. In situ Hybridization of Pig and Mouse Specific DNA Probes to Transplanted Pig Skin Grafts on SCID Mice

*In situ* hybridization of labeled isolated pig or mouse genomic DNA to the nuclear DNA of cut paraffin section was performed to determine the species origin of the vasculature of the porcine STSG. In brief, STSG sections from day 30 mice that previously received Indian Ink I.V. were processed and mounted on double silanated slides. Both pig and mouse genomic DNA (Clonetech, CA, USA) were labeled with biotin using a commercially available biotin dUTP nicked translation labeling kit (Clonetech). Hybridization of the labeled probe to the nuclear DNA of cut sections was performed overnight at 37°C. The sections were then washed and exposed to an avidin/alkaline phosphatase conjugate, and then to NBT/BCIP substrate (Gibco, NY, USA) for visualization of the hybridized probe.

F. Preparation of Human Serum from Human Plasma

Freshly frozen and thawed human AB plasma units were collected by the Canadian Red Cross and obtained from the Hospital for Sick Children. At least 10 units (150-200 ml each) were pooled and clotted by adding CaCl$_2$ to create a 10 mM plasma solution. The plasma was then incubated at 37°C for 30-45 minutes to allow clotting. After a solid clot had formed, the human serum was removed, aliquoted, and frozen at -70°C. The total protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) and the serum was adjusted to 55 mg/ml total protein with physiological phosphate buffered saline. All manipulations of the AB plasma and serum were performed under sterile conditions.
G. Reconstitution of Human immunity into SCID Beige Mice

SCID mice transplanted with pig skin were injected with 1.0 ml of 55 mg/ml total protein concentration AB human serum I.V. on day 3, day 7, and day 30 (n=8). Of the eight animals, five animals were observed for 24 hours for animal health and graft condition then sacrificed for histological and immunohistochemical studies, and three were observed for 7 days post injection to observe any delayed rejection response.

H. Immunohistochemical Analysis of Engrafted Pig Skin

Cut paraffin sections of mice injected with human serum were stained for the presence of human IgG and IgM in the presence of mouse tissues and the STSG as outlined by Hsu (156). In brief, 5 μm paraffin sections were mounted on silanated slides. The sections were dewaxed and rehydrated for digestion in 0.5% pepsin in 0.01 M hydrochloric acid. Rabbit anti-human IgG (1/4000 dilution, Dako, Carpinteria, CA) or rabbit anti-human IgM (1/1000 dilution, Dako) was then applied to each slide for 1 hour. Biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) was then applied to each slide for 30 minutes. The slides were then incubated with a streptavidin (Vector) for 30 minutes. This was followed by incubation with a biotinylated horseradish peroxidase (Vector). 3,3-diaminobenzideine tetrahydrochloride (Sigma, St. Louis, MO) was used as the substrate for the peroxidase. All incubations with antibodies, biotin and streptavidin were performed at room temperature and in a humidified chamber. The slides were counterstained with Harris hematoxylin.
I. Hemolytic Assay for Complement Activity

The complement activity of the human serum was determined by exposing sensitized sheep erythrocytes to doubling dilutions of test serum. In brief, 50 µl of serial dilutions (1:2 to 1:256) of the test serum were pipetted in triplicate into a round bottom 96 well plate kept on ice. Twenty-five microliters of a 1:50 dilution of goat anti-sheep erythrocyte polyclonal sera (Amboceptor 6000, Behring, Marburg, Germany) were pipetted into each well followed by 25 µl of 1% v/v sheep erythrocytes. The plate was sealed using parafilm and incubated at 37°C for 30 minutes, and centrifuged at 50 xg. The classical CH₉₀ is defined as the dilution of test serum that contains 50% pellet of sheep erythrocytes compared to the negative control (heat inactivated sera).

J. Porcine Endothelial Cell Lysis Assay.

All human sera were heated at 56°C for 30 minutes to inactivate complement. Porcine aortic endothelial cells (PAEC) were grown to confluency in 96 well plates and were incubated with either heat inactivated human serum, or PBS diluted in culture medium. After 30 minutes incubation at 37°C, the plates were washed twice with 150 µl of PBS and 100 µl of fresh SCID mouse complement diluted in culture medium was placed in each well for 30 minutes at 37°C. The plates were then washed twice with 150 µl of PBS and incubated with 50 µl of 2.5 mM Calcein AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. The plates were scanned with a fluorescence plate reader (485 nm excitation, 530 nm emission; Cytofluor 2300, Millipore). The percentage of live
cells was calculated by dividing the average of triplicate wells by the average of triplicate control wells (culture medium only) and expressing the value as a percentage.

K. Statistical Analysis

The data are presented as mean ± standard deviation of five samples. To compare multiple treatment groups, an analysis of variance was carried out. If there were significant differences, a t-test for comparison of pairs of groups was then used (157). A 95% confidence interval was used to assess significance.
Results

A. Engraftment of Pig Skin onto SCID Beige mice

STSG were able to engraft onto the flanks of SCID Beige mice and establish continuous blood flow with the recipient as early as 3 days after transplantation. Inflammation of the STSG became evident by day 7 as demonstrated by the development of erythema of the graft (Figure 7. B) and dilation of the skin graft’s microvasculature (Figure 8. B and C). Hematoxylin and eosin staining of cut paraffin embedded tissues revealed an intact and viable epidermis at all stages of engraftment. Between 7 and 14 days a large numbers of fibroblasts could be seen populating the dermis (Figure 8. D and E). This occurred at the same time as increased graft thickness was observed grossly, implying that graft thickening may be due to production of collagen bundles during this time period (Figure 7.). On day 7, all the STSG developed hemorrhage at the interface between the STSG and the mouse body wall. However, STSG were able to survive this and remained attached to the mouse body wall. The hemorrhage found in the STSG resolved between 14 and 30 days.

Cellular infiltrates were seen between days 3-14 (data not shown). The infiltration was primarily composed of neutrophils with few macrophages. Typically, the greatest population of infiltrates were found in the scar and dermis on days 3-7 after the transplant, but receded to populate the scar only by day 14 (Figure 8.).

Intravenous injection of Indian ink revealed ink in the upper vascular plexus of STSG and in the vessels of the mouse body wall as early as day 3 (but not on day 1), and was present in the STSG thereafter indicating perfusion of the graft by the recipient SCID mouse (Figure 9. A-D).
Figure 7. The engraftment of STSG onto the flanks of SCID Beige mice.

The engrafted STSG became inflamed with an accompanied hemorrhagic appearance between days 3-7 (A and B). The hemorrhage cleared between days 14-30 (C and D). Graft thickness increased from 0.5 to 1.0 mm to approximately 2 mm between days 3-14. All photos were taken at approximately X1.25.
Figure 8. The histological morphology of STSG engraftment onto SCID Beige mice.
Figure 8. The histological morphology of STSG engraftment onto SCID Beige mice.

Hematoxylin and eosin staining of cut paraffin embedded STSG revealed an intact and viable epidermis at all stages of engraftment (days 3-30). By day 3 a prominent scar could be seen being formed between the mouse body wall and the STSG (A). Between days 7-14 an inflammatory response was observed, and was characterized by a large number of fibroblasts and neutrophils populating the dermis, and a large number of dilated capillaries extending through the dermis of the graft between days 7-14 (B-D). Local areas of hemorrhage developed on day 7 (C); however the STSG were able to survive to day 14 (D). By day 30 the STSG were free of any signs of inflammation and most of the scar tissue was absorbed (E). All photos were taken at X200.
Figure 9. The assessment of STSG perfusion using intravenous Indian ink injection.

Indian ink injected intravenously is found in the upper vascular plexus of STSG as early as day 3 (B), and was present in the STSG on all days thereafter indicating perfusion of the graft by the SCID mouse (B-E). No Indian ink was present in animals injected on the same day as the skin transplant (A, day 0). All photos were taken at X400.
B. In situ Hybridization

The endothelial cells lining the blood vessels of the STSG stained positively with the pig DNA probe (Figure 10. C and D), but did not stain positively with the mouse DNA probe (Figure 10. A and B). The pig skin graft’s microvasculature containing particles of Indian ink (black particles marked by arrows shown in Figure 10. A-D) are primarily positive for pig DNA probe and not the mouse DNA probe, demonstrating perfusion of blood vessels of porcine origin by the recipient SCID mouse.

C. Assessment of Complement activity of Human Serum and the Assessment of Mouse Complement Activation by Human Preformed Xenoantibody

The freshly thawed human serum had a CH$_{50}$ titer between 1:32 and 1:64 demonstrating that the injected serum is capable of causing the lysis of opsonized cells (Figure 11.). In addition to the presence of active human complement, the activation of endogenous mouse complement could contribute to an antibody mediated rejection process. The data in Figure 12. demonstrates that mouse complement is activated by deposits of human preformed xenogeneic antibody to mediate the lysis of cultured PAEC.
Figure 10. The determination of the species origin of the STSG vasculature by *in situ* hybridization using species specific DNA probes.

Species specific DNA probes were used to identify the origin (pig or mouse) of the graft's microvasculature. (A) day 30 skin graft exposed to mouse DNA probe 200X magnification. (B) day 30 skin graft exposed to mouse DNA probe X400. (C) day 30 skin graft exposed to pig DNA probe X200. (D) day 30 skin graft exposed to pig DNA probe X400. The graft microvasculature containing particles of Indian ink (black particles are primarily positive for the pig DNA probe and not the mouse DNA probe, demonstrating perfusion of pig blood vessels.
Figure 11. The *in vitro* assessment of human serum complement activity.

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The complement activity of freshly thawed human serum was determined by incubating human serum with sheep red blood cells (SRBC) opsonized with goat anti-SRBC antibody. The CH₅₀ for freshly thawed human serum was between 1:32 to 1:64 dilutions. Heat inactivation of the complement activity of the human serum resulted in no hemolysis demonstrating that the destruction of the SRBC was due to complement activation.
Exposure of cultured PAEC to heat inactivated human serum (HIHS) and fresh SCID mouse complement (MC') resulted in a significant increase (p<0.05) in the percentage of lysed cells as compared to results in the presence of HI HS or MC' alone.
D. Immunohistochemistry of Pig Skin

A single injection of whole human sera on day 3, day 7 and day 30 did not result in HAR of STSG, nor did it result in a delayed rejection response. However, immunohistochemistry revealed deposition of both human IgG and IgM within the lumen of porcine vessels in the skin immediately after administration of human serum I.V. (Figure 13. A-D). Immunofluorescent staining for human complement revealed no deposition of C3 in the pig skin graft (data not shown).
Immunohistochemistry of human serum injected SCID mice with day 3 and day 30 pig skin grafts revealed deposition of both human IgG and IgM within the lumen of the porcine vessels in the skin (B and D). This was not observed in pig skin engrafted animals given saline (A and C). Photographs are of day 30 skin xenografts taken at X400.
Discussion

The vascular bed of a transplanted tissue is the primary target for rejection by both humoral and cellular components of the immune system. Loss of the vascular bed inevitably leads to a loss of the donor tissue, thus the prevention of anti-donor endothelium immune reactions is essential for successful transplantation. The use of pig skin as temporary dressing or to correct large defects of the skin may be desirable as an alternative to harvesting large autografts or when there is a lack of available autologous skin graft donor sites. Previous reports have demonstrated that syngeneic or allogeneic skin grafts develop a vascular continuity between days 1 and 2 resulting in perfusion of the graft on day 3 \((147, 149)\). This study demonstrates that the same is true for xenogeneic skin grafts engrafted in an immunoincompetent recipient. This is best illustrated by the deposition of ink particles in the STSG of SCID mice after intravenous injection. In addition, this study demonstrates that the donor endothelium remains the primary constituent of the vascular bed of the STSG, resulting in a sharp demarcation between the graft and the recipient at the base of the STSG. The specific process by which arterioles and venules of the graft are able to locate and form connections with the arterioles and venules of the recipient at this time is unknown. We speculate that the development of focal areas of hemorrhage on day 7, which resolved between day 14 and 30, might have been due to incomplete patency between the microvasculature of the STSG and the circulation of the recipient.

Reconstitution of the SCID mouse with human serum did not result in rejection of STSG despite deposition of both human IgG and IgM in the vessels of the graft and the presence of active exogenous human complement and endogenous mouse complement.
This is in contrast to the administration of anti-serum from animals sensitized against skin graft antigens, which demonstrates that isolated antibody or ascites free of active complement are able to cause rejection of skin grafts presumably by activating endogenous recipient complement (152, 154, 158). In addition, this is contrary to hyperacute rejection of skin grafts observed in presensitized hosts (151). One explanation for the discrepant finding is that preformed human xenoantibody is unable to activate mouse complement. However, our study demonstrates that SCID Beige mouse complement can be activated to lyse PAEC opsonized with human serum xenoantibodies. In addition, the human serum injected into the mice contained active human complement with a CH$_{50}$ titer of 1:32-1:64. A second possibility is that unlike previous studies that used immunosuppression to dampen a normal mouse immune system, SCID Beige mice have no functional T or B cells, and thus cannot contribute to a rejection response initiated by an exogenous immunologically active substance such as human antibody and human complement. Thus, the absence of vascular injury and complement deposition in the pig skin graft may be due to resistance of pig microvascular cells to complement mediated lysis. This hypothesis requires further investigation.

In summary, pig skin can be engrafted onto an immunosuppressed recipient and establish continuity with the circulation of the recipient as early as day 3. The process of engraftment may be complicated by inflammation and hemorrhage; however, the STSG are able to survive this crisis period and remain healthy and functional for the lifetime of the recipient. The microvasculature of the STSG was preserved for the duration of the study (30 days). Interestingly, identification of the origin of the microvasculature of the skin graft revealed a sharp demarcation between the graft and the recipient located at the
base of the STSG. This demonstrates that the microvasculature of the donor survives transplantation and is not replaced by recipient vessels. The presence of human preformed xenoantibody and active complement is unable to cause rejection of the STSG in this model. This is in contrast to previous studies in which a single injection I.P. of antiserum into mice induced delayed rejection in 85% of day 7 xenografts within one week after injection (159). Our results suggest that microvascular endothelial cells may be less susceptible to xenoantibody mediated rejection than other endothelial cells such as aortic endothelial cells, or that xenogeneic endothelial cells develop resistance to complement mediated antibody dependent rejection during the process of delayed, rather than immediate, revascularization. The results of the study imply that maintenance of xenogeneic skin grafts solely requires mitigation of cell-mediated immunological responses.
References


IV. CHAPTER 2: Specific Depletion of Human Xenogeneic IgM Prevents Complement Mediated Injury of Xenogeneic Tissues \textit{in vitro} and Hyperacute Rejection \textit{in vivo}.

Introduction

The antibodies responsible for xenotransplant hyperacute rejection are part of the repertoire of naturally occurring antibodies that are present in individuals who have not previously encountered the corresponding antigen. Naturally occurring antibodies have been thought to help protect against micro-organisms \textit{(160)}, facilitate clearance of damaged blood cells \textit{(161)}, and potentially regulate the activity of some serum borne growth factors \textit{(153)}.

Non-specific depletion of total antibody titers in recipients by plasmapheresis \textit{(162-164)}, or by anti-isotypic antibodies prolongs graft survival time \textit{(165-167)}. However, these methods for reducing xenogeneic antibody result in concomitant depletion of whole classes of antibodies, and other serum proteins that are beneficial to host protection, coagulation, and osmotic balance.

Having recognized the need for specific strategies to antagonize xenogeneic antibody to prevent hyperacute rejection, methods have been developed that exploit the fact that a significant amount of xenogeneic antibody is directed against a single xenoantigen, Galactose $\alpha 1,3$ Galactose ($\text{Gal } \alpha 1,3 \text{ Gal}$) \textit{(25,26)}. Infusion of $\alpha$Gal sugars has prolonged survival times of porcine xenografts in baboons. However, prevention of hyperacute rejection requires large quantities of soluble sugars, which results in significant morbidity of the treated recipients \textit{(20,168)}. More recently, Gal $\alpha 1,3$ Gal columns have been developed to prevent HAR \textit{(169)}. Alternatively, anti-idiotypic
antibodies have been developed that recognize the αGal binding sites of anti-αGal xenogeneic antibodies of both IgM and IgG isotype. The infusion of these anti-idiotypic antibodies is associated with very little morbidity while reducing the cytotoxicity of baboon serum to cultured porcine cells (170).

It has been reported that human xenogeneic antibodies that mediate cytotoxicity are primarily of the IgM isotype (171, 172). IgM’s structure is conducive to complement activation in that a single IgM pentamer is able to activate complement as it changes from a planar to a staple (tent-shaped) conformation upon binding to its antigen. This is in contrast to IgG, which relies on sufficiently dense immune complex formation in order to activate complement. Thus, it has been documented that IgM plays a significant role in mediating hyperacute rejection.

With the objective of specific neutralization of human xenogeneic antibodies to prevent hyperacute rejection with the least impact on the antibody repertoire, we have generated monoclonal antibodies directed against human xenogeneic IgM antibodies isolated ex vivo from porcine aortas (anti-XeIgM). This method does not rely on the isolation, selection, and characterization of porcine xenoantigens, and provides specific depletion of a specific isotype of xenogeneic antibody.

Anti-XeIgM immobilized on solid supports may be clinically useful to deplete xenogeneic antibodies at the time of transplantation. In order to test the efficacy of this therapy, human serum was passed through anti-XeIgM columns ex vivo and tested for its capacity to inhibit xenogeneic antibody binding to cultured porcine aortic endothelial cells in vitro, and their ability to prevent antibody-mediated complement lysis of xenogeneic tissues in vitro and in vivo.
Methods

A. Isolation and Culture of Porcine Aortic Endothelial Cells

PAEC were isolated of the vascular lumen of pig aortas obtained from the abattoir. The isolated cells were confirmed to be endothelial cells by uptake of acetylated LDL and by the cobblestone morphology on phase contrast microscopy. PAEC were cultured in 10% fetal calf serum (Immunocorp, Montreal, QC, Canada) in M199 (Gibco) and passaged using trypsin-EDTA (Gibco) in 100 mm culture dishes (Falcon, Oxnard, CA). Cells from passage 4-10 were passaged into 96 well plates (Linbro) for experiments.

B. Preparation of Human Serum from Human Plasma

The human serum was prepared as outlined in Chapter 1, Method F. Heat inactivated human serum samples were produced by incubating human serum in a 56°C water bath for 30 minutes. All manipulations of the human plasma and serum were performed under sterile conditions. All the human AB plasma units were collected by the Canadian Red Cross.

C. Depletion of Xenogeneic Antibodies with Anti-xenogeneic IgM Antibody Columns

Anti-xenogeneic IgM antibody monoclonal antibodies (anti-XeIgM) was produced as outlined previously (us patent no. 5,785,996) (173). Approximately 25 mg of anti-XeIgM (XM-23, I7-7, I7-18 all of the IgG isotype) were coupled to 3.5 ml of swollen CNBr-activated Sepharose 4 fast flow columns (Pharmacia Biotech, Uppsala,
Sweden) utilizing manufacturer's instructions. In addition, 25 mg of mouse IgG (Sigma) were coupled to 3.5 ml of swollen Sepharose 4 fast flow as an isotype control column (mIgG).

Prior to application of human serum, 30 ml of 10 mM phosphate buffered saline (pH=7.2) were used to equilibrate the column. Four milliliters of filtered (0.45 μm filter, Millipore, Bedford, MA) human serum were passed through each column. The first 3 ml of fluid from the column were discarded. The next 5 ml were collected since this represented approximately 90% of total protein of the applied sample. Protein concentrations were determined by a Bradford protein assay (Bio-Rad Protein Assay, Bio-Rad). After sample collection, the columns were washed thoroughly with filtered 10 mM phosphate buffer. The columns were eluted using filtered 0.1 M glycine buffer pH=2.5. The elutions were neutralized (pH=7.0) using 1 M NaOH and stored for densitometry analysis.

All depleted serum samples and whole serum were concentrated using Centriprep 10 concentrators (Amicon, Oakville, ON, Canada) using standard protocols outlined by the manufacturer. The concentrated serum samples were diluted to 55 mg/ml total protein concentration using 0.9% saline USP (Baxter, Deerfield, IL).

D. SDS-Polyacrylamide Gel Electrophoresis and Densitometry of Anti-xenogeneic IgM Antibody Columns Elutions

SDS-PAGE was performed according to the method of Laemmli (174). Ten micrograms of each sample were loaded per well on a 8-16% gel (Novex, San Diego, CA) and silver stained using a Bio-Rad Silver Stain Plus Kit (Bio-Rad). The stained gel
was scanned using a Bio-Rad Gel Doc 1000 gel (Bio-Rad) documentation system and the densitometry analysis of the image was performed using NIH Image v.1.57 (NIH, Bethesda, MD). The density of each band corresponding to IgM and IgG was compared to the density of a IgM or IgG protein standard using the formula: (Density of Band-Background)/(Density of Standard-Background)x100. Background was determined by measuring the density of an area of the gel that did not have a visible band.

E. Enzyme Linked Immunoabsorption Assay (ELISA)

Cellular ELISA was performed according to the method of Platt (175) with some modifications: 4-methylumbelliferyl phosphate (MUP, 100 μg/ml diluted in 25% diethanolamine in water, pH 10, Sigma) was used as the substrate for alkaline phosphatase and incubated for 1 hr at 37°C then read on a Cytofluor 2300 fluorescent plate reader (360 nm excitation, 460 nm emission, Millipore).

F. Porcine Aortic Endothelial Cell Lysis Assay

All human serum samples were heated inactivated as outlined above. PAEC were grown to confluency in 96 well plates and incubated with either human serum samples, or PBS diluted in cell culture medium. After 30 min incubation at 37°C, the plates were washed twice with 150 μl of PBS and 100 μl of fresh low toxicity rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada) were placed in each well for 30 min at 37°C. The plates were then washed twice with 150 μl of PBS and incubated with 50 μl of 2.5 mM Calcein AM (Molecular Probes) for 30 min at 37°C. The plates were scanned with a fluorescence plate reader (485 nm excitation, 530 nm emission; Cytofluor 2300,
Millipore). The percentage of live cells was calculated by dividing the average of triplicate wells by the average of three control wells exposed to culture media only and expressing the value as a percentage.

G. C57BL/6 Mice

Inbred C57BL/6 mice were obtained by Charles River Canada. The mice were bred and housed in sterile microisolator cages and fed sterile food and water. All handling of the mice was performed in a sterile laminar flow hood. All experiments were performed using 4-6 week old mice.

H. Intravenous Injection of Human Serum into C57BL/6 Mice

C57BL/6 mice (n=30) were warmed under a heat lamp and placed into a restrainer. The mouse’s tail was swabbed with 70% Ethanol and untreated human serum, treated human serum samples, or the equivalent volume intravenous 0.9% saline (Baxter) was injected into the lateral tail vein at approximately 1 ml/ min. The mice were observed at set time points (0 min, 5 min, 15 min, 30 min, 45 min, 1h, 1.5 h, 2 h, 4 h, 6 h, 12 h, 18 h and 24 h post-injection) to monitor them for morbidity and mortality. The hearts of all injected mice were collected and sectioned. Half the biopsy was fixed in 10% formalin and the other half the biopsy was snap frozen in liquid nitrogen. Each treatment group consisted of 5 mice (n=5).
I. Immunohistochemistry of Mouse Tissues

Paraffin sections were stained for human IgG and IgM deposition in mouse hearts as outlined in Chapter 1, Method H. All photos were taken at X400.

Complement staining was performed on flash frozen tissue embedded in O.C.T. Compound (Sakura, Tokyo, Japan). In brief, 5 μm sections were fixed in cold acetone at −20°C for 10 minutes. The slides were then incubated with rabbit anti-human C3c antibody (cross-reactive to mouse C3c, 1/20 dilution, Dako) at room temperature in a light shielded container for 30 minutes. The slides were observed under UV light using a green/yellow filter to visualize fluorescein positively labeled tissue. All fluorescence photos were taken at X200 for an exposure time of 1 minute.

J. Statistical Analysis and Calculations

The data are presented as mean ± standard deviation of at least five experiments. The statistical analysis of the data was performed as outlined in Chapter 1, Method K. The percentage inhibition was calculated as (Standard Value-Sample Value)/Standard ValueX100. Change in inhibition was calculated as (Inhibition of Sample 1-Inhibition of Sample 2)/Standard ValueX100.
Results

A. Anti-XeIgM Column Treatment of Human Serum Decreases IgM Binding but not IgG Binding to PAEC

Antibodies were coupled to sepharose beads and packed in columns. When human serum were passed through columns made from XM-23, 17-7, and 17-18 anti-XeIgM there was a significant (p<0.001 by single factor ANOVA, p<0.001 by t-test) reduction in XeIgM binding to PAEC by 83.2±2.1%, 60.7±2.5%, and 32.2±3.0% respectively, as determined by cellular ELISA. In contrast, the isotype control column, mIgG, did not significantly change XeIgM binding (Figure 14.). The anti-XeIgM columns did not significantly (p>0.05 by t-test) alter the binding of xenogeneic IgG after four passes (Figure 15.).

B. Analysis of Bound Proteins to Anti-XeIgM Columns

To characterize the antibody binding specificity of anti-XeIgM columns proteins eluted from XM-23 and mIgG were analyzed by SDS-PAGE and densitometry. Figure 16. is a representative gel of proteins eluted from a XM-23 column, the anti-XeIgM that was most effective at diminishing XeIgM binding to PAECs, and from a mIgG column. The proteins eluted from the XM-23 column were enriched with human IgM as demonstrated by a significantly (p<0.05 by t-test) greater amount of human IgM (79% of the density of hIgM standard) as compared to mIgG and untreated human serum (Figure 17.). However a significantly (p<0.05) lower amount of human IgG (31%) (Figure 18.) as compared to proteins eluted from a mIgG column (53%, 58%) or with human AB serum (52%, 62%).
Anti-XeIgM columns decreased anti-PAEC IgM binding as assessed by ELISA. Human AB serum was passed through each of the indicated columns four times. Human AB serum passed through a mIgG column did not show any significant depletion of xenoreactivity relative to untreated human AB serum. XM-23, I7-7, and I7-18 columns were all able to significantly (p<0.001) decrease xenoantibody binding to PAEC by approximately 83.2±2.1%, 60.7±2.5%, and 32.2±3.0% relative to untreated AB serum as determined by cellular ELISA.
Human AB serum was passed through the highest XeIgM binding column, XM-23, did not significantly decrease IgG binding to PAEC (p>0.05). Statistical analysis was performed using single factor ANOVA and t-tests for comparison of pairs of groups (p<0.001 by ANOVA and p<0.001 by t-test).
Figure 16. A representative SDS-PAGE of elutions from the XM-23 and mIgG (isotype and species control) columns.

Densitometry analysis was performed on silver stained SDS-PAGE loaded with 10 µg of protein/well to analyze the composition of the proteins that bind to the XM-23 column.
Figure 17. The density of elution IgM heavy chain bands relative to human IgM heavy chain standard (hIgM).

Analysis of bound proteins to anti-XeIgM columns (B) XM-23 elutions contained a significantly (p<0.05) more human IgM (hIgM) as compared to mIgG elutions and untreated human AB serum.
Figure 18. The density of elution IgG heavy chain bands relative to human IgG heavy chain standard (hIgG).

XM-23 elutions contained significantly (p>0.05) less human IgG (hIgG) as compared to mIgG elutions and untreated human AB serum. Statistical analysis was performed using single factor ANOVA and t-tests for comparison of pairs of groups (p<0.05 by t-test).
A comparable quantity of human serum protein, aside from human IgM, bound to both XM-23 and mIgG as these two antibodies are of the same species origin (mouse) and isotype. Human proteins that bind to mouse IgG outside of the bind site of the mouse IgG would bind equally to XM-23 and mIgG equally. This data, in conjunction with the ELISA data, suggests that these anti-XeIgM columns preferentially bind XeIgM over other antibody isotypes.

C. Depletion of XeIgM by Anti-XeIgM Columns Decreases Human Serum Cytotoxicity to Cultured PAEC in vitro

The efficacy of anti-XeIgM columns to prevent activation of complement was tested using an in vitro cell lysis assay. The complement in human serum (HIHS) and in human serum passed through an XM-23 column (HIXM-23) was heat inactivated. There were no significant differences in PAEC lysis produced by HIXM-23, HIHS, fresh rabbit complement alone (RC), and culture media (p>0.05). When PAEC were exposed to HIHS supplemented with RC, there were significantly fewer live cells (13% live cells as compared to culture media alone) than when PAEC were exposed to culture media (100%) (p<0.001). In contrast, HIXM-23 supplemented with RC did not decrease the number of live PAEC (126%) compared to cells treated with media alone, implying XM-23 columns removed complement activating xenogeneic antibodies (Figure 19.).
Depletion of XeIGM by XM-23 column decreases human serum cytotoxicity to cultured PAEC \textit{in vitro}. Exposure of PAEC in the presence of heat inactivated human AB serum and fresh rabbit complement (HIHS+RC) resulted in a significant decrease ($p<0.001$) in the number of live cells as compared to results in the presence of supplemented cell culture media alone (10% FCS M199). In contrast, exposure of PAEC in the presence of heat inactivated human AB serum passed through XM-23 and fresh rabbit complement (HIXM-23+RC) resulted in no significant ($p>0.05$) loss of live cells as compared to 10%FCS M199. Exposure of PAEC to RC, HIHS or HIXM-23 separately did not result in any significant ($p>0.05$) loss of PAEC.
D. Depletion of XeIgM by Anti-XeIgM Columns Prevents Human Serum Mediated Hyperacute Rejection in Mice.

Most animal species with the exception of Old World non-human primates express the major xenoantigen Gal α1,3 Gal on the cell surface of their tissues, and therefore both mouse and pig organs are subject to human serum-mediated HAR (18-22). Human serum was injected into the lateral tail veins of mice to assess the ability of HS to mediate HAR in mice and to establish a small model of human serum rejection of xenogeneic tissues in vivo. Figure 20. demonstrates that human serum causes HAR in C57BL/6 mice a dose dependent manner similar to that observed for pig organs. C57BL/6 mice administered 2.5 ml of human serum had a survival time of 0.74±1.01 h. Animals administered serum treated with human serum treated with negative control columns mIgG had a mean survival time of 2.62±3.50 h (Figure 21.). C57BL/6 mice given human serum pre-treated with XM-23, and I7-7 survived to the end of the study (24 hours) at which time they were sacrificed for histological and immunohistological analysis. This increase in survival time was significantly (p<0.001) increased as compared to mice administered untreated human serum, and mIgG treated human serum. Mice administered human serum treated with I7-18 had an increased survival time to 16±16.8 h which was significantly (p<0.05) increased compared to mice given untreated human serum, and mice given human serum treated with mIgG (Figure 21.).
Figure 20. The lethality of intravenous administration of human serum into mice.
Figure 21. The prevention of hyperacute rejection by anti-XeIgM column treatment of human serum.
Pathological examination of heart biopsies of mice given 2.5 ml of human serum and 2.5 ml of mIgG treated human serum revealed many of the classical characteristics of HAR (Figure 22., HAR and mIgG), including fibrin deposition, vascular thrombosis, and edema. However, these tissues did not have conspicuous hemorrhage that has been seen in other models. Animals given saline and animals given human serum treated with anti-XeIgM displayed no classical signs of HAR (Figure 22., Saline and XM-23).

Two immunohistochemical markers of serum rejection are the deposition of human antibodies and complement in the xenograft. C57BL/6 mice given saline injections did not stain positively for either human IgG or IgM (Figure 23.). Mice given human serum (HAR), mIgG treated, anti-XeIgM had positive staining for human IgG. Immunohistochemical staining for human IgM revealed positive staining in mice treated with human serum (HAR), and mIgG treated human serum. In contrast, mice treated with anti-XeIgM did not stain heavily for human IgM.

Examination for the presence of complement deposition revealed that mice given saline or XM-23 treated human serum did not have deposits of C3c within their myocardium. However, mice given human serum and mIgG treated human serum stained positively for complement along the membranes of the heart tissue (Figure 24.).
Figure 22. The histological morphology of mice exposed to untreated and treated human serum.

The morphology of heart biopsies from C57BL/6 mice treated with human serum. Mice administered human serum (HAR) have fibrin deposition (1), vascular thrombosis (2), and microthrombi located the cardiac mass (3). This is in contrast with heart biopsies from mice administered anti-XeIgM serum (XM-23), which presented morphological characteristics indistinguishable from saline treated animals (Saline). Few cellular infiltrates were found in the biopsies.
Figure 23. The immunostaining of mouse hearts for the deposition of human IgG and IgM.
Figure 23. The immunostaining of mouse hearts for the deposition of human IgG and IgM.

Animals given saline injections did not stain positive for either human IgG or IgM. Animals given untreated human serum (HAR) and mIgG serum intravenously had heavy positive staining of IgG and IgM. In contrast, hearts from mice administered anti-XeIgM serum (XM-23) had heavy IgG staining along the membranes of the mouse cells, but did not stain positively for IgM deposits. All photographs were taken at X400.
Figure 24. The immunofluorescent staining for complement deposition in mouse hearts.

Immunostaining for complement deposition in mouse hearts. Mice given untreated human serum (HAR) and mIgG serum had strong fluorescent staining indicating complement deposition and complement mediated injury to the myocardium and vascular tissue. Mice administered saline or anti-XelgM serum (XM-23) did not have any detectable deposition of complement. All photos were taken at X200 with an exposure time of 1 minute.
Discussion

Xenogeneic antibody binding is a critical event that mediates hyperacute rejection of discordant xenografts. Thus, inhibition of xenogeneic antibody binding or depletion of xenogeneic antibodies prior to xenotransplantation provides an efficacious therapy to prevent hyperacute rejection without major modification to the donor or the recipient (5-7,28-31).

The development of specific therapies directed against xenogeneic antibodies of isotype(s) that are the primary mediators of xenotransplant hyperacute rejection would allow prevention of hyperacute rejection without total depletion of antibodies that facilitate other physiological processes such as anti-microbial activity (176), the removal of damaged blood cells (177), and the regulation of the biological activity of growth factors (178).

This study demonstrates the feasibility utilizing anti-XeIgM therapy. A small panel of anti-XeIgM coupled to a solid substrate can substantially and specifically inhibit human XeIgM binding in vitro. XeIgM binding can be inhibited as much as 83.2±2.1%, which is significantly different from isotype control and untreated human serum. Furthermore, anti-XeIgM columns do not significantly reduce the binding of xenogeneic IgG to PAEC in vitro. Evaluation of the elutions from anti-XeIgM by SDS-PAGE and densitometry revealed an enrichment of IgM as demonstrated by a significant increase in the band density of human IgM heavy chain as compared to mIgG and untreated human serum.
The inhibition of xenogeneic IgM antibody binding alone can prevent antibody-mediated complement dependent lysis of PAEC in vitro. Anti-XeIgM treated serum significantly prevented the cytotoxicity of PAEC as compared to untreated serum.

It is documented that Gal α 1,3 Gal is a major antigen common to many mammals, such as pigs and mice, that is not found in humans and Old World monkeys (24). This antigen is the major target for human xenogeneic antibody, and thus the same xenogeneic antibody directed against Gal α 1,3 Gal mediates complement injury to both mice and pigs (22). C57BL/6 mice injected intravenously with human serum die in a pace consistent with HAR. In addition, histological analysis of the mouse hearts revealed changes consistent with HAR found in other discordant xenotransplant animal models, including fibrin deposition and vascular thrombosis. Thus, the infusion of mice with human serum provides a small animal model of discordant xenotransplantation.

Immunohistochemical analysis revealed deposition of IgM, IgG, and complement fragment C3c. Treatment of human serum ex vivo with XM-23 and 17-7 prior to intravenous injection into C57BL/6 mice significantly increased the survival time of the mice to the end of the study at which time they were sacrificed for pathological analysis (24 h). This is in contrast to isotype control treated serum, which induced HAR by 2.62±3.50 h. Heart biopsies from mice receiving mIgG treated serum had histological and immunohistochemical profiles identical to mice given untreated human serum. In contrast, hearts from mice given serum treated with anti-XeIgM were free of signs of HAR and were indistinguishable from mice given an equivalent volume of saline. Immunohistochemistry revealed deposition of IgG, but not IgM or C3c. These results
suggest that a significant though incomplete depletion of XeIgM alone is sufficient to prevent activation of complement and HAR.

This study provides evidence that XeIgM plays a predominant role in the initiation of hyperacute rejection. Depletion of XeIgM alone was sufficient to prevent cytotoxicity \textit{in vitro}, and HAR \textit{in vivo}. These results support the potential utility of anti-xenogeneic antibodies to achieve highly specific and therapeutically useful depletion of xenogeneic antibodies.
References


CHAPTER 3: Human Serum Induces Apoptosis of Mouse Cardiomyocytes

in vivo.

Introduction

Hyperacute rejection (HAR) leads to massive cell lysis (necrosis) and rapid loss of graft function (5,179,180). Experimental mitigation of HAR by interventions such as the administration of anti-idiotypic antibodies and plasmapheresis (181,182) has revealed the development of a delayed form of vascular rejection. Thus, the xenograft response may be conceptualized to consist of two temporally and pathogenetically distinct phases: an immediate (minutes), or hyperacute rejection (HAR), and a delayed rejection response (hours to days).

Apoptosis is a ubiquitous process that regulates cell survival by mechanisms that are physiologically, morphologically, and biochemically distinct from necrosis. Typically, necrosis is characterized by an accidental loss of cell membrane integrity which triggers inflammation and immune cell recruitment, whereas apoptosis is characterized by a specific set of biochemical and cellular events that lead to a controlled loss of cells without the requirement for inflammation or immune cell recruitment (183-185).

Apoptosis has been demonstrated to occur in cardiac myocytes in response to a number of mechanical and biochemical stimuli (186-189). Recent studies have demonstrated that patients with end-stage heart disease have low but detectable levels of myocardial apoptosis (170,171), supporting the hypothesis that apoptotic deletion of cardiac myocytes may contribute to progressive myocardial dysfunction in certain clinical disorders (171).
Recent studies have implicated a role for apoptosis in xenotransplantation. Bach et al. have recently reported that endothelial cells and smooth muscle cells of accommodated hamster hearts transplanted into rats which were administered cobra venom factor (CVF) and cyclosporine A exhibit upregulated expression of anti-apoptotic genes ("protective genes") such as A20 and bcl-2, in contrast to hamster hearts that undergo delayed xenograft rejection. In addition, Bach et al. provides evidence that apoptotic endothelial cells are found in allografts undergoing chronic rejection (146).

These studies provide evidence that apoptosis is associated with graft injury and therefore may play a role in delayed rejection processes. Our group previously showed that porcine aortic endothelial cells undergo apoptosis and detachment from cell culture plates after 18 hours following xenogeneic stress (192). It has also been speculated that complement is involved in the induction of apoptosis of xenografts; specifically, that the terminal attack complex of complement allows the entry of apoptotic factors so those cells that are not eliminated by necrosis during HAR and thus may be subject to apoptosis (193).

The intravenous injection of human serum into mice is a model of the human xenogeneic response. By utilizing immunocompetent C57BL/6 mice, and nonobese diabetic severe combined immunodeficient (NOD SCID) mice, which do not possess functional T or B cells, have reduced NK cell activity, and have no detectable complement titer (194), one is able to assess the affect of human serum reconstitution in the presence and absence of functional immune factors and cells. In addition, inhibition of the complement-mediated HAR in these models using a graded reduction in human serum dosing sub-lethal dose (SLD) and CVF decolementation of human serum to test
the hypothesis that xenograft tissues are susceptible to human serum induced apoptosis which may mediate a delayed phase of xenograft rejection (DXR).
Methods

A. C57BL/6 Mice and NOD SCID Mice

Inbred C57BL/6 mice were obtained from Charles River Canada and NOD SCID mice were obtained from the animal facility at Mount Sinai Hospital, ON, Canada. The mice were bred and maintained in microisolator cages and fed autoclaved food and water. All manipulations were done in a sterile laminar flow hood. Sixty-five 5 week old mice were used to complete these experiments.

B. Preparation of Human Serum from Human Plasma

Human serum was prepared from human plasma as outlined in Chapter 1, Method F. The heat inactivation of the human serum was performed as outlined in Chapter 2, Method B. The Canadian Red Cross collected all of the human AB plasma units used in these experiments.

C. Intravenous Injection of Human Serum into C57BL/6 and NOD SCID Mice

C57BL/6 mice (n=35) and NOD SCID mice (n=30) were warmed under a heat lamp and placed into a restrainer. The mouse’s tail was swabbed with 70% Ethanol and either a lethal dose (LD, 6.9 mg/g body weight), sub-lethal dose (SLD, 4.6 mg/g body weight), heat inactivated dose (HIHS, 6.9 mg/g body weight) of human AB serum, or the equivalent volume intravenous 0.9% saline was injected into the lateral tail vein at approximately 1 ml/min. The mice were observed at set time points (0 min, 5 min, 15 min, 30 min, 45 min, 1h, 1.5 h, 2 h, 4 h, 6 h, 12 h, 18 h and 24 h) and were sacrificed at 45 minutes and 18 hours for in situ analysis of apoptosis.
D. Immunohistochemistry of Mouse Tissues

Paraffin sections were stained for human IgG and IgM deposition in mouse hearts as outlined in Chapter 1, Method H. All photos were taken at X400.

Complement staining was performed as outlined in Chapter 2, Method H. All fluorescence photos were taken at X200 for an exposure time of 1 minute.

E. In situ Detection of Apoptosis by Terminal Deoxynucleotidyl Transferase Nicked End Labeling (TUNEL)

DNA fragmentation by endonucleases is typically associated with apoptosis. During the process of DNA fragmentation many more 3' and 5'-OH ends become exposed as compared to intact genomic DNA (89, 90). TUNEL involves labeling 3' and 5'-OH ends using digoxigenin labeled UTP in the presence of deoxynucleotidyl transferase (TdT) followed by an anti-digoxigenin antibody fluorescein conjugate (113,114). All TUNEL staining was performed using the Oncor ApopTag Kit (Oncor, Gaithersburg, MD) in accordance with the manufacturer's instructions. In brief, heart, lung, liver and kidney biopsies were fixed in 1% paraformaldehyde and embedded in paraffin wax. Paraffin sections were dewaxed and rehydrated for labeling of 3' and 5'-OH ends. The slides were incubated with terminal deoxynucleotidyl transferase enzyme for 1 hour at 37°C followed by fluorescein conjugated anti-digoxigenin antibody for 30 minutes at room temperature. Propidium Iodide (Molecular Probes) was used to stain the DNA in the sections. The stained sections were examined under UV light using a
green/yellow filter to screen for fluorescein positively labeled cells and a red filter to examine the propidium iodide stained cells.

**F. Complement Inhibition**

Purified cobra venom factor (CVF, Quidel Corporation, San Diego, CA) was used to inactivate the complement activity of human AB serum. In brief, 12.9 IU CVF were added per milliliter of human serum and incubated at 37°C for 4 hours. The complement activity of treated serum was determined by conventional CH₅₀ assay. Human serum samples were inactivated if the test samples were unable to cause hemolysis at a 1:1 dilution.

**G. Carbohydrate Inhibition**

Gal α 1,3 Gal (V Labs, Convington, LO) was dissolved into fresh human AB serum to create a 20 mM solution, and incubated for 4 hours at 4°C. After incubation, the samples were centrifuged at 10,000xg for 30 minutes to remove any precipitate. The supernatants from each sample were collected and tested *in vivo* by injection of C57BL/6 mice at a sub-lethal dose.

**H. Statistical Analysis of *in situ* Detection of Apoptosis and Calculations**

In order to assess the level of apoptosis, four visual fields of the left ventricular free wall and septum were taken at X400 magnification. Each visual field contained at least 150 cells/field. Each field was examined using a green/yellow filter to determine the number of positively TUNEL stained nuclei, and a red filter to confirm that the positive TUNEL stain was labeled DNA, and to determine the number of cells per field.
The percentage of apoptotic cells per tissue was calculated using the formula: \( \frac{\text{Number of (+)fluorescein cells}}{\text{Number of (+)PI cells}} \times 100 \).

The statistical significance between all treatment groups was determined using a one-way ANOVA, and the statistical significance between individual treatment groups was determined using a Student’s t-test (157). The results were considered statistically significant if \( p<0.05 \).
Results

A. Human Serum Injection into C57BL/6 and NOD SCID Mice

Mice and pigs express the major xenoantigen Gal α1,3 Gal, and both mouse and pig organs are subject to human serum-mediated HAR. Human serum was injected into the lateral tail veins of mice to assess the cytotoxicity of HS to xenogeneic tissues in vivo. Human serum causes HAR in C57BL/6 mice and NOD SCID mice in a dose dependent manner similar to that observed for pig organs (Figure 25. and 26.). C57BL/6 mice given a lethal dose of serum pretreated with Gal α 1,3 Gal (20 mM Gal α 1,3 Gal + LD) died at a pace consistent with HAR (0.07±0.04 h, Figure 25.), which was not significantly different (p>0.05) from the survival times of LD (0.74±1.01 h, Figure 26). Heat inactivation and CVF complement inactivation of LD significantly (p<0.05) prolongs the survival of mice to 24 hours indicating that the HAR response to human serum depends on the presence of functionally active complement. Control mice were given an equivalent volume of saline I.V. Animals that survived 24 hours lived out their normal life span.

Pathological examination of heart biopsies of mice given a lethal dose of human serum revealed many of the classical characteristics of HAR (Figure 27. B), including fibrin deposition, vascular thrombosis, and edema. However, these tissues did not have conspicuous hemorrhage that has been seen in other models. Animals given saline, CVF treated human serum, and animals given SLD displayed no classical signs of HAR (Figure 27. A, C, and D).
Figure 25. The survival of C57BL/6 mice infused intravenously with human serum.

Intravenous injection of human serum causes HAR of C57BL/6 mice in a dose dependent manner. C57BL/6 mice given 20 mM Gal α 1,3 Gal + LD died from HAR; thus demonstrating that a concentration of 20 mM is not sufficient to prevent human xenoantibody dependent complement injury of xenografts. Heat inactivation and CVF treated LD significantly (p<0.05) prolongs the survival of mice to 24 hours. Since these methods inactive the complement of the human serum exclusively, these groups demonstrate that endogenous mouse complement in C57BL/6 mice is not sufficiently activated by human antibody to cause HAR. Animals that survived 24 h lived out their normal life span. Intravenous injection of an equivalent volume of saline does not kill the mice. Statistical analysis was carried out by ANOVA and t-test for comparison of pairs of groups (n=5).
Intravenous injection of human serum causes HAR of NOD SCID mice in a dose-dependent manner similar to human serum HAR of C57BL/6 mice. Heat inactivation and CVF treated LD significantly (p<0.05) prolongs the survival of mice to 24 hours. Animals that survived 24 h lived out their normal life span. Intravenous injection of an equivalent volume of saline does not kill the mice. Statistical analysis was carried out by ANOVA and t-test for comparison of pairs of groups (n=5).
The key hallmarks of serum rejection are the deposition of human antibodies and complement in the xenograft. Mice given saline injections did not stain positively for either human IgG or IgM (Figure 28. A). Mice given either LD or SLD, regardless of treatment with heat inactivation, CVF, or 20 mM Gal α 1,3 Gal, (Figure 28. B) had heavy positive staining for human IgG and IgM (data not shown for IgM). Examination for the presence of complement activation revealed that mice given saline or CVF treated human serum did not have deposits of C3c within their myocardium (Figure 29. A and B). However, mice given LD, or 20 mM Gal α 1,3 Gal treated human serum stained positively for complement along the membranes of the heart tissue (Figure 29. C and D).

B. Assessment of Human Serum Induced Apoptosis of C57BL/6 and NOD SCID Hearts in vivo

HAR mediated by human serum is a necrotic process in which preformed xenoreactive serum antibodies are deposited on the vascular endothelium of the graft leading to the activation of complement. Apoptosis is a second potential pathway leading to cell death following xenogeneic stress. Examination of the hearts, lungs, livers and kidneys of mice administered serum revealed that only hearts had a significant (p<0.001 ANOVA) change in the number of apoptotic cells between SLD, LD, and saline treated control animals. Mice given LD (Figure 30. E and F) showed few apoptotic cells as compared to control animals (Figure 30. C and D). TUNEL analysis of histological tissue sections from mice given SLD and sacrificed in a time frame consistent with the pace of HAR (i.e. 45 minute survival, Figure 30. G and H), were also indistinguishable from
Figure 27. The histological morphology of mouse hearts exposed to human serum.

The morphology of heart biopsies from C57BL/6 mice treated with human serum. Mice administered an equivalent volume of saline as LD (A), CVF treated human serum (C), and SLD 18 h (D) showed no detectable changes in morphology. Mice administered LD (B) have fibrin deposition (1), vascular thrombosis (2), edema (3), and little hemorrhage (4). Few cellular infiltrates were found in the biopsies.
Animals given saline injection (A) did not stain positive for either human IgG or IgM. Animals given human serum intravenously (regardless of pretreatment with CVF or 20 mM Gal α 1,3 Gal) (B) had heavy positive staining of IgG. Human IgM staining had similar staining pattern as IgG (data not shown). All photographs were taken at X400.
Figure 29. The immunofluorescent staining of complement deposition in mouse hearts.

Mice administered saline or CVF decomplemented human serum did not have any detectable deposition of complement. Mice given LD or Gal α 1,3 Gal + LD had strong fluorescent staining indicating complement deposition and complement mediated injury to the myocardium and vascular tissue. All photos were taken at X200 with an exposure time of 1 minute.
Figure 30. A-D. The TUNEL of mouse heart biopsies of mice exposed to human serum.
Figure 30. E-H. The TUNEL of mouse heart biopsies of mice exposed to human serum.
Figure 30. I-L. The TUNEL of mouse heart biopsies of mice exposed to human serum.
Figure 30. The TUNEL of mouse heart biopsies of mice exposed to human serum.

A sub-lethal dose of human serum induces apoptosis in mouse myocardium.

Examination of mouse left ventricular myocyte nuclei using TUNEL was performed on five separate individuals per group (n=5). Bright green/yellow nuclei on left indicate presence of nicked ends characteristic of apoptosis (left side of panel). Propidium iodide staining (right side of panel) confirmed fluorescein positive cells to be DNA. SLD produces an increase in the number of apoptotic mouse myocardial cells after 18 h (I and J) as compared to other treatment groups. Animals given LD (E and F), and animals given SLD and sacrificed after 45 min (G and H) do not have a significant difference in the number of apoptotic cells as compared to animals administered an equal volume of saline (C and D). Heat inactivation (56°C for 30 min) is not associated with the induction of apoptosis (K and L). Arrowheads indicate positively labeled nuclei. Rat mammary gland was used as a positive control for TUNEL (A and B). All photos were taken at X400.
mice given saline (Figure 30. C and D). However, mice given SLD and sacrificed after
18 h (Figure 30. I and J) had a number of apoptotic cells scattered throughout the
myocardium. Pretreatment of human serum by heat inactivation completely inhibited
human serum-mediated apoptosis in mice given SLD and sacrificed at 18 h (Figure 30. K
and L).

Statistical analysis of the number of positively TUNEL stained cells per field was
performed on five animals per treatment group (n=5) and the results were expressed as
percentages. Examination of heart tissues revealed that C57BL/6 mice (Figure 31.) given
SLD 18 h had 2.69±1.26% apoptotic cells per field which was significantly higher than
that observed in the control animals after 18 h (0.29±0.40%), SLD 45 min (0.60±0.49%),
HIHS after 18 h (0.63±0.77%), and LD (0.64±0.48%; survival time < 30 min) (ANOVA
p=0.0002; t-test p<0.05 between SLD 18 h and all other groups). NOD SCID mice
(Figure 32) given SLD 18 h had 2.11±0.35% apoptotic cells per field. This was
significantly (p<0.05) greater than NOD SCID mice given saline (0.32±0.47%). SLD 45
min (0.53±0.67%), HIHS (0.19±0.26%), and LD (0.29±0.31%). No significant
differences were found in the apoptotic rates of the treatment groups of the other tissues.
Figure 31. The statistical analysis of apoptosis of C57BL/6 heart biopsies of mice exposed to human serum.

The average number of positively TUNEL stained cells is not significantly different (p>0.05, ANOVA) between saline treated, LD, SLD 45 min, and HIHS groups for C57BL/6 mice. SLD 18 h demonstrated a significant increase (p<0.001, ANOVA, p<0.05, Student’s t-test) in the number of positively TUNEL stained cells as compared to saline treated, LD, and SLD 45 min treated mice. Four separate fields of approximately 150 cells were counted per slide at X400, and each treatment group contained at least five animals (n=5).
Figure 32. The statistical analysis of apoptosis of NOD SCID heart biopsies of mice exposed to human serum.

![Bar chart showing apoptotic cell percentages]

The average number of positively TUNEL stained cells is not significantly different (p>0.05, ANOVA) between saline treated, LD, SLD 45 min, and HIHS groups for NOD SCID mice. SLD 18 h demonstrated a significant increase (p<0.001, ANOVA, p<0.05, Student’s t-test) in the number of positively TUNEL stained cells as compared to saline treated, LD, and SLD 45 min treated mice. Four separate fields of approximately 150 cells were counted per slide at X400, and each treatment group contained at least five animals (n=5).
C. Assessment of Cobra Venom Factor Decomplemented Human Serum Induced Apoptosis of C57BL/6 and NOD SCID Mouse Hearts in vivo.

The role of active complement to induce apoptosis was examined by using CVF to inactivate human serum complement. The administration of an equivalent dose of CVF treated human serum to both C57BL/6 and NOD SCID mice (Figure 33.) did not significantly alter the amount of apoptosis as compared to SLD 18 h (C57BL/6=2.67±1.32% versus 2.68±1.26%, NOD SCID=2.11±0.35% versus 2.28±1.27%, p>0.05).

D. Assessment of Carbohydrate Treatment of Human Serum Apoptosis of C57BL/6 and NOD SCID Hearts in vivo.

Carbohydrate inhibition of apoptosis was examined in vivo (Figure 34.). The administration of a sub-lethal dose of 20 mM of Gal α 1,3 Gal treated human serum to C57BL/6 mice significantly reduced the number of apoptotic cells compared to SLD 18 h (0.52±0.35% versus 2.69±1.26%, p<0.05).
Figure 33. The effect of complement inhibition of human serum on the apoptosis of C57BL/6 and NOD SCID mouse hearts.

The inhibition of complement activity by Cobra Venom Factor (CVF) did not significantly (p>0.05) affect the number of apoptotic cells in vivo between SLD 18 h and SLD 18 h pretreated with CVF. Each treatment group consisted of five animals (n=5).
Figure 34. The effect of Gal α 1,3 Gal treatment of human serum on the apoptosis of C57BL/6 and NOD SCID mouse hearts.

The pretreatment of human serum with 20 mM of Gal α 1,3 Gal resulted in a significant reduction (p<0.05) in the percentage of apoptotic cells as compared to sub-lethal dose after 18 h. Carbohydrate treatment was able to reduce the percentage of apoptotic cells to the same level as saline treated mice. Assessment of apoptosis was performed using TUNEL staining. Four separate fields of approximately 150 cells were counted per slide at X400, and each treatment group contained five animals (n=5).
Discussion

The deposition of preformed xenoreactive antibody directed against Gal α 1,3 Gal epitopes appears to be a central feature of HAR which characterizes discordant xenotransplantation models (18-22). Gal α 1,3 Gal is a widespread xenoantigen found on most mammalian species with the exception of human beings and Old World monkeys (24). Consequently, humans are able to hyperacutely reject the organs from a wide variety of animals. The binding of xenoantibodies ultimately results in the activation of complement via the classical cascade. Hyperacute rejection is characterized by several pathological features, including fibrin deposition, microvascular thrombosis, edema and hemorrhage.

Human serum, acting through the Gal α 1,3 Gal epitope, induces apoptosis of the cardiomyocytes in immunocompetent (C57BL/6) and immunodeficient (NOD SCID) mice. The reason why human serum induced apoptosis is restricted to the myocardium is currently unknown. Both C57BL/6 and NOD SCID mice administered a single LD intravenously invariably died within 1 hour post injection and displayed the pathological features characteristic of HAR. Immunohistochemistry revealed deposition of IgG, IgM and complement throughout the myocardium in HAR mice. The requirement for active complement to mediate HAR in this model is demonstrated by the prolongation of survival of mice administered heat inactivated human serum and CVF treated human serum. The rapid time course of rejection, the requirement for antibody and complement, and the pathological observed features, all support the validity of this model which mimics the human anti-Gal α 1,3 Gal xenotransplant rejection.
Mice given a lethal dose of human serum did not have a significant (p>0.05) increase in the percentage of apoptotic cells (C57BL/6=0.64±0.48%, NOD SCID=0.29±0.31%) as compared to saline treated mice. Administration of SLD 45 min resulted in a low percentage of apoptotic cells (C57BL/6=0.60±0.49%, NOD SCID=0.53±0.67%), and was not significantly (p>0.05) different from saline treated or LD treated mice. This implies that human serum-mediated apoptosis is not an essential event in early phases of rejection such as HAR.

Myocardial remodeling and progressive myocardial failure is associated with apoptosis. Clinical features of myocyte dysfunction can be subtle and not be immediately reflected in overall cardiac function. As a consequence, the impact of myocyte apoptosis is not fully appreciated (195). However, there is recent evidence that apoptosis occurs in the myocardium of patients with end-stage dilated cardiomyopathy (190, 191), and in the border regions of myocardial infarction (196). Animal models have demonstrated that myocyte apoptosis may be induced in response to a variety of stimuli (e.g. mechanical stress, ischemia-reperfusion) (186, 187, 189). Studies of chronic end-stage heart failure have revealed a relatively low (<0.2%) level of apoptotic cells (191); however, unopposed by cell proliferation, this may eventuate in the loss of a substantial percentage of the myocyte mass and the onset of heart failure and/or arrhythmias (197).

Mitigation of the complement-mediated HAR in this model using a graded reduction in human serum dosing and inhibition of complement using CVF allowed us to examine the therapeutically relevant hypothesis that apoptosis participates in the DXR. A single intravenous injection of SLD into C57BL/6 and NOD SCID mice resulted in the appearance at 18 h of apoptotic cells evenly distributed throughout the myocardium. The
percentage of apoptotic cells per field (C57BL/6=2.69±1.26%, NOD SCID=2.28±1.27%) was approximately one fold higher than that observed in animals given an equivalent volume of saline (C57BL/6=0.29±0.4%, NOD SCID=0.32±0.47%). We speculate that, like the studies of chronic end-stage heart failure, the 2.69% apoptotic rate over 18 hours may represent a significant contribution of myocyte dropout over the life-time of the xenograft in the absence of HAR or acute cellular rejection, and therefore an increase in myocyte apoptosis may account for cardiac dysfunction associated with DXR.

The apoptotic activity of human serum is shown to be heat labile, as demonstrated by the low percentage of apoptotic cells observed in mice administered HIHS (C57BL/6=0.63±0.77%, NOD SCID=0.19±0.26%). Importantly, the effects of heat inactivation demonstrate that the induction of apoptosis is not mediated by xenogeneic antibody, since heat inactivation does not affect the capacity of antibodies to bind to their epitopes. In contrast to heat inactivation, specific inactivation of the necrotic complement pathway by CVF treatment did not significantly alter the apoptotic activity of human serum as compared to SLD 18 h (C57BL/6=2.67±1.32% versus 2.69±1.26%, NOD SCID=2.28±1.23% versus 2.11±0.35%, p>0.05). Thus, there is no requirement for the complement activity to induce apoptosis, and therefore xenogeneic antibody and/or complement activation cannot account for the human serum-induced apoptosis observed in this xenotransplant model. Treatment of human serum with Gal α 1,3 Gal inhibits apoptosis of mouse myocyte cells in vivo. The treatment of serum at SLD with 20 mM Gal α 1,3 Gal significantly (p<0.05) reduced the percentage of apoptotic myocytes evident at 18 hours. However, treatment of LD with 20 mM Gal α 1,3 Gal did not significantly (p>0.05) prolong the survival of mice (survival time = 0.07±0.04 h) as
compared to administration of untreated LD (0.74±1.01 h). Thus, 20 mM Gal α 1,3 Gal is not a sufficient concentration to prevent antibody binding, opsonization of xenograft cells or complement activation, and thus does not prevent HAR (198). This is further demonstrated by the presence of antibody and complement deposits in mice given 20 mM Gal α 1,3 Gal treated human serum. More importantly 20 mM Gal α 1,3 Gal inhibition studies demonstrate that human serum apoptosis is inhibitable in the presence of xenoantibody and active complement, and suggest that there are other unknown constituents of human serum that bind Gal α 1,3 Gal, aside from xenoantibody. Since these studies include the injection of human serum into immunodeficient NOD SCID mice, they demonstrate that xenograft apoptosis does not require the participation of xenogeneic lymphocytes or natural killer cells. Studies are underway to identify the potential serum factors that may cause the apoptotic injury associated with DXR.

In summary, this study indicates that human serum, acting through the Gal α 1,3 Gal epitope, induces apoptosis of xenograft cardiomyocytes in vivo. Injection of human serum at doses insufficient to produce HAR revealed the development of a delayed apoptotic myocardial injury response, which is not attributable to the presence of xenogeneic antibody and complement, or cell-mediated events, as demonstrated by the induction of myocyte apoptosis in both C57BL/6 and NOD SCID mice. The ability of Gal α 1,3 Gal to inhibit apoptosis suggests that a putative human serum apoptosis factor that is (1) a heat labile serum factor; (2) a lectin directed against the major Gal α 1,3 Gal xenoepitope; and (3) supports the rationale for the use of anti-apoptotic strategies to preserve graft function.
References


VI. CHAPTER 4: Human Serum Induces Apoptosis of Mouse and Pig Myocytes

in vitro.

Introduction

Xenotransplantation holds the potential to address the critical shortage of viable organs available for transplantation. However, xenotransplants are subject to potent immunological rejection by serum borne factors, which mediate hyperacute rejection (HAR). Hyperacute rejection has been shown to result in the necrosis of the endothelium of the xenograft, by the activation of complement by antibody (179, 5-7). Inhibition of hyperacute rejection by inactivating complement in combination with cellular immunosuppression has lead to increased survival times (182). However, acceptance of xenografts remains elusive due to cytotoxic injury resulting in delayed xenograft rejection. In previous studies, we have demonstrated that infusion of cobra venom factor (CVF) treated human serum (i.e. complement inactivated human serum) into immunocompetent and severe combined immunosuppressed mice results in approximately a ten fold increase in the number of apoptotic cardiomyoctes as compared to saline treated animals, and to mice given untreated human serum, which results in HAR of the animal following I.V. infusion.

Beranek has proposed that apoptosis may participate in serum-mediated rejection. Specifically, the author suggests that the formation of the terminal attack complex of complement allows the entry of apoptotic factors into cells that are not eliminated by necrosis during the process of HAR (193). Bach et al. have reported that endothelial and smooth muscle cells of accommodated hamster hearts transplanted into rats which are
administered CVF and cyclosporine A exhibit upregulation of “protective genes”, A20 and bcl-2, which are known inhibitors of apoptosis (146).

Apoptosis is a cell-regulated process that influences cell survival in response to physiological and pathological conditions. It is distinct from necrosis in that specific cell pathways are activated to mediate cytotoxicity in the absence of inflammation or massive immune cell recruitment (184, 185). Pathological examination of myocardium that has been subject to a variety of mechanical and biochemical stresses has revealed the presence of apoptotic cardiomyocytes. As a result of these findings, many studies have been designed to determine if cardiomyocyte “dropout” by apoptosis contributes to progressive myocardial dysfunction (187, 190, 193). Should apoptosis occur in xenotransplants due to insult by factor(s) is human serum, sufficient injury may occur that would lead to the loss of the transplant.

The first objective of this study is to determine if human serum can stimulate xenogeneic cardiomyocytes to undergo apoptosis outside the context of an intact physiological system, in order to demonstrate that human serum directly induces apoptosis. The second objective of this study is to assess if pig cardiomyocytes, which represent tissues from a more relevant xenograft organ donor, are stimulated to undergo apoptosis by human serum. The third objective is to assess the capacity of normal human TNF-α (a well characterized mediator of apoptosis) concentration is able to stimulate apoptosis of xenogeneic cardiomyocytes.
Methods

A. Isolation of Rod Shaped Cardiomyocytes

Terminally differentiated rod-shaped cardiomyocytes are the basic functional unit of the heart. Moreover, injury and death of these cells result in heart failure that may lead to morbidity and mortality. In order to assess the significance of human serum apoptosis of cardiac xenotransplant tissues, the isolation of rod shaped cardiomyocytes was performed as illustrated in Figure 35. Ventricular biopsies were collected from both adult mice and pigs for separate isolations. The biopsies were washed thoroughly of blood to prevent contamination of red and white cells. The tissue was finely minced using iris scissors and the fragments were washed three times in Joklik’s S-MEM (Gibco) with 5 mM EDTA (Sigma). The fragments were incubated in a digestion solution of 2.5% trypsin (Gibco)/331 IU/ml collagenase type I (Worthington Biochemicals, Lakewood, NJ) in Joklik’s S-MEM with 5 mM EDTA for 15 minutes in a shaking water bath at 37°C. The digestate was pipetted vigorously using a wide bore pipette and the undigested tissue was allowed to settle. The supernatant containing single cells was collected and placed on ice. The pellet of undigested material was incubated with fresh digestion solution. This process was repeated four times to ensure adequate harvesting of rod shaped cardiomyocytes. The rod shaped cardiomyocytes were purified by layering the digestion supernatant on 4%BSA (Sigma)/media, and collecting the pellet after centrifugation at 50xg. The viability of the isolated culture was assessed by Trypan (Sigma) exclusion. Typical isolations yielded rod-shaped cells with visible striations with few
Figure 35. A representative diagram of the primary isolation of mouse and pig cardiomyocytes.

Rod shaped cardiomyocytes were isolated from both mouse and pig cardiac biopsies. The biopsies were finely minced with iris scissors (1.), and the minced pieces were incubated in a trypsin and collagenase digestion solution in a 37°C shaking water bath (2.). The digestate was centrifuged at 50 xg to pellet the undigested pieces from the isolated rod shaped myocytes (3.). The pelleted undigested pieces were placed in fresh digestion solution (4.), and steps 2-4 were repeated until no viable cardiomyocytes could be isolated. All viable rod shaped cardiomyocytes were pooled for testing (5.).
hypercontractile cells. Human fetal cardiomyocyte cultures were kindly provided by Dr. L. Hornberger (Hospital for Sick Children, Toronto, ON, Canada).

B. Production and Handling of Human Serum Samples

The human serum was prepared as described in Chapter 1, Method F. The heat inactivation of the human serum (HIHS) was produced as outline in Chapter 2, Method B. The Canadian Red Cross collected all of the human AB plasma units used in these experiments.

C. In Situ Detection of Apoptosis by Terminal Deoxynucleotidyl Transferase Nicked End Labeling (TUNEL)

TUNEL staining was performed using the Oncor ApopTag Kit (Oncor) in accordance with the manufacturer's instructions. In brief, treated cultures were collected into 1.5 ml tubes and washed three times in saline. The cardiomyocytes were fixed in 1% paraformaldehyde at 4°C overnight. The cardiomyocytes were permeabilized with 2:1 v/v ethanol:acetic acid and incubated with terminal deoxynucleotidyl transferase enzyme for 1 hour at 37°C followed by fluorescein conjugated anti-digoxigenin antibody for 30 minutes at room temperature. Propidium Iodide (Molecular Probes) was used to stain the DNA in the cardiomyocytes. The stained cardiomyocytes were suspended in mounting media (Nova Diagnostics, San Diego, CA) and transferred to glass slides. The slides were examined using UV light microscopy. A green/yellow filter was used to screen for fluorescein positively labeled cells and a red filter was used to visualize propidium iodide stained cells.
D. Inhibition of Human Serum Complement Activity Using Cobra Venom Factor (CVF)

CVF decomplemented human serum was produced as described in Chapter 3, Method D. The human serum samples were considered complement inactivated if the test samples were unable to cause hemolysis of opsonized sheep red blood cells at a 1:1 dilution.

E. Carbohydrate Inhibition of Human Serum Apoptosis

Galactose α 1,3 galactose (V Labs) treatment of human serum samples was performed as described in Chapter 3, Method G. The supernatants from each sample were collected and tested *in vitro*.

F. Measurement of TNF α Levels in Human Serum

Measurement of TNFα levels was performed according to the Protocols of Immunology using WEHI 1640 mouse fibrosarcoma cells kindly supplied by Dr. R. Gorczynski (Toronto General Hospital, Toronto, ON, Canada) (199). In brief, WEHI 1640 were plated in 96 well plates and cultured in 10% fetal calf serum (Immunocorp, Montreal, QC, Canada) in RPMI (Gibco) to confluency. Doubling dilutions of 20% human serum in media and 50 ng/ml human recombinant TNF α (rhTNF, Calbiomed, La Jolla, CA) in RPMI (Gibco) were added to each plate in triplicate, and incubated for 18 hours at 37°C. After washing, the plates were incubated with 2.5 mM Calcein AM (Molecular Probes) for 30 min at 37°C. The percentage of live cells was calculated by
dividing the average of triplicate wells by the average of three control wells exposed to culture media only.

G. Statistical Analysis of in situ Detection of Apoptosis and Calculations

In order to assess the level of apoptosis, four visual fields of at least 150 cells/field were assessed at X400 magnification. Each field was examined using a green/yellow filter to determine the number of positively TUNEL stained nuclei, and a red filter to confirm that the positive TUNEL stain was labeled DNA, and to determine the number of cells per field. The percentage of apoptotic cells per tissue was calculated using the formula: \((\text{Number of (+)fluorescein cells} / \text{Number of (+)PI cells}) \times 100\).

The statistical significance between all treatment groups was determined using a one-way ANOVA, and the statistical significance between individual treatment groups was determined using a Student’s t-test \((t^2)\). The results were considered statistically significant if \(p<0.05\).
Results

A. Assessment of Human Serum Induced Apoptosis of Isolated Mouse and Pig Cardiomyocytes in vitro

Healthy rod-shaped myocytes were isolated and purified from fresh cardiac biopsies, in order to insure that the culture was free of other cell types such as fibroblasts, endothelial cells, and smooth muscle cells. Figure 36. consists of two representative examples of a non-apoptotic rod shaped pig cardiomyocyte from a negative control group and an apoptotic cardiomyocyte as determined by TUNEL from a human serum treated experimental group. Statistical analysis of the treated groups demonstrates that both mouse and pig isolated cardiomyocytes had an increased percentage of apoptotic cardiomyocytes in the treated population upon exposure of 30% v/v human serum in culture medium (mouse = 33.51±4.5%, pig = 39.03±5.31%, Figure 37.). This increase was significant (p<0.05) as compared to the negative control saline treated cardiomyocytes (mouse = 17.98±2.4%, pig = 22.38±5.16%). Heat inactivation of human serum samples prior to exposure to cardiomyocytes restored the % apoptosis back to levels seen in the negative control (mouse = 16.97±4.84%, pig = 22.02±4.37%). In contrast, the allogeneic combination of human cardiomyocytes and human serum did not result in a significant difference between saline (12.21±1.76%), heat inactivated human serum (9.70±1.79%) and human serum (12.98±9.14%) treated samples.
Normal and apoptotic isolated rod shaped cardiomyocytes as seen under fluorescent microscopy after TUNEL. (A) is a normal (non-apoptotic) isolated pig rod shaped cardiomyocyte exposed to saline in culture media for 18 h at 37°C. (B) is an apoptotic isolated pig rod shaped cardiomyocyte exposed to human serum in culture medium for 18 h at 37°C. Apoptosis in TUNEL is demonstrated by strong green/yellow fluorescence of the nuclei indicating the presence of fragmented DNA. All photos were taken at 400X power and the film was exposed for 1.5 min.
Human serum induces apoptosis of isolated mouse, pig, and fetal human cardiomyocytes \textit{in vitro}. Exposure of isolated mouse (33.51\pm4.50\%) and pig (39.03\pm5.31\%) cardiomyocytes to 30\% v/v human serum in culture media (HS) significantly (p<0.05) increased the percentage of apoptotic cells of the culture (% apoptosis) as compared to mouse (17.98\pm2.40\%) and pig (22.38\pm5.16\%) cardiomyocytes exposed to 30\% v/v saline in culture media (Saline). This is in contrast to allogeneic combination of human cardiomyocytes and human serum (12.98\pm9.14\%), which did not significantly (p>0.05) change the % apoptosis as compared to Saline treated human cardiomyocyte cultures (12.21\pm1.76\%). The apoptotic effect of human serum could be abrogated by heat inactivating the serum (HIHS) (mouse = 16.97\pm4.84\%, pig = 22.02\pm4.37\%).
B. Assessment of Cobra Venom Factor Decomplemented Human Serum Induced Apoptosis of Isolated Mouse and Pig Cardiomyocytes \textit{in vitro}.

The role of active complement to induce apoptosis was examined by using CVF to specifically inactivate human serum complement. 30% v/v CVF treated human serum: culture medium did not significantly alter the % apoptosis for both mouse (38.47±7.50%) and pig (44.15±9.12%) cardiomyocytes as compared to saline treated mouse and pig cardiomyocytes (Figure 38.).

C. Assessment of Carbohydrate Treatment of Human Serum Apoptosis of Isolated Mouse and Pig Cardiomyocytes \textit{in vitro}.

Mice and pigs share a common antigen, Gal α 1,3 Gal, which is not expressed by humans. Gal α 1,3 Gal is been demonstrated to be a major antigen that mediates human anti-xenograft responses. The administration of human serum pre-treated with 20 mM of Gal α 1,3 Gal significantly (p<0.05) reduced the number of apoptotic cells for both mouse (22.75±4.91%) and pig (24.59±6.92%) cardiomyocytes as compared to saline treated animals (Figure 39.).
Inactivation of complement by CVF does not affect the ability of human serum to induce apoptosis of xenogeneic cardiomyocytes. CVF was used to specifically inactivate complement activity. CVF treatment (CVF + HS) did not significantly (p>0.05) alter the % apoptosis for mouse (38.47±7.50%) and pig (44.15±9.12%) cardiomyocytes as compared to HS treated mouse (33.51±4.50%) and pig (39.03±5.31%) cardiomyocytes.
Figure 39. The effect of Gal α 1,3 Gal treatment of human serum on the apoptosis of primary isolated mouse and pig cardiomyocytes in vitro.

The pretreatment of human serum with 20 mM Gal α 1,3 Gal inhibits xenogeneic apoptosis of cardiomyocytes. Cardiomyocytes exposed to 20 mM Galα 1,3 Gal treated human serum (20 mM Gal + HS) had a significant (p<0.05) decrease in the % apoptosis for both mouse (22.75±4.91%) and pig (24.59±6.92%) as compared to HS (mouse = 33.51±4.50%, pig = 39.03±5.31%). The % apoptosis of 20 mM Gal + HS was not significantly (p>0.05) different from saline treated samples.
D. TNF-α Induced Apoptosis of Isolated Mouse and Pig Cardiomyocytes in vitro.

One possible mediator of apoptosis that is found in human serum is TNF-α. In order to determine if TNF-α played any role in xenogeneic apoptosis by human serum, the TNF-α levels were measured and then medium supplemented with recombinant human TNF-α was used to induce apoptosis of isolated mouse and pig cardiomyocytes. WEHI 1640 fibrosarcoma cells are sensitive to TNF-α in a concentration dependent manner, and thus may be used to measure the activity of TNF-α in a solution. The human serum used to induce apoptosis of the mouse and pig cardiomyocytes has no detectable level of TNF-α as compared to the standard curve using rhTNF-α (limit of detection is approximately 1 ng/ml, Figure 40.). In addition, mouse (15.48±3.41%) and pig (12.92±4.18%) cardiomyocytes exposed to a concentration of 1 ng/ml rhTNF-α culture medium did not have a significant increase % apoptosis as compared to saline treated cardiomyocytes (Figure 41.). A concentration of 50 ng/ml rhTNF-α uniformly induced apoptosis of both mouse (74.18±25.10%) and pig (86.48±9.08%) cardiomyocytes. This was significantly (p<0.05) increased over saline treated cultures. Pre-treatment of 50 ng/ml rhTNF-α solutions with 20 mM Gal α 1,3 Gal did not significantly change the % apoptosis of mouse (61.56±27.36%) and pig (82.74±15.93%) cardiomyocyte cultures.
The TNF-α activity of normal human serum as measured by a WEHI 1640 cytotoxicity assay. The TNF-α levels of the normal human serum pool (■) used to conduct this study were assessed by comparison to a standard curve generated by assessing the cytotoxic activity of doubling dilutions of 50 ng/ml rhTNF-α to WEHI 1640 cells (♦). There was no TNF-α activity in the normal human serum down to a limit of detection of approximately 1 ng/ml (1:32-1:64).
Figure 41. The induction of apoptosis of primary isolated pig and mouse cardiomyocytes by human recombinant human TNF-α (rhTNF-α) in vitro.

Exposure of mouse (15.48±3.41%) and pig (12.92±4.18%) cardiomyocytes to 1 ng/ml rhTNF-α in vitro did not result in a significant (p>0.05) difference in the % apoptosis as compared to Saline treated groups. A concentration of 50 ng/ml rhTNF-α uniformly caused a significant (p<0.05) increase in the % apoptosis of mouse (74.18±25.10%) and pig (86.48±9.08%) cardiomyocytes as compared to Saline treated groups. Pre-treatment of 50 ng/ml rhTNF-α solutions with 20 mM Gal α 1,3 Gal did not significantly (p>0.05) alter the % apoptosis for both mouse (61.56±27.36%) and pig (82.74±15.93%) cardiomyocytes.
Discussion

HAR of xenografts by human serum is a central feature that complicates the implementation of xenotransplantation as a viable clinical therapy. Several strategies have been developed to prevent HAR. These strategies focus on disabling classical pathway complement activation either by removing xenogeneic antibody or by inactivating the complement system (165, 182, 200-203). In a previous in vivo study, we demonstrated that in the absence of HAR and in the absence of complement activation, approximately a ten-fold increase in apoptotic cardiomyocytes may be detected after an intravenous infusion of human serum into immunocompetent and severe combined immunodeficient mice.

In the absence of HAR and acute rejection, a third form of xenograft injury, delayed xenograft rejection, is revealed. At this time, the mediators and pathway that mediate this form of injury have yet to be determined. Studies by Bach et al. have demonstrated that rats that have accommodated hamster hearts and thus avoided HAR and delayed xenograft rejection, have upregulated expression of “protective genes” that are well known to prevent apoptosis (146). However, this study does not clearly demonstrate that apoptosis plays a role in mediating delayed xenograft injury.

The results of this study demonstrate that human serum is able to induce apoptosis of primary isolates of mouse and pig cardiomyocytes. Mouse (33.51±4.50%) and pig (39.03±5.31%) cardiomyocytes had a significantly (p<0.05) increased percentage of apoptotic cells as compared to saline treated negative control samples (mouse = 17.98±2.40%, pig = 22.38±5.16%, Figure 37). In contrast, the allogeneic combination of human cardiomyocytes and human serum (12.98±9.14%) did not result in a significant
(p>0.05) difference in the % apoptosis as compared to saline treated negative control samples (12.21±1.76%). Heat inactivation of the human serum prior to exposure to cardiomyocyte isolates decreased the % apoptosis to levels comparable to that in the negative controls (mouse = 16.97±4.84%, pig = 22.02±4.37%). This result suggests that the human serum factor(s) that stimulate cardiomyocyte apoptosis is heat labile. To investigate if the heat labile complement pathway was influential in the induction of apoptosis, the alternate method of complement inactivation, namely pre-treatment of human serum with CVF, was employed. The % apoptosis of CVF decomplemented serum (mouse = 38.47±7.50%, pig = 44.15±9.12%) was not significantly (p<0.05) different than untreated human serum exposed mouse (33.51±4.50%) and pig (39.03±5.31%) cardiomyocyte cultures (Figure 39.). All together, these results demonstrate that the human serum factor(s) that stimulate apoptosis are not antibody (a heat stable protein) and complement.

The major xenoantigen that has been proposed to be a focal point of human reactivity to xenografts is Gal α 1,3 Gal (18-21). Gal α 1,3 Gal is expressed by both mice and pigs (22). The treatment of human serum with Gal α 1,3 Gal (mouse = 22.75±4.91%, pig = 24.59±6.92%) results in a significant (p<0.05) decrease in the % apoptosis as compared to untreated human serum. This result, in combination with the absence of an increase in the % apoptosis in the allogeneic combination (i.e. human cardiomyocytes exposed to human serum), suggests that human serum-mediated apoptosis may be restricted to xenogeneic tissues.

TNF-α is a serum borne cytokine that is known to induce apoptosis. The normal human serum pool used in this study had an undetectable level of TNF-α activity using
TNF-α sensitive WEHI 1640 fibrosarcoma cells (limit of detection approximately 1ng/ml, Figure 40.). A solution of 1ng/ml TNF-α in culture medium did not significantly change the % apoptosis of mouse (15.48±3.41%) and pig (12.92±4.18%) cardiomyocytes as compared to saline treated cultures (Figure 41.). These results demonstrate that the endogenous level of TNF-α in the normal human serum pool is insufficient to increase in the % apoptosis of both mouse and pig cardiomyocytes. In contrast, 50 ng/ml TNF-α significantly (p<0.05) increased the % apoptosis of both mouse (74.18±25.10%) and pig (86.48±9.08%) cardiomyocytes as compared to saline treated cultures. This effect was not significantly affected by pre-treatment with 20 mM Gal α 1,3 Gal (mouse = 61.56±27.36, pig = 82.74±15.93).

In summary, this study is the first demonstration that human serum is able to directly induce apoptosis of xenogeneic cardiomyocytes in vitro. More importantly, it demonstrates that human serum induces apoptosis of pig cardiomyocytes, and thus suggests that pig xenografts, which are the proposed donors for clinical xenotransplantation, would be subject to apoptotic injury. The factor(s) present in human serum that initiates apoptosis of xenogeneic cells remains to be identified. Our study indicates that apoptotic injury does not depend on the presence of TNF-α, antibody, active complement, nor effector immune cells.
References


VII. Conclusions:

Xenotransplant rejection is a multifaceted immunologically driven process that is mediated by both humoral (7.25-26) and cellular effectors (5,11,17). Hyperacute rejection is a major barrier to xenotransplant engraftment (6,7,17). This reaction is mediated by xenogeneic antibody deposition on the surfaces of the xenograft tissues and the activation of complement that leads to massive membrane damage and the induction of necrosis (6,7). Cellular effectors also mediate rejection. In vitro and in vivo xenotransplant models demonstrate that cell mediated immunity will cause the destruction of xenogeneic tissue primarily by apoptosis. Fortunately, current immunosuppressive protocols are able to prevent this form of injury and rejection (204-207). Inhibition of hyperacute rejection and cellular rejection has lead to the unmasking of a third form of xenotransplant injury, termed delayed xenograft rejection (141,142,144,145). The mechanism of delayed xenograft rejection is currently unknown. However, under specific circumstances in select xenotransplant models delayed xenograft rejection can be averted in a process termed accommodation. Xenograft tissues that have undergone the process of accommodation have upregulated expression of anti-apoptotic A20, bcl-2, and bcl-XL genes suggesting a role for apoptosis in delayed xenograft rejection (146). However, it is unclear if the apoptotic stimulus is serum borne and part of the humoral immune system or requires the activation and participation of the cellular immune system. The overall objective of this study is to investigate the cytotoxic mechanisms by which xenotransplants may be rejected by human serum.

The investigation into specific human xenogeneic humoral immunity requires the development of an appropriate xenotransplant model that utilizes human immune
constituents instead of relying on a surrogate immunological system. The initial experiments utilized pig skin engrafted SCID mice and the passive transfer of human humoral immunity by intravenous injection of normal human serum. The pig skin grafts were shown to be resistant to human serum HAR and survive for the lifetime of the mouse. The process of pig skin engraftment preserved the donor porcine vascular bed, which could be distinguished from mouse vessels by species specific DNA probes from day 0 to the end of the study. The porcine vasculature remained as the primary vascular bed of the graft from the first day of perfusion (day 3) for the lifetime of the recipient. Reconstitution of the SCID mice by intravenous injection of human serum did not result in the selective rejection of the pig skin. Immunohistochemical analysis revealed the deposition of human IgG and IgM in the vessels of the graft, but the absence of complement deposition. The complement titer of the human serum was 1:32-1:64. Although this study does not provide a strict human anti-pig xenotransplant model, it revealed that mice were subject to hyperacute rejection much like pigs, and thus gave rise to a discordant human anti-xenograft murine model.

The prevention of hyperacute rejection by the specific depletion of human xenogeneic IgM by mouse anti-human xenogeneic IgM columns was tested using the human anti-xenograft murine model. By utilizing a small panel of anti-XeIgM antibodies coupled to a solid substrate, XeIgM binding was inhibited up to 83.2±2.1% relative to untreated serum. This reduction in XeIgM binding significantly reduced the ability of treated serum to mediate cytotoxicity of PAEC in combination with an exogenous source of complement. Intravenous reconstitution of mice with human serum leads to the deposition of IgG, IgM and complement in the tissues of the mice. More importantly,
human serum injection is able to cause hyperacute rejection of the recipient, as assessed histologically (i.e. vascular thrombosis, fibrin deposition, and microthrombi in the tissue mass) and a rapid pace of rejection. Due to the pace of rejection, the route of perfusion of the human serum, and the massive damage to the cardiac mass, we concluded that the mice die of cardiac failure. Intravenous injection of treated serum prevents hyperacute rejection and the deposition of IgM and complement. The histological profile of these tissues is indistinguishable from saline treated animals.

The anti-XeIgM therapy addresses the prevention of complement mediated injury and is shown by these experiments to prevent necrosis. Necrosis appears to be the major mediator of injury in the process of hyperacute rejection. Examination of hyperacutely rejected mouse hearts revealed few apoptotic cells as determined by TUNEL. However, mice that receive a sub-lethal dose of human serum have a significantly increased number of apoptotic cells as compared to normal untreated, saline treated, and hyperacutely rejected hearts. The induction of apoptosis by human serum is mediated by a heat labile molecule as demonstrated by the absence of apoptosis in the heat inactivated treatment group. Thus, heat stable molecules such as antibodies cannot be implicated in the induction of apoptosis in this model. Specific depletion of human serum complement by CVF did not significantly alter the proportion of apoptotic cells as compared to an equivalent dose of untreated serum. The presence of apoptosis in the absence of active complement clearly demonstrates that mediators of necrosis, namely antibodies and complement, are not required for the initiation of xenogeneic apoptosis by human serum. The treatment of human serum with the major xenoantigen, 20 mM Gal α 1,3 Gal, significantly prevented the apoptosis, but did not prevent xenoantibody binding and
hyperacute rejection. In total, these results strongly demonstrate that human serum mediates the apoptosis of xenogeneic cells via the major xenoantigen, and that human serum induced apoptosis is not mediated by traditional humoral immune components such as antibodies and complement.

Consistent with the results of the *in vivo* xenotransplant model, primary isolated mouse and pig cardiomyocytes undergo apoptosis upon exposure to human serum *in vitro*. In contrast, cultured human cardiomyocytes exposed to human serum did not result in a significant increase in the number of apoptotic cells as compared to saline treated cultures. As with the previous *in vivo* experiments, the inhibition of the complement activity by CVF did not alter the number of apoptotic cells as compared to untreated serum, and pretreatment with 20 mM Gal α 1,3 Gal resulted in a significant decrease in the number of apoptotic cells as compared to untreated serum. TNF-α is a known pro-apoptotic cytokine found in serum. Measurement of TNF-α activity in the human serum was undetectable to 1 ng/ml, and supplementation of culture medium with 1 ng/ml of rTNF-α did not increase the number of apoptotic cells in the culture. In contrast, 50 ng/ml rTNF-α was able to significantly increase the number of apoptotic cells. This concentration of rTNF-α was not inhibited by a concentration of 20 mM Gal α 1,3 Gal.

In combination with the in vivo study, these results demonstrate that human serum directly induces the apoptosis of both pig and mouse cells, and that human serum-mediated apoptosis cannot be attributed to complement, antibody, or TNF-α activity.

The significance of these findings impacts the clinical application of xenotransplantation, and helps define the anti-necrotic and anti-apoptotic strategies that should be employed in order to preserve the xenograft in the context of the human
immune system. These results demonstrate that human serum initiates both necrotic and apoptotic mechanisms which can injure the cardiac xenograft. Current investigations that focus on preventing hyperacute rejection and necrotic injury have demonstrated to be inadequate to enable long surviving xenografts. In light of this body of research and past investigations, this thesis embodies several novel findings. First, a novel in vivo model based on passive transfer of human serum immune factors was used to investigate human anti-xenograft immune responses and to elucidate the mechanisms of xenograft injury by human serum. The model has identical characteristics found in traditional xenograft experimental models and clinical xenotransplantation. As the model involves the passive transfer of human serum immunity into an animal, the impact of human immune factors are observed without reliance on surrogate animal immune systems. This is a crucial factor in identifying novel mechanisms of injury as the xenogeneic response is sensitive to differences between two select species, and thus other discordant models may not have the same injury pathways against a given species as the injury pathways found in the human immune system. Second, a novel apoptotic injury to xenogeneic tissues is observed in vivo and in vitro using the human anti-xenograft model. The apoptotic injury is separate from the necrotic injury mediated by the activation of the complement cascade by the deposition of xenogeneic antibody. Third, human serum induced apoptosis of xenogeneic tissues is directed against the major xenoantigen, as soluble antigen was able to inhibit apoptosis in both the in vivo and in vitro models. This is further supported by the absence of apoptosis in allogeneic combinations. Lastly, the human serum apoptosis of xenogeneic tissues does not rely upon the activity of various known immune factors
known immune factors such as, TNF-α, antibody, active complement, or effector immune cells, and thus these finding possibly represent the activity of a novel molecule.

The presence of a novel apoptotic injury requires anti-apoptotic strategies to be employed in order to prevent delayed xenograft rejection. This will require further investigation into the mechanism of human serum induced xenogeneic apoptosis, the isolation of the apoptotic molecule, and the investigation of the relevant apoptosis signal transduction pathways that are crucial in mediating xenogeneic apoptosis.
References


