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Development of an *In Vitro* Model for Accommodation

by

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To Mom, Dad, Joe and Baba.

Thank-you Pluto.

ABSTRACT

Accommodation is characterized by a resistance to complement-mediated lysis in the presence of normal antibody and complement levels. This state can occur, by an unknown mechanism, in xenotransplantation following antibody depletion. This study sought to develop an *in vitro* model to study physiological changes to porcine epithelial cells which result in resistance to complement-mediated lysis.

Porcine epithelial cells (LLC-PK1) were incubated with human group B serum for 1.5-96 hours. MTT cytotoxicity assays using Pel-Freez rabbit and human complement demonstrated a significant decrease in cytotoxicity ($p<0.05$). However, a reduction in cytotoxicity with 3% triton-x (positive control) after 72 and 96 hours may indicate that these findings were artifactual. There was no evidence of a change in antibody specificity as shown by immunoblots. The level of IgM, IgG, and IgA binding did vary over time which may indicate that each antibody class mediates different physiological effects upon binding to the cell surface.

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ABBREVIATIONS AND SYMBOLS

%	Percent
°C	Degree celcius
α1,2-FT	α1,2-fucosyltransferase
α1,3GT	α1,3-glucosyltransferase
αGal	Galα1-3βGal1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH_2)
αGal1-3Gal	Galα1-3βGal1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH_2)
μg	Microgram
μL	Microlitre
A20	Zinc finger A20
ABO	Human blood group antigen system
ADCC	Antibody dependent cellular cytotoxicity
ADP	Adenosine diphosphate
ADPase	Adenosine diphosphatase
ANOVA	Analysis of variance
AT-III	Anti-thrombin III
BRQ	Brequinar sodium
BSA	Bovine serum albumin
C1q	Complement component 1q
C2a	Complement component 2 fragment a
C3	Complement component 3
C3a	Complement component 3 fragment a
C3d	Complement component 3 fragment d
C4b	Complement component 4 fragment b
C5	Complement component 5
C5a	Complement component 5 fragment a
C5b	Complement component 5 fragment b
C5b-9	Membrane attack complex

C6	Complement component 6
C7	Complement component 7
CD16A	FcγRIIIA
CD59	Homologous restriction factor
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
CO_2	Carbon dioxide
cpm	Counts per minute
CRP	Complement regulatory protein
CsA	Cyclosporin A
CVF	Cobra venom factor
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
DSG	15-Deoxyspergualin
DXR	Delayed xenograft rejection
ECs	Endothelial cells
ELISA	Enzyme-linked immunosorbant assay
FVII	Factor VII; human coagulation cascade
FIX	Factor IX; human coagulation cascade
FX	Factor X; human coagulation cascade
F(ab')2	Fragment, antigen-binding (interchain disulfide bond is intact)
Fc	Crystallizable fragment (immunoglobulin non-antigenic binding fragment)
FK506	Tacrolimus
g	gram
Galα(1,3)Gal	Galα1-3βGal1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH_2)
gp 115/135	Triad complex of 115 kD, 125 kD, and 135 kD glycoproteins
gp 115	115 kD glycoprotein; β3 integrin
gp 125	125 kD glycoprotein; αIIb integrin
gp 135	135 kD glycoprotein; α2 integrin

gpIb	Platelet glycoprotein Ib
gpIIbIIIa	Platelet glycoprotein IIbIIIa
H antigen	α-1,2-fucosyl lactosamine (O blood group antigen)
HAR	Hyperacute rejection
HLA	Human leukocyte antigen
hr	Hour
HS	Heparan sulphate
IκBα	NFκB inhibitor
iC3b	Complement component 3bi
ICAM-1	Intracellular adhesion molecule 1
IgA	Immunoglobulin alpha (class A)
IgG	Immunoglobulin gamma (class G)
IgG1	Immunoglobulin gamma subclass 1
IgG2a	Immunoglobulin gamma subclass 2, isotype a
IgG2b	Immunoglobulin gamma subclass 2, isotype b
IgG2c	Immunoglobulin gamma subclass 2, isotype b
IgM	Immunoglobulin mu (class M)
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-13	Interleukin-13
INF-γ	Interferon gamma
kD	kiloDalton
L	Litre
LPS	Lipopolysaccharide
M	Molar
MAC	Membrane attack complex
MCP	Membrane cofactor protein

MCP-1	Monocyte chemotactant protein-1
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
MMF	Mycophenolate mophetil
MPA	Mycophenolic acid
mRNA	Messenger ribonucleic acid
MnSOD	Manganese superoxide dismutase
MTT	3-4,5-dimethyldiazol-2-yl-2,5-diphenyl tetrazolium bromide
NFκB	Nuclear factor κB
NHS	Natural human serum
NK cells	Natural killer cells
OD	Optical density
PAEC	Porcine aortic endothelial cells
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
sCR1	Soluble complement receptor type 1
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TBS	Triphosphate buffered saline
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Th1	T helper cell type 1 cytokine response
Th2	T helper cell type 2 cytokine response
TNF-α	Tumor necrosis factor alpha
TNF-γ	Tumor necrosis factor gamma
v	Volume
V	Volts

VCAM-1	Vascular adhesion molecule 1
VLA-4	Very late expressed antigen 4
vWF	von Willebrand factor
w	Weight
× g	Relative centrifugal force
XNAb	Xenoreactive natural antibodies

INTRODUCTION

Xenotransplantation, transplantation across species, is now being considered as a realistic alternative to allotransplantation in light of an ongoing critical shortage in human donor organs. Presently, the goal of xenotransplantation is to use organs from other species as a means of bridging the patient until a human organ becomes available. One of the primary issues surrounding xenotransplantion is the use of concordant donors versus discordant donors.

Concordant species refers to donors which are phylogenetically close such as baboon or chimpanzee and man. There appears to be a strong correlation between the phylogenetic distance of species and the existence of preformed xenoreactive antibodies which can initiate a rapid rejection process known as hyperacute rejection (HAR) (1). However, this is not to say that HAR between these species could not occur. Normally, there are low to undetectable levels of xenoreactive antibodies between concordant species so rejection is usually cellular and is consistent with allograft rejection (2). However, if antibodies do exist, such as a blood group incompatibility between man and baboon, rapid rejection similar to those of ABO-mismatched blood groups in allotransplantation can be initiated.

Discordant species are phylogenetically distant, for example pigs and man. The preformed naturally occurring xenoreactive antibodies (XNAbs) in the recipient result in HAR and once this process has begun, it is irreversible.

Obviously, the best donors with respect to organ acceptance would be a concordant species and clinical studies using human recipients have been done. Chimpanzee to human renal transplants performed by Reemtsma (3) demonstrated up to a

9 month survival. Rejection was slow and of a cellular nature. In contrast, Starzl (4) used the more phylogenetically distant baboon and only achieved a 19-60 day survival.

Prior to the use of cyclosporin (CsA) therapy, early cardiac xenotransplants were largely unsuccessful (5,6). In 1984, Bailey performed a baboon heart transplant in an infant which functioned for 20 days but was ultimately rejected due in part to an ABO-incompatibility (7).

Chimpanzee to human liver transplants were first attempted by Starzl who achieved graft survival from <1-14 days (8-12). Two baboon to human liver transplants by the same group in the early nineties survived 70 and 26 days (13-15).

Unfortunately, the use of chimpanzees and baboons for donor organs is fraught with difficulty including: small numbers available, expensive and difficult to breed, ethical problems and the possibility of disease transmission. For these reasons, the pig has been considered a possible alternative. Pigs are readily available, breeding pigs to suitable sizes is inexpensive, they are physiologically similar to human, and they can be bred in germ-free environments (16).

To date, there has been one attempt of pig to human orthotopic heart transplantation (17) and one pig to human auxiliary pig to human liver transplantation (18). Both of these experiments proved unsuccessful as both grafts succumbed to HAR. Before pig to human xenotransplantation can become a reality, there are many immunological barriers to overcome. Initially, HAR was the primary focus of investigators, but it now appears that even after HAR is prevented, a delayed rejection process occurs involving cellular infiltration.

MECHANISMS OF REJECTION IN XENOTRANSPLANTATION

In this review, focus shall be given to humoral rejection in xenotransplantation. Specifically; direct antibody-mediated endothelial cell activation, antibody-mediated complement activation, and antibody-dependent cellular cytotoxicity (ADCC).

Hyperacute Rejection

The three primary immunological barriers between pigs and man which lead to HAR are: preformed natural-occurring antibodies, complement activation, and the ineffectiveness of heterologous complement regulatory proteins in the regulation of complement activation.

It is generally thought that IgM class is the most prominent class of antibodies in HAR because it is a more efficient activator of complement. Once xenoreactive antibodies bind to resting endothelial cells (ECs) and complement is activated, ECs retract from one another and expose the subendothelial matrix containing von Willebrand factor (vWF) to platelets (19). Platelet gpIb binds to vWF resulting in platelet activation which includes upregulation of P-selectin and gpIIbIIIa. Platelet fibrinogen receptor gpIIb/IIIa upregulation results in the expression of the platelet adhesion molecule P-selectin (20). P-selectin increases platelet-leukocyte interactions and subsequent TF expression on monocytes (21).

Other changes which promote thrombosis occur on the EC surface. ADPase is associated with the cell surface and it acts to inhibit platelet aggregation. After EC activation, ADPase is lost from the surface and ADP accumulates which then stimulates platelet thrombosis (22).

Thrombomodulin (TM) is also expressed on the cell surface. Following EC activation, transcription of this product is suppressed (23). Normally, TM catalyzes the conversion of inactive protein C to its active form which then serves as a potent anticoagulant and anti-inflammatory agent against monocytes and macrophages.

Heparan sulfate (HS) release from the cell surface is associated with the binding of complement component C5a (24,25). Heparan sulfate is a very significant EC surface proteoglycan which, under normal circumstances, functions to act as a barrier to blood cells and plasma proteins, protects against complement and oxidants (binds extracellular superoxide dismutase and protects against injury by oxygen radicals) (24), and acts as an anticoagulant by binding and activating antithrombin III (AT-III). It is thought that loss of heparan sulfate may make ECs more sensitive to oxidant mediated injury as occurs in organ preservation and reperfusion (24).

The Epitope

Investigations by Platt (26), determined by Western blot analysis using human serum and pig endothelial cells, that the most reactivity occurred at 115kD, 125 kD and 135 kD. It was established that in order for antibody binding to occur, a carbohydrate moiety had to be present on a glycoprotein core of the endothelial cells and platelets (27). It has already been demonstrated that xenoreactive human antibody binding to porcine cells and organs is blocked using purified carbohydrates which have the structure αGal1-3βGal1-4NAcGlu (αGal) (28). Collins showed that by treating porcine aortic endothelial cells (PAECs) with α-galactosidase, IgM binding decreased 70-80% with a similar reduction in IgM binding towards gp115/135 which proved that the αGal epitope is an important component of gp115/135 (29). Finally, transfection of COS cells (Old World monkey cells which do not express the αGal epitope and are not usually reactive with

human serum) with α1,3-galactosyltransferase causes them to express the αGal1-3Gal epitope and makes them reactive with human serum (30).

Vaughan et al. has since found that many molecules are galactosylated by α1,3-galactosyltransferase (α1,3GT) (31). By using two dimensional electrophoresis, it was shown that approximately 20 different surface molecules were αGal1-3Gal positive and upon longer exposure, up to 40 αGal1-3Gal positive molecules appeared. It was also demonstrated that there are αGal1-3Gal positive molecules unique to endothelial cells and lymphocytes.

The αGal epitope is produced via the glycosylation enzyme α1,3-glycosyltransferase which uses uridine diphosphate galactose in the golgi apparatus to produce αGal epitopes on glycolipids and glycoproteins (32). The expression of this epitope appears to be regulated genetically although not through Mendelian inheritance (33). The functional gene encoding for α1,3GT appears to be present in all mammals with the exception of humans, apes, and Old World monkeys (34-36). The cDNA for bovine (35), murine (36), and porcine (37) α1,3-galactosyltransferase has been isolated and was found to be functional as the αGal epitope was expressed to the cell surface. The homologous human gene has also been isolated but it has frameshift mutations which result in a premature stop codon interruption (34,35). There is a reciprocal relationship between the αGal epitope and the anti-αGal antibody. Since humans lack the functional enzyme, they do not express the αGal epitope. It is believed that the reason for this mutation may be evolutionary as a result of an infectious agent endemic to the Old World. Evidence of this is shown by the existence of many viruses, bacteria, and protozoa which express αGal epitopes including normal gastrointestinal bacteria such as *Eschericia coli* and *Klebsiella pneunomiae* (38). These may act as antigenic stimulants for natural

antibody production in the same manner as environmental stimuli cause the production of antibodies to blood groups A and B.

Xenoreactive Natural Antibodies

There is considerable debate as to the role of each antibody class in the rejection of xenografts. Xenoreactive naturally occurring antibodies are believed to be broadly shared amongst species populations since hyperacute rejection occurs every time with some species combinations or not at all with others (1). Most XNAbs are derived from $CD5^+$ B lymphocytes and are polyreactive (39). That is, they react with more than one ligand, such as thyroglobulin and single stranded DNA, both of which block human IgM and IgG xenoreactive antibodies from binding to porcine endothelial cells (40). These antibodies are thought to function as an initial defense against invasive microorganisms, help prevent auto-sensitization, and help clear damaged cells from circulation.

Naturally occurring antibodies are believed to initiate HAR because of the following evidence. First, recipient immunoglobulin is quickly deposited in xenografts and rejected xenografts have very high deposits (41-44). Second, the rate of rejection is slower when recipient antibodies are depleted by such means as plasmapheresis or immunoadsorption (45-48). Third, antigraft antibodies accelerate xenograft rejection after administration to recipient (43,49-51). Fourth, hyperacute allorejection is shown to be caused by antidonor antibodies and it resembles HAR both clinically and pathologically (52-56). Fifth, Newborn baboons (57), which are shown to have very low to undetectable levels of IgM, do not reject pig cardiac grafts hyperacutely. Human newborns have also been shown to have only negligible levels of IgM directed against pig endothelial cells (58). These human neonates did have observable levels of IgG but at lower levels than seen in adults and these IgG antibodies were not cytotoxic towards pig endothelial cells.

Finally, genetically modified animals have demonstrated that by abrogating the antibody-antigen complex from forming, hyperacute rejection will subsequently be prevented. Transgenic animals can be made to produce the α1,2-fucosyltransferase enzyme which competes with α1,3-glucosyltransferase for a common precusor and thus prevents the expression of α-Gal (59,60). Also, α1,3-glucosyltransferase knockout animals can be produced which lack the α-Gal epitope (61).

Naturally-occurring IgM is thought to be the most important antibody class in HAR because of its ability to activate complement (44). C1q requires IgG to be in an aggregated in order to bind which may explain its lower complement-activating efficiency. IgM has been show by Platt and others to bind primarily to gp115/135 kD all of which are N-linked oligosaccharides (62,63). IgG has also been shown to also bind primarily to the αGal epitope (64). However, other investigators have found that epitopes other than αGal may mediate more damage after being bound by IgG antibodies (63,65). These conflicting results may be due to varying titres of IgM and IgG in individuals (65).

Watier et al. found that following αGal removal with α-galactosidase, that antibody-dependent cellular cytotoxicity (ADCC) still occurred suggesting that IgG antibodies other than anti-αGal are involved (66). ADCC via natural killer (NK) cells is dependent upon FcγRIIIA (CD16A). He suggested that targeting anti-αGal antibodies as a means of preventing rejection may not be sufficient as NK cell-mediated cytotoxicity will still occur. Also, the epitopes other than αGal which are recognized by IgG must be elucidated so that subsequent vascular damage can be prevented.

Cooke et al. found that only a minor population of IgG antibodies recognize αGal epitopes (63). He found that following α-galactosidase treatment, IgM binding decreased 80% whereas IgG binding decreased only 45-50%. Cooke also found that IgG antibodies were found to bind to molecules distinct from those recognized by IgM. These include 75

kD, 110 kD, 180 kD and 210 kD, of which only 110 kD and 180 kD are N-linked oligosaccharides.

Complement

In the pig to human species combination, the classical pathway (antibody-directed complement activation) has been shown to be primarily responsible for cell lysis (46), although the role of the alternative pathway without the involvement of antibody may still be involved. Schaapherder et al. have reported the involvement of dimeric IgA in the activation of the alternate pathway in the pig to human species combination (67). In clinically relevant species combinations, the activation of the classical pathway has been shown to be an essential step in endothelial cell activation after the binding of IgM (68-71). Cell lysis occurs after assembly of the membrane attack complex (MAC), however other early complement components also mediate cell damage.

The complex C5b67 causes a change in EC morphology which may contribute to HAR (71). The cells retract from one another after the binding of C5b67, the resultant "gap" formation does not cause cell death, and assembly of the entire MAC results in its closure. This transient interruption of the endothelium exposes tissue factor (TF) to FVII resulting in thrombin formation. Also, vWF is exposed to platelet gpIb causing platelets to adhere to the subendothelial matrix and form aggregates. *In vitro*, endothelial cells with C5b67 bound to the membrane were found to lose their polygonal appearance and their cell-to-cell contact resulting in holes in the monolayer and the cells bulged from the plate. This process required both xenoreactive antibodies and complement to occur (71).

The complement component iC3b contributes to endothelial cell damage by providing binding sites for neutrophils (72) on the EC surface. The close contact of neutrophils with the ECs is sufficient for the activation of the ECs.

In homologous systems, the spontaneous activation of complement is held in check with complement regulatory proteins (CRPs). These proteins function at different points along the complement pathway (73). Decay accelerating factor (DAF) inactivates the C3 convertase, membrane cofactor protein (MCP) inactivates the C5 converatase, and CD59 blocks the formation of the MAC. However, these proteins do not function across the pig to human species barrier due to molecular incompatibilities. Consequently, human complement is spontaneously activated on the porcine endothelial cell surface.

Delayed Xenograft Rejection

Delayed xenograft rejection (DXR) occurs as a result of factors involved in HAR (loss of anticoagulant molecules, incomplete antibody and/or complement inhibition, EC retraction) which were not completely inhibited as well as factors which are expressed days later in the rejection processes, after protein synthesis has taken place. The hallmarks of DXR include the infiltration of macrophages and natural killer cells which lead to inflammation and thrombosis. The study of DXR is possible by first preventing HAR from occurring. This is achieved by inhibiting the activation of complement (74,75) while maintaining antibody levels so that antibody-mediated responses involved in DXR may be studied.

Once ECs are activated, the transcription of genes for adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1) (76), cytokines (IL-1, IL-6, IL-8, MCP-1) (77), and procoagulant molecules (tissue factor (TF)) ensues. The cytokines secreted from the activated ECs recruit macrophages and NK cells to the site of injury.

NK cells bind to the Fc portion of bound IgG antibodies via an Fc receptor (CD16R) resulting in the transcription and secretion of tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) which then further activate ECs and macrophages (78).

NK cells have also been found to bind directly to major histocompatibility complex (MHC) class I molecules (79), αGal epitopes (80), and to E-selectin (81) on the EC surface. This has serious implications in that following complete antibody depletion, mononuclear cell activation and subsequent rejection could still proceed.

The chemokines IL-8 and MCP-1 produced by activated ECs result in the infiltration of monocytes (82-84). The monocytes are subsequently activated and express TNF-α and IL-1β, which cause further EC activation (85). Platelets have a role in the recruitment and activation mononuclear cells. The complement component C1q activates platelets via the platelet fibrinogen receptor gpIIb/IIIa which results in the expression of the platelet adhesion molecule P-selectin (20). P-selectin increases platelet-leukocyte interactions and subsequent TF expression on monocytes (21).

Molecular incompatibilities exist between pigs and humans such that factors which ordinarily regulate the amplification of coagulation in homologous systems, no longer function. One such incompatibility exists with porcine and human tissue factor pathway inhibitor (TFPI). Normally, TF activates FVII to FVIIa which then activates factors X and IX (86). Antithrombin III (AT-III) and TFPI are associated with the EC surface, AT-III binds to VIIa of the VIIa/TF complex and causes it to dissociate (87). TFPI is a much more efficient inhibitor of coagulation, it acts by binding to Xa and forming a quaternary structure with VIIa/TF (88).

Kopp et al. showed that porcine aortic endothelial cell (PAEC) TFPI is not capable of adequately inhibiting human Xa (21). During HAR, TFPI is still expressed and functions to a small degree, but as DXR progressed, monocytes express TF and TFPI is lost from the EC surface. Heparan sulfate is a major anchor for EC TFPI, thus its loss from the EC surface is accompanied by the loss of TFPI.

Although it would seem that the many factors and processes involved in xenotransplantation would ultimately lead to rejection, this is not the case. On rare occasion, a phenomenon referred to as *accommodation* (discussed below) occurs whereby neither HAR nor DXR occur despite the presence of normal antibody and complement levels.

PROLONGATION OF XENOGRAFT SURVIVAL

Therapies aimed at hyperacute rejection

Prolongation of discordant xenograft survival has been attained by interfering with the processes responsible for the progression of HAR. These include; the binding of human antibodies to porcine cells, the expression of the antigenic epitope on porcine cells toward which most human antibodies are directed against, and the activation of human complement on the porcine cell surface.

Currently under investigation are methods involving reduced αGal expression as a means for inhibiting HAR. These include α1,3-glucosyltransferase (α1,3GT) knockout animals and transgenic animals which express the enzyme α1,2-fucosyltransferase which competes with α1,3GT for a common precursor.

αGal knockout mice have been produced using homologous recombination (59). These mice showed 60% less human antibody binding and 30-50% less complement activation. Unfortunately, these animals although viable, developed cataracts and it is unknown if there are other, as yet undetected, negative effects of this treatment.

Currently, αGal knockout pigs using homologous recombination are not available because porcine embryonic stem cells are not yet available. However transgenesis using

pigs is possible. The glycosyltransferase α1,3GT and α1,2-fucosyltransferase (α1,2FT) both compete for the common precursor N-acetyl lactosamine. α1,2FT is preferentially used resulting in the expression of H antigen which is found in nearly all humans (60,61). Sandrin et al. has shown that COS cells preferentially express H antigen after being transfected with both α1,3GT and α1,2 FT, and an increase in H antigen resulted in a decrease in αGal expression. Coexpression of both α1,3GT and α1,2FT decreased both human XAb binding and complement activation on porcine cells *in vitro* (60).

Antibody Depletion

Prevention of HAR has been achieved with xenoreactive natural antibody (XNAb) inhibition. Successful treatments, alone or in combination include plasmapheresis, organ perfusion, immunoadsorption, and immunosuppression.

Plasmapheresis has been shown to effectively remove antibodies from the recipient. However, following plasmapheresis, the antibody titre returns to pretreatment levels or higher within 1-2 weeks (89). This would require continuous treatments so that *accommodation* may occur. Accommodation is a state in which endothelial cells are no longer activated in the presence of XNAb and complement (68). Other drawbacks include the fact that other serum components might also be depleted which may place the recipient at risk for infection.

Organ perfusion is advantageous over plasmapheresis in that natural antiendothelial antibody levels are specifically reduced (90). Fischel et al performed a pig to rhesus monkey cardiac xenograft following organ perfusion, and although only a minor reduction in total antibody levels was noted, graft survival was significantly prolonged compared to plasmapheresis (90).

The method which has met with the most success with regards to specifically reducing XNAbs and preventing HAR is immunoadsorption. The use of specific carbohydrates for this purpose has advanced the study of xenotransplantation two-fold. Not only has this therapy allowed for the prolongation of graft survival, but it has also helped define the specific epitope to which most XNAbs bind.

Immunoadsorption was first utilized as a means to inhibit rejection of ABO-incompatible allografts by using A and B trisaccharides in both humans (91,92) and baboons (93,94). Immunoadsorption can be performed using columns containing specific oligosaccharides (91), using brief intravenous infusions to neutralize antibodies *in vivo* (94,95), and also by continuous intravenous therapy as performed by Cooper et al with ABO-incompatible baboon allografts (96). Cooper's use of this therapy was successful in that one baboon survived 39 days after the treatment was discontinued.

The results from ABO-incompatible allografts prompted the use of immunoadsorption in the treatment of discordant xenografts. Various oligosaccharides have been tested and it has been shown that carbohydrates in the α configuration such as: D-galactose, melibiose (Galα(1-6)Glc), stachyose (Galα(1-6)Galα(1-6)Glcβ(1-2)Fru), and methyl-α-D-galactopyranoside (30), as well as linear B type 2 (αGal(1-3)βGal(1-4)βGlcNAc-R), linear B type 6 (αGal(1-3)βGal(1-4)βGlc-R), and B disaccharide (αGal(1-3)βGal-R) (28) inhibit the reaction of human serum with pig cells specifically. Carbohydrates with β linkage such as D-glucose and methyl-β-D-galactopyranoside did not significantly reduce reactivity (30).

Its been found that melibiose is relatively ineffective in adsorbing human antibodies (97,98) and in baboons it was up to 50-fold less effective than the disaccharides and trisaccharides, and was shown to be toxic (99). Also, B disaccharides (1 mg/mL)

achieved about a 76% reduction in cytotoxicity with human serum against PK15 cells and a 93% reduction in cytotoxicity with baboon serum. Taken together, these results suggested a difference in antibody specificity between humans and baboons which may have implications when using baboons as a model for pig to human transplantation.

Immunosuppression

Studying immunosuppressive therapies in xenotransplantation is much more difficult than in allotransplantation. A small animal model has yet to be found which can parallel the processes which occur between pigs and humans. Some investigators have used concordant models such as hamster to rat (100). However, concordant species lack preformed antibodies which are an important aspect of HAR in the pig to human model. Other studies used discordant models such as guinea pig to rat but it has been suggested that in this model, rejection occurs primarily through the activation of the alternative complement pathway (101) which again, is not an accurate model for pig to human xenotransplantation. It has been proposed that the only relevant species combinations are those which include animals having XNAb against the αGal epitope.

Results from immunosuppression studies using the baboon, which is a concordant species to humans, should also be interpreted cautiously as they have different levels of tolerance towards immunosuppressants. Baboons can withstand CsA doses four times higher than those used in humans without experiencing toxicity (102).

Another consideration with respect to immunosuppression is the fact that delayed xenograft rejection is multifactoral (antibodies, adhesion molecules, procoagulant molecules, macrophages, NK cells, cytokines, platelets, etc). One must select a regimen which inhibits EC activation and damage. This would entail profound immunosuppression which may be just as detrimental to the recipient as rejection. To date, conventional

immunosuppressive therapies are ineffective in preventing eventual rejection in pig to human xenotransplantation.

With this in mind, there has been some progress in determining immunosuppressive regimens for discordant species combinations. Although effective in allotransplantation, Cyclosporine (CsA) and FK506 are ineffective for xenotransplantation. These drugs interfere with the signal transduction of T cells which is antibody-independent. However, both of these drugs, when used in combination with immunosuppressants which inhibit HAR and DXR, have prolonged xenograft survival by inhibiting T cell-mediated rejection.

Rapamycin acts by inhibiting B and T cell activation by blocking the IL-2 receptor-coupled signal transduction pathway. Rapamycin has not been shown to be effective when used alone in the concordant hamster to rat model (103) or in the discordant pig to rabbit model (104). However Rapamycin is effective at inhibiting IgM, IgG and IgA production *in vitro* (105). Also, when combined with Brequinar sodium, Rapamycin is additive (106).

15-deoxyspergualin (DSG) is one immunosuppressant being investigated as part of a regimen for xenotransplantation as it both prevents the production of IgM and the immunoglobulin class switch to IgG (107). Its mechanism of action is not clear but it may involve binding of the drug to heat shock proteins 70 (HSP 70) and 90 (HSP 90). Heat shock proteins ensure the proper folding of antigenic peptides once they are internalized by antigen presenting cells. Therefore, DSG interferes with proper antigen processing and presentation. DSG has been shown to inhibit XNAb production in rats toward human peripheral blood lymphocytes (108) and mouse cardiac xenografts (108).

Brequinar sodium (BRQ) prevents RNA and DNA synthesis by blocking the production of pyrimidine nucleotides. This results in the inhibition of T and B cell

functions and subsequent antibody production inhibition (109). A hamster to rat model demonstrated that BRQ alone increased survival from 4 days (control) to 5.5 days (110). However, when used in combination with CsA, graft survival exceeded 100 days. This survival was associated with the suppression of rat anti-hamster antibody production. This synergism with CsA is not seen with all drugs and it may provide an important tool in pig to human xenotransplantation although more data is required using non-human primates.

The immunosuppressant mycophenolic acid (MPA) is the product resulting from de-estrification of the prodrug mycophenolate mofetil (MMF). MPA acts by inhibiting the enzymes inosine monophosphate dehydrogenase (IMPDH) and guanine phosphate synthetase, both of which are involved in the de novo synthesis of purine nucleotides. Lymphocytes rely upon the de novo pathway for proliferation, thus MMF inhibits the production of XNAbs derived from B cells. MMF also inhibits the proliferation of smooth muscle cells (111,112) which is one of the pathological features of chronic xenograft rejection.

Alone, MMF is relatively ineffective with the hamster to rat (113,114), rat to mouse (115), and hamster to mouse (115) models. MMF was shown to slightly prolong pig to rabbit cardiac survival (104). However, when combined with other immunosuppressants, MMF is shown to act synergistically. The concordant hamster to rat (114,116) model and the discordant guinea pig to rat (114) model both exhibited significantly prolonged graft survival when CsA, BRQ, and MMF were used in combination compared to MMF and BRQ or CsA with either MMF or BRQ. Cardiac survival with the concordant cynmolgus monkey to baboon cardiac model was extended from 3 months in a group receiving CsA, methylprednisolone, and azathioprine to 10 months in a group receiving CsA, methylprednisolone, and MMF (117).

The study of immunosuppression is exceedingly complicated in xenotransplantation. This is due in part to investigators using different animal models, drug combinations, and dosing protocols such that it is difficult to compare results from different studies.

Complement inhibition and depletion

Therapies aimed at complement inhibition which have prolonged graft survival by inhibiting HAR include the use of cobra venom factor (CVF) and soluble complement receptor 1 (sCR1). CVF has been shown to be the more effective of the two in depleting complement (74). However, there are many disadvantages inherent to its use, most important of which is its level of toxicity in therapeutic doses (75). CVF works systemically by consuming host complement by fluid phase activation. By activating complement, anaphylotoxins C3a and C5a and well as opsonic ligands (C3d, iC3b, C4b) are formed which function to recruit and aid in the adherence of neutrophils and macrophages to the endothelium (74). Another disadvantage of CVF therapy which deters its use in the pig to human species combination is the fact that the major oligosaccharide of CVF contains αGal (Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1) which would stimulate production of anti-αGal and thus, repeated injection decreases its efficacy (118).

In contrast to CVF, sCR1 acts locally and does not generate anaphylotoxins. sCR1 inhibits both the classical and alternative pathways by binding to C3b and C4b of the C3 and C5 convertases causing the dissociation of C2a and Bb respectively. sCR1 also acts as a cofactor for serum protease factor I which promotes degradation of C3b and C4b which then releases sCR1 in a functional form (119). Unfortunately, sCR1 does not prevent C3 deposition which allows for the accumulation of neutrophils (75).

Attempts to transfect pigs with human complement regulatory proteins (CRPs), which prevent spontaneous complement activation on the porcine cell membrane, have succeeded in ameliorating HAR (120-123). Currently under investigation is the CRP or combination of CRPs which most effectively inhibit complement activation. One study found that a pig CD59-like molecule is capable of inhibiting human membrane attack complex assembly but does not prevent HAR (124). HAR may ensue in this situation due to the still functional early components of the complement cascade (C3a, C5a, C3bi, C5b67). A 30 hour survival in a pig to baboon heterotopic cardiac model was achieved using a combination of DAF and CD59 (121). The use of these CRPs has the potential to significantly prolong pig to human graft survival especially if used in combination with therapies which prevent other aspects of HAR such as antibody depletion.

Therapies aimed at delayed xenograft rejection

Therapies aimed at the prevention of DXR are now gaining considerable attention. Inhibition of platelet activation and aggregation has been achieved by using a peptidomimetic gpIIbIIIa antagonist (125). This agent was shown to suppress P-selectin expression thus decreasing subsequent leukocyte infiltration.

Another strategy of DXR prevention is to inhibit EC activation. Most of the genes which are activated during DXR are activated after the transcription factor NFκB binds to the reporter region of the gene. The gene products which are controlled by NFκB include TF, adhesion molecules (E-selectin, ICAM-1, VCAM-1) (76), and cytokines (IL-1 and IL-8) (77). Instead of attempting to control the expression of each protein individually, inhibition of NFκB would reduce the expression of many procoagulant/proinflammatory molecules simultaneously. This can be achieved by the overexpression of IκBα in the cytoplasm which holds NFκB in an inactive form and prevents its translocation into the nucleus (126).

Accommodation

Accommodation was first noted in ABO- (60,125) and HLA-incompatible (128) allografts. It was demonstrated that following antibody depletion either by plasmapheresis or immunoadsorption, grafts survived despite the return of antibodies to pretreatment levels and normal complement levels. This process has since been observed with xenotransplantation and although the results have not been as positive as with allografts, the induction of accommodation is a common goal of many involved in the study of xenotransplantation. The primary benefit of this state would be the withdrawal of treatments aimed at antibody and complement depletion, including the massive immunosuppressive regimen which would otherwise be necessary to maintain graft survival. To date, the mechanism by which accommodation occurs is unknown.

Early reports of accommodation by Fischel et al. (90) demonstrated that following plasmapheresis, organ perfusion, and immunosuppression (Minnesota anti-lymphocyte globulin, cyclosporin A, azathioprine, and prednisone) a pig to rhesus monkey transplant functioned 8 days before the monkey had to be sacrificed. Immunoflorescence showed small amounts of IgM deposited and only focal deposition of fibrin and complement.

Proposed mechanisms involved in this state include the following. Naturally occurring antibodies which return to the circulation may differ in affinity/specificity from the XNAbs present before the graft. For example, if a class switch from IgM to IgG took place, complement activation would decrease, resulting in decreased EC activation (68). Also, an isotype switch from complement-binding IgG (IgG1, IgG3,) subclasses to IgG2, which fixes complement poorly, may occur (129).

Another mechanism may be that the susceptibility of the EC to injury may vary over time. Trauma of transplant or injury from the preservation fluid [results in the

generation of toxic oxygen species and release of proteolytic enzymes (130)] may make cells very sensitive to antibody and complement initially, but over time they are conditioned to resist injury. This process has been demonstrated in a study which found that stimulation of cells with an inflammatory agent (IL-1β, TNF-γ, lipopolysaccharides (LPS), or phorbol 12 myristate 13-acetate) induces the release of TF which initiates the extrinsic path of coagulation. Restimulation with the same agent results in self-hyporesponsiveness in which the cells are less sensitive to the stimulant and to cellular injury (131). The reason for this may be to limit the extent of extrinsic coagulation in the presence of inflammatory agents, though the exact mechanism of this observation is unknown.

A third hypothesis involves the target antigens and their alteration (132). This change in the epitopes may result in either an absence of binding or a change in binding such that complement is no longer activated. However, recent experiments have shown comparable antibody binding in both rejected and accommodated xenografts (129,133,134).

And finally, Winkler et al. proposed that ECs may adapt and undergo responsive activation which protects the cell and represses responses caused by XNAbs and complement (132). Two groups of genes may be induced following activation. The first group leads to a pro-inflammatory/pro-coagulant phenotype (135), these include IL-1, IL-6, IL-8, E-selectin, VCAM-1, and TF expression. The second group likely results in the protection of the EC and include A20 (136), ferritin (137), manganese superoxide dismutase (MnSOD) (138), and IκBα (139). A20 is a zinc finger protein which protects ECs from TNF-induced apoptosis, ferritin and MnSOD protect the cell from oxidative damage, and IκBα inhibits the transcription factor NFκB from activating the genes involved in the pro-inflammatory/pro-coagulant response.

Two notable studies have been performed *in vitro* to demonstrate accommodation, both of which reported different findings. Dalmasso et al. (133) preincubated PAECs with human serum for 1-40 hours and reported a resistance to complement-mediated damage which required protein synthesis to occur. It was also found that pretreatment with purified human IgM antibodies for at least 17 hours was required for a dose-dependent reduction in cytotoxicity which progressively decreased to 40 hr. This observation was not apparent when purified human IgG or serum depleted of IgM was used so it was concluded that IgM was responsible for the reduction in cell death.

Dorling et al. (134) preincubated an immortalized porcine endothelial cell line with human polyclonal IgG and found that IgG was responsible for accommodation which began after 72 hours and was optimal after 120 hours. Phenotypic changes to the cell line included downregulation of VCAM and MHC class I which corresponded to a resistance to complement-mediated lysis. It has been shown that porcine VCAM can interact with human VLA-4 on human leukocytes and this interaction allows for transmigration of leukocytes across the monolayer. It was hypothesized that while the binding of IgM induces mRNA expression of proinflammatory genes in PAECs, IgG binding could signal separate events as this class of antibodies clearly has been shown to bind to different epitopes than IgM (140).

The involvement of IgG in accommodation described by Dorling are in agreement with Bach et al. who demonstrated accommodation in a concordant hamster to rat *in vivo* model (133). The rats were treated daily with CsA and given CVF for 6-11 days. Perhaps the most important findings by this group were: the expression of protective genes, the expression of a Th2 cytokine response, and the increased binding of IgG2c over other IgG isotypes. They reported the expression of the protective genes A20, bcl-2, hemoxygenase and bcl-xl which can prevent apoptosis (136,141,142). Also, A20 and bcl-2

have been shown to inhibit NFκB activation (143,144). The preference of a Th2 cytokine response (IL-4, IL-10, IL-13) as opposed to a Th1 response (IL-2, IFN-γ, TNF-α) in accommodation is significant in that Th2 cytokines prevent monocyte (145) and NK cell (146) activation even though mononuclear infiltrates were similar in both rejected and accommodated grafts. Immunoglobulin class switches are mediated by switch-recombinase enzyme (147) which is triggered by cytokines from Th1 and Th2 cells (148-150). Preferential activation of regulatory Th2 cells instead of pro-rejection Th1 cells may result in long-term graft survival due to the difference in Ig class production. Mouse IgG2c is known to be an inefficient activator of complement, it may protect endothelial cells by binding to donor class I MHC and block other antibodies from binding (151). Its predominance in the accommodated hearts suggests that an isotype switch may be required to achieve accommodation.

Accommodation and immunosuppression

The choice of immunosuppression may play a significant role in achieving accommodation. Hancock et al. used a hamster to rat model to demonstrate different results attained when using CsA in combination with Brequinar, Rapamycin, or CVF (100). It was found that the Brequinar and Rapamycin therapies prevented leukocyte infiltration, cytokine expression, deposition of IgM, IgG, C3, and the expression of genes which are thought to protect ECs. As these events are thought to induce accommodation, it is possible that these therapies may prevent accommodation from occurring.

In contrast, Farraresso et al. performed rat cardiac allografts using 0.8 mg/kg/day rapamycin and found that rapamycin allowed for the production of non-cytotoxic IgG2c-blocking antibodies and for the activation of Th2 cells (151). Rapamycin may inhibit specific lymphokine signals which regulate the production of various IgG subclasses.

OBJECTIVES AND RATIONALE

Induction of accommodation is one way xenograft survival can be prolonged. However, the mechanism by which accommodation occurs is presently unknown. An *in vitro* model allows for the study of pig to human xenotransplantation which cannot otherwise be accomplished. As previously discussed, small animal models may not adequately represent the processes involved in pig to human xenorejection. By using an *in vitro* model, standardization which is not possible with animal models can be maintained. Also, it is possible to change single variables *in vitro* without affecting multiple systems as with an *in vivo* model.

The causes of accommodation are still in the early stages of elucidation although it is generally agreed that antibody depletion is a prerequisite (152). In order to demonstrate the involvement of natural xenoreactive antibodies in the induction of accommodation, the objectives of this study are as follows:

1. To determine whether the level of human antibody binding to porcine cells changes significantly with prolonged serum exposure, and if this change correlates with decreased cytotoxicity.

2. To determine whether human antibody specificity to porcine cell epitopes changes with prolonged serum exposure.

3. To determine whether porcine cell activation decreases with prolonged human serum exposure by measuring E-selectin which is upregulated during endothelial cell activation.

METHODS AND MATERIALS

LLC-PK1 Cultures

LLC-PK1 cells, a renal tubular epithelial cell line (American Type Culture Collection, Rockville, MD) were used for all experiments. The cell line was cultured in M199 media (Life Technologies, Burlington, ON) containing L-glutamine, Earl's salts and 2200mg/L sodium bicarbonate. The media was supplemented with 10% (v/v) heat-inactivated (56 °C for 30 min) fetal bovine serum (FBS) (Life Technologies), 600 μL (40 mg/mL) gentamicin (Life Technologies), 0.66% (v/v) human insulin (Nova Nordisk, Mississauga, ON), and 1 mM sodium pyruvate (Life Technologies). Cells were plated at a density of approximately 1×10^4 cells/mL in attachment factor (Cell Systems, Kirkland, WA) coated 75 cm^2 flasks , 96 well plates, 24 well plates and 6 well plates (Falcon, Franklin Lakes, NJ) .

Addition of Human Antibody

The model used in this study consisted of a pool of natural human serum (NHS) from individuals whose blood group was type B. The blood was collected in unit packs lacking anticoagulant by the Canadian Red Cross. The decision to use this blood group was based on evidence that human anti-B antibodies can cross react with the αGal epitope (153). The natural human serum (NHS) was incubated on a transformed porcine renal epithelial cell line (LLC-PK1) for selected time intervals (1.5-96 hours).

For each set of experiments, cells were grown in media for at least 24 hours. Serum and controls were added on day one to the plates and flasks allocated for the 96 hour serum incubation. Media was removed and cells were washed once with fresh media after which heat-inactivated, pooled human group B serum (Canadian Red Cross, Edmonton, AB) and the appropriate controls were added. On day two, serum and

controls were added to the plates and flasks allocated to the 72 hour serum incubation. The same process was performed on day three (48 hour incubation) and day four (24 hour incubation). On day five, serum and controls were added to the plates allocated to the 1.5 hour serum incubation after which all of the plates and flasks were washed twice with fresh media and the appropriate assay was performed. On day three, all media and serum was replaced in all flasks and plates.

^{3}H-Thymidine Assay for Cell Proliferation

Cells were incubated in 6-well plates with 3 mL of either 25% (v/v) heat-inactivated human group B serum, neonatal pig serum, or media for 96, 72, 24 and 1.5 hours. Plates were washed twice with media and 334 μL of Low Tox-H rabbit complement (Cedar Lane, Hornby, ON) was added. Plates were incubated for 1 hour at 37°C in 5% CO_2. Plates were washed twice in fresh media and 1:1000 ^{3}H-thymidine (Amersham Ltd, Oakville, ON) in media was added to each well and incubated for 21 hours. The media was removed and 1 mL trypsin-EDTA 1x (Life Technologies, Burlington, ON) was added to each well and incubated for 10 min. One milliliter of media was added to each well and cells were transferred to 15 mL conical tubes and washed 3 times with saline. Cells were resuspended in 1 mL saline and are lysed by freezing at -20°C overnight.

The *DC* protein assay from BioRad (Hercules, CA) was performed according to manufacturers instructions, then 500 μL of lysate was filtered using a Millipore filtering apparatus with Whatman GF/F glass filters (Fisher, Edmonton, AB). Filters were placed in scintillation fluor and counted on a beta counter (Micromedic systems Inc).

MTT Cytotoxicity Assay

For each time point, duplicate plates were used to assess cytotoxicity as measured by the MTT assay. A modification of the method described by Mosmann et al. (154) was used. At the beginning of each incubation (96, 72, 48, 24 and 1.5 hr), the media was removed and 100 μL of heat-inactivated human serum (concentrations: 100%, 50 %, 25%, 10%, 5%, and 2.5% in phenol red-free M199) was added to quadruplicate wells on each plate. Controls consisted of neonatal pig serum with and without complement, media with and without complement, and 3% Triton-x (Sigma). The plates were incubated at 37°C in 5% CO_2. After the 1.5 hr incubation, serum was removed and the plates were washed twice with media. Rabbit or human complement (20 μL) was added and incubated 1 hour at 37 °C. Both Cedar Lane Low Tox-H (Hornby, ON) and Pel-Freez (Brown Deer,WI) rabbit complement were used in order to compare the two. After incubation, complement was removed and the cells were washed twice with phenol red-free M199, after which 50 μL of a 1 g/L solution of MTT (3-4,5-dimethyldiazol-2-yl-2,5-diphenyl tetrazolium bromide) (Sigma) diluted in media was added. The cells were then incubated for 3 hrs at 37 °C after which the plates were spun at 1000 x g for 5 min with the unconverted MTT being removed by inversion of the plates. Live cells take up the MTT reagent into the mitochondria where formazan crystals are then formed. To dissolve the formazan crystals, 100 μL of propanol was added to each well and mixed to ensure complete dissolution. The optical densities (OD) of the plates were read at 540 nm using an ELISA plate reader (Ortho Diagnostic Systems, Markham, ON). Two negative controls were used consisting of cells treated with pig serum or media and complement in the absence of human serum and a positive control consisted of cells treated with 3% (w/v) solution of Triton-X 100. The percentage of toxicity was calculated as follows:

$$\% \text{ cytotoxicity} = [1-(\text{test OD}/\text{negative control OD})] \times 100.$$

Neutral Red Cytotoxicity Assay

Cells were grown in 24 well plates and incubated with 625 µL of 25% heat-inactivated pooled human group B serum in duplicate for the appropriate time interval. Controls consisted of neonatal pig serum and media incubated with complement and 3% Triton-x 100. The serum was removed and the cells were washed twice with fresh media after which 125 µL of Low Tox-H rabbit complement (Cedarlane, Hornby, ON) was added to appropriate wells and incubated for 1 hr. To the wells in which complement is not added, fresh media was added. After incubation, the complement was removed and cells were washed twice with fresh media and 1 mL of media was added to each well. Then, 60µL of neutral red (2.5 mg/mL; Fisher Scientific Co., Edmonton, AB) was added to each well and plates were incubated 30 min at 37°C. Live cells are permeable to the vital dye. Percent live versus dead cells were assessed by counting 200 cells/well:

$$\% \text{ live cells} = \frac{\text{live cell}}{\text{live cells + dead cells}}$$

Enzyme Linked Immunosorbant Assay (ELISA)

The plates were incubated with 100 µL of primary antibody (human serum or control) for each time interval. Serum concentrations used were 100%, 50%, 25%, 10%, and 2.5% (v/v). Controls consisted of neonatal pig serum and fresh media which was subtracted as a blank. At the end of the incubation, plates were washed three times with wash buffer [0.05 % v/v Tween 20 (Sigma) in PBS]. The plates were then fixed with 0.1% (w/v) glutaraldehyde (Sigma) for 5 min after which they are washed three times with wash buffer. 100 µL/well of blocking agent [1% (w/v) BSA in PBS](Pierce, Rockford, Il) was added and after 30 min at room temperature, it was removed and plates were blotted dry.

For the ELISAs demonstrating human antibody (IgM, IgG, IgA) binding, 100 μL of horseradish peroxidase conjugated goat anti-human IgM, IgG, or IgA (Southern Biotechnology Associates, Inc, Birmingham, AL) diluted 1:2500 in 0.01% (v/v) Tween/PBS was added to each well and incubated for 90 min on a rocking platform at room temperature. For the ELISAs demonstrating upregulation of porcine E-selectin, 100 μL of 1:100 F(ab')2 rabbit anti-mouse IgG (Serotec, Raleigh, NC) was used and 1:100 mouse IgG1 was used as a negative control (Serotec).

After incubation, the cells were washed three times with wash buffer and incubated with 100 μL/well of wash buffer for 5 min. The wash buffer was aspirated from the cells and 100 μL of ABTS developing solution (Pierce, Rockford, Il) was added and plates were incubated for 30 min. The reaction was stopped with 50 μL of 1% SDS (w/v) (Sigma) and the absorbance was read with an ELISA plate reader (405 nm) within 2 hours.

Preparation of LLC-PK1 Membrane Proteins

After 25% serum incubation, confluent monolayers were washed twice with TBS supplemented with 1.0 mM phenymethylsulfonylfluoride (Sigma). Cells were scraped from the flasks in the same buffer and centrifuged at 1600 × g for 5 min at 25°C. Cell pellets were then resuspended 1:2 with lysis buffer [TBS containing 0.5% NP40 (Sigma), 1mM PMSF, 0.5% aprotinin (Sigma), 0.5% sodium azide (Sigma)] and incubated on ice for 1.5 hr with occasional mixing. Solubilized cell suspensions were then centrifuged at 21 000 × g for 30 min at 4°C. The supernatant was aliquoted and stored at -70°C until use. Membrane protein concentration was determined using the *DC* protein assay from BioRad (Hercules, CA).

SDS Polyacrylamide Gel Electrophoresis

Membrane protein extracts (12 μg/well) were resolved using 10% w/v separating gels utilizing a discontinuous buffer system according to the method of Laemmli (155). Proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) at 100 V for 1 hr. Efficiency of transfer was monitored utilizing a prestained molecular weight standard (Bio-Rad). Membranes for IgM, IgG, and IgA detection were blocked with 3% (w/v) milk protein in PBS for 3 hrs.

Immunoblots

The membranes were incubated with 1:1000 pooled human group B serum. Blots were washed with 0.05% (w/v) Tween 20 in PBS (PBS/Tween) and rinsed with PBS. Human antibody binding to porcine proteins were probed with the following horseradish peroxidase conjugates diluted in blocking buffer: 1:5000 goat anti-human IgM (μ-chain specific; Southern Biotechnology Associates Inc, Birmingham, AL), 1:5000 goat anti-human IgG (γ-chain specific; Southern Biotechnology Associates Inc.) and 1:2500 goat anti-human IgA (Southern Biotechnology Inc.). Blots were then washed with PBS/Tween, rinsed with PBS and developed by enhanced chemiluminescence (Pierce, Rockford, Il), according to manufacturer's instructions with exposure on Kodak X-omat AR Film (Kodak Inc, Rochester, NY). Broad range molecular weight standards (Bio-Rad.Hercules, CA) were used to determine molecular weights of samples.

Statistics

Jandel Scientific software was used for statistical analysis. One way analysis of variation (ANOVA) was used to assess the signficance of each treatment group over the specified time intervals. If either the normality test or the equal variance test failed, the Kruskal-Wallis ANOVA on ranks was performed. A statistically significant difference with either test warranted the use of the Dunnett's multiple comparison procedure using

the 24 hour time point as a control for the cytotoxicity assays since maximal cell lysis occurred at this time for the majority of the assays. The 1.5 hour time point was used as the control for the ELISAs demonstrating IgM, IgG, and IgA binding as well as for E-selectin expression in order to demonstrate a significant change in each over the course of the entire experiment. A p value of less than 0.05 was considered statistically significant.

RESULTS

^{3}H-Thymidine Cell Proliferation Assay

The incorporation of ^{3}H-thymidine during DNA synthesis was used to assess cell proliferation. The proliferation of the cells exposed to 25% human serum was comparable to cells exposed to media and pig serum (Fig 1). This control validates that the test cells are alive and proliferating similar to the controls. The decreased incorporation of ^{3}H-thymidine over the course of the experiment is an indication that cellular proliferation is slowing down, which may result from the cells being confluent. It may also reflect the media becoming less mitogenic over time, perhaps due to labile cytokines or growth factors. It may also be that human serum results in a short-term peak in cell proliferation.

The ^{3}H-thymidine was added to the cells following serum incubation. Therefore, the peak in cell proliferation may have occurred during the first 24 hours after human serum addition and thus incorporation of ^{3}H-thymidine would appear to be less with the 72 and 96 hour time points because the peak proliferation had occurred early in the experiment.

Cytotoxicity Assays

Two methods were used to assess cell viability using human heat-inactivated group B serum. The neutral red vital dye assay was used as a comparison to test the validity of the MTT cytotoxicity assay. The neutral red assay demonstrated a trend in that a greater degree of cell death at 96 hours occurred following Cedar Lane Low-Tox H rabbit complement (Fig. 2a) compared to Pel-Freez rabbit complement (Fig.2b). This difference may be due to the fact that each test group was run only in duplicate. Also, dead cells were often lysed and the debris could not be counted which may have introduced bias in the neutral red results.

Subtraction of spontaneous cell death resulting from incubation with Pel-Freez rabbit complement from the neutral red assay does show a decreasing trend in cytotoxicity (Fig. 2b) over time using both pig serum and media as negative controls but not at the same rate as seen with the MTT assay using Pel-Freez complement (Fig. 3). The MTT shows maximal cell death at 24 hour and a subsequent decrease at 48,72 and 96 hours. In contrast, the neutral red assay showed maximal cell death at 1.5 hours. The MTT cytotoxicity assay using Pel-Freez rabbit complement showed a similar reduction in cytotoxicity when using both media and pig serum as negative controls

The MTT assay using Cedar Lane rabbit complement shows varying degrees of cytotoxicity depending on whether pig serum or media are used as a negative control to account for spontaneous cell lysis due to complement (Fig. 4). When media is used as a negative control, no significant decrease in cell lysis is seen. In contrast, when pig serum is used as a negative control, there is a significant decrease ($p<0.05$) with all NHS concentrations compared to the 24 hour control. This may be in part due to the fact that cell death increases with prolonged exposure to pig serum particularly at 96 hours (Fig. 5). When test optical densities are compared relative to pig serum and complement negative control, it appears as though more cells are alive. This may warrant the use of media as a negative control instead of pig serum.

Human serum as a source of complement was used for one set of MTT experiments. The results demonstrated a significant decrease with 50%, 25%, and 10% human serum with both the neonatal pig serum and the media negative controls. However, it was found that after accounting for spontaneous cell death using media or pig serum (Fig. 6), the cytotoxicity remaining was negligible (<25%). Although the use of human serum would more closely reflect the pig to human system, many investigators prefer the use of heterologous complement with lytic assays as it is not regulated as

efficiently as homologous complement (156). Also, due to the scale of this experiment, the quantity of fresh human serum with a low anti-αGal titre was difficult to obtain.

An observation which was noted with virtually all the MTT cytotoxicity assays was the reduction in cell death due to 3% Triton-x after 72 and 96 hour incubation intervals. The one exception of this was when using Cedar Lane Low-Tox H rabbit complement with media as the negative control. Interestingly, this was the only cytotoxicity assay which did not demonstrate a significant reduction in cytotoxicity. The fact that Triton-x also showed a reduction in cell death over time may indicate that the resistance in cell death noted in this study may not be due to exposure to human antibody but instead the development of resistance to noxious stimuli in general. Alternatively, the results of the positive control may suggest an artifact introduced by subtracting the negative control (media or neonatal pig serum). If there is an increase in cell death with the negative control over the course of the experiment, by comparison, there would appear to be an decrease in cell death with the test wells when using the following equation:

$$\% \text{ cytotoxicity} = [1-(\text{test OD}/\text{negative control OD})] \times 100.$$

Antibody binding

The level of antibody binding is important, as the reduction in cytotoxicity has been previously shown to be dose-dependent (133). In this study, the level of binding of each antibody class varied considerably. The levels of IgM bound to the epithelial cells as measured by ELISA did fluctuate with 50% (all time points) and 25% (24 hr time point) NHS but the other concentrations did not change significantly when compared to the 1.5 hour control time (Fig. 7a). IgG levels decreased significantly over time with 50% (24,

72, 96 hr time points), 25% (24 and 96 hr time points), and 2.5% (all time points) NHS (Fig. 7b). In contrast, IgA levels increased significantly with 50% serum (72 and 96 hours), 25% serum (24, 48, 72, 96 hours) , 10% serum (24, 48, 72, 96 hours) and 2.5% serum (96 hours) (Fig. 7c). The decrease in IgM and IgG binding may reflect a depletion of free antibody over time as cells proliferate.

Immunoblots using cells preincubated with human serum showed no observable change in antibody specificity over time (Fig. 8). As described by others (63), different classes of antibodies have different epitope specificity (Table 1). This may have relevance when one considers the difference in pathogenesis attributed to each class in xenograft rejection. Glycoproteins at 115 kD, 125 kD, and 135 kD were not prominent on the immunoblots as described by Platt (62), this may be due to the cell line used. However, Aspeslet et al. (157) found that although there were some differences in the antigens identified using porcine aortic endothelial cells (PAECs) compared to LLC-PK1 cells, these differences were said to be minor (Table 1). In this study, there were found to be both shared and unique epitopes bound by each antibody class. IgM and IgG both bound to epitopes of approximately 98 kD, 77 kD, 64 kD, 61 kD, 44kD, and 32 kD. IgA also bound to epitopes of approximately 99 kD and 65 kD. Epitopes unique to each antibody class are summarized on Table 1.

E-selectin

E-selectin (CD62E) is an adhesion molecule which is upregulated on the EC surface during inflammation. E-selectin on the surface of ECs interacts with carbohydrate groups on circulating leukocytes such that they roll along the endothelium and interact with chemokines, chemotactic molecules and other cell adhesion molecules which are also expressed on the EC surface.

In order for E-selectin to be expressed, protein synthesis must first occur which takes hours to days. Therefore, the expression of E-selectin is associated with delayed xenograft rejection and is used as a cell marker for this later phase of rejection.

Although E-selectin is expressed on endothelial cells and not epithelial cells, the monoclonal E-selectin antibody is known to cross-react with other glycoproteins. As suitable markers for porcine epithelial cell activation were lacking, the E-selectin monoclonal antibody was used to demonstrate whether there could be a cross-reactive epitope on the epithelial surface which is upregulated during cell activation.

The expression of an as yet unidentified epitope was significantly increased ($p<0.05$) after 24 hours with 100%, 50%, 25%, and 10% NHS compared to the 1.5 hour control (Fig. 9). Interestingly, the highest expression of this epitope was following incubation with pig serum. Neonatal porcine serum was meant to act as a negative control. However, the cytotoxicity assays and the upregulation of the epitope indicate that neonatal porcine serum caused cell activation and death. The serum lacked IgM antibodies but may have contained maternal IgG which could contribute to cellular activation in much the same manner as a blood group-incompatible allograft response. Further study into the characterization of this epitope is required.

DISCUSSION

Accommodation is defined as a resistance to complement-mediated lysis in the presence of normal antibody and complement levels. The mechanism by which accommodation occurs is unknown at present, though the pretransplant depletion of xenoreactive antibodies is believed to be a prerequisite (152). Probable mechanisms include the "healing-in" of the graft during the presence of low concentrations of XNAbs. Or, the returning XNAbs may be of a different isotype which mediates the upregulation of protective genes as opposed to proinflammatory genes. Some have also suggested changes in the core structure of the antigens towards which XNAbs bind.

This study sought to explore humoral involvement in accommodation and if human antibodies are sufficient in achieving an accommodated state. The MTT cytotoxicity assay using Pel-Freez rabbit complement and human complement (with media as the negative control) did indicate a reduction in cytotoxicity over time. There was no evidence of a change in the antigenic determinant being responsible for the reduction in cytotoxicity as the immunoblots did not detect any major changes in the epitopes that bound XNAb over the course of the experiment. There were however, changes in the level of XNAb over time. IgM levels fluctuated with 50% and 25% NHS but did not significantly change with any other concentration of human serum. IgG levels decreased significantly over the course of the experiment in contrast to IgA which significantly increased over time. Future experiments should normalize for cell number for each treatment group as cells may peel from the plate during prolonged culture. This can be achieved by using MHC class I as a marker which is expressed on epithelial cells.

Previous studies reported an increase or similar levels in antibody binding to the endothelium over time (133). If these antibodies are a different isotype than the original

antibodies, they may have a higher affinity towards the porcine epitopes and act to saturate the epitopes, thus protecting the cells from antibodies which mediate cellular activation. This *in vitro* assay does not allow for an isotype switch which may account for the lack of a significant increase in IgM and IgG antibody binding over time. Also, if IgM and IgG have a high affinity towards the cellular epitopes and saturate these sites early in the experiment, there may not be sufficient quantities of antibodies in the supernatant to further saturate proliferating cells later in the experiment. Conversely, if IgA has a low affinity or less binding sites than either IgM or IgG, there may be excess antibody in the supernatant which would be available as cells proliferate, resulting in an increased binding over time.

Liu et al. also demonstrated the binding of purified human IgM, IgG and IgA to porcine endothelium but found that only IgM and IgG were cytotoxic in the presence of rabbit complement (158). Conversely, Schaapherder et al. (67) found that 1 mg/mL purified human dIgA caused the deposition of C3 but not C4 on porcine endothelial cells resulting in 28% cell lysis compared to 31% with 1 mg/mL purified IgM. Agammaglobulinemic serum, purified IgG or purified monomeric IgA showed negligible cell lysis. The difference in these results may be due to the different species of complement used. IgA may also be involved in other effector mechanisms such as Fc receptor binding on macrophages and neutrophils (159,160).

IgM is considered to be the primary antibody class involved in the progression of HAR in the pig to human species combination due to its ability to activate complement. IgG, in contrast, may be the primary antibody involved in DXR as its Fc portion is used as opsonization for leukocytes involved in the cellular infiltration characteristic of DXR.

Dorling et al. (134) demonstrated the significance of IgG in accommodation by preincubating immortalized porcine endothelial cells (IPECs) with polyclonal IgG for up to 144 hours followed by incubation with human AB serum. A decrease in IgG binding was observed over time with no significant change in the level of IgM. Accommodation as indicated by a change in IPEC phenotype leading to resistance to complement-mediated lysis was maximal after 120 hr preincubation. It was suggested that the expression of non-αGal epitopes were reduced during preincubation which lead to decreased IgG binding.

Dorling also reported no consistent or significant changes in E-selectin expression. Phenotypic changes included the downregulation of both MHC class I and VCAM which the investigators attributed to an accommodated state.

Dalmasso et al. (133) reported no significant change in IgM or IgG binding following a 40 hr preincubation using PAECs. Investigators attributed a reduction in cytotoxicity after 17 hours to IgM, as the same reduction did not occur with either purified IgG or serum depleted of IgM. There was no loss of antigens reported in this study.

The contribution of IgM and IgG towards achieving accommodation may differ considerably as suggested by the combined results of Dorling and Dalmasso. Dalmasso found that purified IgM caused a reduction in complement-mediated lysis beginning at 17 hours and maximal at 40 hours. Dorling showed that by using purified IgG during preincubation, a decrease in VCAM and MHC class I expression began after 72 hours and was maximal after 120 hours. The different rates and effects of these two classes of antibodies in the induction of accommodation may be based on the observation that these antibodies target different epitopes on the cell surface as well as targeting some of the

same epitopes albeit to different degrees. The differences in antibody specificity on the epithelial cell surface were made apparent in this study using immunoblots (Fig. 8).

Although epitopes of the approximate MW as the prominent triad described by Platt (gp 115/135) were detected by the human IgM in this study, the bands were not prominent. Aspeslet et al. (157) also failed to detect Platts triad using LLC-PK1 cells which suggest that this cell line differs from PAECs with respect to the degree of expression of these glycoproteins. The results from this study are in agreement with those of Aspeslet though bands of higher molecular weight were detected in this study with both IgM (193 kD and 139 kD) and IgG (166 kD). Cooke sought to define endothelial epitopes which were bound exclusively by IgG. Bands of approximately 210 kD, 180 kD, 110 kD, and 75 kD were described of which, 110 kD and 75 kD were also observed on the LLC-PK1 cells in this study. The bands unique to IgG are a significant finding in that once bound to the cell surface, these antibodies may mediate different effects than those by IgM.

The degree of contribution towards accommodation by both IgM and IgG may prove significant as it has been shown that in addition to there being interindividual variation in the titre of each, the ratio of IgM to IgG can also be significantly different between individuals (65). If, for example, an individual has a significantly higher level of IgG XNAbs than IgM, the effects mediated by the IgG class may be more pronounced in that there may be a higher degree of NK cell infiltration as a result of opsonization.

Both Dorling and Dalmasso stress the concentration-dependence of each antibody class in achieving accommodation. This study did not purify and quantitate each class. It has been suggested that antibodies alone can transduce signals directly into cells via integrins thus affecting cytokine gene expression or adhesion molecule expression.

(140,161). Dorling demonstrated that the protecting phenotype induced by IgG began after approximately 72 hours preincubation. In contrast, Dalmasso observed a reduction in cytotoxicity after 17 hours with IgM. It is possible that the proinflammatory, procoagulant phenotype induced by IgG may overlap the time interval for which IgM is inducing protective genes. Should this be the case, using natural human serum for the duration of the preincubation may add to the complexity of studying accommodation *in vitro* and may explain differences in the results between this study and others.

Other inconsistencies may be due to either the cell line used or the complement source. LLC-PK1 cells, a porcine renal epithelial cell line, were used in this experiment because they were an immortalized cell line which would be amenable to the conditions of the experiment. That is, they do not require cell-to-cell contact to thrive after initial plating, unlike PAECs. This was important due to the low concentration of cells (approximately 1×10^4 cells/mL) which were used over the duration of the experiment. Aspeslet (157) compared immunoblots of both PAEC and LLC-PK1 lysates (Table 1). It was observed that although there was a change in the number of epitopes bound by both IgM (17 by LLC-PK1 and 11 by PAECs) and IgG (8 by LLC-PK1 and 11 by PAECs), the prominent bands were common to both cell lines.

Dorling et al. (134) justified the use of IPECs rather than PAECs by comparing the quantity and kinetics of VCAM and E-selectin expression following exposure to rTNFα. The expression of both VCAM and E-selectin were higher on IPECs than PAECs and the kinetics were delayed with IPECs. Peak expression of E-selectin occurred 4 hr on PAECs after rTNFα exposure compared to 24 hr with IPECs, and VCAM expression peaked at 24 hr with PAECs in contrast to 72 hr with IPECs.

The differences between the cell lines described above are subtle, but obvious physiological differences do exist. It is possible then, that other, as yet undefined differences exist which may warrant distinct conditions in order to induce accommodation. Also, prolonged cell culture may cause alterations in the expression of some cell membrane epitopes. This may warrant the use of primary cell cultures.

The type of complement used can also affect assay results. Human serum as a complement source would be optimal when using a pig to human model. However, very little cytotoxicity was noted with human complement (< 25%) after subtraction of either the media and complement or neonatal pig serum and complement negative control (Fig. 6). Also, fresh human complement was not readily available in the quantities required. It is because of these reasons that lyophilized rabbit complement was used as a complement source for the cell viability assays. Two different types of rabbit complement were used; Pel-Freez rabbit complement and Cedar Lane Low-Tox H rabbit complement. The Cedar Lane complement was selected based on the fact that it is intended for use in assays which utilize cells normally sensitive to lysis by normal pooled rabbit serum. It has been selected for low toxicity to human peripheral blood B lymphocytes. Pel-Freez rabbit complement, which is obtained from rabbits 8-12 weeks old, has been tested for high titre and low background toxicity. There was a difference noted in the reduction of cytotoxicity with the two types of rabbit complement. When media was used as a negative control, complement-mediated cytotoxicity was reduced with all serum concentrations with Pel-Freez rabbit complement whereas there was no significant decrease in cytotoxicity when Cedar Lane rabbit complement was used. The lot of complement used, the strain of rabbit from which the complement was obtained, as well as the length of time the complement was stored could all have an effect on the results.

This study explored the possibility of developing an *in vitro* model for accommodation. Such a model would be a valuable tool in further investigating the complex processes involved in xenotransplantation as well as being an ethical alternative to animal studies. However, this study observed potential inconsistencies which may occur in an *in vitro* system leading to artifactual results. Results may differ depending on cell culture technique, source of complement, cell line used, or assay method used. Despite these inconsistencies, the results obtained with Pel-Freez rabbit complement using media as the negative control strongly indicate a reduction in cytotoxicity due prolonged antibody exposure. This result suggests that an accommodated state may have occurred. If this is the case, these results are significant in that they indicate that an isotype switch or a T h response may not be necessary to induce accommodation. Instead, direct transduction of a signal by the bound XNAb into the cell may be sufficient in some situations. However, further investigation is required so that possible artifacts discussed earlier may be excluded.

Some experiments which could further this area of research would be to characterize various porcine cell lines using flow cytometry to quantitate the expression of key adhesion molecules following cell activation. This would delineate any major differences between cell lines and assist in choosing appropriate cells for further experiments. In addition to standardization of the cell line, purification and quantitation of each antibody class to be run in tandem experiments would be the optimal method for monitoring physiological changes to the cells. In this way, the relative contribution of each antibody class alone and in combination in achieving accommodation *in vitro* can be assessed.

Although various cell markers (E-selectin, VCAM, and MHC class I) have been used as an indication that accommodation has occurred, it would be interesting to

investigate which of these changes are responsible for an accommodated phenotype. That is, to see if there appears to be a "master switch" (ie. NFκB) which results in several phenotypic changes over the course of time required to achieve accommodation. Also, the connection between the activation of genes which inhibit the expression of NFκB and result in the induction of accommodation should be further explored using the discordant pig to human model. If the essential factors involved in accommodation are described, the conditions in which these factors are regulated could be studied, standardized and later incorporated into an *in vivo* model.

Future experiments may include the hu-PBL SCID (severe combined immune deficient) mouse model in which human lymphocytes are injected i.p. into the SCID mouse. The goal of this model is to engraft SCID lymphoid tissues (spleen and lymph nodes) with human lymphocytes. Sandhu et al. demonstrated that human T helper cells, antigen presenting cells and B cells are functional in the SCID environment (162) and Naziruddin et al. found that hu-PBL SCIDS produce XNAb that are biochemically and functionally similar to XNAb found in natural human serum (163). If studying accommodation in this model one must evaluate whether the hu-PBL SCID is in fact representative of the human immune system. That is, whether a significant cross section of human lymphocyte clones are colonizing the SCID mouse.

CONCLUSIONS

The results from this study indicate that further standardization of the assays used is required such that true accommodation can be distinguished from artifacts introduced through experimental procedure. Although there was a significant decrease in cytotoxicity noted with some of the cytotoxicity assays (MTT assay using Pel-Freez rabbit complement with both media and neonatal pig serum as negative controls and using Cedar Lane Low-Tox H rabbit complement with neonatal pig serum as a negative control), there were inconsistencies which affect the interpretation of these results. These inconsistencies include the fact that the positive control (3% Triton-x) also exhibited a decrease in cytotoxicity over the course of the experiment. Also, the type of complement used and the negative control employed were also shown to affect results. Finally, markers of epithelial cell activation must be explored further.

There are many inconsistencies which can arise from using an *in vitro* system in addition to it not being true to an actual *in vivo* system. At this time, an *in vivo* model may provide more information about accommodation than can be obtained using an *in vitro* model. It may prove useful to apply the *in vivo* strategy such as the hu-PBL SCID mouse model or the model which was employed by Bach et al (129) using the concordant hamster to rat model. The strategy could be employed using the discordant pig to non-human primate species combination. These two models reject organs through different processes and it would be interesting to see whether the antibody isotype switch to IgG2, the Th2 cytokine response, and the upregulation of genes which prevent apoptosis and the translocation of NFκB contribute to accommodation in the pig to non-human primate as well. If so, an *in vitro* model more complex than designed herein would be required to study the mechanisms at a molecular level.

In the last 10 years several important observations have been made regarding pig to human xenotransplantation. These include the predominant epitopes targeted on the porcine endothelium, the processes involved in HAR and DXR, and the prevention of HAR by inhibiting either antibody binding or complement activation. However, 100% graft survival is still not attainable. Immunosuppressants routinely used in allotransplantation are not effective in xenotransplantation due to the intensity of the rejection process. This warrants alternate therapies, one of which includes the induction of accommodation. The mechanism of accommodation is currently not defined but due to the vast quantity of information gained in recent studies, it is possible that this is a realistic goal in prolonging xenograft survival and ultimately enabling xenotransplantation to serve as an alternative to allotransplantation.

APPENDIX

FIGURES AND TABLES

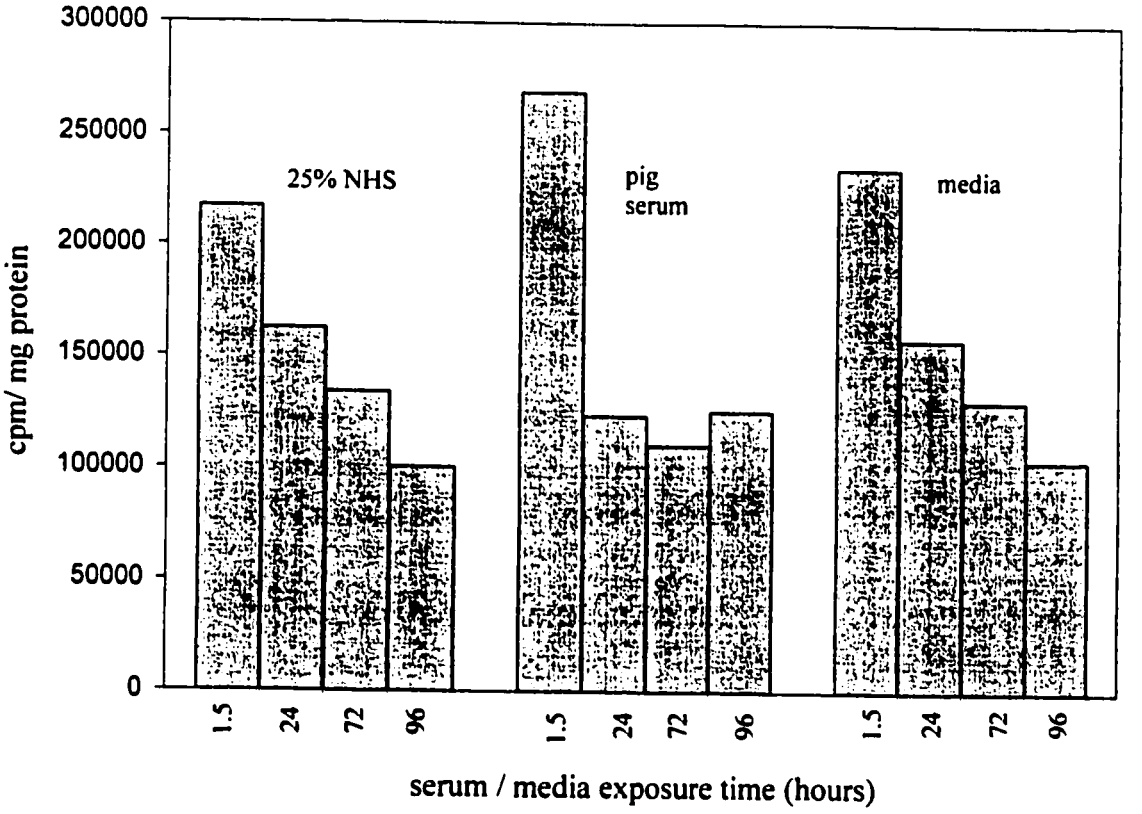
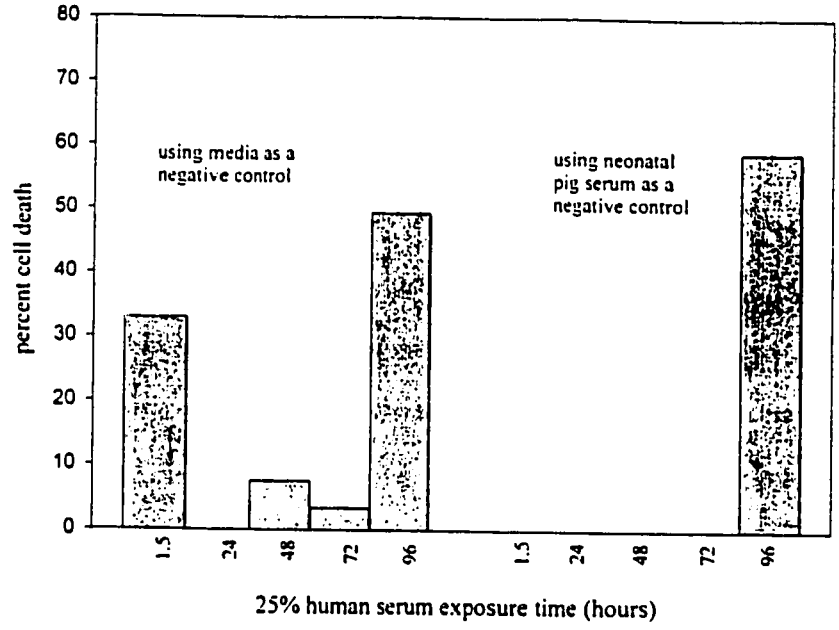


Figure 1. ^{3}H-Thymidine viability assay. Cells were incubated for 1.5-96 hours with either 25% NHS, neonatal pig serum or media (n=2) followed by a 1 hour incubation with rabbit complement. ^{3}H-Thymidine diluted was 1: 1000 with enriched media was incubated with the cells for 21 hours. Incorporation of ^{3}H-Thymidine into the DNA was measured using a β-counter. Protein was quantitated using cell lysates and samples were normalized by dividing cpm by mg protein.

Figure 2. Neutral red cell viability assay using either Cedar Lane Low-Tox H rabbit complement (a) or Pel-Freez rabbit complement (b). Cells were counted and both media and neonatal pig serum were used as negative controls, the spontaneous cell death from each was subtracted from the 25% human serum test wells (N=2).

a)


80
70
60
50
40
30
20
10
0
percent cell death
using media as a negative control
using neonatal pig serum as a negative control
1.5
24
48
72
96
1.5
24
48
72
96
25% human serum exposure time (hours)

b)

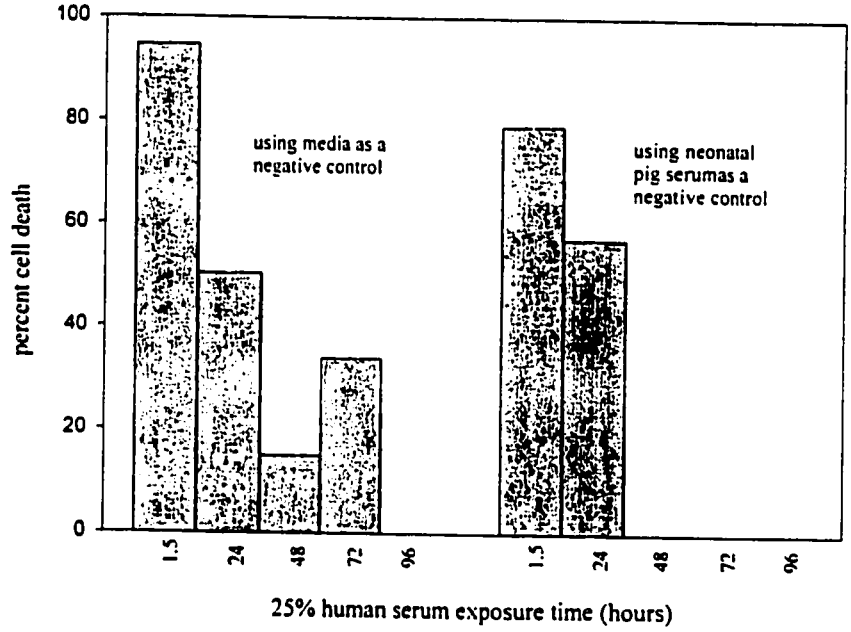
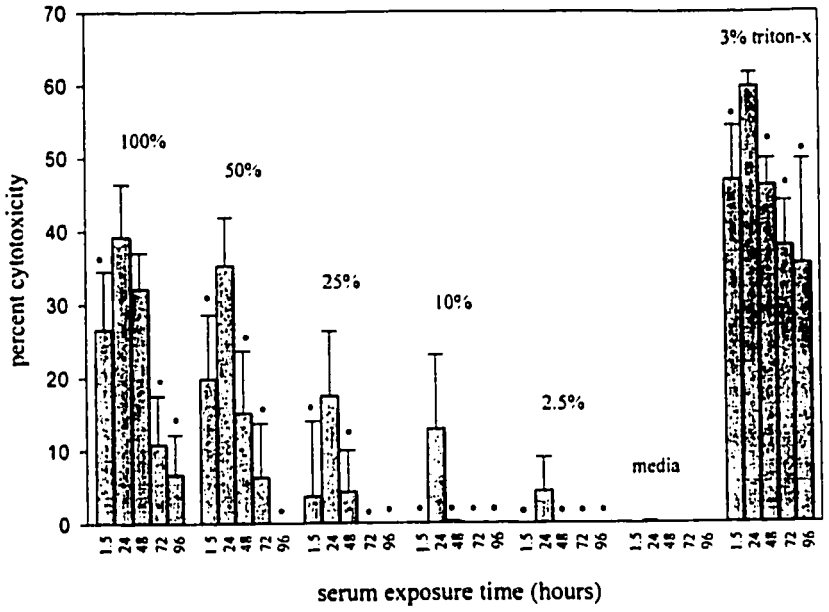

100
80
60
40
20
0
percent cell death
using media as a negative control
using neonatal pig serumas a negative control
1.5
24
48
72
96
1.5
24
48
72
96
25% human serum exposure time (hours)

Figure 3. MTT cytotoxicity assay using Pel-Freez rabbit complement. Cells were incubated with 100%, 50%, 25%, 10%, and 2.5% NHS for 1.5-96 hours. Following serum incubation, cells were incubated for 1 hour with rabbit complement. 3% triton-x was used as a positive control and either neonatal pig serum (a) or media (b) was used as a negative control. The OD of the negative control was subtracted from the test OD in order to account for spontaneous cell death over the course of the experiment. OD was measured at 540 nm. N=8, error bars represent the mean ± SD. Time points which are statistically significant compared to the control (24 hr) are indicated by *.

a)


percent cytotoxicity
0
10
20
30
40
50
60
70
100%
50%
25%
10%
2.5%
media
3% triton-x
1.5
24
48
72
96
serum exposure time (hours)

b)

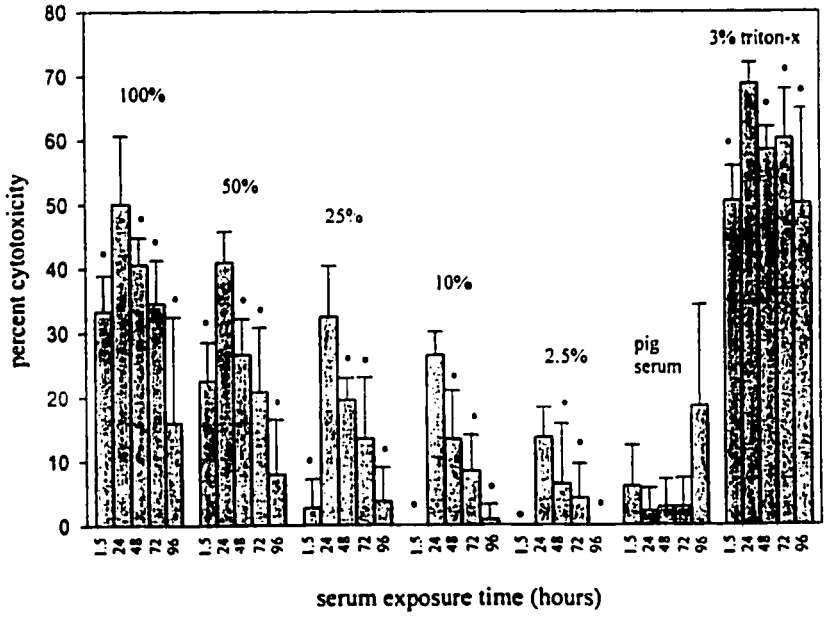
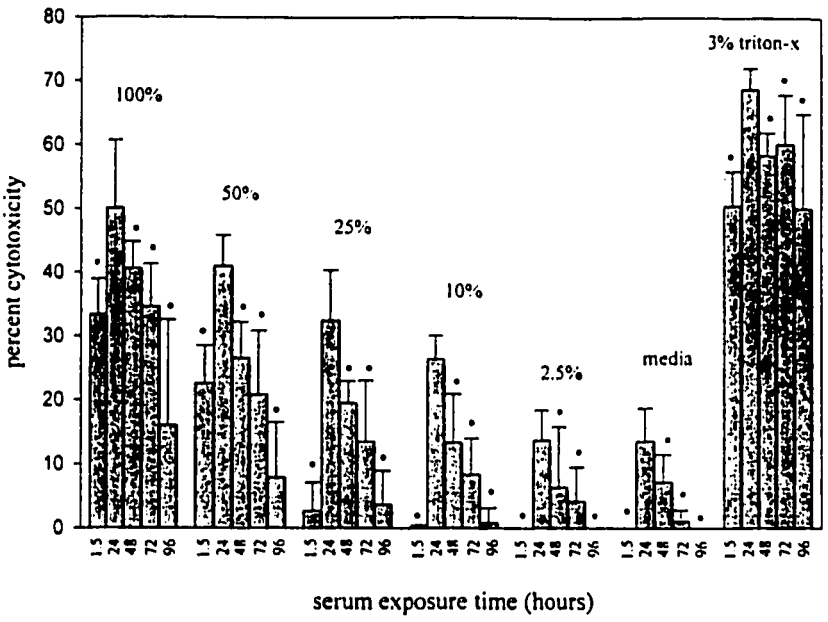
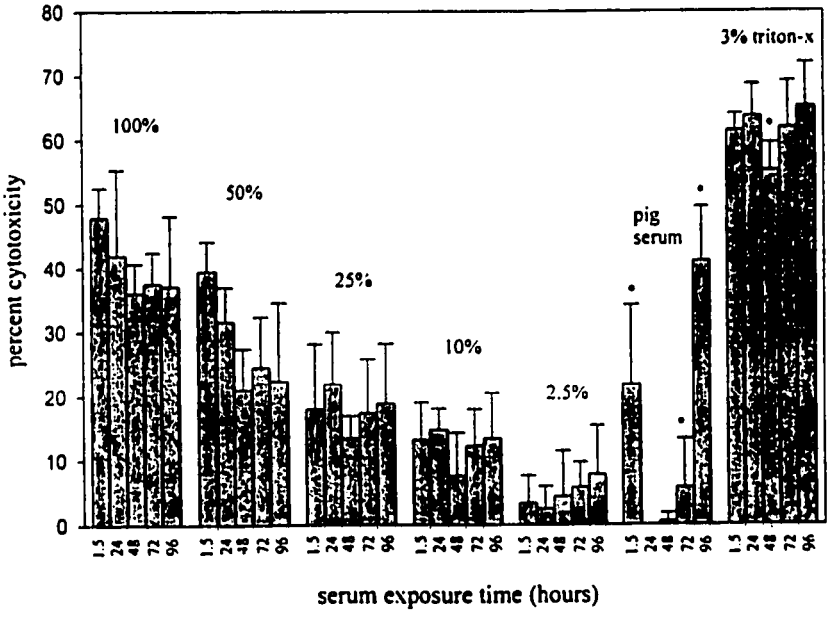

percent cytotoxicity
0
10
20
30
40
50
60
70
80
100%
50%
25%
10%
2.5%
pig serum
3% triton-x
1.5
24
48
72
96
serum exposure time (hours)

Figure 4. MTT cytotoxicity assay using Cedar Lane Low-Tox rabbit complement. Cells were incubated with 100%, 50%, 25%, 10%, and 2.5% NHS for 1.5-96 hours. Following serum incubation, cells were incubated for 1 hour with rabbit complement. 3% triton-x was used as a positive control and either neonatal pig serum (a) or media (b) was used as a negative control. The OD of the negative control was subtracted from the test OD in order to account for spontaneous cell death over the course of the experiment. OD was measured at 540 nm. N=8, error bars represent the mean ± SD. Time points which are statistically significant compared to the control (24 hr) are indicated by *.

a)


percent cytotoxicity
100%
50%
25%
10%
2.5%
media
3% triton-x
serum exposure time (hours)

b)


percent cytotoxicity
100%
50%
25%
10%
2.5%
pig serum
3% triton-x
serum exposure time (hours)

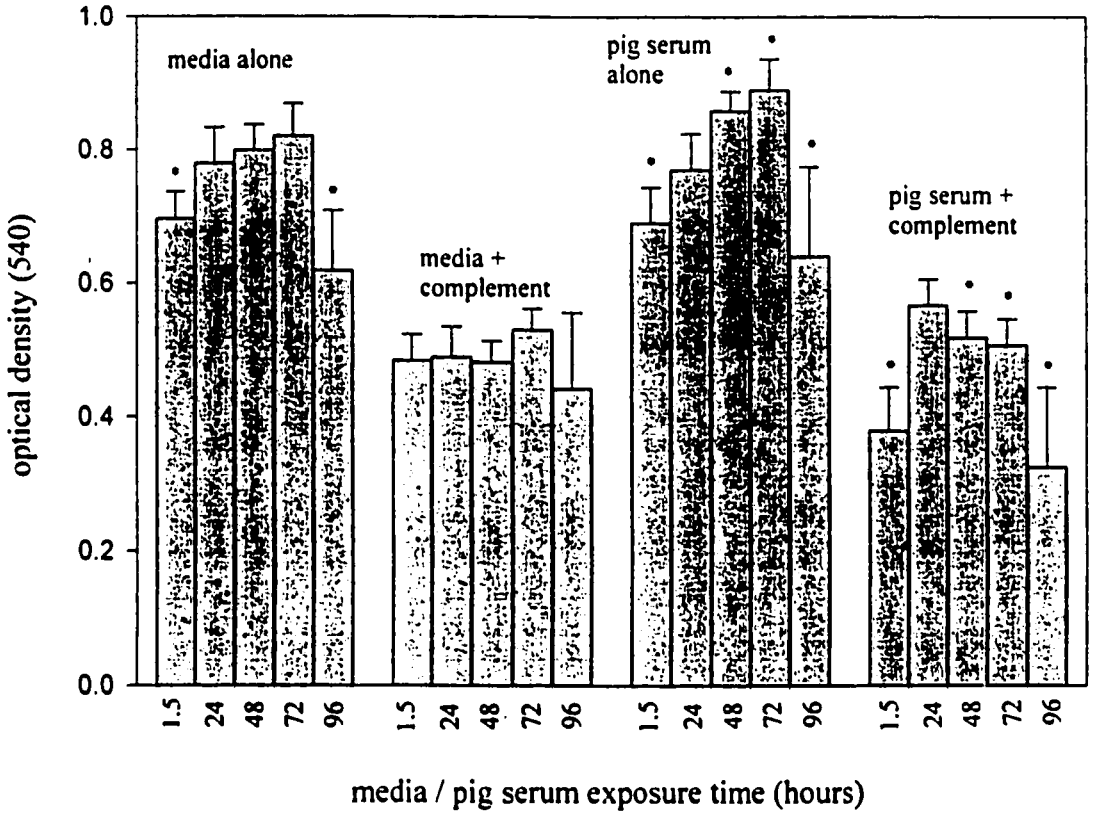
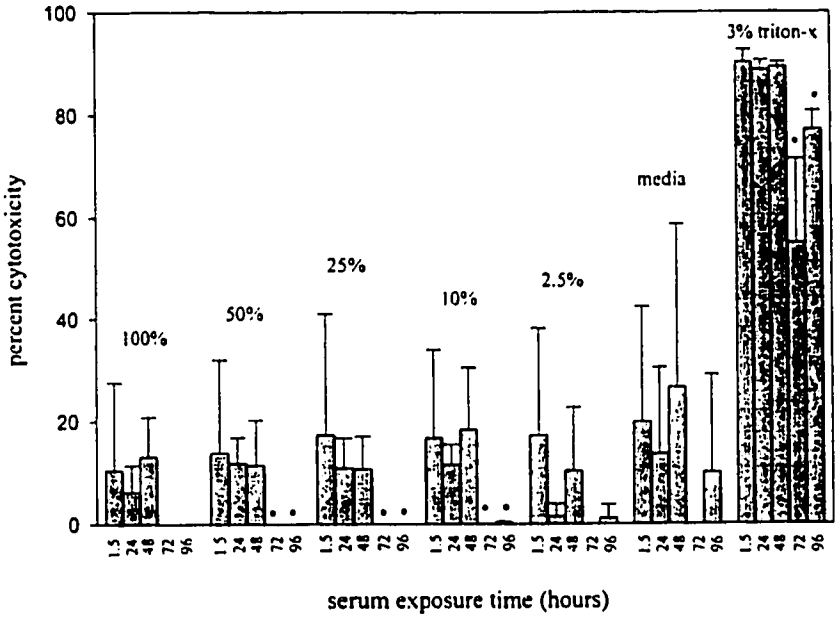


Figure 5. MTT cytotoxicity assay demonstrating spontaneous cell death. Cells were incubated with either enriched media or neonatal pig serum for 1.5-96 hours followed by a 1 hour incubation with Cedar Lane Low-Tox H rabbit complement. Optical density was measured at 540 nm. N=8, error bars represent the mean ± SD. Time points which are statistically significant compared to the control (24 hr) are indicated by *.

Figure 6. MTT cytotoxicity assay using human complement. Cells were incubated with 100%, 50%, 25%, 10%, and 2.5% NHS for 1.5-96 hours. Following serum incubation, cells were incubated for 1 hour with rabbit complement. 3% triton-x was used as a positive control and either neonatal pig serum (a) or media (b) was used as a negative control. The OD of the negative control was subtracted from the test OD in order to account for spontaneous cell death over the course of the experiment. OD was measured at 540 nm. N=6, error bars represent the mean ± SD. Time points which are statistically significant compared to the control (24 hr) are indicated by *.

a)


percent cytotoxicity
100
80
60
40
20
0
100%
50%
25%
10%
2.5%
media
3% triton-x
1.5 24 48 72 96
serum exposure time (hours)

b)

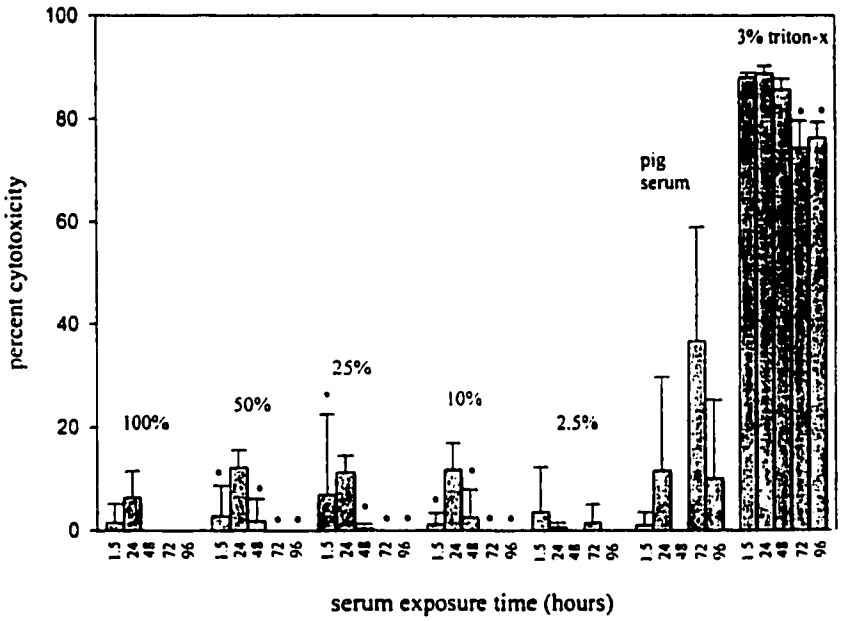
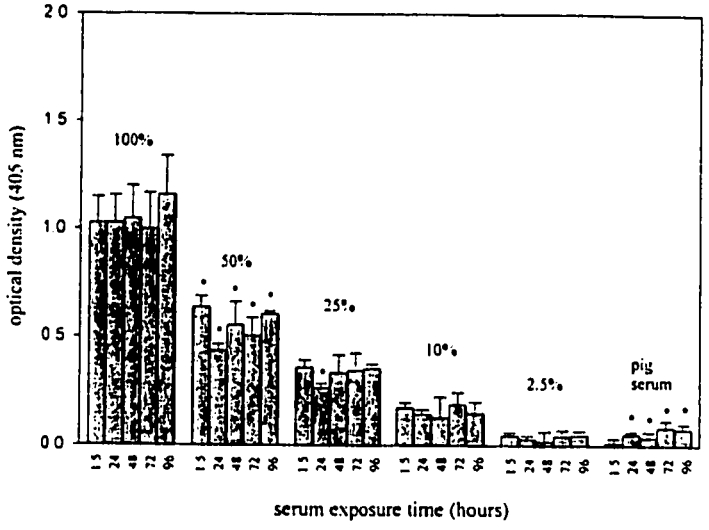
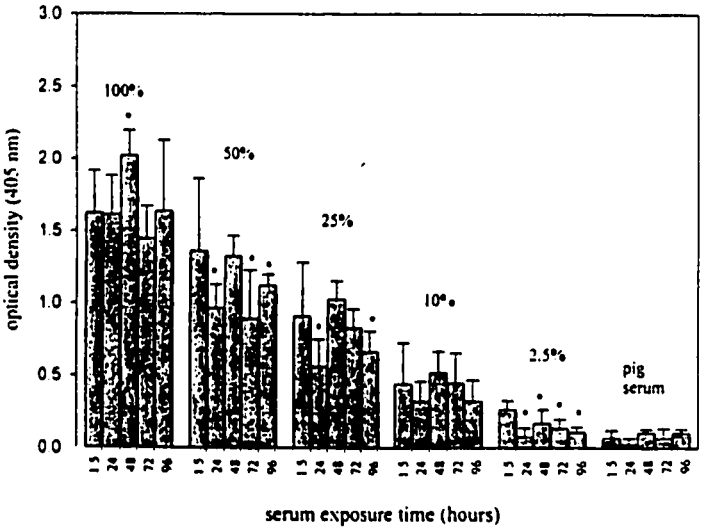

percent cytotoxicity
100
80
60
40
20
0
100%
50%
25%
10%
2.5%
pig serum
3% triton-x
1.5 24 48 72 96
serum exposure time (hours)

Figure 7. Enzyme-linked immunosorbant assay (ELISA). Cells were incubated with 100%, 50%, 25%, 10%, and 2.5% NHS for 1.5-96 hours. Following serum incubation, labelled anti-human IgM (a), IgG (b),or IgA (c) was incubated with cells for 1.5 hours. After development, optical density was measured at 405 nm. N=8, error bars represent the mean ± SD. Time points which are statistically significant compared to the control (1.5 hr) are indicated by *.

a)


optical density (405 nm)
2.0
1.5
1.0
0.5
0.0
100%
50%
25%
10%
2.5%
pig serum
1.5 24 48 72 96
serum exposure time (hours)

b)


optical density (405 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100%
50%
25%
10%
2.5%
pig serum
1.5 24 48 72 96
serum exposure time (hours)

c)

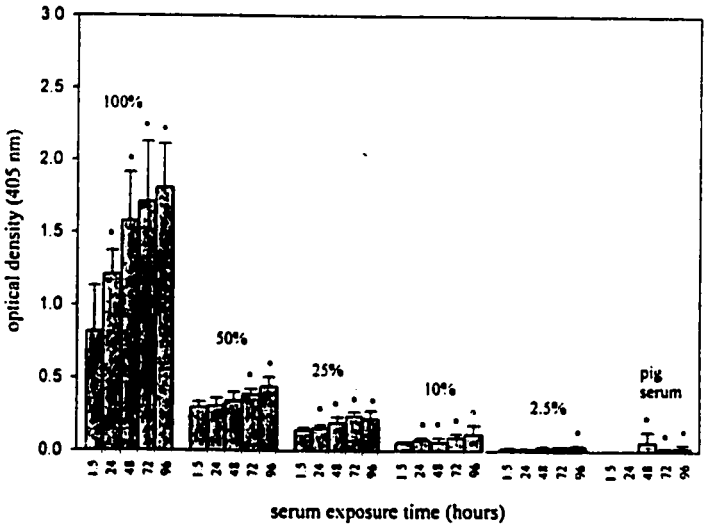
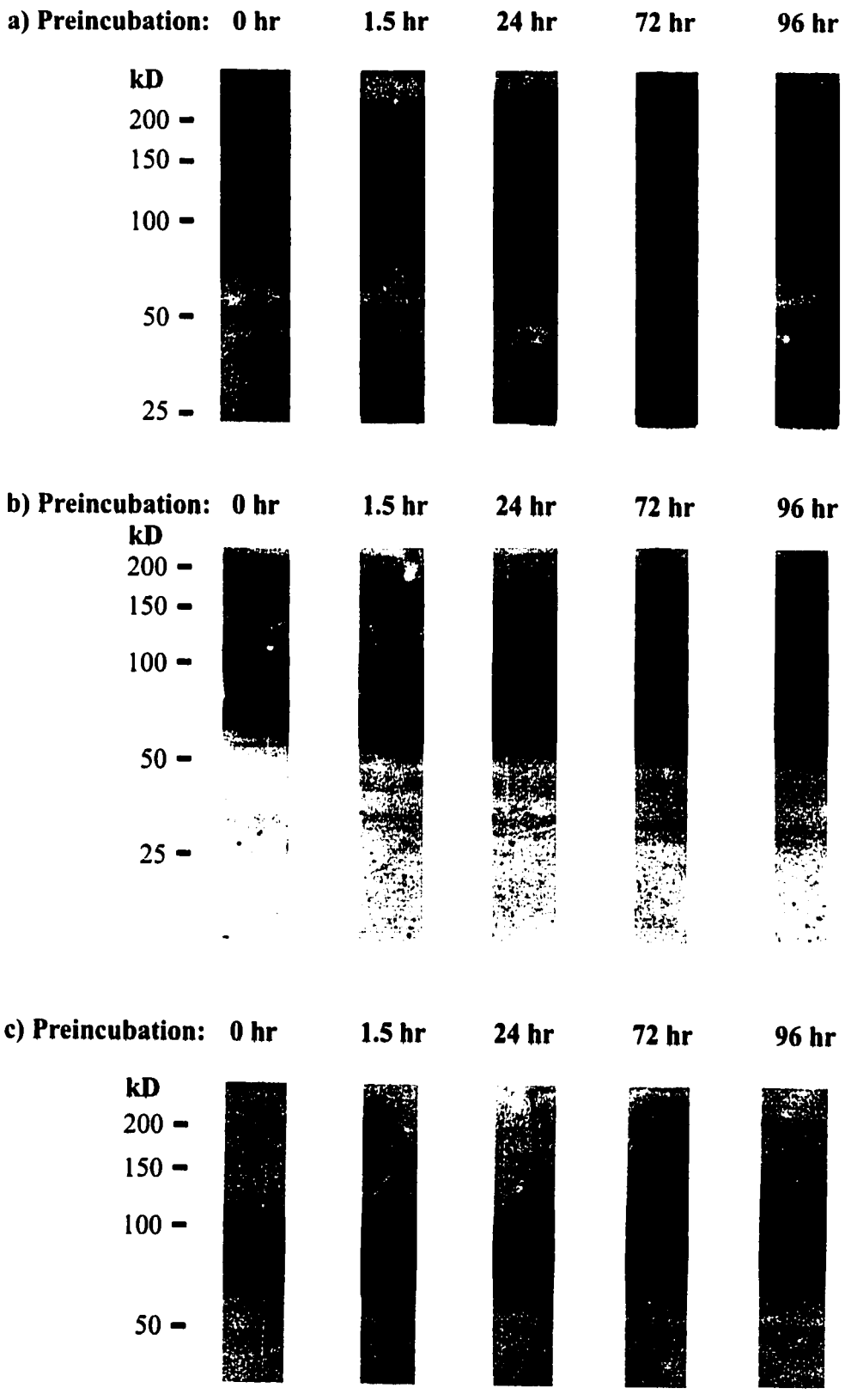

optical density (405 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100%
50%
25%
10%
2.5%
pig serum
1.5 24 48 72 96
serum exposure time (hours)

Figure 8. Immunoblots from LLC-PK1 lysates. Cells were preincubated with either media (0 hr) or 25% NHS for 1.5, 24, 72, or 96 hours. After electrophoresis, immunoblots were incubated with 1:1000 NHS followed by secondary antibody which was either 1:5000 anti-IgM (a), 1:5000 anti-IgG (b), or 1:2500 anti-IgA (c). Immunoblots were developed using enhanced chemiluminescence.


a) Preincubation: 0 hr
1.5 hr
24 hr
72 hr
96 hr
kD
200
150
100
50
25
b) Preincubation: 0 hr
1.5 hr
24 hr
72 hr
96 hr
kD
200
150
100
50
25
c) Preincubation: 0 hr
1.5 hr
24 hr
72 hr
96 hr
kD
200
150
100
50

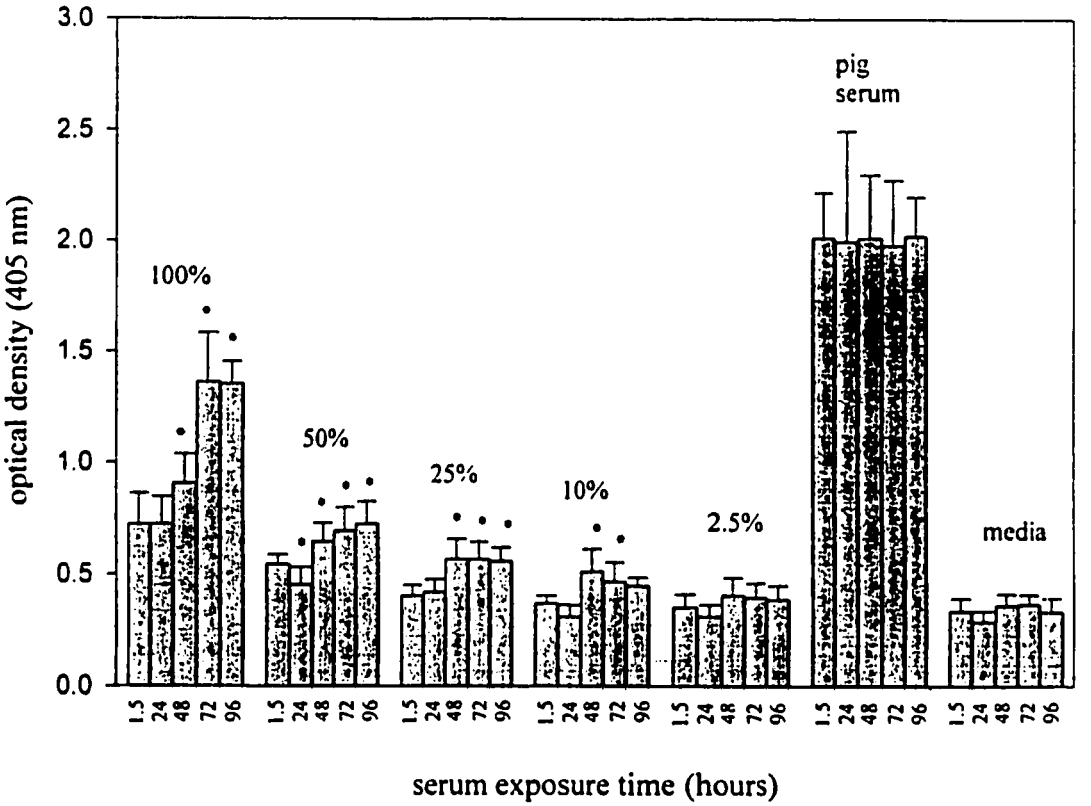


Figure 9. E-selectin ELISA. Cells were incubated with 100%, 50%, 25%, 10%, and 2.5% NHS for 1.5-96 hours. Following serum incubation, the secondary anti-E-selectin antibody was incubated with the cells for 1.5 hr. After development, OD was read at 405 nm. N=8, error bars represent the mean ± SD. Time points which are statistically significant compared to the control (24 hr) are indicated by *.

Table 1. Comparison of epitopes detected by anti-IgM, -IgG, and -IgA from this study and others using either LLC-PK1 or PAEC lysates.

			Cooke (56)	Aspeslet (150)			
LLC-PK1			PAECs	LLC-PK1		PAECs	
IgM (kD)	IgG (kD)	IgA (kD)	IgG (kD)	IgM (kD)	IgG (kD)	IgM (kD)	IgG (kD)
193	166	124	210	126	126	133	123
139	110	114	180	100	120	126	120
135	99	107	110	82	117	120	117
116	92	99	75	75	111	103	106
97	76	89		63	101	91	101
87	63	80		49	91	82	93
78	60	70		44	72	72	82
65	43	65		36	64	65	74
62	32			30	56	59	
44				25	41	55	
32				20	36	44	
						36	
						30	
						26	
						24	
						23	
						20	

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IMAGE EVALUATION
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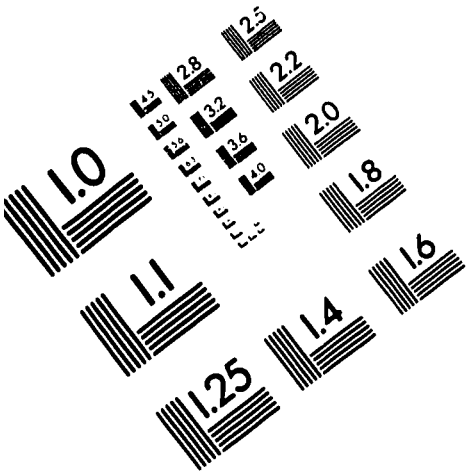

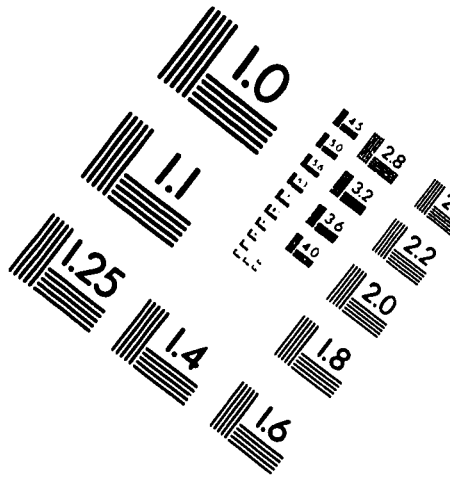

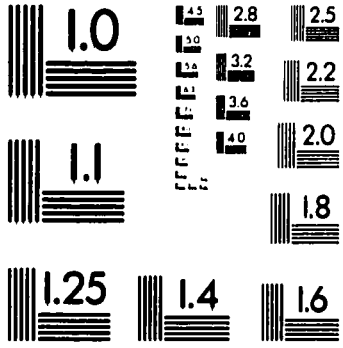

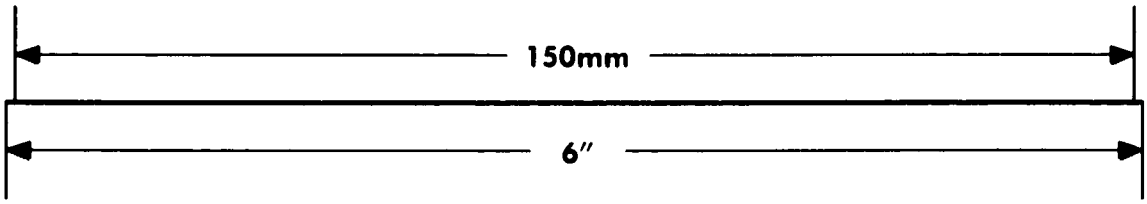

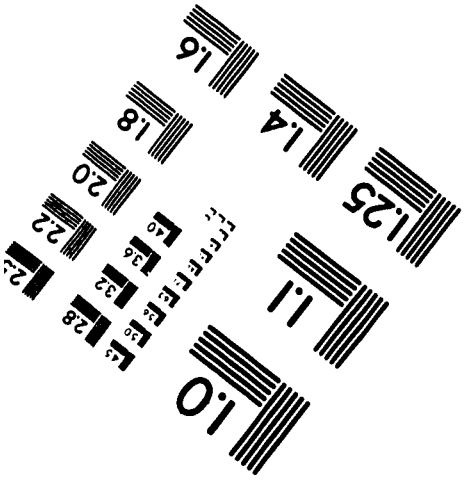

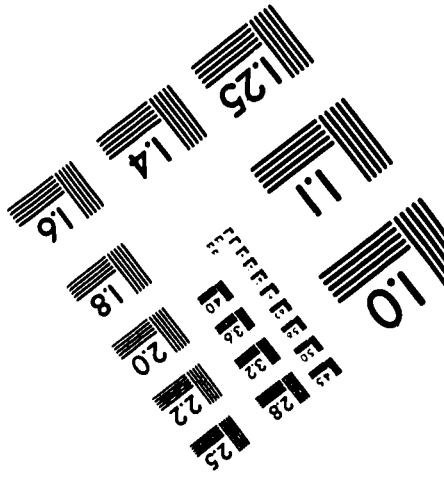

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