INDUCTION OF IMMUNOLOGICAL TOLERANCE IN THE PIG-TO-BABOON XENOTRANSPLANTATION MODEL

Studies aimed at achieving mixed hematopoietic chimerism and preventing associated thrombotic complications

ISBN 90-9014712-8

Printed by Pasmans Offsetdrukkerij b.v., Den Haag

INDUCTION OF IMMUNOLOGICAL TOLERANCE IN THE PIG-TO-BABOON XENOTRANSPLANTATION MODEL

Studies aimed at achieving mixed hematopoietic chimerism and preventing associated thrombotic complications

DE INDUCTIE VAN IMMUNOLOGISCHE TOLERANTIE IN HET VARKEN-NAAR-BAVIAAN XENOTRANSPLANTATIE MODEL

Studies gericht op het bereiken van gemengd hematopoietisch chimerisme en de preventie van geassocieerde thrombotische complicaties

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties

de openbare verdediging zal plaatsvinden op
woensdag 11 april 2001 om 15.30 uur

door

Ian Patrick Joseph Alwayn

geboren te Leidschendam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. J. Jeekel
	Prof.dr. D.K.C. Cooper
Overige leden:	Prof.dr. R. Benner
	Prof.dr. F.G. Grosveld
	Prof.dr. W. Weimar
Copromotor:	Dr. J.N.M. IJzermans

The studies presented in this dissertation were performed at the Transplantation Biology Research Center of Massachusetts General Hospital / Harvard Medical School, Boston, U.S.A.

The work was financially supported by grants from the 'Ter Meulen Fund' of the Royal Dutch Academy of Arts and Sciences and from the Professor Michaël van Vloten Fund.

Voor mijn moeder,

In memory of my father

CONTENTS

Section I	**Introduction**	
Chapter 1	Introduction - aims of this dissertation	11
Section II	**Attempts to induce mixed hematopoietic chimerism in pig-to-baboon xenotransplantation**	
Chapter 2	Depletion of macrophages in baboons delays the clearance of mobilized pig leukocytes	35
Section III	**The importance of suppressing xenoreactive antibody production**	
Chapter 3	Current and novel methods of depletion and/or suppression of production of anti-pig antibodies	59
Chapter 4	Effects of specific anti-B and/or anti-plasma cell immuno-therapy on xenoreactive antibody production in baboons	83
Section IV	**Understanding and preventing the thrombotic complications associated with pig-to-baboon xenotransplantation**	
Chapter 5	Coagulation and thrombotic disorders associated with xenotransplantation	115
Chapter 6	Mechanisms of thrombocytopenia following xenogeneic hematopoietic progenitor cell transplantation	137
Chapter 7	Immunosuppressive therapies and platelet aggregation in baboons	155

Chapter 8 Pharmacologic inhibition of platelet aggregation in baboons 179

Section V Discussion

Chapter 9 General discussion 203
Chapter 10 Summary in Dutch 213

List of abbreviations 223
List of publications 225
Acknowledgements 231
Curriculum vitae auctoris 233

I

Introduction

1

Introduction - aims of this dissertation

Adapted from:
Alwayn IPJ, Buhler L, Basker M, and Cooper DKC. Immunomodulation strategies in xenotransplantation. In: Current and Future Immunosuppressive therapies following Transplantation. *Sayegh M and Remuzzi G (Eds.). In press.*

1.1 THE NEED FOR XENOTRANSPLANTATION

The outcome of clinical organ transplantation has dramatically improved since the introduction of cyclosporine (CyA) in 1979 and of other, more recently introduced, immunosuppressive agents such as azathioprine, mycophenolate mofetil, tacrolimus and sirolimus. Furthermore, due to more refined surgical techniques and perioperative management, prolonged survival of allografts is achieved. Due to its relative success, the inclusion criteria for potential organ transplant recipients have been broadened, resulting in an even greater shortage of donor organs. The number of patients with end-stage organ failure that die awaiting organ transplantation is steadily increasing, mainly because of the shortage of appropriate donor organs (1,2,3). Many clinicians and investigators believe that strategies directed at expanding the allogeneic donor pool will not be sufficient to resolve this problem of organ shortage. In contrast, successful xenotransplantation - the transplantation of tissues and/or organs between two different species - has the potential of providing an unlimited supply of donor organs that would be available when required (4). However, major immunological barriers, as well as other important issues, at present prevent the implementation of xenotransplantation clinically (5).

Concordant xenografts - tissues/organs transplanted between phylogenetically close species, i.e., mouse and rat, monkey and baboon, or nonhuman primate and human - reject more vigorously than allografts. The mechanism of rejection is thought to be mediated by a combination of humoral and cellular factors. Conventional immunosuppressive therapy cannot guarantee long-term graft survival because of the potent induced antibody response in concordant xenotransplantation. However, with judicious pharmacological immunosuppressive therapy, the reported survival of concordant heart grafts in nonhuman primates exceeds 500 days (6).

Discordant xenotransplantation - xenotransplantation between phylogenetically widely disparate species, i.e., guinea pig and rat, pig and baboon, or pig and human - is characterized by the occurrence of hyperacute rejection (HAR) (7, 8). This type of

rejection, mediated in primates by preformed (natural) antibodies (directed against the Galα1-3Gal (α-Gal) epitopes) (9), which activate complement, takes place within minutes to hours after transplantation (10). Histological examination of these rejected grafts reveals intravascular thrombosis and interstitial hemorrhage (11, 12).

It has recently become possible to overcome HAR through antibody and/or complement depletion or by utilizing organs from pigs transgenic for human complement regulatory proteins (8, 13, 14, 15). If HAR is successfully prevented, a delayed type of rejection termed acute humoral xenograft rejection (AHXR) occurs within days. Cellular infiltrates are invariably found throughout the xenograft, consisting of macrophages, NK cells, and monocytes (16, 17). Data suggest that this type of rejection is most probably antibody-mediated but complement-independent. It has thus far not been possible to overcome AHXR in the pig-to-primate model. Survival of xenografts to date has generally been less than 30 days (18, 19), and has not exceeded 99 days (White DJG, personal communication).

These major immunological differences notwithstanding, it is widely thought that the pig will be the most appropriate organ source (20, 21), as pigs have a number of advantages over nonhuman primates as a potential source of organs for humans (Table 1). Furthermore, pigs can be bred in specific pathogen-free environments, thus eliminating to a large extent the risk of transfer of disease from pig to human. Certain herds of miniature swine have fully documented MHC antigens, which may facilitate certain manipulations aimed at the induction of tolerance in the recipient, and pigs may be genetically modified to become less immunogenic to humans. Finally, since pigs are currently being bred for human consumption, it is likely that the general public will be amenable to using pigs for human organ xenotransplantation.

Many studies have been directed at attempting to understand the mechanisms of pig-to-nonhuman primate xenograft rejection. Much progress in this field has been made in the last decade, although the pathophysiology of xenograft rejection has not yet been fully elucidated. This chapter will briefly summarize our current knowledge of the mechanisms involved in discordant xenograft rejection and describe the

techniques being explored to prevent rejection from occurring. Emphasis will be placed on our experience in the pig-to-nonhuman primate preclinical model. Various

TABLE 1

RELATIVE ADVANTAGES AND DISADVANTAGES OF PIGS AND BABOONS AS POTENTIAL SOURCES OF ORGANS AND TISSUES.

	Baboon	Pig
Availability	Restricted	Unlimited
Breeding potential	Poor	Good
Reproductive age	3-5 years	4-8 months
Length of pregnancy	173-193 days	114 +/- 2 days
Number of offspring	1-2	5-12
Rate of growth	Slow	Rapid
Size of adult organs	Inadequate	Adequate
Maintenance cost	High	Low
Similarity		
Anatomical	Similar	Similar
Physiological	Very similar	Less similar
Immunological	Close	Distant
Knowledge of tissue typing	Limited	Considerable (in selected herds)
Experience with genetic engineering	None	Considerable
Risk of transfer of infection (xenozoonosis)	High	Low
Availability of specific pathogen-free animals	No	Yes
Public opinion	Mixed	More favorable

methods of immunomodulation will be discussed, including i) those that deplete or inhibit complement, ii) pharmacological immunosuppressive therapy aimed at suppression of both T and B cell function, iii) genetic engineering of the donor animal, and iv) the induction of tolerance and/or accommodation.

1.2 REJECTION OF XENOGRAFTS

Hyperacute Rejection

HAR is an antibody-dependant, complement-mediated process that occurs within minutes to hours after revascularization of the transplanted discordant xenograft. Perper and Najarian were among the first to describe this process in 1966 in the pig-to-dog model (7). All nonprimate mammals, including the pig, express terminal Galα1-3Gal oligosaccharides (αGal) as surface antigens on many cells, including the vascular endothelium (22, 23). In 1984 Galili et al. reported that humans possess natural antibodies with anti-αGal specificity (24), but it was not until 7 years later that Good et al. demonstrated that these antibodies (anti-αGal) play a major role in the HAR of pig xenografts in primates (9). Anti-αGal are of IgG, IgM, and IgA subclasses and account for more than 85% of circulating natural anti-pig antibody in humans and 100% in baboons (24, 25, 26, 27, 28). IgM is thought to be the major contributor to HAR (29). Binding of these antibodies to the αGal epitopes on porcine tissue results in activation of complement through the classical pathway. This initiates a chain-reaction involving multiple complement components and leads to the formation of complexes, known as the membrane attack complex of complement, which causes lysis and opsonisation of cell membranes, resulting in rapid graft destruction.

Acute Vascular Rejection

Several methods have been developed to successfully prevent HAR, including extracorporeal immunoadsorption (EIA) of anti-αGal antibody, complement depletion or inhibition, and/or the use of pigs transgenic for human complement regulatory proteins. These approaches are discussed below. Once HAR has been averted, however, acute vascular rejection (AHXR) develops, most probably as a result of the development of high levels of high-affinity antibody directed against αGal and other pig epitopes (30). IgG seems to be the antibody subclass mostly responsible as increases in anti-αGal IgG of 100-300-fold have been documented after experimental pig organ and bone marrow transplantation in humans and nonhuman primates (31,

32). These induced antibodies initiate AHXR by mechanisms that appear to be independent of complement, although complement fractions may play a role (17,30).

Cells, such as macrophages, NK cells, and monocytes are present in the rejected xenografts at the time of AHXR, suggesting that they may be important to the rejection process (16, 33, 34). Macrophages, in particular, play an important role in the production, mobilization, activation, and regulation of immune effector cells. They participate in the activation of B and T cells. They process and present antigens, secrete various cytokines and chemokines, and phagocytose apoptotic and necrotic cells as well as various pathogens. It is not clear, however, if these cells, as well as NK cells, play a direct role in mediating or effecting AHXR, or if they migrate to the graft as a result of the presence of antibody.

Whatever the mechanism of AHXR, there is growing evidence that it is associated with a state of disseminated intravascular coagulation in the recipient, which may develop before histopathological evidence of AHXR is advanced (32, 35). This phenomenon will de discussed in more detail in chapter 7.

Immunological Events following Antibody-Mediated Rejection

Since it has to date not been possible to routinely avert AHXR, discussion of the immunological processes following AHXR is mostly hypothetical. However, in vitro and in vivo studies involving discordant xenogeneic cells or grafts have shown that pig tissues are also rejected by cellular (antibody-independent) mechanisms (36, 37, 38, 39, 40). The transplantation of pig pancreatic islets may be a useful model in that these are transplanted in the absence of a vascular endothelium, and therefore do not express αGal. Nevertheless, porcine islets are rejected by a cellular mechanism in which macrophages and NK cells play a significant role. Although this model cannot be fully extrapolated to the transplantation of a vascularized organ, it seems likely that, if AHXR is prevented, a cellular form of rejection will take place in the vascularized organ. Furthermore, chronic rejection, e.g. graft atherosclerosis, is likely to develop early.

1.3 MODULATION OF THE XENOGENEIC IMMUNE RESPONSE

HAR can be prevented by i) depletion/inhibition of anti-αGal, ii) depletion or inhibition of components of complement, or iii) the use of organs from pigs transgenic for human complement regulatory proteins. To date, it has not been possible to prevent AHXR in the pig-to-nonhuman primate model. Several methods, largely aimed at depletion/inhibition of anti-αGal combined with prevention of production of induced antibody, are currently being studied. Since non-specific cells, such as macrophages and NK cells, appear to be important in the pathogenesis of AHXR, agents targeting these cells may also be essential in preventing AHXR.

Present progress with these various therapeutic modalities are presented and discussed in this chapter. The necessity for, and current methods of, depletion and suppression of production of anti-αGal antibodies will be discussed in Chapters 3.

Depletion or Inhibition of Complement

In the 1960s, cobra venom was important in elucidating the activation of the complement pathway (41). Cobra venom factor (CVF) was purified and was found to activate the complement system as a functional analogue of C3b (42, 43). Similar to C3b, CVF can bind to factor B, but is approximately 5 times more stable than C3bBb and is resistant to decay acceleration and proteolytic inactivation (44). The administration of CVF therefore leads to continuous complement activation, resulting in depletion of one or more complement components. Complement depletion by CVF can prolong discordant xenograft survival from minutes to days, but AHXR eventually develops by complement-independent mechanisms (14, 45, 46, 47).

Inhibition of complement activation can be achieved successfully by administering human soluble complement receptor 1 (sCR1). This leads to acceleration of the proteolytic cleavage of C3/C5-convertases, thus preventing development of MAC and proinflammatory cytokines. sCR1 does not activate and deplete complement components and may therefore be less toxic than CVF. Administered alone, sCR1 can also prolong discordant cardiac xenograft survival in the guinea pig-to-rat model to 32

hours (48) and in the pig-to-primate model to 7 days (49). When combined with CPP, cyclosporine and steroids, survival of pig cardiac xenografts was further prolonged to a maximum of 6 weeks (50, 51).

Genetic Engineering of the Organ-Source Pig

To date, it has proven rather difficult to maintain depletion of anti-αGal antibodies in the recipient primate. A different approach would be to prevent expression of the αGal epitope on the vascular endothelial cells of the pig by genetic manipulation. It is currently not possible to create an αGal-knockout pig (i.e., to disrupt the α1,3galactosyl-transferase gene (αGT) by homologous recombination, thereby preventing αGal expression) because pig embryonic stem cells are not yet available. Furthermore, studies in αGal-knockout mice indicate that the absence of αGal may induce expression of underlying 'cryptic' oliogosaccharide epitopes to which humans may have antibodies (52). However, several advances have been made in the field of genetic engineering and are discussed below.

Transgenesis for human complement regulatory proteins

Since complement activation in humans is regulated by membrane bound complement regulatory proteins, such as CD46 (membrane cofactor protein, MCP), CD55 (decay accelerating factor, DAF), and CD59, inducing expression of these human proteins in pigs to inhibit complement activation seems to be a logical approach. Complement regulatory proteins are largely species-specific, i.e., complement regulatory proteins expressed on porcine tissue do not modulate human complement and vice-versa. The creation of pigs transgenic for human complement regulatory proteins therefore seems desirable. White's group in Cambridge has successfully created pigs transgenic for hDAF. When organs from these pigs are transplanted into nonhuman primates, they are protected from HAR and, in association with intensive immunosuppressive therapy, graft survival of up to 99 days has been reported (White, personal communication). Median graft survival of life-supporting pig kidneys transplanted into cynomolgus monkeys is approximately 30 days and of pig hearts transplanted orthotopically into baboons about 15 days (18,19). Other groups have created pigs

transgenic for more than one human complement regulatory protein but with less prolonged graft survival (53), probably due to differences in the immunosuppressive protocol administered.

Competitive glycosylation

As it is not yet possible to create an αGal-knockout pig, alternatives have been proposed to decrease the αGal expression on donor pig organs. The introduction of a gene that would compete with αGT for its substrate, N-acetyllactosamine, is one such approach (54) (Figure 1). In vitro studies by Sandrin et al. (55, 56) in COS cells demonstrated that competition takes place between αGT and α1,2fucosyltransferase (αFT) in the Golgi apparatus for this substrate, and that αFT takes precedence, thereby resulting in a cell that expresses more H blood group antigen than Gal. COS cells simultaneously transfected with cDNA clones encoding for either αGT or αFT showed greater expression of the αFT-product (the H epitope) than the αGT-product

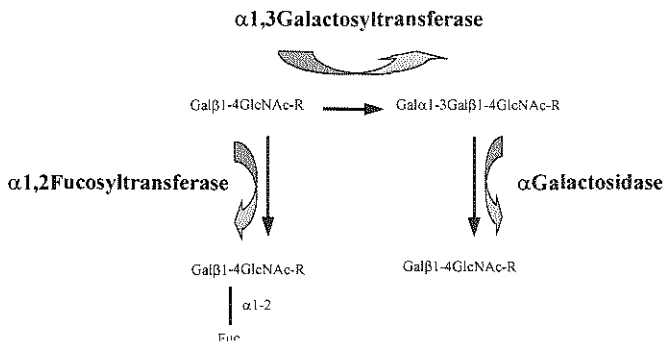

Figure 1.

Natural biosynthetic pathway for synthesis of the Gal epitope (Galα1-3Gal), and methods by which this can be modified by transgenic techniques. Galactose is added to the N-acetyllactosamine (Galß1-4GlcNAc) substrate by the α1,3 galactosyltransferase enzyme to form Galα1-3Gal. Galß1-4GlcNAc can also form the substrate for the H (O) histo-blood group epitope when the gene for the α1,2 fucosyltransferase enzyme is transgenically introduced. Furthermore, cleavage of Galα1-3Gal occurs when the gene for the α-galactosidase enzyme is introduced. Modification of the natural pathway has been demonstrated in cells in culture and in αGal-knockout (αGal-negative) mice by transgenic techniques, but has not yet been successfully achieved in pigs. (Modified from Sandrin MS, et al. (123)).

(αGal) (56). Mice transgenic for αFT demonstrated a major decrease in αGal expression and reduction of reactivity of these cells when challenged with human serum (57). More recently, evidence has been presented that high-level expression of the H antigen on porcine cells reduces human monocyte adhesion and activation (58). It would be necessary, however, to ensure that all the αGal epitopes are replaced by the H epitopes, or the transplanted organ may still be susceptible to HAR or AHXR. It has therefore been proposed to add another gene, namely α-galactosidase, to the donor pig in addition to that for αFT (59). αGalactosidase removes terminal αGal rather than adding it, and would ensure that those αGal epitopes that are not competitively replaced with H epitopes by αFT are removed by α-galactosidase. Sandrin's group has provided data demonstrating a complete absence of αGal expression in cell cultures containing the genes for both αFT and α-galactosidase (60).

Nuclear transfer

Our knowledge of and ability to genetically manipulate embryonic and adult cells of large mammals has greatly increased in the last few years (61). The recent successful cloning of pigs by PPL Therapeutics (62) holds considerable potential for the field of xenotransplantation. Although the current cloned pigs are genetically unmodified, this technology may allow the cloning of pigs that are genetically altered to render their organs less susceptible to rejection by humans recipients. The technique by which PPL Therapeutics achieved this success, nuclear transfer, had already led to the cloning of the unmodified sheep, Dolly, in 1996 (61) and the genetically-modified sheep, Polly, in 1997 (63). In Polly, the gene encoding the human clotting factor IX had been inserted.

Nuclear transfer entails the *in vitro* removal of the nucleus from an unfertilized egg, and its replacement with a nucleus removed from another cell of interest. This latter cell can be a normal, unmodified embryonic, foetal or adult cell or a cell in which the genome has been modified. The newly-created egg is initially cultured for a few days

to allow cell division to begin, and the developing embryo is then implanted into the uterus of a surrogate mother. The offspring is a (modified or unmodified) clone of the pig that donated the original nuclear material. The potential benefit for xenotransplantation lies in the possibility of deleting or inserting genes of interest in the genome before its transfer, and thus create a genetically-modified pig. Advantages over other methods of genetic modification are that (i) it does not require embryonic stem cells (that to date remain unavailable in pigs), (ii) it allows for the assessment of success of the genetic manipulation at an early (cellular) stage, thus negating the costly wait until the offspring is born (as is the case with current transgenic techniques), and (iii) it has the potential to produce an almost instantaneous herd of identical modified swine.

In xenotransplantation, modification of the genome and nuclear transfer could, for example, enable the production of αGal-knockout pigs by deleting the gene for α1,3galactosyltransferase in the nucleus before transfer, theoretically rendering the resulting pig's organs resistant to a human recipient's antibody-mediated immune response as discussed above. It remains uncertain, however, whether an αGal-knockout pig will be viable as so much αGal is present in the pig that some authorities have questioned whether it may be essential to sustain life (64). Alternatively or additionally, certain protective genes might be inserted, e.g., those for one or more complement regulatory proteins, those known to be associated with the development of accommodation (65), as discussed below, or those that might promote thromboregulation (66). Physiologic barriers could also be surmounted, e.g., by the introduction of a gene responsible for the production of a human protein, enzyme or hormone (whenever the porcine equivalent is found not to function satisfactorily in primates).

The technique of nuclear transfer will need considerable refinement and testing before its implementation into clinical xenotransplantation, but its recent successful attainment in pigs brings optimism for the future.

Accommodation

Under certain circumstances, ABO-incompatible allografts or allografts in HLA-sensitized recipients are able to survive despite the presence of circulating antibodies directed against determinants on the grafted organ (67). This process has been termed accommodation, and can be summarized as the absence of antibody-mediated rejection of a primary vascularized organ despite the presence of circulating antibodies that are potentially reactive with antigens on the vascular endothelium of that graft (68). The phenomenon has not yet been documented to conclusively occur in Gal-incompatible large animal models. Possible explanations for the phenomenon of accommodation have been proposed by Bach et al. (68) and are briefly reviewed. Firstly, the returning antibodies, i.e. the antibodies that are induced post-transplant following pre-transplant depletion, may be different in isotype, affinity, and/or specificity, and are thus unable to initiate rejection. Secondly, the surface antigens on the vascular endothelium may show subtle changes during the absence of natural antibodies, thus preventing recognition by the returning, induced antibodies. Finally, during the return of antibodies, the endothelial cells may adapt and either respond differently to these antibodies or become 'desensitized'. It has been proposed that, during accommodation, 'beneficial' genes are upregulated and 'detrimental' genes are downregulated (65).

Induction of Tolerance

Many investigators believe that the ideal method for avoiding both HAR and AHXR is to induce B cell tolerance. Additionally, if T cell tolerance were also induced, the subsequent cellular response would be avoided. This would prevent the complications associated with long-term pharmacological immunosuppressive therapy. Since tolerance can be defined as a state of permanent specific unresponsiveness to donor antigens (but not to other antigens) by the recipient in the absence of maintenance immunosuppressive therapy, immune responses to pathogens would be normal. Sachs and Sykes have developed tolerance in small and large animal allotransplantation models (31, 32, 69, 70).

Molecular chimerism

One approach to the induction of tolerance is by gene therapy in an attempt to induce what has been termed molecular chimerism. For example, B cell tolerance might be achieved if the primate recipient could be induced to express the Gal epitope on its tissues, which might lead to the suppression of production of anti-Gal antibody. Autologous transplantation of bone marrow from αGal-knockout mice transduced ex vivo with the gene for αgalactosyltransferase (αGT, the enzyme that leads to the production of the Gal epitopes) resulted in suppression of production of anti-αGal and the achievement of B cell tolerance to αGal (71). Preliminary studies in baboons, however, demonstrated only transient expression of αGal following the infusion of transduced autologous bone marrow cells. The transduction efficiency of baboon bone marrow cells is currently being optimized with the use of improved vectors and culture parameters.

T cell tolerance might be achieved by the introduction into the recipient of a gene encoding a swine MHC (SLA) class II antigen. The presence of a donor-specific class II antigen in the recipient (following gene transduction of bone marrow cells) has been demonstrated to lead to tolerance to a kidney allograft in miniature swine (72), and its importance has been tested in the pig-to-baboon model (73). Ex vivo transduction of baboon bone marrow cells with an SLA class II gene of a specific MHC-inbred miniature swine genotype was performed. Autologous transplantation of these transduced cells led to detection of the transgene in blood and bone marrow, but the transcription was transient. Subsequent pig skin or organ grafts (from a pig matched to the transgene) were rejected within 8 to 22 days from an antibody-mediated mechanism. In contrast to control baboons, however, the induction of IgG against non-αGal antigens was prevented, suggesting prevention of a T cell response.

Thymic transplantation

Zhao et al. demonstrated that the transplantation of fetal pig thymus and liver tissue (as a source of hematopoietic cells) under the kidney capsule of thymectomized and T and NK cell-depleted mouse recipients can induce donor-specific tolerance and

restore immune competence (74). Furthermore, when donor-matched pig skin grafts were transplanted to these mice, permanent graft survival was achieved, whereas allogeneic skin grafts rejected within 26 days (75). These studies were expanded by Wu et al., who demonstrated in vitro unresponsiveness following fetal porcine thymic tissue grafting in thymectomized, T cell-depleted baboons (76). Further studies are underway in this model.

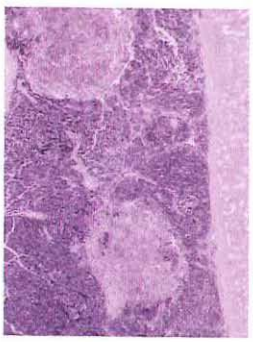

Figure 2.

Microscopic appearance (100x) of pig thymic tissue 3 months after transplantation under the capsule of an autologous kidney. Kidney tissue can be seen at the right margin of the figure; note the complete reorganization of the thymic tissue, which allowed normal thymopoiesis.

Our center is also involved with the induction of T cell tolerance by the transplantation of a vascularized thymic graft from the donor. Yamada et al. have demonstrated in our MHC-inbred herd of pigs that autologous thymic tissue, when transplanted under the renal capsule, becomes vascularized, regenerates, and functions normally (77) (Figure 2). When these "thymo-kidney" composite grafts are transplanted across a fully-mismatched allogeneic barrier, in a T cell depleted and thymectomized recipient, they are able to induce T cell tolerance (78). If anti-αGal could be successfully depleted for a limited period of time, the transplantation of a pig thymokidney could potentially induce T cell tolerance in the pig-to-primate model. The problem of providing an anti-Gal-free environment that would allow T cell tolerance to develop, however, remains unresolved at the present time.

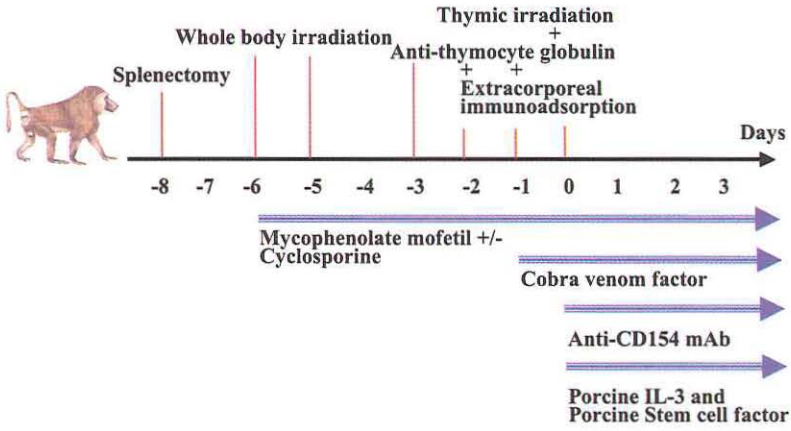

Figure 3.

Standard non-myeloablative regimen aimed at inducing immunological tolerance. The induction therapy for baboons consists of a splenectomy on day –8, followed by whole body irradiation 150 cGy x2 on days –6 and –5 to facilitate engraftment of porcine cells. T cell depletion is achieved with antithymocyte globulin on days –3, –2 , and –1 and thymic irradiation 700 cGy on day –1. Extracorporeal immunoadsorption of anti-αGal Ab is performed on days –2, –1, and 0. The maintenance therapy consists of mycophenolate mofetil at 110 mg/kg/day by continuous iv infusion from days –6 to 21, complement depletion with cobra venom factor from days –1 to 28, costimulatory blockade with anti-CD154 mAb from days 0 to 14 at 20 mg/kg on alternate days, either with or without cyclosporine at 15 mg/kg/day by continuous iv infusion. PBPC are transplanted on days 0, 1, and 2 (not shown). Porcine growth factors (IL-3 400 μg/kg/day and stem cell factor 2000 μg/kg/day) are administered from days 0 to 21 by continuous iv infusion to promote engraftment of porcine cells.

Mixed hematopoietic cell chimerism

Another approach to inducing tolerance to a transplanted xenogeneic organ would be to first induce mixed hematopoietic cell chimerism between pig and primate (32). To induce this state in the pig-to-primate model, a combination of (at least temporary) (i) depletion or inhibition of anti-αGal, (ii) depletion or inhibition of complement, (iii) T cell depletion (and/or costimulatory blockade), and (iv) pig hematopoietic cell engraftment, would appear to be necessary. Our laboratory is involved in studies aimed at inducing mixed hematopoietic chimerism and thus a state of both B and T cell tolerance in this model. We infuse large quantities of cytokine-mobilized peripheral blood progenitor cells (approximately $2 - 4 \times 10^{10}$ cells/kg), obtained from

our MHC-inbred herd of miniature swine, into a pre-conditioned recipient baboon. Our current conditioning regimen consists of splenectomy, WBI, thymic irradiation, anti-thymocyte globulin, MMF, CyA, CVF, anti-CD154 mAb, and multiple EIAs (Figure 3).

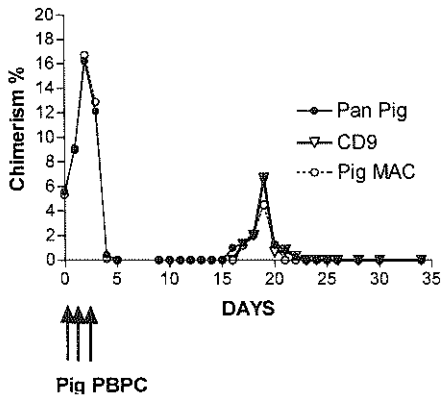

Figure 4.

Pig cell chimerism detected by flow cytometry in the blood of a baboon treated with our immunomodulatory regimen (see Figure 3) . Porcine PBPC were infused on days 0 – 2, during which up to 16% of the white blood cells in the baboon were of pig origin. From days 5 through 15 no pig cells could be detected, but on days 16 through 21 a pig monocyte population (stained with markers for pig (pan pig), pig CD9 (platelets, early B cells, activated T cells, and basophils), and pig MAC (monocytes and granulocytes)) could again be detected, indicative of transient pig cell engraftment in the baboon.

With this regimen, we are able to detect porcine cells by flow cytometry until day 6 following infusion, and by polymerase chain reaction consistently through day 30 and intermittently up to 140 days. Although engraftment of porcine cells, measurable by flow cytometry, has been definitively documented only twice, a state of donor-specific hyporesponsiveness, detected by mixed lymphocyte reaction from day 40 – 80 (Figure 3), has been observed in several cases, indicating that the induction of tolerance through mixed hematopoietic chimerism seems feasible if anti-Gal can be removed for a sufficient period of time.

In our experience, the experiments aimed at inducing immunological tolerance through mixed chimerism, have been associated with severe thrombotic complications. Most baboons that underwent our tolerance inducing protocols developed a thrombotic microangiopathy, resulting in massive bleeding complications and even death of several baboons. Although these thrombotic complications appear to be quite different than the disseminated intravascular coagulation that is observed after solid organ pig-to-baboon xenotransplantation, it is possible that the mechanisms that play a role in the etiology of these events may be similar.

1.4 AIMS OF THIS DISSERTATION

Xenotransplantation has the potential to change transplantation medicine as it is currently known. No longer will patients with organ failure need to wait weeks, months or years for an organ as a limitless number of animal organs will be readily available. The surgical procedure can be planned electively, during normal working hours, while the patient is still in reasonable health. The use of animals as the source of organs also presents us with new opportunities to manipulate and/or modify the "donor" using techniques such as genetic engineering and nuclear transfer. Manipulation of the donor has not been possible in allotransplantation.

The rejection processes that take place following the transplantation of a vascularized organ from a pig to a primate present formidable barriers. Much progress has been made in the last decade in elucidating the pathophysiology of these phenomena. Although major immunological hurdles (such as the prevention of HAR) have been overcome, many others remain. It is unlikely that any of the therapeutic modalities discussed in this chapter will be successful in preventing rejection when used alone. A combination of therapies will almost certainly be necessary, and we believe that the successful induction of immunological tolerance will greatly facilitate clinical implementation of xenotransplantation.

The aims of this dissertation are to discuss the current status of the induction of immunological tolerance in pig-to-baboon xenotransplantation through mixed hematopoietic chimerism, and to investigate some of the hurdles and complications associated with this approach. The author wishes to stress that only immunological aspects of xenotransplantation are presented, and that no attempts will be made to discuss the equally important issues of infectious hazards, ethical considerations, and physiological barriers that are inherent to xenotransplantation, as these latter items fall beyond the scope of this dissertation.

The results obtained from our attempts to induce mixed hematopoietic chimerism by depleting, or inhibiting the function of, macrophages in the pig-to-baboon model are presented in this thesis (**Section II, Chapter 2**).

In order to achieve mixed hematopoietic chimerism, anti-αGal depletion, and maintenance of depletion for a significant period of time, possibly several weeks, is required. We have summarized current and novel methods of depletion or suppression of production of anti-pig antibodies in pig-to-primate xenotransplantation (**Chapter 3**). Furthermore, the effect of depletion of B cells by specific immunotherapy on anti-αGal production in baboons is presented (**Chapter 4**).

The potentially lethal thrombotic microangiopathy that is associated with the transplantation of large quantities of porcine hematopoietic cells is discussed in **Section III, Chapter 5**, and various etiology is presented. The direct effect of porcine cells on baboon platelet aggregation is investigated (**Chapter 6**), and potential beneficial or detrimental aspects of our conditioning regimen aimed at inducing mixed chimerism are examined (**Chapter 7**). Finally, several agents that may prevent platelet aggregation in baboons are examined (**Chapter 8**).

These data are summarized in **Section IV, Chapter 9**.

REFERENCES

1 Harper AM, Rosendale JD. The UNOS OPTN waiting list and donor registry. Clin Transpl 1997: 61
2 Delmonico FL, Harmon WE, Lorber MI, et al. A new allocation plan for renal transplantation. Transplantation 1999; 67:303
3 Aaronson KD, Mancini DM. Mortality remains high for outpatient transplant candidates with prolonged (>6 months) waiting list time. J Am Coll Cardiol 1999; 33(5): 1189
4 Cooper DKC. Xenografting: how great is the clinical need? Xeno 1993; 1: 25
5 Lambrigts D, Sachs DH, Cooper DKC. Discordant organ xenotransplantation in primates. World experience and current status. Tranplantation 1998; 66: 547
6 Bailey LL, Gundry SR. Survival following orthotopic cardiac xenotransplantation between juvenile baboon recipients and concordant and discordant donor species: foundation for clinical trials. World J Surg 1997; 21:943
7 Perper RJ, Najarian JS. Experimental renal heterotransplantation. I. In widely divergent species. Transplantation 1966; 4: 377
8 Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. J Heart Lung Transplant 1988; 7: 238
9 Good AH, Cooper DKC, Malcolm AJ, et al. Identification of carbohydrate structures that bind human anti-porcine antibodies: implications for discordant xenografting in humans. Transplant Proc 1992; 24: 559
10 Platt JL, Fischel RJ, Matas AJ, et al. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation 1991; 52: 514
11 Rose AG, Cooper DKC, Human PA, et al. Histopathology of hyperacute rejection of the heart: experimental and clinical observations in allografts and xenografts. J Heart Lung Transplant 1991; 10: 223
12 Rose AG, Cooper DK. A histopathologic grading system of hyperacute (humoral, antibody-mediated) cardiac xenograft and allograft rejection. J Heart Lung Transplant 1996; 15:804
13 Alexandre GPJ, Gianello P, Latinne D, et al. Plasmapheresis and splenectomy in experimental renal xenotransplantation. In: Hardy MA (ed). Xenograft 25. New York, Elsevier, 1989. p 28.
14 Leventhal JR, Dalmasso AP, Cromwell JW, et al. Prolongation of cardiac xenograft survival by depletion of complement. Transplantation. 1993; 55: 857
15 Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. Nat Med 1995; 1: 964
16 Fryer JP, Leventhal JR, Dalmasso AP, et al. Cellular rejection in discordant xenografts when hyperacute rejection is prevented: analysis using adoptive and passive transfer. Transpl Immunol 1994; 2:87
17 Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nat Med 1995; 1: 869
18 Schmoeckel M, Bhatti FN, Zaidi A, et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. Transplantation 1998; 65: 1570
19 Zaidi A, Schmoeckel M, Bhatti F, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584
20 Cooper DKC, Ye Y, Rolf LL Jr, et al. The pig as a potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, et al. (eds.). Xenotranplantation. Heidelberg, Springer 1991. p481
21 Sachs DH. The pig as a potential xenograft donor. Vet Imm Immunopath 1994; 43: 185
22 Galili U, Shohet SB, Kobrin E, et al. Man, apes, and Old World monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J Biol Chem 1988; 263: 17755
23 Oriol R, Ye Y, Koren E, et al. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433
24 Galili U, Rachmilewitz EA, Peleg A, et al. A unique natural human IgG antibody with anti-α-galactosyl specificity. J Exp Med 1984; 260: 1519
25 Cooper DKC, Good AH, Koren E, et al. Identification of α-galatosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transplant Immunol 1993; 1: 198
26 Cooper DKC, Koren E, Oriol R. Oligosaccharides and discordant xenotransplantation. Immunol

Rev 1994; 141: 31
27 Kujundzic M, Koren E, Neethling FA, et al. Variability of anti-αGal antibodies in human serum and their relation to serum cytotoxicity against pig cells. Xenotransplantation 1994; 1: 58
28 McMorrow IM, Comrack CA, Nazarey PP, et al. Relationship between ABO blood group and levels of Gal alpha,3galactose-reactive human immunoglobulin G. Transplantation 1997; 64: 546
29 Sandrin MS, Vaughan HA, Dabkowski PL, et al. Anti-pig IgM antibodies in human serum react predominantly with Galα(1-3)Gal epitopes. Proc Natl Acad Sci USA 1993; 90: 11391
30 Galili U. Anti-αgalactosyl (anti-Gal) antibody damage beyond hyperacute rejection. In: Cooper DKC, Kemp E, Platt JL, et al. (eds). Xenotransplantation, 2nd edition. Heidelberg, Springer 1997. p 95
31 Kozlowski T, Monroy R, Xu, Y, et al. Anti-Galα1-3Gal antibody response to porcine bone marrow in unmodified baboons and baboons conditioned for tolerance induction. Transplantation 1998; 66: 176
32 Kozlowski T, Shimuzu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody adsorption. Transplantation 1999; 67:18
33 Candinas D, Belliveau S, Koyamada N, et al. T cell independence of macrophage and natural killer cell infiltration, cytokine production, and endothelial activation during delayed xenograft rejection. Transplantation 1996; 62:1920
34 Marquet RL, van Overdam K, Boudesteijn EA, et al. Immunobiology of delayed xenograft rejection. Transplant Proc 1997; 29:955
35 Ierino FL, Kozlowski T, Siegel JB, et al. Disseminated intravascular coagulation in association with the delayed rejection of pig-to-baboon renal xenografts. Transplantation 1998; 66:1439
36 Moses RD, Auchincloss H. Mechanism of cellular xenograft rejection. In: Cooper DKC, Kemp E, Platt JL, et al. (ed.), Xenotransplantation 2nd edition. Heidelberg, Springer 1997. p140
37 Vallee I, Guillaumin JM, Thibault G, et al. Human T lymphocyte proliferative response to resting porcine endothelial cells results from an HLA-restricted, IL-10 sensitive, indirect presentation pathway but also depends on endothelial-specific costimulatory factors. J Immunol 1998; 161:1652
38 Simeonovic CJ, Townsend MJ, Morris CF, et al. Immune mechanisms associated with the rejection of fetal murine proislets allografts and pig proislet xenografts: comparison of intragraft cytokine mRNA profiles. Transplantation 1999; 67:963
39 Soderlund J, Wennberg L, Castanos-Velez E, et al. Fetal porcine islet-like cell clusters transplanted to cynomolgus monkeys: an immunohistochemical study. Transplantation 1999; 67:784
40 Siebers U, Horcher A, Brandhorst H, et al. Analysis of the cellular reaction towards microencapsulated xenogeneic islets after intraperitoneal transplantation. J Mol Med 1999; 77:215
41 Muller Eberhard HJ, Nilsson UR, Dalmasso AP, et al. A molecular concept of immune cytolysis. Arch Path 1966; 82:205
42 Alper CA, Balatovich D. Cobra venom factor: evidence for its being altered cobra C3 (the third component of complement). Science 1976; 191:1275
43 Vogel CW, Smith CA, Muller Eberhard HJ. Cobra venom factor: structural homology with the third component of human complement. J Immunol 1984; 133:3235
44 Lachmann PJ, Halbwachs L. The influence of C3b inactivator (KAF) concentration on the ability of serum to support complement activation. Clin Exp Immunol 1975; 21:109
45 Adachi H, Rosengard BR, Hutchins GM, et al. Effects of cyclosporine, aspirin, and cobra venom factor on discordant cardiac xenograft survival in rats. Tranplant Proc 1987; 19:1145-1148
46 Scheringa M, Schraa EO, Bouwman E, et al. Prolongation of survival of guinea pig heart grafts in cobra venom factor-treated rats by splenectomy. No additional effect of cyclosporine. Transplantation 1995; 60:1350
47 Kobayashi T, Taniguchi S, Neethling FA, et al. Delayed xenograft rejection of pig-to-baboon cardiac transplants after cobra venom factor therapy. Transplantation 1997; 64: 1255
48 Candinas D, Lesnikoski BA, Robson SC, et al. Effect of repetitive high-dose treatment with soluble complement receptor type 1 and cobra venom factor on discordant xenograft survival. Transplantataion 1996; 62:336
49 Pruitt SK, Bollinger RR, Collins BH, et al. Effect of continuous complement inhibition using soluble complement receptor type 1 on survival of pig-to-primate cardiac xenografts. Transplantation 1997; 63:900

50 Davis EA, Pruitt SK, Greene PS, et al. Inhibition of complement, evoked antibody, and cellular response prevents rejection of pig-to-primate cardiac xenografts. Transplantation 1996; 62:1018
51 Marsh HC Jr, Ryan US. Therapeutic effect of soluble complement receptor type 1 in xenotransplantation. In: Cooper DKC, Kemp E, et al. eds. Xenotransplantation, 2nd edition. Heidelberg, Springer 1997. p 437
52 Shinkel TA, Chen CG, Salvaris E, et al. Changes in cell surface glycosylation in alpha 1,3-galactosyltransferase knockout and alpha 1,2-fucosyltransferase transgenic mice. Transplantation 1997; 64:197
53 Lin SS, Weidner BC, Byrne GW, et al. The role of antibodies in acute vascular rejection of pig-to-primate cardiac transplants. J Clin Invest 1998; 101:1745
54 Cooper DKC, Koren E, Oriol R. Genetically engineerd pigs (Letter). The Lancet 1993; 342:682
55 Sandrin MS, Fodor WL, Mouhtouris E, et al. Enzymatic remodelling of the carbohydrate surface of a xenogenic cell substantially reduces human antibody binding and complement-mediated cytolysis. Nature Med 1995; 1:1261
56 Sandrin MS, Cohney S, Osman N, et al. Overcoming the anti-Galα1-3Gal reaction to avoid hyperacute rejection: molecular genetic approaches. In: Cooper DKC, Kemp E, Platt JL, et al. (eds.) Xenotransplantation, 2nd edition. Heidelberg, Springer 1997. p683
57 Chen CG, Salvaris EJ, Romanella M, et al. Transgenic expression of human alpha1,2-fucosyltransferase (H-transferase) prolongs mouse heart survival in an ex vivo model of xenograft rejection. Transplantation 1998; 65:832
58 Kwiatkowski P, Artrip JH, Edwards NM, et al. High-level porcine endothelial cell expression of alpha(1,2)-fucosyltransferase reduces human monocyte adhesion and activation. Transplantation 1999; 67:219
59 Cooper DKC, Koren E, Oriol R. Manipulation of the anti-αGal antibody-αGal epitope system in experimental discordant xenotransplantation.Xenotransplantation 1996; 3:102
60 Osman N, McKenzie IF, Ostenried K, et al. Combined transgenic expression of alpha-galactosidase and alpha 1,2-fucosyltransferase leads to optimal reduction in the major xenoepitope Galalpha (1,3) Gal. Proc Natl Acad Sci USA 1997; 94:14677
61 Wilmut I, Schnieke AE, McWhir J, et al. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810
62 Dobson R. Cloning of pigs bring xenotransplants closer. BMJ 2000; 320: 826
63 Schneike AE, Kind AJ, Ritchie WA, et al. Human factor IX transgenic sheep produced by transfer of nuclei from fetal fibroblasts. Science 1997; 278: 2130
64 Tanemura M, Maruyama S, Galili U. Differential expression of alpha-Gal epitopes (Galalpha1-3Galbeta1-4GlcNAc-R) on pig and mouse organs. Transplantation 2000; 69: 187
65 Bach FH, Ferran C, Hechenleitner P, et al. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med. 1997; 3: 196
66 Chen D, Riesbeck K, McVey JH, et al. Regulated inhibition of coagulation by porcine endothelial cells expressing P-selectin-tagged hirudin and tissue factor pathway inhibitor fusion proteins. Transplantation 1999; 68: 832
67 Chopek MW, Simmons RL, Platt JL. ABO incompatible renal transplantation: initial immunopathologic evaluation. Transplant Proc 1987; 19: 4553
68 Bach FH, Platt JL, Cooper DKC. Accommodation - the role of natural antibody and complement in discordant xenograft rejection. In: Cooper DKC, Kemp E, Reemtsma K, et al. (eds). Xenotransplantation. Heidelberg, Springer 1991. p 81
69 Yang YG, DeGoma E, Ohdan H, et al. Tolerization of anti-Galα1-3Gal natural antibody-forming B cells by induction of mixed chimerism. J Exp Med 1998; 187:1335-1342
70 Ohdan H, Yang YG, Shimizu A, et al. Mixed chimerism induced without lethal conditioning prevents T cell- and anti-Gal alpha 1,3Gal-mediated graft rejection. J Clin Invest 1999; 104:281
71 Bracy JL, Sachs DH, Iacomini J. Inhibition of xenoreactive natural antibody production by retroviral gene therapy. Science 1998; 281:1845
72 Emery DW, Sablinski T, Shimada H, et al. Expression of an allogeneic MHC DRB transgene, through retroviral transduction of bone marrow, induces specific reduction of alloreactivity. Transplantation 1997; 64:1414

73 Ierino FL, Gojo S, Banerjee PT, et al. Transfer of swine major histocompatibility complex class II genes into autologous bone marrow cells of baboons for the induction of tolerance across xenogeneic barriers. Transplantation 1999; 67:1119
74 Zhao Y, Fishman JA, Sergio JJ, et al. Immune restoration by fetal pig thymus grafts in T cell-depleted, thymectomized mice. J Immunol 1997; 158:1641
75 Zhao Y, Swenson K, Sergio JJ, et al. Skin graft tolerance across a discordant xenogeneic barrier. Nat Med 1996; 2:1211
76 Wu A, Esnaola NF, Yamada K, et al. Xenogeneic thymic transplantation in a pig-to-nonhuman primate model. Transplant Proc 1999; 31:957
77 Yamada K, Shimizu A, Ierino FL, et al. Thymic transplantation in miniature swine: I. Development and function of the "thymokidney". Transplantation 1999; 68:1684
78 Yamada K, Shimizu A, Ierino FL, et al. Allogeneic thymokidney transplants induce stable tolerance in miniature swine. Transplant Proc 1999; 31:1199

II

Attempts to induce mixed hematopoietic chimerism in pig-to-baboon xenotransplantation

2

Depletion of macrophages in baboons delays the clearance of mobilized pig leukocytes

Adapted from:
Murali Basker*, Ian P.J. Alwayn*, Leo Buhler, David Harper, Sonny Abraham, Huw Kruger Gray, Michel Awwad, Julian Down, Robert Rieben, Mary E. White-Scharf, David H. Sachs, Aron Thall, and David K. C. Cooper. Clearance of mobilized porcine peripheral blood progenitor cells is delayed by depletion of the mononuclear phagocyte system in baboons. *Transplantation 2001. In press.*
* Authors contributed equally.

ABSTRACT

Introduction. Attempts to achieve immunological tolerance to porcine tissues in nonhuman primates through establishment of mixed hematopoietic chimerism are hindered by the rapid clearance of mobilized porcine leukocytes, containing progenitor cells (pPBPC), from the circulation. Eighteen hours after infusing 1-2x10^{10} pPBPC/kg into baboons that had been depleted of circulating anti-αGal and complement, these cells are almost undetectable by flow cytometry. The aim of the present study was to identify mechanisms that contribute to rapid clearance of pPBPC in the baboon. This was achieved by depleting, or blocking the Fc-receptors of, cells of the mononuclear phagocyte system (MPS) using medronate liposomes (ML) or intravenous immunoglobulin (IVIg), respectively.

Methods. Baboons (Preliminary studies, n=4) were used in a dose-finding and toxicity study to assess the effect of ML on macrophage depletion in vivo. Furthermore, baboons (n=9) received a non-myeloablative conditioning regimen (NMCR) aimed at inducing immunological tolerance, including splenectomy, whole body irradiation (300 cGy) or cyclophosphamide (80 mg/kg), thymic irradiation (700 cGy), T cell depletion, complement depletion with cobra venom factor, mycophenolate mofetil, anti-CD154L mAb, and multiple extracorporeal immunoadsorptions of anti-αGal antibodies. Group 1 (n=5) NMCR + pPBPC Tx. Group 2 (n=2) NMCR + ML + pPBPC Tx. Group 3 (n=2) NMCR + IVIg + pPBPC Tx. Detection of pig cells in the blood was assessed by FACS and PCR.

Results. Preliminary studies: ML effectively depleted macrophages from the circulation in a dose-dependent manner. Group 1: On average, 14% pig cells were detected 2 hours post-infusion of 1x10^{10} pPBPC/kg. After 18 hours, there were generally less than 1.5% pig cells detectable. Group 2: Substantially higher levels of pig cell chimerism (55-78%) were detected 2 hours post-infusion, even when a smaller number (0.5-1x10^{10}/kg) of pPBPC had been infused, and these levels were better sustained 18 hours later (10-52%). Group 3: In one baboon, 4.4% pig cells were detected 2 hours after infusion of 1x10^{10} pPBPC/kg. After 18 hours, however, 7.4% pig cells were detected. A second baboon died 2 hours after infusing 4x10^{10} pPBPC/kg, with a total white blood cell count of 90,000 of which 70% were pig cells.

No differences in microchimerism could be detected between the groups as determined by PCR.

Conclusions. This is the first study to report an efficient decrease of phagocytic function by depletion of macrophages with ML in a large animal model. Depletion of macrophages with ML led to initial higher chimerism and prolonged the survival of circulating pig cells in baboons. Blockade of macrophage function with IVIg had a more modest effect. Cells of the MPS, therefore, play a major role in clearing pPBPC from the circulation in baboons. Depletion or blockade of the MPS may contribute towards achieving mixed hematopoietic chimerism and induction of tolerance to a discordant xenograft.

INTRODUCTION

The induction of stable mixed hematopoietic chimerism has led to transplantation tolerance in several models of rodent (1,2,3,4), porcine (5), and primate (6,7) allotransplantation, as well as in concordant rodent (8,9) and primate (10) xenotransplantation. It has, however, not yet proven possible to achieve stable mixed hematopoietic chimerism in the highly relevant pig-to-primate discordant xenotransplantation model. Our laboratory has developed a non-myeloablative regimen (NMCR) aimed at inducing mixed hematopoietic chimerism in this model that is based on protocols that have previously been successful in the allograft and xenograft studies mentioned above. Large numbers of mobilized porcine peripheral blood leukocytes, containing progenitors cells (pPBPC), are transplanted into baboons pre-conditioned with a NMCR. Most pPBPC are, however, rapidly cleared from the circulation of these baboons, as determined by flow cytometric analysis of the peripheral blood. As our NMCR includes therapies that deplete and block the function of T cells, and deplete complement and circulating xenoreactive natural anti-Galα1-3Gal antibodies (anti-αGal Ab), we hypothesized that elements of the innate immune system may be responsible for the rapid clearance of pPBPC.

It has been well described that macrophages, as well as other cells of the phagocytic reticulo-endothelial system, are associated with allo- and xenograft rejection

(11,12,13). Several groups have reported that depletion of these cells led to increased graft survival (14,15,16,17). Van Rooijen et al. have extensively studied the depletion of macrophages using liposomes that contain the diphosphonate, clodronate (18,19,20). These liposomes have distinct dimensions and properties, that render them susceptible to phagocytosis by macrophages. Once phagocytosed, clodronate interferes with the cell's metabolism and causes cell death. We have made similar observations using liposomes containing medronate (ML), the parent compound of clodronate, to which it has similar properties. We have previously demonstrated that effective depletion of macrophages from the peripheral blood and other tissues is achieved when ML are administered to mice (Cheng J, et al. manuscript in preparation). To date, however, ML have not been studied in large animals, including nonhuman primates.

Instead of depleting macrophages, an alternative would be to inhibit the function of macrophages. Intravenous immunoglobulin (IVIg) is widely used in the treatment of various autoimmune diseases, including idiopathic thrombocytopenic purpura (21). Although the mechanism of action of IVIg has not been fully elucidated, it is thought that one of the mechanisms through which IVIg may act is to bind to the Fc-gamma receptors of macrophages, thus inhibiting their ability to mediate antibody-dependent cellular cytotoxicity (22). Furthermore, as IVIg may also inhibit complement activity (23), and has been used in the treatment of humoral rejection in allotransplantation (24,25), this action could be of additional benefit in xenotransplantation.

The present study was, therefore, designed to determine (i) whether ML are able to deplete macrophages in baboons using a modified in vivo phagocytosis assay (26), and (ii) whether the depletion (by ML) or blockade (by IVIg) of the cells of the MPS delays the clearance of pPBPC following their transplantation in pre-conditioned baboons.

MATERIAL & METHODS

Animals

Baboons (Papio anubis, n=13, Biomedical Resources Foundation, Houston, TX), of known ABO blood group and weighing 9-15 kg, were used to assess the effect of ML on macrophage depletion in vivo (n=4), or as recipients of pPBPC (n=9). Massachusetts General Hospital MHC-inbred miniature swine (n=6, Charles River Laboratories, Wilmington, MA), of blood group O and weighing 18-40 kg, served as donors of pPBPC.

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical Procedures

Details of anesthesia, intravenous (iv) catheter placement in pigs and baboons, and intra-arterial line placement, splenectomy, and extracorporeal immunoadsorption of anti-αGal antibodies in baboons have been described previously (27,28).

Non-myeloablative Conditioning Regimen (NMCR)

This regimen consists of induction therapy with splenectomy, whole body irradiation (150 cGy x2), thymic irradiation (700 cGy), anti-thymocyte globulin (50 mg/kg x3, Atgam, Upjohn, Kalamazoo, MI), and multiple extracorporeal immunoadsorptions of anti-αGal antibodies. The maintenance therapy consists of treatment with anti-CD154 monoclonal antibody (mAb) +/- cyclosporine (Sandimmune iv, 15 mg/kg/day by continuous iv infusion, generously donated by Novartis Pharmaceuticals, East Hanover, NJ), mycophenolate mofetil (Cellcept iv, 110 mg/kg/day by continuous iv infusion, generously donated by Roche Laboratories, Nutley, NJ), and cobra venom factor (Advanced Research Technologies, San Diego, CA). Details of this regimen can be found in Chapter 1, figure 3. All baboons received cefazolin, 500 mg iv daily, as prophylaxis against infection and ranitidine, 12.5 mg iv twice daily (Zantac iv, Glaxo-Wellcome, Research Triangle Park, NC).

Mobilization and Leukapheresis of pPBPC

Our methodology has been described previously (29). Briefly, pigs were treated with recombinant hematopoietic growth factors before and during the period of collection of pPBPC. Leukapheresis was carried out on days 5-9 and 12-16 following commencement of growth factor mobilization using a Cobe Spectra apheresis machine (Cobe, Lakewood, CO). Plasma was removed from the product by centrifugation at 920g for 7 minutes. The collected leukocytes (pPBPC), containing progenitor cells, were washed, frozen, and stored until transplantation.

Preparation and Transplantation of pPBPC

The pPBPC were thawed by immersion in a 37 – 40^0 C water bath with gentle shaking for 1 – 2 minutes, washed twice with Hanks' balanced salt solution (calcium-, magnesium-, and phenol red-free) (Mediatech, Herndon, VA), and resuspended in Hanks' solution for immediate transplantation through a central systemic iv catheter to the recipient baboon. Viability was assessed with trypan blue staining; approximately 70-80% of pPBPC remained viable. The baboons received additional therapy to prevent thrombotic complications associated with the transplantation of pPBPC (30). This consisted of prostacyclin, 20 ng/kg/min, and heparin, 10 U/kg/hour by continuous iv infusion, and methylprednisolone, 2 mg/kg iv twice daily for 7 days, tapered to daily for a further 7–21 days.

Preparation of Medronate Liposomes (ML)

This followed the method described by others (19). Briefly, 75mg phosphotidyl choline (Avanti Polar Lipids, Alabaster, AL) and 11mg cholesterol (Sigma, St. Louis, MO) were dissolved in chloroform and mixed to form a film. After low vacuum rotary evaporation at 37^0 C, the film was dispersed by gentle rotation in 10ml phosphate buffered saline in which 2.5 mg medronate was dissolved. The resulting liposomes were centrifuged twice at 100,000 g for 30 minutes to remove free, non-entrapped medronate. The ML were then resuspended in phosphate buffered saline and ultrasonicated for 2 minutes before administration (Bransonic 2210, Branson

Ultrasonics Corporation, Danbury, CT). Blank liposomes were formed using the same protocol with the omission of medronate.

Preparation of Fluorescent Latex Beads

Polystyrene latex beads, with a diameter of 2 μm (Polyscience, Warrington, PA), were impregnated with fluorescein isothiocyanate and added to tubes coated with bovine serum albumin and rinsed with 70% ethanol. The resulting beads had dimensions that made them particularly amenable to phagocytosis by macrophages. They were stored in the dark in sterile normal saline at 4° C until use.

Preparation of Intravenous Immunoglobulin (IVIg)

Two preparations of human IVIg were used in these experiments. Pentaglobin (Biotest A.G., Dreieich, Germany), consisted of IgG (approximately 76%), IgM (approximately 12%), and IgA (approximately 12%). Iveegam (Immuno U.S., Rochester, MI), contained IgA (0.2 μg/ml) and IgG (>95%). Both preparations were depleted of anti-αGal Ab by immunoadsorption using synthetic αGal columns (Alberta Research Council, Alberta, Canada).

Flow Cytometric Analysis

Baboon blood samples were obtained, water lysed, and washed twice. For determination of fluorescent latex bead clearance kinetics, the FACScan (Becton Dickinson, Franklin Lakes, NJ) was calibrated to sample a fixed volume over a given period of time (60μl ± 7μl/minute). Freshly prepared fluorescent latex beads were used as positive control. For the detection of pig cells, 10 μl human immunoglobulin was added to $1x10^6$ cells to block non-specific binding of subsequently added monoclonal Ab to Fc-receptors. The cells were then stained in 100 μl FACS medium (1% bovine serum albumin and 0.1% azide in phosphate-buffered saline) using the following conjugated monoclonal antibodies (mAb): W6/32 FITC (mouse anti-primate MHC Class I, IgG), 1030H1-19 PE (pan-pig, mouse anti-porcine leukocyte, IgM), 36-7-5 FITC (mouse anti-mouse MHC Class I, IgG) and 12-2-2 PE (mouse anti-mouse MHC class I, IgM), the latter two as isotypic controls. Dead cells were

excluded based on propidium iodide staining. Acquisition was performed under hi-flow and samples were analyzed using WinList (Verity Software House, Topsham, ME).

Polymerase chain reaction

A PCR assay that amplifies the porcine cytochrome-B gene was used to detect pig microchimerism in baboon samples. An internal PCR standard was created by adding the porcine cytochrome-B primer sequence (Cyt-F1) to either side of a fragment of human A-2 phospholipase followed by cloning into a plasmid vector and quantitation using limiting dilution PCR. Peripheral blood and BM cells were purified using Histopaque 1077 (Sigma). The cells (2 - 10 x 10^5) were pelleted and frozen at -80°C until use. Genomic DNA was extracted using the Qiamp Blood Kit® (Qiagen, Valencia, CA), quantified using Hoechst 33258 dye and a Hoeffer TKO-100 Fluorometer (Hoeffer Scientific Instruments, San Francisco, CA), and denatured. The microchimerism assay consisted of 250 ng of sample DNA added to a total PCR reaction volume of 100 µl. The final reagent mixture consisted of 1X GeneAmp® PCR Buffer II, 2.0 mM $MgCl_2$, 0.4mM dNTP, and 2.5 units of Amplitaq Gold® (Perkin-Elmer, Philadelphia, PA). Eighty picomoles each of Cyt-F1 and Cyt-R1 primers were added to the reactions. The PCR tubes were placed in a Perkin-Elmer GeneAmp 9600 Thermal Cycler. The first denaturing step was 9 minutes at 95°C followed by 40 cycles of 96°C for 10 seconds, 59°C for 30 seconds, and 72°C for 30 seconds. The program concluded with a 5 minute incubation at 72°C. The internal standard was added to PCR reactions along with sample DNA to produce a 240 bp product that was compared to the 210 bp product produced by the porcine cytochrome-B gene through ethidium bromide-stained agarose gels. Samples that produced a cytochrome-B product that was greater than or equal to the product from 5 copies of the standard were considered to be positive for microchimerism.

Hematology and Chemistry Parameters

Serial blood samples were taken for measurement of platelet count, prothrombin time, partial thromboplastin time, fibrinogen, and fibrinogen degradation products

(Hematology Laboratory, Massachusetts General Hospital). Serum samples were obtained for the measurement of lactate dehydrogenase (LDH) and basic renal and liver function tests (Chemistry Laboratory, Massachusetts General Hospital).

Experimental Groups

All baboons were monitored for toxic side effects, and hematological parameters and serum chemistries were measured.

Preliminary studies (n=4). The effect of ML on macrophages was assessed by measuring the clearance kinetics of fluorescent latex beads from the circulation in baboons. Fluorescent latex beads ($1x10^{10}$) were infused iv on day 0 into baboons that were either untreated, had received blank liposomes, or had received ML at various dosages (200 mg twice daily x 2; 200 mg twice daily x 6; 800 mg once daily x 1; or 800 mg twice daily x 2). Treatment with ML was timed such that the last dose was administered on day –1 before bead infusion. In one experiment, when treatment was continued for 6 days, ML was administered daily from days –2 to 3. Baboons received 25mg diphenhydramine prior to ML or blank liposomes administration to prevent potential allergic reactions. Blood was drawn at various time points (0, 1, 5, 15, 30, 60, 120 and 240 minutes) after the beads were administered and flow cytometric analysis was performed (as described earlier) to determine the number of fluorescent latex beads remaining in the circulation.

Group 1 (controls, n=5). Baboons underwent the NMCR with transplantation of a total of 2-4 x 10^{10} pPBPC/kg on days 0 – 2. No ML or IVIg was administered.

Group 2 (n=2). Baboons underwent the NMCR with transplantation of a total of 2-4 x 10^{10} pPBPC/kg on days 0 – 2 or 0 – 3. Additionally, one baboon (B130-94) received ML at 40 mg/kg on days –2 and –1, followed by 20 mg/kg on days 1, 3, and 4, and 40 mg/kg on day 7. A second baboon (B57-309) received ML at 80 mg/kg on days –2 and –1.

Group 3 (n=2). Baboons underwent the NMCR with transplantation of a total of 2-4 x 10^{10} pPBPC/kg on days 0 – 2 or 0 – 3. Additionally, one baboon (B68-11) received IVIg at 500 mg/kg/day from days 0 – 8, with a double dose (total 1000 mg/kg) on day 1. A second baboon (B68-55) received IVIg at 500 mg/kg on days 0 – 2. IVIg was administered immediately before pPBPC transplantation on each occasion.

RESULTS

Preliminary studies

Clearance of fluorescent latex beads from the circulation in an untreated baboon and in a baboon that had received blank liposomes occurred within 4 hours (Fig. 1). In contrast, treatment with ML led to substantially delayed clearance of fluorescent latex beads. Four hours following treatment with one dose of ML at 800 mg, 5% of the beads remained in the circulation, but after 24 hours, >99% of the beads had been cleared. Administration of ML at 200 mg twice daily x 2 resulted in 6% persistence of fluorescent latex beads 4 hours following infusion, and 5% after 24 hours. With this regimen, the beads remained in the circulation for 4 days. When ML were infused at 200 mg twice daily x 6, 7% of the beads could be detected after 4 hours, with 4% remaining after 24 hours, and 2% detectable after 5 days. When ML were administered at 800 mg twice daily x 2, 28% of the fluorescent latex beads could be detected after 4 hours, 17% after 72 hours, and <1% at 12 days.

All baboons that received ML developed elevated LDH levels (maximum approximately 3000 U/l). The increase in LDH closely correlated with the dose and duration of treatment of ML. This was probably related to release of LDH by dying macrophages. No major changes were observed in renal, liver, or hematological parameters.

These observations demonstrate that fluorescent latex beads are effectively removed from the circulation in untreated baboons, and that treatment with ML results in delayed clearance. In turn, this indicates a decrease of phagocytic function that is most likely due to depletion of macrophages.

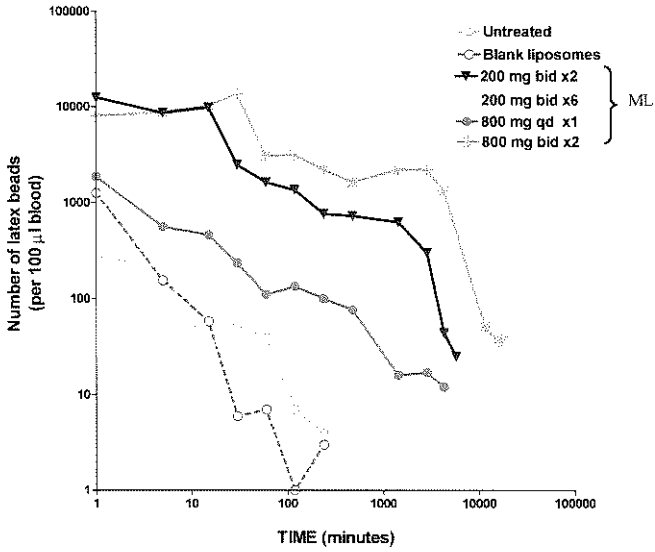

Figure 1 (Preliminary studies).

Clearance kinetics of fluorescent latex beads infused into baboons (data are presented in log scales). The number of beads in circulation at 1 minute post infusion is designated as the baseline (100%). In untreated baboons, or baboons that received blank liposomes, fluorescent latex beads were cleared from circulation within 4 hours. In contrast, all baboons receiving medronate liposomes (ML) demonstrated a substantially delayed clearance of the beads from the circulation. Treatment with ML at 200 mg or 800 mg twice daily x2 doses gave the most optimal results, with, respectively, 6% and 28% persistence of fluorescent latex beads in the circulation 4 hours following infusion, 2% and 28% remaining after 48 hours, and <1% and 17% remaining after 72 hours. Treatment with 6 doses of ML led to 7% persistence of the beads after 4 hours, 1% after 72 hours, and 3% after 72 hours. No beads were detectable 5 – 12 days after administration in any baboon receiving ML.

Group 1 (controls)

These baboons received the NMCR with transplantation of a total of 2 – 4 x 10^{10} pPBPC/kg on days 0 – 3 without ML or IVIg administration. The percentage of porcine cell chimerism as detected by flow cytometry is depicted in Figure 2. On average, pig cells made up approximately 14% (0.5% – 50%) of circulating cells 2 hours following transplantation of 1 x 10^{10} pPBPC / kg. Eighteen hours after pPBPC transplantation, virtually no pig cells could be detected. This pattern was observed after each day of pPBPC transplantation. After day 3, no porcine cells could be detected.

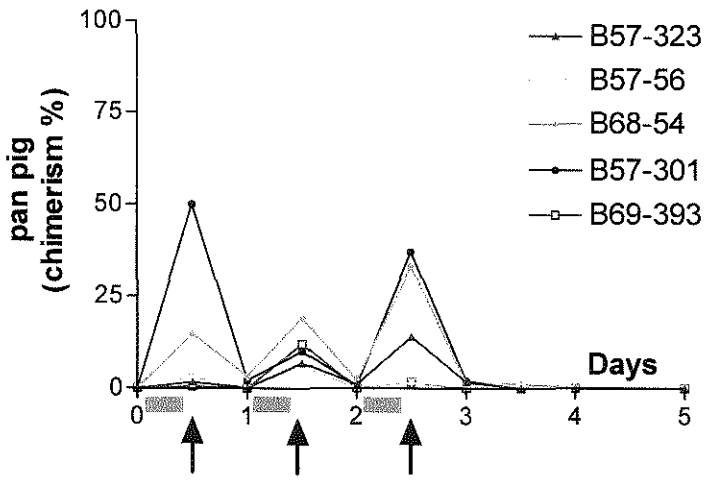

Figure 2.

Pig cell chimerism detected by flow cytometry in 5 baboons in Group 2, using a pan-pig leukocyte marker. All baboons received the NMR with transplantation of approximately 2 – 4 x10^{10} pPBPC/kg divided equally over days 0, 1, and 2. No medronate liposomes or IVIg was administered. Horizontal bars indicate the periods during which pPBPC were infused. Arrows indicate sampling timepoints 2 hours after transplantation. Two hours after transplantation of pPBPC on day 0, a mean of 14% circulating pig cells was detected. Eighteen hours later (marked by timepoint 1), virtually no pig cells (1.1%) were detectable. On day 1, 2 hours following transplantation, pig cell chimerism reached 10.7%, falling to 0.9% eighteen hours later. On day 2, 17.4% of all cells were of porcine origin 2 hours post transplantation, decreasing to near-undetectable levels (1.2%) on day 3. By day 4, no pig cells were detectable.

Porcine DNA was detected by PCR continuously in the blood for approximately 19 (7 – 28) days and intermittently up to 263 days. Porcine DNA was detected intermittently in the bone marrow in 4 out of 5 baboons between 16 and 365 days.

All baboons developed a thrombotic microangiopathic state, with elevated LDH (>1,500 U/l) and thrombocytopenia (<20,000 /μl) necessitating platelet transfusions in most instances. This state of microangiopathic thrombosis has been described and discussed elsewhere (30).

These data indicate that, using this NMCR, transplanted pPBPC are rapidly cleared from the circulation of baboons.

Group 2

In addition to the NMCR, these two baboons received ML before, or during, pPBPC transplantation. In B130-94, 2 hours after the first pPBPC transplantation, pig cells made up 49% of the cells detected in the circulation (Fig. 3). The following morning, this percentage had increased to 53%. The percentage of pig cells increased to reach a maximum of 78% 2 hours after the second pPBPC transplantation (Fig. 4), after which pig cell chimerism was slowly lost, despite continued administration of ML. A last peak of circulating pig cells was observed on day 4, after the final $1x10^{10}$ pPBPC/kg were transplanted. After day 5, no pig cells were detectable. This baboon died of infectious complications on day 12. A similar pattern was observed in B57-309, where 2 hours after the first transplantation of pPBPC, 30% of circulating cells were of porcine origin. The following day, this percentage was sustained at 30%. Two hours after the second transplantation, the percentage of pig cells increased to 33% and reached a maximum of 55% 18 hours later. After the third, and last, day of pPBPC transplantation, however, the percentage of pig cells decreased to 25% and, by day 4, no pig cells were detected.

Porcine DNA was detected continuously for 16 days in the blood of B57-309 and intermittently through day 96. No porcine DNA was detected in the bone marrow of

this baboon. Porcine DNA was detected in all tissues of B130-94 at autopsy on day 12.

Both baboons developed increased LDH levels (7900 U/l and >10,000 U/l for B130-94 and B57-309, respectively), probably limiting any potential increase in the dosage of ML. Furthermore, a microangiopathic state similar to that in Group 1 was observed, with a marked thrombocytopenia (<20,000 /μl), necessitating platelet transfusions.

Treatment with ML, therefore, led to substantially higher initial levels of circulating pig cells, and sustained these cells in the circulation for 4 – 5 days.

Group 3

These two baboons underwent the NMCR before pPBPC transplantation, and also received daily infusions of IVIg. In B68-11 the initial percentage of circulating porcine cells (9.3% at 2 hours after the first transplantation of pPBPC) was equivalent to that in the Group 2 baboons (Fig. 5). Two hours after the second transplantation of pPBPC (day 1), 4.4% of the cells were of porcine origin. Eighteen hours later, this percentage had increased to 7.4%. There was, therefore, slightly better maintenance of pig cells in this baboon than in the Group 2 baboons. Over the next days, however, pig cells were lost from the circulation. In the second baboon (B68-55), the percentage of pig cells was also similar to that of the Group 2 baboons during the first two days of pPBPC transplantation (2 hours post transplantation, 8% and 12%, respectively). Before the third day of pPBPC transplantation, 5% porcine cells remained detectable. After the third transplantation of pPBPC, this baboon developed a leukocytosis of 90,000 /μl, of which 70% (63,000 /μl) were of porcine origin. The baboon died suddenly 2 hours after the third transplantation of pPBPC. Autopsy revealed a widespread thrombotic microangiopathy, with thromboses in the microvasculature of the heart, lungs, and kidneys. These findings were consistent with previous findings when death occurred after high-dose pPBPC transplantation (30).

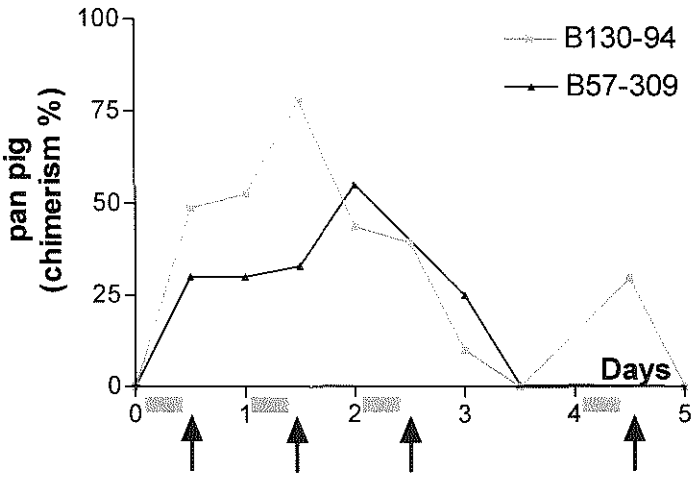

Figure 3.

Pig cell chimerism detected by flow cytometry in 2 baboons in Group 3, using a pan-pig leukocyte marker.

B130-94 received the standard NMR with transplantation of approximately 2 – 4 $x10^{10}$ pPBPC/kg divided over days 0, 1, 2, and 3. Horizontal bars indicate periods of pPBPC transplantation. Arrows indicate sampling timepoints 2 hours after transplantation. B130-94 received medronate liposomes (ML) at 40 mg/kg on days –2 and –1, followed by 20 mg/kg on days 1, 3, and 4, and 40 mg/kg on day 7. Two hours after pPBPC transplantation on day 0, pig cells made up 49% of the total cells in the circulation. Eighteen hours later, this percentage had increased to 53%. Two hours after transplantation of pPBPC on day 1, the pig cell percentage increased to 78% (see Fig. 4). On days 2 and 3, even after transplantation of more pPBPC, pig cell chimerism was lost. On day 4, after a further $1x10^{10}$ pPBPC/kg were transplanted, a final peak of pig cells was observed. These cells were rapidly cleared from the circulation and, after day 5, no pig cells were detectable.

B57-309 underwent the NMR and pPBPC transplantation on days 0, 1, and 2 (to a total of 3 $x10^{10}$ pPBPC/kg) and received ML at 80 mg/kg on days –2 and –1. Two hours after pPBPC transplantation on day 0, 30% of cells were of porcine origin. The following day, this percentage was sustained at 30% and increased to 33% two hours following pPBPC transplantation on day 1 and to 55% eighteen hours later. After pPBPC transplantation on day 2, the percentage of pig cells decreased to 25% and, by day 4, no pig cells remained.

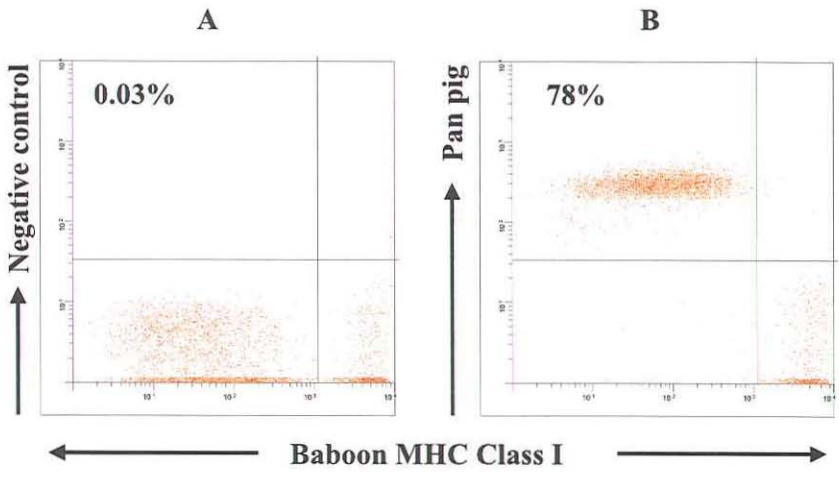

Figure 4.

Two-color flow cytometric analysis of circulating cells in B130-94, 2 hours after pPBPC transplantation on day 1. Staining was performed for baboon MHC Class I (W6/32 FITC), negative isotype control (12.2.2 PE, Fig. A), and pan-pig leukocyte (1030H1.19 PE, Fig. B). At this timepoint, 78% of cells were of porcine origin.

Porcine DNA was detected in the blood of B68-11 continuously through day 9 and intermittently through day 43. In the bone marrow, porcine DNA was detected only on day 14. All tissues of B68-55 at autopsy on day 2 contained porcine DNA.

B68-11 developed a thrombotic microangiopathic state, with elevated LDH (>1,500 U/l) and thrombocytopenia (<20,000 /μl) comparable to Group 1 baboons.

The addition of IVIg to the NMCR, therefore, led to a marginally delayed rate of clearance of pPBPC and, in one case, to a very high level of chimerism (that was probably a major factor in the death of the baboon). These differences may be explained by differences in the preparations of IVIg used. Alternatively, treatment with IVIg in B68-55 may have been more effective in saturating all Fc-gamma receptors of the macrophages, thereby preventing opsonization and phagocytosis of porcine cells, and leading to higher chimerism.

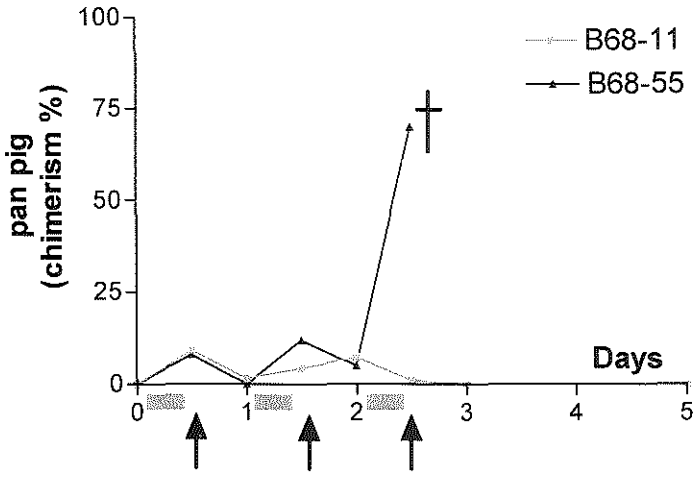

Figure 5.

Pig cell chimerism detected by flow cytometry in 2 baboons in Group 4, using a pan-pig leukocyte marker. Both baboons received the NMR with transplantation of approximately 2 – 4 x10^{10} pPBPC/kg divided over days 0, 1, and 2. Horizontal bars indicate period of pPBPC transplantation, arrows indicate sampling timepoints 2 hours after transplantation. B68-11 received IVIg at 500 mg/kg/day from days 0 – 8, with a double dose (total 1000 mg/kg) on day 1. The initial pig cell chimerism 2 hours post pPBPC transplantation on day 0 was 9.3%, which decreased 18 hours later to 1.6%. Two hours after pPBPC transplantation on day 1, 4.4% of cells were of porcine origin, with an increase to 7.4% eighteen hours later. Pig cell chimerism decreased and became undetectable over the next few days. B68-55 received IgM-containing IVIg (Pentaglobin) at 500 mg/kg on days 0 – 2. After the first 2 days of pPBPC transplantation, pig cells were detected at 8% and 12%, respectively. On day 2, before the last transplantation of pPBPC, pig cell chimerism was sustained at 5%. Two hours after transplantation of pPBPC on this day, the pig cell chimerism was 70%, with a leukocytosis of 90,000 /µl, at which time the baboon died from thrombotic complications.

DISCUSSION

The protocols developed at our laboratory aimed at inducing immunological tolerance through establishing stable mixed hematopoietic chimerism have not proven successful in the discordant pig-to-baboon model (31). Our NMCR, which includes non-myeloablative irradiation, T cell depletion, costimulatory blockade, complement

depletion, and (temporary) depletion of xenoreactive anti-αGal Ab, has thus far been insufficient to allow engraftment of pPBPC in baboons.

It has been suggested that macrophages may pose barriers to hematopoietic engraftment of xenogeneic cells, and that depletion of macrophages can improve chimerism in human-to-SCID-mouse xenotransplantation models (14,32). We are the first to report successful depletion of macrophages without significant side-effects in a large animal model using ML. Furthermore, to assess the negative effect of the MPS on the induction of chimerism in the pig-to-baboon model, we tested macrophage depletion with ML, or inhibition of the ability of macrophages to opsonize and phagocytose particles with IVIg, in our NMCR.

When ML were added to our protocol, and to a lesser extent when IVIg was added, substantially higher initial levels of circulating pig cells were observed, and pig cells were sustained in the circulation for longer periods of time. These data indicate that macrophages, indeed, play an important role in the clearance of porcine cells from the circulation of baboons. This short-term improvement in pig cell survival, however, did not lead to engraftment of these cells or to the development of immunological tolerance. Experiments in rodents indicate that depletion of macrophages with ML is temporary, and that a rebound in the number and/or the activity of macrophages can occur within days of discontinuing ML therapy (Cheng J, et al., manuscript in preparation). It is not clear, therefore, whether depletion of macrophages for only a limited period of time will be sufficient to establish hematopoietic cell chimerism. One may postulate that macrophages will need to be depleted for an extended period of time, sufficient to allow the porcine cells to home to and engraft in the recipient baboon's bone marrow.

One of the mechanisms through which baboon macrophages may clear porcine cells is through antibody-dependant cellular cytotoxicity. Although anti-αGal Ab can be efficiently removed from the circulation (33), continued production of anti-αGal Ab cannot be prevented (34). This is illustrated by the observation that, within hours of extracorporeal immunoadsorption of antiαGal Ab and pPBPC transplantation, the

pPBPC in the circulation are largely coated with anti-αGal IgM and IgG Ab (Awwad M, et al., unpublished data). Coating of pPBPC by anti-αGal IgG may facilitate phagocytosis of pPBPC by cells of the MPS (i.e., by macrophages), thus reducing the potential of engraftment. Methods that successfully maintain depletion of anti-αGal Ab, perhaps only for a limited period of time, may, therefore, be of benefit. However, it is possible that pPBPC may be phagocytosed by macrophages in the absence of coating.

Depletion of cells of the MPS may, however, be counterproductive, as these cells may be required to transport porcine antigen to the thymus if central tolerance is to be induced (35). Furthermore, recent data from our laboratory suggest that macrophages are essential in maintaining the state of humoral unresponsiveness to porcine antigens induced by treatment with anti-CD154 mAb (Buhler L, et al., manuscript in preparation), and may thereby be associated with the induction of peripheral tolerance. Depletion of macrophages resulted in an elicited Ab response to porcine antigens following hematopoietic cell transplantation that was previously prevented with costimulatory blockade with anti-CD154 mAb. Sustained depletion of anti-αGal Ab, macrophages, and T cells may allow hematopoietic chimerism and immunological tolerance to be achieved.

In addition to the effect IVIg may have on macrophages, other mechanisms of action of IVIg may be beneficial in pig-to-primate xenotransplantation. Proposed actions of IVIg include reducing complement-mediated injury by modifying the antibody-antigen ratio (23) and, particularly for IgM-containing IVIg (such as Pentaglobin), by 'scavenging' of activated complement fragments (36). Furthermore, down-regulation of B cells by binding to B cell immunoglobulin, functioning as anti-idiotypic antibodies and binding of xenoreactive antibodies (37,38), or binding to IgG receptors on $CD8^+$ T cells, thereby altering T-helper functions (39), have also been determined.

In conclusion, it appears that cells of the MPS play an integral role in the clearance of porcine cells from the circulation of baboons. Depletion of the cells of the MPS, or inhibition of their function, leads to higher levels of chimerism that are better

sustained, and may potentially facilitate the induction of mixed hematopoietic chimerism and immunological tolerance to porcine antigens in baboons.

REFERENCES

1 Sharabi Y and Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a non-lethal preparative regimen. J Exp Med 1989; 169: 493

2 Lee LA, Sergio JJ, Sykes M. Natural killer cells weakly resist engraftment of allogeneic long-term multilineage-repopulating hematopoietic stem cells. Transplantation 1996; 61: 125

3 Wekerle T, Sayegh MH, Hill J, et al. Anti-CD154 or CTLA4Ig obviates the need for thymic irradiation in a non-myeloablative conditioning regimen for the induction of mixed hematopoietic chimerism and tolerance. Transplantation 1999; 68: 1348

4 Wekerle T, Kurtz J, Ito H, et al. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. Nat Med 2000; 6: 464

5 Huang CA, Fuchimoto Y, Scheier-Dolberg R, et al. Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. J Clin Invest 2000; 105:173

6 Kawai T, Cosimi AB, Colvin RB, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. Transplantation 1995; 59: 256

7 Kimikawa M, Sachs DH, Colvin RB, et al. Modifications of the conditioning regimen for achieving mixed chimerism and donor-specific tolerance in cynomolgus monkeys. Transplantation 1997; 64: 709

8 Sharabi Y, Aksentijevich I, Sundt III TM, et al. Specific tolerance induction across a xenogeneic barrier: production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. J Exp Med 1990; 172: 195

9 Ildstad ST, Wren SM. Cross-species bone marrow transplantation: evidence for tolerance induction, stem cell engraftment, and maturation of T lymphocytes in a xenogeneic stromal environment (rat -> mouse). J Exp Med 1991; 174: 67

10 Ko DS, Bartholomew A, Poncelet AJet al. Demonstration of multilineage chimerism in a nonhuman primate concordant xenograft model. Xenotransplantation 1998; 5: 298

11 Alwayn IP, van Bockel HJ, Daha MR, et al. Hyperacute rejection in the guinea pig-to-rat model without formation of the membrane attack complex. Transpl Immunol 1999; 7: 177

12 Qian Z, Wasowska BA, Behrens E, et al. C6 produced by macrophages contributes to cardiac allograft rejection. Am J Pathol 1999; 155: 1293

13 Leonard CT, Soccal PM, Singer L, et al. Dendritic cells and macrophages in lung allografts. A role in chronic rejection? Am J Respir Crit Care Med 2000; 161: 1349

14Terpstra W, Leenen PJ, van den Bos C, et al. Facilitated engraftment of human hematopoietic cells in severe combined immunodeficient mice following a single injection of Cl2MDP liposomes. Leukemia 1997; 11: 1049

15 Fox A, Koulmanda M, Mandel TE, et al. Evidence that macrophages are required for T-cell infiltration and rejection of fetal pig pancreas xenografts in nonobese diabetic mice.Transplantation 1998; 66: 1407

16 Wu G, Korsgren O, van Rooijen N, et al. The effect of macrophage depletion on delayed xenograft rejection: studies in the guinea pig-to-C6-deficient rat heart transplantation model. Xenotransplantation. 1999 Nov;6(4):262

17 Feng X, Zheng XX, Yi S, et al. IL-10/Fc inhibits macrophage function and prolongs pancreatic islet xenograft survival. Transplantation 1999; 68: 1775

18 van Rooijen N, van Nieuwmegen R. Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study. Cell Tissue Res 1984; 238: 355

19 Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994; 174: 83

20 Van Rooijen N. Minireview: The liposome-mediated macrophage suicide technique. J Immunol Methods 1989: 124; 1

21 Fehr J, Hofmann V, Kappeler U. Transient reversal of thrombocytopenia in idiopathic thrombocytopenic purpura by high-dose intravenous gamma globulin. N Engl J Med 1982; 306: 1254
22 Bussel JB. Treatment effects on chronic idiopathic thrombocytopenic purpura. In: Imbach P; ed. Immunotherapy with Intravenous Immunoglobulins. San Diego: Academic Press 1991; 253
23 Dalakkas MC, Illa I, Dambrosia JM, et al. A controlled trial of high-dose intravenous immune globulin infusions as treatment for dermatomyositis. N Engl J Med 1993; 329: 1993
24 Jordan SC, Quartel AW, Czer LS, et al. Posttransplant therapy using high-dose human immunoglobulin (intravenous gammaglobulin) to control acute humoral rejection in renal and cardiac allograft recipients and potential mechanism of action. Transplantation 1998; 66: 800
25 Furth S, Neu AM, Hart J, et al. Plasmapheresis, intravenous cytomegalovirus-specific immunoglobulin and reversal of antibody-mediated rejection in a pediatric renal transplant recipient: a case report. Pediatr Transplant 1999; 3: 146
26 Singer JM, Adlersberg L, Hoenig EM, et al. Radiolabeled latex particles in the investigation of phagocytosis in vivo: clearance curves and histological observations. J Reticuloendothel Soc 1969; 6:561
27 Kozlowski T, Shimuzu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody adsorption. Transplantation 1999; 67: 18
28 Alwayn IPJ, Appel JZ III, Goepfert C, et al. Inhibition of platelet aggregation in baboons: therapeutic implications for xenotransplantation. Xenotransplantation 2000; 7: 247.
29 Nash K, Chang Q, Watts A, et al. Peripheral blood progenitor cell mobilization and leukapheresis in pigs. Lab Anim Sci 1999; 49: 645
30 Buhler L, Basker M, Alwayn IPJ, et al. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. Transplantation 2000; 70: 1323.
31 Kozlowski T, Monroy R, Giovino M, et al. Effect of pig-specific cytokines on mobilization of hematopoietic progenitor cells in pigs and on pig bone marrow engraftment in baboons. Xenotransplantation 1999; 6: 17
32 Verstegen MM, van Hennik PB, Terpstra W, et al. Transplantation of human umbilical cord blood cells in macrophage-depleted SCID mice: evidence for accessory cell involvement in expansion of immature CD34+CD38- cells. Blood 1998; 91: 1966
33 Watts A, Foley A, Awwad M, et al. Plasma perfusion by apheresis through a Gal immunoaffinity column successfully depletes anti-Gal antibody – experience with 320 aphereses in baboons. Xenotransplantation 2000; 7: 181.
34 Alwayn IP, Basker M, Buhler L, et al. The problem of anti-pig antibodies in pig-to-primate xenografting: current and novel methods of depletion and/or suppression of production of anti-pig antibodies. Xenotransplantation 1999; 6: 157
35 Wekerle T and Sykes M. Mixed Chimerism as an approach for the induction of transplantation tolerance. Transplantation 1999; 68: 459
36 Rieben R, Roos A, Muizert Y, et al. Immunoglobulin M-enriched human intravenous immunoglobulin prevents complement activation in vitro and in vivo in a rat model of acute inflammation. Blood 1999; 93: 942
37 Dietrich G and Kazatchkine MD. Normal immunoglobulin G (IgG) for therapeutic use (Intravenous Ig) contain antiidiotypic specificities against an immunodominant, disease-associated, cross-reactive idiotype of human anti-thyroglobulin autoantibodies. J Clin Invest 1990; 85: 620
38 Nydegger UE, Sultan Y, Kazatchkine MD. The concept of anti-idiotypic regulation of selected autoimmune diseases by intravenous immunoglogulin. Clin Immunol Immunopathol 1989; 53:S72
39 Ruiz de Souza V, Carreno MP, Cavailon JM, et al. Modulation of cytokine production by mmunoglobulins for intravenous use (IVIg). FASEB J 1984; 8: 1238

III

The importance of suppressing xenoreactive antibody production

3

Current and novel methods of depletion and/or suppression of anti-pig antibodies

Adapted from:
Alwayn IPJ, Basker M, Buhler L, and Cooper DKC. The problem of anti-pig antibodies in pig-to-primate xenografting: current and novel methods of depletion and/or suppression of production of anti-pig antibodies. *Xenotransplantation 1999;6:157-68*

ABSTRACT

The role of antibodies directed against Galα1-3Gal (αGal) epitopes in porcine-to-primate xenotransplantation has been widely studied during the past few years. These antibodies (anti-αGal) have been associated with both hyperacute rejection and acute vascular rejection of vascularized organs. Depletion and (temporary or permanent) suppression of production of anti-αGal seems essential for obtaining long-term survival of these organs, even when the ultimate aim is the achievement of accommodation or tolerance. Although greater than 95% depletion of anti-αGal can be realized by the use of immunoaffinity column technology, to date no regimen has been successful in preventing the return of anti-αGal from continuing production. In this review, we discuss current and novel methods for achieving depletion or inhibition (i.e., extracorporeal immunoadsorption, anti-idiotypic antibodies, the intravenous infusion of immunoglobulin or oligosaccharides) and suppression of production (i.e., irradiation, pharmacologic agents, specific monoclonal antibodies, immunotoxins) of anti-αGal antibodies.

INTRODUCTION

In 1966, Perper and Najarian were among the first to recognize that both antibodies and complement were involved in the hyperacute rejection (HAR) of organs transplanted between widely disparate species, such as pig-to-dog (1). It has been known for more than 10 years that pigs and other nonprimate mammals express terminal Galα1-3Gal oligosaccharides (αGal) as surface antigens on many cells, including the vascular endothelium (2). Galili et al. showed in 1984 that humans possess antibodies that have anti-αGal specificity (3), and that its IgG component comprises 1% of circulating IgG in human serum. It was not until 1991, however, that Good et al. demonstrated that these natural antibodies (anti-αGal) play a major role in the HAR of pig xenografts in primates (4-6). Furthermore, they determined that anti-αGal accounts for more than 85% of circulating natural anti-pig antibody in humans and that these antibodies are primarily of IgG and IgM subclasses (4-8). Galili and

coworkers have postulated that anti-αGal develop soon after birth, similar to ABO antibodies in humans, due to exposure to colonizing microorganisms in the gastrointestinal tract that express αGal surface antigens (9). There is now clear evidence that anti-αGal are responsible for complement-mediated HAR of porcine vascularized organs transplanted into human or nonhuman primate recipients (4-6,10,11).

Even if complement activation is inhibited (e.g. by the use of complement regulatory agents or porcine organs transgenic for human complement regulatory proteins), anti-αGal still plays a major role in a delayed rejection response, variously termed delayed xenograft rejection or acute vascular rejection (AVR), which is most probably complement-independent (12,13). Since it is now widely accepted that pigs (that express Gal epitopes in abundance) are the most likely animals to provide organs for xenotransplantation in humans (who have high levels of anti-αGal), the importance of the αGal antigen-anti-αGal antibody complex in xenotransplantation cannot be overemphasized.

In this chapter, current knowledge of anti-αGal and its importance in the rejection of vascularized organs in the pig-to-primate model is discussed. We summarize various methods being explored of achieving depletion or inhibition of anti-αGal and, furthermore, of suppressing production.

Anti-αGal Antibodies

Oriol et al. have presented detailed data on the presence of αGal epitopes in different porcine tissues (14) and have demonstrated that αGal is expressed uniformly on vascular endothelium of all pig breeds (15). This contrasts with humans, who express ABO blood group epitopes rather than αGal, even though both humans and pigs express N-acetyllactosamine and a sialic acid on their vascular endothelium (Chapter 1, Table 1) (16). It has long been considered that anti-αGal and anti-A and anti-B blood group antibodies are members of the same family of natural antibodies (17), and further evidence to this effect has recently been presented by Parker et al (18).

The concentration of αGal on most pig cells approximates 10^7 epitopes per cell (2). The IgM subclass of anti-αGal is of special importance in HAR since IgM is often found to be deposited on graft endothelium before, and sometimes in the absence of, IgG (10,19). In addition, depletion of anti-αGal IgM eliminates complement-mediated cytotoxicity of human serum on porcine endothelial cells (20). However, evidence has also been presented that IgG can play a role in HAR as well as in AVR (11,21).

Humans and nonhuman primates (with the exception of New World monkeys) have a nonfunctional α-galactosyltransferase pseudogene, hence lack the encoded α1,3-galactosyltransferase enzyme, and therefore do not make αGal (22). In the absence of αGal, primates develop anti-αGal. It has been suggested that nonhuman primates were exposed to an αGal-expressing pathogen at an evolutionary stage after the divergence of New from Old World monkeys, and that the survival of Old World primates depended on adapting by suppressing αGal expression and producing anti-αGal antibody (23). Since αGal epitopes have been found on the surface of certain parasites, bacteria, and viruses (reviewed in 6), anti-αGal may play a role in defense mechanisms against these organisms in humans and nonhuman primates. These observations suggest that anti-αGal may be essential to primates in providing natural immunity to a wide variety of pathogens.

Anti-αGal Antibodies: Role in Hyperacute Rejection

When vascularized organs are transplanted between pigs and primates, HAR almost uniformly occurs (24). The development of HAR in the pig-to-primate model depends on the activation of complement, mostly, if not entirely, through the classical pathway. Activation of this pathway is initiated by the binding of anti-αGal to the corresponding antigens (i.e. αGal) (19,25). When anti-αGal is depleted from xenograft recipients, prolongation of graft survival is seen (26-29). When porcine hearts are transplanted into newborn baboons (that lack anti-αGal), HAR does not occur (30). The importance of the αGal antigen-anti-αGal antibody system was confirmed by Collins et al, who demonstrated that HAR occurred when an organ from

a New World monkey (expressing αGal) was transplanted into a baboon (that produces anti-αGal) (31).

Anti-αGal Antibodies: Role in Acute Vascular Rejection

HAR can be prevented by antibody depletion or inhibition (26-29), complement depletion or inhibition (32,33), or by transplanting organs from pigs transgenic for human complement regulatory proteins (34-37). However, a delayed rejection phenomenon (AVR) occurs and leads to destruction of the xenograft (13). It has been proposed that this destruction results from the development of high levels of high affinity antibody, largely IgG, directed against αGal and possibly also new pig epitopes (12). This response occurs despite high doses of immunosuppressive therapy. Increases in anti-αGal IgG of 100-300-fold have been documented after experimental organ and bone marrow transplantation (38,39) and after clinical pancreatic islet cell transplants (40). Even after the transplantation of relatively avascular porcine meniscus cartilage to cynomolgus monkeys, anti-αGal titers (IgM and IgG) rose 10-fold (41). These induced antibodies appear to be the major cause of AVR (42,43).

Accommodation

It has been described that ABO- or human leukocyte antigen (HLA)-incompatible allografts are able to survive despite the presence of circulating antibodies directed against these determinants (44-46). This process has been termed accommodation, and can be applied to any system in which antibodies exist that are potentially reactive with antigens on the endothelium of a primarily vascularized organ graft and yet where antibody-mediated rejection does not occur (47,48). The phenomenon has not yet been documented to occur in Gal-incompatible large animal models. Several possible explanations for the phenomenon of accommodation have been proposed (47). Firstly. the returning antibodies may be different in isotype, affinity, and/or specificity, and are thus unable to initiate rejection. Secondly, the surface antigens on the vascular endothelium may show subtle changes during the absence of natural antibodies. Finally, the endothelial cells may adapt during the return of antibodies and respond differently or become 'desensitized'. Bach et al have presented evidence that

changes occur in the endothelial cells, with upregulation of 'beneficial' genes and downregulation of 'detrimental' genes (48).

Tolerance

Perhaps the ultimate method for avoiding HAR and AVR would be to induce B cell tolerance. If T cell tolerance were also induced, the subsequent cellular response (and the complications associated with long-term high-dose immunosuppression) would also be avoided. This option is being studied at our center, and involves the induction of mixed hematopoietic cell chimerism between pig and primate (38,39,49). Tolerance is achieved when there is permanent specific unresponsiveness to donor antigens (but not to other antigens) in the absence of maintenance immunosuppressive therapy. To induce this state in the pig-to-primate model, a combination of (at least temporary) (i) anti-αGal depletion or inhibition, (ii) depletion or inhibition of complement, (iii) T cell depletion (and/or costimulatory blockade), and (iv) pig hematopoietic cell engraftment, would appear to be necessary. The use of organs from genetically engineered pigs transgenic for human complement regulatory proteins may also prove an advantage, although this remains uncertain.

Whether accommodation is to be achieved or tolerance is to be induced, however, it seems essential to devise strategies that lead to the depletion of anti-αGal and to the suppression of production of anti-αGal for a period of time.

METHODS OF DEPLETION OR INHIBITION OF ANTI-αGAL ANTIBODIES

Initial experiments in the 60s and 70s directed against removing xenoreactive antibodies were performed by perfusion of the recipient's blood or plasma through a donor-specific organ, such as a pig kidney or liver (50-53). Antibodies were adsorbed by the perfused organ's vascular endothelium, thus temporarily depleting the anti-species antibody and enabling modestly prolonged survival of a subsequently transplanted organ. Initial experiments in the pig-to-non-human primate model (26,54) showed the pig liver to be a better immunoadsorbent than the kidney, a

finding confirmed recently by Azimzadeh et al (55). Evidence has also been presented that perfusion of human blood through pig lungs is more effective in removing anti-pig antibody than perfusion through pig liver or spleen (56). Other methods for depleting anti-αGal include (i) plasma exchange, (ii) plasma perfusion through non-specific immunoadsorbants (e.g., protein columns), and (iii) plasma perfusion through specific antibody sorbents (e.g., synthetic αGal immunoaffinity columns).

Plasma Exchange

Plasma exchange - the (complete) removal of the subject's plasma (with replacement of volume by other fluids) - is utilized as a treatment for numerous conditions, such as myasthenia gravis, thrombotic microangiopathies, and cryoglobulinemias. All or most circulating antibodies, including anti-αGal, are removed. Because of this temporary state of agammaglobulinemia, the patient is at risk for infection. Furthermore, the patient may be depleted of plasma proteins important to the coagulation cascade. Alexandre and coworkers were successful in using repeated plasma exchange to remove circulating anti-A and anti-B blood group antibodies from potential kidney transplant recipients (45), and demonstrated that the phenomenon of accommodation could result. This technique was also moderately successful in depleting xenoreactive natural antibodies from baboons in a pig-to-baboon kidney transplant model (27) but, although the porcine grafts functioned for up to 22 days, accommodation did not occur, and the grafts were lost to AVR.

Nonspecific Antibody Sorbents

Nonspecific antibody adsorption differs from plasma exchange in that the plasma is passed through an immunoaffinity column containing proteins that remove immunoglobulins from the plasma. The remaining plasma is then returned to the patient, reducing the need for replacement fluids. The proteins (such as staphylococcal protein A or protein G) are efficient in removing antibody by binding to the Fc portion of the antibody. Since the proteins are non-specific, all antibodies are removed, resulting again in hypogammaglobulinemia and predisposition for infectious complications. Palmer et al. reported some success in the use of protein A

columns to deplete anti-HLA antibodies in potential renal transplant recipients (46), and this technique has been used by several groups in xenotransplantation studies.

Specific Antibody Sorbents

Specific antibody sorbents for anti-αGal utilize columns that remove anti-αGal from plasma by binding them to natural or synthetic αGal oligosaccharides. Significant hypogammaglobulinemia does not occur. Initial studies were by Ye et al. (57), who demonstrated that extracorporeal immunoadsorption (EIA, Figure 1) of baboon plasma, utilizing immunoaffinity columns of melibiose on 4 consecutive days, could reduce cytotoxicity to pig kidney (PK15) cells to 20%.

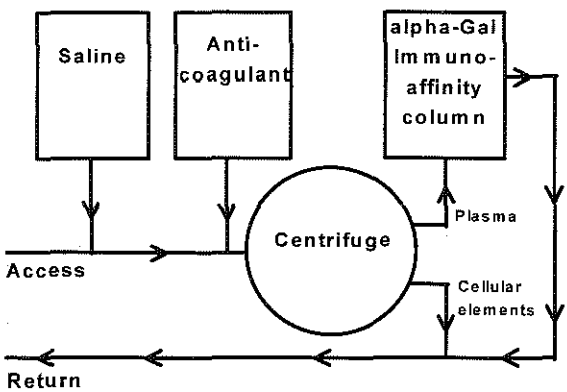

Figure 1.

Diagram of extracorporeal immunoadsorption circuit, with access through the internal jugular vein and return through the saphenous vein.

When more specific αGal oligosaccharides became available, a course of EIA resulted in successful depletion of anti-αGal for 4-5 days after which anti-αGal returned and led to rejection of a transplanted porcine organ (28,29,58,59). Kozłowski et al. (60) provided evidence indicating that EIAs should be carried out approximately 24 – 48 hours apart in order to enable equilibration of anti-αGal between the intra-

and extravascular spaces to occur. Although αGal immunoaffinity columns are efficient in depleting anti-αGal, in order to maintain depletion, other therapeutic modalities need to be employed, such as inhibiting the production of anti-αGal. This has proved much more difficult, and is perhaps the major barrier to successful xenotransplantation facing us at the present time (see below).

Antiidiotypic Antibodies

An alternative approach to using a synthetic oligosaccharide column is the use of antiidiotypic antibodies (AIA) directed against idiotypes expressed on anti-αGal antibodies. AIAs recognize specific idiotypes that are antigenic determinants within the variable regions of immunoglobulins. AIAs are also directed against the B lymphocyte subpopulations that bear the same idiotypes as surface receptors. Koren et al. (61) produced AIAs in mice by the injection into the mouse of human anti-pig antibodies (eluted from pig organs after repeated perfusion with human plasma) and by the subsequent creation of hybridomas. Several of these AIAs, when incubated with human serum, had a major inhibitory effect on serum cytotoxicity towards pig PK15 cells *in vitro*. The AIAs could be made up as an immunoaffinity column or could be infused intravenously into the recipient (to be bound by anti-αGal, thus inhibiting binding of anti-αGal to a transplanted organ). When infused into baboons, serum cytotoxicity was markedly reduced (to approximately 10%). Recently, we have produced a pig polyclonal AIA by immunizing a pig with human anti-αGal antibodies. The purified final preparation contained 1-2 % AIA. After repeated administration to a baboon (following repeated EIA of anti-αGal), a delayed return of anti-αGal and reduced cytotoxicity to pig cells was observed. Furthermore, at this time point, the baboon serum was able to partially inhibit the cytotoxicity of other highly cytotoxic sera.

Intravenous Infusion of Immunoglobulin

The intravenous (i.v.) infusion of concentrated human immunoglobulin (IVIG) has been used successfully in the treatment of certain autoimmune disorders, e.g., idiopathic thrombocytopenic purpura (62), and more recently as therapy to reduce

anti-HLA antibodies in highly-sensitized patients awaiting organ transplantation (63). Magee et al. (20) demonstrated that the i.v. infusion of immunoglobulin could prevent the HAR of pig organs transplanted into baboons. Different hypotheses have been proposed to explain the mechanism of action of IVIG, including the presence of AIAs or complement inhibition. Recent studies suggest that IVIG accelerates the physiological catabolism of IgG by saturating specific intracellular receptors, allowing degradation of IgG in proportion to its plasma concentration (65). If IVIG depleted of anti-αGal is repeatedly infused, the level of anti-αGal should steadily fall. Our preliminary data using IVIG (depleted of anti-αGal) in experiments involving the i.v. infusion of porcine peripheral blood progenitor cells into baboons indicate that significantly greater hematopoietic cell chimerism can be obtained than when IVIG is not administered (see Section II, Chapter 2). We believe this beneficial effect is brought about, at least in part, by inhibition of macrophage function.

Intravenous Infusion of Oligosaccharides

Yet another approach to 'neutralize' anti-αGal is to administer a large quantity of soluble antigen, thus blocking anti-αGal *in vivo*. It has been demonstrated that many oligosaccharides terminating in αGalα1 can inhibit the action of anti-αGal (4-6,57,66-68). Ye et al. (57) demonstrated that the i.v. infusion of melibiose and/or arabinogalactan in a very high concentration was effective in (partially) eliminating cytotoxicity of the serum to PK15 cells in baboons. Infusion of such high concentrations, however, resulted in severe toxicity. More recently, i.v. administration of different oligosaccharides has proved non-toxic and also inhibited anti-αGal activity. HAR was delayed, but not abolished (67,68).

METHODS OF SUPPRESSION OF PRODUCTION OF ANTI-αGAL ANTIBODIES

Antibodies are produced by B lymphocytes and plasma cells. If it were possible to suppress selectively the cells responsible for the production of anti-αGal, a major step would have been taken in achieving long-term xenograft acceptance. As this is not yet

a reality, present studies at our center are directed towards temporarily destroying or suppressing all B cells and/or plasma cells by means of (i) irradiation, (ii) conventional or novel pharmacologic agents, (iii) anti-B cell or plasma cell monoclonal antibodies, or (iv) immunotoxins.

Whole Body Irradiation (WBI)

Irradiation causes death preferentially of rapidly dividing cells, such as malignant cells and cells involved in hematopoiesis, and is currently being used as an element in the conditioning regimen for bone marrow transplantation (69). It has therefore been included in some protocols aimed at achieving mixed hematopoeitic chimerism and the induction of tolerance (39,49). WBI of 300cGy (which is a non-lethal dose from which the bone marrow can spontaneously recover) leads to the temporary ablation of B cells (but not plasma cells (70)) (Figs. 2A & 2B), but is associated with only a small and temporary reduction in the production of antibody, including anti-αGal (38,39,49,70). B cell recovery is fairly rapid (Figure 2C). WBI, therefore, although creating "space" in the bone marrow to facilitate engraftment of transplanted hematopoietic cells, has only a minimal and transient effect on antibody production.

Pharmacologic Agents

Pharmacologic immunosuppressive therapy combined with EIA in the depletion and suppression of production of anti-αGal has been investigated by Lambrigts et al. (72). Without immunosuppressive therapy, anti-αGal returned to pre-EIA levels within 48 hours after the last of 3 consecutive EIA procedures. Various combinations of agents were administered in an effort to slow the rate of recovery of anti-αGal, but none suppressed anti-αGal production to a level of clinical relevance. However, rebound of anti-αGal to levels greater than or equal to pre-treatment levels was successfully prevented. Of the agents investigated, mycophenolate mofetil was judged to provide most suppression of antibody production in the absence of toxic side effects. We have continued this investigation in baboons by assessing the efficacy of other agents, such as zidovudine, methotrexate and cladribine (see below). Some agents are worthy of further discussion.

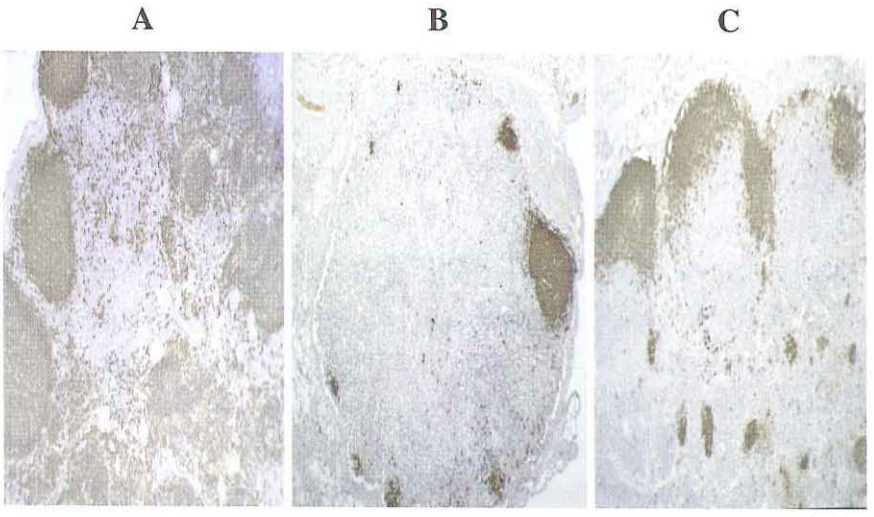

Figure 2.

Photomicrographs of baboon LNs at various timepoints pre and post WBI (300 cGy) immunohistochemically stained with the mouse anti-human monoclonal antibody ∝CD20.

A. LN before WBI showing normal distribution of B cells in the follicles.

B. LN 5 days after WBI. Note the near complete depletion of B cells.

C. LN 15 days after WBI. Recovery of B cells can be seen, beginning at the periphery of the follicles.

Cyclophosphamide

Cyclophosphamide, an alkylating metabolite, has been associated with suppression of both T and B lymphocytes. Its administration in large doses results in a significant reduction in the rate of return of anti-αGal after EIA (72). White and coworkers have used cyclophosphamide extensively, along with cyclosporine and steroids, in their hDAF transgenic pig-to-primate heart and kidney transplant models (36,37). Its use has been associated with significant prolongation of xenograft function for up to several weeks, although AVR remained difficult to prevent. It is a difficult drug to use long-term as it can result in severe leukopenia and other side effects which contribute to morbidity.

Mycophenolate mofetil (MMF)

MMF is an inhibitor of purine synthesis that inhibits T and B cell proliferation and antibody production. It reduced anti-αGal return after EIA to a comparable extent to that obtained by cyclophosphamide, when this latter drug was given in moderate and safe dosage (72). Minanov et al. (73) described modestly prolonged graft survival of pig-to-newborn heart transplants treated with a combination of MMF, cyclosporine, and methylprednisolone (from 3.6 to 6.2 days).

Melphalan

This alkylating agent has been used as mainstay treatment for multiple myeloma, often in combination with steroids. It would appear to be a promising drug for use in xenotransplantation in view of its effect on plasma cells, which are almost certainly the major source of anti-αGal production. However, Lambrigts et al. found it rather less effective in depleting anti-αGal than cyclophosphamide or MMF (72), although there would appear to be a delayed effect on antibody production that may be important. Our impression is that prolonged therapy with melphalan (over several weeks) combined with intermittent courses of EIA may slowly reduce the number of anti-αGal antibody-producing cells.

Zidovudine (AZT)

Zidovudine is a purine analogue used in the treatment of HIV infection (74). It inhibits viral reverse transcriptase, but also inhibits the mammalian DNA-polymerase that is responsible for DNA synthesis of mitochondria. Our hypothesis was that, since antibody production by B and plasma cells is a highly energy-dependant process, depletion of cellular mitochondria might prevent antibody from being produced. We have administered zidovudine to baboons while depleting anti-αGal by EIA. There was significant reduction of anti-αGal, particularly of IgG, suggesting some suppressive effect of the drug (Fig. 3). However, this reduction would not have been sufficient by itself to be of clinical impact.

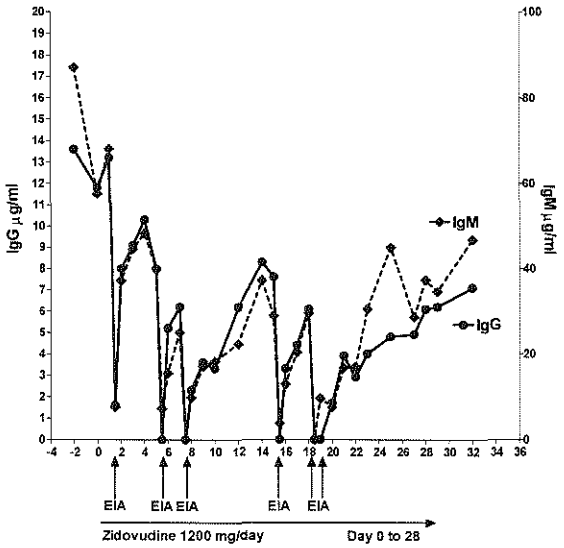

Figure 3.

Graph depicting the anti-αGal IgG and IgM profiles in a naïve baboon receiving zidovudine from day 0 through day 28. EIA was performed on the days indicated by short arrows. Following successful removal of anti-αGal by repeated EIA, the rate of return of antibody was slow, but return to pre-treatment level was not significantly prevented.

Methotrexate

Methotrexate is a dihydrofolate reductase inhibitor, and is another drug that has been used in the treatment of malignant lymphoid tumors. It has also been used as adjunctive therapy following organ transplantation in patients with steroid-resistant or recurrent acute rejection (75). We have assessed its effect on anti-αGal production in baboons. Although it had no apparent myelosuppressive effect (in contrast to its effect in humans), anti-αGal levels remained low after EIA for more than 2 weeks.

Cladribine (Leustatin)

Cladribine (leustatin) is a novel antineoplastic drug used for therapy in lymphoproliferative malignancies. It is being explored in experimental transplantation

as an immunosuppressive agent in both small and large animal models (76). Unlike other chemotherapeutic agents affecting purine metabolism, it is not only toxic to actively dividing lymphocytes but also to quiescent lymphocytes and monocytes, in which it induces apoptosis. In our studies, its efficacy as a suppressor of antibody production is at least equal to that of most of the other agents tested. Following EIA, baboons receiving cladribine maintained a decrease in anti-αGal levels of approximately 50% – 80% for approximately 2 weeks.

Anti-B Cell Monoclonal Antibodies (mAbs)

B cells can be targeted with mAbs directed against B cell-specific surface antigens. Several such mAbs have proven successful in reducing the mass of B cell lymphomas in *in vitro*, preclinical, and clinical studies (76-81). Following coating by mAb, the lymphoma cells are cleared mainly through antibody-dependant cell-mediated cytotoxicity. Such mAbs may prove useful in depleting normal B cells and thus perhaps lead to reduced antibody production. CD20, expressed on the surface of normal B cells, is targeted by the human-mouse chimeric antibody IDEC-C2B8. This mAb is effective in depleting malignant B cells in the blood of patients with B cell lymphoma; tumor regression has been documented (77-81). In our laboratory, baboons have been treated with a 4 week course (x1 weekly) of anti-CD20 mAb, after which no B cells could be detected in the blood or bone marrow for up to 3 months. Lymph nodes showed an 80% decrease in B cells 5 weeks after the initial dose, with recovery beginning by week 6. After a course of EIAs, anti-αGal levels remained reduced for 3-4 weeks but the reduction was relatively modest when compared with the extent of B cell deletion.

Immunotoxins

Some anti-B cell mAbs can be conjugated to toxins, such as ricin A or sapporin, and become more efficient in depleting B cells (82,83). CD22 is involved with the generation of mature B cells within the bone marrow, blood, and marginal zones of lymphoid tissues. An anti-CD22 mAb has been successful in the treatment of non-Hodgkin lymphoma (84). An anti-CD22 mAb was conjugated to the ricin A chain (kindly provided by Ellen Vitetta) and administered to a baboon. Its administration

led to a rapid reduction of B cells in the blood, bone marrow and lymph nodes, but its prolonged effect was impaired by the development of anti-murine and anti-ricin antibodies. It was therefore difficult to assess its true effect on anti-αGal antibody levels but, even with impairment of its efficacy by the development of antibodies, its immediate effect looked encouraging.

Prevention of Induced Antibodies

Induced anti-αGal IgG, and possibly antibody directed against new porcine (non-αGal) antigenic determinants, are considered to play a major role in AVR. These newly-synthesized antibodies are almost certainly produced by a T cell-dependent mechanism. Prevention of this induced reponse may therefore prove to be a major step in the prevention of AVR. The costimulatory pathway of CD40 and the T cell ligand, CD40L (or CD154), is crucial for effective activation of T cells to antigen (85) and plays an important role in establishing T cell-dependent B cell activity (86). Blockade of this pathway alone or in combination with blockade of the B7/CD28 pathway effectively prolongs survival of skin and organ allografts in rodents (87) and of kidney allografts in monkeys (88). At our center, costimulatory blockade has been shown to facilitate the establishment of mixed chimerism and tolerance to skin allografts in rodents when combined with a nonmyeloablative regimen (89). In xenotransplantation, costimulatory blockade has allowed prolonged survival of rat-to-mouse skin and heart grafts, as well as pig-to-mouse skin grafts (90). Recently, we have incorporated murine anti-human CD40L mAb therapy in a regimen aimed towards the induction of mixed hematopoietic chimerism in baboons (91). Without anti-CD40L mAb, high doses of porcine blood progenitor cells transplanted into baboons lead to sensitization to αGal (with a 100-fold increase in anti-αGal IgG) and the development of antibody to new (non-αGal) epitopes Fig. 4).

When anti-CD40L mAb was added to the regimen, sensitization to all pig antigens, including αGal, was prevented (Fig. 5). Although there was a return of αGal-reactive IgM and IgG to pretransplant levels, there was no increase of either immunoglobulin above those levels. The development of antibody to other pig antigens was also

prevented. These data suggest that anti-CD40L mAb may be of great value in preventing AVR in pig-to-primate xenotransplantation.

CONCLUSIONS / FUTURE INVESTIGATIONS

Anti-αGal plays a major role in complement-mediated HAR of a porcine vascularized organ. If this is avoided, the development of AVR would appear to be related to induced high affinity anti-αGal IgG and antibody to non-αGal porcine determinants. Unless accommodation develops, or tolerance is achieved during a window when there is no antibody being produced, it would appear to be necessary to suppress anti-αGal production permanently, which would clearly be difficult, if not impossible. If tolerance is the goal, it would at least appear to be necessary to suppress anti-αGal production for a period of time sufficient to allow for apoptosis of αGal-reactive B/plasma cells by the presence of pig cells expressing the αGal epitope.

Several strategies have been developed for depletion and maintenance of depletion of anti-αGal. EIA with an αGal-specific immunoaffinity column is currently effective in depleting anti-αGal, of both IgG and IgM subclasses. Our experience with almost 300 EIAs in baboons indicates that a course of 3 EIAs results in 99% and 97% reductions in IgG and IgM, respectively. Unfortunately, depletion cannot be maintained without repeated EIA.

The combination of EIA with agents such as cyclophosphamide, MMF, zidovudine, melphalan, and cladribine have contributed to a reduced rate of return of anti-αGal, but not to the extent to be clinically relevant. In the absence of an effective anti-plasma cell mAb, current attention is directed towards B cells. The normal life-span of human plasma cells remains uncertain but, if B cells could be eliminated, no new plasma cells would be produced. Following the death of existing plasma cells, a state of hypogammaglobulinemia might be reached. During this period, newly-developing αGal-reactive B cells could be tolerized by successful porcine hematopoietic cell

transplantation. Although this temporary state of hypogammaglobulinemia would not be without risk to the subject, steps could be taken to limit this risk.

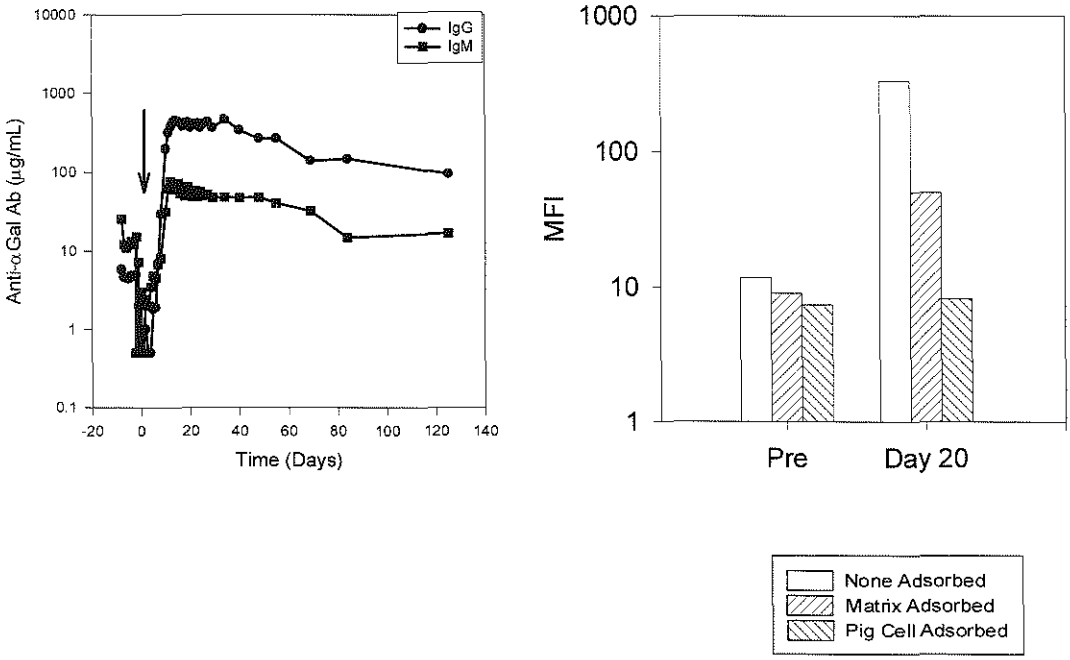

Figure 4.

Anti-αGal IgG and IgM (left) and non-αGal-reactive antibody (right) responses as median fluorescence intensity (MFI) following porcine peripheral blood progenitor cell (PBPC) transplantation in representative baboons receiving a tolerance inducing regimen **without** anti-CD40L mAb. The arrows (left) indicate the first day of porcine PBPC transplantation, which was administered after the third and final EIA (day 0). In (right), column 1 represents the anti-pig antibody level, column 2 represents the same serum after immunoadsorption of anti-αGal antibody, and column 3 represents this serum depleted of both αGal-reactive and non-αGal-reactive antibodies. The difference between columns 1 and 2 therefore indicates the amount of anti-αGal antibody, and the difference between columns 2 and 3 indicates the amount of anti-nonαGal antibody.

Left. A rise in both anti-αGal IgG and IgM occurred by day 10, indicating sensitization to the Gal determinants on the PBPC.

Right. Antibody directed to porcine nonαGal determinants on the PBPC developed within 20 days.

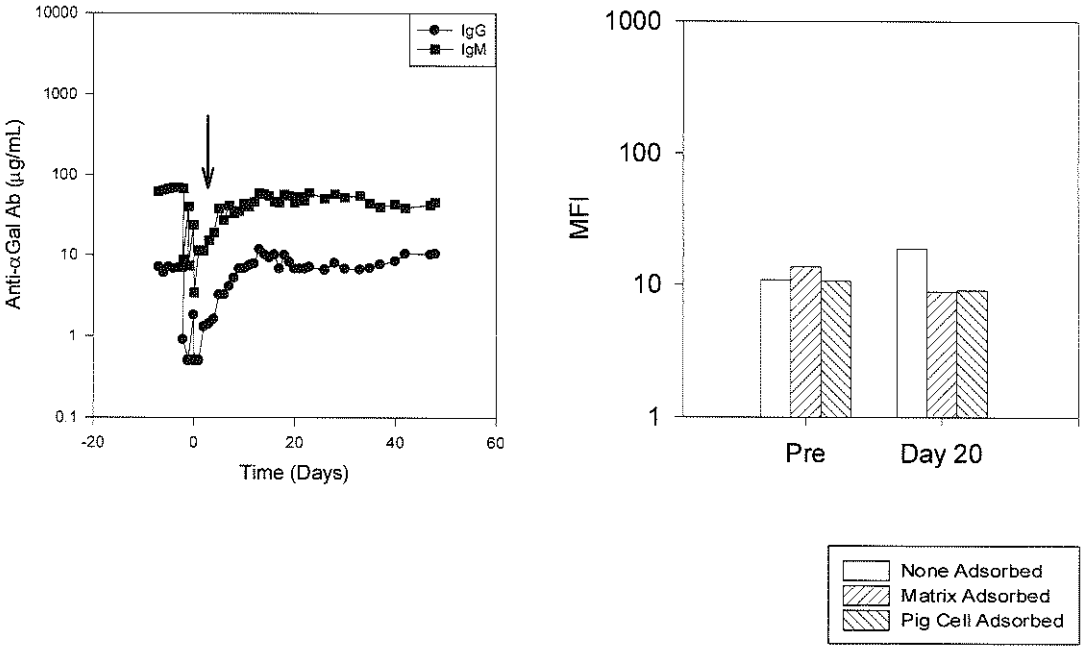

Figure 5.

Anti-αGal IgG and IgM (left) and non-αGal-reactive antibody (right) responses as median fluorescence intensity (MFI) following porcine peripheral blood progenitor cell (PBPC) transplantation in representative baboons receiving a tolerance inducing regimen **with** anti-CD40L mAb.

Left. No rise in anti-αGal IgG or IgM over baseline occurred.

Right. Antibody directed to porcine nonαGal determinants on the PBPC did not develop

With regard to current progress, however, the ability of anti-CD40L mAb to prevent the induced antibody response to pig cells represents a significant step in overcoming the immunologic barriers to xenotransplantation, and will undoubtedly facilitate the survival of transplanted pig organs in primates and the induction of tolerance.

REFERENCES

1. Perper RJ, Najarian JS. Experimental renal heterotransplantation. I. In widely divergent species. Transplantation 1966; 4: 377

2. Galili U, Shohet SB, Kobrin E, et al. Man, apes, and Old World monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J Biol Chem 1988; 263: 17755
3. Galili U, Rachmilewitz EA, Peleg A, et al. A unique natural human IgG antibody with anti-α-galactosyl specificity. J Exp Med 1984; 260: 1519
4. Good AH, Cooper DKC, Malcolm AJ, et al. Identification of carbohydrate structures that bind human anti-porcine antibodies: implications for discordant xenografting in humans. Transplant Proc 1992; 24: 559
5. Cooper DKC, Good AH, Koren E, et al. Identification of α-galatosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transplant Immunol 1993; 1: 198
6. Cooper DKC, Koren E, Oriol R. Oligosaccharides and discordant xenotransplantation. Immunol Rev 1994; 141: 31
7. Koren E, Neethling FA, Richards S, et al. Binding and specificity of major immunoglobulin classes of preformed human anti-pig heart antibodies. Transplant Int. 1993; 6: 351
8. Kujundzic M, Koren E, Neethling FA, et al. Variability of anti-αGal antibodies in human serum and their relation to serum cytotoxicity against pig cells. Xenotransplantation 1994; 1: 58
9. Galili U, Mandrell RE, Hamadeh RM, et al. The interaction between the human anti-α-galactosyl IgG (anti-Gal) and bacteria of the human flora. Infect Immun 1988; 57: 1730
10. Sandrin MS, Vaughan HA, Dabkowski PL, et al. Anti-pig IgM antibodies in human serum react predominantly with Galα(1-3)Gal epitopes. Proc Natl Acad Sci USA 1993; 90: 11391
11. Galili U. Interaction of the natural anti-Gal antibody with αgalactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol Today 1993; 14: 480
12. Galili U. Anti-αgalactosyl (anti-Gal) antibody damage beyond hyperacute rejection. In: Cooper DKC, Kemp E, Platt JL, White DJG (eds). Xenotransplantation, 2nd ed. Heidelberg: Springer 1997. p 95
13. Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nat Med 1995; 1: 869
14. Oriol R, Ye Y, Koren E, et al. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433
15. Oriol R, Barthold F, Bergemer A-M, et al. Monomorphic and polymorphic carbohydrate antigens on pig tissues: Implications for organ xenotransplantation in the pig-to-man model. Transplant Int 1994; 7: 405
16. Cooper DKC. Xenoantigens and xenoantibodies. Xenotransplantation 1998; 5: 6
17. McMorrow IM, Comrack CA, Nazarey PP, et al. Relationship between ABO bloodgroup and levels of Gal alpha,3galactose-reactive human immunoglobulin G. Transplantation 1997; 64: 546
18. Parker W, Lundberg-Swanson KL, Holzknecht ZE, et al. Isohemagglutinins and xenoreactive antibodies: members of a distinct family of natural antibodies. Hum Immunol 1996; 45: 94
19. Platt JL, Fischel RJ, Matas AJ, et al. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation 1991; 52: 514
20. Platt JL, Vercelotti GM, Lindman BJ, et al. Release of heparan sulfate from endothelial cells: Implications for pathogenesis of hyperacute rejection. J Exp Med 1990; 171:1363
21. Koren E, Kujundzic M, Koscec M, et al. Cytotoxic effects of human preformed anti-Gal IgG and complement on cultured pig cells. Transplant Proc 1994; 26: 1336
22. Joziasse DH, Shaper JH, Jabs EW, et al. Characterization of an α1,3-galactosyltransferase homologue on human chromosome 12 that is organized as a processed pseudogene. J Biol Chem 1991; 266: 6991
23. Galili U, Andrews P. Suppression of α-galactosyl epitopes synthesis and production of the natural anti-Gal antibody: a major evolutionary event in ancestral Old World primates. J Hum Evol 1995; 29: 433
24. Lambrigts D, Sachs DH, Cooper DKC. Discordant organ xenotransplantation in primates. World experience and current status. Tranplantation 1998; 66: 547
25. Rose AG, Cooper DKC, Human PA, et al. Histopathology of hyperacute rejection of the heart: experimental and clinical observations in allografts and xenografts. J Heart Lung Transplant 1991; 10: 223

26. Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. J Heart Lung Transplant 1988; 7: 238
27. Alexandre GPJ, Gianello P, Latinne D, et al. Plasmapheresis and splenectomy in experimental renal xenotransplantation. In: Hardy MA (ed). Xenograft 25. New York, Elsevier, 1989. p 28.
28. Sablinski T, Latinne D, Gianello P, et al. Xenotransplantation of pig kidneys to nonhuman primates: I. Development of the model. Xenotransplantation. 1995; 2: 264
29. Cooper DKC, Cairns TDH, Taube DH. Extracorporeal immunoadsorption of anti-pig antibody in pigs using αGal oligosaccharide immunoaffinity columns. Xeno 1996; 4: 27
30. Kaplon RJ, Michler RE, Xu H, et al. Absence of hyperacute rejection in newborn pig-to-baboon cardiac xenografts. Transplantation 1994; 59: 1
31. Collins BH, Cotterell AH, McCurry KR, et al. Cardiac xenografts between primate species provide evidence for the importance of the α-galactosyl determinant in hyperacute rejection. J Immunol 1995; 154: 5500
32. Leventhal JR, Dalmasso AP, Cromwell JW, et al. Prolongation of cardiac xenograft survival by depletion of complement. Transplantation. 1993; 55: 857
33. Kobayashi T, Taniguchi S, Neethling FA, et al. Delayed xenograft rejection of pig-to-baboon cardiac transplants after cobra venom factor therapy. Transplantation 1997; 64: 1255
34. Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. Nat Med 1995; 1: 964
35. McCurry KR, Kooyman DL, Alvarado CG, et al. Human complement regulatory proteins protect swine to primate cardiac xenografts from human injury. Nat Med 1995: 1; 123
36. Schmoeckel M, Bhatti FN, Zaidi A, et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. Transplantation 1998; 65: 1570
37. Zaidi A, Schmoeckel M, Bhatti F, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584
38. Kozlowski T, Monroy R, Xu, Y, et al. Anti-Galα1-3Gal antibody response to porcine bone marrow in unmodified baboons and baboons conditioned for tolerance induction. Transplantation 1998; 66: 176
39. Kozlowski T, Shimizu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance inducing regimen and antibody adsorption. Transplantation 1999; 67: 18
40. Groth CG, Korgsen O, Tibell A, et al. Transplantation of fetal porcine pancreas to diabetic patients: Biochemical and histological evidence for graft survival. Lancet 1994; 344: 1402
41. Galili U, Latemple DC, Walgenbach AW, et al. Porcine and bovine cartilage transplants in cynomolgus monkeys: II. Changes in anti-gal response during chronic rejection. Transplantation 1997; 63: 646
42. Goodman DL, Millan MT, Ferran C, et al. Mechanisms of delayed xenograft rejection. In: Cooper DKC, Kemp E, Platt JL, White DJG (eds). Xenotransplantation, 2nd ed. Heidelberg,Springer, 1997. p 77
43. Buhler L, Friedman T, Iacomini J, Cooper DKC. Xenotransplantation - state of the art - update 1999. Front Biosci 1999; 4: D416
44. Chopek MW, Simmons RL, Platt JL. ABO incompatible renal transplantation: initial immunopathologic evaluation. Transplant Proc 1987; 19: 4553
45. Alexandre GPJ, Squifflet JP, De Bruyere M, et al. Present experiences in a series of 26 ABO-incompatible living donor renal allografts. Transplant Proc 1987; 19: 4538
46. Palmer A, Welsh K, Gjorstrup P, et al. Removal of HLA antibodies by extracorporeal immunoadsorption to enable renal transplantation. Lancet 1989; 1: 10
47. Bach FH, Platt JL, Cooper DKC. Accommodation - the role of natural antibody and complement in discordant xenograft rejection. In: Cooper DKC, Kemp E, Reemtsma K, White DJG (eds). Xenotransplantation, 1st ed. Heidelberg, Springer, 1991. P 81
48. Bach FH, Ferran C, Hechenleitner P, et al. Accomodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med 1997; 3: 196
49. Sykes M, Sachs DH. Xenogeneic tolerance through hematopoietic cell and thymic transplantation. In: Cooper DKC, Kemp E, Platt JL, White DJG (eds). Xenotransplantation, 2nd ed. Heidelberg, Springer,: 1997. p 496

50. Moberg AW, Shons AR, Gerwurz H, et al. Prolongation of renal xenografts by the simultaneous sequestration of preformed antibody, inhibition of complement, coagulation, and antibody synthesis. Transplant Proc 1971; 3: 538
51. Slapak M, Greenbaum M, Bardawil W, et al. Effect of heparin, arvin, liver perfusion, and heterologous antiplatelet serum on rejection of pig kidney to dog. Transplant Proc 1971; 3: 558
52. Shons AR, Beir M, Jetzer J, et al. Techniques of *in vivo* plasma modification for the treatment of hyperacute rejection. Surgery 1973; 73: 28
53. Terman DS, Garcia-Rinaldi R, McCalmon R, et al. Modification of hyperacute renal xenograft rejection after extracorporeal immunoadsorption of heterospecific antibody. Int J Artif Organs 1979; 2: 35
54. Lexer G, Cooper DKC, Rose AG, et al. Hyperacute rejection in a discordant (pig-to-baboon) cardiac xenograft model. J Heart Transplant 1986; 5: 411
55. Azimzadeh A, Meyer C, Watier H, et al. Removal of primate xenoreactive natural antibodies by extracorporeal perfusion of pig kidneys and livers. Transpl Immunol 1998; 1: 13
56. Macchiarini P, Oriol R, Azimzadeh A, et al. Evidence of human non-alpha-galactosyl antibodies involved in the hyperacute rejection of pig lungs and their removal by pig organ perfusion. J Thorac Cardiovasc Surg 1998; 5: 831
57. Ye Y, Neethling FA, Niekrasz M, et al. Evidence that intravenously administered alpha-galactosyl carbohydrates reduce baboon serum cytotoxicity to pig kidney cells (PK15) and transplanted pig hearts. Transplantation 1994; 58: 330
58. Taniguchi S, Neethling FA, Korchagina EY, et al. *In vivo* immunoadsorption of antipig antibodies in baboons using a specific Galα1-3Gal column. Transplantation 1996; 10: 1379
59. Xu Y, Lorf T, Sablinski T, et al. Removal of anti-porcine natural antibodies from huma and nonhuman primate plasma *in vitro* and *in vivo* by a Galα1-3Galβ1-4Glc-X immunoaffinity column. Transplantation 1998; 65: 172
60. Kozlowski T, Ierino FL, Lambrigts D, et al. Depletion of anti-αGal1-3Gal Antibody in baboons by specific immunoaffinity columns. Xenotransplantation 1998; 5: 122
61. Koren E, Milotic F, Neethling FA, et al. Monoclonal antiidiotypic antibodies neutralize cytotoxic effects of anti-αGal antibodies. Transplantation 1996; 62: 837
62. Wordell CJ. Use of intravenous immune globulin therapy: an overview. DICP 1991; 25: 805
63. Jordan SC, Tyan D, Czer L, et al. Immunomodulatory actions of intravenous immunoglobulin (IVIG): potential applications in solid organ transplant recipients. Pediatr Transplant 1998; 2: 92
64. Magee JC, Collins BH, Harland RC, et al. Immunoglobulin prevents complement mediated hyperacute rejection in swine-to-primate xenotransplantation. J Clin Invest 1995; 96: 2404
65. Yu Z, Lennon VA. Mechanism of intravenous immune globulin therapy in antibody-mediated autoimmune diseases. N Engl J Med 1999; 340: 227
66. Neethling FA, Joziasse D, Bovin N, et al. The reducing end of αGal oligosaccharides contributes to their efficiency in blocking natural antibodies of human and baboon sera. Transplant Int. 1996; 9: 98
67. Simon PM, Neethling FA, Taniguchi S, et al. Intravenous infusion of αGal oligosaccharides in baboons delays hyperacute rejection of porcine heart xenografts. Transplantation. 1998; 63: 346
68. Romano E, Neethling FA, Nilsson KGI, et al. Intravenous synthetic αGal saccharides delays hyperacute rejection following pig-to-baboon heart transplantation. Xenotransplantation. In press.
69. Lindsley K, Deeg HJ. Total body irradiation for marrow or stem-cell transplantation. Cancer Invest 1998; 16: 424
70. Kimikawa M, Kawai T, Sachs DH, et al. Mixed chimerism and transplantation tolerance induced by a nonlethal preparative regimen in cynomolgus monkeys. Transplant Proc 1997; 29: 1218
71. Clave E, Socie G, Cosset JM. Multicolor flow cytometry analysis of blood cell subsets in patients given total body irradiation before bone marrow transplantation. Int J Radiat Oncol Biol Phys 1995; 34: 881
72. Lambrigts D, Van Calster P, Xu Y, et al. Pharmacologic immunosuppressive therapy and extracorporeal immunoadsorption in the suppression of anti-αGal antibody in the baboon. Xenotransplantation 1998; 5: 274
73. Minanov OP, Artrip JH, Szabolcs M, et al. Triple immunosuppression reduces mononuclear cell infiltration and prolongs graft life in pig-to-newborn baboon cardiac xenotransplantation. J Thorac Cardiovasc Surg 1998; 115: 998
74. Lindback S, Vizzard J, Cooper DA, et al. Long-term prognosis following zidovudine monotherapy

in primary human immunodeficiency virus type 1 infection. J Infect Dis 1999; 179: 1549
75. Costanzo-Nordin M, Grusk B, Silver M, et al. Reversal of recalcitrant cardiac allograft rejection with methotrexate. Circulation 1988; 78:III 47
76. Gorski A, Grieb P, Korczak-Kowalska G, et al. Cladribine (2-chloro-deoxyadenosine, CDA): an inhibitor of human B and T cell activation *in vitro*. Immunopharmacology 1993; 26: 197
77. Reff ME, Carner K, Chambers KS, et al. Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. Blood 1994; 83: 435
78. Maloney DG, Grillo-Lopez AJ, Bodkin DJ, et al. IDEC-C2B8: results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma. J Clin Oncol 1997; 15: 3266
79. Tobinai K, Kobayashi Y, Narabayashi M, et al. Feasibility and pharmacokinitic study of a chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in relapsed B cell lymphoma. The IDEC-C2B8 study group. Ann Oncol 1998; 9: 527
80. Coiffier B, Haioun C, Ketterer N, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. Blood 1998; 92: 1927
81. McLaughlin P, Grillo-Lopez AJ, Link BK, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four dose treatment program. J Clin Oncol 1998; 16: 2825
82. Bolognessi A, Tazzari PL, Olivieri F, et al. Evaluation of immunotoxin containing single-chain ribosome-inactivating proteins and an anti-CD22 monoclonal antibody (OM124): in vitro and in vivo studies. Br J Haematol 1998; 101: 179
83. Sausville EA, Headlee D, Stetler-Stevenson M, et al. Continuous infusion of the anti-CD22 immunotoxin IgG-RFB4-SMPT-dgA in patients with B cell lymphoma: a phase I study. Blood 1995; 85: 3457
84. Tedder TF, Tuscano J, Sato S, et al. CD22, a B lymphocyte –specific adhesion molecule that regulates antigen receptor signaling. Annu Rev Immunol 1997; 15: 481
85. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological characterization of a murine ligand for CD40. Nature 1992; 357: 80
86. Noelle R, Roy M, Shepherd D, et al. A 39-kDa protein on activated helper T cells binds CD40and transduces the signal for cognate activation of B cells. Proc Natl Acad Sci USA 1992; 89: 6550
87. Larsen CP, Elwood ET, Alexander DZ, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 ligand. Nature 1996; 381: 434
88. Kirk AD, Harlan DM, Armstrong NN, et al. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. Proc Natl Acad Sci USA 1997; 94: 8789
89. Wekerle T, Sayegh MH, Hill J, et al. Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. J Exp Med 1998; 187: 2037
90. Elwood ET, Larsen CP, Cho HR, et al. Prolonged acceptance of concordant and discordant xenografts with combined CD40 and CD28 pathway blockade. Transplantation 1998; 65: 1422
91. Buhler L, Awwad M, Basker M, et al. A nonmyeloablative regimen with CD40L blockade leads to humoral and cellular hyporesponsiveness to pig hematopoietic cells in baboons. Transplant Proc. 2000; 32: 1100.

4

Effects of specific anti-B and/or anti-plasma cell immunotherapy on xenoreactive antibody production in baboons

Adapted from:

Alwayn IPJ*, Xu Y*, Basker M*, Wu C, Buhler L, Lambrigts D, Treter S, Harper D, Kitamura H, Vitetta E, Abraham S, Awwad M, White-Scharf ME, Sachs DH, Thall A, and Cooper DKC. Effects of specific anti-B and/or anti-plasma cell immunotherapy on antibody production in baboons: depletion of CD20- and CD22-positive B cells does not result in significant decreased production of anti-αGal antibody. *Xenotransplantation 2001. In press.*

&

Basker M, Alwayn IPJ, Treter S, Harper D, Buhler L, Andrews D, Thall A, Lambrigts D, Awwad M, White-Scharf M, Sachs DH, and Cooper DKC. Effects of B cell/plasma cell depletion or suppression on anti-Gal antibody in the baboon. *Transpl. Proc. 2000;32(5):1009.*

*Authors contributed equally.

ABSTRACT

Background. Anti-Galα1-3Gal antibodies (anti-αGal Ab) are a major barrier to clinical xenotransplantation as they are believed to initiate both hyperacute and acute humoral rejection. Extracorporeal immunoadsorption (EIA) with αGal oligosaccharide columns temporarily depletes anti-αGal Ab, but their return is ultimately associated with graft destruction. We, therefore, assessed the ability of two immunotoxins (IT) and two monoclonal antibodies (mAb) to deplete B and/or plasma cells both in vivo and in vitro in baboons, and to observe the rate of return of anti-αGal Ab following EIA.

Methods. The effects of the mouse anti-human IT anti-CD22-ricin A (∝CD22-IT, directed against a B cell determinant) and anti-CD38-ricin A (∝CD38-IT, B and plasma cell determinant) and the mouse anti-human anti-CD38 mAb (∝CD38 mAb) and mouse/human chimeric anti-human anti-CD20 mAb (∝CD20 mAb, Rituximab, B cell determinant) on B and plasma cell depletion and anti-αGal Ab production were assessed in vivo in baboons (n=9) that had previously undergone splenectomy. For comparison, two baboons received nonmyeloablative whole body irradiation (WBI) (300 cGy), and one received myeloablative WBI (900 cGy). Five baboons were administered either ∝CD22-IT (at 0.125 – 0.19 mg/kg x 4 doses; n=2) or ∝CD38-IT (0.1 – 0.6 mg/kg x 14 doses; n=3). One baboon received ∝CD38 mAb at 0.3 mg/kg x 21 doses. Three baboons received ∝CD20 mAb at 20 mg/kg x 4 doses, either with (n=1) or without (n=2) WBI (150 cGY). Depletion of B cells was monitored by flow cytometry of blood, bone marrow (BM) and lymph nodes (LN), and by histology of LN. EIA was carried out after the therapy and anti-αGal Ab levels were measured daily. These agents were further analyzed in vitro.

Results. In vivo, WBI (300 or 900 cGy) resulted in 100% B cell depletion in blood and BM, > 80% depletion in LN, with substantial recovery of B cells after 21 days and only transient reduction in anti-αGal Ab after EIA. The effect of both murine ITs was in part limited by the development of ∝murine and/or ∝ricin antibodies. However, ∝CD22-IT depleted B cells by >97% in blood and BM, and by 60% in LN,

but a rebound of B cells was observed after 14 and 62 days in LN and blood, respectively. At 7 days, serum anti-αGal IgG and IgM Ab levels were reduced by a maximum of 40 - 45% followed by a rebound to levels up to 12-fold that of baseline anti-αGal Ab by day 83 in one baboon. The results obtained with ∝CD38-IT were inconclusive. This may have been, in part, due to inadequate conjugation of the toxin. Cell coating was 100% with ∝CD38 mAb, but no changes in anti-αGal Ab production were observed. ∝CD20 mAb resulted in 100% depletion of B cells in blood and BM, and 80% in LN, with recovery of B cells starting at day 42. Adding 150cGy WBI at this time led to 100% depletion of B cells in the BM and LN. Although B cell depletion in blood and BM persisted for >3 months, the reduction of serum anti-αGal IgG or IgM Ab levels was not sustained beyond 2 days. In vitro, ∝CD22-IT inhibited protein synthesis in the human Daudi B cell line more effectively than ∝CD38-IT. Upon differentiation of B cells into plasma cells, however, less inhibition of protein synthesis after ∝CD22-IT treatment was observed. Depleting CD20-positive cells in vitro from a baboon spleen cell population already depleted of granulocytes, monocytes, and T cells led to a relative enrichment of CD20-negative cells, i.e., plasma cells, and consequently resulted in a significant increase in anti-αGal Ab production by the remaining cells, whereas depleting CD38-positive cells resulted in a significant decrease in anti-αGal Ab production.

Conclusions. ∝CD20 mAb + WBI totally and efficiently depleted B cells in blood, BM, and LN for >3 months in vivo, but there was no sustained clinically significant reduction in serum anti-αGal Ab. The majority of antibody secretors are CD38-positive cells, but targeting these cells in vitro or in vivo with ∝CD38-IT was not very effective. These observations suggest that B cells are not the major source of anti-αGal Ab production. Future efforts will be directed towards suppression of plasma cell function.

INTRODUCTION

Xenotransplantation of porcine organs is viewed by many clinicians and investigators as a possible solution to the increasing shortage of donor organs for transplantation

(1,2,3). Although many advances have been made in understanding the immunologic and inflammatory phenomena associated with xenograft rejection (4,5,6), porcine vascularized xenografts do not survive beyond a median of approximately one month in nonhuman primate recipients (7,8). There is clear evidence that primate antibodies directed against terminal Galα1-3Gal oligosaccharides (anti-αGal Ab) on porcine vascular endothelium (9,10,11,12) are responsible for complement-mediated hyperacute rejection of porcine organs (13). Furthermore, even if hyperacute rejection is averted by depletion of anti-αGal Ab, the use of complement regulatory agents or of porcine organs transgenic for human complement regulatory proteins, anti-αGal Ab still plays a major role in a delayed rejection response, known variously as acute vascular rejection, delayed xenograft rejection, or acute humoral xenograft rejection, which is possibly complement-independent (14).

Specific depletion of anti-αGal Ab by extracorporeal immunoadsorption (EIA) of baboon plasma through columns containing synthetic αGal oligosaccharides (15,16) has resulted in prolonged xenograft survival (17,18,19). However, extended survival is limited by the return of anti-αGal Ab and by the development of antibodies directed against other porcine (non-αGal) determinants (19,20). These develop despite intensive pharmacologic immunosuppressive therapy. Specific therapies that result in a sustained depletion of anti-αGal Ab and other antibodies will almost certainly prove beneficial in overcoming acute vascular rejection.

Since antibodies are produced by B and plasma cells, depleting (or inhibiting the function of) these cells should result in diminished antibody production. Several pharmacologic agents targeting B cells were assessed for their ability to suppress production of anti-αGal Ab without significant success (6,21). Fuelled by the advances made in the treatment of B cell neoplasias using immunotherapy (22,23,24,25), the effects of specific anti-B and/or plasma cell immunotherapy on B cell depletion and anti-αGal Ab production in vivo and in vitro in baboons were investigated. These effects were compared to those achieved in baboons receiving myeloablative or nonmyeloablative whole body irradiation (WBI), as this has been

shown to deplete the majority of B cells in blood, bone marrow (BM), and lymph nodes (LN) (6) by inducing apoptosis in rapidly dividing cells such as lymphocytes (26).

We selected deglycosylated (dg) ricin A (RTA) for the construction of specific anti-B and/or plasma cell immunotoxins (IT). The dgRTA chain inhibits protein synthesis by destroying 60S ribosomal RNA. dgRTA is not efficiently internalized without the ricin B chain but, once conjugated to a desired monoclonal antibody (mAb), internalization can be facilitated and specific cell toxicity achieved. Other criteria used to select effective ITs include: (i) high affinity of the antibody for antigen; (ii) limited distribution of the targeting antigen in order to avoid non-specific toxicity; (iii) inhibition of protein synthesis by the toxin at relatively low concentrations due to effective internalization and intracellular routing.

Four agents were tested in vivo and/or in vitro in baboons.

1. A mouse anti-human CD22 monoclonal antibody (RFB4) conjugated to dgRTA (∝CD22-IT) was prepared as described previously (27). This IT can inhibit protein synthesis and antibody production in vitro and give multi-log depletion of human B cell lymphomas in vivo (28). CD22 is expressed in the cytoplasm of all B cells and as surface antigen on mature B cells (29). The CD22 antigen is present on lymphoplasmacytoid cells and is expressed in most B cell lymphomas, but is diminished on the fully-differentiated plasma cell (29).

2. The unconjugated mouse anti-human CD38 mAb (∝CD38 mAb, OKT10, American Type Culture Collection, Manassas, VA). CD38 is expressed on all pre-B and B cells, plasma cells, and thymocytes and is also present on activated T cells, natural killer cells, myeloblasts, erythroblasts, and BM cells (30).

3. The ∝CD38 mAb was conjugated to dgRTA (∝CD38-IT) as described previously (27). The purity of the IT was assessed by sodium dodecylsulphate–

polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions (31). The preparation contained approximately 60% unconjugated mAb.

4. A chimeric mouse-human mAb directed at the human CD20 surface antigen (∝CD20 mAb, Rituximab, kindly provided by IDEC Pharmaceuticals, San Diego, CA). CD20 is expressed on both resting and activated B cells, but is down-regulated on plasma cells (32). CD20 may also be expressed on follicular dendritic cells in germinal centers (33).

MATERIAL AND METHODS

In Vivo Experiments

Animals

Baboons (Papio anubis, n=12), of both sexes and weighing approximately 9 – 15 kg, were obtained from Biomedical Resources Foundation, Houston, TX. In view of the shortage and expense of nonhuman primates, five of the 12 baboons had been used previously (>2 months) for other experiments, but recovery of all study parameters (i.e., B cell counts and anti-αGal Ab levels) had occurred. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical procedures

Splenectomy, intravenous (iv) and intra-arterial catheterization, and EIA were carried out under inhalation anesthesia. A splenectomy is routinely performed in our baboons because there is evidence that this leads to prolonged survival of xenografts (34, 35) and, furthermore, we have found that spleens contain a large number of anti-αGal Ab-secreting cells (Xu Y, et al. unpublished data). Baboons were premedicated with atropine (0.05 mg/kg im) and ketamine (10 mg/kg im) prior to surgery and were intubated and ventilated through a closed circuit with oxygen (1 – 2 L/min), nitrous oxide (2 – 4 L/min), and isoflurane (0.5 – 3.0 %). Splenectomy was performed as

described previously (17). Catheters were inserted in the internal and external jugular veins and carotid artery. The catheters were tunneled subcutaneously to the back, and brought out through a jacket and tethering system allowing monitoring of arterial blood pressure, access to blood withdrawal, and continuous or intermittent iv administration of fluids and/or drugs. Cefazolin (500 mg iv daily) was given as prophylaxis against infection.

Extracorporeal immunoadsorption (EIA) of anti-αGal Ab

EIA was performed using an immunoaffinity column (Alberta Research Council, Alberta, Canada) containing synthetic αGal trisaccharide type VI as previously described (19,36). Briefly, using a COBE-Spectra apheresis unit (Gambro BCT International, Lakewood, CO), 3 plasma volumes were immunoadsorbed through an αGal immunoaffinity column at an average perfusion rate of 20 ml/min. Anticoagulation was achieved with citrate phosphate dextrose adenine, and hemodynamic stability maintained by volume replacement +/- phenylephrine infusion. The anti-αGal Ab-depleted plasma was returned to the baboon. Three consecutive daily EIAs were performed at the time of optimal B cell and/or plasma cell depletion.

Whole body irradiation (WBI)

Baboons received nonmyeloablative WBI 2x150 cGy (n=2) or myeloablative WBI 2x450 cGy (n=1) using a Cobalt 60 teletherapy unit (Therapy Services Inc., Frederick, MD) without specific anti-B and/or anti-plasma cell immunotherapy. Furthermore, one baboon received 150 cGy WBI 20 days after treatment with ∝CD20 mAb (see below).

Administration of experimental agents in vivo

Treatment with ITs / mAbs was preceded by the iv infusion of 25 mg diphenhydramine to prevent potential anaphylactic reactions. Commencing on day 0, the ITs / mAb were administered as bolus iv infusions to baboons. We selected the dosages for ∝CD22-IT (37) and ∝CD20 mAb (38) based on maximum tolerated

doses from previous clinical studies. The dose of the ∝CD38 mAb and the ∝CD38-IT we had were extrapolated from the maximum tolerated dose used for the ∝CD22-IT, since there was no previous clinical experience with this agent.

∝CD22-IT (n=2) at 0.125 – 0.19 mg/kg every other day x 4 doses.
∝CD38 mAb (n=1) at 0.3 mg/kg/day x 21 doses.
∝CD38-IT (n=3) at 0.1 – 0.6 mg/kg/day x 14 doses.
∝CD20 mAb (Rituximab) (n=3) at 20 mg/kg/week x 4 doses.

In Vitro Assays

Tissue and cell preparation

Baboon spleen cells were surgically obtained by splenectomy as described above. Cells were harvested in RPMI (Gibco BRL, Grand Island, NY), homogenized, and filtered sequentially through a flow-mesh FM-100 (PGL Scientifics, Frederick, MD). Peripheral blood mononuclear cells were prepared from heparinized peripheral blood obtained from healthy baboons. BM cells were obtained and prepared as described previously (39). Cells were additionally purified by separation over a Ficoll density gradient (Histopaque, Sigma, St.Louis, MO).

B cell enrichment

Red blood cells from cell preparations were lysed with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA.2H_2O$). Cells were washed and resuspended in RPMI containing 10% fetal calf serum and gentamicin (Gibco BRL). The average total yield per spleen was 3-5 x 10^9 cells. Monocytes were depleted by adherence at 37°C with 5% CO_2 in RPMI with 10% fetal calf serum overnight. T cells were depleted by E-rosetting using AET- (Sigma) treated sheep red blood cells (Cocalico Biologicals, Inc., Reamstown, PA) following standard procedures. Granulocytes were removed during the E-rosetting procedure. Alternatively, T cells were depleted with anti-CD2 (Leu5b, Becton Dickinson, San Jose, CA) followed by goat anti-mouse IgG magnetic beads (Dynal, Lake Success, NY). B cells were enriched from an initial

average of 25-50% to 80-90%, as was assessed by flow cytometry (described below). The final preparation contained approximately 10% CD2-positive cells.

In vitro activation / differentiation of plasma cells

B cells ($2x10^6$ cells/ml in cell suspension medium) were activated with 0.04% Pansorbin (fixed *Staphylococcus Aureus*, Calbiochem-Novabiochem Corp., La Jolla, CA) and 2.5 ng/ml of IL2 (R&D Systems, Minneapolis, MN) for 2 – 3 days at 37°C with 5% CO_2. Cells were washed and subsequently cultured in 2.5 ng/ml IL-2 and 100 ng/ml IL-10 (R&D Systems) for up to 8 days, according to a modified protocol (40). These cells were then maintained in hybridoma medium (IMDM medium with 10% fetal calf serum, 5 mg/L bovine insulin, 10 mg/L bovine transferrin, 17.3 µg/L sodium selenite, and 5.5 $x10^{-5}$M 2ME) for up to 30 days. Plasma cell morphology was confirmed by cytospin (differential staining and intracellular immunoglobulin), ELISPOT and flow cytometry (both described below). Plasma cells at different stages of differentiation were harvested for further study.

^{3}H-Leucine (^{3}H-Leu) incorporation assay to measure total protein synthesis after IT treatment

A total of 10^5 cells/well were incubated with ∝CD22-IT, ∝CD38-IT, or ∝CD25-IT (as a control) at concentrations from 10^{-13}M to 10^{-8}M for 1 hour at 4°C. Cells were washed and cultured overnight in RPMI with 10% fetal calf serum for B cells or with addition of 2.5 ng/ml IL-2 and 100 ng/ml IL-10 for plasma cells at 37°C with 5% CO_2. Cells were washed again, and pulsed in Leucine-free RPMI with 5 µCi/well ^{3}H-Leu for 5hr (for Daudi cells) or 18 hours (for B and plasma cells) at 37°C. ^{3}H-Leu incorporation was examined by harvesting cells on to filters and counting the radioactivity by beta-counter (Wallac, Gathersburg, MD).

ELISPOT for detection of anti-αGal Ab and total immunoglobulin production

For detection of anti-αGal Ab production, 96-well MultiScreen-HA plates (MAHAS 4510 mixed cellulose esters, Millipore, Bedford, MA) were coated with 100 µl/well (5 µg/ml in PBS) of αGal-BSA or control BSA (Alberta Research Council) at 4°C

overnight. For detection of total IgM or IgG production, goat anti-human IgM or IgG (Southern Biotech, Birmingham, AL) were used as coating reagents with goat anti-mouse IgM or IgG as negative controls. Plates were washed with PBS and blocked with IMDM and 0.4% BSA for 1 hr at 37°C. A total of $1x10^6$ cells/well, with a serial 5-fold dilution, were cultured overnight in a modified hybridoma medium (10% fetal calf serum replaced by 0.4% BSA) at 37°C with 5% CO_2. The plates were then washed with PBS and PBS plus 0.1% Tween-20. Antibody production was detected with 100 µl goat anti-human IgM or IgG conjugated to HRP (Southern Biotech) at 1:1000 dilution in PBS with 1% BSA and 0.5% Tween at 4°C for 1 hour. Plates were washed with PBS plus 0.1% Tween and PBS. Spots were visualized with substrate AEC or 4CN (Sigma) under a stereomicroscope (Nikon SMZ-U) equipped with a vertical white light. Data were presented as spot forming units (SFU) per 10^6 cells.

Flow cytometry

Flow cytometry to detect B and T cells was performed on blood, BM (aspirates obtained from the iliac crests), and LN (biopsies obtained from either inguinal or axillary regions). The direct conjugated antibodies anti-CD3 FITC (FN18, kindly provided by Dr. David Neville, Bethesda, MD) and anti-CD2 PE (Leu-5b, Becton Dickinson) were used as T cell markers, and anti-CD20 FITC (Leu-16, Becton Dickinson) and anti-CD22 PE (Clone RFB4, Caltag Laboratories, Burlingame, CA) as B cell markers. Blood and BM were incubated at 4° C, lysed at room temperature with ACK-lysing buffer (Bio-Whittaker, Walkersville, MD), and washed and resuspended in 500 µl FACS Medium (1% BSA and 0.1% azide in phosphate-buffered saline). LNs were mashed, filtered, and resuspended in 500 µl FACS medium. Cell count was approximately 1 x 10^6 / 100 µl in all samples. The samples were stained using the aforementioned T and B cell-specific antibodies. Acquisition was performed under hi-flow using the FACScan (Becton Dickinson), and samples were analyzed using WinList (Verity Software House, Inc., Topsham, ME).

Cell depletion using magnetic beads

One million cells (10^6/ml) were incubated with 10 μg mAb (targeting the cells that were to be depleted) at 4°C for 1 hour. Cells were then washed and incubated with secondary goat-anti-mouse IgG magnetic beads following the manufacturer's protocol (Dynal). The cells that bound to the mAb were removed, and the remaining cells were assessed by flow cytometry and for antibody production by ELISPOT.

Measurement of anti-αGal antibody (anti-αGal Ab) by ELISA

Daily serum samples were obtained and anti-αGal Ab levels were measured by ELISA (19). This consisted of loading a 0.016%-2% concentration of baboon serum on Maxisorb plates (Nunc, Naperville, IL, USA) coated with 5 μg/mL of αGal-BSA (Alberta Research Council). Bound antibodies were detected using polyclonal donkey anti-human IgG (Accurate, Westbury, NY) or rabbit anti-human IgM (DAKO, Copenhagen, Denmark) conjugated to horseradish peroxidase. Color development was achieved by using o-phenylenediamine dihydrochloride (Sigma) as a substrate at 0.9mg/mL in PBS with urea hydrogen peroxide (Sigma). Absorbance at 490 nm was determined by a THERMOmax plate reader (Molecular Devices, Menlo Park, CA) after blocking the reaction with 2N H_2SO_4.

Immunohistochemistry

For in vitro experiments, a total of 100,000 cells in 200 μl PBS was centrifuged onto slides by Cytospin. The slides were either immediately stained with Giemsa or fixed overnight in 0.25% paraformaldehyde at 4° C for intracellular immunoglobulin staining. Goat anti-human IgM and/or IgG conjugated to peroxidase (Southern Biotech) at 1:1000 was added to the slides after which they were incubated at room temperature for 1 hour in a humidified chamber. After slides were washed, a DAB substrate kit (Vector Laboratories, Burlingame, CA) was used to visualize the staining.

For in vivo experiments, depletion of CD20 positive cells in LNs was assessed using the anti-CD22 anti-human mAb (Caltag) as primary antibody on frozen samples in a standard immunohistochemical assay. Depletion of CD22-positive cells was

examined in paraffin-embedded tissue using the anti-CD20 anti-human mAb (DAKO, Carpinteria, CA).

RESULTS

In vivo experiments

Effect of WBI on B cell depletion and anti-αGal Ab production

Administration of 2x150 cGy WBI led to rapid and complete B cell depletion as detected by flow cytometric analysis of blood and BM by day 2, with recovery starting by day 28 in the blood and BM (data not shown). B cell depletion in the LN was 75 – 100% between days 7 and 14, with recovery by day 15 (data not shown). This finding was confirmed by immunohistochemistry, where complete depletion of B cells in the LN was observed at day 9, with recovery starting at day 15 in the periphery of the follicles (See Chapter 3, Figure 2). In these baboons, a thrombocytopenia (<20,000/μl) was noted between days 6 and 13, and a leukocytopenia (< 1000/μl) between days 13 and 17, most probably due to BM depression. The anti-αGal Ab levels in these baboons remained unchanged, in normal ranges (data not shown), although no EIAs were performed. When EIAs were performed after myeloablative WBI (900 cGy), the rate of recovery of anti-αGal Ab was unchanged when compared to EIA treatment alone (data not shown). These data confirm that B cells are readily depleted by WBI in blood and BM for 28 days, and in LN for 15 days, but that this depletion is not associated with a significant decrease in anti-αGal Ab production.

Effect of the ∝CD22-IT on B cell depletion and anti-αGal Ab production

At the doses given, ∝CD22-IT was efficient in depleting B cells in blood and BM (86-99% (Fig. 1 - top) and 80-97%, respectively) between days 6 and 8. B cell depletion in LN was less marked, the maximum percentage of depletion ranging between 50 and 58% on days 7-12 (data not shown). T cell counts in blood, BM, and LN remained unchanged. However, the recovery of B cells was swift. In blood, evidence of recovery was observed at day 12 in both baboons, with increases up to 6-

fold baseline levels between days 62 and 85 (Fig 1 - top). In BM, recovery was first observed between days 12 – 15, with recovery to baseline levels by day 62 (Data not shown).

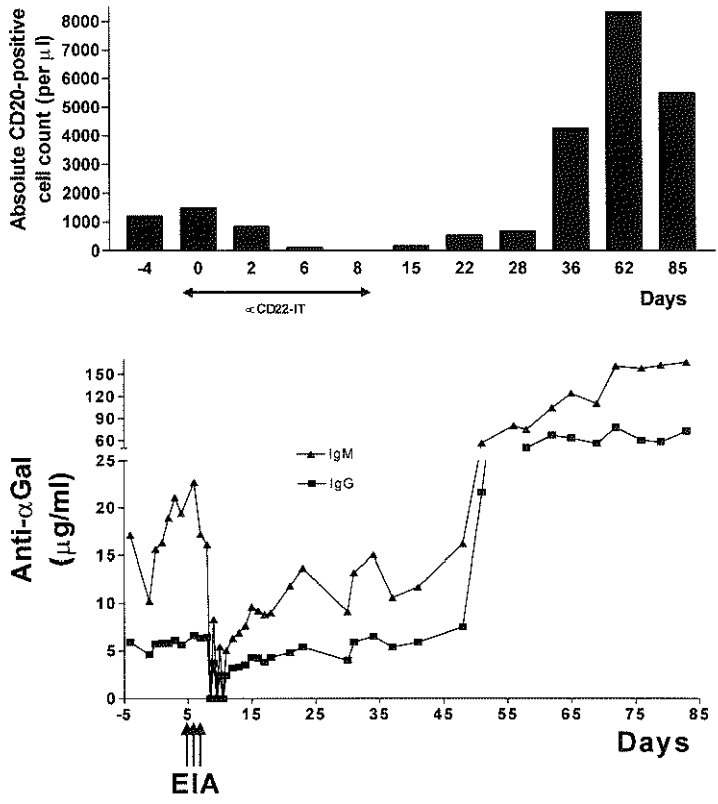

Figure 1.

The effect of ∝CD22-IT in vivo on B cell depletion and anti-αGal Ab production in a baboon.

Top. Bar chart demonstrating the depletion of CD20-positive B cells in blood (as absolute cell count) before, during, and after treatment with four iv doses of 0.19 mg/kg every other day of ∝CD22-IT commencing on day 0. Complete depletion of B cells was seen immediately following treatment at day

Bottom. Anti-αGal Ab profile of this baboon. EIAs were performed at time of maximum B cell depletion (days 8, 9, and 10). Note the complete depletion of anti-αGal IgG and IgM Ab following each consecutive EIA. The maximum depletion was 40% and 45% 7 days following EIA for IgG and IgM, respectively. Rebound of anti-αGal IgG and IgM Ab to levels 12- and 9-fold higher than baseline, respectively, was observed by day 83.

A rebound of B cells to numbers higher than baseline was also observed in LN in one baboon by day 22 (2-fold), while in a second baboon return to baseline levels was seen after 18 days (data not shown). Immunohistochemistry of LN correlated well with the flow cytometry data (data not shown). In one baboon, the initial anti-αGal Ab profiles were encouraging with depletion of IgG and IgM 48 hours post EIA sustained at 70 and 100%, respectively. However, by day 7, the depletion of IgG and IgM was 17 and 34%, respectively (data not shown). In the second baboon, the maximum depletion of anti-αGal Ab was 40 – 45% at 7 days, with rebound above baseline levels by day 55 (Fig 1 - bottom). Both baboons developed anti-murine antibodies and one baboon developed anti-ricin A chain antibodies, as determined by ELISA (data not shown). No changes in platelet or white cell count were observed.

These data indicate that although the ∝CD22-IT was effective in the short-term depletion of B cells from blood, BM, and LN, this was not associated with a significant decrease in the rate of return of anti-αGal Ab. Furthermore, treatment with this IT led to the development of anti-murine and anti-ricin antibodies.

Effect of the ∝CD38 mAb on B cell depletion and anti-αGal Ab production

Cell coating with the ∝CD38 mAb was 100% throughout the treatment in the one baboon studied. However, no substantial changes occurred in anti-αGal Ab levels following EIA (data not shown). Although blood levels of anti-CD38 mAb approximated 1000 ng/ml, no sensitization to the murine component of the monoclonal antibody occurred. Conjugation to the ricin A toxin would, therefore, seem necessary to successfully deplete B cells and plasma cells in vivo.

Effect of the ∝CD38-IT on B cell depletion and anti-αGal Ab production

∝CD38-IT was first tried in a baboon which, 18 months before, had received myeloablative WBI and had undergone treatment with melphalan for several weeks. However, by the time of treatment with the ∝CD38-IT, anti-αGal Ab levels, as well as T and B cell counts, had returned to baseline levels (i.e., pre WBI). Following

depletion by EIA, anti-αGal IgG and IgM Ab remained at undetectable levels for 1 week. After an additional EIA on day 26, anti-αGal IgM Ab was undetectable for 10 days while the IgG returned to baseline values by day 4 (Fig. 2). In a second, previously untreated baboon, anti-αGal IgG Ab increased to levels greater than baseline within 48 hours after the last of 3 consecutive EIAs (data not shown). Coating of CD38-positive cells with ∝CD38-IT was 100% in both cases. Sensitization occurred in both baboons, with the development of anti-murine and anti-ricin antibodies. Treatment with the ∝CD38-IT in a third baboon did not result in coating of CD38-positive cells and subtherapeutic plasma levels of ∝CD38 were measured (data not shown). The reasons for failure in this animal were unclear.

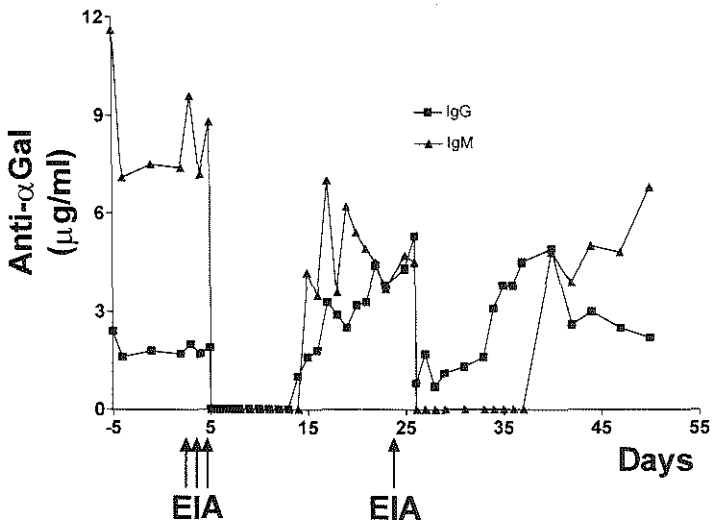

Figure 2.

Anti-αGal Ab profile in baboon receiving ∝CD38-IT. During iv treatment with ∝CD38-IT (0.1 mg/kg/day for 14 days commencing on day 0), 3 consecutive EIA were performed, effectively removing all anti-αGal IgG and IgM Ab from circulation. Levels of anti-αGal Ab remained undetectable for 7 days after which recovery of both IgG and IgM occurred, with rebound above baseline of IgG. After a fourth EIA was performed on day 26, IgM was again undetectable for 10 days, whereas IgG returned to previous levels after 6 days.

These results indicate that in one experiment ∝CD38-IT may have been effective in depleting antibody-producing cells, as the levels of anti-αGal Ab remained at low levels following EIA. The reason for our failure to duplicate these results in subsequent baboons are unclear. It is possible that the development of anti-murine and/or anti-ricin A chain antibodies played a role. Alternatively, it is possible that the positive effect observed with ∝CD38-IT in one baboon may have been associated with previous treatment (which includes melphalan), even though the drug has been discontinued > 2 months previously.

Effect of ∝CD20 mAb and/or WBI on B cell depletion and anti-αGal Ab production

Following the first dose of ∝CD20 mAb, B cells were rapidly depleted from the blood in all baboons, and remained at low levels through day 112 (Fig. 3 - top). Depletion of B cells in BM increased following each dose of ∝CD20 mAb, with a maximum depletion of 92% at day 40 in one baboon, after which recovery was observed (data not shown). The effect of ∝CD20 mAb on B cell depletion in LN was less impressive, with a maximum depletion of 70% B cells/gram LN at day 35, and recovery to baseline values by day 57. In one baboon, because some return of B cells in BM was observed at day 35, 150 cGy WBI was administered at day 42. Complete depletion of B cells in BM was achieved until day 105, with 40% recovery at day 287. The depletion in LN was complete until day 70, with recovery by day 105 (data not shown). Although EIA resulted in complete depletion of anti-αGal IgG and IgM Ab, recovery of anti-αGal Ab could be observed within 72 hours following the last EIA (Fig. 3 - bottom). Three days following EIA, the reduction in anti-αGal IgG Ab levels was 80%, with recovery to 75% of pre-treatment levels within 2 days thereafter. The reduction in anti-αGal IgM Ab levels was less marked (60% 3 days following EIA), with recovery to pre-treatment levels within 1 day, and increase above baseline levels (1-2 fold) within 5 days.

In vitro experiments

Morphology of B and plasma cells in culture

The anti-human ∝CD20 mAb, ∝CD22 mAb, surface immunoglobulin, and ∝CD38 mAb exhibited good cross-reactivity in binding to baboon spleen B cells when compared with binding to human spleen B cells (data not shown). During plasma cell differentiation, CD20, CD22, and surface immunoglobulin were down-modulated whereas CD38 expression remained high (Fig. 5A). Morphologically, plasma cells could readily be distinguished from B cells by size and cell-cell interactions (Fig. 5B). Furthermore, plasma cells secreted immunoglobulin, as was confirmed by intracellular immunoglobulin staining (data not shown).

These results indicate that the anti-human ITs and mAb used in these studies cross-react with baboon cells, and that efficient CD38 targeting may be effective in depleting both B and plasma cells.

Inhibition of protein synthesis

The ability of ∝CD22-IT and ∝CD38-IT, along with the negative control ricin A-conjugated anti-CD25 mAb (∝CD25-IT), to inhibit protein synthesis using the human Burkitt's lymphoma, Daudi, cell line was evaluated. Flow cytometric analysis of Daudi cells demonstrated that they are positive for both CD22 (RFB4, 66%) and CD38 (OKT10, 74%), and that CD38 stains brighter than CD22 (data not shown). Using the ^{3}H-Leucine incorporation assay, 100% inhibition of protein synthesis was achieved with ∝CD22-IT at 10^{-9} – 10^{-8} M, whereas ∝CD38-IT led to a maximum of 60% inhibition of protein synthesis at 10^{-8} M (Fig. 4). Furthermore, the slope of the curve for ∝CD38-IT is much less steep than that for ∝CD22-IT. This suggests that ∝CD38-IT has either a slow rate of internalization and intracellular routing, or slow kinetics, leading to less effective inhibition of protein synthesis.

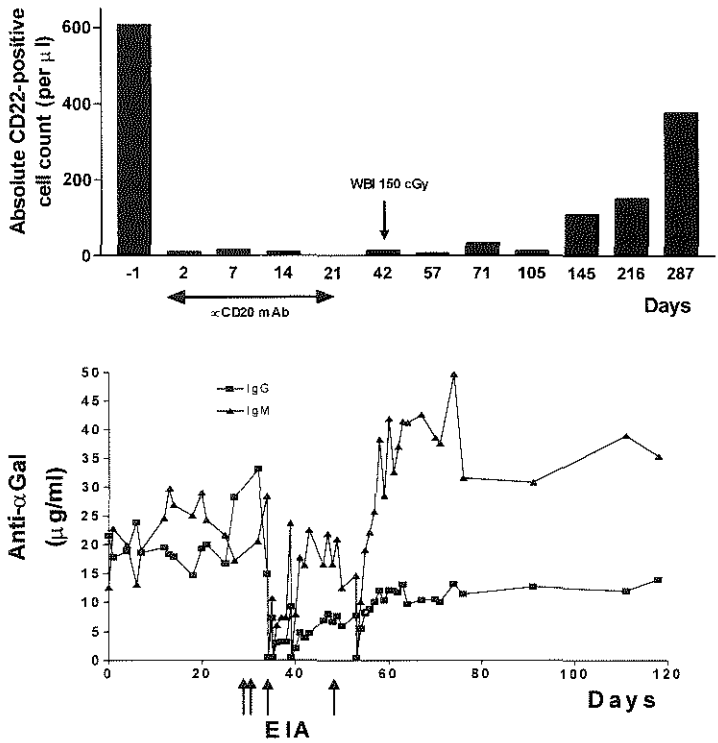

Figure 3.

The effect of ∝CD20 mAb in vivo on B cell depletion and anti-αGal Ab production in a baboon.

Top. Bar chart demonstrating the depletion of CD22-positive B cells in blood (as absolute cell count) before, during, and after treatment with four iv doses of 20 mg/kg of ∝CD20 mAb, at weekly intervals commencing on day 0. Almost total depletion of B cells was seen immediately after initiating the course of ∝CD20 mAb therapy. As some return of B cells was seen in the BM by day 35, WBI (150 cGy) was administered – this led to almost complete depletion of B cells > 3 months.

Bottom. Anti-αGal Ab profile in this baboon. EIAs were performed at time of maximum B cell depletion. Note the complete depletion of anti-αGal Ab following each consecutive EIA. Return of anti-αGal Ab was not significantly inhibited following treatment with ∝CD20 mAb, with anti-αGal IgG Ab returning to baseline values, and anti-αGal IgM Ab increasing to levels above baseline. The maximum reduction in anti-αGal IgG Ab was 80% 72 hours following EIA, with recovery to 75% of pre-treatment levels within 2 days. The maximum reduction in anti-αGal IgM Ab was less marked, reaching 60% 72 hours following EIA, with recovery to pre-treatment levels within 1 day, and increase above baseline levels (1-2 fold) within 5 days.

Activated B cells and/or early plasma cells had high levels of protein synthesis (in contrast to resting B cells). Protein synthesis was efficiently inhibited by the ∝CD22-IT by approximately 60% at 10^{-10} M in suspensions containing 80% CD22-positive cells (Fig. 5). This inhibition was less marked than that observed with the ∝CD22-IT on human Daudi cells, presumably due to the presence of a CD22-negative population in the baboon cell preparation. Plasma cells down-modulated surface CD22 expression during differentiation. Consequently, at day 8 (22% CD22-positive cells) and day 15 (6% CD22-positive cells) of in vitro differentiation, protein synthesis was not inhibited by the ∝CD22-IT.

These data suggest that ∝CD22-IT is more effective than ∝CD38-IT in inhibiting protein (e.g., antibody) synthesis in vitro, but, as predicted, that it is not effective in inhibiting protein synthesis of differentiated B cells (e.g., plasma cells).

Anti-αGal Ab production by baboon spleen cells after depleting CD20-, CD22-, or CD38-positive cells

Baboon spleen cells, depleted of T cells, monocytes, and granulocytes, were used in this assay. Removal of CD20-positive B cells from baboon spleen cells with magnetic beads resulted in an increased frequency of anti-αGal IgM Ab and total immunoglobulin secretors (Fig. 6A). This effect was also seen when CD22-positive cells were depleted (data not shown). Depletion of CD38 cells by magnetic beads led to a significant decreased frequency of anti-αGal Ab and total immunoglobulin secretors (Fig. 6B). Flow cytometry confirmed the efficient removal of CD20-, CD22-, or CD38-positive cells (data not shown).

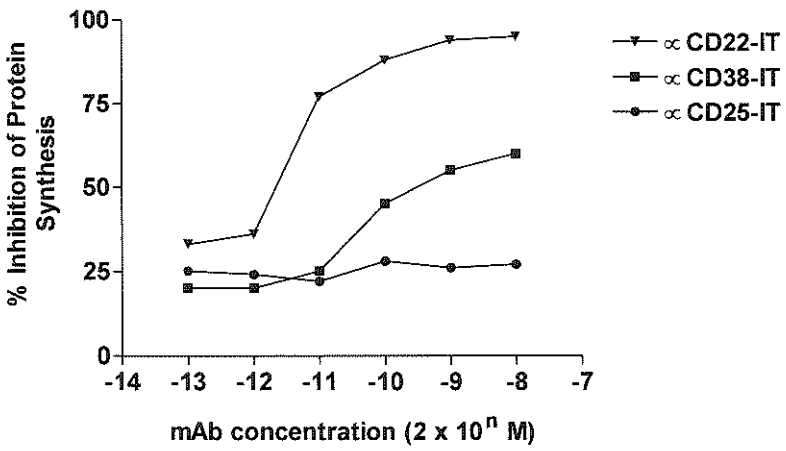

Figure 4.

Protein synthesis by human Daudi cells in the presence of ∝CD22-IT, ∝CD38-IT, and the negative control ∝CD25-IT. Data are presented as percentage protein synthesis (i.e., (^{3}H-Leu incorporation by cells treated with IT / ^{3}H-Leu incorporation by cells treated with unconjugated mAb) x 100%).

The negative control ∝CD25-IT demonstrates a maximum and stable 75% protein synthesis at all concentrations of antibody. Treatment with ∝CD22-IT leads to an efficient decrease in protein synthesis, approaching 0% at concentrations between 10^{-10}-10^{-8}M, whereas treatment with ∝CD38-IT is not as efficient. The minimum protein synthesis is 40% at a concentration of 10^{-8}M.

These data indicate that enrichment for plasma cells (by depleting CD20- or CD22-positive cells) leads to a relative increase in the frequency of anti-αGal Ab and total immunoglobulin producing cells, and that depletion of CD38-positive cells (i.e., B cell blasts and plasma cells) removes anti-αGal Ab and total immunoglobulin secretors. Plasma cells and B cell blasts therefore appear to be responsible for the majority of anti-αGal Ab production in baboon spleens.

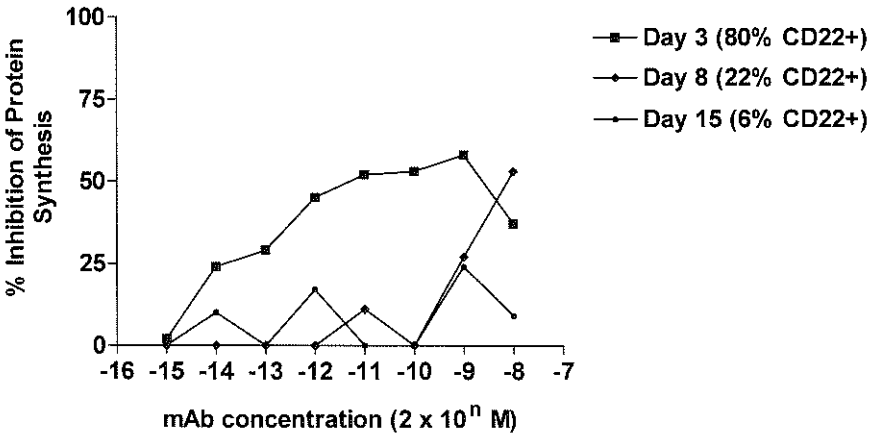

Figure 5.

Protein synthesis by baboon spleen cells in the presence of ∝CD22, ∝CD22-IT, on days 3, 8, and 15 after in vitro activation and differentiation as measured by ^{3}H-Leu incorporation. Data are presented as percentage protein synthesis (i.e., (^{3}H-Leu incorporation by cells treated with ∝CD22-IT / ^{3}H-Leu incorporation by cells treated with ∝CD22) x 100%). The minimum percentage protein synthesis was achieved at day 3 after in vitro differentiation, correlating with 80% CD22-positive cells. At days 8 and 15 after in vitro differentiation, protein synthesis was not significantly changed. At these timepoints, only 22% and 6% CD22-positive cells, respectively, were measured.

DISCUSSION

It is well recognized that anti-αGal Ab are the major barriers to clinical implementation of xenotransplantation. Anti-αGal Ab are believed to be responsible for both hyperacute and acute vascular rejection of vascularized, discordant xenografts (9,14). Although it is possible to remove anti-αGal Ab from the circulation of nonhuman primates, either by perfusing the recipient's blood through porcine organs or through an extracorporeal immunoaffinity column specific for anti-αGal Ab, sustained depletion of anti-αGal Ab has not been achieved due to ongoing antibody production (6). Treatment modalities aimed at reducing the production of anti-αGal Ab have to date been unsuccessful. Various pharmacologic agents, including brequinar sodium, methotrexate, melphalan, cladribine, zidovudine, cyclophosphamide, and mycophenolate mofetil, have been evaluated in our laboratory

without clinically significant reduction of antibody production (6,21). In our pig-to-primate model aimed at inducing immunological tolerance, we have recently demonstrated that the anti-CD154 mAb effectively prevents the induced (T cell-dependent) antibody response to porcine antigens, but the return to baseline levels of (T cell-independent) anti-αGal Ab, in particular anti-αGal IgM Ab, is not prevented (6,20). Following depletion, the return of anti-αGal Ab in vascularized organ transplant models is invariably associated with destruction of the transplanted graft (18,19,41).

Our center has been interested in studying the cellular origin of anti-αGal Ab. We have examined numerous tissues obtained from naïve baboons and baboons previously exposed to porcine tissue to determine whether anti-αGal Ab-producing cells are specific for, or restricted to, certain anatomical regions of the body. ELISPOT analyses of these tissues were performed and we observed the highest frequencies of anti-αGal Ab (predominantly IgM) secretors in naïve baboons in the spleen, tonsils, BM, and LN. Anti-αGal IgG was primarily found in the LN, whereas anti-αGal IgA was mostly seen in the small intestine in Peyer's patches. In splenectomized baboons sensitized to porcine antigens, however, these secretors (especially IgM and IgG) were primarily observed in the BM (Xu Y et al., unpublished data). We believe that, although treatment aimed specifically at cells producing anti-αGal Ab is highly desirable, the technology to do this is not yet available. Attention was, therefore, directed at less specific therapies targeting all antibody-producing cells.

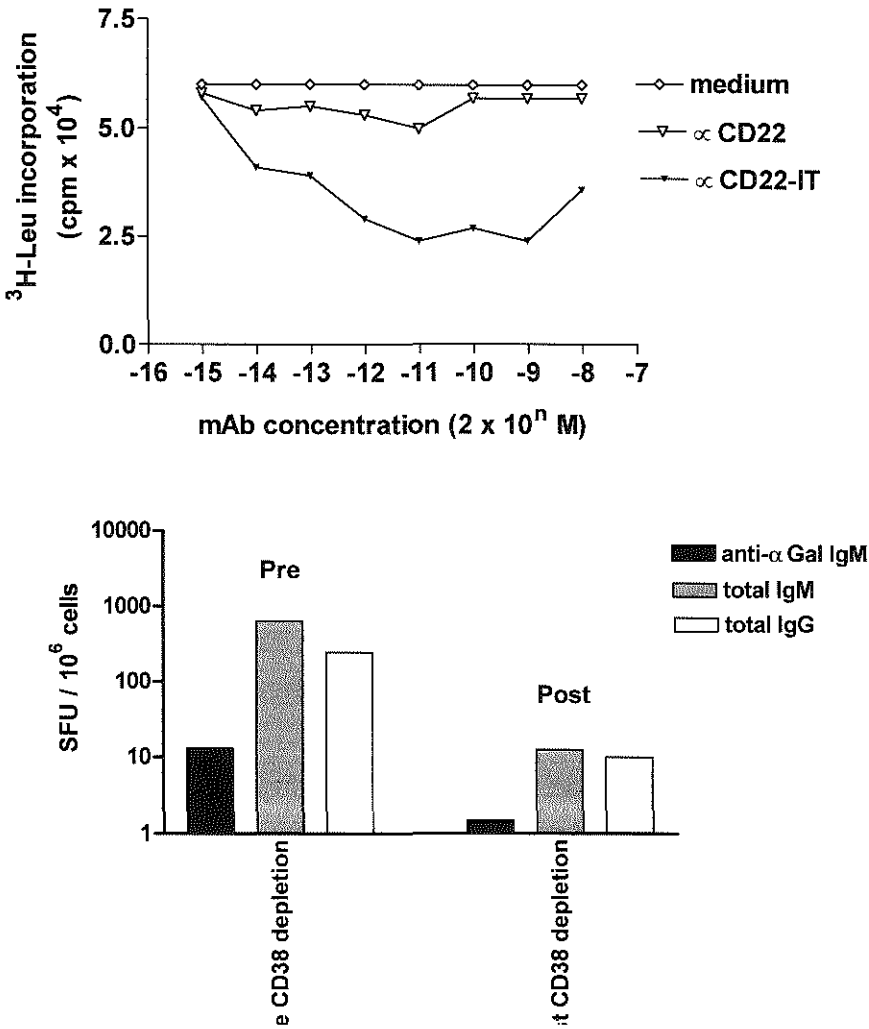

Figure 6

ELISPOT assay demonstrating the number of spot forming units (SFU) / 10^6 cells of anti-αGal IgM Ab, and total IgG and IgM in baboon spleens after depletion of CD20- or CD38-positive cells by magnetic beads.

A. Depletion of CD20-positive cells resulted in an approximate 30-fold increase of anti-αGal IgM Ab production over baseline, and a 5-9 fold increase of total IgG and IgM. This increase indicated that the non-depleted (CD20-negative) cells were responsible for the majority of antibody production.

B. Depletion of CD38-positive cells led to an 88% decrease of anti-αGal IgM Ab, and a 96-98% decrease of total IgG and IgM production, respectively. These data suggest that CD38-positive cells are mainly responsible for antibody production.

Recently, significant progress has been made in the treatment of several hematologic malignancies. Several in vitro and in vivo pre-clinical and clinical studies have shown promising data using specific anti-B and/or plasma cell mAbs to reduce the tumor load of B cell malignancies (22,23,24,25,42). We, therefore, assessed the capacity of several of these mAbs and ITs to deplete normal (non-malignant) B and/or plasma cells and to reduce production of anti-αGal Ab in baboons.

Both treatment with the ∝CD22-IT in vivo and WBI led to a complete, but transient, depletion of CD22- and CD20-positive cells in blood and BM. Furthermore, there was no substantially reduced anti-αGal Ab production following EIA. In fact, after the ∝CD22-IT treatment, as ∝murine antibodies developed, the levels of anti-αGal Ab increased significantly above baseline. It is not clear if this was due to sensitization to murine-αGal, or a rebound phenomenon after depletion of B cells. The latter seems more likely as the amount of total immunoglobulin also increased.

Sustained B cell depletion was achieved when ∝CD20 mAb was combined with 150 cGy WBI. B cells were virtually undetectable in the circulation for > 3 months, and were almost completely depleted from BM and LN for at least 70 days. Recovery in the blood and BM had not yet reached 25 and 40 % baseline, respectively, at day 216. Despite this medium-term, near-complete depletion of B cells, reduction of anti-αGal Ab levels was not sustained. The maximum depletion of anti-αGal IgG and IgM Ab following 72 hours following EIA was 80% and 60%, respectively, and was sustained for only 2 days, after which there was a return to baseline levels of IgM. Levels of IgG, however, remained at 66% of those pretreatment. Since depletion of B cells with the ∝CD22-IT was not as effective as that obtained with the ∝CD20 mAb, we did not combine the ∝CD22-IT with WBI.

These findings suggest that CD20- or CD22-positive B cells are not the major source of anti-αGal Ab production, and that other cells, presumably plasma cells or B cell blasts, are mainly responsible for antibody production. These observations are also in keeping with data presented by other groups, suggesting that antibody-producing cells

are long-lived (43,44) and radiation-resistant (45). Unfortunately, in vivo treatment of baboons with ∝CD38-IT, reportedly targeting plasma cells, did not yield reproducible results. Only in one of 3 baboons was treatment with ∝CD38-IT followed by reduced production of anti-αGal Ab. It is unclear, however, whether this reduction in production of anti-αGal Ab was due solely to the treatment with ∝CD38-IT, or to some extent due to a late effect of extensive prior immunosuppressive treatment. In two other cases, when naïve baboons were used, no effect on antibody production was observed. One potential explanation is that the ∝CD38 mAb interaction with the CD38 molecule expressed on the cell surface did not efficiently mediate internalization of the mAb – receptor complex, which is required for effective killing by dgRTA. Furthermore, as CD38 is also expressed on cells other than B or plasma cells (e.g., activated T cells, natural killer cells, and BM cells), this could interfere with the specific binding of ∝CD38-IT to B cells and plasma cells, reducing its efficacy. However, this explanation is not likely, since all cells were coated with ∝CD38. In these experiments, higher doses of ∝CD38-IT may have been more effective by providing greater cross-linking of receptors which could lead to increased internalization of the IT.

The results of these in vivo studies prompted us to carry out further in vitro investigations. We first demonstrated that expression of CD20, CD22, and CD38 by baboon B cells is highly dependent on the state of differentiation / maturation of the cells. Most B cells express immunoglobulin, CD20, CD22, and CD38 on their surface. Plasma cells, however, have down-modulated surface immunoglobulin, CD20, and CD22 expression, although expression of CD38 is maintained on baboon plasma cells. This suggests that mAbs or ITs directed against CD20 or CD22 will potentially deplete B cells but, if both B- and plasma cells are to be depleted, a mAb or IT specific for a plasma cell marker, such as CD38, would be necessary.

We confirmed this in our subsequent experiments. Removal of CD38-positive cells from baboon spleen B lineage cells by magnetic beads led to a greatly diminished frequency of anti-αGal IgM Ab and total immunoglobulin secretors. In contrast,

depletion of CD20- or CD22-positive cells from baboon spleen B lineage cells led to an effective enrichment of CD20- or CD22-negative cells and to an increased frequency of anti-αGal IgM Ab and total immunoglobulin secretors. When the inhibition of protein synthesis by human Daudi cells with ∝CD38-IT was compared to the inhibition obtained with ∝CD22-IT, however, we found that ∝CD38-IT was less effective than ∝CD22-IT. These data may correlate with our inconsistent in vivo experience with ∝CD38-IT. It appears that, although ∝CD38-IT targets the desired population of (plasma) cells, it is not particularly efficient at killing those cells, possibly due to low affinity interaction of ∝CD38 mAb or slower internalization of the CD38 receptor. Unfortunately, we have not been able to find other anti-human anti-CD38 mAb that cross-react with baboons. In contrast, ∝CD22-IT is very effective at killing CD22-positive cells but, unfortunately, these cells are not the major secretors of antibody, including anti-αGal Ab.

In none of the in vivo experiments described above, where administration of anti-B cell agents combined with EIA +/- WBI was the only therapy, were any infections noted. In one case (not included in this study), treatment with ∝CD20 mAb was incorporated in our protocol aimed at inducing immunological tolerance (20,41,46). This protocol consists of induction therapy with splenectomy, WBI 300 cGy, ATG, and EIAs, and maintenance therapy with anti-CD154 mAb +/- CyA, mycophenolate mofetil, corticosteroids, and cobra venom factor. Although this is an intensive protocol that renders the baboon severely immunosuppressed, viral infections have rarely been observed. However, when pretreatment with ∝CD20 mAb was added, a viral hepatitis (confirmed by inclusion bodies on histology) resulted in death of the baboon within 20 days. It is conceivable that pretreatment with ∝CD20 mAb reduced the ability of the baboon to mount a humoral response to the viral antigen load. Close monitoring would, therefore, be necessary if ∝CD20 mAb therapy were to be combined with other intensive immunosuppressive therapies.

More recently, several groups have demonstrated that combining anti-B ITs, e.g., anti-CD19 and anti-CD22 (47), or combining anti-B cell and anti-plasma cell ITs, e.g.,

anti-CD19, anti-CD22, and anti-CD38 (48), can cure B cell neoplasias in a mouse tumor xenograft model, while the individual ITs are unable to do so alone. This treatment could overcome the heterogeneity of surface antigen expression on B cells and plasma cells and prove valuable in our goals to deplete antibody-producing cells.

An interesting observation was also recently made by Treon et al (49). They found that treatment with interferon-gamma caused significant upregulation of CD20-expression on plasma cells of patients with multiple myeloma. This approach (i.e., pretreatment with interferon-gamma) could, therefore, render some plasma cells more susceptible to treatment with ∝CD20 mAb. In vitro studies at our center, however, have so far failed to demonstrate significant upregulation of CD20 on baboon plasma cells by interferon-gamma therapy (Thall A, unpublished data).

In conclusion, the above data demonstrate that baboon B cells can be efficiently depleted with WBI, ∝CD22-IT, or ∝CD20 mAb, and that medium-term (>3 months) depletion of B cells can be achieved with ∝CD20 mAb combined with WBI. However, depletion of B cells does not lead to a clinically significant reduction in the rate or extent of return of anti-αGal Ab following EIA. These observations suggest that B cells are not the major source of anti-αGal Ab production, and that attention must be directed towards suppressing plasma cell and B cell blast function. We have demonstrated in vitro that CD38-positive cells are the major secretors of anti-αGal Ab. Treatment with ∝CD38-IT, however, did not yield consistent results in vivo, possibly due to pre-existing anti-ricin mAbs, low-affinity or slow internalization of the antibody, sub-optimal conjugation of ricin-A, and/or relatively low specificity of CD38. Nevertheless, further studies focussed on this IT or on the development of new and refined anti-plasma cell agents are clearly warranted and are currently in progress at our center. Furthermore, combinations of ∝CD20 mAb or ∝CD22-IT with an optimized anti-plasma cell IT may be of benefit.

REFERENCES

1 Cooper DKC. Xenografting: how great is the clinical need? Xeno 1993 ;1: 25

2 Cooper DKC, Ye Y, Rolf LL Jr, et al. The pig as a potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, et al. (eds.). Xenotranplantation. Heidelberg, Springer 1991. p481

3 Sachs DH. The pig as a potential xenograft donor. Vet Imm Immunopath 1994 ;43: 185

4 Platt JL, Fischel RJ, Matas AJ, et al. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation 1991; 52: 514

5 Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nat Med 1995; 1: 869

6 Alwayn IPJ, Basker M, Buhler L, Cooper DKC. The problem of anti-pig antibodies in pig-to-primate xenografting: current and novel methods of depletion and/or suppression of production of anti-pig antibodies. Xenotransplantation 1999; 6: 157

7 Schmoeckel M, Bhatti FN, Zaidi A, et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. Transplantation 1998; 65: 1570

8 Zaidi A, Schmoeckel M, Bhatti F, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584

9 Good AH, Cooper DKC, Malcolm AJ, et al. Identification of carbohydrate structures that bind human anti-porcine antibodies: implications for discordant xenografting in humans. Transplant Proc 1992; 24: 559

10 Oriol R, Ye Y, Koren E, et al. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433

11 Galili U. Interaction of the natural anti-Gal antibody with αgalactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol Today 1993; 14: 480

12 Galili U, Clark MR, Shohet SB, et al. Evolutionary relationship between the natural anti-Gal antibody and the Galα1→3Gal epitope in primates. Proc. Natl. Acad. Sci. USA 1987; 84: 1369

13 Rose AG, Cooper DK. A histopathologic grading system of hyperacute (humoral, antibody-mediated) cardiac xenograft and allograft rejection. J Heart Lung Transplant 1996; 15: 804

14 Galili U. Anti-αgalactosyl (anti-Gal) antibody damage beyond hyperacute rejection. In: Cooper DKC, Kemp E, Platt JL, et al. (eds). Xenotransplantation, 2nd edition. Heidelberg, Springer 1997. p 95

15 Taniguchi S, Neethling FA, Korchagina EY, et al. *In vivo* immunoadsorption of antipig antibodies in baboons using a specific Galα1-3Gal column. Transplantation 1996; 10: 1379

16 Kozlowski T, Ierino FL, Lambrigts D, et al. Depletion of anti-αGal1-3Gal antibody in baboons by specific immunoaffinity columns. Xenotransplantation 1998; 5: 122

17 Sablinski T, Latinne D, Gianello P, et al. Xenotransplantation of pig kidneys to nonhuman primates: I. Development of the model. Xenotransplantation. 1995; 2: 264

18 Cooper DKC, Cairns TDH, Taube DH. Extracorporeal immunoadsorption of anti-pig antibody in pigs using αGal oligosaccharide immunoaffinity columns. Xeno 1996; 4: 27

19 Xu Y, Lorf T, Sablinski T, et al. Removal of anti-porcine natural antibodies from human and nonhuman primate plasma *in vitro* and *in vivo* by a Galα1-3Galβ1-4Glc-X immunoaffinity column. Transplantation 1998; 65: 172

20 Buhler L, Awwad M, Basker M, et al. High-dose porcine hematopoietic cell transplantation combined with CD40L blockade in baboons prevents an induced anti-pig humoral response. Transplantation 2000. In press

21 Lambrigts D, Van Calster P, Xu Y, et al. Pharmacologic immunosuppressive therapy and extracorporeal immunoadsorption in the suppression of anti-αGal antibody in the baboon. Xenotransplantation 1998; 5: 274

22 Vitetta ES, Fulton RJ, May RD, et al. Redesigning nature's poisons to create anti-tumor reagents. Science 1987; 238: 1098

23 Ghetie MA, Tucker K, Richardson J, et al. The antitimor activity of an anti-CD22 immunotoxin in SCID mice with disseminated Daudi lymphoma is enhanced by either an anti-CD19 antibody or an anti-CD19 immunotoxin. Blood 1992; 80: 2315

24 Grossbard ML, Press OW, Appelbaum FR, et al. Monoclonal antibody-based therapies of leukemia and lymphoma. Blood 1992; 80: 863

25 Pastan I and Fitzgerald D. Recombinant toxins for cancer treatment. Science 1991; 254: 1173

26 Dewey WC, Ling CC, Meyn RE. Radiation-induced apoptosis: Relevance to radiotherapy. Int J Rad Oncol Biol Phys 1995; 33: 781
27 Thorpe PE, Wallace PM, Knowles PP, et al. Improved antitumor effects of immunotoxins prepared with deglycosylated ricin A-chain and hindered disulfide linkages. Cancer Res 1988; 48: 6396
28 Sausville EA, Headlee D, Stetler-Stevenson M, et al. Continuous infusion of the anti-CD22 immunotoxin IgG-RFB4-SMPT-dgA in patients with B-cell lymphoma: a phase I study. Blood 1995; 85: 3457
29 Campana D, Janossy G, Bofill M, et al. Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 1985; 134: 1524
30 Malavasi F, Funaro A, Roggero S, et al. Human CD38: a glycoprotein in search of a function. Immunol Today 1994; 15: 95
31 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680
32 Stashenko P, Nadler LM, Hardy R, Schlossman SF. Characterization of a human B lymphocyte-specific antigen. J Immunol 1980; 125: 1678
33 Zhong RK. Human blood dendritic cell-like B cells isolated by the 5G9 monoclonal antibody reactive with a novel 220-kDa antigen. J Immunol 1999; 163: 1354
34 Scheringa M, Schraa EO, Bouwman E, et al. Prolongation of survival of guinea pig heart grafts in cobra venom factor-treated rats by splenectomy. No additional effect of cyclosporine. Transplantation 1995; 60: 1350
35 Schmoeckel M, Bhatti FN, Zaidi A, et al. Splenectomy improves survival of hDAF transgenic pig kidneys in primates. Transplant Proc 1999; 31: 961
36 Watts A, Foley A, Awwad M, et al. Plasma perfusion by apheresis through a Gal immunoaffinity column successfully depletes anti-Gal antibody – experience with 320 aphereses in baboons. Xenotransplantation 2000; 7: 181.
37 Vitetta ES, Stone M, Amlot P, et al. Phase I immunotoxin trial in patients with B-cell lymphoma. Cancer Res 1991; 51: 4052
38 Zompi S, Tulliez M, Conti F, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with clonal lymphoproliferative disorders after orthotopic liver transplantation: a report of three cases. J Hepatol 2000 Mar; 32: 521
39 Giovino MA, Hawley RJ, Dickerson MW, et al. Xenogeneic bone marrow transplantation: II. Porcine-specific growth factors enhance porcine bone marrow engraftment in an in vitro primate microenvironment. Xenotransplantation 1997; 4: 112
40 Agematsu K, Nagumo H, Oguchi Y, et al. Generation of plasma cells from peripheral blood memory B cells: synergistic effects of interleukin-10 and CD27/CD70 interaction. Blood 1998; 91: 173
41 Kozlowski T, Shimizu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody adsorption. Transplantation 1999; 67: 18
42 Senderowicz AM, Vitetta E, Headlee D. Complete sustained response of a refractory, post-transplantation, large B-cell lymphoma to an anti-CD22 immunotoxin. Ann Intern Med 1997; 126: 883
43 Slifka MK, Antia R, Whitmire JK, et al. Humoral immunity due to long-lived plasma cells. Immunity 1998; 8: 363
44 Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. Nature 1997; 388: 133
45 Miller JJ, Cole LJ. The radiation resistance of long-lived lymphocytes and plasma cells in mouse and rat lymph nodes. J Immunol. 1967; 98: 982
46 Kozlowski T, Monroy R, Xu Y, Glaser R, Awwad M, Cooper DK, Sachs DH. Anti-Gal(alpha)1-3Gal antibody response to porcine bone marrow in unmodified baboons and baboons conditioned for tolerance induction. Transplantation 1998; 66: 176
47 Ghetie MA, Tucker K, Richardson J, et al. Eradication of minimal disease in severe combined immunodeficient mice with disseminated Daudi lymphoma using chemotherapy and an immunotoxin cocktail. Blood 1994; 84: 702
48 Flavell DJ, Noss A, Pulford KAF, et al. Systemic therapy with 3BIT, a triple combination cocktail of anti-CD19, -CD22, and –CD38-saporin immunotoxins, is curative of human B-cell lymphoma in severe combined immunodeficient mice. Cancer Research 1997; 57: 4824

49 Treon SP, Shima Y, Preffer FI, et al. Treatment of plasma cell dyscrasias by antibody-mediated immunotherapy. Semin Oncol 1999; 26: 97

IV

Understanding and preventing the thrombotic complications associated with pig-to-baboon xenotransplantation

5

Coagulation and thrombotic disorders associated with xenotransplantation

Adapted from:
Alwayn IPJ, Buhler L, Basker M, Goepfert C, Kawai T, Kozlowski T, Ierino F, Sachs DH, Sackstein R, Robson SC, and Cooper DKC. Coagulation / Thrombotic disorders associated with organ and cell xenotransplantation. *Transpl. Proc. 2000;32(5):1099.*
&
Buhler L*, Basker M*, Alwayn IPJ, Goepfert C, Kitamura H, Kawai T, Gojo S, Kozlowski T, Ierino FL, Awwad M, Sachs DH, Sackstein R, Robson SC, and Cooper DKC. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. *Transplantation 2000;70(9):1323-1331.*
* Authors contributed equally

ABSTRACT

Background. Efforts to achieve tolerance to transplanted pig organs in nonhuman primates by the induction of a state of mixed hematopoietic chimerism have been associated with disorders of

coagulation and thrombosis. Activation of recipient vascular endothelium and platelets by porcine hematopoietic cells and/or activation of donor organ vascular endothelium. Molecular differences between species with respect to regulation of coagulation and hemostasis may play a role. Irradiation or drug therapy could possibly potentiate endothelial cell activation and/or injury.

Methods. We have investigated parameters of coagulation and platelet activation in nonhuman primates following (i) a regimen aimed at inducing mixed hematopoietic chimerism and tolerance (NMCR that included total body irradiation, T cell depletion and splenectomy, (ii) pig bone marrow or pig peripheral blood mobilized progenitor cell transplantation (PCTx, and/or (iii) pig organ transplantation (POTx). Five experimental groups were studied. Baboons were the recipient subjects in all groups except Group 1. Group 1 Cynomolgus monkeys (n=6) underwent NMCR + allotransplantation of hematopoietic cells and a kidney or heart or NMCR + concordant xenotransplantation (using baboons as donors) of cells and a kidney; Group 2 Baboons (n=4) underwent NMCR with or without (+/-) autologous hematopoietic cell infusion; Group 3 (n=12) PCTx +/- NMCR; Group 4 (n=5) POTx +/- NMCR; Group 5 (n=4) NMCR + PCTx + POTx. Platelet counts, with plasma prothrombin time, partial thromboplastin time, fibrinogen levels, fibrin split products and/or D-dimer were measured.

Results. In the absence of a discordant (porcine) cellular or organ transplant (Groups 1 and 2), NMCR resulted in transient thrombocytopenia only, in keeping with bone marrow depression from irradiation. PCTx alone (Group 3) was associated with the rapid development of a thrombotic thrombocytopenic (TTP)-like microangiopathic state, that persisted longer when PCTx was combined with NMCR. POTx (+/- NMCR) (Group 4) was associated with a gradual fall (over several days) in platelet counts and fibrinogen with disseminated intravascular coagulation (DIC); after graft excision, the DIC generally resolved. When NMCR, PCTx and POTx were combined (Group 5), an

initial TTP-like state was superceded by a consumptive picture of DIC within the first week, necessitating graft removal.

Conclusions. Both PCTx and POTx lead to profound alterations in hemostasis and coagulation parameters that must be overcome if discordant xenotransplantation of hematopoietic cells and organs is to be fully successful. Disordered thromboregulation could exacerbate vascular damage and potentiate activation of coagulation pathways following exposure to xenogeneic cells or a vascularized xenograft.

INTRODUCTION

The current shortage of organs for transplantation could be overcome if pig organs could be transplanted successfully. However, a number of barriers have to be overcome. Hyperacute rejection can be prevented by various procedures, such as depletion of anti-pig antibody (1-3) or complement (4-6) in the recipient primate or by the use of organs from pigs transgenic for one or more human complement regulatory proteins (7,8). However, even under these circumstances, a delayed form of rejection occurs that is characterized by endothelial cell activation, platelet adhesion and aggregation, with thrombin and fibrin deposition (9,10). Severe coagulation disturbances have been documented in nonhuman primates following the transplantation of pig hematopoietic cells and/or organs (3,11-13, and Buhler, L., et al, manuscript submitted). Many factors could contribute to these disturbances, including the therapeutic interventions or procedures used to suppress the recipient immune system. Activation of host vascular endothelium, circulating leukocytes and platelets by infused porcine hematopoietic cells is an additional possibility. Alternatively, the vascular endothelium within the transplanted porcine organ may be directly perturbed as a result of the effects of xenoreactive antibodies (13).

Molecular incompatibilities may also be important factors in both situations (9,10,13-16). Relevant examples documented include the inability of porcine tissue factor pathway inhibitor to adequately neutralize human factor Xa, activation of both human prothrombin and factor X by porcine endothelial cells, the failure of porcine thrombomodulin to bind human thrombin and hence activate human protein C, and the

enhanced potential of porcine von Willebrand factor to associate with human platelet GPIb (9-19).

We have reviewed our own data in nonhuman primate auto-, allo-, and concordant xeno-transplantation models and in the miniature swine-to-nonhuman primate discordant xenotransplantation model in an effort to clarify the relative roles of a regimen aimed to induce mixed chimerism and tolerance (tolerance-inducing regimen; NMCR), porcine hematopoietic cell transplantation (PCTx), and porcine organ transplantation (POTx) in the development of coagulation/thrombotic disturbances. Our observations indicate that NMCR +/- autologous, allogeneic, or concordant xenogeneic cell or organ transplantation have minimal disturbances in coagulation. However, in the discordant combination using comparable NMCR, PCTx results in a thrombotic thrombocytopenic microangiopathic (TTP)-like state (20, and Bühler, L., et al, manuscript submitted), and the development of delayed rejection following POTx leads to disseminated intravascular coagulation (DIC) (3,11) which progresses unless the organ is excised.

MATERIALS AND METHODS

Animals

Cynomolgus monkeys (Macaca fascicularis) (n=6) of both sexes, weighing 4-6 kg, and baboons (Papio anubis) (n=24) of both sexes, weighing 10-15 kg (all from Biomedical Resources Foundation, Houston, TX) were the recipient animals used in these studies. Cynomolgus monkeys, baboons or Massachusetts General Hospital MHC-inbred miniature swine (Charles River Laboratories, Wilmington, MA), weighing 9-17 kg, were donors of hematopoietic cells and organs. Cynomolgus monkeys and baboons were of AB, B or A blood type. All pigs were of O blood type.

Care of animals was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Anesthesia and Surgical Procedures

Sedation, anesthesia, intravenous line placement, splenectomy, porcine kidney and heterotopic heart transplantation have been described previously in baboons (1,3,21) and cynomolgus monkeys (22), as have the harvesting and processing of donor bone marrow (BM) (22,23) and the mobilization and leukapheresis of porcine peripheral blood progenitor cells (PBPC) (24).

At the time of kidney transplantation, in general one native kidney was excised and the ureter of the remaining kidney was ligated. In the event of DIC or rejection developing within three weeks, necessitating excision of the transplanted kidney, the ligature around the native ureter was released allowing survival of the recipient.

Conditioning Regimen for Tolerance Induction (NMCR)

The NMCR (See Chapter 1, figure 3) consisted of (i) non-myeloablative whole body irradiation in either two or three fractions (150 or 100 cGy each, respectively) on days -6 and -5 (total dose 300 cGy); (ii) horse anti-human antithymocyte globulin (ATG) 50 mg/kg i.v. daily on days -3, -2 and -1; (iii) thymic irradiation (TI) of 700 cGy on day -1; and (iv) splenectomy on day -8 or 0 (3,20). In baboons undergoing PCTx and/or POTx, extracorporeal immunoadsorption (EIA) x 3 or 4 was carried out during the previous week (3,25), and cobra venom factor (approximately 100 units/kg/day) was administered (and titrated to CH50) throughout the post-transplant course (6,20).

After hematopoietic cell and/or organ transplantation, pharmacologic immunosuppressive therapy varied slightly between the different experimental groups. All recipients received cyclosporine (CyA; Novartis, Basel, Switzerland) 10-20 mg/kg/day i.m. from day 0 in cynomolgus monkeys or 15-20 mg/kg/day by continuous i.v. infusion from day -5 in baboons, except where stated to the contrary. A second agent was added whenever xenotransplantation was being undertaken. This was either 15-deoxyspergualin (Novartis) 6 mg/kg/day i.v. on days 0-13 or mycophenolate mofetil (MMF) 100 mg/kg/day i.v. from day -9. Baboons in Groups 3 and 5 also received methylprednisolone 4 mg/kg/day reducing to 0.5 mg/kg/day over the first four weeks. Two baboons receiving autologous BM transplantation received a myeloablative

regimen that has been described previously (26). An anti-CD40L mAb (20 mg/kg/i.v. on alternate days for 8 doses) was substituted for CYA in some baboons in Groups 3-5 (20). There was no direct correlation between the immunosuppressive regimen administered and the changes seen in coagulation parameters.

Extracorporeal Immunoadsorption (EIA)

When PCTx or POTx was to be undertaken, anti-Galα1-3Gal (αGal) antibody was depleted from the baboon's circulation by the perfusion of blood or plasma through immunoadsorption columns containing synthetic Galα1-3Galß1-4ßGlc-X-Y (αGal type VI trisaccharide; Alberta Research Council, Edmonton, Alberta, Canada), as described previously (25).

Supportive Therapy

Kidney transplant recipients were monitored daily by complete blood count, serum creatinine, blood urea nitrogen, total protein, and electrolytes. In addition, heart transplant recipients were monitored by daily observation/palpation of the donor heart contractions. Ofloxacin (50 mg/day) or cefazolin (500 mg/day) was administered daily as prophylaxis against infection in all baboons of Groups 2-5. Twice weekly blood cultures were performed to detect systemic bacterial or fungal infection. To correct anemia, thrombocytopenia and coagulopathy, baboon ABO-matched packed red blood cells, platelets (collected by apheresis or by manual processing), and fresh frozen plasma (depleted of anti-pig antibody using adsorption columns or pig red blood cells) were administered to baboons in Groups 2-5. The indication for blood transfusion was a hematocrit of <20%, for platelets a platelet count of $<10 \times 10^3/mm^3$ and/or clinical evidence of bleeding, and for plasma, clinical bleeding or a persistent prothrombin time of >35 sec.

Measurement of Coagulation Parameters

White blood cell and platelet counts were determined daily by standard methodology (Excell, Danam Electronics, TX). If the platelet count were $<100,000/mm^3$, a blood smear was prepared, fixed with methanol, air dried, and stained with Wright Giemsa (Fisher Scientific, Pittsburgh, PA). Platelets were counted under oil immersion.

Erythrocyte morphology was also observed. Blood for special assays was collected in Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing 0.015 M citrate or heparin for plasma, and plain tubes for serum. Blood samples were centrifuged at 800 g for 10 min. Plasma and serum samples were immediately stored at -80°C until used in assays.

Prothrombin time (PT), partial thromboplastin time (PTT) and fibrinogen determinations were performed on plasma samples at the Hematology Laboratory at the Massachusetts General Hospital. PT and PTT were assayed by standard methods, and the fibrinogen measured by the Clauss method (27), using the MDA-180 automated coagulation analyzer (Organon Teknika, Durham, NC). Fibrin split products were measured by standard techniques at the Massachusetts General Hospital laboratory. Ranges of D-dimer were measured using a semiquantitative assay kit, as described in the manufacturer's instructions (Diagnostica Stago, Asnieres, France), supplemented by specific enzyme-linked immunosorbent assay (ELISA) (Gold-Dimertest, American Diagnostica, Greenwich, CT).

Histopathology of Transplanted Organs or Native Organs at Autopsy

Tissues were (i) examined by light microscopy after being formalin-fixed and stained with hematoxylin and eosin or periodic acid-Schiff and (ii) processed for immunohistology/fluorescence. To detect platelet aggregates, an indirect immunoperoxidase technique was applied with a murine monoclonal antibody directed to human CD62P (P-selectin) (Becton Dickinson, San Jose, CA). For vWF detection, a rabbit polyclonal antibody to human vWF crossreactive with baboon vWF was used (DAKO, Glastrup, Denmark). For the detection of IgG, IgM, C3 and fibrin deposition, frozen tissue sections were stained using direct immunofluorescence with FITC-conjugated rabbit polyclonal antibody to human IgG, IgM, C3 and fibrin (DAKO), all of which were crossreactive with baboon antigens. Controls consisted of tissue staining with an irrelevant murine monoclonal antibody or rabbit-anti-human albumin.

Experimental Groups

Five experimental groups were studied (Table 1).

Group 1 (n=6). Cynomolgus monkeys received NMCR and allo (n=3) or concordant xeno (n=3) BM cells and kidney (n=2) or heart (n=1) transplants on day 0.

Group 2 (n=4). Baboons received either non-myeloablative NMCR alone (n=2) or myeloablative NMCR and autologous BM transduced with the porcine α1,3 galactosyltransferase gene which was infused intravenously on days 0, 1 and 2 (n=2).

Group 3 (n=12). PCTx+/-NMCR. Two baboons received pig PBPC (3 x 10^{10} cells/kg) and one received pig BM (14 x 10^{8}/kg) without NMCR. Nine baboons received NMCR followed by PBPC (3 x 10^{10}/kg).

Group 4 (n=4). POTx+/-NMCR. Non-myeloablative NMCR was carried out in one baboon which received a pig kidney transplant. Three other baboons received kidney (n=2) or heterotopic heart (n=1) transplants without NMCR.

Group 5 (n=4). NMCR+PCTx+POTx. Following non-myeloablative NMCR, 3 baboons received kidney transplants and one a heterotopic heart transplant. All received either PBPC (3 x 10^{10}/kg) (n=1) or pig BM (8-26 x 10^{8}/kg) (n=3), the hematopoietic cell infusion beginning either immediately after the organ transplant or 24 hours later (Figure 1).

RESULTS

The results are summarized in Table 1.

Effects of NMCR +/- non-discordant cellular/organ Tx (Groups 1 and 2)

Whenever NMCR was administered without PCTx or POTx, i.e. either NMCR alone or when accompanied by hematopoietic autologous, allo or concordant xeno cells and organs, there was a *gradual* decrease in platelet numbers over 7-12 days with full

recovery to normal levels between days 15-18 (Figure 1). The thrombocytopenia was more prolonged following myeloablative NMCR (Group 2) (not shown). However, in all cases, PT, PTT, fibrinogen, and fibrin split products remained within the normal ranges or were transiently deranged to a minor extent.

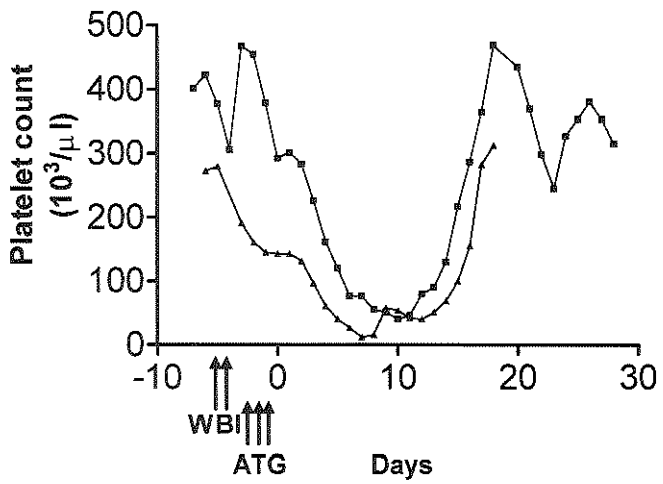

Figure 1.

Changes in platelet count in 2 representative recipient baboons from Group 2 that had received NMCR alone or NMCR and autologous hematopoietic cell transplantation. NMCR alone or NMCR + autologous cells resulted in a steady fall in platelet count for 10-12 days, with subsequently a rapid recovery phase.

Effects of discordant xenogeneic cellular Tx with or without NMCR (PCTx+/- NMCR) (Group 3)

Whenever PCTx was carried out, a TTP-like state developed. When PCTx was carried out *without* NMCR, a *rapid* decrease in platelets (to <20,000 mm^3) occurred (within 3 days) (Figure 2) with a slight and transient prolongation of PT (<17 sec) and PTT (<45 sec) and decrease in fibrinogen level (to 30% of baseline). On blood smear, schistocytosis was marked (>12 hpf) and persisted for several weeks. A massive increase in lactate dehydrogenase (to >10,000 units) was seen. Fibrin split products and D-dimer

increased to high levels but rapidly normalized. Platelets recovered within 5-9 days and fibrinogen by day 15. In one baboon, after recovery of coagulation parameters, a second set of PBPCs was infused, resulting in similar changes.

When NMCR preceded PCTx, there was a rapid and profound fall in platelet count (to <20 mm^3) following the first infusion of porcine cells (even after as few cells as 1 x 10(/kg) (Figure 2). Thrombocytopenia persisted for approximately two weeks, during which platelet transfusions were required. Thereafter, recovery of platelet count was relatively rapid. The initial rapid onset of thrombocytopenia was temporally associated with the PCTx. In contrast to when PCTx was carried out alone, the delayed recovery was associated with the effects of myelosuppression brought about by NMCR. Schistocytosis was again marked (>12 hpf) unless prophylactic therapy was given to reduce platelet and endothelial cell activation (20) and lactate dehydrogenase rose. In some baboons there was mild purpura, transient neurologic disorder (transient collapse, lethargy), or transient renal dysfunction (in one baboon).

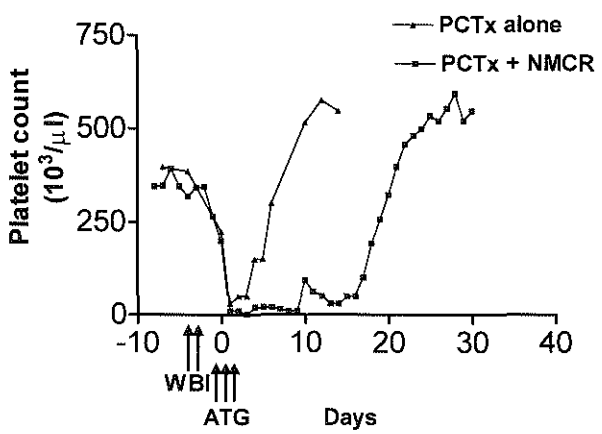

Figure 2.

Changes in platelet count in 2 representative recipient baboons in Group 3 that had received pig hematopoietic cells alone or following NMCR. The infusion of pig hematopoietic cells resulted in an immediate thrombocytopenia, which recovered quickly unless NMCR had also been administered and bone marrow suppression had been induced.

One of two baboons that received PBPC alone died after receiving a second infusion of PBPC. After the second PBPC infusion, severe thrombocytopenia developed, with an increase in LDH to >25,000 units/L. PT, PTT and fibrinogen level remained relatively stable. The baboon died with features of cardiorespiratory failure within 4 days. Autopsy showed gross hemorrhage in the lungs and heart and in other essential organs. Histological examination confirmed interstitial hemorrhage, particularly in the heart, the lungs, kidneys, and adrenal glands. Immunohistochemistry showed deposition of platelets in small vessels, expression of vWF on the capillary walls, with fibrin deposition (data not shown).

TABLE 1

EXPERIMENTAL GROUPS AND SUMMARY OF OBSERVED CHANGES IN COAGULATION PARAMETERS

GROUPS	REGIMEN	PLATELETS	PT	PTT	FIBRINOGEN	D-DIMER
1 (n=6)	NMCR + allo or concordant xeno HCs and organ Tx	↓	-	-	↑	↑
2 (n=4)	NMCR +/- auto-logous HCs	↓/↓↓	-	-	-	-
3 (n=12)	PCTx +/- NMCR	↓↓	↑	↑	↓	↑
4 (n=5)	POTx +/- NMCR	↓	↑↑	↑↑	↓↓	↑
5 (n=4)	NMCR + PCTx +POTx	↓↓↓	↑↑↑	↑↑↑	↓↓↓	↑↑

NMCR = tolerance-inducing regimen

HCs = hematopoietic cells (bone marrow or peripheral blood mobilized progenitor cells)

PCTx = porcine hematopoietic cell transplantation

POTx = porcine organ transplantation

Change in parameter:

Decrease ↓ mild, ↓↓ moderate, ↓↓↓ profound

Increase ↑ mild, ↑↑ moderate, ↑↑↑ profound

One baboon that received NMCR + PBPC died from acute cardiorespiratory failure on day 5 with an undetectable level of circulating platelets, despite platelet transfusion. There was a transient increase in PT to twice the baseline level, but this persisted for only 1-2 days before normalizing. PTT was not significantly altered. The fibrinogen level fell modestly 4-6 days after the initial PCTx. D-dimer levels increased between days 1-7, but again normalized rapidly. At autopsy, the changes were similar to those above. In addition, however, there was massive hemorrhage in all mesenteric lymph nodes.

Effects of discordant xenogeneic organ Tx with or without NMCR (POTx+/- NMCR) (Group 4)

Whenever POTx was performed, an apparent consumptive coagulopathy and DIC occurred concurrently with the development of delayed antibody-mediated rejection. When POTx was performed +/- NMCR, gradual but steady falls in both platelet count and fibrinogen level occurred. The first indication that DIC was developing was frequently an increase in fibrinolytic activity indicated by a rise in fibrin split products or D-dimer (Table 2).

After 1-3 days, in 4 of the 5 cases there was a precipitate rise in PT (data not shown). Clinical bleeding occurred in 2 baboons with hematuria, and both died on day 9 with massive intraperitoneal hemorrhage. Rejected xenografts revealed microthrombi in glomeruli and microvasculature with hemorrhagic and necrotic ureters, particularly in the distal portion; sequential deposits of platelets (platelet microthrombi) and fibrin were also seen (data not shown). The changes of DIC resolved only in those baboons where the transplanted porcine organ was excised as an emergency procedure (Figure 3, and Table 2). Histopathological examination of the excised organ demonstrated variable features of rejection with sometimes only 20% of the organ affected by interstitial and deposition of platelets and fibrin. (Fibrin deposition was not seen in native organs, indicating a localised response.) IgM deposition was seen in the grafted organ at the time of excision, but IgG deposition was variable (sometimes absent or minimal) depending

on the pre and post-transplant therapy given. Complement deposition was frequently absent as a resuly of therapy with cobra venom factor.

TABLE 2
D-DIMER LEVELS IN TWO GROUP 4 BABOONS FOLLOWING PIG KIDNEY TRANSPLANTATION

DAY	B75-18	B75-34
Pre POTx	0	0.5
Post-POTx		
1	<2	2-8
2	2-8	0.5-2
5	2-8	2-8
At GTx	>8	>8
48h post-GTx	0.5	0.5

POTx = porcine organ transplantation
GTx = graftectomy

Effects of combined discordant xenogeneic cellular and organ Tx with NMCR (NMCR+PCTx+POTx) (Group 5)

When NMCR was combined with both PCTx and POTx, all baboons developed immediate and profound thrombocytopenia following the first PCTx, which extended for 12-15 days (Figure 3), and required a greater number of platelet transfusions compared to Group 3. This was followed by a progressive fall in fibrinogen and a prolongation of PT, indicating consumption of coagulation factors such as seen in DIC. As with POTx alone, these coagulation parameters resolved only if graftectomy was performed.

All of the transplanted organs had to be removed before day 15 to prevent worsening DIC. Histopathological examination of the rejected organ, however, again showed considerable variability in the extent of immune injury, with patchy localized injury in some cases. At excision of the graft, there was variable IgM, IgG and complement deposition as in Group 4. When NMCR was followed by both PCTx and POTx,

therefore, the features seen were a combination of the thrombotic microangiopathy that occurred following PCTx alone and the consumptive state that occurred following POTx alone.

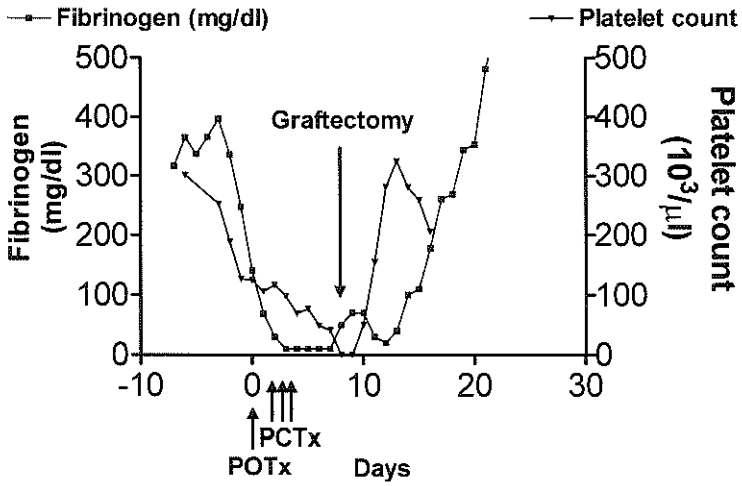

Figure 3.

Changes in platelet count and plasma fibrinogen concentration in a representative recipient baboon from Group 5 (NMCR+PCTx+POTx). When both pig hematopoietic cells and a kidney were transplanted in combination in a baboon which had also received NMCR, there was an immediate thrombocytopenia which persisted for 14 days until the effects of myelosuppression had worn off. There was also a steady reduction in fibrinogen until the pig organ was excised (graftectomy), following which there was rapid recovery.

DISCUSSION

We have presented evidence for profound perturbations in hemostasis, coagulation and thromboregulation that occur consistently with the transplantation of discordant xenogeneic cells and/or organs in the the miniature swine-to-nonhuman primate model. The coagulopathies observed after discordant organ xenotransplantation in this model were first documented by Ierino (11) and Kozlowski (3) with their respective colleagues, and have also been described in the pig-to-baboon model by Meyer et al (12). The present study, which includes a small number of the experiments reported by Ierino and

Kozlowski (3,11,26), contributes additional information on this topic and further elucidates this important consequence of discordant xenograft rejection.

In particular, we demonstrate that NMCR alone or when combined with autologous, allo or xeno hematopoietic cell transplantation +/- organ allo- or concordant xeno-transplantation resulted in relatively minimal and transient changes in coagulation parameters of the recipient animal (either cynomolgus monkey or baboon). The NMCR utilized at our center, therefore, although prolonging the duration of thrombocytopenia following PCTx, would not otherwise appear to play a direct role in the development of either the thrombotic angiopathic state seen following PCTx or the consumptive coagulopathy seen after POTx. However, it is possible that whole body irradiation could 'sensitize' the vascular endothelium making it more susceptible to activation by PBPC.

PCTx, whether alone or in combination with NMCR, caused a profound thrombocytopenia, limiting the number of porcine cells that could be transfused into the recipient baboon. In the absence of NMCR, recovery of platelet count following PCTx occurred within 5-9 days. The myelosuppression caused by NMCR delayed recovery of platelets for approximately two weeks. A TTP-like state developed, which has been investigated further and will be fully described elsewhere (Bühler, L., et al. manuscript submitted). In brief, animals rapidly became thrombocytopenic, developed schistocytes and demonstrated features of microangiopathy, associated with a rise in lactate dehydrogenase. Only minor fluctuations in plasma levels of von Willebrand factor were seen, with transient shifts in von Willebrand factor multimeric patterns. No changes in von Willebrand factor-cleaving protease activity were noted (data not shown). TTP can progress to a state where widespread organ damage can trigger release of thromboplastin and a consumptive coagulopathy (DIC); this was not a feature in the present studies except under agonal circumstances in 2 baboons in Group 3.

This TTP-like state is therefore comparable to that seen complicating autologous or allogeneic hematopoietic stem cell transplantation in humans (28). As this complication was seen in baboons receiving cobra venom factor (as well as in baboons receiving hematopoietic cells taken from pigs transgenic for human decay accelerating factor -

data not shown), this indicates that depletion of complement factors does not preclude the TTP-like state.

We are currently investigating the mechanisms whereby PBPC activate primate endothelial cells, leukocytes and/or platelets. Many of the natural anticoagulants and complement regulators are expressed by endothelial cells and monocyte-macrophages. These anticoagulant proteins are largely ineffective across discordant xenogeneic species barriers (19). Such molecular incompatibilities may lead to activation of coagulation following the infusion of xenogeneic leukocytes and could result in (i) excessive fibrin deposition upon the infused porcine cells with consequent platelet interactions mediated via GPIIbIIIa or (ii) direct upregulation of adhesive proteins with cellular activation, predisposing to leukocyte-platelet aggregates.

Some of the baboons being studied, particularly in Groups 3 and 5, received therapy aimed at reducing the activation of the vascular endothelium and platelets of the host. This therapy (consisting of prostacyclin, heparin and methylprednisolone) had some beneficial effect in reducing the features of the TTP-like state, particularly in reducing the need for platelet transfusion (data not shown), but was not completely successful (20).

The manifestations of TTP were quite different from the widespread coagulation changes seen when POTx was performed. Here coagulation-factor consumption and DIC developed, generally within 1-2 weeks and concurrent with the development of xenograft rejection. This was exemplified by a steady fall in fibrinogen and platelets and a late rise in PT. In some cases, after several days, PT rose acutely to a high level, followed by clinical hemorrhage leading to death of the recipient unless the graft was removed as an emergency. Following graftectomy, the coagulopathic state generally, but not always, rapidly resolved. When both PCTx and POTx were combined, an initial TTP-like state was seen, which was exacerbated by the development of xenograft rejection and the evolution of DIC. The consumptive coagulopathy then became the predominant feature.

Of significance was the fact that when graftectomy was necessitated by a rapidly deteriorating coagulopathic state, on occasion the transplanted porcine organ looked relatively normal macroscopically, and histological examination showed only moderate immune injury. The extent of histopathological change in the form of interstitial hemorrhage or fibrin deposition could involve as little as 20% of the organ. We suggest, therefore, that the DIC may not necessarily be a result of the organ damage linked to the acute vascular rejection that is developing, but may be associated with factors common to both the development of coagulation disturbances and acute vascular rejection, such as vascular endothelial cell activation.

Quiescent endothelial cells express potent thromboregulatory factors, including the vascular ATP-diphosphohydrolase (or CD39), tissue factor pathway inhibitor, and thrombomodulin (29). In addition to heparan sulphate (30), these proteins are lost in association with xenograft rejection. Hence the activated endothelial cell becomes thrombophilic, and may initiate and perpetuate the thrombotic reactions that result in systemic depletion of coagulation factors seen in DIC (9,10,18,29). Tissue factor is an important initiating factor of coagulation and is expressed by activated endothelial cells and denuded vasculature. In vitro assays have shown that porcine endothelial cells stimulated by human serum are able to increase tissue factor via IL-1α production and require the presence of sub-lytic complement Membrane Attack Complex (MAC) components (31). Despite cobra venom factor therapy in our POTx experiments, such sub-lytic complement activation is still likely and may have induced increased expression of tissue factor on the activated endothelium of the xenografts. In addition, porcine endothelial cells, including hDAF transgenic cells, have the capacity to upregulate tissue factor following exposure to other inflammatory mediators, e.g. tumor necrosis factor released by infiltrating mononuclear cells (32).

The development of DIC in these xenografts has parallels to the consumptive coagulopathy seen with the Shwartzman phenomenon in antibody-mediated vascular injury of allografts (33). Removal of the activated endothelium (with the graft) leads to a greater response to therapy in the form of fresh frozen plasma and platelet transfusions.

We are not alone in identifying DIC following xenotransplantation experiments. This was first described in the 1960s (34,35), although there have been few reports in the pig-to-nonhuman primate model. In the pig-to-baboon renal transplant model, Meyer et al. (12) have also shown evidence for platelet activation, formation of thrombin-antithrombin III complexes, a fall in fibrinogen, and an increase in fibrinolytic activity, all suggesting DIC. They also demonstrated that the transplantation of organs from pigs with homozygous von Willebrand's disease, that were severely deficient in von Willebrand factor, did not prevent platelet and coagulation activation. None of their animals, either control or with von Willebrand disease, presented with overt bleeding. d'Apice's group in Australia has recently observed a DIC-like state in unmodified (non-immunosuppressed) baboons receiving pig kidneys transgenic for human CD55 and CD59 (36). Other groups have not reported major coagulation disorders following pig-to-nonhuman primate organ transplantation. Indeed, it was not observed by the Cape Town and Oklahoma groups of which one of us (DKCC) was a member (6,37,38).

Numerous potential differences in experimental protocols may account for this discrepancy, including, for example, differences in (i) the species of nonhuman primate used as recipient (e.g. cynomolgus monkey or baboon), (ii) the breed of organ-donor pig (e.g. large white or miniature swine), (iii) whether the pig has been genetically modified or not (e.g. the presence of hDAF on its vascular endothelium), (iv) the organ transplanted (kidney or heart), (v) the immunosuppressive regimen (where there are numerous variables, including alkylating agents at different dosages), and (vi) any adjunctive therapy administered (e.g. the concomitant infusion of hematopoietic cells or the intravenous administration of prostacyclin). However, we have observed DIC both in cynomolgus monkeys and baboons, after the transplantation of pig kidneys and hearts, when induction therapy was with whole body irradiation or with cyclophosphamide, and with and without cobra venom factor. Apart from the report from d'Apice's group mentioned above, DIC does not seem to have been observed by those using organs from pigs transgenic for one or more complement regulatory proteins (7,8,39). However, we have recently seen this complication in 2 of 3 baboons receiving hDAF kidneys at our center (Buhler, L., et

al, manuscript in preparation); all 3 had received induction therapy with cyclophosphamide and not whole body irradiation. As we routinely adsorb out anti-Gal antibody before the transplant, it is possible that it is the slow return of antibody, with gradual binding to the graft vascular endothelium (rather than immediate and massive antibody binding as occurs when antibody is not removed), that is important in the development of a procoagulant state. Furthermore, procedures that deplete antibody may also deplete or perturb labile coagulation factors. Others, however, have included antibody adsorption in their protocols (by similar but not identical techniques) but have not reported consumptive coagulopathy as a complication (39,40). It is therefore difficult to explain why this complication has been observed frequently at some laboratories but not at other centers using similar or even identical protocols.

We conclude that both PCTx and POTx result in significant changes in coagulation that must be further investigated and overcome if discordant xenotransplantation of hematopoietic cells and organs is to be fully successful. The TTP-like state that follows PCTx limits the number of pig hematopoietic cells that can be infused and therefore may be a factor in the extent of pig cell chimerism that can be obtained. Under the circumstances of the experiments reported here, rejection of a porcine organ xenotransplant was consistently associated with the development of DIC, necessitating excision of the transplanted organ, whether it were a kidney or a heart. Importantly, in some cases, DIC developed before there were widespread changes of immunological injury to the transplanted organ.

DIC might therefore be considered to be a surrogate marker for developing antibody-mediated rejection. Our observations, and those of others, suggest that the discordant xenogeneic vasculature may be predisposed under certain circumstances to initiate thrombotic reactions *in vivo*.

REFERENCES

1. Sablinski T, Latinne D, Gianello P, et al. Xenotransplantation of pig kidneys to nonhuman primates. I. Development of the model. Xenotransplantation 1995; 2: 264
2. Cooper DKC, Cairns TDH, Taube DH. Extracorporeal immunoadsorption of anti-pig antibody in baboons using αGal oligosaccharide immunoaffinity columns. Xeno 1996; 4: 27
3. Kozlowski T, Shimizu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody adsorption. Transplantation 1999; 67: 18
4. Leventhal JR, Dalmasso AP, Cromwell JW, et al. Prolongation of cardiac xenograft survival by depletion of complement. Transplantation 1993; 55: 857
5. Pruitt SK, Kirk AD, Bollinger RR, et al. The effect of soluble complement receptor type 1 on hyperacute rejection of porcine xenografts. Transplantation 1994; 57: 363
6. Kobayashi T, Taniguchi S, Neethling FA, et al. Delayed xenograft rejection of pig-to-baboon cardiac transplants after cobra venom factor therapy. Transplantation 1997; 64: 1255
7. Schmoeckel M, Bhatti FNK, Zaidi A., et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. Transplantation 1998; 65: 1570
8. Zaidi A, Scmoeckel M, Bhatti FNK, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584
9. Bach FH, Robson SC, Ferran C, et al. Endothelial cell activation and thromboregulation during xenograft rejection (review). Immunol Rev 1994; 141: 5
10. Bach FH, Winkler H, Derran C, et al. Delayed xenograft rejection. Immunol Today 1996; 17: 379
11. Ierino FL, Kozlowski T, Siegel JB, et al. Disseminated intravascular coagulation in association with the delayed rejection of pig-to-baboon renal xenografts. Transplantation 1998; 66: 1439
12. Meyer C, Wolf P, Romain N, et al. Use of von Willebrand diseased kidney as donor in a pig-to-primate model of xenotransplantation. Transplantation 1999; 67: 38
13. Robson SC, Schulte am Esch II J, Bach FH. Factors in xenograft rejection. Ann NY Acad Sci 1999; 875: 261
14. Jurd KM, Gibbs RV, Hunt BJ. Activation of human prothrombin by porcine aortic endothelial cells - a potential barrier to pig-to-human xenotransplantation. Blood Coagul Fibrinolysis 1996; 7: 336
15. Jurd KM, Lee J, Cairns T, et al. Effect of Galα1,3Galß1,4 GlcNAc and complement depletion on haemostatic activation in an in vitro model of the pig-to-human xenograft reaction. Transplant Proc 1996; 28: 637
16. Siegel JB, Grey ST, Lesnikoski BA, et al. Xenogeneic endothelial cells activate human prothrombin. Transplantation 1997; 64: 888
17. Kopp CW, Siegel JB, Hancock WW, et al. Effect of porcine endothelial tissue factor pathway inhibitor on human coagulation factors. Transplantation 1997; 63:749
18. Lawson JH, Daniels LJ, Platt JL. The evaluation of thrombomodulin activity in porcine to human xenotransplantation. Transplant Proc 1997; 29: 884
19. Schulte J, Siegel JB, Bach FH, Robson SC. The A1 domain of von Willebrand factor expressed on cell membranes directly activates platelets. Blood 1996; 88(Suppl): 81
20. Buhler L, Awwad M, Basker M, et al. High-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade in baboons prevents an induced anti-pig humoral response. Transplantation. Accepted for publication.
20. Cooper DKC, Ye Y, Niekrasz M. Heart transplantation in primates. In: Handbook of Animal Models in Transplantation Research. Cramer DV, Podesta L, Makowka L (eds). Boca Raton, CRC, 1993, p. 173
21. Kawai T, Cosimi B, Colvin R, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. Transplantation 1995; 59: 2561
23. Pennington LR, Sakamoto K, Popitz-Bergez FA, et al. Bone marrow transplantation in miniature swine. I. Development of the model. Transplantation 1988; 45: 21
24. Nash K, Chang Q, Watts A., et al. Peripheral blood progenitor cell mobilization and leukapheresis in pigs. Lab Anim Sci. 1999; 49: 645
25. Kozlowski T, Ierino FL, Lambrigts D, et al. Depletion of anti-Galα1-3Gal antibody in baboons by specific α-Gal immunoaffinity columns. Xenotransplantation 1998; 5: 122
26. Ierino FL, Gojo S, Banerjee PT, et al. Transfer of swine major histocompatibility complex Class II

genes into autologous bone marrow cells of baboons for the induction of tolerance across xenogeneic barriers. Transplantation 1999; 67: 1119
27. Clauss A. Rapid physiological coagulation method in the determination of fibrinogen. Acta Hematol (Basel) 1957; 17: 237
28. Pettitt AR, Clark RE. Thrombotic microangiopathy following bone marrow transplantation. Bone Marrow Transplant 1994; 14: 495
29. Robson SC, Kopp C. Disordered thromboregulation in discordant xenograft rejection. Life Sci 1996; 6: 34
30. Platt JL, Vercelloti GM, Lindman BJ, et al. Release of heparan sulphate from endothelial cells. J.Exp.Med. 1990; 171:1363.
31. Saadi S, Holzknecht RA, Patte PP, et al. Complement-mediated regulation of tissue factor activity in endothelium. J Exp Med 1995; 182: 1807
32. Tucker AW, Carrington CA, Richards AC et al. Endothelial cells from human decay acceleration factor transgenic pigs are protected against complement mediated tissue factor expression in vitro. Transplant Proc 1997; 29; 888
33. Starzl TE, Boehmig HJ, Amemiya H, et al. Clotting changes, including disseminated intravascular coagulation, during rapid renal homograft rejection. N Engl J.Med 1970; 278: 642
34. Rosenberg JC, Broersma RJ, Bullemer G, et al. Relationship of platelets, blood coagulation, and fibrinolysis to hyperacute rejection of renal xenografts. Transplantation 1969; 8: 152
35. Broersma RJ, Bullemer GD, Rosenberg JC, et al. Coagulation changes in hyperacute rejection of renal xenografts. Thromb Diath Haem.-Supplementum 1969; 36: 333
36. Cowan P, Goodman D, Aminian A, et al. Disseminated intravascular coagulation in non-immunosuppressed baboons receiving transgenic pig renal xenografts. (Abstract 0185). 5th Congress of the International Xenotransplantation Association, Nagoya, 1999
37. Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. J Heart Transplant 1988; 7: 238
38. Cooper DKC, Cairns TDH , Taube DH. Extracorporeal immunoadsorption of αGal antibodies. Xeno 1996; 427
39. Lin SS, Weidner BC, Byrne GW et al. The role of antibodies in acute vascular rejection of pig-to-baboon cardiac transplants. J Clin Invest 1998; 101: 1745
40. Leventhal JR, Sakiyalak P, Witson J, et al. The synergistic effect of combined antibody and complement depletion on discordant cardiac xenograft survival in nonhuman primates. Transplantation 1994; 57: 974

6

Mechanisms of thrombocytopenia following xenogeneic hematopoietic progenitor cell transplantation

Adapted from:
Alwayn IPJ, Buhler L, Appel JZ III, Csizmadia E, Harper D, Down J, Awwad M, Kitamura H, Sackstein R, Cooper DKC[♦], and Robson SC[♦]. Mechanisms of thrombocytopenia following xenogeneic hematopoietic progenitor cell transplantation. *Transplantation 2001. In press.*
[♦] Joint senior authors

ABSTRACT

Introduction. Attempts to induce tolerance though mixed hematopoietic chimerism in the discordant pig-to-baboon xenotransplantation model are complicated by a potentially fatal thrombotic microangiopathy in the recipient baboons. This state develops immediately following the infusion of porcine mobilized peripheral blood leukocytes, containing progenitor cells (PBPC). In the present study, we examined the interaction of infused porcine PBPC with recipient platelets in vivo in baboons and investigated the underlying mechanisms using an in vitro model.

Methods. Two naïve baboons and six baboons pre-conditioned with irradiation and immunosuppression that received porcine PBPC were evaluated in vivo. The interaction of porcine and baboon PBPC with baboon platelets was investigated by an in vitro platelet aggregation assay. Fresh and cryopreserved PBPC were evaluated as well as PBPC obtained from growth-factor-mobilized and unmobilized pigs. Furthermore, cellular subsets of PBPC were assessed for potential to induce platelet aggregation. Immunohistochemical staining was performed on platelet-leukocyte aggregates and potential inhibition of aggregation with anti-P-selectin and anti-CD154 mAbs, or eptifibatide (a GPIIb/IIIa receptor antagonist), was tested.

Results. All baboons that received porcine PBPC rapidly developed marked thrombocytopenia (<20,000/μl), elevated serum lactate dehydrogenase (>1,500U/l), schistocytosis, and platelet aggregates on blood smear. Three baboons died (two untreated and one pre-conditioned), and substantive platelet aggregates containing porcine leukocytes were observed in the microvasculature of lungs and kidneys. In vitro, porcine, but not baboon, PBPC induced aggregation of baboon platelets in a dose-dependent manner. Immunohistological examination of these aggregates confirmed the incorporation of porcine leukocytes. Cryopreserved PBPC caused less aggregation than fresh PBPC, and growth-factor-mobilized PBPC induced less aggregation than unmobilized PBPC. Aggregation was fully abrogated by the addition of eptifibatide, and modulated by anti-P-selectin and anti-CD154 monoclonal antibodies that recognize adhesion receptors on activated platelets. Purified fractions (granulocytes, $CD2^+$, and $CD2^-$ cells) of porcine PBPC did not initiate aggregation,

whereas addition of exogenous porcine PBPC membranes (erythrocytes, dead cells, and/or platelets) to the purified fractions exacerbated the aggregation response.
Conclusions. These data indicate that porcine PBPC mediate aggregation of baboon platelets. This process likely contributes to the thrombotic microangiopathy observed following PBPC transplantation in the pig-to-baboon model. Eptifibatide can fully abrogate platelet aggregation induced by porcine PBPC in vitro. Purification of the precursors from porcine PBPC and/or treatment of baboons with eptifibatide may be beneficial.

INTRODUCTION

The use of porcine xenografts as an alternative for human cadaveric organs is viewed by many as a potential solution to the increasing shortage of allografts (1,2). Although many advances have recently been made with respect to understanding the immunological basis of rejection of highly disparate organs when transplanted into nonhuman primates, xenografts generally do not survive beyond approximately one month (3,4). This process is, at least in part, related to host immunological responses to the xenograft.

In our efforts to induce tolerance to porcine organs transplanted to nonhuman primates, we have attempted to induce a state of mixed hematopoietic chimerism (5). Such an approach has been successful, without major complications, in rodent allogeneic and xenogeneic transplantation models, and also in swine and primate allogeneic models. In these models, immunological tolerance is associated with indefinite survival of the grafts (6, 7, 8, 9). When extending this approach to the discordant pig-to-baboon model, using large numbers of mobilized porcine leukocytes, containing approximately 2% progenitor cells (PBPC), however, a thrombotic microangiopathy has been observed (10). This phenomenon did not occur when smaller numbers of porcine bone marrow cells were transplanted into baboons (11) and, importantly, when a comparable number of cells were transplanted in a swine allotransplantation model (8). The clinicopathological entity included neurological and renal disturbances with fever, immediate thrombocytopenia,

markedly elevated serum lactate dehydrogenase levels, schistocytosis on blood smear, with microvascular thrombosis of lungs, heart, and kidneys on autopsy (12).

Thrombotic microangiopathy has also been observed following clinical autologous and allogeneic bone marrow transplantation. Here the underlying pathophysiology is thought to be widespread endothelial cell damage associated with several factors, including inflammation, cyclosporine, or graft-versus-host disease (13). This state, however, does not generally develop before the transplanted marrow has engrafted, whereas the thrombotic microangiopathy in our studies developed rapidly after porcine PBPC (pPBPC) transplantation. We therefore postulated that the etiology of these two pathologic states may differ, in that porcine leukocytes may be directly interacting with baboon platelets in vivo. This process may be accentuated by the existence of molecular incompatibilities that would facilitate these interactions (14).

We have therefore evaluated such interactions in pre-conditioned and unconditioned baboons receiving large numbers of pPBPC ($1\text{-}3x10^{10}$ cells/kg) in vivo, and correlated these observations with pPBPC–platelet interactions in vitro. Furthermore, several agents aimed at preventing these interactions were studied in vitro. Effects of these individual components of the conditioning regimen on platelet aggregation are discussed elsewhere (Appel JZ III, et al., manuscript in preparation).

MATERIAL AND METHODS

In vivo experiments

Animals

Naïve baboons (Papio anubis, n=8, Biomedical Resources Foundation, Houston, TX), of known ABO blood group and weighing 9-15 kg, were used as recipients of pPBPC, as donors of platelet-rich plasma (PRP), or as donors of baboon PBPC. Massachusetts General Hospital MHC-inbred miniature swine (n=6, Charles River Laboratories, Wilmington, MA), of blood group O and weighing 18-40 kg, served as donors of pPBPC. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of

Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical procedures

Details of anesthesia, intravenous (iv) catheter placement in pigs and baboons, and intra-arterial line placement, splenectomy, and extracorporeal immunoadsorptions of anti-αGal antibodies in baboons have been described elsewhere (15, 16, 17).

Non-myeloablative conditioning regimen (NMCR)

This regimen has been described previously (18) and consists of induction therapy with splenectomy, whole body irradiation, thymic irradiation, anti-thymocyte globulin, and multiple extracorporeal immunoadsorptions of anti-αGal antibodies. Maintenance therapy consists of anti-CD154 monoclonal antibody (mAb) +/- cyclosporine, mycophenolate mofetil, cobra venom factor, prostacylin, heparin, and methylprednisolone. Porcine growth factors (IL-3 and stem cell factor) were administered to promote pig cell engraftment.

Mobilization and leukapheresis of PBPC in pigs and baboon

This procedure was performed as previously described (19). In summary, pigs were treated with recombinant hematopoietic growth factors before and during the period of collection of PBPC. Leukapheresis was carried out on days 5-9 and 12-16 following commencement of growth factor mobilization using a Cobe Spectra apheresis machine (Cobe, Lakewood, CO). Baboons were treated with recombinant hematopoietic growth factors (as for the pigs) and leukapheresis was carried out on day 7 alone. Plasma was removed from the product by centrifugation at 920 g for 7 minutes. The collected cells were washed and used for in vitro assays, or frozen and stored in 5% DMSO until transplantation or until used for in vitro assays.

Preparation of pPBPC for transplantation

Cryopreserved PBPC were thawed by immersion in a 37 – 40^0 C water bath with gentle agitation for 1 – 2 minutes, washed twice with calcium-, magnesium, and phenol red-free Hanks' balanced salt solution (HBSS, Mediatech, Herndon, VA), and

resuspended in the same solution for immediate transplantation to the recipient baboon. Viability was assessed with trypan blue staining.

Hematologic and serum chemistry parameters

Daily blood samples were taken for measurement of platelet count (Excell, Danam Electronics, Dallas, TX), and prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, and fibrinogen degradation products (Hematology Laboratory, Massachusetts General Hospital). Blood smears were also prepared on a daily basis, fixed with methanol, air dried, and stained with Wright Giemsa (Fisher Scientific, Pittsburgh, PA). Platelets were counted under oil immersion, and erythrocyte morphology was observed. Daily serum samples were obtained for the measurement of lactate dehydrogenase levels (Chemistry Laboratory, Massachusetts General Hospital).

Immunopathology

Tissues were (i) formalin-fixed, stained with hematoxylin and eosin for evaluation by light microscopy, or (ii) processed for immunohistology using standard methodology. Baboon platelets and porcine cells were detected by standard indirect immunoperoxidase technique, using the murine anti-CD62 (P-selectin) mAb cross-reactive with baboon (Becton Dickinson, San Jose, CA) and the porcine pan-leukocyte mAb (1030H-1-19), respectively.

<u>In vitro</u> experiments

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma

Blood was drawn from naïve baboons (10 ml), citrated, and centrifuged at 280 g for 15 minutes at 22°C. The supernatant, consisting of PRP containing approximately $1x10^8$ platelets/ml (as measured with a Coulter counter, Beckman Coulter, Inc., Fullerton, CA), was transferred in 400 µl aliquots to siliconized glass cuvettes and used as experimental samples. The remaining pellet was further centrifuged at 2,400g for 2 minutes at 22°C, the resulting supernatant, platelet-poor plasma (containing less than $1x10^3$ platelets/ml), was transferred in a 400 µl aliquot to a siliconized glass cuvette and used as reference sample.

Preparation of peripheral blood progenitor cells (PBPC)

Fresh pPBPC, and frozen baboon or pPBPC, were processed as described above. The cells were resuspended in HBSS to reach a final concentration of approximately $1x10^4$ cells/μl.

Cell sorting

A MoFlo Cytomation High Speed Cell Sorter (Cytomation, Fort Collins, CO) was used to purify and sort fractions of fresh or cryopreserved pPBPC. The product was washed twice in phosphate buffered saline containing 2% fetal bovine serum, but without calcium or magnesium, and $1x10^8$ cells were resuspended at $1x10^7$/ml. The cells were then incubated with the murine biotinylated anti-porcine CD2 (MSA4) mAb, and subsequently labeled with streptavidin phycoerythrin. Cells were then washed, resuspended at $5x10^7$/ml and run through the cell sorter. The pPBPC product was sorted for $CD2^+$ and $CD2^-$ cells based on lymphocyte and fluorescence gating. Sorting of granulocytes and exogenous membranes (debris, consisting of erythrocytes, platelets, or dead cells) was based on forward-side scatter. Approximately $2x10^6$ $CD2^+$ and $CD2^-$ cells, $2x10^5$ granulocytes, and $2x10^6$ exogenous membrane fragments (as measured per event) were used for each assay. Data acquisition and analysis were performed with Cyclops Summit software (Cytomation, CO).

Platelet aggregation assay

Platelet aggregation in PRP was measured in a platelet aggregometer (two-sample, four-channel, model 560 Ca Lumi-aggregometer, Chronolog Corp., Havertown, PA), according to the manufacturer's specifications, as a percentage change in light optical density from baseline (set to 0%) as compared to a reference sample. The maximum platelet aggregation induced by a potential stimulator was determined after 6 minutes.

Fresh pPBPC, cryopreserved pPBPC, or cryopreserved baboon PBPC were added to the baboon PRP in increasing quantities, ranging from $1x10^5$ – $1x10^7$ cells, and platelet aggregation was measured. When PBPC were added, an initial increase in optical density was observed dependent on the final number of particles in solution.

Baseline aggregation was then reset at 0%. In other experiments, pPBPC ($1x10^6$ – $1x10^7$) from growth factor-mobilized and unmobilized miniature swine, or individual and combined fractions of pPBPC ($2x10^5$ – $2x10^6$, as described above), were added to the PRP. Positive controls of aggregation were baboon PRP to which exogenous collagen (5 μg/ml) was added. Negative controls consisted of experimental samples of baboon PRP alone, or to which HBSS was added. Continuous stirring of the sample with a magnet prevented sedimentation of cells during the assay.

Competitive blocking experiments were performed by incubating baboon PRP with an anti-human P-selectin mAb (R&D Systems) (2.5 – 5.0 μg/ml), an anti-CD154 mAb (100 – 300 μg/ml) that would recognize adhesion receptors linked to platelet-cellular interactions (ref), or eptifibatide (GPIIb/IIIa receptor antagonist) (0.5 – 5.0 μg/ml), at room temperature for 15 minutes. Following this incubation, the extent of baboon platelet aggregation in the presence of pPBPC was measured.

Immunohistology

The experimental samples, obtained after platelet aggregation assays with baboon or pPBPC, were centrifuged at room temperature for 10 seconds. The pellet was resuspended in 20 μl supernatant, and snap-frozen in isopentane embedded in TBS tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Five μm sections were prepared and stained with standard immunohistochemical staining techniques using the murine anti-CD62 mAb (P-selectin, Becton-Dickinson), or the murine anti-GPIIbIIIa (CD31a, R&D Systems, both cross-reactive with baboon), and porcine pan-leukocyte mAb (1030H-1-19), as well as with a hematoxylin counterstain.

RESULTS

In vivo experiments

Approximately 70-80% of PBPC remained viable after the freezing/thawing process. All baboons developed a marked thrombocytopenia (<20,000/μl) almost immediately

following transplantation of pPBPC that necessitated platelet transfusions on several occasions. In baboons that received pPBPC only, platelet recovery was observed within 5 – 9 days (data not shown), whereas pPBPC transplantation with the NMCR resulted in sustained thrombocytopenia for 2 – 3 weeks (Fig. 1 – top left). These differences were in keeping, at least in part, with the myelosuppression associated with whole body irradiation. All baboons developed an increase in serum lactate dehydrogenase level, which was more marked in the baboons receiving pPBPC alone than in those receiving pPBPC with the NMCR (>10,000U/l and <1,700U/l, respectively, data not shown). Components of the NMCR (i.e. heparin, prostacyclin, and methylprednisolone) appear responsible for this protective effect (Appel JZ III, manuscript in preparation). Increases in schistocytes and platelet aggregates on blood smear were also observed (Fig. 1 – top right). These features were consistent with a thrombotic microangiopathy. In the three baboons that died, microvascular thrombosis and interstitial hemorrhage was observed in lungs, kidneys, heart, and other organs. Immunohistochemical examination revealed deposition of platelets closely associated with porcine cells (Fig. 1 – bottom).

In vitro experiments

The addition of baboon PBPC ($1x10^5$ – $2x10^6$) did not result in measurable aggregation of baboon platelets as assayed by light aggregometry (Fig. 2 – left). Increased baseline optical density was, however, observed depending on the number of baboon PBPC added. The more sensitive immunohistochemical examination of sedimented samples demonstrated small baboon platelet aggregates in conjunction with $1x10^6$ baboon PBPC (Fig. 2 – right). Examination of baboon platelets alone, or platelets plus HBSS (data not shown), did not demonstrate any aggregates. These data indicate that minimal platelet aggregates are observed in conjunction with baboon PBPC. These are most likely due to limited interaction between the baboon PBPC products and platelets under shear stress.

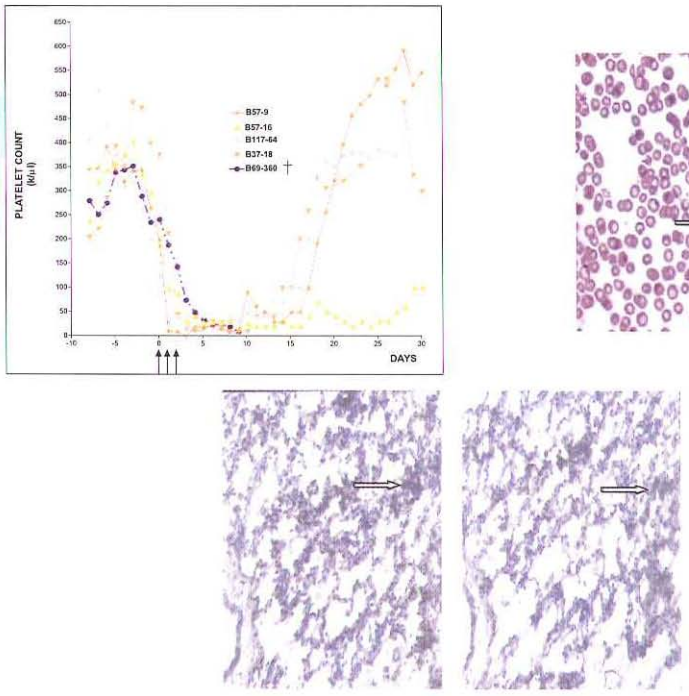

Figure 1.

Top left. Immediate thrombocytopenia following pPBPC infusion in baboons receiving NMCR. The platelet counts (x $1x10^{-3}/\mu l$) are plotted against time. Porcine PBPC (total $3x10^{10}$ cells/kg) were infused on days 0, 1, and 2 (depicted by arrows). Note the rapid fall in platelet count to <100,000/μl immediately after the first pPBPC are infused. The thrombocytopenia is sustained around 10,000 – 20,000/μl for approximately 14 days, in keeping at least in part with bone marrow depression resulting from whole body irradiation, during which platelet transfusions are required (not shown). Recovery of platelets to pre-PBPC infusion levels generally took place within 3 – 4 weeks. † signifies the demise of baboon B69-360 from thrombotic complications.

Top right. Platelet aggregates and schistocytes on blood smear from a representative baboon that underwent the NMCR and pPBPC transplantation ($3x10^{10}$ /kg). The horizontal arrow indicates baboon platelet aggregates. The 2 vertical arrows indicate typical schistocytes.

Bottom. Immunohistochemical examination of frozen lung tissue obtained at autopsy from B69-360 on day 9 (see Fig. 1 – top left). The two photomicrographs are serial sections (note the blood vessel in the lower left corner) at 100x magnification.

Left. Stained with an anti-CD62 (P-selectin) mAb. Arrow depicts conglomerate of baboon platelets in microvasculature.

Right. Stained with anti-1030H1-19 (pan-pig leukocyte) mAb. Arrow depicts porcine leukocytes in identical location as baboon platelet aggregate.

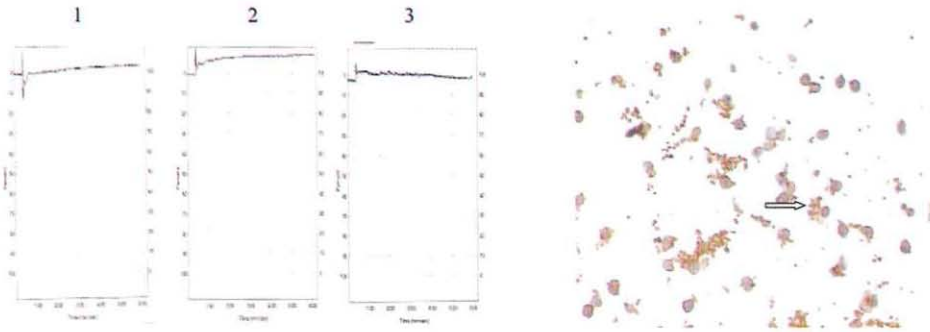

Figure 2.

Left. Graphs depicting the effect of baboon (allo) PBPC on baboon platelet aggregation as measured by light aggregometry. The percentage aggregation is calculated by subtracting the optical density observed immediately after the PBPC have been added (set to 0%) from the optical density observed after 6 minutes. Baboon PBPC are added in increasing numbers. The addition of $1x10^5$ (1), $1x10^6$ (2), or $2x10^6$ (3) baboon PBPC did not cause macroscopic aggregation of baboon platelets.

Right. Immunohistochemical examination of experimental sample from Fig. 2 – left – 2 ($1x10^6$ baboon PBPC + PRP). Specific staining of platelets was performed with an anti-CD62 mAb and leukocytes were visualized by hematoxylin counterstain. The horizontal arrow indicates several aggregated platelets around an activated baboon leukocyte.

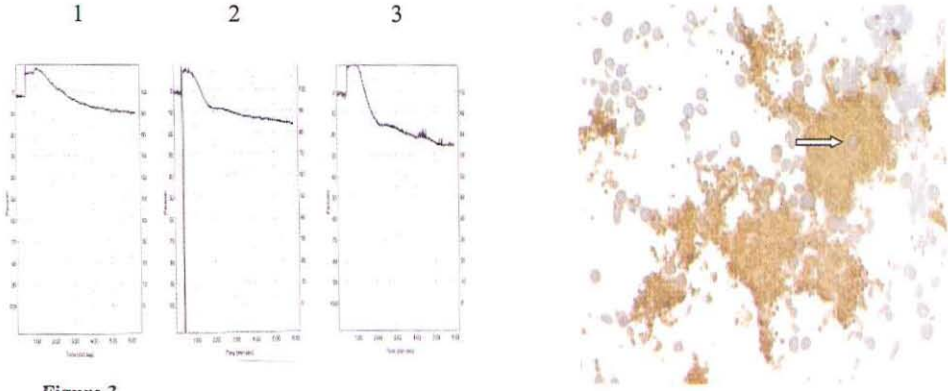

Figure 3.

Left. Graphs depicting the effect of porcine (xeno) PBPC on baboon platelet aggregation as measured by light aggregometry. Porcine PBPC were added in increasing numbers. The addition of $1x10^5$ (1), $1x10^6$ (2), or $2x10^6$ (3) pPBPC induced 17%, 26%, and 35% aggregation of baboon platelets, respectively.

Right. Immunohistochemical examination of experimental sample from Fig. 3 – left – 2 ($1x10^6$ pPBPC + PRP). Specific staining of baboon platelets was performed with an anti-CD62 mAb and of porcine leukocytes with anti-1030H1-19 mAb. Note the extensive platelet conglomerates incorporating porcine leukocytes as indicated by the horizontal arrow.

Addition of cryopreserved pPBPC ($1x10^5$ – $2x10^6$) led to marked aggregation of baboon platelets that was further determined to be dose-dependent (Fig. 3 – left). Platelet aggregation approximated 17%, 26%, and 35% with addition of $1x10^5$, $1x10^6$, and $2x10^6$ pPBPC, respectively. Furthermore, in these samples increased optical density was observed that was dependent on the number of pPBPC added. Variations in platelet aggregation could be observed depending on the pPBPC product and the source of the PRP. In 5 assays, $1x10^6$ pPBPC induced 14% – 63% baboon platelet aggregation, with a mean of 28% (data not shown).

Immunohistochemistry demonstrated multiple large platelet aggregates in conjunction with $1x10^6$ pPBPC (Fig. 3 – right), when compared to baboon PBPC.

Fresh pPBPC induced more platelet aggregation than cryopreserved PBPC (Fig. 4). In 4 assays, $1x10^6$ fresh pPBPC induced 30 – 79% (mean 48%) platelet aggregation. Fresh pPBPC contained more granulocytes and platelets than cryopreserved pPBPC, whereas the latter contained more dead cells (data not shown). The highest percentage of platelet aggregation (79%) was observed with fresh pPBPC that were obtained from a pig that had not been mobilized with porcine growth factors. The main difference found between unmobilized and mobilized pPBPC was the higher proportion of monocytes in mobilized pPBPC that increased relative to the duration of growth factor-mobilization (data not shown).

Fresh or cryopreserved porcine granulocytes, $CD2^+$ cells, $CD2^-$ cells in isolation, or porcine membranes alone were not able to induce aggregation of baboon platelets (Table 1). When all subsets of fresh or cryopreserved pPBPC including the membranes were combined, however, 48% and 14% platelet aggregation was induced, respectively. The omission of exogenous porcine membranes from the preparation consistently failed to induce aggregation. Unsorted fresh or cryopreserved PBPC resulted in 52% and 22% aggregation, respectively.

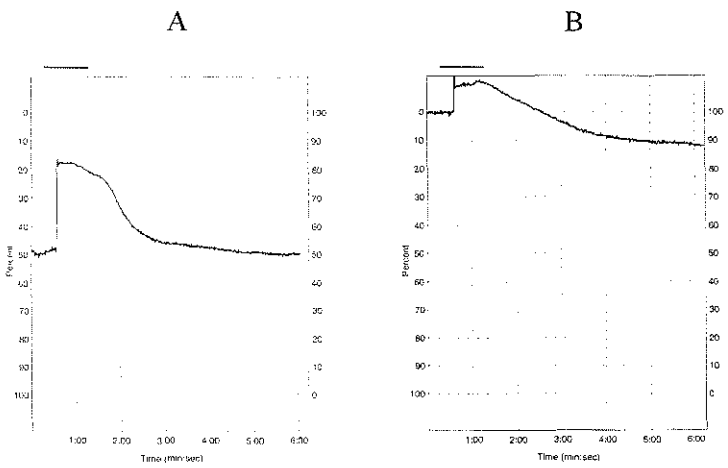

Figure 4.

Graphs comparing the effect of $1x10^6$ fresh and cryopreserved pPBPC (from the same leukapheresis product) on platelet aggregation as measured by light aggregometry. Representative experiments are shown. Fresh pPBPC induced 32% platelet aggregation, whereas cryopreserved pPBPC induced only 21% aggregation. Note that the increase in optical density following the addition of frozen/thawed pPBPC is less than that following the addition of fresh pPBPC, suggesting less exogenous particulate matter in the cryopreserved product.

Pre-incubation of PRP with anti-P-selectin mAb led to a 60% inhibition of platelet aggregation when $1x10^5$ cryopreserved pPBPC were added, when compared to aggregation in the absence of P-selectin (Table 2). Pre-incubation of PRP with anti-CD154 mAb or eptifibatide resulted in 51% and 93% inhibition of platelet aggregation, respectively, after $1x10^6$ cryopreserved pPBPC were added (Table 2), when compared to controls.

TABLE 1

BABOON PLATELET AGGREGATION RESPONSES ARE NOT INDUCED BY PURIFIED FRACTIONS OF PORCINE PBPC

Fractions of pPBPC	Cryopreserved aggregation response (%)	Fresh aggregation response (%)
$CD2^+$	1	2
$CD2^-$	4	1
Granulocytes *	2	0
Exogenous membranes	2	2
Combined fractions **	48	14
Combined fractions w/o membranes	4	0
Total product pre-cell sorting	52	22

Fractions of pPBPC were obtained by cell sorting (as described).

$2x10^6$ cells/fractions are used in all assays except when otherwise noted.

* **$2x10^5$ cells were used in this assay as fewer could be obtained.**

** **Combination of all fractions of pPBPC in equal percentages.**

DISCUSSION

Thrombotic microangiopathy in baboons following the transplantation of large numbers of pPBPC is a serious and potential fatal complication. Furthermore, it also limits the number of pPBPC that could be transplanted, thereby decreasing the possibility of induction of mixed hematopoietic chimerism and anticipated immunologic tolerance. In contrast to the thrombotic microangiopathy following autologous or allogeneic bone marrow transplantation, the onset of thrombocytopenia and other thrombotic events was directly related to the infusion of pPBPC to the baboon, suggesting a direct interaction between pPBPC and baboon platelets. This was confirmed in the baboons that died, where, at autopsy, baboon platelets were demonstrated in close proximity to porcine leukocytes in the microvasculature of lungs and kidneys.

TABLE 2

THE INDUCTION OF BABOON PLATELET AGGREGATION RESPONSES BY CRYOPRESERVED PORCINE PBPC IS MODULATED BY ANTI-P-SELECTIN AND ANTI-CD154 MONOCLONAL ANTIBODIES AND FULLY ABROGATED BY EPTIFIBATIDE

pPBPC	Aggregation response (%)	Decrease from baseline (%)
Anti-P-selectin mAb *	8	60
Anti-CD154 mAb **	22	51
Eptifibatide ***	3	93

$1x10^6$ cells are used in all assays except when otherwise noted.

Baseline aggregation response is obtained in the absence of blocking agents (anti-P-selectin mAb, anti-CD154 mAb, or eptifibatide).

Blocking agents were pre-incubated with PRP for 15 minutes at room temperature.

* **$1x10^5$ cells were used in this assay (for baseline as well as blocking experiment) as fewer could be obtained. Concentration of anti-P-selectin mAb was 5.0 μg/ml.**

** **Concentration of anti-CD154 mAb was 300 μg/ml.**

*** **Concentration of eptifibatide was 5.0 μg/ml.**

In our in vitro studies, porcine PBPC, but not baboon PBPC were able to induce baboon platelet aggregation in a dose-dependent manner. Immunohistological examination demonstrated incorporation of porcine leukocytes into baboon platelet aggregates. Further analysis of the pPBPC product revealed that heightened aggregation was initiated by fresh rather than frozen pPBPC. Fragmented porcine erythrocytes, dead cells, and/or platelets also appeared to contribute to this process.

Based on these data, we have hypothesized several mechanisms that may contribute to this thrombotic microangiopathy. Firstly, dead cells and platelets present in the pPBPC preparations could provide a greater number of xenogeneic membranes, and thereby potentiate complement activation (20). Although suppression of complement activity in baboons undergoing our NMCR is attempted by administering cobra venom factor, we realize that the complement depletion may be incomplete, and, due to the mechanism of action of cobra venom factor, residual complement components

may be in an activated state. Complement activation has been shown to induce and increase the conversion of prothrombin to thrombin, that can subsequently induce platelet aggregation (14, 21, 22, 23) and result in the described thrombotic complications. Secondly, complement has been demonstrated to be associated with the adhesion of human leukocytes to porcine aortic endothelial cells through P-selectin (24). These interactions may be relevant to our model. As adhesion through selectin molecules is conserved across mammals (25), porcine leukocytes may interact with baboon platelets *via* such adhesion molecules. We have presented further evidence to this effect by decreasing the induction of pPBPC-mediated baboon platelet interactions with an anti-P-selectin mAb. Furthermore, since the activation of complement can lead to upregulation of P-selectin (26) and β_2-integrin (27), porcine leukocytes may thus adhere to baboon platelets (28, 29). Aggregation of these activated platelets, initiated by GPIIbIIIa cross-linking, could then result. A third possibility is that porcine monocytes become activated and upregulate adhesion molecules in the absence of overt complement activation or in the presence of sub-lytic levels of complement. Inflammatory cytokines, such as those released from dead cells, are associated with an increased expression of adhesion molecules (30). These cytokines (such as tumor necrosis factor-α) may activate leukocytes, increase the expression of adhesion molecules (such as P-selectin and β-integrins) on the cell surface, consequently activate platelets and induce aggregation responses. These potential mechanisms and others need to be further elucidated.

We have demonstrated that a thrombotic microangiopathy occurs following the transplantation of pPBPC in nonhuman primates, and that this is associated with porcine leukocyte – baboon platelet interaction. We can now present evidence that pPBPC can directly induce the aggregation of baboon platelets in vitro. Aggregation responses can be modulated by anti-CD154 and anti-P-selectin mAbs, and are fully abrogated by eptifibatide (a potent GPIIbIIIa antagonist). The severe thrombotic microangiopathy that is associated with the transplantation of pPBPC into baboons may therefore be ameliorated by stringent purification of the pPBPC product and/or including eptifibatide to the conditioning regimen. Such an approach may allow for the transplantation of larger numbers of pPBPC and facilitate the induction of mixed

chimerism and immunological tolerance. These studies are currently underway in our laboratory.

REFERENCES

1 Cooper DKC, Ye Y, Rolf LL Jr, et al. The pig as a potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, et al. (eds.). Xenotranplantation. Heidelberg, Springer 1991. p481
2 Sachs DH. The pig as a potential xenograft donor. Vet Imm Immunopath 1994; 43: 185
3 Schmoeckel M, Bhatti FN, Zaidi A, et al. Orthotopic heart transplantation in a transgenic pig-to-primate model. Transplantation 1998; 65: 1570
4 Zaidi A, Schmoeckel M, Bhatti F, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584
5 Sablinski T, Emery DW, Monroy R, et al. Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. Transplantation 1999; 67: 972
6 Tomita Y, Yoshikawa M, Zhang QW, et al. Induction of permanent mixed chimerism and skin allograft tolerance across fully MHC-mismatched barriers by the additional myelosuppressive treatments in mice primed with allogeneic spleen cells followed by cyclophosphamide. J Immunol 2000; 165: 34
7 Wekerle T, Kurtz J, Ito H, et al. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. Nat Med 2000; 6: 464
8 Huang CA, Fuchimoto Y, Scheier-Dolberg R, et al. Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. J Clin Invest 2000; 105: 173
9 Kawai T, Poncelet A, Sachs DH, et al. Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance. Transplantation 1999; 68: 1767
10 Alwayn IPJ, Buhler L, Basker M, et al. Coagulation / Thrombotic disorders associated with organ and cell xenotransplantation. Transpl Proc 2000; 32: 1099.
11 Sablinski T, Emery DW, Monroy R, et al. Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. Transplantation 1999; 67: 972
12 Buhler L, Basker M, Alwayn IPJ, et al. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. Transplantation 2000; 70: 1323
13 Holler E, Kolb HJ, Hiller E, et al. Microangiopathy in patients on cyclosporine prophylaxis who developed acute graft-versus-host disease after HLA-identical bone marrow transplantation. Blood 1989; 73: 2018
14 Robson SC, Schulte am Esch J 2nd, Bach FH. Factors in xenograft rejection. Ann N Y Acad Sci 1999; 875: 261
15 Kozlowski T, Shimuzu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody adsorption. Transplantation 1999; 67: 18
16 Buhler L, Awwad M, Basker M, et al. High-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade in baboons prevents an induced anti-pig humoral response. Transplantation 2000; 69: 2296
17 Watts A, Foley A, Awwad M, et al. Plasma perfusion by apheresis through a Gal immunoaffinity column successfully depletes anti-Gal antibody – experience with 320 aphereses in baboons. Xenotransplantation 2000; 7: 181
18 Kozlowski T, Monroy R, Xu Y, et al. Anti-αGal antibody response to porcine bone marrow in unmodified baboons and baboons conditioned for tolerance induction. Transplantation 1998; 66: 176
19 Nash K, Chang Q, Watts A, et al. Peripheral blood progenitor cell mobilization and leukapheresis in pigs. Lab Anim Sci 1999; 49: 645
20 Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 1997; 89: 1121

21 Robinson RA, Worfolk L, Tracy PB. Endotoxin enhances the expression of monocyte prothrombinase activity. Blood 1992; 79: 406
22 Minnema MC, Pajkrt D, Wuillemin WA, et al. Activation of clotting factor XI without detectable contact activation in experimental human endotoxemia. Blood 1998; 92: 3294
23 Peerschke EI, Jesty J, Reid KB, et al. The soluble recombinant form of a binding protein/receptor for the globular domain of C1q (gC1qR) enhances blood coagulation. Blood Coagul Fibrinolysis 1998; 9: 29
24 Rollins SA, Johnson KK, Li L, et al. Role of porcine P-selectin in complement-dependent adhesion of human leukocytes to porcine endothelial cells. Transplantation 2000; 69: 1659
25 Jalkanen S, Reichert RA, Gallatin WM, et al. Homing receptors and the control of lymphocyte migration. Immunol Rev 1986; 91: 39
26 Mulligan MS, Schmid E, Till GO, et al. C5a-dependent up-regulation in vivo of lung vascular P-selectin. J Immunol 1997; 158: 1857
27 Jagels MA, Daffern PJ, Hugli TE. C3a and C5a enhance granulocyte adhesion to endothelial and epithelial cell monolayers: epithelial and endothelial priming is required for C3a-induced eosinophil adhesion. Immunopharmacology 2000; 46: 209
28 Brown KK, Henson PM, Maclouf J, et al. Neutrophil-platelet adhesions: relative roles of platelet P-selectin and neutrophil β2 (CD18) integrins. Am J Resp Cell Mol Biol 1998; 18: 100
29 Diacovo TG, Roth SJ, Buccola JM, et al. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood 1996; 88: 146
30 Teplyakov AI, Pryschepova EV, Kruchinsky NG, et al. Cytokines and soluble cell adhesion molecules. Possible markers of inflammatory response in atherosclerosis Ann N Y Acad Sci 2000; 902: 320

7

Immunosuppressive therapies and platelet aggregation in baboons

Adapted from:
Appel JZ III[*], Alwayn IPJ[*], Buhler L, DeAngelis HA, Cooper DKC[◆], and Robson SC[◆]. Modulation of platelet aggregation in baboons: implications for mixed chimerism in xenotransplantation. 1. The roles of individual components of a transplantation conditioning regimen and of pig peripheral blood progenitor cells. *Transplantation 2001. In press.*
&
Appel JZ III, Alwayn IPJ, Buhler L, Correa LE, Cooper DKC[◆], and Robson SC[◆]. Modulation of platelet aggregation in baboons: implications for mixed chimerism in xenotransplantation. 2. The effects of cyclophosphamide on pig peripheral blood progenitor cell-induced aggregation. *Transplantation 2001. In press.*
[*] Authors contributed equally
[◆] Joint senior authors.

ABSTRACT

Introduction. The induction of tolerance to pig antigens in primates should facilitate the development of successful clinical xenotransplantation protocols. The infusion of mobilized porcine peripheral blood leukocytes (PBPC) into splenectomized pre-conditioned baboons, intended to induce mixed hematopoietic cell chimerism, however, results in thrombocytopenia and a severe thrombotic microangiopathy (TM). As the mechanisms responsible for this phenomenon are unclear, we have explored the effects of individual components of the conditioning regimen, of therapeutic adjuncts and of PBPCs on platelet aggregation.

Methods. Groups of splenectomized baboons (n=2 in each group) were treated with single components of the conditioning regimen - whole body irradiation (WBI), anti-thymocyte globulin (ATG), extracorporeal immunoadsorption (EIA), mycophenolate mofetil (MMF), anti-CD40L mAb, cobra venom factor (CVF), pig hematopoietic growth factors (interleukin-3 (pIL3) and stem cell factor (pSCF)) – or with potential adjuncts, cyclophosphamide (CPP), prostacyclin (PGI2), heparin, methylprednisolone, and eptifibatide. Blood samples were collected and platelet-rich plasma (PRP) was prepared. Using light transmission aggregometry, the extent of aggregation induced by platelet agonists (Thrombin, adenosine diphosphate (ADP), collagen, ristocetin, and arachidonic acid) was determined in vitro. PRP was also prepared from untreated baboons and baboons receiving either eptifibatide (a GPIIb/IIIa antagonist) or CPP and PBPC were added and platelet aggregation was measured in the absence of exogenous platelet agonists.

Results. WBI, ATG, MMF, anti-CD40L mAb, CVF, pIL3 + pSCF, and PGI2 had no effect on purified baboon platelet aggregation profiles in vitro. CPP or eptifibatide markedly inhibited platelet aggregation induced by all standard agonists. Thrombin-induced platelet aggregation was inhibited by EIA or heparin, and ADP-induced aggregation was inhibited to some extent by methylprednisolone. In vitro addition of PBPC to PRP stimulated platelet aggregation in the absence of any agonists. Prior treatment of baboons with eptifibatide or CPP, however, inhibited this effect by 70-80%

and 55%, respectively. TM was not evident in baboons receiving a conditioning regimen that included CPP instead of WBI.

Conclusions. Aggregation of baboon platelets and TM is directly induced by PBPC, but not by individual components of the conditioning regimen. CPP has direct anti-aggregatory properties and may provide an alternative strategy to WBI. GPIIb/IIIa antagonists, such as eptifibatide, interfere directly with xenogeneic PBPC-platelet interactions and may further ameliorate TM in the pig-to-primate model.

INTRODUCTION

Because of the limited availability of suitable human organ donors, xenotransplantation has received considerable attention in recent years. For several reasons, the pig is considered the best candidate for this purpose (1-3). However, the transplantation of porcine tissue into humans or non-human primates elicits a severe and rapid rejection response, resulting in graft loss, even when intensive immunosuppressive therapy is administered (4).

One approach that would overcome the pig-to-primate xenogeneic barrier is the development of donor-specific unresponsiveness, or transplantation tolerance (5). Tolerance has been induced in several allogeneic models using a variety of approaches. Of these, perhaps the most successful protocols are those that establish mixed hematopoietic cell chimerism by infusing donor bone marrow or hematopoietic progenitor cells into conditioned recipients (6).

Our center has infused porcine hematopoietic progenitor cells into conditioned primates in an attempt to induce a state of pig-primate mixed chimerism to promote xenogeneic tolerance (5). Leukocytes are mobilized from porcine bone marrow in vivo using pig-specific hematopoietic growth factors (7-9). These mobilized peripheral blood leukocytes (PBPC), that contain approximately 2% hematopoietic progenitor cells, are collected by leukapheresis (9). The PBPC are then infused into baboons that have undergone a conditioning regimen modified from those that have met with success in allogeneic models (See Chapter 1, Figure 3). This preparative

regimen entails thymic irradiation and non-lethal myelosuppression in the form of whole body irradiation (WBI). Baboons receive anti-thymocyte globulin (ATG) and undergo a process of extracorporeal immunoadsorption (EIA) of anti-Gal (α1-3) Gal (Gal) natural antibodies. Baboons also receive immunosuppression, in the form of mycophenolate mofetil (MMF) and anti-CD40L mAb, cobra venom factor (CVF), and pig-specific hematopoietic growth factors, pig interleukin-3 (pIL3) and pig stem cell factor (pSCF) (10). Once this regimen has been completed, large numbers of porcine PBPC (2-4 x 10^{10}/kg) are infused intravenously.

Although transient engraftment of pig hematopoietic progenitor cells (detectable in the blood by polymerase chain reaction and by flow-activated cell sorting) has been achieved in two of eight baboons receiving this regimen, a severe thrombotic microangiopathy (TM) inevitably develops shortly after the infusion of PBPC (7). This is manifest by pronounced thrombocytopenia (platelet count < 20,000) (See Chapter 5, Figure 2), schistocytosis, and elevation of lactate dehydrogenase. In many regards, this syndrome resembles the TM observed in approximately 14% of bone marrow transplant recipients (11) but, in the xenogeneic combination, develops much earlier (i.e. long before, not after, engraftment). Prothrombin time (PT), partial thromboplastin time (PTT), and fibrinogen levels, although transiently disturbed by EIA, typically remain unaffected. The condition can be fatal in experimental animals, and autopsies reveal microvascular thrombosis and vascular injury in several major organs, including the heart, lungs, and kidneys (Figure 1) (7).

TM represents a considerable barrier to the achievement of xenotransplantation tolerance in this model because it may be associated with significant morbidity and it limits the total number of PBPC that can be infused, therefore reducing the likelihood of establishing mixed hematopoietic cell chimerism. Thus, an improved understanding of the mechanisms underlying the platelet activation/aggregation observed in baboons receiving WBI and PBPC may suggest ways of preventing these sequelae, thereby facilitating mixed chimerism and the induction of xenotransplantation tolerance.

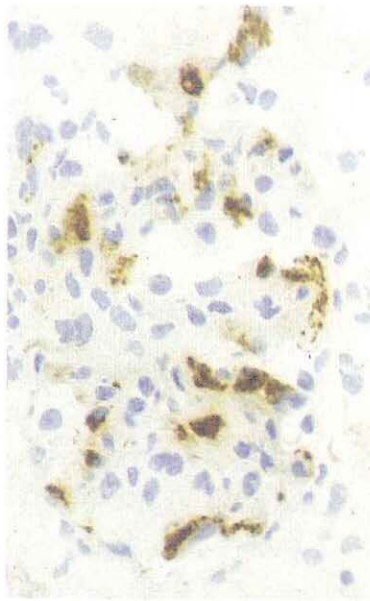

Figure 1.

Baboons receiving WBI develop thrombotic microangiopathy after infusion of pig PBPC. Microvascular thrombi with platelet sequestration develop in organs such as the kidney (stained for P-selectin).

Cyclophosphamide (CPP) has been used as an alternative to irradiation in some instances prior to clinical bone marrow transplantaion (12). A prodrug, CPP is biotransformed by a microsomal oxidase system in the liver to active metabolites. Some of these species inhibit the growth of rapidly proliferating cells, most likely by cross-linking DNA, thereby exerting an immunosuppressive and, possibly, myelosuppressive effect. Additionally, the administration of CPP to some patients with TM of various etiologies has proved therapeutic (13-16). Thus, CPP may be a favorable alternative to WBI in this pig-to-primate model of mixed chimerism and tolerance induction.

In this study, standard light transmission aggregometry was utilized to assess the effects of the individual components of this complex conditioning regimen and of PBPC on platelet aggregation in vitro. Furthermore, the anti-thrombotic properties of CPP and of several potential adjuncts to the current conditioning regimen, including

PGI2, heparin, methylprednisolone, and eptifibatide (a GPIIb/IIIa platelet receptor antagonist) were examined.

MATERIAL & METHODS

Animals

Baboons (Papio anubis, n=12, Biomedical Resources Foundation, Houston, TX), weighing 9-13 kg, were used to test experimental agents in vivo and as a source of platelets for in vitro experiments. All animals had been previously splenectomized through a midline laparotomy. Normal platelet counts and coagulation parameters were present in all baboons at the onset of the present study. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical Cannulation of Vessels

Baboons were anesthetized and the carotid artery and internal jugular vein were cannulated with silastic catheters as described previously (17). A jacket and tethering system was utilized, which allowed continuous or intermittent administration of intravenous (IV) fluids and/or drugs, withdrawal of blood, monitoring of arterial blood pressure, and sedation (as needed) for the baboon. Cefazolin sodium (500 mg IV) was given daily as prophylaxis against infection.

Individual Components of the Conditioning Regimen

All immunomodulatory procedures, agents, or combinations of agents, were tested in vivo in at least two baboons (Table 1). Serial blood samples were taken in order to monitor platelet count, PT, PTT, fibrinogen, fibrinogen degradation products, white blood cell counts, and hematocrit (Hematology Laboratory, Massachusetts General Hospital). Seven baboons were used to test more than one agent, but platelet aggregation and coagulation parameters were allowed to return to baseline before

subsequent drugs were administered. Most agents were infused continuously although bolus injections were used in some instances, as indicated in Table 1.

Collection of Mobilized Peripheral Blood Leukocytes (PBPC)

PBPC were collected from miniature swine using a 16-day leukapheresis protocol described previously (9). Pigs were treated with recombinant hematopoietic growth factors (8-10) before and during the period of leukapheresis. On days 0-15, pIL3 and pSCF were administered daily by subcutaneous injection, each at a dose of 100 μg/kg/d. Human granulocyte colony stimulating factor (Amgen, Thousand Oaks, CA) was administered subcutaneously at 10 μg/kg/d on the evening before each leukapheresis procedure. Leukapheresis was performed on days 5-9 and 12-16 using a Cobe Spectra apheresis machine (Cobe, Lakewood, CO). This leukapheresis product was washed and, following lysis of red blood cells, the remaining PBPC were frozen in 5% dimethylsulfoxide.

Platelet Aggregation Assay

At intervals before, during and after the administration of each agent, or before and after a procedure (e.g. EIA, WBI), 10 ml citrated blood was drawn from the baboon and centrifuged at 280 g for 15 minutes at 22 °C. The supernatant, consisting of platelet-rich plasma (PRP), was removed and the remaining pellet was centrifuged at 2,400 g for 2 minutes at 22 °C to obtain platelet-poor plasma. The final PRP was transferred in 400 μl aliquots to siliconized glass cuvettes, comprising the experimental samples. A 400-μl aliquot of platelet-poor plasma was used as a reference sample, to which platelet aggregation in PRP samples could be compared.

TABLE 1

AGENTS / PROCEDURES ADMINISTERED TO BABOONS

Agent/Procedure Tested	Dose Administered	References Suggesting Pro-/Anti-Thrombotic Properties
Whole body irradiation	150 cGy/d x 2d	18-21
Anti-thymocyte globulin	50 mg/kg/d CIV x 3d	22
Extracorporeal Immunoadsorption	Daily x 3d	23, 24
Mycophenolate mofetil	110 mg/kg/d CIV	22, 25
Anti-CD40L mAb	40 mg/kg x 1	26-29
Cobra venom factor	100-200 U/kg/d x 3d	30-32
Recombinant pIL3 + Recombinant pSCF	200 μg/kg bolus, 400 μg/kg/d CIV x 2d 1 mg/kg bolus, 2 mg/kg/d CIV x 2d	
Cyclophosphamide	40 mg/kg/d x 3d	12-16, 33, 34
Prostacyclin (PGI2)	20-30 ng/kg/min CIV x 3d	35-37
Heparin sodium	10 – 50 U/kg/hr CIV x 5d	38, 39
Methylprednisolone	2 mg/kg twice daily x 3d	40
Eptifibatide (GPIIb/IIIa antagonist)	180 μg/kg bolus, 2.0-7.5 μg/kg/min CIV x 1d	41, 42

pIL3 = porcine interleukin 3, pSCF = porcine stem cell factor, CIV = continuous intravenous infusion

Four of five standard platelet agonists (thrombin (1.25 U/ml), adenosine diphosphate (ADP) (20 μM), collagen (10 μg/ml), and ristocetin (1.25 μg/ml)) were added to the experimental samples, and the extent of aggregation over a period of six minutes was measured using a platelet aggregometer (two-sample, four-channel, model 560 Ca Lumi-aggregometer, Chronolog Corp., Havertown, PA). Because ristocetin (which acts via GPIb receptors in association with vWF) was found to be a less potent agonist, arachidonic acid (which is converted into pro-aggregatory metabolites PGH2

and TXA2) (1.2 mM) was used in some instances. By adding a small rotating magnet to each sample, shear stress was created in order to simulate intravascular flow conditions.

Whenever PBPC were to be added to baboon platelets in vitro, blood was drawn from untreated baboons and, in the manner described above, PRP was prepared. Immediately prior to use, PBPC were rapidly thawed in a water bath at 37 °C and were washed twice with calcium-, magnesium-, and phenol red-free Hanks balanced salt solution. In the absence of any agonists, increasing numbers of PBPC (1×10^5, 2×10^5, 1×10^6, and 2×10^6) were added to separate PRP samples and aggregation was measured.

Immunohistochemical Staining of PBPC-stimulated Aggregates

Experimental PRP samples to which PBPC had been added were centrifuged at 10,000 rpm for 20 seconds. The supernatant was aspirated from these and the remaining pellet was resuspended in 20 µl supernatant, and frozen in TBS tissue freezing medium (American Mater-Tech Scientific, Lodi, CA). From these frozen samples, 5 µm-thick sections were prepared and subsequently stained with anti-P-selectin or 1030H-1-19, a pan-pig monoclonal antibody developed by our laboratory.

RESULTS

Effects of Individual Components of the Conditioning Regimen on Platelet Aggregation

Table 2 summarizes platelet aggregation (relative to baseline) measured in PRP prepared from baboons treated with each of the individual components of the conditioning regimen. A change in platelet aggregation was considered to be substantial if it differed by more than 20% from baseline measurements and occurred in both baboons tested.

Exposure to WBI did not affect platelet aggregation, which was assessed 48 hours following the second fractionated dose. Following WBI, platelet counts in both

baboons decreased gradually, reaching values approximating 20% of baseline measurements after 8 and 10 days, respectively. Coagulation parameters and hematocrit remained stable during this period. Administration of CPP on three consecutive days (a total dose of 120 mg/kg), however, resulted in a progressive inhibition of agonist-stimulated platelet aggregation that was most pronounced after approximately 6-8 days (Figure 2). Aggregation induced by thrombin, ADP, collagen, and arachidonic acid was decreased from baseline by as much as 62%, 85%, 84%, and 95% respectively, in one baboon and by 74%, 98%, 83%, and 98% in another. Fourteen days after administration of the drug, platelet aggregation returned to pre-treatment levels in both baboons. Coagulation parameters were not altered significantly during the experiment. CPP-treated baboons experienced a progressive, but transient, decrease of white blood cells (from approximately 9,000/μl to 900/μl and from 12,000/μl to 800/μl over 7 days) and of platelets (from approximately 310,000/μl to 66,000/μl and from 300,000/μl to 170,000/μl over 11 days), which returned to pre-treatment values 16 days following administration of the drug.

To verify that the reduction in platelet aggregation was independent of platelet count, PRP was prepared from untreated baboons. Baseline platelet aggregation induced by thrombin, ADP, collagen, and arachidonic acid was 27%, 30%, 62%, and 20%, respectively. When diluted (from an initial count of 540,000 platelets/μl) as much as eight-fold with calcium-, magnesium-, and phenol red-free Hanks balanced salt solution, platelet aggregation induced by thrombin, ADP, and collagen was unaffected (22%, 33%, and 65%, respectively). Similar results were obtained when PRP was diluted with platelet-poor plasma.

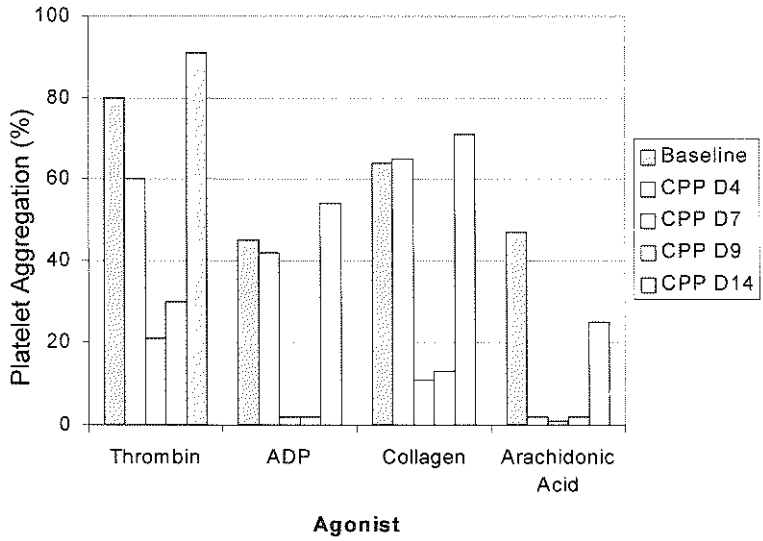

Figure 2.

Effect of the in vivo administration of cyclophosphamide (total dose 120 mg/kg) on in vitro baboon platelet aggregation. Platelet aggregation by all agonists was partially inhibited 6-11 days after cyclophosphamide administration. ADP = adenosine diphosphate.

The process of EIA had a transient inhibitory effect on thrombin-induced platelet aggregation (by 98%) (Table 2). Coagulation parameters, measured at the same time, were prolonged (PT >30 s, PTT > 80 s) but recovered over a period of 12-18 hours. Administration of ATG, MMF, anti-CD40L mAb, or CVF to individual baboons, however, had no substantial effects on the aggregation of baboon platelets. As anticipated, baboons receiving ATG developed leukopenia, but no thrombocytopenia, and this was rapidly reversed upon discontinuation of treatment. Laboratory parameters were otherwise unchanged in these baboons.

Inconsistent effects on platelet aggregation were observed in baboons receiving either hematopoietic growth factors (pIL3 + pSCF) or PGI2. Ristocetin-induced platelet aggregation was slightly increased (by 25%) in one baboon receiving pIL3 and pSCF. Platelet aggregation was unaffected in baboons receiving PGI2 20 ng/kg/min,

although ADP- and collagen-induced platelet aggregation was inhibited by 24% and 23%, respectively, in one baboon receiving 30 ng/kg/min. No disturbances of vital signs, coagulation parameters, or other adverse effects were noted in these baboons.

As predicted, heparin reduced thrombin-induced platelet aggregation in a dose-dependent manner. Little effect on global platelet parameters and aggregation responses was apparent in baboons maintained on a continuous infusion of 10 U/kg/hour. However, considerable inhibition of thrombin-induced aggregation (by 90%) was evident at 50 U/kg/hr. PTT was prolonged to >150 seconds in contrast to baseline parameters of < 30 seconds.

In both baboons that received methylprednisolone, platelet aggregation induced by ADP was inhibited (by 43% and by 50%). Arachidonic acid-induced platelet aggregation was inhibited in one baboon by up to 93% on two separate days, but was unaffected in a second baboon.

The administration of eptifibatide resulted in potent and global inhibition of platelet aggregation (Figure 3). This was dose-dependent, such that continuous infusion at 2.0 μg/kg/min had little effect (0-48% inhibition), whereas 7.5 μg/kg/min inhibited aggregation induced by all agonists tested (by 84 - 99%). The onset of this effect was rapid, evident as early as 30 minutes after initiation of the drug infusion and was rapidly reversible, platelet aggregation returning to baseline levels within two hours after discontinuation of therapy.

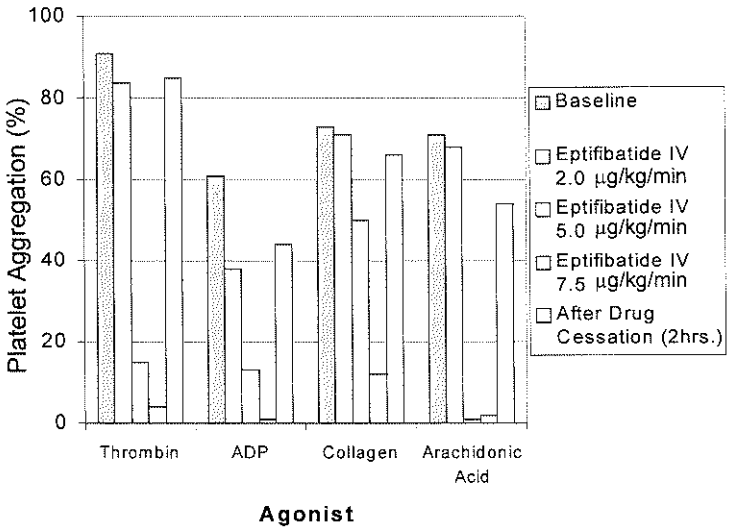

Figure 3.

Effect of the in vivo administration of eptifibatide on in vitro baboon platelet aggregation. Eptifibatide inhibited platelet aggregation induced by all agonists, and had an increasing inhibitory effect at higher dosages. ADP = adenosine diphosphate.

Vital signs, coagulation, and other laboratory parameters were unaffected at all doses tested, and bleeding episodes or other adverse effects were not observed.

Effect of PBPC on Platelet Aggregation

In vitro, the addition of porcine PBPC to PRP from untreated baboons stimulated platelet aggregation in the absence of any agonist, and increased with the number of PBPC added (Figure 4A & B). Although no aggregation was detected when 1 x 10^5 cells were added, the addition of 2 x 10^5, 1 x 10^6, and 2 x 10^6 PBPC induced aggregation by as much as 17%, 26%, and 35%, respectively, in one baboon. This trend was apparent in a total of four experiments using PBPC and PRP from different pigs and baboons. Immunocytochemistry using anti-P-selectin and/or the pan-pig preparation, 1030H-1-19, demonstrated the aggregation of baboon platelets around porcine leukocytes (See Chapter 6, Figure 3). When PBPC were added in vitro to

PRP prepared from baboons treated with eptifibatide (7.5 μg/kg/min IV), platelet aggregation was prevented by > 70% (Figure 4C). Similarly, PBPC-induced platelet aggregation was inhibited by 55% in PRP prepared from a baboon 6 days after the administration of CPP (120 mg/kg total dose) (Figure 4D). In baboons treated with a CPP-based conditioning regimen instead of WBI, TM was not evident following the infusion of PBPC. Although platelet counts in CPP-treated baboons decreased to some extent, the profound thrombocytopenia and thrombotic complications characteristic of WBI-treated baboons did not develop and platelet counts recovered earlier (Figure 5 – Top). Schistocytosis was not detected and lactate dehydrogenase levels were unaffected in these CPP-treated baboons (Figure 5 – Bottom).

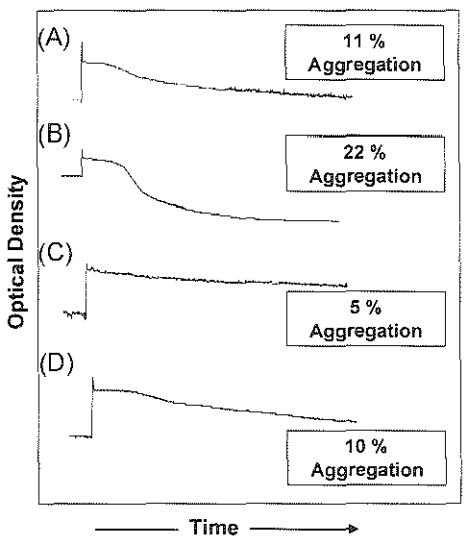

Figure 4.

Platelet aggregation curves of naïve baboons obtained after in vitro addition of porcine PBPC.

(A) Addition of $1x10^5$ porcine PBPC to baboon PRP containing approxiamtely $1x10^8$ platelets led to 11% aggregation. (B) Addition of 2 x 10^6 PBPC resulted in 22% platelet aggregation. (C) When platelets were prepared from the same baboon after treatment with eptifibatide, platelet aggregation induced by 2 x 10^6 PBPC was inhibited by > 70% (to 5%). (D) Platelet aggregation induced by 2 x 10^6 PBPC was inhibited by 55% (to 10%) in PRP prepared from the same baboon 6 days after treatment with CPP.

TABLE 2

EFFECT OF VARIOUS IN VIVO AGENTS/PROCEDURES ON IN VITRO BABOON PLATELET AGGREGATION

Agent/Procedure Tested	N	Platelet Agonist				
		Thrombin	ADP	Collagen	Ristocetin	Arachidonic Acid
WBI	2	-	-	-	-	NA
Anti-thymocyte globulin	2	-	-	-/↑	-	NA
EIA	2	↓	-	-	-	NA
Mycophenolate mofetil	2	-	-/↓	-	-	NA
Anti-CD40L mAb	2	-	-	-	-	NA
Cobra venom factor	2	-	-	-/↓	-	NA
pIL3 + Pscf	2	-	-	-	-/↑	NA
Cyclophosphamide	2	↓	↓	↓	NA	↓
PGI2 (20 ng/kg/min)	2	-	-	-	NA	-
PGI2 (30 ng/kg/min)	2	-	-/↓	-/↓	NA	-
Heparin	2	↓	-	-	NA	-
Methylprednisolone	2	-	↓	-	NA	-/↓
Eptifibatide	2	↓	↓	↓	NA	↓

↓ or ↑ indicates a change in platelet aggregation >20% from baseline.

– indicates a change of <20%. NA indicates agonist not used in corresponding experiment. WBI = whole body irradiation, EIA = extracorporeal immunoadsorption, pIL3 = porcine interleukin 3, pSCF = porcine stem cell factor, PGI2 = prostacyclin, ADP = adenosine diphosphate.

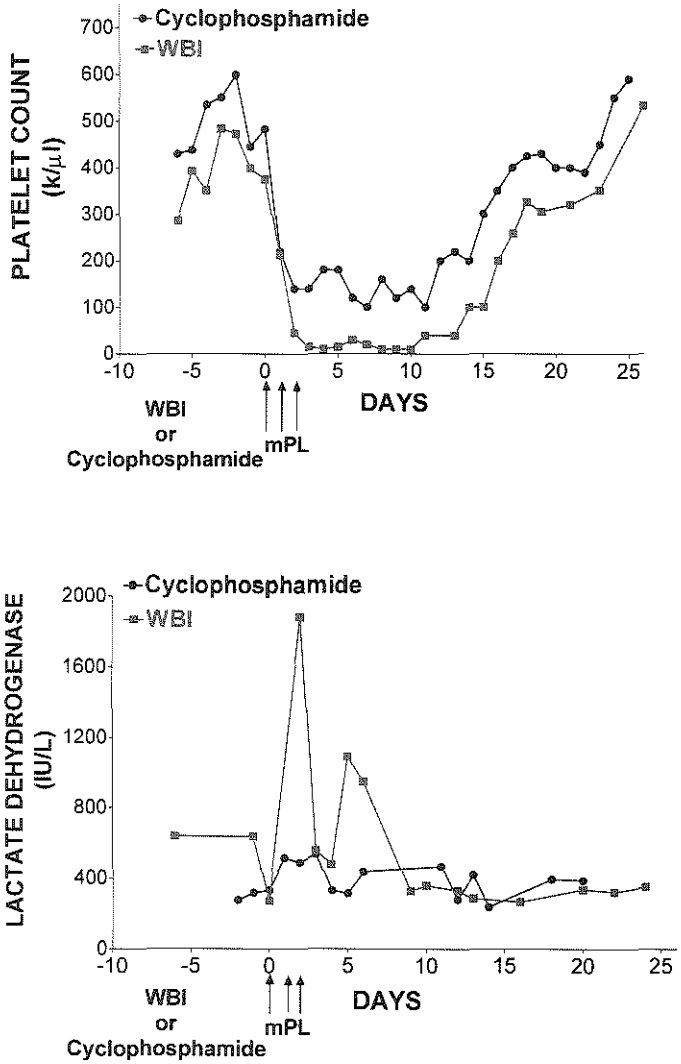

Figure 5.

Platelet count and lactate dehydrogenase levels in baboon recipients of PBPC treated with WBI- or cyclophosphamide-based conditioning regimens. (Top) Thrombocytopenia is much less pronounced and (Bottom) lactate dehydrogenase levels are more stable in baboons receiving cyclophosphamide than in those undergoing WBI.

DISCUSSION

Substantial advances in xenotransplantation have enabled hyperacute rejection to be overcome (4). The onset of acute vascular rejection to be delayed in part by the administration of intensive immunosuppressive therapy. However, even if acute vascular rejection (delayed xenograft rejection) can be prevented, the acute cellular response is likely to be strong. Long-term survival of discordant xenografts may therefore depend on the successful induction of transplantation tolerance.

By infusing donor hematopoietic progenitor cells into recipients this way, and inducing a state of mixed hematopoietic cell chimerism, tolerance has been achieved in allogeneic animal models (6) and, recently, in a human renal allograft recipient (43). Using a complex conditioning regimen (as described above) (4, 7), transient bone marrow engraftment of pig progenitor cells has been achieved in some baboons pretreated with WBI and thymic irradiation. However, baboons undergoing WBI exhibit a severe TM immediately after the infusion of PBPC (7).

The current study investigated the roles of the individual components of the conditioning regimen, and of PBPC, in the aggregation of baboon platelets. Furthermore, it assessed the anti-thrombotic properties of CPP, an alternative to WBI, and of several potential adjuvants, including PGI2, heparin, methylprednisolone, and eptifibatide, a GPIIb/IIIa platelet receptor antagonist.

When administered individually to baboons, no single component of the WBI-based conditioning regimen increased platelet aggregation. Porcine PBPC, when added to baboon platelets in vitro, initiated aggregation in the absence of any platelet agonists. Immunocytochemistry, using anti-human P-selectin and 1030H-1-19 (a pig-specific stain), confirmed the formation of PBPC-platelet aggregates. This phenomen increased with larger numbers of PBPC and suggests that they play a primary role in the formation of platelet microthrombi in vivo. It is likely that the aggregation of platelets and the sequestration in the microvasculature contribute to the thrombocytopenia and TM observed in conditioned baboons after PBPC infusion.

The observation that WBI had no effect on platelet aggregation is of interest as the formation of platelet microthrombi can occur following irradiation in vivo (20, 21). The detrimental effects of irradiation on the vascular endothelium are well documented (18, 19) and include endothelial cell activation (indicated by upregulation of ICAM-1 and P-selectin), vacuolization, and separation from the basement membrane (20). As WBI does not affect the inherent tendency of platelets to aggregate, as indicated by the results of this study, the formation of microthrombi in irradiated patients may depend largely on endothelial damage. In contrast to the TM observed in baboon recipients of porcine PBPC, the clinical sequelae of bone marrow allotransplantation typically require several days as opposed to hours to become manifest (11). Although irradiation may inflict endothelial damage in the baboons in our studies, which may lead to the formation of microthrombi in some instances, it is unlikely that this contributes primarily to the TM observed immediately following the administration of PBPC.

When administered to baboons, CPP inhibited platelet aggregation induced by all agonists. This effect may explain why treatment with CPP instead of WBI prior to the infusion of PBPC protected baboons against TM. In contrast to WBI-treated baboons, those receiving CPP appeared to tolerate the infusion of PBPC without major complications and with only a minor reduction in platelet count (Figure 5 - Top). However, engraftment may be compromised as it was not observed in baboons receiving CPP. CPP may be a less effective mode of myelosuppression in this model, or it may not create the bone marrow "space" necessary for PBPC engraftment. However, the use of CPP as an alternative, or in combination with a lower dose of WBI, may yield certain advantages.

Although not reported here, the infusion of PBPC into baboons in the absence of the conditioning regimen initiated a state nearly identical to the TM seen in baboons receiving the full regimen, confirming the primary role of PBPC in this syndrome (See Section 4, Chapters 5 & 6). This finding also supports the conclusion that CPP is protective against TM, since this syndrome develops in baboons (with or without

WBI), but is not evident in those receiving CPP. This also demonstrates that WBI is not necessary for the initiation of TM, although it may exacerbate this state by delaying marrow regeneration. Moreover, additional effects of WBI, such as endothelial cell damage, may also contribute to the formation of microthrombi.

The inhibition of platelet aggregation by CPP was not apparent until approximately one week after infusion of the drug. Thus, the direct actions of one or several metabolites of CPP on platelets could contribute. Two of these, 4-hydroxy-CPP and acrolein, have been implicated in at least one other report (17). Data from preliminary experiments in our laboratory suggest that the effects of CPP on baboon platelets result from the modification of signal transduction pathways that are involved in normal aggregation (data not shown).

Because the cytotoxic effect of CPP metabolites has been attributed to their ability to cross-link DNA nucleotides, our laboratory has hypothesized that such complexes could bind purinergic receptors resulting in blockade at the platelet surface. However, using serotonin and epinepherine, which would bypass purinergic receptor blockade, indicate that this is not the case (data not shown).

The maximal CPP-induced inhibition of platelet aggregation was apparent on days when platelet counts approached their nadirs (66,000/μl and 170,000/μl, respectively). Previous reports have indicated that ADP- or collagen-induced platelet aggregation is sustained at platelet counts as low as 50,000/μl (44). Our control studies, in which PRP (with an initial platelet count of 540,000/μl) was diluted as much as eight- to twelve-fold, confirmed these findings for ADP and collagen, as well as for thrombin. Thus, a low platelet count alone would not account for this decrease in platelet aggregation. It is possible that this decrease in platelet count reflects CPP-induced megakaryocyte depletion or inhibition, resulting in temporarily diminished platelet regeneration. Subsequent recovery of megakaryocyte function and production of new platelets would account for the concomitant increase in platelet count and return of platelet aggregation to baseline.

The EIA of anti-Gal antibodies transiently prevented thrombin-induced platelet aggregation. The activation of platelets and coagulation factors by circulation through the immunoadsorption apparatus could contribute to this effect. This may have been exacerbated further, however, by the high concentration of citrate used as an anticoagulant during EIA, which may have had marginal effects on blood coagulation and platelet activation (45, 46).

Of the potential therapeutic agents administered to baboons, eptifibatide was the most effective platelet antagonist, inhibiting platelet aggregation by all agonists tested. This effect was related to the dose of the drug administered (0-48% inhibition at 2.0 μg/kg/min. and 84-99% inhibition at 7.5 μg/kg/min.). The effect was rapidly induced, requiring administration for only 30 minutes, and readily reversible, returning to near-baseline values within 2 hours after cessation. As expected, heparin inhibited the aggregation of baboon platelets by thrombin alone, as heparin catalyzes thrombin degradation by associating with anti-thrombin. Methylprednisolone inhibited ADP-induced platelet aggregation by as much as 55%, an effect that, to our knowledge, has not been reported previously, but that could relate to membrane stabilization and the inhibition of phospholipases (40). In contrast to results in other animal models (36, 37), PGI2 did not inhibit platelet aggregation substantially. This may indicate that this preparation of PGI2 is less effective in baboons than in humans. Alternatively, as PGI2 also acts on the endothelium, the antiplatelet properties of this compound may require the presence of a functioning endothelium and thus may not be apparent in this model of platelet aggregation.

When PRP was prepared from baboons treated with eptifibatide, PBPC-induced in vitro platelet aggregation was inhibited by > 70%. This suggests that eptifibatide, or other GPIIb/IIIa antagonists, would be beneficial adjuncts to the conditioning regimen currently used. For this reason, trials investigating the role of eptifibatide in baboons receiving porcine PBPC have begun. Furthermore, PBPC-induced platelet aggregation was also inhibited (by 55%) in PRP prepared from a baboon 6 days after administration of CPP. The ability of CPP to abrogate PBPC-induced platelet

aggregation provides further evidence that CPP may protect baboons receiving PBPC against TM.

The experimental model of platelet aggregation used in the present study provides considerable information on the ability of various agents to alter the threshold for platelet activation and aggregation in baboons. Most molecular interactions would continue to occur in vivo after the administration of each agent or procedure prior to withdrawal of blood samples. Additionally, since the PRP subsequently prepared contains most of the components of blood, many molecular interactions would also occur during in vitro assays. By inducing an element of low shear stress, the platelet aggregometer mimics flow conditions in the venous (but not the arterial) vasculature to some extent. However, this model is limited in that it does not account for the contributions imparted by the endothelium (whether intact and functional or in a pathological state) including cell-cell interactions, the release of soluble mediators, and vasoconstrictive/vasodilatory responses. Thus, in future endeavors, it would be useful to utilize assays that better assess the role of the endothelium, such as intravital video microscopy.

Although this study demonstrates that porcine PBPC initiate the formation of baboon platelet aggregates in vitro, resulting in thrombocytopenia when infused in vivo, it does not elucidate which component of the leukapheresis product is primarily responsible. Flow-activated cell sorting studies demonstrate that the PBPC leukapheresis product (after one freezing/thawing cycle) contains approximately 30% T cells, 45% B cells and monocytes, 20% debris (e.g. dead cells and cellular fragments), and <1% granulocytes. Any of these cellular subsets, or even soluble factors that they have released, could contribute to this phenomenon. To determine whether or not specific cellular subsets of porcine PBPC are responsible for TM, in vitro studies have begun to assess which, if any, specific fractions induce platelet aggregation and/or endothelial cell activation. It may be that aggregation is induced by a fraction that is unnecessary for the engraftment of porcine hematopoietic progenitor cells, in which case the removal of this subset from PBPC may eliminate

the morbidity currently associated with PBPC infusion and facilitate mixed chimerism.

Substantial PBPC-induced platelet aggregation has only been observed between discordant species in our laboratory. The addition of baboon PBPC to baboon PRP, for example, does not result in substantial platelet aggregation (see Chapter 6). Thus, it will be important to determine if immunological factors or molecular barriers contribute. It is unlikely that aggregation is mediated by baboon complement since the depletion of complement by CVF has no effect on the course of TM. Likewise, it is improbable that preformed xenoreactive antibodies play a major role since the depletion of anti-Gal (by >95%) by EIA does not affect PBPC-induced platelet aggregation (Appel, J.Z., et al. – unpublished observations). It may be that specific porcine PBPC-baboon platelet ligands interact more extensively than species-specific ligands and therefore result in more substantial platelet aggregation. Specific inhibition of these interactions (e.g. by CPP or GPIIb/IIIa antagonists) may abrogate this syndrome.

Nevertheless, it is likely that the TM observed in baboons receiving PBPC results from a multifactorial process. It is possible that the combination of some components of the conditioning regimen exacerbate TM in baboons. Although platelet aggregation is not increased after treatment with the full WBI-based conditioning regimen (without PBPC) (Alwayn, I.P.J. – unpublished observations), these baboons may exhibit a dysfunction in platelet-endothelial interactions not detected in this in vitro model. Furthermore, it is likely that the mechanisms involved in TM extend beyond simple platelet-platelet and platelet-leukocyte interactions. It will be important to assess the tendency of porcine PBPC and of PBPC-induced platelet aggregates to activate primate endothelial cells and studies to investigate this have already been initiated.

REFERENCES

1. Cooper DKC, Ye Y, Rolf LL Jr., Zuhdi N. The pig as potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, White DJG, eds. *Xenotransplantation*. 1st ed. Heidelberg, Germany: Springer, 1991: 481.
2. Sachs DH. The pig as a potential xenograft donor. *Vet Immunol Immunolopathol*. 1994; 43: 185.
3. Appel JZ III, Buhler L, Cooper DKC. The pig as a source of cardiac xenografts. *J Cardiac Surg*. (In press).
4. Buhler L, Friedman T, Iacomini J, Cooper DKC. Xenotransplantation – state of the art - update 1999. *Frontiers in Bioscience*. 1999: 4: d416. http://www.bioscience.org/1999/v4/d/buhler/fulltext.htm
5. Sykes M, Sachs DH. Xenogeneic tolerance through hematopoietic cell and thymic transplantation. In: Cooper DKC, et al, eds. *Xenotransplantation*. 2nd ed. Heidelberg, Germany: Springer; 1997: 496.
6. Kawai T, Poncelet A, Sachs DH, et al. Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance. *Transplantation*. 1999; 68: 1767.
7. Buhler L, Basker M, Alwayn IPJ, et al. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. *Transplantation*. (In press).
8. Colby C, Chang Q, Fuchimoto Y, et al. Cytokine-mobilized peripheral blood progenitor cells for allogeneic reconstitution of miniature swine. *Transplantation*. 1999; 69: 135.
9. Nash K, Chang Q, Watts A, et al. Peripheral blood progenitor cell mobilization and leukapheresis in pigs. *Lab Anim Sci*. 1999; 49: 645.
10. Kozlowski T, Monroy R, Giovino M, et al. Effect of pig-specific cytokines on mobilization of hematopoietic progenitor cells in pigs and on pig bone marrow engraftment in baboons. *Xenotransplantation*. 1999; 6: 17.
11. Pettit AR, Clark RE. Thrombotic microangiopathy following bone marrow transplantation. *Bone Marrow Transplant*. 1994; 14: 495.
12. Bimalangshu D, Sykes M, Spitzer TR. Outcomes of recipients of both bone marrow and solid organ transplants. *Medicine*. 1998; 77: 355.
13. Perez-Sanchez I, Anguita J, Pintado T. Use of cyclophosphamide in the treatment of thrombotic thrombocytopenia purpura complicating systemic lupus erythematous: report of two cases. *Ann Hematol*. 1999; 78: 285.
14. Ruggenenti P, Remuzzi G. Thrombotic thrombocytopenia and related disorders. *Hem. – Onc. Clin. N. Amer*. 1990; 4: 219.
15. Strutz F, Wieneke U, Braess J, et al. Treatment of relapsing thrombotic thrombocytopenia purpura with cyclophosphamide pulse therapy. *Nephrology, Dialysis, Transplantation*. 1998; 13: 1320.
16. Nomura M, Okada J, Tateno S, et al. Renal thrombotic microangiopathy in a patient with rheumatoid arthritis and antiphospholipid syndrome: successful treatment with cyclophosphamide pulse therapy and anticoagulant. *Int. Med*. 1994; 33: 484.
17. Cooper DKC, Ye Y, and Niekrasz M. Heart transplantation in primates. In: Cramer DV, Podesta L, and Makowka L, eds. *Handbook of Animal Models in Transplantation Research*. Boca Raton: CRC Press. 1994: 173.
18. Hallahan DE, Virudachalam S. Ionizing radiation mediates expression of cell adhesion molecules in distinct patterns within the lung. *Cancer Res*. 1997; 57: 2096.
19. Hallahan DE, Kuchibhotla J, Wyble C. Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium. Cancer Res. 1996; 56: 5150.
20. Phillips TL. An ultrastructural study of the development of radiation injury in the lung. *Radiology*. 1966; 87: 49.
21. Sporn LA, Rubin P, Marder VJ, Wagner DD. Irradiation induces release of von willebrand protein from endothelial cells in culture. *Blood*. 1984; 64: 567.
22. In: Schrefer J, et al., eds. *Mosby's GenRx*. 10th ed. St. Louis, Missouri: Mosby. 2000.
23. Xu Y, Lorf T, Sablinski T, et al. Removal of anti-porcine natural antibodies from human and nonhuman primate plasma *in vitro* and *in vivo* by a Gal α1-3Galß1-4ßGlc-X immunoaffinity column. *Transplantation*. 1998; 65: 172.

24. Kozlowski T, Shimizu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody absorption. *Transplantation.* 1999; 67: 18.
25. Blaheta RA, Nelson K, Opperman E, et al. Mycophenolate mofetil decreases endothelial prostaglandin E2 in response to allogenic T cells or cytokines. *Transplantation.* 2000; 69: 1977.
26. Kobayashi T, Taniguchi S, Neethling FA, et al. Delayed xenograft rejection of pig-to-baboon cardiac transplants after cobra venom factor therapy. *Transplantation.* 1997; 64: 1255.
27. Leventhal JR, Dalmasso AP, Cromwell JW, et al. Prolongation of cardiac xenograft survival by depletion of complement. *Transplantation.* 1993; 55: 857.
28. Candinas D, Lesnikoski B, Hancock W, et al. Inhibition of platelet integrin GPIIbIIIa prolongs survival of discordant cardiac xenografts. *Transplantation.* 1996; 62: 1.
29. Kawai, T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody and CD40 ligand. *Nature Med.* 2000, 6: 114.
30. Kirk, A.D., Harlan, D.M. Reply to Kawai, T., et al. *Nature Med.* 2000, 6: 114.
31. Biogen, Inc. Biogen says it has halted several tirals of anti-CD40 ligand monoclonal antibody (Press release). http://www.biogen.com/index2.html. Oct. 21, 1999.
32. Henn V, Slupsky JR, Grafe M, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature.* 1998; 391: 591.
33. Karolak L, Chandra A, Khan W, et al. High-dose chemotherapy-induced platelet defect: inhibition of platelet signal transduction pathways. *Mol. Pharm.* 1993; 43: 37.
34. Panella TJ, Peters W, White JG, Hannun YA, Greenberg CS. Platelets acquire a secretion defect after high-dose chemotherapy. *Cancer.* 1990; 65: 1711.
35. Dormandy JA. Rationale for antiplatelet therapy in patients with atherothrombotic disease. *Vasc Med.* 1998; 3: 253.
36. Whittle BJ, Moncada S, Vane JR. Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species. *Prostaglandins.* 1978; 16: 373.
37. Nishimura H, Naritomi H, Iwamoto Y, et al. In vivo evaluation of antiplatelet agents in gerbil model of carotid artery thrombosis. *Stroke.* 1996; 27: 1099.
38. Boneu B. New antithrombotic agents for the prevention and treatment of deep vein thrombosis. *Haemostasis.* 1996; 26: 368.
39. Sakuragawa N. Regulation of thrombosis and hemostasis by antithrombin. *Semin Thromb Hemost.* 1997; 23: 557.
40. Samuelsson B, Dahler SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science.* 1987; 237: 1171.
41. Kleiman NS, Lincoff AM, Flaker GC, et al. Early percutaneous coronary intervention, platelet inhibition with eptifibatide, and clinical outcomes in patients with acute coronary syndromes. PURSUIT Investigators. *Circulation.* 2000; 101: 751.
42. Stringer KA. The evolving role of platelet glycoprotein IIb/IIIa inhibitors in the management of acute coronary syndromes. *Ann. Pharmacother.* 1999; 33: 712.
43. Spitzer TR, Delmonico F, Tolkoff-Rubin N, et al. Combined histocompatibility leukocyte antigen-matched donor bone marrow and renal transplantation for multiple myeloma with end stage renal disease: the induction of allograft tolerance through mixed lymphohematopoietic chimerism. *Transplantation.* 1999; 68: 480.
44. Levine PH. The effect of thrombocytopenia on platelet aggregation. *Am. J. Clin. Pathol.* 1976; 65: 79.
45. Loftus JC, Smith JW, Ginsberg, MH. Integrin-mediated cell adhesion: the extracellular face. *J. Biol. Chem.* 1994; 269: 25235.
46. Marguerie GA, Edington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J. Biol. Chem.* 1980, 255: 154.

8

Pharmacologic inhibition of platelet aggregation in baboons

Adapted from:
Alwayn IPJ*, Appel JZ III*, Goepfert C, Buhler L, Cooper DKC, and Robson SC. Inhibition of platelet aggregation in baboons: therapeutic implications for xenotransplantation. *Xenotransplantation 2000;7(4)247-257*

* Authors contributed equally.

ABSTRACT

Introduction. Activation of endothelial cells and platelet sequestration play a major role in rejection of xenografts. The histopathology of both hyperacute and acute vascular or delayed rejection of vascularized discordant xenografts is characterized by interstitial hemorrhage and intravascular thrombosis. Agents that prevent platelet activation and consequent microthrombus formation have proven beneficial in xenograft rejection but do not fully preclude vascular thrombosis. Recently, several new antiplatelet therapies have undergone extensive clinical testing for atherosclerotic thrombotic vascular disorders; other putative therapies are undergoing preclinical evaluation. We have investigated the effect of several of these novel agents on platelet aggregation in baboons in order to screen for future potential in xenograft rejection models.

Methods. Drugs tested in these experiments were aurintricarboxylic acid (ATA, von Willebrand Factor-GPIb inhibitor), fucoidin (selectin-inhibitor), 1-benzylimidazole (1-BI, thromboxane synthase antagonist), prostacyclin (PGI_2, endothelial stabilizer), heparin (thrombin antagonist), nitroprusside sodium or nicotinamide (NPN or NA, both NO-donors), and eptifibatide (EFT, GPIIb/IIIa receptor antagonist). These were infused intravenously to 9 baboons. Coagulation parameters and platelet counts were monitored and baboons were observed for adverse side effects. The efficacy of these agents in inhibiting platelet aggregation was assayed in a platelet aggregometer.

Results. Treatment with ATA and fucoidin resulted in complete inhibition of platelet aggregation but also in major perturbation of coagulation parameters. 1-BI and PGI_2 had no effect when administered alone, but in combination resulted in moderate inhibition of aggregation without disturbance in PT or PTT. NPN and NA had no substantive effects on platelet aggregation. Heparin resulted in specific inhibition of thrombin-induced platelet aggregation and, as anticipated, was associated with moderate prolongation of PTT. Importantly, EFT caused complete inhibition of platelet aggregation without changes in coagulation. Platelet counts, fibrinogen levels, and fibrinogen degradation products remained within the normal ranges in all experiments.

Conclusions. Although excellent inhibition of platelet activation was obtained with ATA and fucoidin, clinical use may be precluded by concomitant disturbances of coagulation. Combinations of heparin and EFT may prove beneficial in preventing the thrombotic disorders associated with xenograft rejection while maintaining adequate hemostatic responses. These agents are to be evaluated in our pig-to-primate xenotransplantation models.

INTRODUCTION

Transplantation of vascularized porcine organs in untreated primates results in hyperacute rejection within minutes to hours after reperfusion (1,2,3). When hyperacute rejection is averted, either by depletion of xenoreactive antibodies or complement or by using donors transgenic for human complement-regulatory proteins, rejection usually occurs within days to weeks and is termed acute vascular or delayed xenograft rejection (4,5,6,7). All forms of rejection are associated with vascular injury resulting at least in part from platelet activation (8,9,10,11,12). Rejected xenografts invariably demonstrate intravascular thrombosis and interstitial hemorrhage (2,3,4,13,14). In addition, following miniature swine-to-baboon kidney transplantation, disseminated intravascular coagulation has developed within 6-10 days. This process had been manifest by steadily decreasing fibrinogen and platelet counts, with increased prothrombin time (PT) and fibrinogen degradation products (15,16,17). This state has developed before histopathological evidence of rejection was advanced (17).

In our attempts to induce immunological tolerance in the miniature swine-to-baboon model by achieving mixed hematopoietic chimerism, a thrombotic microangiopathic state has been observed after the systemic infusion of mobilized porcine peripheral blood progenitor cells. Thrombocytopenia, high levels of lactate dehydrogenase, and erythrocyte schistocytosis have been observed (17,18).

The potential benefit of antithrombotic agents in the treatment of transplantation rejection is well recognized, and has been the subject of investigation for numerous

years (9,19,20,21,22). Inhibition of platelet activation, recruitment, and sequestration might inhibit development of the thrombotic microangiopathy and/or of the disseminated intravascular coagulation. There have been major advances in understanding mechanisms of platelet activation that have resulted in the development of new therapeutic strategies to block the formation of platelet microthrombi (9,23). We have therefore investigated the effect of various agents on platelet aggregation. Eight drugs were selected and then administered to baboons by intravenous (iv) infusion; the effect of each agent on platelet aggregation was tested in vitro.

MATERIALS AND METHODS

Animals

Baboons (Papio Anubis, n=9, Biomedical Resources Foundation, Houston, TX), weighing approximately 9-15 kg, were used for testing the experimental agents. All animals had been previously splenectomized through a midline laparotomy. Five baboons had previously been treated with a regimen aimed at inducing immunological tolerance, either with or without infusion of mobilized porcine peripheral blood progenitor cells > 4 months prior to the current experiments (15). Three baboons had previously (>3 months) received pharmacologic immunosuppressive therapy. Each baboon was administered at least two of the agents tested, but coagulation parameters and platelet aggregation were allowed to return to baseline before a second drug was tested.

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical Procedures

Baboons were given atropine (0.05 mg/kg im) and ketamine (10 mg/kg im) as premedication, and were intubated and ventilated through a closed circuit with oxygen (1-2 L/min), nitrous oxide (2-4 L/min), and isoflurane (0.5-3.0 %). The baboon's

carotid artery and internal jugular vein were cannulated, the cannulae were tunneled subcutaneously towards the scapula, and brought out through a jacket and tethering system. This allowed monitoring of arterial blood pressure, access to blood withdrawal, and continuous or intermittent iv administration of fluids and/or drugs. Cefazolin (500 mg iv daily) was given as prophylaxis against infection.

Experimental Agents

All agents, or combination of agents, were tested in at least two baboons, and were administered through continuous iv infusion unless otherwise described. Bolus injections are specified when used.

Aurintricarboxylic acid (ATA, Sigma-Alldrich, St. Louis, MO), administered at 0.5 – 8.0 mg/kg/hour, is known to inhibit binding of von Willebrand factor (vWF) to platelet glycoprotein Ib (GP Ib) in vitro and has been shown to significantly inhibit platelet adhesion and platelet aggregation in several small animal models (24,25,26). The observed antithrombotic activity possibly arises from inhibitory effects on thrombin activity and the vWF-GPIb pathway.

Fucoidin (Fluka Chemie AG, Buchs, Switzerland), administered at 0.5 – 1.0 mg/kg/hour, is a non-toxic sulfated fucose oligosaccharide derived from seaweed that blocks platelet (P)-selectin and may prevent platelet adhesion and associated aggregation responses (27,28,29,30). Selectins are adhesion molecules found on leukocytes (L-selectin), endothelium (P- and E-selectin), and platelets (P-selectin) that bind to oligosaccharide ligands containing fucose and sialic acid to mediate leukocyte rolling on, and platelet adhesion to, the endothelium. Inhibition of these selectins may result in reduced platelet adhesion and/or aggregation (29,30).

Thromboxane A2, an enzymatic product of arachidonic acid, has been implicated in platelet aggregation and the initiation of vasoconstriction in various organ systems (31,32). Selective inhibition of thromboxane synthase, which converts prostaglandin H2 to thromboxane A2, using 1-benzylimidazole (1-BI, Cayman Chemical Company, Ann Arbor, MI) has been shown to reduce thromboxane A2 concentration in vivo in

rat brains (33). 1-BI was administered at 10 – 40 μg/kg/hour as a single agent or in conjunction with PGI_2 (see below).

Prostacyclin (PGI_2, Glaxo Wellcome Inc, Research Triangle Park, NC) is an endothelial-derived vasodilator with reported anti-thrombotic effects (34). It has been documented to inhibit platelet aggregation in vitro in small animal models (35,36). PGI_2 was administered at 20 – 40 ng/kg/min as a single agent, and in conjunction with 1-BI at 30 ng/kg/min.

Heparin has well-known actions in potentiating antithrombin III and thereby influencing thrombin-mediated platelet aggregation (37,38). Thrombin catalyzes the formation of fibrin from fibrinogen, resulting in thrombus formation, and strongly induces platelet activation and recruitment (39). Furthermore, thrombin promotes chemotaxis by monocytes, and stimulates P-selectin activity in endothelial cells (40). Heparin sodium (Heparin, Elkins-Sinn, Inc., Cherry Hill, NJ) was administered at 10 – 50 Units/kg/hour.

Nitroprusside sodium (NPN, Ohmeda PPD Inc., Liberty Corner, NJ), administered at 1.0 – 10 μg/kg/min, is a well–described nitric oxide (NO)-donor that may also have an inhibitory effect on platelet aggregation. NO, formally referred to as the endothelium-derived relaxation factor, is a potent vasodilator that maintains endothelial integrity (41,42). It has also been described to have anti-platelet effects through increasing the intracellular cyclic guanidine monophosphate in platelets (43). Nicotinamide (NA, Sigma Chemical Co., St, Louis, MO), administered at 0.25 – 0.5 mg/kg/hour, is a B-complex vitamin that is also associated with increased NO production and vasodilatation (44). NA may therefore be effective in inhibiting platelet aggregation for the same reasons as NPN.

Eptifibatide (EFT, Cor Therapeutics, Inc., South San Francisco, CA) was administered as a bolus infusion of 180 μg/kg iv followed by continuous iv infusion at 2.0 – 7.5 μg/kg/min. EFT is a synthetic GP IIb/IIIa receptor antagonist currently used in the treatment of acute myocardial infarction and for post-angioplasty restenosis

(45,46). By preventing GP IIb/IIIa receptor binding to fibrinogen, crosslinking of platelets is precluded and aggregation is specifically inhibited.

Coagulation Parameters

Serial blood samples were taken for measurement of platelet count, prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, and fibrinogen degradation products (FDP) (Hematology Laboratory, Massachusetts General Hospital).

Platelet Aggregation Assay (Figure 1)

Ten milliliters citrated blood were drawn from the baboon and centrifuged at 280g for 15 minutes at 22°C. The supernatant, consisting of platelet-rich plasma, was transferred in 400 µl aliquots to siliconized glass cuvettes, and formed the experimental samples. The platelets were counted in a Coulter counter (Beckman Coulter, Inc., Fullerton, CA) and, if necessary, the experimental samples were diluted to a final concentration of $1x10^8$ platelets/ml with a calcium-, magnesium-, and phenol red-free Hanks buffer solution (Mediatech, Inc., Herndon, VA). The reference sample, consisting of platelet-poor plasma, was used to set the baseline level of aggregation to 0%. This was obtained by further centrifuging the remaining pellet at 2,400g for 2 minutes at 22°C.

Different platelet agonists/stimulators (agents that are known to initiate platelet aggregation) were added to the experimental samples in the platelet aggregometer (two-sample, four-channel, model 560 Ca Lumi-aggregometer, Chronolog Corp., Havertown, PA) at set concentrations, and aggregation was induced with a magnet causing shear stress. The agonists used were thrombin (1 – 5 U/ml, causing platelet activation and aggregation by binding to platelet GP Ib and cleaving platelet GP V),

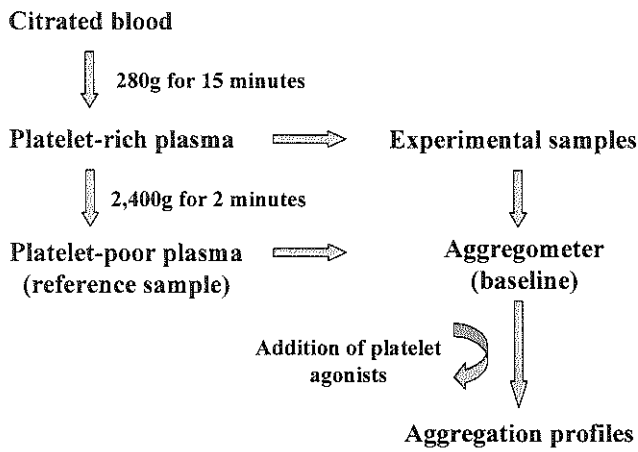

Figure 1.

Platelet aggregation assay. Citrated blood collected from baboons was centrifuged for 15 minutes at 280g at room temperature. The supernatant, platelet-rich plasma, was then used as experimental samples. Changes in optical density after adding platelet agonists (thrombin, collagen, ADP, ristocetin, and arachidonic acid) were measured in the aggregometer against the optical density reference sample, platelet-poor plasma (see methods).

adenosine diphosphate (ADP) (5 – 20 μM, inducing platelet shape change and aggregation by binding to multiple high-affinity ADP-specific receptors on platelets), collagen (5 – 10 μg/ml, inducing platelet aggregation by interacting with multiple platelet receptors such as GP Ib and fibronectin), ristocetin (1.25 μg/ml, initiating platelet aggregation by interacting with the GP Ib-vWF binding site). Arachidonic acid (1.2 mM) was substituted for ristocetin when an agent was tested that was not expected to interact with the GP Ib receptor. Platelet aggregation was measured as a percentage change in light optical density from baseline (with baseline being the measurement obtained in the experimental sample in the absence of the platelet aggregation agonist when compared with the reference sample (see Fig. 1)). Typical aggregation curves obtained from a naïve, untreated baboon are depicted in Fig. 2.

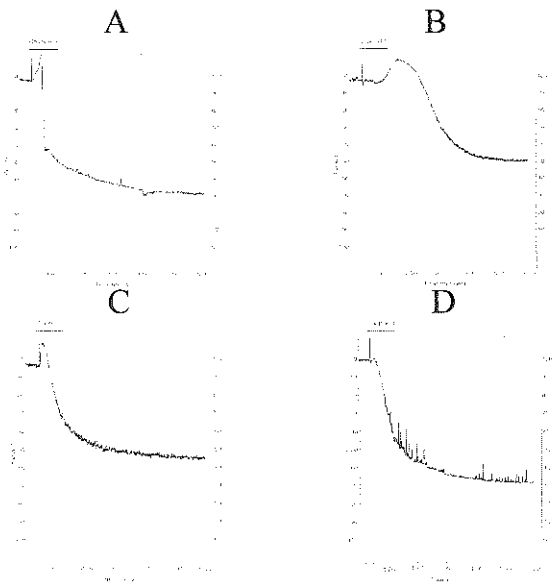

Figure 2.

Control baboon platelet aggregation curves. Agonists were added to platelet-rich plasma in the aggregometer. Results are from a representative experiment. Initial increase in percentage aggregation is due to shape changes of activated platelets.

A. Addition of thrombin resulted in 68% aggregation.

B. Addition of collagen led to 48% aggregation.

C. Addition of ADP led to 53% aggregation.

D. Addition of ristocetin resulted in 68% aggregation.

RESULTS

The clinical results and in vitro assays are summarized in Table 1. Coagulation parameters (PT, PTT, fibrinogen, and circulating FDP levels), platelet count and aggregation profiles were all within normal ranges at the onset of each experiment, and none of the agents tested induced any changes in platelet count, fibrinogen, or FDP levels.

Aurintricarboxylic acid (ATA)

ATA was administered in increasing doses from 0.5– 8.0 mg/kg/hour iv. At a cumulative dose of 15 mg/kg, vomiting was observed as well as an increased heart rate to >200 bpm with a simultaneous drop in blood pressure to 65/35 mmHg. Cessation of the drug resulted in rapid recovery. When re-administered after an interval of several days at 0.5 mg/kg/hour, no effect on platelet aggregation or changes in coagulation were observed (data not shown). Increasing the dose to 1.0 mg/kg/hour resulted in decreased platelet aggregation induced by ristocetin (Fig. 3 – left). This was accompanied by a prolongation in PT from 14.0 to >50 seconds (Fig. 3 – right). Discontinuing the drug resulted in recovery of the coagulation parameters within 3 days, but one baboon died from intra-abdominal bleeding 2 days after cessation of the drug. Infusion of ATA at 2.0 mg/kg/hour completely abrogated aggregation induced by ristocetin (Table 1). The other agonists (i.e. thrombin, collagen, and ADP) were not influenced by ATA therapy.

Fucoidin

Fucoidin at 0.5 μg/kg/hour did not alter the platelet aggregation or coagulation profile. At 1 μg/kg/hour for 4 hours, near-complete inhibition of aggregation induced by all agonists was achieved (Table 1). This also resulted in an increase in PT from 18 to 65 seconds. No other side effects were documented and recovery of coagulation parameters occurred rapidly following discontinuation of the drug.

1-benzylimidazole (1-BI)

1-BI at 20 or 40μg/kg/hour did not substantively change platelet aggregation induced by any of the platelet agonists (Table 1). Coagulation parameters remained unchanged, and there were no untoward side effects.

Prostacyclin (PGI_2)

Inconsistent changes in platelet aggregation were observed with this agent. The maximum inhibition of aggregation obtained with PGI_2 was 40 and 70% for ADP and arachidonic acid, respectively (Table 1). No changes in coagulation parameters and no additional toxic effects were observed at doses between 10 – 50 ng/kg/min.

TABLE 1

CHANGE IN PLATELET AGGREGATION AND COAGULATION PARAMETERS IN BABOONS

	Number of Baboons	Inhibition of Platelet Aggregation					Disturbance of Coagulation Parameters	Comments
		Thrombin	ADP	Collagen	Ristocetin	Arach acid		
ATA	2	+	+	++	+++	N/A	Yes	Clinical bleeding, major increase in PT.
Fucoidin	2	+++	+++	+++	++	N/A	Yes	Major increase in PT.
1-BI	4	-	-	-	-	N/A	No	-
PGI_2	2	-	-	-	N/A	++	No	-
1-BI + PGI_2	2	+	-	-	-	N/A	No	Moderate inhibition of platelet aggregation.
Heparin	2	+++	-	-	-	N/A	Yes	Anticipated increase in PTT.
NPN	2	+	-	-	N/A	+	No	-
NA	2	-	-	-	N/A	+	No	-
EFT	2	+++	+++	+++	N/A	+++	No	Dose-dependent inhibition of platelet aggregation by all agonists.

Average percentage inhibition of platelet aggregation: - = 0-20; + = 20-50; ++ = 50-80; +++ = >80. N/A: not applicable

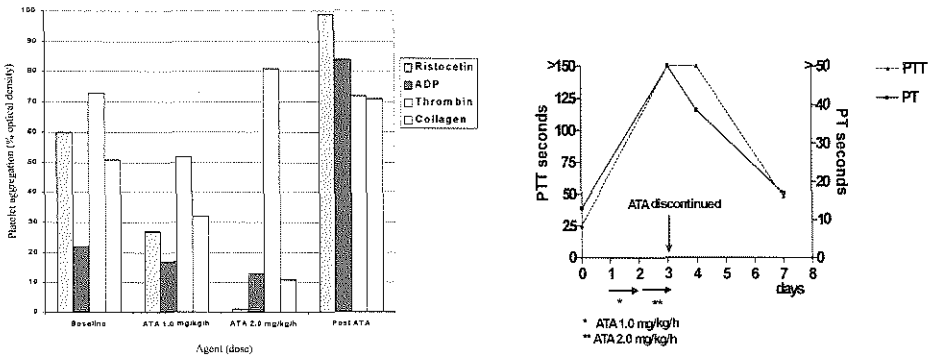

Figure 3.

Aurintricarboxylic acid (ATA)

Left. Administration of ATA at 1.0 mg/kg/h and 2.0 mg/kg/h by continuous iv infusion. A dose-dependent inhibition of aggregation induced by ristocetin and, to a less extent, by collagen was achieved, with little response to ADP and thrombin.

Right. At an ATA infusion of 2.0 mg/kg/h, an increase in PT and PTT to >50 and >150 seconds, respectively, was observed. Cessation of ATA led to recovery of coagulation parameters.

1-benzylimidazole (1-BI) and prostacyclin (PGI_2)

The combination of 1-BI (at 10 μg/kg/hour) with PGI_2 (at 30 ng/kg/min) in one baboon resulted in progressive blockade of platelet aggregation induced by thrombin. Increasing 1-BI to 20 μg/kg/hour led to complete inhibition of aggregation induced by thrombin. Aggregation induced by other agonists was not significantly changed (Table 1). A further increase of 1-BI to 40 μg/kg/hour in a second baboon, however, resulted in only minimal inhibition of platelet aggregation. In combination, the two agents did not result in any substantial changes in coagulation parameters.

Heparin

Heparin at 10 U/kg/hour led to a predicted increase in PTT from 29 seconds to 100 seconds in one baboon, and was associated with complete inhibition of platelet aggregation induced by thrombin (Table 1). Aggregation profiles induced by collagen, ristocetin, and ADP remained unchanged. In a second baboon, however, heparin at 10

U/kg/hour did not alter the coagulation parameters nor inhibited platelet aggregation. At an incremented dose of 50 U/kg/hour, an increase in PTT to 100 seconds was observed. This was accompanied by inhibition of aggregation induced by thrombin. No clinical signs of bleeding or other side effects were observed at this time and heparin dosage.

Nitroprusside sodium (NPN)

NPN (at 1.0 – 15 μg/kg/min) resulted in a modest decrease in platelet aggregation induced by thrombin and arachidonic acid in one baboon, but did not affect aggregation in a second baboon, suggesting individual sensitivity (Table 1). At doses higher than 10 μg/kg/min, vomiting was observed without changes in blood pressure or coagulation parameters.

Nicotinamide (NA)

NA (at 0.25 – 0.5 mg/kg/hr) did not cause significant inhibition of platelet aggregation although aggregation induced by arachidonic acid was decreased to some extent (Table 1). No other effects of the drug were noted.

Eptifibatide (EFT)

EFT was administered as an iv bolus of 180 μg/kg followed by continuous infusions at dosages ranging from 2.0 – 7.5 μg/kg/min. Platelet aggregation induced by all agonists used (i.e., thrombin, ADP, collagen, and arachidonic acid) was effectively inhibited with increasing dosages of EFT (Table 1 and Fig. 4). EFT at 2.0 μg/kg/min had no effect on aggregation. Increasing the dose to 5.0 and 7.5 μg/kg/min led to a dose-dependent complete inhibition of aggregation induced by ADP (Fig. 5) and by other agonists. Cessation of the drug led to full recovery of platelet aggregation within 2 – 4 hours. During treatment with EFT, no side effects were noted, and no changes in coagulation parameters were observed.

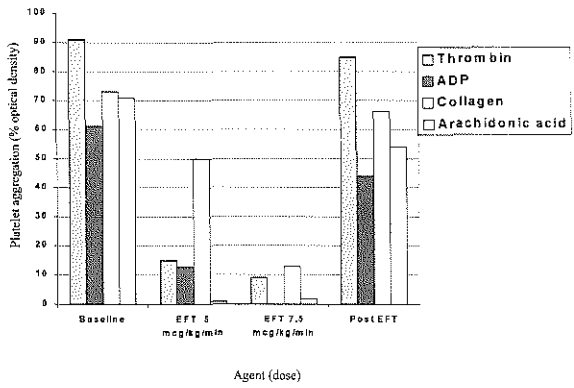

Figure 4.

Eptifibatide (EFT). EFT effectively inhibited platelet aggregation in a dose-dependent manner. At 2 μg/kg/min, no response was noted (data not shown). At 5 μg/kg/min, aggregation responses induced by thrombin, ADP, collagen, and arachidonic acid decreased markedly. EFT when administered at 7.5 μg/kg/min resulted in a further, near-complete decrease in aggregation induced by all agonists. Two hours following discontinuation of EFT, aggregation returned to baseline levels.

DISCUSSION

There are two major reasons related to xenotransplantation why we have investigated drugs that inhibit platelet aggregation.

Firstly, in our experience, the histopathological features of hyperacute and acute vascular rejection of vascularized xenografts in the pig-to-nonhuman primate model invariably demonstrate intravascular thrombosis and interstitial hemorrhage. Miniature swine kidney transplants into baboon recipients frequently show features of disseminated intravascular coagulation, even before rejection is histopathologically advanced (15,16,17). We believe that activation of recipient platelets and/or donor vascular endothelium play major roles. Prevention of these thrombotic events may extend xenograft survival by preserving adequate perfusion and maintaining

oxygenation of the graft even though the underlying inflammatory mechanisms may not be fully prevented.

Secondly, the transplantation of mobilized porcine peripheral blood progenitor cells to baboons in an attempt to induce immunological tolerance through mixed hematopoietic chimerism has been associated with a thrombotic microangiopathy, characterized by thrombocytopenia (platelet count < 20,000/μl), elevated lactate dehydrogenase, and erythrocyte schistocytosis (18). In our laboratory, we have observed the formation of baboon platelet aggregates in the presence of mobilized porcine peripheral blood progenitor cells in vitro (Alwayn IPJ, et al., unpublished data). Drugs that modulate formation of these aggregates may allow the infusion of more porcine progenitor cells, thus facilitating engraftment of these cells and the development of mixed hematopoietic chimerism.

It has become clear that the binding of agonists/stimulators to receptors on the platelet surface plays a central role in inducing platelet activation. Platelet adhesion to subendothelium leads to activation of the platelets, after which platelet sequestration and formation of aggregates can occur. When circulating platelets come into contact with exposed subendothelium (i.e. after endothelial cell retraction), adhesion occurs through binding of subendothelial platelet agonists, such as vWF, to platelet GP Ib under shear stress (47,48). This results in activation of platelets with release/generation of more platelet mediators, such as thrombin, ADP, and arachidonic acid (a precursor of thromboxane A2). These agonists generally have their own specific receptors on platelets and cause the platelets to change shape, form pseudopodia, and activate the GPIIb/IIIa receptor on the platelet surface. Cross-linking of these receptors with fibrinogen or soluble vWF results in platelet aggregation (49,50). Recent data suggest that platelets may also bind directly to endothelial cells and subsequently become activated (51). Other platelet receptors include $\alpha 2\beta 1$ integrin, GP IV, and GP VI (all receptors for collagen). Although there are many diverse substances that can activate platelets, our interest was in testing agents that interact with platelets at different points of application.

Although treatment with ATA or fucoidin was very effective at inhibiting platelet aggregation, both gave rise to severe disturbances of coagulation parameters, which may preclude their use in a clinical setting. The effects of ATA and fucoidin on coagulation parameters were not anticipated and could not directly be explained by the mechanisms by which these agents interact with platelets. It is, however, possible that, in addition to inhibiting receptors on platelets, they interact with other components of the coagulation pathway that have not yet been delineated.

1-BI was brought to our attention by Pierson and his colleagues, who have used thromboxane synthase inhibitors in a pig-to-human xenoperfusion model in an effort to prevent pulmonary vasoconstriction (52). This agent has also been shown to improve perfusion in a rat cerebrovascular ischaemia model by preventing the production of thromboxane A2, a potent platelet agonist (35). When administered to a baboon, we observed no significant changes in platelet aggregation even when dosages in excess of those recommended in other experimental models were used. It is possible, though unlikely, that therapeutic levels of 1-BI necessary to effectively inhibit platelet aggregation were not achieved. In these experimental settings, no changes in PT or PTT were observed.

PGI_2, a metabolite of arachidonic acid, has potent vasodilatory effects on pulmonary and systemic arterial beds (53,54) and has been reported to be an inhibitor of platelet aggregation in humans (55). We administered this drug in doses exceeding those recommended for humans without any significant change in blood pressure, heart rate or coagulation parameters. In contrast to the human data, no suppression of platelet aggregation in vitro could be obtained. When PGI_2 was combined with a high dose of 1-BI, however, moderate inhibition of platelet aggregation induced by thrombin was obtained, unaccompanied by changes in PT or PTT.

Heparin has been used in several models in an attempt to prolong survival of transplanted allo- or xeno-grafts, with varying results (21,56,57). In the latter two experiments, heparin was effective in preventing platelet aggregation induced by

thrombin in vitro. This effect was only observed when the PTT had reached a therapeutic level of >100 seconds, and was not associated with clinical bleeding.

It has been shown that NO has protective effects on the endothelium (43,44,45) and can prevent platelet aggregation by increasing intracellular cyclic guanidine monophosphate (cGMP) levels in platelets. In our model, however, NPN, an NO-donor, did not lead to any substantive decrease in aggregation. One explanation may be that insufficient levels of NO were obtained in our dosing regimen, as no associated vasodilatory effects, such as hypotension, were noted. Furthermore, since NO has both inflammatory oxidant-mediated effects (58) as well as platelet aggregation inhibitory potential (45), titrating towards a therapeutic dose will be challenging. Additionally, exogenous NO may exacerbate inflammation through synthesis of platelet-activating factor and mobilization of P-selectin (59). NA, also associated with increased NO production and vasodilatation, may have a similar mode of action as NO-donors (46). However, we observed no inhibitory effect on platelet aggregation in the studies presented here. It is possible that endothelial cells may be more susceptible to NO protection than platelets and that these potentially beneficial effects of NO may be more apparent in an in vitro model of platelet-endothelium interaction.

The last and most promising agent tested was EFT. This agent is a synthetic GP IIb/IIIa receptor antagonist that has been shown to have potent inhibitory effects on platelet aggregation in humans (47,48). We were able to confirm these results in baboons. EFT was studied instead of the monoclonal antibody abciximab, which also targets the GP IIb/IIIa receptor, because of the lower incidence of intracerebral hemorrhage, decreased potential for Fc-interactions, and absent risk for cross-immunization associated with treatment with EFT. No side effects were noted at the dosages administered. The complete inhibition of platelet aggregation in the absence of any changes of coagulation parameters may be of benefit in preventing the thrombotic disorders associated with the transplantation of porcine vascularized organs or hematopoietic progenitor cells. In the discordant guinea pig-to-rat cardiac xenograft model Candinas et al. demonstrated prolonged xenograft survival and

diminution of intragraft platelet microthrombi using the GPIIb/IIIa antagonist GPI 562. These results were not confirmed in a recent ex vivo discordant perfusion model of a porcine kidney with human blood using the GPIIb/IIIa antagonist abciximab (60). In this model, however, as no measures were undertaken to prevent hyperacute rejection, the effect of inhibition of platelet aggregation post-hyperacute rejection was not addressed.

We recently administered EFT as an initial iv bolus of 180 μg/kg, followed by continuous iv infusion at 7.5 μg/kg/min, to one baboon receiving our conditioning regimen aimed at inducing immunological tolerance. This non-myeloablative regimen has been described elsewhere (15), and consists of splenectomy, whole body (300 cGy) and thymic (700 cGy) irradiation, multiple extracorporeal immunoadsorptions of anti-αGal antibodies, T cell depletion, pharmacological immunosuppressive therapy, and costimulatory blockade, prior to the infusion of large numbers of mobilized porcine peripheral blood progenitor cells. The infusion of pig cells is always followed by an immediate and profound thrombocytopenia that can be ameliorated by steroids, PGI_2, and heparin. When EFT was infused, in the absence of heparin, the onset of thrombocytopenia was delayed by one day, but not prevented. These data suggest that, although inhibition of platelet-platelet aggregates may provide some benefit, other factors (e.g. platelet-leukocyte, or platelet-(sub)endothelial interactions) contribute to these thrombotic complications, and that receptors other than GPIIb/IIIa may also be involved.

In the context of solid vascularized organ xenotransplantation, in one experiment in our pig-to-baboon model, the combined administration of heparin and EFT appeared to reverse the early features of disseminated intravascular coagulation (increasing thrombocytopenia and steadily falling fibrinogen levels) (data not shown).

Other platelet-inhibitors have been described and remain under experimental evaluation in other models. The vascular NTP-diphosphohydrolase (CD39) is another candidate for study, as are ADP-receptor antagonists, such as ticlopidine. The lack of a fully active truncated form of CD39 has delayed testing of this agent, and ADP-

receptor antagonists have been implicated in the initiation of thrombotic thrombocytopenic purpura (61).

These data lead us to conclude that, although ATA and fucoidin are potent inhibitors of platelet aggregation, the experimental use of these agents in baboons may be prohibited by an associated high risk of bleeding. The combination of 1-BI and PGI_2 results in moderate inhibition of platelet aggregation, without changes in coagulation or other side effects, but its effect may be insufficient to be of clinical value. Heparin leads to good inhibition of thrombin-induced platelet aggregation at doses that cause only minor prolongation of PTT, allowing for its use in xenotransplantation models. NPN and NA do not appear to effectively inhibit platelet aggregation in these studies. EFT clearly was efficient in inhibiting platelet aggregation without any untoward side effects and will be considered for incorporation into our pig-to-primate transplantation model for further study.

REFERENCES

1 Perper RJ, Najarian JS. Experimental renal heterotransplantation. I. In widely divergent species. Transplantation 1966; 4: 377
2 Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. J Heart Lung Transplant 1988; 7: 238
3 Kobayashi T, Taniguchi S, Neethling FA, et al. Delayed xenograft rejection of pig-to-baboon cardiac transplants after cobra venom factor therapy. Transplantation 1997; 64: 1255
4 Parker W, Saadi S, Lin SS, et al. Transplantation of discordant xenografts: a challenge revisited. Immunol Today 1996; 17:373
5 Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nature Med 1995; 1: 869
6 Chen RH, Naficy S, Logan JS, et al. Hearts from transgenic pigs constructed with CD59/DAF genomic clones demonstrate improved survival in primates. Xenotransplantation 1999; 6: 194
7 Marquet RL, van Overdam K, Boudesteijn EA, et al. Immunobiology of delayed xenograft rejection. Transplant Proc 1997; 29: 955
8 Rosenberg JC, Broersma RJ, Bullemer G, et al. Relationship of platelets, blood coagulation, and fibrinolysis to hyperacute rejection of renal xenografts. Transplantation 1969;8:152
9 Robson SC, Schulte am Esch J 2nd, Bach FH. Factors in xenograft rejection. Ann N Y Acad Sci 1999; 875: 261
10 Schulte am Esch J 2nd, Cruz MA, Siegel JB, et al. Activation of human platelets by the membrane-expressed A1 domain of von Willebrand factor. Blood 1997; 90: 4425
11 Siegel JB, Grey ST, Lesnikoski BA, et al. Xenogeneic endothelial cells activate human prothrombin. Transplantation 1997; 64: 888
12 Koyamada N, Miyatake T, Candinas D, et al. Apyrase administration prolongs discordant xenograft survival. Transplantation 1996; 62: 1739
13 Platt JL, Fischel RJ, Matas AJ, et al. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation 1991; 52: 514
14 Romano E, Neethling FA, Nilsson K, et al. Intravenous synthetic alphaGal saccharides delay hyperacute rejection following pig-to-baboon heart transplantation. Xenotransplantation 1999; 6: 36

15 Ierino FL, Kozlowski T, Siegel JB, et al. Disseminated intravascular coagulation in association with the delayed rejection of pig-to-baboon renal xenografts. Transplantation 1998; 66: 1439
16 Kozlowski T, Shimuzu A, Lambrigts D, et al. Porcine kidney and heart transplantation in baboons undergoing a tolerance induction regimen and antibody adsorption. Transplantation 1999; 67: 18
17 Buhler L, Basker M, Alwayn IPJ, et al. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. Transplantation 2000; 70: 1323
18 Buhler L, Goepfert C, Kitamura H, et al. Porcine hematopoietic cell transplantation in nonhuman primates is complicated by thrombotic microangiopathy. Submitted for publication.
19 Moberg AW, Shons AR, Gewurz H, et al. Prolongation of renal xenografts by the simultaneous sequestration of preformed antibody, inhibition of complement, coagulation and antibody synthesis. Transpl Proc 1971;3:538
20 Ghilchik MW and Morris AS. Platelet trapping by sheep kidney and heart xenografts in the dog. J Surg Research 1974;17:434
21 Kincaid-Smith P. Modification of the vascular lesions of rejection in cadaveric renal allografts by dipridamole and anticoagulants. Lancet 1969;2:920
22 Lesnikoski BA, Candinas D, Otsu I, et al. Thrombin inhibition in discordant xenotransplantation. Xenotransplantation 1997;4:140
23 Marcus AJ. Pathogenesis of atherosclerosis: special pathogenetic factors-inflammation and immunity: platelets. In Atherosclerosis and Coronary Artery Disease. Vol 1. Fuster V, Ross R, and Topol EJ, editors. Lippincott-Raven, Philadelphia 1996: 607
24 Ito T, Matsuno H, Kozawa O, et al. Comparison of the antithrombotic effects and bleeding risk of fractionated aurin tricarboxylic acid and the GPIIb/IIIa antagonist GR144053 in a hamster model of stenosis. Thromb Res 1999; 95: 49
25 Kawasaki T, Kaku S, Kohinata T, et al. Inhibition by aurintricarboxylic acid of von Willebrand factor binding to platelet GPIb, platelet retention, and thrombus formation in vivo. Am J Hematol 1994; 47: 6
26 Yamamoto H, Vreys I, Stassen JM, et al. Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 1998; 79: 202
27 Nishino T, Fukuda A, Nagumo T, et al. Inhibition of the generation of thrombin and factor Xa by a fucoidan from the brown seaweed Ecklonia kurome. Thromb Res 1999; 96: 37
28 Millet J, Jouault SC, Mauray S, et al. Antithrombotic and anticoagulant activities of a low molecular weight fucoidan by the subcutanaous route. Thromb Haemost 1999; 81: 391
29 Dardik R, Savion N, Kaufmann Y, et al. Thrombin promotes platelet-mediated melanoma cell adhesion to endothelial cells under flow conditions: role of platelet glycoproteins P-selectin and GPIIb-IIIa. Br J Cancer 1998; 77: 2069
30 Merhi Y, Provost P, Chauvet P, et al. Selectin blockade reduces neutrophil interaction with platelets at the site of deep arterial injury by angioplasty in pigs. Arterioscler Thromb Vasc Biol 1999; 19: 372
31 Thomas DW, Mannon RB, Mannon PJ, et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 1998; 102: 1994
32 Hirata T, Ushikubi F, Kakizuka A, et al. Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. J Clin Invest 1996; 97: 949
33 Pettigrew LC, Grotta JC, Rhoades HM, et al. Effect of thromboxane synthase inhibition on eicosanoid levels and blood flow in ischemic rat brain. Stroke 1989; 20: 627
34 Dormandy JA. Rationale for antiplatelet therapy in patients with atherothrombotic disease. Vasc Med 1998; 3: 253
35 Wu KK. Endothelial prostaglandin and nitric oxide synthesis in atherogenesis and thrombosis. J Formos Med Assoc 1996; 95: 661
36 Nishimura H, Naritomi H, Iwamoto Y, et al. In vivo evaluation of antiplatelet agents in gerbil model of carotid artery thrombosis. Stroke 1996; 27: 1099
37 Boneu B. New antithrombotic agents for the prevention and treatment of deep vein thrombosis. Haemostasis 1996; 26: 368
38 Sakuragawa N. Regulation of thrombosis and hemostasis by antithrombin. Semin Thromb Hemost 1997; 23: 557
39 Narayanan S. Multifunctional roles of thrombin. Ann Clin Lab Sci 199; 29: 275

40 Coughlin SR. How the protease thrombin talks to cells. Proc Natl Acad Sci 1999; 96: 11023
41 Vinten-Johansen J, Zhao ZQ, Nakamura M, et al. Nitric oxide and the vascular endothelium in myocardial ischaemia-reperfusion injury. Ann N Y Acad Sci 1999; 874: 354
42 Goodwin DC, Landino LM, and Marnett LJ. Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. FASEB J 1999; 13: 1121
43 Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109
44 Nishikawa Y, Kanki H, Ogawa S. Differential effects of N-acetylcysteine on nitroglycerin- and nicorandil-induced vasodilation in human coronary circulation. J Cardiovasc Pharmacol 1998; 32: 21
45 Kereiakes DJ, Broderick TM, Roth EM, et al. Time course, magnitude, and consistency of platelet inhibition by abciximab, tirofiba, or eptifibatide in patients with unstable angina pectoris undergoing percutaneous coronary intervention. Am J Cardiol 1999; 84: 391
46 The PURSUIT Trial Investigators. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. NEJM 1998; 339: 436
47 Body SC. Platelet activation and interactions with the microvasculature. J Cardiovasc Pharmacol 1996; 27: S13
48 Marcus AJ, Safier LB. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. FASEB J 1993; 7: 516
49 Shattil SJ. Regulation of platelet anchorage and signaling by integrin alpha IIb beta 3. Thromb Haemost 1993; 70: 224
50 Phillips DR, Fitzgerald LA, Charo IF, et al. The platelet membrane glycoprotein IIb/IIIa complex. Structure, function, and relationship to adhesive protein receptors in nucleated cells. Ann N Y Acad Sci 1987; 509: 177
51 Bombeli T, Schwartz BR, Harlan JM. Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. Blood 1999; 93: 3831
52 Pierson RN III and Parker RE. Thromboxane mediates pulmonary vasoconstriction and contributes to cytotoxicity in pig lungs perfused with fresh human blood. Transplant Proc 1996; 28: 625
53 Horton R, Nadler J, Manogian C, et al. Prostacyclin is a key mediator of the vasodilator action of dopamine in humans. Adv Prostaglandin Thromboxane Leukot Res 1987; 17: 753
54 Haraldson A, Kieler-Jensen N, Nathorst-Westfelt U, et al. Comparison of inhaled nitric oxide and inhaled aerosolized prostacyclin in the evaluation of heart transplant candidates with elevated pulmonary vascular resistance. Chest 1998; 114: 780
55 Rolland PH, Bory M, Leca F, et al. Evidence for isosorbide dinitrate (ISDN) promoting effect on prostacyclin release by the lung and prostacyclin implication in ISDN-induced inhibition of platelet aggregation in humans. Prostaglandins Leukot Med 1984; 16: 333
56 Shapira OM, Rene H, Lider O, et al. Prolongation of rat skin and cardiac allograft survival by low molecular weight heparin. J Surg Res 1999; 85: 83
57 Dalmasso AP and Platt JL. Potentiation of C1 inhibitor plus heparin in prevention of complement-mediated activation of endothelial cells in a model of xenograft hyperacute rejection. Transplant Proc 1994; 26: 1246
58 Miller MJ and Sandoval M. Nitric Oxide. III. A molecular prelude to intestinal inflammation. Am J Physiol. 1999; 276: G795
59 Okayama N, Ichikawa H, Coe L, et al. Exogenous NO enhances hydrogen peroxide-mediated neutrophil adherence to cultured endothelial cells. Am J Physiol 1998; 274: L820
60 Fiane AE, Videm V, Mollnes TE, et al. Inhibition of platelet aggregation by the GPIIb/IIIa antagonist reopro does not significantly prolong xenograft survival in an ex vivo model. Transplant Int 1999; 12: 323
61 Imai M, Kaczmarek E, Koziak K, et al. Suppression of ATP diphosphohydrolase/CD39 in human vascular endothelial cells. Biochemistry 1999; 38: 13473

V

Discussion

9

General discussion

GENERAL DISCUSSION

The potential clinical implementation of xenotransplantation, or the transplantation of tissues and/or organs between species, has received considerable attention from both governmental institutions and media in numerous countries over the last few years. Renewed interest in xenotransplantation research has been fuelled by an increasing shortage of donor organs and mortality of patients awaiting a donor organ. The measures that have been undertaken to increase the donor-pool have thusfar not been successful, and alternatives to xenotransplantation – i.e. the use of bioartificial organs, stem cells, or gene therapy for organ failure – are still in early development.

Most investigators believe that, if xenotransplantation is to become a clinical reality, the pig will be the most suitable source of organs for a number of reasons (1). However, xenografts between widely disparate species i.e. discordant xenografts, such as pig-to-primate, are rapidly rejected within minutes to hours by a humoral, complement-dependent, mechanism, known as hyperacute rejection. Once hyperacute rejection is averted, the rejection responses that follow are also thought to be antibody-mediated, but most likely complement-independent, and may involve cellular mechanisms (2). Although much progress has been made in overcoming some of the immunological hurdles associated with pig-to-nonhuman primate xenotransplantation, porcine organs do not routinely survive beyond three months in nonhuman primates (3). As current evidence suggests that suppression of both the humoral and cellular response to a discordant xenograft will require extremely intensive immunosuppressive therapy, with its associated increased risks, it seems likely that xenotransplantation will only prove successful if immunologic tolerance to the transplanted pig organs can be achieved (4).

This dissertation describes our attempts at inducing immunological tolerance in the pig-to-baboon xenotransplantation model through mixed chimerism. The condition regimen we used was modified from others that have proven successful in various models of allotransplantation and concordant xenotransplantation. The importance of preformed, xenoreactive antibodies in these experiments is emphasized, and several

methods of depleting, or suppressing the production of, these antibodies are discussed in detail. Furthermore, thrombotic complications were observed in baboons that received porcine cells or organs. Additional experiments were designed to address these complications; these are also included in this dissertation.

An introduction to xenotransplantation is presented in **Chapter 1**. The needs for xenotransplantation, as well as the current understanding of the rejection responses and potential modulation of these responses are discussed in detail. From this chapter we conclude that the discussed therapeutic modalities alone will not be able to achieve long term, clinically relevant survival of xenografts. In this context, the phenomenon of accomodation deserves some more attention. Although this state has been described in sensitized humans receiving kidney allotransplants (5), as well as in various small animal xenotransplantation combinations (6), it has to date not been described in a pig-to-nonhuman primate xenotransplantation model. In fact, with our conditioning regimen aimed at inducing immunological tolerance, we have created an environment that theoretically would be ideal to induce a state of accommodation. The baboon recipients in these experiments are temporarily depleted of anti-Gal antibodies and of terminal complement components when a porcine kidney is transplanted. The returning anti-Gal antibodies, especially IgM, however, remain capable of mediating graft rejection (Buhler et al., submitted for publication). It appears that the porcine endothelial cells, rather than becoming accommodated, become activated by the induced antibody response and that accommodation may not be achieved in pig-to-primate xenotransplantation. We therefore believe that combinations of current therapies, further refinement of these therapies, and new technologies designed to prevent or treat rejection (especially those aimed at inducing immunological tolerance), need to be explored and developed.

Our protocol aimed at achieving mixed hematopoietic chimerism and thereby inducing immunological tolerance in the pig-to-baboon model is presented in **Chapter 2**. This protocol consists of induction therapy with splenectomy, whole body irradiation, thymic irradiation, anti-thymocyte globulin, and multiple extracorporeal immunoadsorptions of anti-Gal antibodies. The maintenance therapy consists of

treatment with anti-CD154 mAb +/- cyclosporine, mycophenolate mofetil, and cobra venom factor, after which a large number of growth factor-mobilized porcine leukocytes (1×10^{10} cells/kg) are infused into recipient baboons on each of 3 consecutive days (total 3×10^{10} cells/kg). In this chapter we present, for the first time in a large animal model, successful depletion of baboon macrophages using medronate liposomes. The distinct dimensions and properties of these liposomes render them susceptible to phagocytosis by macrophages. Once phagocytosed, medronate interferes with the cell's metabolism and causes cell death. When macrophage depletion is added to our conditioning regimen for tolerance induction, significantly higher levels of porcine chimerism are achieved in the recipient baboons, underlining the importance of recipient macrophages in the clearance of donor porcine hematopoietic cells and the high potential of medronate liposomes for other experimental xenotransplantation protocols.

In addition, observations from other experiments suggest that an absence of anti-Gal IgG may also be an important factor in allowing the development of chimerism, as these antibodies are able to destroy target cells by antibody-dependent cell-mediated cytotoxicity, independent of complement. Although anti-Gal antibodies are effectively depleted from circulation by multiple extracorporeal immunoadsorptions, the return of anti-Gal antibodies cannot be prevented, thereby reducing the possibility of achieving mixed hematopoietic chimerism and immunological tolerance. The importance of anti-Gal antibodies in mixed chimerism protocols and vascularized organ xenotransplantation, as well as current methods for depleting, or suppressing the function of these antibodies are described in **Chapter 3**. We conclude, based on a comprehensive review of the literature, as well as on our own experience, that, although anti-Gal antibodies may be successfully but temporarily depleted by extracorporeal immunoadsorption, none of the immunosuppressive agents currently employed are significantly successful at preventing their return following depletion. However, the ability of anti-CD154 mAb to prevent the induced antibody response to pig cells does represent a significant step in overcoming the immunologic barriers to xenotransplantation, and will undoubtedly facilitate the survival of transplanted pig organs in primates and the induction of tolerance.

Some of the future studies described in chapter 3 with respect to the development of specific anti-B cell or anti-plasma cell antibodies in an effort to prevent the production of anti-Gal antibodies are explored in **Chapter 4**. In extensive in vivo and in vitro experiments, we demonstrated that baboon B cells can be efficiently depleted with whole body irradiation or with specific anti-B cell mAbs or immunotoxins. Additionally, combining irradiation with a mAb directed against the B cell-specific CD20 determinant led to depletion of B cells for > 3 months. However, this does not lead to a clinically significant reduction in the rate or extent of return of anti-Gal antibodies following immunoadsorption. These observations suggest that B cells are not the major source of anti-Gal antibody production in baboons, and that attention must be directed towards suppressing plasma cell and B cell blast function. This was confirmed by our findings that the major secretors of anti-Gal antibodies in baboons are CD38-positive cells, or plasma cells. Unfortunately, the anti-CD38 immunotoxin we studied was not effective in vivo, potentially due to various reasons, including suboptimal conjugation of the mAb to the toxin.

The next chapters focus on the thrombotic complications that are associated with pig-to-baboon xenotransplantation. There are historical precedents for the development of coagulation abnormalities and thrombocytopenia in association with solid organ xenograft rejection, and these data are confirmed by the studies described in **Chapter 5**. In this chapter, we also demonstrate that the infusion of porcine hematopoietic cells is associated with widespread thrombotic vascular injury with deleterious consequences for the recipient. The mechanisms underlying the thrombotic complications in these settings are still unclear. It is possible that varying levels of immune mediators within the vascularized xenograft could promote vascular thrombosis, as a component of the inflammatory response *ab initio* with endothelial cell activation. With respect to porcine hematopoietic cell transplantation, we hypothesized that these cells directly induce platelet aggregation and subsequent sequestration of platelets in the microvasculature of recipient baboons, or that the conditioning regimen aimed at inducing mixed chimerism contains components that may initiate platelet aggregation and thrombocytopenia.

In **Chapter 6** we demonstrate that porcine hematopoietic cells mediate aggregation of baboon platelets. Furthermore, we present evidence that eptifibatide, a substance that prevents the GPIIb/IIIa platelet-receptor from binding to fibrinogen, thereby precluding crosslinking of platelets, can fully abrogate platelet aggregation induced by these cells in vitro. Purification of the precursors from porcine PBPC and/or treatment of baboons with eptifibatide may be beneficial in preventing these sequelae.

These findings are confirmed in **Chapter 7** where, in addition, each of the individual components of the conditioning regimen are assessed for their ability to induce platelet aggregation in baboons. Interestingly, none of the components of the conditioning regimen are found to be pro-aggregatory, but cyclophosphamide appears to have protective, anti-aggregatory properties and may therefore provide an alternative strategy to whole body irradiation in our attempts to achieve mixed hematopoietic chimerism and the induction of tolerance.

Finally, in **Chapter 8** other pharmacologic agents aimed at preventing platelet aggregation in baboons are examined. We demonstrate that, although aurintricarboxylic acid and fucoidin are potent inhibitors of platelet aggregation, the experimental use of these agents in baboons may be prohibited by an associated high risk of bleeding. Heparin leads to good inhibition of thrombin-induced platelet aggregation at doses that cause only minor prolongation of the partial thromboplastin time, allowing for its use in xenotransplantation models. Eptifibatide leads to excellent inhibition of platelet aggregation without any untoward side effects and is therefore a good candidate to be incorporated into our future pig-to-nonhuman primate studies.

The successful induction of stable immunological tolerance in pig-to-baboon xenotransplantation will, beyond any doubt, pave the way to clinical xenotransplantation. The above discussion clearly points out that many factors, both immunological and not, stand in the way of mixed chimerism and tolerance. Based on the previous studies, it has become evident that anti-Gal antibodies remain a major

problem in xenotransplantation. Recent experiments performed at our own laboratory (not included in this dissertation) with in vivo depletion of anti-Gal antibodies by infusion of bovine serum albumin conjugated to Gal (BSA-Gal) to naïve baboons, baboons undergoing our tolerance induction protocol, or baboons receiving porcine kidney xenografts (in conjunction with extracorporeal immunoadsorption and costimulatory blockade with an anti-CD154 mAb), demonstrated complete or near complete depletion of anti-Gal antibodies sustained for periods of up to 30 days (Teranishi and Gollackner, unpublished data). The return of anti-Gal antibodies following cessation of the BSA-Gal was more delayed than observed in controls that did not receive BSA-Gal. In addition to neutralizing anti-Gal antibodies, this agent may, therefore, possibly tolerize anti-Gal antibody-producing cells by binding to their antigen receptors. We therefore believe that future studies must focus on the refinement of BSA-Gal, as well as on the development of agents that prevent the production of anti-Gal antibodies, such as efficient anti-plasma cell immunotoxins.

Additionally, the potential of macrophage depletion in pig-to-nonhuman primate tolerance induction needs to be further explored and possibly implemented in refined protocols, Furthermore, the etiology of the associated thrombotic complications must be elucidated.

In conclusion, this dissertation discusses some of the major immunological hurdles that preclude clinical implementation of xenotransplantation, and offers insight into current attempts at inducing immunological tolerance through mixed hematopoietic chimerism. The problem of anti-pig antibodies is addressed, as well as associated thrombotic disorders, and current and novel therapeutic modalities are presented. Based on the results presented in this dissertation, the addition of a specific and effective anti-plasma cell immunotoxin, macrophage depletion with medronate liposomes, and prevention of platelet aggregation with eptifibatide, to the standard conditioning regimen as discussed in Chapter 2, may facilitate the induction of mixed chimerism and immunological tolerance. Further refinement of BSA-Gal, to achieve in vivo depletion of anti-Gal antibodies, as well as using composite thymo-organs for T cell tolerance may also be beneficial. The author hopes that the experiments and

findings described will be of benefit to the future of xenotransplantation, and that this exciting field will eventually become a part of clinical medicine.

REFERENCES

1 Sachs DH. The pig as a potential xenograft donor. Vet Imm Immunopath 1994; 43: 185
2 Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nat Med 1995; 1: 869
3 Zaidi A, Schmoeckel M, Bhatti F, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584
4 Wekerle T, Sykes M. Mixed chimerism as an approach for the induction of transplantation tolerance. Transplantation 1999; 68: 459
5 Alexandre GPJ, Squifflet JP, De Bruyere M, et al. Present experiences in a series of 26 ABO-incompatible living donor renal allografts. Transplant Proc 1987; 19: 4538
6 Bach FH, Ferran C, Hechenleitner P, et al. Accomodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med 1997; 3: 196

10

Summary in Dutch

DISCUSSIE

Het begrip xenotransplantatie, ofwel het transplanteren van weefsel en/of organen tussen diersoorten, is de laatste jaren onderwerp van discussie geweest op nationaal en internationaal niveau, zowel bij overheidsinstanties als in de publieke media. Vanwege het steeds groter wordend tekort aan orgaandonoren, met daarbij een stijgende mortaliteit van patiënten met terminaal orgaanfalen die op de wachtlijst staan voor een donororgaan, is hernieuwde belangstelling voor xenotransplantatie-onderzoek ontstaan. De huidige maatregelen welke getroffen zijn om het aantal donoren te vergroten schieten helaas tekort, en alternatieven voor transplantatie – zoals het gebruik van bioartificiële organen, stamcellen, of gentherapie bij orgaanfalen – zijn nog niet voldoende ontwikkeld.

Het varken wordt door veel wetenschappers gezien als de ideale leverancier voor donororganen (1). Het transplanteren van organen tussen diersoorten die fylogenetisch ver van elkaar zijn verwijderd, zoals van het varken naar de primaat, gaat echter gepaard met een zeer snelle en heftige afstotingsreactie. Deze afstoting, ook wel hyperacute afstoting genoemd, is antilichaam-gemedieerd en complement-afhankelijk, en treedt binnen enkele minuten tot uren na transplantatie op. Indien het lukt deze afstotingsvorm af te wenden, bijvoorbeeld door antilichaam of complement depletie of door varkens te gebruiken die transgeen zijn voor humaan complement regulerende eiwitten, treden andere afstotingsverschijnselen op. Deze zijn eveneens antilichaam-gemedieerd maar waarschijnlijk complement-onafhankelijk, en bevatten daarnaast cellulaire componenten (2). Hoewel veel vooruitgang is geboekt in het begrijpen en behandelen van sommige van deze immunologische barrières, lukt het niet routinematig overleving van meer dan drie maanden te verkrijgen van varkensorganen in primaten (3). De huidige bewijslast suggereert dat de humorale en cellulaire afstotingsresponsen tegen xenotransplantaten in het varken-naar-primaat model dermate hevig zullen zijn dat deze alleen te behandelen zullen zijn met bijzonder hoge doseringen immuunsuppressiva, hetgeen gepaard zal gaan met onaanvaardbaar hoge risico's op infecties en maligniteiten. Het verkrijgen van immunologische tolerantie in dit model, waarbij een specifieke hyporesponsiviteit voor varkensantigenen in de

primaat-ontvanger optreedt, is derhalve essentieel voordat xenotransplantatie klinisch geïmplementeerd zal worden (4).

Dit proefschrift beschrijft onze pogingen immunologische tolerantie te induceren in het varken-naar-baviaan xenotransplantatie model via het verkrijgen van gemengd hematopoietisch chimerisme. Het gebruikte conditioneringsprotocol is gemodelleerd naar succesvolle protocollen binnen allotransplantatie en concordante xenotransplantatie. Het belang van voorgevormde, xenoreactieve antilichamen in deze experimenten wordt benadrukt en diverse methoden van depleteren of onderdrukken van de produktie van deze antilichamen worden in detail besproken. Hiernaast worden de thrombotische complicaties welke zijn gesignaleerd in bavianen die varkensorganen of hematopoietische cellen ontvangen gepresenteerd. Tevens worden de aanvullende experimenten die zijn uitgevoerd om deze complicaties te analyseren en beperken beschreven.

Een introductie tot xenotransplantatie wordt gegeven in **Hoofdstuk 1**. Het belang en de noodzaak van deze vorm van transplantatie, alsmede het huidig begrip en de behandelingsmogelijkheden van de afstotingsverschijnselen, worden in detail besproken. Wij concluderen uit dit hoofdstuk dat de huidige therapie op zichzelf ontoereikend is om langdurige, klinisch relevante overleving van xenotransplantaten te verkrijgen. In dit verband verdient het begrip accommodatie enige toelichting. Het houdt in dat de getransplanteerde organen overleven ondanks de aanwezigheid van antilichamen gericht tegen antigenen van deze transplantaten. Accommodatie is beschreven bij gesensitiseerde patiënten die een niertransplantatie ondergaan (5) alsmede in verscheidene kleine proefdiermodellen van xenotransplantatie (6). Tot heden is dit fenomeen echter niet aangetoond in het varken-naar-primaat xenotransplantatie model. In het door ons bestudeerde tolerantie-inductie protocol wordt een omgeving gecreëerd welke theoretisch ideaal zou zijn voor het optreden van accommodatie. Immers, de bavianen in deze experimenten zijn gedepleteerd van anti-Gal antilichamen en terminale complement componenten op het moment dat een varkensnier wordt getransplanteerd. De anti-Gal antilichamen welke terugkeren na depletie, in het bijzonder IgM antilichamen, blijven echter in staat afstoting te

veroorzaken (Buhler et al., ingestuurd voor publikatie). Kennelijk ondergaan de varkensendotheelcellen in plaats van accommodatie, activatie door de geïnduceerde antilichaamrespons in dit model en is het mogelijk dat accommodatie niet te verkrijgen zal zijn in varken-naar-primaat xenotransplantatie. Wij denken derhalve dat wellicht combinaties van therapieën, verdere verfijning van bestaande behandelingen en het ontwikkelen van nieuwe strategieën gericht op het voorkómen danwel behandelen van afstoting dienen te worden ontwikkeld. In het bijzonder dient de inductie van immunologische tolerantie aandacht te krijgen.

Het protocol gericht op het verkrijgen van gemengd hematopoiëtisch chimerisme met hierbij de inductie van immunologische tolerantie in het varken-naar-baviaan model wordt gepresenteerd in **Hoofdstuk 2**. Dit protocol bestaat uit inductietherapie van de baviaan met behulp van splenectomie, totale lichaams- en thymus bestraling, het toedienen van anti-thymocyten globuline, en multipele extracorporele immunoadsorpties van anti-Gal antilichamen. De onderhoudsbehandeling bestaat uit het toedienen van anti-CD154 monoklonale antilichamen +/- cyclosporine, mycofenolaat mofetil en cobra gif, waarna verspreid over drie dagen een grote hoeveelheid groeifactor-gemobiliseerde varkens leukocyten (totaal 3 x 10^{10} cellen/kg) wordt toegediend aan de baviaan. Hier presenteren wij voor de eerste keer in een grote proefdieren model succesvolle depletie van bavianen macrofagen met behulp van medronaatliposomen. Deze liposomen hebben specifieke kenmerken en afmetingen welke ze ontvankelijk maken voor fagocytose door macrofagen. Het gefagocyteerde medronaat verstoort vervolgens het metabolisme van de cel en leidt tot celdood. Wanneer macrofaagdepletie wordt toegevoegd aan het basis-conditionerings protocol zoals hierboven uiteengezet worden beduidend hogere waarden van varkenschimerisme in de ontvangende bavianen aangetoond. Hiermee wordt het belang van macrofagen in het wegvangen van varkenscellen uit de circulatie van bavianen onderstreept en lijkt het interessant macrofaagdepletie ook in andere xenotransplantatiemodellen te bestuderen.

Belangrijk voor het verkrijgen van gemengd hematopoietisch chimerisme is de afwezigheid van anti-Gal antilichamen, in het bijzonder anti-Gal IgG. Uit eerdere studies is gebleken dat deze antilichamen cellen kunnen destrueren middels

antilichaam-afhankelijke cel-gemedieerde cytotoxiciteit, onafhankelijk van complement. Anti-Gal antilichamen kunnen effectief worden verwijderd uit de circulatie middels achtereenvolgende extracorporele immunoadsorpties. De terugkeer van anti-Gal antilichamen wordt hiermee echter niet voorkomen waardoor de kans op het verkijgen van chimerisme en immunologische tolerantie wordt verminderd. Het belang van anti-Gal antilichamen in gemengd-chimerisme protocollen en xenotransplantatie van gevasculariseerde organen, alsmede huidige behandelingsmethoden voor het depleteren of verminderen van de functie van deze antilichamen worden beschreven in **Hoofdstuk 3**. Wij concluderen, gebaseerd op de meest recente literatuur en onze eigen ervaring dat, hoewel tijdelijke depletie van anti-Gal antilichamen zeer goed mogelijk is middels extracorporele immunoadsorptie, niet één van de gangbare immuunsuppressiva de terugkeer van antilichamen na depletie kan voorkomen. Het vermogen van het anti-CD154 antilichaam om de geïnduceerde antilichaamrespons tegen varkenscellen te voorkomen is echter wel een significante bevinding en zal zeker de overleving van varkensorganen in primaten verbeteren en de inductie van tolerantie vergemakkelijken.

Sommige van de in hoofdstuk 3 beschreven vervolgstudies met betrekking tot het ontwikkelen van specifieke anti-B cel of anti-plasmacel antilichamen met als doel anti-Gal antilichaamproduktie te voorkomen, worden bestudeerd in **Hoofdstuk 4**. In uitgebreide in vivo en in vitro experimenten demonstreren wij dat B cellen in bavianen efficiënt kunnen worden gedepleteerd middels totale lichaamsbestraling of door toediening van specifieke anti-B cel monoclonale antilichamen of immunotoxines. De combinatie van bestraling met een monoclonaal antilichaam gericht tegen de B cel-specifieke determinant CD20 resulteert in depletie van B cellen voor meer dan 3 maanden. Dit leidt echter niet tot vermindering van anti-Gal antilichaamproduktie. Deze observaties suggereren dat B cellen niet de voornaamst verantwoordelijke cellen zijn voor antilichaam produktie in bavianen en dat aandacht dient te worden besteed aan het onderdrukken van de funktie van plasmacellen. Dit blijkt ook uit in vitro experimenten waarin het merendeel van anti-Gal antilichaam secreterende cellen CD38-positief zijn, een marker voor plasmacellen. Helaas was het anti-CD38 immunotoxine dat wij hanteerden in onze in vivo experimenten niet

effectief, onder andere vanwege een suboptimale conjugatie van het antilichaam met het toxine.

In de volgende hoofdstukken wordt aandacht besteed aan de thrombotische complicaties die geassocieerd worden met varken-naar-baviaan xenotransplantatie. Het ontstaan van stollingsstoornissen en thrombocytopenie samenhangend met de xenotransplantatie van gevascullariseerde organen is in het verleden reeds beschreven en wordt door onze onderzoeksresultaten bevestigd in **Hoofdstuk 5**. In dit hoofdstuk demonstreren wij ook dat het transplanteren van varkenshematopoietische cellen geassocieerd is met indrukwekkende thrombotische vasculaire schade met schadelijke gevolgen voor de ontvanger. Het hieraan ten grondslag liggend mechanisme is onduidelijk. Mogelijk spelen immuunmediatoren binnen het xenotransplantaat een rol. Deze zouden bijvoorbeeld thrombose kunnen bevorderen als onderdeel van een inflammatoire reactie welke optreedt na endotheelcelactivatie. In het geval van hematopoietische celtransplantatie is het mogelijk dat deze cellen direct bloedplaatjesaggregatie veroorzaken met een hieropvolgende sequestratie van bloedplaatjes in de microcirculatie van de baviaan ontvangers. Ook kunnen individuele componenten van het conditioneringsregime bloedplaatjesaggregatie met thrombocytopenie veroorzaken.

In **Hoofdstuk 6** tonen wij dat varkenshematopoietische cellen aggregatie kunnen medieren van bavianenbloedplaatjes. Tevens presenteren wij dat eptifibatide, een GPIIb/IIIa receptor antagonist, deze aggregatie in vitro volledig kan voorkomen. Het verwijderen van debris uit het varkenshematopoietische stamcellen produkt en/of het behandelen van bavianen met eptifibatide kan helpen deze complicaties te voorkomen.

Deze bevindingen worden nogmaals bevestigd in **Hoofdstuk 7**. Tevens worden de individuele componenten van het conditioneringsregime beoordeeld op hun vermogen thrombocyten aggregatie te induceren in bavianen. Naast het feit dat geen van de componenten inductie van aggregatie veroorzaakt, wordt aangetoond dat cyclofosfamide beschermende, anti-aggregatoire eigenschappen bezit. Dit middel kan

derhalve in onze pogingen gemengd hematopoietisch chimerisme te bereiken een alternatief betekenen voor totale lichaamsbestraling.

Tenslotte worden in **Hoofdstuk 8** andere farmacologische middelen getest op hun vermogen thrombocytenaggregatie te remmen in bavianen. Middelen als aurintricarboxylzuur en fucoidin blijken krachtige remmers van aggregatie te zijn. Het gebruik hiervan wordt echter verhinderd door een onaanvaardbaar hoog risico op bloedingscomplicaties. Heparine is een bekende remmer van thrombine-geïnduceerde bloedplaatjesaggregatie en kan worden gebruikt in doseringen die slechts een kleine verlenging van geactiveerde partiële thromboplastinetijd veroorzaken. Eptifibatide leidt tot een zeer goede inhibitie van thrombocytenaggregatie zonder duidelijke bijwerkingen en is hierdoor een goede kandidaat om te gebruiken binnen onze toekomstige xenotransplantatieprotocollen.

De succesvolle inductie van stabiele immunologische tolerantie in varken-naar-baviaan xenotransplantatie zal ongetwijfeld de voorwaarde scheppen voor klinische xenotransplantatie. Het bovenstaande illustreert dat vele factoren, zowel immunologische als andere, in de weg staan van gemengd chimerisme en tolerantie voor varkensorganen. Gebaseerd op voorgaande studies is duidelijk geworden dat anti-Gal antilichamen een groot probleem vormen in xenotransplantatie. Wij hebben recent experimenten uitgevoerd (niet beschreven in dit proefschrift) om in vivo depletie van anti-Gal antilichamen te verkrijgen in naïve bavianen, in bavianen die ons tolerantie-inductieprotocol ondergaan en in bavianen die varkensniertransplantaten ontvangen, door albumine van kalveren geconjugeerd met Gal (BSA-Gal) toe te dienen. Met dit middel wordt (bijna) complete depletie van anti-Gal antilichamen verkregen tot 30 dagen (Teranishi and Gollackner, niet gepubliceerde data) na toediening. De terugkeer van anti-Gal antilichamen, nadat behandeling met BSA-Gal is gestaakt, verloopt vervolgens langzamer dan bij bavianen die niet met BSA-Gal worden behandeld. Naast het vermogen van BSA-Gal om anti-Gal antilichamen te neutralizeren, kan het waarschijnlijk ook anti-Gal antilichaamproducerende cellen tolerizeren door hun antigeenreceptoren te binden. Wij vinden derhalve dat, naast de al eerder genoemde studies gericht op het

ontwikkelen van middelen die de produktie van anti-Gal antilichamen kunnen remmen, zoals efficiënte anti-plasmacel immunotoxinen, toekomstige studies gericht moeten zijn op het verfijnen van het gebruik van BSA-Gal.

Tevens dient het potentieel van macrofaagdepletie in varken-naar-primaat tolerantie-inductie modellen nader onderzocht te worden en zo mogelijk geïmplementeerd te worden in verbeterde protocollen. Ook zal de etiologie van het vóórkomen van thrombotische complicaties in deze modellen bestudeerd moeten worden.

Samenvattend kan worden gesteld dat in dit proefschrift een aantal van de belangrijkste immunologische obstakels voor klinische implementatie van xenotransplantatie wordt besproken. Tevens wordt inzicht gegeven in de huidige mogelijkheden om immunologische tolerantie te verkijgen in het pre-klinische varken-naar-baviaan gemengd-chimerisme model. Het probleem van anti-varkens antilichamen wordt toegelicht, begeleidende thrombotische complicaties worden beschreven en huidige en nieuwe behandelingsmogelijkheden hiervan worden voorgesteld. Op grond van de resultaten beschreven in dit proefschrift zal een protocol dat, naast de componenten van het conditioneringsregime zoals beschreven in Hoofdstuk 2, tevens een specifieke en effectieve anti-plasmacel immunotoxine, medronaatliposomen voor macrofaagdepletie, alsmede eptifibatide ter voorkoming van thrombocytenaggregatie, bevat, mogelijk de inductie van gemengd chimerisme en immunologische tolerantie faciliteren. De auteur hoopt dat de experimenten en resultaten welke beschreven worden in dit proefschrift, van belang zijn voor de toekomst van xenotransplantatie en dat dit boeiende vakgebied deel zal gaan uitmaken van de transplantatiegeneeskunde.

REFERENCES

1 Sachs DH. The pig as a potential xenograft donor. Vet Imm Immunopath 1994; 43: 185
2 Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nat Med 1995; 1: 869
3 Zaidi A, Schmoeckel M, Bhatti F, et al. Life-supporting pig-to-primate renal xenotransplantation using genetically modified donors. Transplantation 1998; 65: 1584

4 Wekerle T, Sykes M. Mixed chimerism as an approach for the induction of transplantation tolerance. Transplantation 1999; 68: 459
5 Alexandre GPJ, Squifflet JP, De Bruyere M, et al. Present experiences in a series of 26 ABO-incompatible living donor renal allografts. Transplant Proc 1987; 19: 4538
6 Bach FH, Ferran C, Hechenleitner P, et al. Accomodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med 1997; 3: 196

LIST OF ABBREVIATIONS

1-BI	1-benzylimidazole
^{3}H-Leu	^{3}H-Leucine
αGal	galα1-3Gal epitope
∝CD20 mAb	anti-CD20 monoclonal antibody Rituximab
∝CD22-IT	anti-CD22-ricin A immunotoxin
∝CD25-IT	anti-CD25-ricin A immunotoxin
∝CD38 mAb	anti-CD38 monoclonal antibody OKT10
∝CD38-IT	anti-CD38-ricin A immunotoxin
ADP	adenosine diphosphate
Anti-αGal Ab:	anti-Galα1-3Gal antibodies
ATA	aurintricarboxylic acid
ATG	antithymocyte globulin
AVR	acute vascular rejection
BM	bone marrow
CPP	cyclophosphamide
CVF	cobra venom factor
dg	deglycosylated
DIC	disseminated intravascular coagulation
EFT	eptifibatide
EIA	extracorporeal immunoadsorption
FDP	fibrinogen degradation products
GP	glycoprotein
HAR	hyperacute rejection
HBSS	Hank's balanced salt solution
IT	immunotoxin
i.v.	intravenous
IVIg	intravenous immunoglobulin
LDH	lactate dehydrogenase
LN	lymph node

mAb	monoclonal antibody
ML	medronate liposomes
MPS	mononuclear phagocyte system
NA	nicotinamide
NMCR	non-myeloablative conditioning regimen
NO	nitric oxide
NPN	nitroprusside sodium
PCTx	pig cell transplantation
PGI_2	prostacyclin
pIL3	porcine interleukin 3
POTx	pig organ (kidney or heart) transplantation
pPBPC	porcine hematopoietic progenitor cells
PRP	baboon platelet-rich plasma
pSCF	porcine stem cell factor
PT	prothrombin time
PTT	partial thromboplastin time
ricin A	RTA
SFU	spot forming units
TM	thrombotic microangiopathy
TTP	thrombotic thrombocytopenic purpura (microangiopathy)
vWF	von Willebrand factor
WBI	whole body irradiation

LIST OF PUBLICATIONS

Publications related to this dissertation:

Alwayn IPJ, Buhler L, Basker M, and Cooper DKC. Immunomodulation strategies in xenotransplantation. In: *Current and Future Immunosuppressive therapies following Transplantation. Sayegh M and Remuzzi G (Eds.). In press.*

Alwayn IPJ, Basker M, Buhler L, Harper D, Abraham S, Kruger Gray H, Awwad M, Down J, Rieben R, White-Scharf ME, Sachs DH, Thall A, and Cooper DKC. Clearance of mobilized porcine peripheral blood progenitor cells is delayed by depletion of the phagocytic reticuloendothelial system in baboons. *Transplantation 2001. In press.*

Alwayn IPJ, Basker M, Buhler L, and Cooper DKC. The problem of anti-pig antibodies in pig-to-primate xenografting: current and novel methods of depletion and/or suppression of production of anti-pig antibodies. *Xenotransplantation 1999;6(3):157-168.*

Basker M, **Alwayn IPJ**, Treter S, Harper D, Buhler L, Andrews D, Thall A, Lambrigts D, Awwad M, White-Scharf M, Sachs DH, and Cooper DKC. Effects of B cell/plasma cell depletion or suppression on anti-Gal antibody in the baboon. *Transpl. Proc. 2000;32(5):1009.*

Alwayn IPJ, Xu Y, Basker M, Wu C, Buhler L, Lambrigts D, Treter S, Harper D, Kitamura H, Vitetta E, Awwad M, White-Scharf ME, Sachs DH, Thall A, and Cooper DKC. Effects of specific anti-B and/or anti-plasma cell immunotherapy on antibody production in baboons: depletion of CD20- and CD22-positive B cells does not result in significantly decreased production of anti-αGal antibody. *Xenotransplantation 2001. In press.*

Alwayn IPJ, Buhler L, Basker M, Goepfert C, Kawai T, Kozlowski T, Ierino F, Sachs DH, Sackstein R, Robson SC, and Cooper DKC. Coagulation / Thrombotic disorders associated with organ and cell xenotransplantation. *Transpl. Proc. 2000;32(5):1099.*

Buhler L, Basker M, **Alwayn IPJ**, Goepfert C, Kitamura H, Kawai T, Gojo S, Kozlowski T, Ierino FL, Awwad M, Sachs DH, Sackstein R, Robson SC, and Cooper DKC. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. *Transplantation 2000;70(9):1323-1331.*

Alwayn IPJ, Buhler L, Appel JZ III, Goepfert C, Eva Csizmadia, Hiroshi Kitamura, Sackstein R, Cooper DKC, and Robson SC. Mechanisms of thrombocytopenia following xenogeneic hematopoietic progenitor cell transplantation. *Transplantation 2001. In press.*

Alwayn IPJ, Appel JZ III, Buhler L, DeAngelis HA, Cooper DKC, and Robson SC. Modulation of platelet aggregation in baboons: implications for mixed chimerism in xenotransplantation. 1. The roles of individual components of a transplantation conditioning regimen and of pig peripheral blood progenitor cells. *Transplantation 2001. In press.*

Appel JZ III, **Alwayn IPJ**, Buhler L, Correa LE, Cooper DKC, and Robson SC. Modulation of platelet aggregation in baboons: implications for mixed chimerism in xenotransplantation. 2. The effects of cyclophosphamide on pig peripheral blood progenitor cell-induced aggregation. *Transplantation 2001. In press.*

Alwayn IPJ, Appel JZ III, Goepfert C, Buhler L, Cooper DKC, and Robson SC. Inhibition of platelet aggregation in baboons: therapeutic implications for xenotransplantation. *Xenotransplantation 2000;7(4):247-257.*

Other publications:

Alwayn IPJ, Xu R, Adler WH, and Kittur DS. Does high MHC class II gene expression in normal lungs account for the strong immunogenicity of lung allografts? *Transpl. Int. 1994;7(1):43-46.*

Scheringa M, **Alwayn IPJ**, and Marquet RL. Chronic rejection after xenotransplantation. *Xeno 1996;4(1):13.*

Scheringa M, **Alwayn IPJ**, Bouwman E, IJzermans JNM, and van Bockel JH. Aorta transplantation as a model to study hyperacute, acute, and chronic rejection of xenografts. *Xenotransplantation 1996;3(3):231-236.*

Alwayn IPJ, van Bockel HJ, Daha MR, and Scheringa M. Hyperacute rejection in the guinea pig-to-rat model without formation of the membrane attack complex. *Transpl. Immunol. 1999;7(3):177-182.*

Basker M, Buhler L, **Alwayn IPJ**, Appel JZ III, and Cooper DKC. Pharmacotherapeutic agents in xenotransplantation. *Exp. Opin. Pharmacother. 2000; 1(4):757-769.*

Buhler L, Treter S, McMorrow I, Neethling FA, **Alwayn I**, Awwad M, Thall A, Cooper DK, LeGuern C, Sachs DH. Injection of porcine anti-idiotypic antibodies to primate anti-Gal antibodies leads to active inhibition of serum cytotoxicity in a baboon. *Transpl. Proc. 2000;32(5):1102.*

Goepfert C, Buhler L, Wise R, Basker M, Gojo S, Imai M, **Alwayn I**, Sachs DH, Sackstein R, Cooper DK, Robson SC. Von willebrand factor concentration, multimeric patterns, and cleaving protease activity in baboons undergoing xenogeneic peripheral blood stem cell transplantation. *Transpl. Proc. 2000;32(5):990.*

Watts A, Foley A, Awwad M, Treter S, Oravec G, Buhler L, **Alwayn IPJ**, Kozlowski T, Lambrigts D, Gojo S, Basker M, White-Scharf ME, Andrews D, Sachs DH, and Cooper DKC. Plasma perfusion by apheresis through a Gal immunoaffinity column successfully depletes anti-Gal antibody – experience with 320 aphereses in baboons. *Xenotransplantation 2000;7(3):181-185.*

Buhler L, Basker M, **Alwayn IPJ**, and Cooper DKC. Therapeutic strategies for xenotransplantation. In: *Xenotransplantation. Platt JL (Ed.). AMS Press, Washington, DC.pp117-135.*

Appel JZ III, **Alwayn IPJ**, and Cooper DKC. Xenotransplantation – the challenge to current psychosocial attitudes. *Progress in Transplantation 2000;10:217.*

Buhler L, **Alwayn IPJ**, Appel JZ III, Robson SC, and Cooper DKC. Anti-CD154 monoclonal antibody and thromboembolism (letter). *Transplantation 2001;71:491.*

Buhler L, **Alwayn IPJ**, et al. Pig hematopoietic cell chimerism in baboons conditioned with a nonmyeloablative regimen and CD154 blockade. *Transplantation 2001. In press.*

Alwayn IPJ, Buhler L, Teranishi K, Gollackner B, and Cooper DKC. Immunosuppression for transgenic pig-to-nonhuman primate organ grafting. *Current Opinions in Transplantation 2001. In press.*

Alwayn IPJ and Robson SC. Understanding and preventing the coagulation disorders associated with xenograft rejection. *Graft 2001. In press.*

Alwayn IPJ, Basker M, and Cooper DKC. Suppressing natural antibody production. *Graft 2001. In press.*

Buhler L, Goepfert C, Kitamura H, **Alwayn IPJ**, Basker M, Gojo S, Chang Q, Down J, Tsai H, Sachs DH, Cooper DKC, Robson SC, and Sackstein R. Porcine hematopoietic cell xenotransplantation in nonhuman primates is complicated by thrombotic microangiopathy. *Bone Marrow Transplantation 2001. In press.*

Buhler L, Yamada K, Kitamura H, **Alwayn IPJ**, Basker M, Appel JZ III, Colvin RB, White-Scharf ME, Sachs DH, Robson SC, Awwad M, and Cooper DKC. Pig kidney transplantation in baboons: IgM is associated with acute humoral xenograft rejection and disseminated intravascular coagulation. *Submitted for publication.*

McMorrow IM, Buhler L, Treter S, Neethling F, **Alwayn IPJ**, Comrack CA, Kitamura H, Sachs DH, Awwad M, DerSimonian H, Cooper DKC, and LeGuern C. Down-regulation of the anti-Gal xenogeneic response in vivo through anti-idiotypic modulation. *Submitted for publication.*

Buhler L, **Alwayn IPJ**, Basker M, Oravec G, Thall A, White-Scharf ME, Sachs DH, Awwad M, and Cooper DKC. CD40-CD154 pathway blockade requires host macrophages to induce humoral unresponsiveness to pig hematopoietic cells in baboons. *Submitted for publication.*

Buhler L, Deng S, O'Neil J, Kitamura H, Koulmanda M, **Alwayn IPJ**, Appel JZ, Awwad M, Sachs DH, Weir G, Squifflet JP, Cooper DKC, Morel P. Adult porcine islet transplantation in baboons treated with a nonmyeloablative regimen and CD154 blockade. *Submitted for publication.*

ACKNOWLEDGEMENTS

Many people have contributed to the realization of this dissertation. In particular, I would like to acknowledge the support, motivation, inspiration, and friendship of the following people:

Prof.dr. D.K.C. Cooper, prof.dr. D.H. Sachs, prof.dr. J. Jeekel, dr. J.N.M. IJzermans, prof.dr. R. Benner, prof.dr. F. Grosveld, prof.dr. W. Weimar, the TBRC fellows, techs, and staff, the RdGG surgical staff and residents, Patrick Vriens, Hein Stockmann, the Alwayn family, and :)

CURRICULUM VITAE AUCTORIS

The author was born on September 30th, 1968 in Leidschendam, The Netherlands. After graduating from high school at the Huygens Lyceum in Voorburg in 1986, he attended medical school at the University of Leiden. During medical school, he worked as a student assistant at the Thoracic Intensive Care Unit, Leiden University Medical Center (prof.dr. H.A. Huysmans), performed research electives at the Laboratory for Experimental Surgery, University Hospital Rotterdam 'Dijkzigt' (dr. R.L. Marquet) and the Division of Transplantation Surgery, Johns Hopkins Hospital, Baltimore, U.S.A. (dr. D.S. Kittur), and performed a clinical subinternship at the G.I. Gold Service, Department of Surgery, Johns Hopkins Hospital, Baltimore, U.S.A. (prof.dr. J. Cameron). In 1994, he graduated from medical school *cum laude*.

From December 1994 through December 1995, he worked as an intern at the General Surgical Intensive Care Unit, Leiden University Medical Center (prof.dr. O.T. Terpstra). During this time he performed research under the supervision of prof.dr. H.J. van Bockel. In February 1996, he became a resident at the Department of Surgery (chairman prof.dr. J. Jeekel), University Hospital Rotterdam 'Dijkzigt' (prof.dr. H.A. Bruining / prof.dr. H.J. Bonjer).

From February 1999 through July 2000, he worked as a research fellow in surgery at the Transplantation Biology Research Center, Massachusetts General Hospital / Harvard Medical School, Boston, U.S.A. (prof.dr. D.K.C. Cooper / prof.dr. D.H. Sachs). It is at this institution that the research outlined in this dissertation was performed. Financial support was obtained from the 'Ter Meulen Fund' of the Royal Dutch Academy of Arts and Sciences and from the Professor Michaël van Vloten Fund.

Since August 2000, the author is completing his surgical residency at the Reinier de Graaf Gasthuis in Delft (dr. P.W. de Graaf).