Preface

No field of medicine has engendered greater excitement or enjoyed greater success than the field of transplantation. Organ transplantation allows the "cure" of disease by replacing failing organs with physiologically normal organs. Tissue transplantation and tissue engineering allow not only the replacement of abnormal cells, such as bone marrow cells, but also the possibility of using a transplant to impart novel physiologic functions. The major limitation to applying transplantation for the treatment of disease is a shortage of human donors. This shortage limits transplant procedures to as few as five percent of those that would be carried out if the supply of organs and tissues were unlimited. Because of this shortage and because of recent advances in fundamental knowledge, there has been a crescendo of interest in xenotransplantation, the use of animals in lieu of humans as organ and tissue donors.

For many years, xenotransplantation has seemed only a distant prospect because of the severe immune responses of the host against the graft. Recent studies, however, have revealed the molecular basis of these immune responses and have given rise to novel therapeutic approaches for circumventing them. For example, the generation of transgenic animals expressing human complement regulatory proteins or human glycosyltransferases raises the prospect that the severest type of rejection can be avoided without manipulating the xenograft recipient. Thus, xenotransplantation has quickly moved to center stage in the field of transplantation, engaging the interest of clinicians, basic scientists, and academicians.

Xenotransplantation: Basic Research and Clinical Applications compiles and explains the fundamental molecular and cell biology that has been applied with such advantage in the emerging fields of transplant immunology and xenotransplantation. The contributors to this book are established authorities in transplant immunology and molecular and cell biology. This book provides a base of knowledge for the practitioner, fellow, and student, and those involved in biotechnology and related sciences.

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2 Pathological Responses to Xenotransplantation

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INTRODUCTION

The idea of transplanting animal organs into patients with organ failure is not new. When the development of vascular anastomosis made organ transplantation feasible from a surgical perspective, a few clinical renal xenografts were attempted. In 1906, Jaboulay *(1)* described the xenotransplantation of pig and goat grafts into humans. Neither pig nor goat grafts functioned, and the failure of the xenograft did not allow vascular thrombosis to be observed. At the same time, Unger performed xenotransplantation using organs from nonhuman primates with similar results *(2)*. In 1923, Harol Neuhof affirmed that thrombosis or hemorrhage in the xenotransplant could be prevented *(2)*. However, technical imperfection and the lack of understanding of immunological host reactivity led to waning interest in xenotransplantation.

The first reports of successful clinical xenotransplantation appeared in the literature as recently as 1960. The initial attempts were performed with monkeys and baboons as donors. Reemtsma et al. *(3)* utilized chimpanzees and Starzl et al. *(4)* reported use of a series of baboons-to-human renal xenografts. These transplants did not suffer immediate failure like those performed by Jaboulay, and, indeed, some of the transplants functioned for months. However, the outcome of the transplant was generally unsatisfactory, as the recipients suffered repeated episodes of rejection or transplantation infection and all eventually died. The pathological changes in cross-species xenotransplantation are described by Porter et al. *(5)* as interstitial cellular infiltrates with edema, patchy hemorrhage, and patchy infarction. Although these early attempts

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failed from a clinical perspective, today they are perhaps responsible for the rise of interest in xenotransplantation.

In 1966, Kissmeyer-Nielson et al. *(6)* describe "hyperacute rejection" of clinical allotransplants as a cause of early graft failure. By this time, Perper and Najarian *(7)* find that xenotransplantation might have two different outcomes. Organs transplanted between closely related species such as sheep-to-goat or chimpanzee-to-human function for a period of days before rejection ensues, and the characteristics of rejection resemble those of allografts. However, species that are phylogenetically distant, such as guinea pig-to-rat and pig-to-human, exhibit a course dramatically different owing to a hyperacute rejection reaction much like that described by Kissmeyer-Nelson *(6)*. In 1970, Calne *(8)* formalized this concept. Species combinations in which xenografts are not subject to hyperacute rejection are called "concordant" and species combinations in which xenografts are subject to hyperacute rejection are called "discordant."

It would seem logical that the best xenograft donor from a physiologic and immunologic perspective would be phylogenetically close to the recipient (concordant xenograft). However, although these procedures may help individual patients, they will not solve the overall problem of donor shortage because few nonhuman primates of appropriate size can be found. The transplantation of pig organs is preferred because organs of appropriate size might be available in large numbers at low cost and because the transplantation of porcine organs engenders less risk of zoonosis than the transplantation of primate organs *(9)*. These advantages have prompted surgeons and scientists to focus on a pig-to-primate model as the final preclinical model. However, the use of porcine organs represents a discordant model owing to the presence of preformed natural antibodies to pig antigens. In the study of discordant xenografts, small animal models, such as guinea pig-to-rat models, constitute the most abundant source of information about histologic and immunologic changes. The different antibody–antigen systems and complement activation pathways involved in small animal models differ significantly from the processes in humans and nonhuman primates. Thus, the information from small animals is insufficient for clinical application.

The following section describes the pathology of xenografts in a pig-to-baboon model with insights into the causes and pathogenesis of vari-

ous types of xenograft rejection and suggests new rational therapeutic strategies for future clinical application of xenotransplantation.

HYPERACUTE REJECTION

An organ transplanted into unmanipulated, phylogenetically disparate recipients is subject to hyperacute rejection, which destroys the graft. Hyperacute rejection begins immediately on reperfusion of a xenogeneic organ graft, destroying the graft between minutes to hours. The first clinical and pathological description of hyperacute rejection is commonly credited to Kissmeyer-Nielsen in 1966 *(6)*. The pathological features of the rejected organs contained extensive microvascular thrombosis and neutrophil infiltration similar to what is seen in a generalized Schwartzman reaction. The recipients had titers of antibodies directed against donor kidney extracts. Based on these findings, Kissmeyer-Nelson et al. *(6)* concluded that the rejection reaction is caused by preexisting antibodies directed against foreign antigens in the graft. Thus, what Kissmeyer-Nelson et al. provided for the first time is not only the pathologic description of hyperacute rejection, but they proposed that antibodies against tissue antigens of the donor could mediate a form of rejection that is unique clinically and pathologically.

Platt et al. *(10)* describe this kind of rejection in heart, kidney, and lung xenografts. Macroscopically, blood flow to the transplant organ begins to decline and changes in coloration of the external surface of the xenograft are evident. The tempo of hyperacute rejection varies from experiment to experiment and in the combination of donor and recipient. In species combinations such as pig-to-primate, in which hyperacute rejection is initiated by natural antibodies, the titer of these antibodies is probably the most important factor in determining the rate of rejection. In other species such as guinea pig-to-rat, where hyperacute rejection does not depend on natural antibodies but rather reflects direct activation of the recipients' complement system on donor cells, hyperacute rejection is especially rapid and explosive. Microscopically, hyperacute rejection is characterized by platelet aggregates and erythrocyte sludge in the lumen of blood vessels. As rejection progresses, the pathological features are dominated by interstitial hemorrhage and thrombosis with posterior destruction of vessels. At ultrastructural levels, the damage to endothelial cells becomes more evident, showing alteration in cellular junctions, with platelet attachment to blood vessels

and small vessels appearing to be collapsed. In the evolution of this picture of hyperacute rejection, electron microscopy shows a distortion of the endothelium with irregular surfaces and separation from the underlying matrix. At this point, many capillaries are often found to be occluded by platelets with some erythrocytes. Advanced lesions show rupturing of vessels with extravasation to the interstitium. The immunopathology of hyperacute rejection has been described in detail by Platt et al. *(10)*. Platt and colleagues reveal classical pathway components C1q, C2, and C4 deposited along blood vessels. The alternative pathway components factor B or properdin are observed in some, but not in all, tissues. Also, the presence of immunoglobulin deposits of recipient origin is found along the endothelial cell surfaces of graft blood vessels. The immunopathological studies suggested by Platt et al. are as follows:

1. The endothelial cells constitute the primary target of the immune reaction.
2. In most cases, complement activation in pig-to-primate xenografts is initiated by activation of the classical complement pathway.

ACUTE VASCULAR REJECTION

Experimental approaches to the prevention of discordant xenograft hyperacute rejection are explored in pig-to-primate experimental models (Table 1). All of these manipulations combined with heavy pharmacologic immunosuppressive therapy extend graft survival. Although hyperacute rejection can be prevented by those approaches, another kind of rejection can also occur, namely, acute vascular rejection *(11)*. Acute vascular rejection has also been referred to as delayed hyperacute rejection by others *(12)*.

When hyperacute rejection is averted according to approaches that have been mentioned, the xenograft becomes subject to acute vascular rejection, which destroys the graft over a period of hours to days. This type of rejection is now viewed as a major immunologic barrier to the clinical application of xenotransplantation. Although acute vascular rejection might be considered to be a delayed form of hyperacute rejection, there is much evidence that suggests acute vascular rejection is distinct from hyperacute rejection because the pathogenesis and the pathology of acute vascular rejection are different from that of hyperacute rejection.

Table 1

Therapeutic target	*Therapy*	*Mechanism*	*References*
Xenoreactive antibodies	Plasmaphersis	Depletion of Ab+C	*14*
	Absorbent columns	Depletion of Ab+C	
	Anti-idiotype antibody	Inhibition of Ab production/binding	
	Anti-B-cells agents	Inhibition of Ab production	
	Soluble antigen	Inhibition of Ab binding	
Complement (C)	Cobra venom factor	Depletion of complement	*11, 23–26*
	SCR1	Inhibition of complement	
	Gamma globulin	Diversion of binding	
Donor modification	Transgenic for human complement regulatory proteins H-transferase transgenic pigs		*27,28*

Acute vascular rejection may be related pathogenetically to the activation of graft endothelial cells, but the events that incite endothelial cell activation are subject to controversy. Bach and co-workers *(12)* propose that acute vascular rejection is caused by biological processes that occur independently of the immune reaction of the host against the graft. Based on four lines of evidence, Platt and co-workers *(13)* propose that acute vascular rejection is triggered by persistent interaction of xenoreactive antibodies with graft tissue as follows:

1. Primates from which xenografts are removed after rejection have a sudden increase in antidonor antibody levels, implying that the xenograft is continually exposed to xenoreactive antibodies and is actively absorbing them from circulation.

2. Acute vascular rejection of allografts and concordant xenografts is associated with the presence of antidonor antibodies in the blood or can be induced by administration of antidonor antibodies.
3. Patients exposed to porcine antigens from extracorporeal circulation through porcine livers experience an increase in the titer of xenoreactive antibodies within a few days, coinciding with the time when a xenograft is subject to acute vascular rejection, and suggesting that immune stimulation has occurred.
4. Cytotoxic agents such as cyclophosphamide that inhibit the synthesis of antibodies appear to delay or avert acute vascular rejection.

Although the importance of antibodies in the development of acute vascular rejection seems evident, the exact nature of those antibodies is less certain *(14)*.

The histopathological changes observed on transgenic porcine organs after transplantation into baboons begin as soon as 1 h after transplantation. The most common change is prominent endothelial cell swelling in the capillaries with some red cells trapped within the lumen of the vessels. All the vessels are intact and myocytes are well preserved. After 24 h, endothelial cells exhibit marked swelling with increased nuclear size. Some capillaries appear to be occluded with a "rope-like" appearance that is described as typical of acute vascular rejection in allografts. Some vessels appear congested. In general, cardiomyocytes are preserved at 24 h, although in some areas myocytes show shrinkage with moderate nuclear pyknosis. Biopsies taken 72 h after transplantation and at later times, close to the time of rejection, have capillaries with the same features as at earlier times. Some capillaries remain open but some vessels show fibrin in the lumen and are occluded by swelling of endothelial cells and by various types of blood cells. Cardiomyocytes that lack striations and vacuolization of the cytoplasm are seen. Swelling of endothelial cells remain the main feature, whereas destruction of the vascular wall is not often observed. Cardiomyocytes appear to be damaged, having a wavy shape and pyknotic nucleus in areas associated with infiltrate of mononuclear cells, and in other areas, mild infiltrate of neutrophils. Infiltration of mononuclear cells appears around the blood vessels first and later in the interstitium, destroying the cardiac cells.

Electron microscopy confirms, at the ultrastructural level, the findings by light microscopy and the events shown by immunofluorescence. Moreover, electron microscopy allows the study of vascular structure in detail. In biopsies taken in the first hour, endothelial cells show slight swelling without inflammatory cells in the interstitium. No fibrin is seen

in the vessels or subendothelial domains. After 24 h, biopsies reveal histologic features of various grades of "damage" to endothelial cells. At the beginning, the normal flat cytoplasm of endothelial cells is disrupted by the appearance of multiple pinocytic vesicles. The vesicles appear along the luminal surface as well as central and peripheral aspects of the cell. In contrast to normal endothelial cells, which contain few organelles, the cytoplasm of endothelial cells in the organ transplants contain numerous ribosomes. At this stage, the basal membrane remains intact. The endothelial cells are thicker than in normal cells. Moreover, the flattened nucleus of the normal cell is changed by a protrudent nucleus into the lumen, giving the vessel a general undulant appearance. The interendothelial junctions are dense, long, and irregular.

The most prominent characteristic in electron micrographs is an irregular lumen surface that contrasts with the smooth surface on normal endothelial cells. Cytoplasmic blebs or evaginations of the plasma membrane are a common feature of the lumen. This change appears more prominent at early time points. During this process of blebbing, cytoplasmic material appears to be lost. In these early lesions, the interstitium is increased in area, but inflammatory cells are not observed. Vessels are surrounded by edema (with fibroblasts and collagen). Myocytes show some damage in patches observed by lack of striations.

Biopsies taken 3–7 d after transplantation, before the organ is rejected, show invariable changes present on the endothelial cells. The cytoplasmic volume of endothelial cells is increased. Endothelial cells protrude into the capillary lumen and, because of severe swelling, endothelial cells appear to be enfolding and occluding almost the total lumen of the capillaries. The cytoplasm reveals pallor, probably owing to excess water uptake diluting the cytoplasm matrix, and appears relatively structureless. Organelles and inclusions are separated by electron-lucent areas of cytoplasm. The endothelial cells in other larger vessels reveal moderate swelling of the cytoplasm with irregular and undulant surfaces. The endothelial cell surfaces develop long projections, called filopodia, important for binding blood cells. Platelets and white cells appear to be trapped or attached to the endothelium. The lumen of capillaries in advanced stages of rejection appears to be occupied by fibrin strands and sometimes the lumen is occluded by fibrin clots containing white cells and platelets. Platelets appear to be degranulated and in contact with fibrin and white cells. Subendothelial fibrin is observed and sometimes there is evidence of discontinuity between endothelial cells.

It is important to mention that the process of rejection is dynamic, and it is common to see the juxtaposition of moderately damaged vessels next to severely damaged vessels. Ultrastructural changes in the stages of rejection show many necrotic endothelial cells and myocytes. Between the myocytes it is possible to observe fibrin strands that disrupt the cardiac cells.

The immunopathological study of acute vascular rejection reveals the presence of IgM on the vessels within 1 h after transplantation. In some cases, IgM fluorescence decreases at 24 h and remains low for as long as 3 d. In other cases, IgM deposition remains at the same intensity as seen in the first hour. Deposition of IgG is not observed at 1 h after transplant; however, after 24 h, and especially after 3 d, IgG staining is apparent. The presence of IgG is seen even in the interstitium, suggesting that IgG is leaking from the vessels.

Endothelial cells in normal porcine tissues are positive for MHC class I and MHC class II. MHC class I protein levels remain the same until 3 d after transplantation, at which time MHC class I expression on the surface of cardiomyocytes increases. The increase of MHC I is coincident with the presence of cellular infiltration, although the infiltration appears around vessels first. After 3 d, blood vessels are strongly MHC II positive, while a mild cellular infiltrate outside the vessels is also positive for MHC II. The presence of MHC II fluorescence around the vessels correlates with the presence of infiltrate. Biopsies taken early after transplantation do not show cellular infiltration. Infiltration by $CD16^+$ cells is occasionally seen, and the presence of $CD2^+$ cells appears around the vessels at d 7 and later in the interstitium. The influx of macrophages and PMN is present in biopsies associated with the presence of ischemia. Although platelet thrombi in capillaries are a typical feature of hyperacute and acute vascular rejection, the presence of platelets along the vessels is observed as a small component with the presence of fibrin. The analysis of vessels shows progressive deposition of fibrin, with small fibrin thrombi in the vessels by the third day. As the lesion progresses, the presence of fibrin is detected in the interstitium, reflecting barrier failure provided by endothelial cells.

DELAYED HYPERACUTE REJECTION

Although acute vascular rejection might be considered to be a delayed form of hyperacute rejection *(12)*, there is much evidence that acute vascular rejection is distinct from hyperacute rejection. First, acute vascular rejection is observed in allografts and concordant xenografts in

which hyperacute rejection normally does not occur *(15,16)*. Second, the pathology of acute vascular rejection differs from that of hyperacute rejection *(16,17)*. Third, although the pathogenesis of acute vascular rejection is generally thought to reflect activation of endothelial cells in the transplant *(18,19)*, the course of hyperacute rejection proceeds too rapidly to allow significant effects from endothelial cell activation. Fourth, acute vascular rejection develops when the complement system of the recipient is inactivated, a condition that invariably precludes the development of hyperacute rejection. Thus, we think that the term of delayed hyperacute rejection could be reserved to the pathological picture dominated by occlusion of erythrocytes, venular and capillary thrombi, interstitial hemorrhage, and influx of neutrophils in the same proportion to the extravasated erythrocytes and disruption of the capillaries. The pathological features of this condition are thus indistinguishable from hyperacute rejection.

CHRONIC REJECTION

The possibility that pig-to-primate xenografts may be subject to chronic rejection, as allografts, remains to be explored. A limited number of studies in small animal models suggest that graft vascular disease may be an important impediment to long-term xenograft survival. Scheringa et al. *(20)*, using a hamster-to-rat aorta transplantation model (concordant xenograft), show that features common to allograft chronic rejection, namely, intimal proliferation and infiltrating macrophages and T-cells, are the same with this xenograft model. Recently, Shen et al. *(21)* induced chronic rejection in hamster hearts transplanted into Lewis rats treated with leflunomide. Such lesions in xenografts involve arterial tree damage with histological similarities as well as differences with allografts. In summary, they describe differences in the injury pattern mainly involved with larger sized arteries in xenografts with morphologically more aggressive lesions in xenografts than allografts, such as fibrinoid necrosis, marked intimal edema with a large accumulation of extracellular matrix with or without mononuclear cell infiltration. Thus, xenografts represent a more intensive and aggressive process of arterial injury that is less favorable to long-term graft survival.

At least two considerations have to be made in the interpretation of the above descriptions. First, the model used in the description of chronic rejection is a concordant xenograft, and, second, the use of small animal models cannot address many of the problems seen in the large animal discordant xenografts. For instance, the significance of anti-Gal anti-

bodies (IgM or IgG) in chronic rejection could not be evaluated properly in these models.

Understanding the role of these antibodies could provide important information in the search for new immunosuppressive drugs or an approach to tolerance induction. Galili *(22)* evaluates the role of anti-Gal IgG in chronic xenograft rejection and the association between α-Gal epitope expression and inflammatory infiltrates. Galili concludes that anti-Gal IgG would induce xenograft destruction by antibody dependent cell-mediated cytotoxicity (ADCC) by activation of endothelial cells and by increasing activation of T cells against xenograft antigens. Although the histopathology of chronic rejection in pig-to-primate transplants is unknown, one can easily imagine that this kind of rejection could be more intense and aggressive than seen in allografts and would justify a search for new immunologic approaches to overcome.

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