Tolerance and chronic rejection

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The most common cause of chronic allograft loss is an incompletely understood clinicopathological entity called chronic rejection (CR). Recent reports suggest an improvement in long-term renal allograft survival, although it is not clear from these data whether a true reduction of biopsy-proven CR has occurred. Although newer immunosuppressive medications have greatly reduced the incidence of acute rejection (AR) in the early post-transplantation period, the ideal therapy for both AR and CR would be to achieve a state of tolerance. By definition, such a state should allow for indefinite allograft survival, with no histopathological evidence of CR, despite immunocompetence in the host (i.e. without the need for chronic immunosuppression). Although several experimental studies are able to achieve tolerance, with clear improvement in allograft survival, detailed studies on graft function and morphology are often not included. This review will discuss possible ways that tolerance induction could lead to a CR-free state. General mechanisms of CR and transplantation tolerance induction are discussed as well as the difficulties in translating small animals studies into large animals and humans.

Keywords: chronic allograft nephropathy; chronic allograft dysfunction; transplantation; tolerance; chronic rejection; alloantigen

1. INTRODUCTION

The most common cause of chronic allograft loss is an incompletely understood clinicopathological entity sometimes called chronic rejection (CR), chronic allograft dysfunction or in the case of kidneys, chronic allograft nephropathy (CAN). Morphological findings associated with chronic allograft dysfunction include progressive narrowing of hollow structures within the graft, regardless of whether they are vessels, bile ducts or bronchioles. These findings are usually associated with variable degrees of interstitial mononuclear cellular infiltration and fibrosis (Demetris 1998). In the kidney, CAN is characterized histopathologically by widespread obliterative vasculopathy, glomerulosclerosis and interstitial fibrosis with tubular atrophy. Rarely, transplant glomerulopathy, with splitting of the glomerular basement membrane, is seen. The incidence of these histopathological changes in the modern era of kidney transplantation is 62–72%, as determined by protocol biopsies at two years post-transplantation in a recent series of 96 recipients of cadaveric kidney transplants (Solez *et al.* 1998). Recent reports suggest an improvement in long-term renal allograft survival, although it is not clear from these data whether a true reduction of biopsy-proven CR has occurred (Hariharan *et al.* 2000).

Although newer immunosuppressive medications have greatly reduced the incidence of acute rejection (AR) in the early post-transplantation period (Denton *et al.* 1999), the ideal therapy for both AR and CR would be to achieve a state of tolerance (Dong *et al.* 1999;

Suthanthiran 1996; Waldmann 1999). By definition, such a state should allow for indefinite allograft survival, with no histopathological evidence of CR, despite immunocompetence in the host (i.e. without the need for chronic immunosuppression). Although several experimental studies are able to achieve tolerance, with a clear improvement in allograft survival, detailed studies on graft function and morphology are often not included. Clinical data have shown a correlation between reactivity to donor-specific antigens and CR in recipients of kidney (Vella *et al.* 1997a), heart (Ciubotariu *et al.* 1998) and lung (SivaSai *et al.* 1999) transplants. Even more striking correlations exist between donor-specific hyporeactivity and allograft survival (Reinsmoen *et al.* 1994, 1995; Reinsmoen & Matas 1993), suggesting that donor-specific tolerance may indeed protect from CR in humans.

This review will discuss possible ways that tolerance induction could lead to a CR-free state. First, the factors, both antigen dependent and antigen independent, felt to be responsible for the pathogenesis of CR will be reviewed. The general mechanisms of transplantation tolerance, as they relate to CR, will then be discussed in the context of rodent models. Finally, translational studies in large animal models and humans will be presented.

2. CHRONIC ALLOGRAFT DYSFUNCTION

It is generally accepted that both alloantigen-dependent and alloantigen-independent factors contribute to the pathogenesis of CR (Tullius & Tilney 1995). Some of the best evidence supporting the role of alloantigen-dependent factors in the development of chronic allograft dysfunction are the observations that the incidence

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increases (i) after episodes of AR, (ii) with inadequate immunosuppression, and (iii) and with increased human leucocyte antigen (HLA) mismatching (Womer *et al.* 2000b). In fact, HLA-identical allografts may survive indefinitely in the absence of other intervening insults. Both the cellular and humoral arms of the immune response probably act in concert to cause the changes characteristic of CR. These will be briefly reviewed in this section. There is intense interest now in the role of antigen-independent causes of chronic allograft dysfunction, which include organ injury secondary to brain death in the donor, donor–recipient size mismatch, ischaemia–reperfusion events, cytomegalovirus (CMV) infection, hypertension, hyperlipidaemia, cellular senescence, and toxicity of immunosuppressive medications. These factors probably serve to amplify the alloimmune response and may not themselves be sufficient to effect the characteristic changes seen in CR (Humar *et al.* 1999b), particularly with a tolerant state.

(a) *Alloantigen-dependent factors*

(i) *T-cell allorecognition and effector function*

Extensive data from animal studies indicate that alloantigen-activated T cells play a key role in initiating the process of CR. T-cell recognition initiates the effector mechanisms of rejection, including CD4⁺ T cells and macrophages mediating delayed-type hypersensitivity responses, CD8⁺ T cells and alloantibody-producing B cells. It is hypothesized that activated CD4⁺ T cells produce key cytokines which activate macrophages and B cells. Chemoattractants further recruit circulating mononuclear cells to the graft (Nadeau *et al.* 1995), which, along with endothelial cells, secrete growth factors that cause smooth muscle cell proliferation leading to vascular intimal occlusion and interstitial fibrosis (Womer *et al.* 2000b). Both experimental (Russell *et al.* 1994) and clinical data (Kerman *et al.* 1997) confirm the association of alloantibody production with CR.

As discussed in more detail in the review by Womer *et al.* (this issue), there are at least two distinct, although not mutually exclusive, pathways of T-cell allorecognition, each of which generates different sets of T-cell clones (Womer *et al.* 2000a). The direct pathway is characterized by T-cell recognition of intact allo-major histocompatibility complex (MHC) on the surface of donor antigen-presenting cells (APCs). The indirect pathway is characterized by T-cell recognition of processed, donor-derived MHC alloantigen presented as peptides by self. It has been suggested that direct allorecognition may mediate early AR, since newly engrafted tissue typically contains a high density of passenger leucocytes, mostly dendritic cells, that are capable of presenting antigen by this pathway. The corollary of this hypothesis is that direct allorecognition becomes less important as passenger leucocytes are depleted with time after transplantation (Sayegh & Carpenter 1996). Although it was previously thought that allopeptide-induced T-cell responses are restricted to a limited repertoire of peptides representing immunodominant epitopes (Gallon *et al.* 1995; Watschinger *et al.* 1994), it is now clear that a change in the pattern of T-cell responses, termed epitope shifting or spreading, can occur to peptides representing alternative regions within a given MHC β -chain hyper-

variable region (intramolecular spreading), or to peptides representing different MHC chains (intermolecular spreading) (Ciubotariu *et al.* 1998; Vella *et al.* 1997a,b). These data suggest that diversification of the immune response against the graft contributes to CR, supporting the hypothesis that chronic allograft vasculopathy results from continual injury initiated by the allopeptide-reactive T cells. In support of this hypothesis, recent data from Hornick *et al.* (1998) in heart transplant recipients who developed chronic allograft vasculopathy show that both CD4⁺ as well as CD8⁺ T cells with direct allospecificity are hyporesponsive to donor alloantigens, while such patients show increased frequency of CD4⁺ T cells with reactivity to donor HLA peptides presented by self-APCs (Hornick *et al.* 2000). Therefore, in order to prevent CR, tolerance regimens should tolerize T cells with direct and indirect allospecificity and prevent epitope shifting or spreading.

(ii) *HLA matching*

Observations that the half-lives of cadaveric renal and cardiac allografts from HLA-matched donors are longer than those of organs from corresponding HLA-mismatched donors support the notion that CR is, at least in part, alloantigen mediated (Cecka 1991). Further support comes from protocol renal allograft biopsies at two years post-transplantation in patients without episodes of AR, in which histopathological CR is absent in recipients of HLA-identical but not of HLA-mismatched kidneys (Legendre *et al.* 1998). Among 3562 pairs of cadaveric kidneys, in which one went to an HLA-matched recipient and the other to an HLA-mismatched recipient, the rates of functional graft survival were significantly higher for the HLA-matched kidneys (Takemoto *et al.* 2000).

(iii) *AR and inadequate immunosuppression*

AR has been confirmed as one of the strongest predictors for the development of chronic allograft loss in renal transplant recipients. Numerous studies have shown that the risk is directly related to the number as well as the timing of AR episodes (Cecka 1991; Gulinkaker *et al.* 1992; Lindholm *et al.* 1993; Matas *et al.* 1993, 1994), with a higher incidence of CR (biopsy proven) in patients having more than one episode, particularly when AR occurs greater than 60 days post-transplantation (Basadonna *et al.* 1993). Other studies show a correlation between the severity of AR episodes (decline in glomerular filtration rate, histological grade on biopsy, response to therapy) (Humar *et al.* 1999c; Ishikawa *et al.* 1999), while some have found no increased incidence of CR if the graft function returns to normal by one year (Opelz 1997). Attempting to explain why not all clinical AR episodes lead to ultimate graft demise, recent experimental work in a heart transplant model suggests that the relationship between AR and CR may be dependent on the MHC mismatch (Hosenpud *et al.* 2000).

Long identified as a risk factor for AR, inadequate immunosuppression has also been shown to predispose to the development of CR, whether from patient non-compliance or through attempts by physicians to reduce toxic medications such as cyclosporin A (CsA) or corticosteroids. Two large series of renal allograft recipients

have identified a regimen with CsA dosage less than $5 \text{ mg kg}^{-1} \text{ day}^{-1}$ at one year as a risk factor (Almond *et al.* 1993; Guyot *et al.* 1996). Most studies using steroid-free immunosuppressive regimens have noted an increased incidence of both AR (Hricik *et al.* 1993) and chronic allograft dysfunction (Ratcliffe *et al.* 1996; Sinclair 1992). More recent attempts in patients receiving CsA and mycophenolate mofetil suggest that steroids may be safely withdrawn in non-black patients without a history of AR at 90 days, although longer follow-up is warranted (Ahsan *et al.* 1999). The long-term impact of target of rapamycin inhibitors (rapamycin) on CR and on facilitating the withdrawal of calcineurin inhibitors remains to be seen (Denton *et al.* 1999).

(b) *Alloantigen-independent factors*

(i) *The marginal donor*

With increasing demands for organs, the pressure to expand criteria for donor acceptance has intensified. This, in turn, has prompted many to study the relationship between 'marginal' donors and the development of CR. Of particular concern are kidneys with a low renal mass to body weight of the recipient, including those from donors of advanced age. Indeed, a recent retrospective multivariate analysis of 522 consecutive cadaveric transplants confirmed a donor age of more than 55 years as the only risk factor for renal allograft loss greater than five years post-transplantation (Prommool *et al.* 2000). Since no HLA effect was seen in kidneys from older donors, it is not clear whether donor age increases immune recognition and therefore whether this could be successfully treated through tolerance-inducing strategies. However, it is possible that subclinical activation or rejection, not measured by current assays, is occurring in these patients as a result of earlier injury to a marginal organ.

(ii) *Brain death*

The primary source for solid organ transplants are cadavers who have suffered extensive and irreversible central nervous system damage secondary to haemorrhage, infarction or trauma. The clinical observations that organs from living donors, regardless of their relationship to the recipient, are superior to those of cadavers (Cecka 2000; Gjertson & Cecka 2000) implicate an impact of brain death itself on their quality. It has been suggested that organs experiencing warm ischaemia due to haemodynamic instability in brain-dead donors express higher levels of cytokines than those from ideal living donors. Experimentally, kidneys and hearts from brain-dead donor animals experience accelerated AR compared with controls (Pratchke *et al.* 1999), which emphasizes clinical data showing a greater incidence and severity of AR in cadaveric organs over those from living related sources (Terasaki *et al.* 1995). It is plausible, therefore, that effective tolerance-inducing strategies would be sufficient to overcome this immune activation, although the concept has not been directly studied.

(iii) *Ischaemia and reperfusion*

Although the mechanisms by which ischaemic insults may contribute to ultimate graft demise are still

unclear, evidence is growing that the events surrounding organ removal, storage, and engraftment may increase graft immunogenicity by upregulating MHC antigens and triggering the cytokine adhesion molecule cascade to influence the eventual development of obliterative vasculopathy and fibrosis. Experimental models of cold ischaemia (isografts) have demonstrated early upregulation of selectins, which are one of a series of adhesion molecules responsible for leucocyte-endothelial interactions (Takada *et al.* 1997b). MHC class II is also upregulated, suggesting increased immunogenicity of the ischaemic organ. In the same model, upregulation of B7 has been demonstrated, and B7 blockade by monoclonal antibody (mAb) inhibits T-cell and macrophage infiltration (Chandraker *et al.* 1997b; Takada *et al.* 1997a). Similar results have been seen after therapy with low-dose CsA as well (Kouwenhoven *et al.* 1999). Furthermore, in a rat model of CR, B7 mAb blockade prevents the development of CR when given at the time of transplantation (Russell *et al.* 1996) and can interrupt the progression of CR when administered several weeks after transplantation (Chandraker *et al.* 1998). It is difficult to dissect, however, the alloantigen-specific from non-specific effects of this therapy in such a model. In a rat model of tolerance, however, protection against CR is not abrogated by prolonged ischaemia in a second challenge graft, suggesting tolerance is sufficient to protect from non-immunological injury (Akyurek *et al.* 1998).

(iv) *CMV infection*

There has been keen interest recently in the effects of CMV infection on the development of chronic allograft dysfunction. Numerous experimental and clinical studies suggest that CMV-linked vascular wall inflammation might play a role in the immune injury towards allograft vascular structures and thus contribute to allograft arteriosclerosis (Hruban *et al.* 1990; Koskinen *et al.* 1994, 1996; Levisetti *et al.* 1997; McDonald *et al.* 1989; Normann *et al.* 1991). Several explanations as to how the virus may contribute to this process exist. A protein encoded by the IE-2 region of human CMV contains sequence homology and shows immunological cross-reactivity with a conserved domain of HLA-DR, which could potentially enhance the alloimmune response to donor antigens (Fujinami *et al.* 1988). In addition, CMV encodes a glycoprotein homologous to the heavy chain of MHC class I antigen, which has been shown to bind $\beta 2$ -microglobulin, a protein that is normally found associated with the class I MHC antigens necessary for self-non-self recognition in the immune response (Beck & Barrell 1988). Active CMV infection is strongly associated with expression of vascular cell adhesion molecule 1 on the capillary endothelium in heart transplant recipients, suggesting a possible role of this molecule in the adhesion of lymphocytes and monocytes to vascular endothelium during infection (Koskinen 1993). Recent clinical data in renal transplant patients suggest that CMV disease may play a role in the pathogenesis of CR, but only in the presence of AR (Humar *et al.* 1999a). These findings might mean that in a tolerant state, CMV disease no longer predisposes to the vascular obliteration seen in CR.

(v) *Hyperlipidaemia*

Hyperlipidaemia is an important complication of solid organ transplantation, affecting up to 80% of heart (Ballantyne *et al.* 1992) and 75% of kidney (Ong *et al.* 1994) transplant recipients. Recent experimental work suggests that hypercholesterolaemia and the allogeneic state act in concert to augment allograft atherosclerosis, T-cell accumulation, intimal neovascularization, local cytokine expression and indices of cell activation in transplanted arteries (Raisanen-Sokolowski *et al.* 1994; Tanaka *et al.* 1994). Moreover, two randomized, controlled trials in heart transplant recipients demonstrated that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors improve allograft survival and decrease the amount of graft coronary artery disease (Kobashigawa *et al.* 1995; Wenke *et al.* 1997). Although cholesterol reduction may have been responsible for the beneficial effect, other mechanisms have been proposed. In one of the studies, cytotoxicity of natural killer (NK) cells was found to be lower in patients treated with the HMG-CoA reductase inhibitors compared with controls (Kobashigawa *et al.* 1995). In fact, *in vitro* studies have shown inhibition of NK cell activity by HMG-CoA reductase inhibitors that can be reversed by treatment with interleukin 2 (IL-2) (Cutts & Bankhurst 1990; Cutts *et al.* 1989). It is also possible that lowering plasma lipids enhanced the uptake of the lipophilic drug, CsA, resulting in enhanced immunosuppression. These studies taken together suggest that tolerance-inducing strategies might prevent cholesterol-related transplant vasculopathy through attenuation of the alloresponse, and hence, vascular wall inflammation.

(vi) *Calcineurin inhibitors*

Calcineurin inhibitors, particularly CsA, are often implicated in the development of CR, based in large part on early observations made in the Stanford Cardiac Transplantation Program (Myers *et al.* 1984). Myers and colleagues demonstrated renal insufficiency and even end-stage renal disease in recipients of heart transplants immunosuppressed with CsA. Similarly, the histopathological results of two-year protocol biopsies from patients who were enrolled in the US FK506 Kidney Transplant Study Group revealed a significantly higher occurrence of CAN in patients who experienced presumed CsA or tacrolimus nephrotoxicity (Solez *et al.* 1998). Evidence that CsA and tacrolimus activate genes leading to the synthesis of the fibrogenic growth factor, transforming growth factor- β 1, has provided a mechanistic explanation, in part, for this hypothesis (Han *et al.* 1995). More recently, Kahan *et al.* (1996) examined retrospectively the serial pharmacological profiling of a cohort of 240 patients who had been treated for up to five years with CsA. When regression models were analysed, it was clearly shown that those patients with the most variable drug exposure had the highest serum creatinines, the greatest organ dysfunction, and when available, biopsies with the histological findings of CAN. Indeed, those patients experiencing greater than 20% coefficient of variation had the highest risk for CR. Although the full impact of calcineurin inhibitors on CR is probably not fully characterized, it is clear that a state of tolerance, without the need for immunosuppressive medications, would render this question moot.

(vii) *Hypertension*

The causal role of hypertension in the development and progression of chronic renal disease has been well established, and blood pressure control reduces the rate of decline in function in various renal diseases (Giatras *et al.* 1997; Lewis *et al.* 1993). In renal transplantation, clinical studies have confirmed an association between post-transplantation hypertension and poorer graft outcome (Cheigh *et al.* 1989). However, from these studies, it appears that graft dysfunction rather than the presence of hypertension is the major determinant of graft prognosis. Experimental models suggest that angiotensin-converting enzyme (ACE) inhibitors may be of benefit in the treatment of CR, presumably through the reduction of glomerular capillary pressure (Benediktsson *et al.* 1996). However, recent studies have shown a reduction of growth factors and cytokines as well as diminished leucocyte infiltration in allografts after ACE inhibition (Szabo *et al.* 2000). These findings suggest the salutary effect of antihypertensive therapy, at least with ACE inhibitors, may be due to an immunosuppressive effect of these medications on the vessel walls in the graft. What role, if any, tolerance induction would have on the effects of hypertension on allograft function remains unclear.

(viii) *Cellular senescence*

An alternative hypothesis has been proposed by Halloran (1998), who argues that the ultimate effect of the non-immune and immune interactions in chronic allograft dysfunction is to accelerate the process of senescence in key cell populations necessary to heal and to resolve injury. Like whole organisms, cells, tissues and organs are mortal. Key cells, including vascular endothelial cells, renal epithelial cells and bronchial epithelial cells in transplanted organs lose their ability to proliferate to repair injury, leading to focal atrophy, fibrosis and loss of reserve and function. Inflammation due to unresolved injury or rejection induces stress, which eventually exhausts repair capacity and induces cell senescence in rate-limiting cells. Those organs with exhausted healing may continue to have active antigen presentation and hence, ultimate graft destruction. Tolerance-inducing strategies would conceivably reduce initial injury through the absence of early AR episodes and later in the course, through reduced antigen presentation and activation.

3. TRANSPLANTATION TOLERANCE

(a) *Mechanisms*

Transplantation tolerance involves several mechanisms, including deletion, anergy and suppressor or regulatory cells (Dong *et al.* 1999). Although the mechanisms of tolerance induction are best classified as central or peripheral, there is probably some overlap between the two. These mechanisms will be reviewed next in the context of both *in vitro* and small animal models, followed by recent translational studies in large animal models and in humans (summarized in table 1).

(i) *Central tolerance*

Central tolerance involves primarily deletional mechanisms analogous to self-tolerance, whereby developing T cells strongly reactive to self in the thymus undergo

Table 1. *Summary of tolerance strategies*

type	animal model	tolerizing regimen	notes	references
central	rat heart	irradiation; T-cell-depleted BM infusion	prevention of CR; chimerism present	Orloff <i>et al.</i> (1995)
	rat small bowel	irradiation; T-cell-depleted BM infusion	prevention of CR; chimerism present	Orloff <i>et al.</i> (2000)
	rat heart	irradiation; donor BM or PBMC infusion	lack of CR and chimerism only with BM infusion	Hayamizu <i>et al.</i> (1999)
	rat heart	IT injection of donor BM	prevention of CR	Orloff <i>et al.</i> (1995)
	rat heart	IT injection of donor splenocytes; ALS	prevention of CR only when given with ALS	Shin <i>et al.</i> (1995)
	rat kidney	syngeneic BM transplant; IT injection of donor BM	prevention of CR; chimerism not required	Blom <i>et al.</i> (1998)
	rat heart	IT injection of class I donor MHC peptides	evidence of CR despite chimerism	Shirwan <i>et al.</i> (1997)
	monkey kidney	irradiation; anti-thymocyte globulin; donor BM infusion	no CR in 9 out of 11 animals demonstrating chimerism	Kawai <i>et al.</i> (1999)
peripheral	mouse aorta	combined CTLA-4-Ig and anti-CD154 mAb	lack of CR only when given together or chronically	Sun <i>et al.</i> (1997)
	mouse heart	combined CTLA-4-Ig and anti-CD154 mAb	lack of CR only when given together	Larsen <i>et al.</i> (1996)
	mouse heart	CD154-deficient host	donor-specific tolerance does not prevent CR	Shimizu <i>et al.</i> (2000)
	rat kidney	CTLA-4-Ig	prevention of CR	Azuma <i>et al.</i> (1996)
	mouse liver	AICD <i>de novo</i>	corticosteroids abrogate tolerance induction	Bishop <i>et al.</i> (1996)
	monkey kidney	T-lymphocyte depletion	CR limits long-term tolerance	Knechtle <i>et al.</i> (1998)
	monkey kidney	CD3 ⁺ T-cell depletion and deoxyspergualin	more than 3.5 year survival without CR or anti-donor antibodies	Thomas <i>et al.</i> (2000)
	monkey kidney	anti-CD154 mAb	presence of anti-donor antibodies and CR	Kirk <i>et al.</i> (1999)
	swine heart	combined heart and kidney transplantation	'tolerance by association' conferred to heart	Madsen <i>et al.</i> (1998)

programmed cell death (apoptosis) and are removed from the T-cell repertoire. This form of tolerance is achieved through haemopoietic chimerism by donor bone marrow (BM) transplantation (Wekerle & Sykes 1999), although mechanisms other than central deletion may be operative (Wekerle *et al.* 1998). In the minor antigen-disparate Lewis to F-344 rat model of CR, BM chimeras were found to be tolerant to donor-strain cardiac allografts at 120 days post-operatively with no histological evidence of allograft vasculopathy (Orloff *et al.* 1995). Non-chimeric control rats, however, developed allograft vasculopathy at the same time-point. BM chimerism has also been shown to induce donor-specific tolerance to small bowel allografts and prevent CR with this same strain combination (Orloff *et al.* 2000). Other studies have examined the necessity of chimerism for the prevention of CR. Using three different sources of donor cells, Hayamizu *et al.* (1999) demonstrated that while peripheral blood mononuclear cells (PBMCs), granulocyte colony stimulating factor-mobilized PBMCs, and BM cells all led to long-term survival of cardiac allografts, only the injection of BM cells prevented chronic allograft vasculopathy. The BM cell protocol was the only one out of the three that actually induced long-term chimerism (Hayamizu *et al.* 1999).

Central mechanisms may also explain tolerance induction by intrathymic (IT) injection of donor alloantigen, a strategy which has also been effective in preventing or

attenuating the development of CR. IT injection of allogeneic BM cells (Orloff *et al.* 2000) or donor splenocytes plus anti-lymphocyte serum (ALS) treatment (Shin *et al.* 1995) has been shown to prevent or ameliorate CR in the Lewis to F-344 rat models of small bowel and heart transplantation. IT injection of Lewis BM cells into F-344 rats can also induce tolerance to donor renal grafts and prevent chronic dysfunction (Blom *et al.* 1998). Contrary to these reports are studies demonstrating that IT injection of donor splenocytes and donor polymorphic class I peptides is unsuccessful in preventing CR of class I disparate cardiac allografts in the PVG.R8 to PVG.1U rat model (Shirwan *et al.* 1997). Interestingly, the presence of microchimerism in this study did not correlate with prevention of CR.

(ii) *Peripheral tolerance*

Mechanisms of peripheral tolerance include anergy and suppressor or regulatory cells, although cell death probably plays a role as well (Dong *et al.* 1999). Anergy is a state in which antigen-specific T cells are present but functionally inert, with failure of proliferation *in vitro* (Schwartz 1990) and of clonal expansion *in vivo* (Chen *et al.* 1997; Judge *et al.* 1996; Kearney *et al.* 1995). It is now clear that T cells require two distinct signals for full activation. The first signal is provided by the engagement of the T-cell receptor (TCR) with the MHC-peptide complex on APCs, and the second co-stimulatory signal is

provided by engagement of one or more T-cell surface receptors with their specific ligands on APCs. The best characterized co-stimulatory signal involves binding of the T-cell surface molecule CD28 to either of its ligands, CD80 (B7-1) or CD86 (B7-2), expressed on the surface of professional BM-derived APCs (Sayegh & Turka 1998). Engagement of the TCR with donor MHC-peptide in the absence of co-stimulation leads to T-cell anergy *in vitro* (Gimmi *et al.* 1993). Such a condition probably exists *in vivo* once donor dendritic cells have left the graft post-transplantation as donor-reactive T cells encounter antigen on non-professional APCs of the graft (endothelial and epithelial cells) lacking effective co-stimulatory molecules (Lombardi *et al.* 1997; Marelli-Berg *et al.* 1997). These findings are particularly relevant considering recent evidence that peripheral tolerance induction by co-stimulatory blockade is inhibited by conventional immunosuppression, which blocks signal 1 of T-cell activation (Li *et al.* 1999). Pharmacological T-cell co-stimulatory blockade has been shown to induce anergy *in vivo* (Judge *et al.* 1996; Kearney *et al.* 1995), although the mechanisms in transplantation models may also involve T-cell deletion (Li *et al.* 1999; Wells *et al.* 1999) and regulation (Honey *et al.* 1999; Sayegh *et al.* 1995).

Recent rodent studies have demonstrated the powerful ability of co-stimulatory blockade to prolong graft survival and induce tolerance in some models. Blockade of the CD28-CD80-CD86 and/or CD40-CD154 co-stimulatory pathways can prevent AR (Larsen *et al.* 1996) and graft-versus-host disease (Blazar *et al.* 1998), and aid in the establishment of haemopoietic chimerism (Wekerle *et al.* 1998; Wekerle & Sykes 1999). Prolonged treatment of mice with cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4-Ig) and anti-CD154 mAb prevents vascular intimal thickening in a mouse aortic allograft model (Sun *et al.* 1997), although vasculopathy was only prevented when both agents were used simultaneously and given chronically. The administration of CTLA-4-Ig alone or in conjunction with anti-CD154 mAb or donor-specific transfusion in rodents has also been shown to prevent CR of cardiac (Larsen *et al.* 1996; Sayegh *et al.* 1997) and renal (Azuma *et al.* 1996) allografts. Hancock *et al.* (1998) demonstrated permanent mouse cardiac allograft survival in animals treated with mAb to CD4 or CD154 plus donor cells. Freedom from CR, which correlated with vascular expression of the 'protective' genes, *haem oxygenase-1*, *Bcl-xL* and *A20*, was only seen in the animals treated with mAb to CD154 plus donor cells. Although these results are encouraging, contrary findings have been seen in a murine cardiac allograft model using CD154-deficient recipient mice, where long-term allograft survival and donor-specific tolerance do not prevent graft arteriosclerosis (Shimizu *et al.* 2000).

More recently attention has focused on conditions that may lead to deletion of alloreactive T cells in the periphery (Li *et al.* 1999; Wells *et al.* 1999). T cells may undergo apoptosis by two distinct mechanisms: passive cell death and activation-induced cell death (AICD). AICD is dependent on the presence of IL-2 and is mediated by Fas-Fas ligand (FasL) interactions, which serve to eliminate activated cells after repeated antigenic stimulation. Passive cell death eliminates mature, non-activated T cells due to cytokine or growth factor

withdrawal (Van Parijs & Abbas 1998). Animals whose T cells cannot undergo AICD, such as IL-2 knockout (KO) animals and animals with T cells transgenic for the expression of the anti-apoptotic gene, *Bcl-xL*, are resistant to transplant tolerance induction by T-cell co-stimulatory blockade (Dai *et al.* 1998; Li *et al.* 1999; Wells *et al.* 1999). Indeed, CsA, by inhibiting IL-2 secretion and AICD, was reported to inhibit induction of transplantation tolerance (Li *et al.* 1999) and protection from CR (Chandraker *et al.* 1997a) by T-cell co-stimulatory blockade.

Experimentally, high levels of T-cell apoptosis within graft-infiltrating cell populations correlate with liver allograft survival, which occurs in the absence of immunosuppression in several models (Qian *et al.* 1997). Such a phenomenon may be explained by Fas-FasL interactions that are responsible for immune-privileged sites such as the testis and the anterior chamber of the eye (Bellgrau *et al.* 1995; Griffith *et al.* 1995). AICD of donor-reactive T cells has also been demonstrated after liver transplantation in peripheral lymphoid tissues coincident with migration of donor leucocytes to these sites and subsequent upregulation of IL-2 and interferon- γ (IFN- γ) (Bishop *et al.* 1996). A model of high-dose tolerance has been proposed, where large doses of donor antigen induce rapid activation and subsequent deletion of alloreactive cells, whose IL-2 requirements may exhaust endogenous production (Bishop *et al.* 1997). Although complete elimination of alloreactive T cells in the periphery is probably not realistic, the number of T cells may be reduced to numbers that can then be managed by other mechanisms such as regulatory or suppressor cells.

Several reports have established the unequivocal existence of regulatory or suppressor T cells in models of transplantation tolerance (Honey *et al.* 1999; Onodera *et al.* 1996; Qin *et al.* 1993). However, the exact phenotype and mechanisms of action of regulatory T cells remain controversial. The T helper cell 1 (Th1) and T helper cell 2 (Th2) paradigm which holds that rejection is dependent on the expression of Th1 cytokines (IL-2, IFN- γ and IL-12) and that tolerance is dependent on Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) has been recently challenged. The existence of specific cytokine gene KO mice indicates that Th1 cytokines may function not only to initiate cell-mediated immune responses but also to regulate T-cell responses. Indeed, both IL-2 and IFN- γ KO mice are capable of rejecting grafts and are somewhat resistant to induction of tolerance (Dai *et al.* 1998; Konieczny *et al.* 1998; Li *et al.* 1999; Steiger *et al.* 1995). Th2 cytokine gene KOs (such as the IL-4 KO) reject grafts with the same tempo as wild-type mice and can be tolerized (Lakkis *et al.* 1997), although the latter is not a universal finding in all models studied (Sirak *et al.* 1998). More recent studies from our group examined the rejection patterns and effect of T-cell co-stimulatory blockade in signal transducer and activator of transcription 4 (STAT-4) (unable to mount normal Th1 responses) and STAT-6 (unable to mount normal Th2 responses) KO mice. Both these KOs reject grafts with the same tempo as wild-type recipients but are differentially regulated by CD28-B7 versus CD154-CD40 co-stimulation (Kishimoto *et al.* 2000). Interestingly, STAT-4 KOs develop less severe graft arteriosclerosis (Koglin *et al.* 2000), similar to what has been reported for

IFN- γ -deficient animals (Raisanen-Sokolowski *et al.* 1998).

Another controversial issue is whether Th2 cells can function as regulatory T cells in allogeneic models. While several models established the association between a Th2 switch and transplantation tolerance (Hancock *et al.* 1996, 1998; Onodera *et al.* 1997; Sayegh *et al.* 1995), a causal relationship has not been established (Field *et al.* 1997; Nickerson *et al.* 1997; Piccotti *et al.* 1997). Given the unfolding evidence of the importance of AICD, it seems likely that Th1 cytokines are necessary for tolerance induction to the high precursor frequency of T cells responding via the direct pathway during the early phase of rejection. Th2 cytokines, on the other hand, may assist with the maintenance of tolerance later when the indirect pathway with significantly lower precursor frequency predominates (Li *et al.* 1998). Such a model implies a dual role for indirect allorecognition of donor peptides, with presentation to Th1 cells leading to CR and presentation to Th2 cells as a requirement for long-term tolerance. The clinical implications of this new paradigm for development of tolerance and prevention of CR require further investigation.

(b) *Translational studies*

There are a limited number of studies performed in preclinical, large animal models of transplantation that shed more light on the relationship between tolerance and CR. T-lymphocyte depletion with the immunotoxin FN 18-CRM9 reliably prolongs renal allograft survival in rhesus monkeys when started on the day of transplantation (Knechtle *et al.* 1998). However, interstitial nephritis and CR limit the development of long-term tolerance. More recent studies by Thomas *et al.* (2000) demonstrated that depletion of CD3⁺ T cells and administration of deoxyspergualin (days 0–14) lead to tolerance of renal allografts in rhesus macaques, from 268 days to more than 3.5 years. Recipient animals showed significant prolongation of donor skin grafts while rejecting third-party skin grafts and maintaining the ability to accept second kidney transplants from the original donors. Furthermore, tolerant animals did not produce anti-donor antibodies and histological analysis demonstrated that renal allografts from the tolerant animals lacked any of the stigmata of CR. While chimerism was not required for tolerance induction in the aforementioned study, another report using a non-myeloablative regimen in cynomolgus monkeys demonstrated that chimerism, albeit transient, was necessary for long-term renal allograft survival (Kawai *et al.* 1999). No evidence of CR was seen in 9 out of 11 animals demonstrating chimerism in this study. Co-stimulatory blockade has also been studied in non-human primate models of transplantation with the administration of anti-CD154 mAb leading to long-term survival of renal allografts in rhesus monkeys (more than ten months) (Kirk *et al.* 1999). Evaluation of long-term survivors, however, revealed the presence of anti-donor antibodies, and grafts ultimately demonstrated some signs of CR, which argues against a true state of tolerance.

Studies using MHC-inbred miniature swine have produced additional data concerning the relationship between tolerance and CR. In the miniature swine, donor-specific tolerance to class I disparate renal allografts

is easily achieved after a 12 day course of CsA (Rosengard *et al.* 1992), while cardiac allografts are uniformly rejected (Madsen *et al.* 1998). However, when a heart and a kidney from the same donor were co-transplanted with a 12-day course of CsA into a class I-disparate recipient, tolerance was conferred to the heart in addition to the kidney. Thus, tolerance to the kidney induced tolerance to the donor-strain heart, a type of 'tolerance by association'. With this combination, no vasculopathy is seen in either organ. However, removal of the kidney leads to CR of the heart (K. Yamada, personal communication), and when the recipient thymus is removed, both the heart and kidney are rejected with typical chronic vasculopathy changes seen in the heart (Yamada *et al.* 1999).

Our understanding of the relationship between tolerance and clinical CR is hindered by the lack of reliable immune-monitoring assays in humans to determine when a true state of tolerance exists and hence, when withdrawal of immunosuppression is feasible. Clinical studies, however, have provided us with some insight into the factors that contribute to CR. A detailed analysis of 199 kidney transplant recipients revealed that *in vitro* donor-specific hyporesponsiveness of T cells correlates with fewer episodes of AR and CR. Only 1 out of the 35 patients with donor-specific hyporesponsiveness developed histological evidence of CR, while 12% of the patients with anti-donor reactivity developed CR at more than 12 months (Reinsmoen & Matas 1993). Those patients with hyporesponsiveness also demonstrated longer graft survival than patients with donor reactivity, although none of the patients could be considered truly tolerant given the need for immunosuppression. In a recent study of 11 kidney recipients, 9 patients were found to demonstrate indirect reactivity to donor MHC class II peptides, with a tenfold increase in precursor frequencies, while patients without chronic dysfunction lacked such indirect donor-reactivity (Vella *et al.* 1997a). In support of this study of kidney recipients, analyses of heart transplant patients have demonstrated that patients with histological chronic vasculopathy demonstrate elevated frequencies of T cells with indirect anti-donor specificity, while patients free of CR lacked such reactivity (Hornick *et al.* 2000).

4. CONCLUSIONS

A true state of tolerance implies a rejection-free state, both acute and chronic, in the absence of chronic immunosuppression. However, conflicting reports generated from both central and peripheral models of tolerance have raised the question of whether tolerance will be enough of a cure for CR. One could easily argue that these were not 'true' tolerance models, and thus strategies that do not lead to normal graft function and prevention of CR should not be considered true tolerance strategies. Other possible explanations may be mechanistic in nature. For example, although T-cell activation is the central and primary event in allograft rejection, partial clonal anergy or elimination may be sufficient to prevent AR and prolong survival but not sufficient to prevent indolent destruction of the graft. A related issue is the possibility that tolerance strategies which preferentially block the direct pathway of allorecognition may leave the indirect pathway

unchecked (Hornick *et al.* 2000). Therefore, successful strategies may need to take into account both pathways as well as possibly important interactions between the two. It is also possible that the non-immunological factors, as discussed previously (§ 2(b)), contribute to the failure to prevent CR. Future studies will need to specifically address the role of these factors once tolerance to the antigens of the graft has been achieved.

The studies described above clearly indicate the importance of monitoring graft function and morphology in animals and humans subjected to tolerogenic therapies. Finally, before any truly meaningful evaluations of tolerance can be made in humans, effective immune monitoring tools will need to be developed to assess the state of tolerance, allowing the safe withdrawal of immunosuppression (Strom & Suthanthiran 2000).

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