Alloantigen-induced specific immunological unresponsiveness

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When the immune system encounters alloantigen it can respond in any one of a number of different ways. The choice that is made will take into account factors such as where, when and how the contact with the alloantigen takes place, as well as the environmental conditions that prevail at the time the alloantigen is encountered. Alloantigen administration before transplantation either alone or in combination with therapeutic agents that modulate the functional activity of the responding leucocytes can be a powerful way of inducing specific unresponsiveness to alloantigens *in vivo*. The molecular mechanisms that influence the way the outcome of the immune response to alloantigen develops, either activation or unresponsiveness to the triggering antigen, hold the key to our ability to manipulate the immune system effectively by exposing it to donor antigen for therapeutic purposes. This review will focus on alloantigen-induced immunological unresponsiveness and how insights into the mechanisms of unresponsiveness have driven the development of novel tolerance-induction strategies that show promise for translation into the clinic in the future.

Keywords: immune tolerance; transplantation; alloantigen; mechanisms

1. INTRODUCTION

When the immune system encounters alloantigen it can respond in any one of a number of different ways. The choice that is made will take into account factors such as where, when and how the contact with the alloantigen takes place, as well as the environmental conditions that prevail at the time the alloantigen is encountered. The molecular mechanisms that influence both the decision and the way the outcome develops, either activation or unresponsiveness to the triggering antigen, hold the key to our ability to manipulate the immune system effectively by exposing it to donor antigen for therapeutic purposes.

Alloantigen delivery either before or at the time of transplantation has been shown to have the potential of switching off the immune response to donor antigens. If unresponsiveness rather than sensitization could be ensured, alloantigen delivery would provide an effective strategy for promoting specific immunological unresponsiveness to donor antigens either before or in the early post-transplant period.

In this review we examine strategies that use alloantigen delivery to induce specific immunological unresponsiveness to a defined set of alloantigens. In many cases, to maximize the impact of alloantigen pretreatment it is combined with other immunotherapeutic agents including some of those that are reviewed in other contributions in this issue. The molecular mechanisms that are involved in this form of immunological unresponsiveness

will be highlighted here but are discussed in more detail in other articles in this issue (Adams *et al.*; Kirk *et al.*).

2. ALLOANTIGEN PRETREATMENT

The induction of specific unresponsiveness to alloantigen by the administration of antigen from the subsequent tissue donor before transplantation was first demonstrated to be a mechanism for deliberately inducing tolerance to alloantigen *in vivo* by Medawar and his colleagues (Billingham *et al.* 1953). By administering semi-allogeneic bone marrow and spleen cells to neonatal mice, thus taking advantage of the immaturity of the immune system in the new-born recipients, they were able to achieve long-term survival of skin allografts in adult mice without the need for further immunosuppression at the time of transplantation. This work was stimulated as a result of the key observation made by Ray Owen that dizygotic cattle twins shared a common placental circulation and as a result were tolerant of one another due to the exchange of cells *in utero* (Owen 1945).

The plasticity of the immature immune system was one of the keys to the success of the neonatal tolerance-induction strategy developed by Billingham, Brent and Medawar. Unfortunately, from the perspective of direct translation of their approach to the clinical setting, the majority of patients in need of a transplant are not identified until their immune system is already mature. Reprogramming the immune system to induce specific unresponsiveness by exposing it to a defined set of alloantigens when it is already mature obviously presents a much greater challenge. Nevertheless, the administration of donor antigen, either alone or in combination with other immunomodulating agents, to recipients of an

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allograft has been shown to result in improved graft survival in a variety of experimental and clinical situations (see, for example, Halasz *et al.* 1964; Monaco *et al.* 1966; Dossetor *et al.* 1967; Morris *et al.* 1968; Fabre & Morris 1972; Opelz *et al.* 1973; Van Es *et al.* 1977; Wood & Monaco 1980; Salvatierra *et al.* 1983; Sharabi & Sachs 1989). These observations highlight how powerful the administration of donor antigen can be, particularly in terms of promoting donor-antigen-specific immunological unresponsiveness.

The beneficial effects of alloantigen pretreatment on graft survival were considered to be somewhat surprising when they were first described, as intuitively one might have predicted that exposing the mature immune system to donor antigens would have sensitized the recipient to the subsequent transplant rather than induce unresponsiveness (Morris *et al.* 1968). While some patients were sensitized by pretransplant blood transfusions, others were not, and instead showed less rejection and improved graft survival compared with untransfused patients (Dossetor *et al.* 1967; Morris *et al.* 1968; Opelz *et al.* 1973). As mentioned in § 1 the outcome of any immune response depends upon the context in which the antigen is recognized, the status of the recipient's immune system being a major factor. Thus there are mechanisms by which the immune response to a set of antigens can be switched off rather than on when the antigen is first encountered. The trick in the context of transplantation is to work out the rules to ensure that alloantigen pretreatment results in unresponsiveness rather than sensitization in every patient.

Examination of the molecular interactions that occur when a lymphocyte encounters an antigen-presenting cell (APC) has revealed that T cells require multiple signals to become fully activated (Bretscher & Cohn 1970; Lafferty *et al.* 1983). For T cells, signal 1 is delivered by the T-cell receptor (TCR)–CD3 complex as a direct consequence of antigen recognition. The additional signals that are necessary for activation are provided when accessory and co-stimulatory molecules engage their ligands. Sometimes these are referred to collectively as signal 2 or co-stimulation (Schwartz 1990, 1992). As these events are taking place, the cell-surface and intracellular molecules involved in the APC–T-cell interaction are reorganized in the plasma membranes of both the APC and the responding T cell to ensure that molecules that are participating in the signalling events are brought into the immunological synapse (Dustin & Shaw 1999).

For alloantigens, signal 1 can be delivered by more than one pathway of antigen recognition, the direct and indirect pathways of allorecognition. In direct pathway presentation, an intact allogeneic major histocompatibility complex (MHC) molecule together with peptide can be recognized by a host T cell, whereas for presentation via the indirect pathway allomolecules are presented as peptides bound to host MHC molecules by host APCs (Shoskes & Wood 1994). Both pathways can trigger graft rejection when T cells are fully activated after antigen recognition (Gould & Auchincloss 1999; Womer *et al.*, this issue, pp. 639–647). Both pathways are also important in the induction of unresponsiveness after alloantigen delivery.

In some circumstances T cells do not receive all the signals that they require for activation. Partial signalling, such as can occur when alloantigen is recognized (signal 1)

in the absence of co-stimulation, leads to T-cell unresponsiveness (Schwartz 1990, 1992). There are many ways in which alloantigen delivery might result in T cells receiving only signal 1. These include presentation of alloantigens by non-professional APCs or immature dendritic cells that lack the capacity to provide co-stimulatory signals, antigen recognition in the presence of agents that block the delivery of second signals from either accessory molecules such as CD4 or co-stimulatory molecules such as CD28, and antigen recognition in the presence of 'immunosuppressive' cytokines such as interleukin 10 (IL-10) or transforming growth factor- β (TGF- β).

If ways of promoting the development of unresponsiveness following alloantigen delivery could be identified this would be of major benefit in the context of transplantation, as only antigen can provide the specificity required to induce unresponsiveness that is antigen-specific before transplantation. A mechanism-driven approach has enabled strategies to be identified and optimized. Harnessing the potential of natural mechanisms that are used to maintain tolerance to self-antigens or control homeostasis in the immune system offers many potential advantages for controlling the immune response to a transplant.

3. MECHANISMS OF SPECIFIC UNRESPONSIVENESS INDUCED BY ALLOANTIGEN PRETREATMENT

The mechanisms of tolerance that have been reported to be responsible for either the induction or maintenance of tolerance after alloantigen pretreatment include the following: deletion of donor-reactive cells both centrally in the thymus and in the periphery; active regulation of the immune response to donor antigens by suppressor or immunoregulatory T cells; anergy, a state of unresponsiveness which is refractory to further stimulation; immune deviation by alteration of the cytokine micro-environment in the recipient, particularly in the graft itself; and exhaustion, where the ability of donor-reactive cells to proliferate is simply eliminated as a result of overstimulation.

It is important to remember that the induction and maintenance of tolerance to alloantigens is a dynamic process and very often it may not be possible to assign a single mechanism as responsible for either the induction or the maintenance of the tolerant state. More likely, multiple mechanisms will operate in concert with one another with each taking a more or less significant role as tolerance develops. The mechanisms used in any situation will depend on the strategy that is used for the induction of unresponsiveness.

Each of these mechanisms is reviewed in detail in other articles in this issue (Lechler *et al.*; Waldmann *et al.*; Sykes & Sachs). None of the mechanisms outlined below is unique to the deliberate induction of unresponsiveness after exposure to alloantigen. The same mechanism is used to induce and maintain tolerance to self-antigens thereby preventing the onset of autoimmune disease (Mason & Powrie 1998). Integration of data coming from analysing these different situations should enable more effective strategies for tolerance induction to be developed in the future.

(a) *Deletion of donor-reactive leucocytes*

Deletion of donor-reactive leucocytes both centrally in the thymus and/or in the periphery is clearly an effective

way of ensuring the absence of donor-reactive leucocytes from the recipient's immune repertoire and inducing donor-specific tolerance (see Sprent, this issue). This mechanism of tolerance could be sufficient in itself for both inducing and maintaining tolerance in the context of transplantation providing that the process of deletion continued after transplantation and resulted in either complete deletion of donor-reactive cells or a reduction in the number of donor-reactive cells below the threshold required to trigger rejection of each tissue (Jones *et al.* 2001).

(i) *Central deletion*

Therapeutic strategies for inducing tolerance to donor alloantigens by central deletion of donor-reactive cells include mixed chimerism (see Sykes & Sachs, this issue) and intrathymic injection of donor antigen (see below). Clonal deletion in the thymus depends critically on the affinity or avidity of the TCR for the selecting ligand (Jameson *et al.* 1995). This implies that there will be a spectrum of responses depending on the 'strength of interaction' between the T cell and the MHC-peptide complex. Thus T cells with high affinity or avidity for the ligand will be deleted, while those with a lower affinity or avidity will not. Thus T cells with a lower affinity or avidity for the ligand present in the thymus will still be present in the periphery and therefore have the potential to react at a later stage (Kawai & Ohashi 1995). Providing that the number of these potentially donor-reactive cells is maintained below the threshold required to trigger rejection throughout the post-transplant course, central deletion is an effective mechanism for inducing and maintaining tolerance to donor alloantigens.

For central deletion of alloantigen-reactive cells to be the only mechanism used to maintain tolerance, donor antigens have to persist in the thymus throughout the post-transplant course. In mixed allogeneic chimeras in the mouse, donor-derived dendritic cells have been shown to be resident and persist in the recipient thymus (Tomita *et al.* 1994; Manilay *et al.* 1998). As a result there is continuous deletion of donor-reactive thymocytes resulting in the absence of donor-reactive T cells in the periphery and hence tolerance. For this strategy to be effective a high level of stable chimerism, so-called macrochimerism, is required (Wood & Sachs 1996).

Injection of donor antigen, either in the form of donor cells or as soluble MHC molecules including allopeptides, directly into the thymus of young rodents also results in the deletion of donor-reactive cells (Markmann *et al.* 1993; Jones *et al.* 1997, 1998). In the majority of cases intrathymic injection is combined with leucocyte or T-cell depletion in the periphery and leads to the induction of operational donor-specific tolerance (Posselt *et al.* 1990; Remuzzi *et al.* 1991; Jones *et al.* 1997). In contrast to the situation that occurs after establishing a stable mixed chimera, the deletion of donor-reactive cells in the thymus following intrathymic injection is transient (Jones *et al.* 1998). The period of deletion is defined by the persistence of the injected antigen in the thymus (Jones *et al.* 1997, 1998). Therefore intrathymic delivery of donor antigen in combination with the depletion of peripheral T cells provides a window of opportunity in which to transplant a solid organ graft rather than producing

persistent deletion of thymocytes in the long term (Jones *et al.* 1998).

(ii) *Peripheral deletion*

Antigen-reactive T cells can also be deleted in the periphery (Webb *et al.* 1990; Wells *et al.*, this issue). The introduction of very high doses of defined antigens either intravenously or orally has been shown to result in deletion of mature T cells in peripheral lymphoid organs (Kearney *et al.* 1994; Weiner 1997; Bemelman *et al.* 1998). In addition, the introduction of donor antigen in the presence of T-cell modulating agents, such as those that inhibit the function of accessory or co-stimulatory molecules required for T-cell activation, has also been shown to result in the deletion of donor-reactive T cells from the periphery (Qin *et al.* 1989; Pearson *et al.* 1992; Y. Li *et al.* 1999; Wells *et al.* 1999).

Different molecular mechanisms can operate to induce deletion of antigen-reactive cells in the periphery (Van Parijs & Abbas 1998). These include both passive cell death (death by neglect) and activation-induced cell death (AICD). Both pathways use the same terminal effector phase that leads to the loss of cells by apoptosis, but the route to induction and the molecular controls that regulate the responses are distinct.

After alloantigen pretreatment, as well as at the time of transplantation, a very high dose of donor antigen enters the recipient, as mentioned above (§ 2). This will result in T-cell activation unless the activation process is inhibited. As a result of this activation process some of the T cells responding to the donor antigens will undergo AICD and die by apoptosis. If AICD could be promoted without the differentiation of harmful effector cells that could destroy the graft this would reduce the number of donor-reactive leucocytes present in the periphery and potentially promote the development of specific unresponsiveness to the graft (Li *et al.* 1999; Wells *et al.* 1999; Wells *et al.*, this issue). The process of deletion may promote the development of specific unresponsiveness and therefore may be a key component in the development of tolerance in some situations.

(b) *Suppression or immunoregulation*

Suppression was first described in the 1970s following the demonstration that antigen-specific unresponsiveness to sheep red blood cells could be transferred from one recipient to another by leucocytes (Gershon & Kondo 1971). When unresponsiveness can be adoptively transferred it is clear that mechanisms other than deletion of antigen-reactive cells are in operation, and that a population or populations of cells present amongst those adoptively transferred must be capable of regulating the response of naive cells to the same antigen.

Active regulation or suppression of immune responses has been described as a mechanism for both inducing and maintaining tolerance to donor antigens in the context of transplantation (for reviews, see Hutchinson 1986; Waldmann & Cobbold 1998), as well as for maintaining unresponsiveness to self-antigens, thereby preventing the onset of autoimmune disease (Mason & Powrie 1998). After alloantigen pretreatment, there is growing evidence that regulatory or suppressor cells play a key role in setting up the immune system for specific unresponsiveness

at the time of transplantation (Quigley *et al.* 1989b; Bushell *et al.* 1995b; C. Kingsley, M. Karim, A. R. Bushell and K. J. Wood, unpublished data).

In the majority of experimental transplant models in rodents the regulatory or suppressor cells reside in the CD4⁺ T-cell compartment (Hall *et al.* 1985; Quigley *et al.* 1989; Bushell *et al.* 1995). CD8⁺ regulatory T cells are also described but appear to be less common, except perhaps in humans (Hutchinson 1986; Li *et al.* 1999).

Leucocytes with immunoregulatory properties can be enriched using cell-surface markers, but at present there is no marker that uniquely defines regulatory or suppressor T cells. The markers that have been found to be useful to date include particular isoforms of CD45 (CD45RB^{low} (mouse), CD45RC^{low} (rat)) (Powrie & Mason 1990; Josien *et al.* 1995; Yang *et al.* 1995; Hara *et al.* 2001), CD25 (Sakaguchi *et al.* 1995; Gao *et al.* 1999) and most recently intracellular CD152 (cytotoxic T-lymphocyte antigen 4, CTLA-4) (Read *et al.* 2000; Takahashi *et al.* 2000). However, there are conflicting data, in that for example T cells with immunoregulatory activity have been shown to be present in the CD25-negative fraction of leucocytes from lymph node and spleen in the rat (Fowell & Mason 1993; Stephens & Mason 2000).

Suppression or immunoregulation *in vivo* could be operating through a number of different mechanisms. It may promote apoptosis of antigen-reactive cells (see §3(a)), but at present there is only limited evidence to support this as the dominant mechanism for active immunoregulation or suppression in tolerance to donor antigens (Zhang *et al.* 2000). Indeed, if the mechanisms of specific unresponsiveness induced after alloantigen exposure involve immunoregulation, then AICD as outlined above (§3(a)) would need to be selective and only result in the death of potentially aggressive donor-reactive leucocytes. We would propose that leucocytes that have the potential to regulate immune responses would not be susceptible to AICD, i.e. would be preserved during this phase of the response to enable them to exert their regulatory functions in the long term after transplantation. This could be achieved by the upregulation of anti-apoptotic genes after antigen recognition such that they are not susceptible to AICD (B. Sawitzki and H. D. Volk, personal communication).

Results from our laboratory (N. D. Jones and K. J. Wood, unpublished data) together with those reported by Zhang *et al.* (2000) show that regulatory T cells can acquire alloantigen from APCs. The acquisition of antigen from APCs has also been reported in other systems (Huang *et al.* 1999). The outcome of acquiring antigen would appear to be different in different systems. In some cases the acquisition of antigen sensitized the cells themselves for lysis by neighbouring T cells (Huang *et al.* 1999) while in others the acquisition allowed the T cells to kill other cells (Zhang *et al.* 2000). This suggests that immunoregulation as a result of antigen acquisition by T cells may operate by more than one mechanism, depending on the setting.

The demonstration that immunoregulatory cells can be used to adoptively transfer unresponsiveness from a transplant recipient with a long-term surviving graft to a fresh naive recipient through many generations of cells, the process known as infectious tolerance, demonstrates that

these types of regulatory or suppressor cells can generate further cohorts by influencing the differentiation patterns of naive cells *in vivo* (Qin *et al.* 1993; Waldmann & Cobbold 1998). These cells could function by either eliminating donor-reactive aggressive leucocytes and/or by silencing their functional activity *in vivo*. One hypothesis that is attracting particular interest at present is the possibility that these cells represent a third subset of T cells with a specialized cytokine and functional profile.

A role for TGF- β and IL-10, either alone or in combination with each other or cell-surface molecules such as CD152, has been proposed in a number of different models of immunoregulation and anergy including the anterior chamber of the eye, after oral or nasal delivery of antigen and in models of tolerance to self- or alloantigen (Khoury *et al.* 1992; Wilbanks *et al.* 1992; Powrie *et al.* 1996; Josien *et al.* 1998; Asseman *et al.* 1999). TGF- β has been reported to modulate the function of the APCs promoting T helper (Th) type 2 cell responses (Bridoux *et al.* 1997; King *et al.* 1998). IL-10 has been shown to inhibit antigen-induced proliferation and cytokine synthesis by T cells, most probably through its effects on APCs, particularly down-regulation of molecules involved in T-cell co-stimulation (Moore *et al.* 1993). However, both molecules are pleiotropic and contradictory results concerning their role in the regulation of immune responses to transplantation antigens have been reported (Bejarano *et al.* 1992; Qian *et al.* 1996). These conflicting data may be explained by the observation that there are dose-dependent differences in the effect of IL-10 *in vivo* (Blazar *et al.* 1998).

The relationship between TGF- β and IL-10 in the development of tolerance is still being characterized, as different models show differential requirements for one or both of these mediators at particular stages in the response (Miller *et al.* 1999). From these data it would seem reasonable to propose that there are certain soluble mediators that can promote the development of unresponsiveness when present in the correct micro-environment, TGF- β and IL-10 being two examples. As with many immunological mediators, their presence in the right place at a certain concentration is key to the way in which they function.

A complementary hypothesis, for which there is some evidence *in vitro*, to explain the action of regulatory T cells suggests that regulatory T cells control the ability of APCs to trigger T-cell activation through either cell-cell contact or the release of cytokines (Taams *et al.* 1998; Li *et al.* 1999). APCs have been shown to become 'licensed' to initiate effector cell activation once they have encountered an activated T helper cell (Bennett *et al.* 1998; Lanzavecchia 1998; Ridge *et al.* 1998). This hypothesis eliminates the need for clusters of helper and cytotoxic T cells to be brought together in the vicinity of the APC at the same time to ensure that only effector cells with the appropriate antigen specificity are activated. Rather the hypothesis suggests that once an APC has presented antigen and activated a T cell, the T cell changes the functional activity of the APC to enable it to trigger activation of an effector T cell in its absence. It has also been shown that regulatory T cells can inhibit the upregulation of co-stimulatory molecules on APCs when they are present in these cultures (Taams *et al.* 1998; Li *et al.* 1999). These and other data suggest that regulatory cells can also change the

function of APCs preventing them from triggering T-cell activation.

(i) *Linked unresponsiveness*

One potentially very powerful effect of regulatory or suppressor cells is their ability to link the unresponsive state to more than one antigen, the phenomenon known as linked unresponsiveness (Madsen *et al.* 1988; Davies *et al.* 1996a; Wong *et al.* 1996). This phenomenon has been described in both transplantation and autoimmune models, albeit with opposite outcomes. Linked unresponsiveness in the context of transplantation is desirable as it reinforces the development of tolerance. However, in patients with autoimmune disease, epitope spreading can accelerate autoimmunity as tolerance breaks down.

The data in transplantation demonstrate that it is possible to manipulate the immune response to a variety of different antigens by initially targeting just one. For example, if the recipient's immune system is exposed to a defined alloantigen or set of alloantigens before transplantation, either alone or in combination with a T-cell modulating agent, then the response to that antigen can be switched off *in vivo* (Lake & Mitchison 1977; Madsen *et al.* 1988; Saitovitch *et al.* 1996; Wong *et al.* 1997a,b). Interestingly, as the unresponsive state to this defined antigen develops one can then link it to other molecules present on the graft providing the initiating antigen is present. In other words, if one transplants an organ graft expressing the initial antigen but other alloantigens in addition, unresponsiveness to both the triggering antigen as well as those expressed by the transplant will develop in the long term after transplantation (Wong *et al.* 1997a).

The mechanisms underlying linked unresponsiveness are being actively investigated at present. Data from the analysis of T-cell clones anergized *in vitro* and regulatory cells isolated *in vivo* demonstrate very clearly that the process is active and requires cell-to-cell contact (Lombardi *et al.* 1994; Hara *et al.* 2001). It would also appear that in many transplant models the initiating antigen is recognized via the indirect pathway of allorecognition, i.e. by cross-priming, by the recipient's immune system (Wise *et al.* 1998; Hara *et al.* 2001); in other words after processing of the donor molecule by recipient APCs. Moreover, the leucocytes which have been described as being responsible for the development of linked unresponsiveness have the phenotype of regulatory or suppressor cells, as they can function in adoptive transfer systems, are enriched when sorted using markers such as CD45RB^{low} and CD25 and are inhibited by anti-CD152.

(c) *T-cell anergy*

Anergy is a state where T cells become refractory to further stimulation by antigen. This mechanism of specific unresponsiveness is discussed in detail by Lechler *et al.* (this issue).

The majority of studies on T-cell anergy have been carried out *in vitro* and the relationship between anergy and *in vivo* states of specific unresponsiveness has been questioned. However, recently, *in vitro* and *in vivo* studies have confirmed a link between T-cell anergy and immunoregulation or suppression (Schwartz 1990; Lombardi *et al.* 1994; Frasca *et al.* 1997; Takahashi *et al.* 1998; Chai *et al.* 1999). T-cell anergy may therefore be a

form of immunoregulation. Further data on the relationship between anergic T cells generated *in vitro* and their ability to regulate immune responses *in vivo* are required.

(d) *Immune deviation from a Th1 to a Th2 response*

In the context of organ transplantation, much of the supporting evidence for the hypothesis that Th1 responses are aggressive and promote rejection whereas Th2 responses favour graft prolongation has come from reports of an absence of Th1 cytokines in animals with long-term graft survival (Mottram *et al.* 1995; Sayegh *et al.* 1995; Onodera *et al.* 1997). However, the presence of Th2 cells in tolerant recipients is not a uniform finding (Hall *et al.* 1998). As yet, studies have failed to demonstrate that Th2 cytokines actively promote the development of tolerance or that Th1 cytokines always promote graft rejection (Qian *et al.* 1996; Bushell *et al.* 1999). Indeed the absence of the Th1 cytokines interferon- γ and IL-2 has been shown to prevent the induction of tolerance (Dai *et al.* 1998; Konieczny *et al.* 1998).

Studies where some of the signature cytokines of Th2 cells, particularly IL-4, have been neutralized have also failed to demonstrate convincingly that this cytokine, and by inference Th2 immune deviation, is key in promoting long-term graft survival and tolerance (Davies *et al.* 1996b; Bushell *et al.* 1999). Indeed there are many reports that the presence of Th2 cytokines within the micro-environment of the graft actively promotes the development of long-term chronic changes (Russell *et al.* 1994). These and other data suggest that immune deviation alone cannot account for peripheral tolerance, although it cannot be ruled out that deviation of cytokine production towards a Th2 phenotype promotes the recruitment and activation of other mechanisms that all contribute to the tolerance state (see §3(b)).

Taken together the data available suggest that interplay within the cytokine network is clearly critical for controlled unresponsiveness, but that immune deviation is unlikely to act as the only mechanism to maintain tolerance *in vivo*.

(e) *Persistence of donor antigen*

The presence and persistence of donor antigen are required for both the induction and/or maintenance of tolerance, either before or after transplantation, irrespective of the precise nature of the mechanism that is operational. This has been demonstrated very clearly in a number of experimental models using alloantigen delivery both before and after transplantation (e.g. Bushell *et al.* 1994; Scully *et al.* 1994; Tomita *et al.* 1994; Hamano *et al.* 1996; Khan *et al.* 1996).

In the absence of donor antigen, unresponsiveness either fails to be induced (Bushell *et al.* 1995a) or is gradually lost as the mechanisms responsible for maintaining tolerance are no longer stimulated (Scully *et al.* 1994; Hamano *et al.* 1996; Khan *et al.* 1996). One obvious interpretation of these data is that the mechanisms responsible for inducing or maintaining tolerance are no longer stimulated once the donor antigen is removed. In the case of deletion this would result in the immediate reappearance of donor-reactive cells in the immune repertoire, whereas in the case of immune regulation the regulatory cells would gradually become

outnumbered by potentially aggressive donor-reactive cells (see above).

Based on current understanding of the immunological mechanisms that lead to the development of transplantation tolerance, strategies that promote the early deletion of donor-reactive cells while simultaneously creating an environment that facilitates the development of regulatory or suppressor cells may offer an effective approach.

(f) Requirements for the induction of specific unresponsiveness following alloantigen delivery

(i) Experimental studies

Alloantigen pretreatment alone

Experimental effort has been directed at determining if it is possible to eliminate the risk of sensitization after alloantigen pretreatment. One of the approaches investigated was to determine if all blood components were equally effective at inducing unresponsiveness. Experiments in rodent and large animal models suggested that blood components expressing MHC class I antigens in the absence of MHC class II were able to induce unresponsiveness to donor alloantigen, but were less likely to sensitize the recipient as a result of alloantigen administration before transplantation (Jenkins & Woodruff 1971; Welsh *et al.* 1977; Hibberd & Scott 1983; Wood & Morris 1985; Foster 1989).

In many cases, the data obtained from studies trying to assess whether exposure to MHC class I alloantigen was more or less effective than exposure to MHC class II or both, were often difficult to interpret as direct comparisons were not made (Soulillou *et al.* 1984). Gene-transfer technology allowed different MHC genes to be introduced and expressed in cells of recipient origin, overcoming some of these problems. Using this approach we have shown that it is not necessary to expose recipients to all of the alloantigens subsequently represented on the organ transplant to induce unresponsiveness (Madsen *et al.* 1988). Recipient cells expressing single donor MHC molecules, class I or class II, were able to induce unresponsiveness to a vascularized organ allograft (Madsen *et al.* 1988; Sykes *et al.* 1993; Wong *et al.* 1996, 1997a; Yasamoto *et al.* 1997). However, the ability of an individual alloantigen to induce the unresponsive state is dependent on both its intrinsic immunogenicity in the recipient and the antigen load delivered during pretreatment (Madsen *et al.* 1988). These data also supported the idea that transfusions from randomly selected blood donors were originally successful in improving renal allograft survival in clinical transplantation because of fortuitous sharing of some human leucocyte antigens (HLAs) or peptides between the blood donors and the subsequent organ donors (Morris *et al.* 1968; Bushell *et al.* 1994). In other words, linked unresponsiveness is a key feature of alloantigen-induced hyporesponsiveness.

Analysis of the other requirements for antigen-induced unresponsiveness has demonstrated that the form of the antigen administered, the route of administration and the time of inoculation relative to transplantation are all important factors in determining whether specific unresponsiveness to alloantigen is induced. It has been shown in a variety of experimental systems that soluble class I antigen delivered intravenously in the absence of other immunosuppressive agents is unable to induce the

unresponsive state (Spencer & Fabre 1987; Foster *et al.* 1989; Arnold *et al.* 1990) unless it is delivered continuously over a long period (Kamada *et al.* 1981) or injected directly into the thymus (Oluwole *et al.* 1993, 1994). In contrast, cell-associated antigen is more effective, but it does not appear to be necessary for the antigen to be presented by viable cells for prolonged graft survival to be induced as a result of alloantigen administration (Foster *et al.* 1988, 1989). Indeed, intracellular MHC class I antigen has been shown to be able to induce the unresponsive state (B. Spriewald and K. J. Wood, unpublished data). This suggests that the indirect pathway of allorecognition or cross-priming may be very important for the induction of specific unresponsiveness (Shoskes & Wood 1994).

The intravenous route is undoubtedly the most effective way to introduce antigen *in vivo* for the induction of unresponsiveness in the adult if no other form of immunosuppressive therapy is added into the system. Subcutaneous administration of antigen inocula invariably leads to sensitization. There have been reports suggesting that delivery of alloantigen into the portal vein is more effective than other intravenous routes (Kennick *et al.* 1987), but any added benefits have to be set against the added difficulty of accessing the portal vein. Oral delivery of alloantigen has also been shown to be capable of inducing unresponsiveness (Hancock *et al.* 1993; Niimi *et al.* 2001b).

In the 1990s there was renewed interest in intrathymic injection of antigen before transplantation for the induction of tolerance. Very encouraging data have been obtained following intrathymic inoculation of islets of Langerhans, isolated glomeruli or splenocytes in combination with anti-lymphocyte serum (ALS) or anti-CD4 immunotherapy to induce indefinite survival of islet, kidney, heart and skin grafts in rodent models (Posselt *et al.* 1990; Remuzzi *et al.* 1991; Markmann *et al.* 1993; Jones *et al.* 1997). However, attempts to use intrathymic delivery in large animals have been less encouraging.

Alloantigen pretreatment in combination with T-cell modulating agents

Although the administration of donor antigen before transplantation is undoubtedly an effective strategy for inducing unresponsiveness, and in some cases, peripheral tolerance to alloantigen at the time of organ transplantation in adult recipients, the approach may not induce tolerance to alloantigen in every situation. It may therefore be more effective to combine the benefits of immunological specificity achieved with donor antigen with low levels of a less-specific immunosuppressive agent, such as T-cell modulating agents including ALS, anti-CD4, CTLA-4 immunoglobulin (CTLA-4-Ig), anti-CD154 (CD40L), rapamycin or cyclosporine. This strategy relies on the properties of the T-cell modulating agent to create a suitable environment for alloantigen recognition that promotes the induction of specific unresponsiveness to the graft. Under these conditions alloantigen can be introduced by a variety of different routes, including intravenously (Pearson *et al.* 1992), intrathymically (Jones *et al.* 1997) and orally (Niimi *et al.* 2000b) and still induce graft prolongation.

This approach has been explored in a number of different ways, early data being produced by Monaco *et al.*

(1966) using donor bone marrow (DBM) and ALS to prolong the survival of skin allografts in mice. ALS, as its name suggests, potentially affects the activity of all the recipient's lymphocytes, as does total lymphoid irradiation (Myburgh 1985), which has also been used in combination with antigen. Monoclonal antibodies or soluble recombinant ligands that target subsets of leucocytes more selectively in combination with donor antigen have allowed more subtle approaches to be developed, albeit with the equivalent long-term outcome. For example, anti-CD4 was shown to create an environment which facilitated tolerance induction to soluble antigens (Wofsy *et al.* 1985; Benjamin & Waldmann 1986) and alloantigens before transplantation in the adult (Qin *et al.* 1989; Pearson *et al.* 1992; Bushell *et al.* 1995a).

CD4 is not the only T-cell molecule that can be targeted at the time of alloantigen delivery to induce specific unresponsiveness. Anti-leucocyte function-associated molecule 1 (Benjamin *et al.* 1988), co-stimulation blockade using either CTLA-4-Ig (Lin *et al.* 1993; Pearson *et al.* 1996) or anti-CD154 (Markees *et al.* 1997; S. J. Billing and K. J. Wood, unpublished data) have also been reported to facilitate the development of specific unresponsiveness when combined with alloantigen, although to date the long-term efficacy of these different approaches has not been compared directly.

Immunosuppressive drugs might also be effective in combination with donor antigen for the induction of specific unresponsiveness. Soluble donor MHC antigen in combination with short-term treatment with cyclosporine has been shown to be capable of inducing long-term graft survival in the rat (Didlake *et al.* 1988; Foster *et al.* 1992; Goto *et al.* 1992; Ghobrial *et al.* 1996). The idea of using soluble antigen is attractive, as it is less immunogenic than particulate antigen (Medawar 1961) and would theoretically reduce the risks of sensitization associated with antigen-pretreatment protocols. This strategy might also be effective with some of the new immunosuppressive drugs currently in development.

The persistence of the donor antigen administered in combination with agents that create a more immature environment may be an important factor in determining the efficacy of these strategies (see § 3(e)). If alloantigen persists in the short term there will be a window of opportunity created for transplantation (Bushell *et al.* 1995a; Jones *et al.* 1998). Although not essential for the induction of tolerance (Wood & Sachs 1996), the establishment of stable macrochimerism facilitates the long-term duration of the tolerant state. To this end, creating mixed allogeneic chimeras before transplantation by the infusion of allogeneic bone marrow in combination with T-cell modulating agents and manipulations has been shown to be very successful (Wekerle & Sykes 1999).

DBM as a vehicle for alloantigen pretreatment

DBM can be given before, at the time or after transplantation and still have a beneficial effect (e.g. Thomas *et al.* 1983; Wood & Monaco 1984; Qin *et al.* 1989; Sharabi & Sachs 1989; Barber 1990; Wong *et al.* 1996). These observations, together with increasing concerns about the risk of infection transmitted as a consequence of blood transfusion have stimulated a great deal of interest in using bone marrow as the vehicle for

alloantigen delivery. The way in which the recipient is manipulated before or at the time of bone marrow infusion has a marked effect on whether the infused allogeneic bone marrow persists in the long term, developing a state of chimerism (macro or micro) (Wood & Sachs 1996), and results in different mechanisms of tolerance being brought into play in the different systems (see Sykes & Sachs, this issue). A non-myeloablative treatment regimen using sub-lethal irradiation in combination with T-cell-depleting antibodies or co-stimulation blockade is sufficient to achieve a persistent level of chimerism that is essential for the induction of tolerance using this approach (Sharabi & Sachs 1989; Tomita *et al.* 1994; Kawai *et al.* 1995). In this situation the chimerism is clearly the cause of tolerance, as transplantation of tissues or organs from the bone marrow donor are uniformly accepted without the requirement for long-term additional immunosuppression.

The infusion of bone marrow in combination with T-cell modulating agents or immunosuppressive drugs at the time of transplantation can also facilitate the induction of tolerance (Monaco *et al.* 1966; Thomas *et al.* 1983; Qin *et al.* 1989; Wong *et al.* 1996). While the presence of donor-derived leucocytes in the early phase post-transplant has been shown to have a beneficial influence on graft survival (Ko *et al.* 1999) leading to the deletion of donor-reactive leucocytes in some cases through a Fas-dependent mechanism (George *et al.* 1998), the microchimerism is not responsible for the long-term maintenance of tolerance (Shizuru *et al.* 1990; Scully *et al.* 1994; Hamano *et al.* 1996). Therefore this approach is likely to be beneficial, but the persistence of the bone marrow-derived cells is not required for the maintenance of tolerance in the long term.

Donor antigen combined with CD4 monoclonal antibody therapy leads to operational tolerance that is based on specific T-cell immunoregulation

The potential of CD4 monoclonal antibody therapy to promote the development of specific unresponsiveness in the long term after transplantation (Cobbald & Waldmann 1986; Madsen *et al.* 1987; Shizuru *et al.* 1987), and the historical impact of pretransplant blood transfusion in both experimental (Fabre & Morris 1972; Wood & Morris 1985) and clinical transplantation (Morris *et al.* 1968; Opelz *et al.* 1973), led our group to examine whether a combined treatment approach in which blood transfusion was given under the cover of anti-CD4 antibody would induce specific unresponsiveness to donor antigens at the time of transplantation (Pearson *et al.* 1992).

In this protocol, recipient mice are pretreated with two small doses (typically 25–50 µg) of depleting anti-CD4 or 200 µg of non-depleting anti-CD4 to partially inhibit T-cell function combined with a single donor-specific blood transfusion (DST) or DBM infusion to provide a source of donor MHC alloantigen. An important feature of this protocol is that the animals are then rested without further treatment (usually for 28 days) to allow the immune system to recover from the non-specific effects of the antibody therapy and are only transplanted when general immune responses have otherwise returned to normal. Recipients pretreated in this way are specifically unresponsive to donor alloantigens, in that donor-specific

hearts are accepted indefinitely (median survival time (MST) of more than 100 days), while those of an unrelated third-party strain are rejected. Indefinite graft survival is entirely dependent on the combined anti-CD4–DST or anti-CD4–DBM treatment since mice pretreated with either DST, DBM or anti-CD4 alone reject their grafts with an MST of *ca.* 20 days (Pearson *et al.* 1992; Saitovitch *et al.* 1995; Wong *et al.* 1996).

Regulatory or suppressor T cells have been shown to develop in recipients after pretreatment (Bushell *et al.* 1995*b*, 1999) as well as after transplantation (Pearson *et al.* 1992; Hara *et al.* 2001). The regulatory cells identified in both phases of the response are alloantigen specific and can be enriched by sorting CD4⁺ T cells by expression of CD45RB^{low} and CD25 (Hara *et al.* 2001).

Anti-CD4 is not the only T-cell modulating agent that can be used in this alloantigen-pretreatment protocol. We and others have shown in a variety of different systems that anti-CD3, CTLA-4-Ig (Lin *et al.* 1993) and anti-CD154 (Markees *et al.* 1997) can all facilitate the development of tolerance after antigen pretreatment. We have compared directly the ability of anti-CD4 and anti-CD154 to promote the development of unresponsiveness to cardiac allografts. While both are effective in prolonging graft survival the quality of graft function in the long term after transplantation is distinct. Grafts transplanted into mice pretreated with anti-CD4–DST do not develop transplant arteriosclerosis while those treated with anti-CD154–DST develop the disease (S. Ensminger, S. J. Billing and K. J. Wood, unpublished data). These data suggest that anti-CD154 may be less effective in this pretreatment protocol. We are currently following up this observation to determine if this is related to the frequency of regulatory T cells present in recipients with long-term surviving grafts.

While the addition of donor antigen to any therapeutic strategy designed to induce specific immunological unresponsiveness is logical and would be expected to improve the specificity of the unresponsiveness state achieved in a shorter time-frame, the requirement of donor antigen places limitations on the manipulations that can be carried out before transplantation unless living donors are used. However, we have also shown that it is not necessary to pretreat the recipient with every donor alloantigen before transplantation to induce the unresponsive state (Madsen *et al.* 1988). When cells expressing a single donor MHC molecule are combined with anti-CD4 therapy before transplantation, specific unresponsiveness to a cardiac allograft expressing several additional MHC molecules develops (Saitovitch *et al.* 1996; Wong *et al.* 1996). Unresponsiveness to the additional donor alloantigens on the graft develops through the phenomenon of linked unresponsiveness (Wong *et al.* 1997*a*). When CBA mice are pretreated with CBK bone marrow (CBK mice express H-2K^b in addition to the normal CBA MHC molecules (H-2K^k, L^k, D^k, I-A^k and I-E^k)) in combination with anti-CD4 the mice become unresponsive to H-2K^b. Heart grafts expressing H-2K^b irrespective of the additional alloantigens the heart expresses, such as H-2D^b and I-A^b or even H-2^d alloantigens, will be accepted indefinitely. Thus, tolerance to H-2K^b results in tolerance to additional MHC molecules through linked epitope suppression.

Mice with long-term surviving grafts in this model exhibit operational tolerance to donor alloantigens since continued graft survival is independent of any further immunosuppressive treatment, they show prolonged survival of second donor type but not third-party heart and skin grafts and adoptive transfer of spleen cells transfers tolerance to untreated secondary recipients (Pearson *et al.* 1992; Bushell *et al.* 1999). Tolerance in this model involves regulatory CD4⁺ T cells both in the induction, i.e. before transplantation, and maintenance phases of the response (Bushell *et al.* 1995*b*, 1999; Hara *et al.* 2001). Moreover, we have shown recently that this protocol, namely pretreatment with anti-CD4–DST, inhibits the development of transplant arteriosclerosis (A. Lieder and K. J. Wood, unpublished data).

Our working hypothesis to explain the induction of specific unresponsiveness by the anti-CD4–DST protocol proposes that during the initial contact between the recipient's immune system and donor antigen, activation of donor-reactive cells is compromised by the presence of anti-CD4. Thus only partial activation signals are received by the responding cells, which promotes the development of a population of regulatory T cells. Antigen persistence during the CD4 blockade is essential to allow the development of the regulatory cells (Bushell *et al.* 1995). The population of donor antigen-specific regulatory cells is therefore present in the recipient at the time of transplantation. When the cardiac allograft is transplanted donor antigens expressed by the graft promote the expansion of the regulatory T-cell population. Providing the graft continues to function immunoregulation persists. In mice with long-term surviving grafts the regulatory T cells are found within a subset that expresses CD4, CD45RB^{low} and CD25⁺. These cells require IL-10 but not IL-4 to function when adoptively transferred, suggesting that, at least in part, they are dependent on IL-10 to switch off immune responses to donor antigens *in vivo* (Hara *et al.* 2001).

The ability of this protocol to induce the development of regulatory T cells before transplantation has many advantages. Not least, it allows the unresponsive state to develop in the absence of non-specific immunosuppression in contrast to protocols that use novel approaches at the time of transplantation. We have shown in this model that once specific unresponsiveness to donor antigens has been induced the administration of the calcineurin inhibitors, cyclosporine or tacrolimus, has no detrimental effect on graft survival (E. Dover, A. R. Bushell and K. J. Wood, unpublished data). However, the simultaneous administration of the calcineurin inhibitors, cyclosporine or tacrolimus, at the time of anti-CD4–DST treatment results in failure to develop specific unresponsiveness and graft rejection. The presence of the calcineurin inhibitors at the time of the first alloantigen encounter prevents the development of unresponsiveness by inhibiting the generation of regulatory T cells. These data are very important as they suggest that for translation of novel protocols into clinical transplantation at present it may be necessary to establish unresponsiveness before transplantation if calcineurin inhibitors are used or to eliminate calcineurin inhibitors from the protocol at the time of transplantation.

Based on these experimental data a pilot clinical study has been carried out at the Oxford Transplant Centre

investigating the effect of combining pretransplant blood transfusions from HLA-typed blood donors with non-depleting anti-CD4 therapy before transplantation. The study was designed to investigate if regulatory T cells specific for the mismatched alloantigens in the blood transfusion developed as a result of the pretreatment. Preliminary data show that sensitization was not an issue following transfusion, and precursor frequency analyses show nonlinear kinetics suggesting that more than one population of cells is responding to the blood donor antigens in the assay (N. Young, K. J. Wood and P. J. Morris, unpublished data).

These findings are very encouraging but progress in translation of novel strategies to the clinic is hampered by the lack of a reliable tolerance assay (see § 4).

(ii) *Clinical studies using alloantigen pretreatment* *The blood transfusion effect*

In the late 1970s and early 1980s, pretransplant blood transfusion was widely acknowledged as having a significant beneficial effect on the survival of renal allografts from both living-related and cadaver donors (Opelz *et al.* 1973). However, the data gathered in the late 1980s from many transplant centres suggested that the transfusion effect is no longer as striking as it was in the pre-cyclosporine era and in some centres may have disappeared altogether (Ahmed & Terasaki 1991; Opelz 1991). These observations, together with the increased risks associated with the use of blood products and the availability of erythropoietin resulted in transfusions being discontinued altogether at some transplant centres.

The reasons for the loss of the transfusion effect in clinical transplantation are far from clear. It has been proposed that the introduction of cyclosporine has led to the change, but this explanation is likely to be too simplistic as many other factors have also been changing over the same period of time. Indeed a prospective randomized blood transfusion trial carried out subsequently has shown that the transfusion effect is still demonstrable even when cyclosporine-based immunosuppression is used (Opelz *et al.* 1997).

The relationship between the MHC antigens of the blood donor and the transplant recipient might also be an important factor in determining the effect of pretransplant transfusions. A comprehensive survey of transfused patients by Eurotransplant revealed that not all transfusions have an equally beneficial effect in the induction of specific unresponsiveness in clinical transplantation. When the recipient shared at least one HLA-DR antigen in common with the blood donors, pretransplant blood transfusion was found to be most effective (Lagaaij *et al.* 1989). Transfusion of blood mismatched for two HLA-DR antigens appeared to be contraindicated in this analysis because of the increased risk of sensitization and the lack of improvement in graft survival. Experimental data in a mouse model confirmed that the sharing of class II antigens between the blood donor and the recipient promoted the development of specific unresponsiveness (Niimi *et al.* 2000a).

The mechanisms by which HLA-DR-matched transfusions have a beneficial effect on graft survival have not been elucidated. In one study, *in vitro* analysis of the ability of recipients to generate cytotoxic T lymphocytes

specific for donor alloantigens before and after transfusion showed that only recipients receiving transfusions where the blood donor and the recipient had one haplotype or at least one HLA-B and HLA-DR antigen in common had reduced reactivity to donor antigens (Van Twuyver *et al.* 1991). However, these data were not been reproduced when other cohorts of transfused patients were been analysed (Baudouin *et al.* 1997; Van der Mast *et al.* 1997; Young *et al.* 1997). One hypothesis that has been proposed to explain this phenomenon suggests that indirect presentation of donor allopeptides by MHC class II-positive cells in the transfusion inoculum will establish a population of regulatory T cells (see § 3(b)) that can control subsequent reactivity to the graft (Claas *et al.* 1993).

In order to try and reduce the risk of sensitization after pretransplant blood transfusion, platelet transfusions were evaluated as a substitute for whole-blood transfusions in the 1980s. Platelets express MHC class I but not class II antigens in all species. One study carried out at Hôpital Edouard, Herriot, Lyon, France showed that purified platelet transfusions did not lead to sensitization in patients without previous transfusions or pregnancy and that graft survival was equivalent to that obtained in a comparable study group receiving whole-blood transfusions (Pouteil-Noble *et al.* 1991). In contrast, a platelet transfusion study carried out at the Oxford Transplant Centre, Churchill Hospital, Oxford, UK found that sensitization was not completely eliminated by the use of relatively impure platelets, but was if highly purified platelets were used. However, the clinical outcome was poor in both groups (Chapman *et al.* 1986). Interestingly, in both of these clinical studies it was noted that rejection crises tended to occur earlier and to be more severe in the platelet-transfused patients. Data from experimental studies also suggested that accelerated activation of alloreactive cells may be important for the development of specific unresponsiveness (Webb *et al.* 1990; Dallman *et al.* 1991). Therefore, although more difficult to manage clinically, this outcome may have indicated the potential for the induction of unresponsiveness in the platelet-transfused patients.

DBM and anti-lymphocyte globulin

In 1989 Barber and his colleagues undertook a randomized controlled clinical trial to evaluate the efficacy of anti-lymphocyte globulin (ALG) combined with the transfusion of DBM for the induction of specific unresponsiveness to renal allografts. As mentioned above this approach had been shown to be extremely successful for the induction of tolerance in both rodent and primate models (Monaco *et al.* 1966; Thomas *et al.* 1983; Wood & Monaco 1984). Each pair of patients entered into the trial were transplanted with kidneys harvested from the same organ donor and randomized to receive either the standard quadruple immunosuppressive therapy protocol in use at the Alabama centre or quadruple therapy plus a transfusion of cryopreserved bone marrow from the organ donor 10–14 days after transplantation and on the seventh day after the last dose of ALG (Barber *et al.* 1989, 1991). The preliminary data reported from the trial in 1991 were encouraging, and showed that the graft survival rates in the group receiving bone marrow transfusion were higher than in the control group at 12 and 18 months after

transplantation, and that less steroid was used in the marrow-transfused group. In mixed leucocyte culture using donor-specific or third-party stimulators a trend towards reduced donor-specific activity has been observed in the bone marrow group. However, the longer-term follow-up of this group of patients has not shown any significant improvement in graft survival compared with that in the control group. A number of possible explanations for this have been proposed including the use of cryopreserved rather than fresh bone marrow and the inclusion of cyclosporine in the immunosuppression used. However, as there has been no follow-up trial using this strategy to date it is very difficult to draw clear conclusions.

Mixed allogeneic chimerism

Mixed chimerism has been shown to be a very effective way of inducing tolerance to allo- and xenoantigens in rodent and primate models (Ildstad & Sachs 1984; Sharabi & Sachs 1989; Kawai *et al.* 1995; Wekerle & Sykes 1999). This approach has many advantages over bone marrow transplantation even though bone marrow replacement also results in tolerance to a subsequent organ graft from the same donor (Sayegh *et al.* 1991). These include a reduced toxicity to the host as a consequence of the myeloablative conditioning necessary to allow bone marrow engraftment as well as the problems of graft-versus-host disease and failure of the bone marrow to engraft (Clift & Storb 1987). The less-toxic host conditioning required to establish mixed chimerism has resulted in this strategy being developed for use in clinical transplantation.

In a modification of the original non-myeloablative conditioning protocol developed by Sharabi & Sachs (1989) cyclophosphamide was given instead of total body irradiation and leucocyte infusion was delayed to determine if mixed chimerism could mediate a graft-versus-leukaemia effect without causing severe graft-versus-host disease in patients with refractory haematological malignancies (Sykes *et al.* 1999). Recipient treatment included many of the elements that had been shown in the primate studies to be critical for the development of macrochimerism, including thymic irradiation, administration of anti-thymocyte globulin pre- and post-transplantation and cyclosporine in the early post-transplant period. Long-lasting chimerism was induced in a series of HLA-matched DBM-infused transplant recipients as well as a small series of mismatched recipients. These encouraging preliminary data have prompted a study in patients with multiple myeloma who require a renal transplant to treat end-stage renal failure (Spitzer *et al.* 1999).

4. DEVELOPING THE OPTIMAL STRATEGY FOR THE INDUCTION OF TOLERANCE TO DONOR ALLOANTIGENS

When considering any novel strategy for translation to the clinic one of the challenges is how to integrate the new approach into currently accepted protocols. The data suggesting that some immunosuppressive drugs interfere with the induction of tolerance clearly present a challenge (Larsen *et al.* 1996; Kirk *et al.* 1999; Wells *et al.* 1999). However, it may be possible to stage the introduction of

calcineurin inhibitors or anti-proliferative agents such that they do not inhibit the early events that are essential for tolerance induction. Indeed there are already data from trials performed using the T-cell depleting agent OKT3 that suggest that simultaneously delivery of cyclosporine along with the monoclonal antibody may have been less effective in promoting long-term graft survival than when cyclosporine was introduced in a delayed fashion (Opelz 1995). These data support that idea that IL-2 is critical to the early phase of any response that may lead to the development of specific unresponsiveness (see §3). Interestingly, the pilot study using Campath-1H to deplete leucocytes from patients at the time of transplantation was designed so that Campath-1H would be administered alone for the first two days with cyclosporine being introduced on day 3 (Calne *et al.* 1998).

The other major challenge when introducing novel tolerance-induction strategies into the clinic is how to measure the development or the degree of unresponsiveness induced. In many experimental studies where lymphocytes have been shown to have regulatory properties *in vivo* the same functional attributes have not been replicated when the cells have been analysed *in vitro* (Batchelor & Welsh 1976; Pearce *et al.* 1993; Young *et al.* 1997). In other words the standard assays designed to investigate the functional activity of donor-reactive cells *in vitro*, for example mixed leucocyte cultures or by the generation of cytotoxic responses, have failed to reveal the tolerant state. In fact under the conditions routinely used for these assays, cells from tolerant recipients appear to respond as if they were naive. This suggests that the conditions in the *in vitro* assays do not reflect accurately the micro-environment that exists either within the organ graft or within the periphery of tolerant recipients that enable the cells responsible for maintaining tolerance to operate efficiently in this setting. Work trying to identify markers that can be used to enrich regulatory T cells may allow a reliable tolerance assay to be developed in the future. This is really essential if novel strategies for tolerance induction are to be translated effectively to clinical transplantation.

The use at the time of transplantation of T-cell modulating agents that either deplete leucocytes (Cobbold *et al.* 1986; Knechtle *et al.* 1997; Calne *et al.* 1998) or modulate their function (Qin *et al.* 1987; Darby *et al.* 1992; Lenschow *et al.* 1992; Larsen *et al.* 1996; Kirk *et al.* 1999) shows promise, as already discussed (in §3(f)). In this situation the recipient will be non-specifically immunosuppressed in the early post-transplant period as all of these agents target any cell expressing the appropriate marker irrespective of its antigen specificity. However, in this setting the continuing presence of antigen from the graft can promote the development of donor-specific unresponsiveness in the long term after transplantation (Pearson *et al.* 1993). Moreover, the longer the graft is functioning the more profound the unresponsive state becomes (Pearson *et al.* 1993). In these systems tolerance is a dynamic process involving more than one of the mechanisms outlined above. The precise combination of mechanisms will depend in the induction phase, on which T-cell molecule or molecules were targeted and whether this resulted in depletion or functional inactivation of the leucocytes. However, as the unresponsive state develops there are data supporting the

idea that in the absence of continuing T-cell deletion a common mechanism of immunoregulation emerges and is responsible for maintaining tolerance in the long term.

The addition of donor antigen, or cells manipulated to express donor antigen, to strategies using T-cell depleting or T-cell modulating agents appears to offer one of the most effective approaches at present. In the majority of experimental studies, infusing donor antigen either at the time of transplantation or in a pretransplant protocol leads to improved graft survival and reduces the risk of transplant arteriosclerosis (Qin *et al.* 1989; Pearson *et al.* 1992, 1996; Sayegh *et al.* 1997).

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