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Promotion of cell chimerism by immunosuppressive drugs: a possible basis for tolerance induction following organ transplantation

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Advances in the immunosuppressive therapy of organ graft rejection achieved using cyclosporin A (CsA) and, more recently, FK506, have been ascribed to the selective and precise molecular actions of these molecules in inhibiting signal transduction in alloactivated T cells. However, recent evidence indicates that, following organ transplantation, the promotion of peripheral T cell tolerance to alloantigens using these and other drugs may be associated, just as significantly, with a permissive effect of each drug on two-way (donor–recipient) leukocyte migration. This leads to a state of mixed allogeneic cell microchimerism in both the graft and the host. It has been proposed that this cellular chimerism is a natural consequence of organ transplantation under the umbrella of immunosuppressive drug therapy. Moreover, it is plausible that donor-derived cells in the periphery of chimeras play an important role in achieving allotolerance, as many recent reports show that mature T cells can be tolerised after encountering antigen outside the thymus. The cell chimerism observed following organ transplantation in man may persist for many years after the time of grafting, even in patients who have discontinued all forms of immunosuppressive therapy. It seemed to us appropriate to conclude this book with a unifying hypothesis that places less emphasis on the prevention of organ rejection by various immunosuppressive agents in terms of the molecular site of disruption of the alloactivated T cell response, and more on the permissive effect of these drugs on a two-way host–graft leukocyte migration and establishment of mixed microchimerism.

Experimental and clinical observations

It has been four decades since Billingham, Brent, and Medawar showed the causal relationship of haematopoietic chimerism to drug-free immunological tolerance.[1,2] Almost immediately[3] and during succeeding years,[4–9] the adjuvant use of donor bone marrow or cells from the spleen or other lymphoid organs has been advocated to facilitate the transplantation of whole organs, while minimising the liability of immunosuppression. We have recently provided evidence that such strategies are an augmentation of a previously unrecognised natural process of two-way migration of tissue leukocytes of bone marrow origin.[10–15] Although multilineage, the most prominent of these migratory cells, once called 'passenger leukocytes,'[16] were the dendritic cells delineated as a special white cell lineage in 1973 by Steinam and Cohn[17–20] and which are normally correlated with organ immunogenicity.[21–23]

In these recent studies, the dendritic and other donor cells were detected in the tissues of all functioning human kidney[14] and liver recipients[15] ten to thirty years post-transplantation, and in recipients of other kinds of grafts who were studied after shorter follow-up periods. There was no implication of drug specificity in these findings, be-

cause the chimeric state had been induced under azathioprine-, cyclophosphamide-, cyclosporin A-(CsA), and FK506-based protocols with further immune modulation by steroids, poly- or monoclonal antilymphoid globulins, splenectomy, and even thymectomy. A number of these patients had been off all immunosuppressive medications for years. When on or off maintenance therapy, essentially all of the patients tested with mixed lymphocyte reaction (MLR) or cell-mediated lymphocytotoxicity methods had some element of donor-specific non-reactivity. The variable ability of different organs to produce chimerism and consequently maintain for themselves such narrow nonreactivity, ultimately allowing drug independence in many of these cases, was explained by their comparative content of the migratory leukocytes. This is thought to be greatest with the liver and smallest with the heart and kidney.[15]

The conclusion from these clinical studies was that cell migration and subsequent chimerism might be an integral requirement for graft acceptance, as well as the seminal step in tolerance induction for whole organs. Little, however, was known of the events between the transplant operation and the observations made years later. Information which helps to fill this gap has been provided by studies in rats.[24] Although cell migration is a generic phenomenon after the transplantation of all organs, the liver transplant model was selected for the animal experiments because the voluminous traffic to and from this organ is ideal for study of the participating cells. While underscoring the role of dendritic cells, the results demonstrated that both the acute leukocyte migration and the ensuing long-term chimerism were probably multilineage. Donor T and B lymphocytes, as well as dendritic cells and macrophages from the transplanted liver localised promptly to the spleen, lymph nodes, and thymus of the recipient. B lymphocytes homed to the B cell zones, while T cells migrated to those areas where recipient T lymphocytes were normally concentrated. In essence, the traffic routes approximated those utilised by phenotypically identical recipient cells.[25] For the first three to five days post-transplant, these patterns were not obviously different with the use or omission of systemic immunosuppressive therapy. After a further few days, however, the emigrant donor cells disappeared in the untreated recipients.

In contrast, the various donor cells in the rat liver graft recipients immunosuppressed with a 28-day course of FK506 persisted in the locations expected of phenotypically identical recipient lymphoid organs. In addition, a ubiquitous spread was evident after two to four weeks, with the arrival of the donor cells in the skin and heart – in the same way as after bone marrow transplantation,[12] and after allogeneic foetal liver transplantation.[26] After this time, the process proceeded and was sustained without the need for intensive maintenance therapy, or in the absence of all further treatment.

Accommodation of earlier experimental findings

The results of earlier experimental animal work (the so-called 'parked' kidney experiments) have provided a counter argument to the view that cellular chimerism may be *the cause rather than the result* of sustained allograft transplantation. We will discuss these briefly.

In the two stage kidney graft parking models,[27,28] stage one consists of the induction of permanent organ acceptance in the rat using drug or irradiation treatment. Following replacement of passenger leukocytes by host migratory cells, the composite organ is retransplanted into naive recipients (second stage). It is rejected by naive animals of the donor but not the recipient strain. These results, however, can only be accomplished using immunologically 'easy' rat strain combinations[27,28] or with perfect major histocompatibility complex (MHC) matching in larger animals.[29] Even under these circumstances, the outcome tends to be variable.

Although the parking model has provided a useful research tool, the results cannot be extrapolated freely to a discussion of the cell migration concept. This is because neither the host immunocytes (including those that 'home' to the parked organ) nor the donor leukocytes (which are seeded ubiquitously in the recipient) remain the same. The non-responsiveness induced after cell migration involves both graft versus host (GVH) and host versus graft (HVG) reactions. The reciprocal, educational process of donor and recipient leukocytes and its perpetuation in either direction resembles the 'infectious' transplantation tolerance described by Waldmann and colleagues[30] that can be passed on to naive lymphocytes and is self-sustaining in some circumstances. In successful cases, the graft mini immune system is incorporated into the existing

immunological network of the recipient, compatible with the hypothesis of Coutinho (see below). Incomplete assimilation on the HVG limb is monitored by evidence of rejection, which has been the sole measurable end point of all parking experiments.

Assimilation on the GVH limb is also ordinarily silent. It can however be unmasked in transplantation experiments using the LEW to BN rat strain combination that is inherently prone to GVH disease (GVHD).[31,32] The experiments we have conducted consisted of simulating the natural cell migration that occurs after organ transplantation by the administration of bone marrow infusion. At the same time or later, the migratory passenger leukocyte load brought in with a contemporaneous or delayed liver or heart allograft was added.[24] In these rat experiments, liver transplantation plus donor strain bone marrow did not cause GVHD when both engraftments were done simultaneously under immunosuppression. When, however, chimerism was induced with preliminary bone marrow transplantation, followed by a 28-day course of FK506, then a drug free interval of 18 days, subsequent liver transplantation invariably caused lethal GVHD.

The outcome with delayed hepatic transplantation resembled that of a parent to offspring F_1 hybrid experiment in that the liver, including its virgin migratory cells, was seen as self by the altered host immune system. Not having gone through the process of modification, however, the hepatic passenger leukocytes reciprocated by rejecting the defenseless recipient. In contrast, heterotopic hearts transplanted under the same circumstances of prior bone marrow preparation were accepted, without causing clinical GVHD, presumably reflecting the smaller supply of cardiac passenger leukocytes.

The common effect of immunosuppressive drugs

These experimental studies and the preceding clinical ones emphasise how a variety of potentially immunogenic and/or tolerogenic signals are delivered after whole organ transplantation to all of the lymphoid organs and then throughout the recipient by donor leukocytes. The consequences are drastically different in untreated compared with treated animals, but the ultimate therapeutic effect is obviously not defined by drug action alone. The testing of every genuinely potent immunosuppressant during the last 30 years has been followed by claims of tolerance induction in experimental animals, defined by the permanent acceptance of organ grafts after a course of immunosuppression without further treatment or with minimal maintenance therapy[33–43] (Table 17.1).

The exact site of the drug action has not seemed critical, – whether this be at the level of antigen presentation (deoxyspergualin), gene transcription (FK506 and CsA), interdiction of cytokine action (rapamycin), or prevention of clonal expansion by compounds that inhibit DNA synthesis (azathioprine or mizoribine). This generalisation

Table 17.1 Tolerance induction by various immunosuppressive agents

Agent (structure)	Reference
Inhibitor of monocyte-macrophage function	
Deoxyspergualin (semisynthetic polyamine)	Engemann *et al.* (1988)[33]
Monoclonal antibodies against 'adhesion molecules'	
Anti-CD4	Shizuru *et al.* (1990)[34]
Anti-LFA-1	Nakakura *et al.* (1993)[35]
Anti-ICAM-1 (in combination with anti-LFA-1)	Isobe *et al.* (1992)[36]
Inhibitors of cytokine (IL-2) production	
CsA (cyclic peptide)	White *et al.* (1980)[37]
FK506 (carboxycyclic lactone)	Ochiai *et al.* (1987)[38]
Inhibitors of cytokine (IL-2) action	
Rapamycin (carboxycyclic lactone)	Kahan *et al.* (1991)[39]
Anti-IL-2R (p55 α-chain) monoclonal antibody	Kirkman *et al.* (1985)[40]
Inhibitors of DNA synthesis	
Azathioprine (6-mercaptopurine derivative)	
Cyclophosphamide	Mayumi and Good (1989)[41]
Mycophenolate mofetil* (RS-61443) (morpholinoethyl ester of mycophenolic acid)	Hao *et al.* (1992)[42]
Brequinar sodium (quinoline carboxylic acid derivative)	Cramer *et al.* (1992)[43]

*'New' immunosuppressive drugs such as mycophenolate mofetil (RS-61443) or SK&F 105685 may also induce immune suppression by modulation of adhesion molecule expression.

applies also to the biological compounds that include not only those that are T cell depleting, antilymphocyte globulins, but also non-T cell depleting monoclonal antibodies, such as those directed against the cell surface CD4 antigen or against intercellular adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function associated antigen-1 (LFA-1). Thus, variably non-specific immunosuppression can lead to donor-specific tolerance.

We have postulated previously[13] that the commonality of the end result with all of these diverse agents is their *permissive* as opposed to their direct effect, allowing the cell chimerism and consequent bodywide engagement of donor and recipient cells. The chimerism in this hypothesis may be the cause of variable non-reactivity involving the donor–recipient relation that may or may not require lifetime immunosuppression for stability. How the non-reactivity occurs remains speculative, but it is clear that the alteration affects both the reactivity of the recipient immune apparatus toward the passenger leukocytes as well as the other way round.

Peripheral T cell anergy

It is not known how co-existing donor and recipient immunocyte populations in mixed chimerism come to regard each other 'in a revised light' (functional silencing). On the basis of recent experimental observations in animal models and man, however, it has been proposed that a state of T cell anergy can arise in the absence of clonal deletion (the most efficient way of ensuring tolerance, as predicted by Burnet's clonal selection theory). Recent evidence indicates the existence of various (multiple) levels of peripheral T cell tolerance, characterised by modulation (down-regulation) of the T cell receptor (TCR) and accessory molecules, such as CD8.[44] We define anergy in this context as a lack of clonal deletion, with non-reactivity to MHC class II antigens in *in vitro* MLR. The mechanisms inducing this form of extrathymic (peripheral) tolerance are strong enough to overcome even such powerful reactions as those mounted against MHC class I molecules, which otherwise lead either to allograft rejection or lethal GVHD.

Generation of a T cell-mediated immune response, which leads to graft destruction under normal circumstances, requires effective antigen presentation and recognition in its initial phase, together with receipt of a second co-stimulatory signal and the response of T helper 1 (T_{HI}) cells to the combined signal.[45] Both of these signals are normally delivered to T cells by professional antigen-presenting cells (APC), including (activated) B cells and above all, the dendritic cells that ultimately dominated the migration patterns in our experimental animal studies and in the human observations. The dendritic cell (and perhaps other cells, in particular B cells) is critical because it can modify the expression of cell interaction, adhesion and MHC molecules, all of which determine how antigen signals are heeded by T cells (Figure 17.1). Unless activated, B cells appear unable to provide co-stimulatory signals and can induce transplantation tolerance.[46] Molecules that can promote adhesion and can deliver activation signals to T cells include LFA-1, CD2 and CD28, the ligands of which are ICAM-1, LFA-3 and B7, respectively.

Clues to possible mechanisms underlying a state of mixed cellular unresponsiveness come from studies of alloantigen-pretreated, graft-tolerant animals in which induction of T cell anergy has

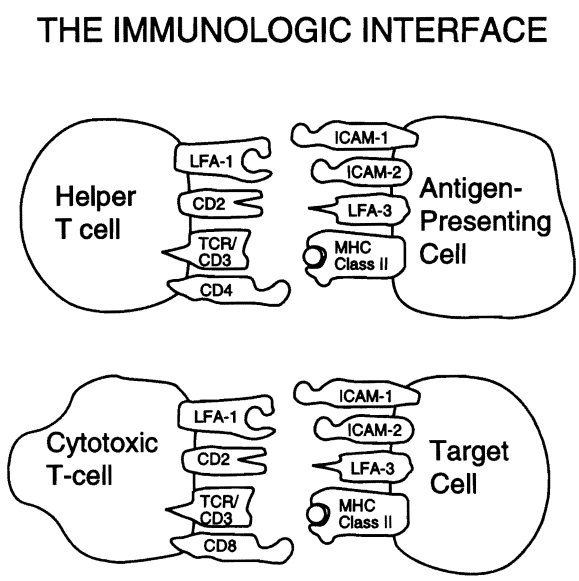

Fig. 17.1 The molecular interface between the T_H cell and an APC and between a cytotoxic T cell and an allogeneic target cell. Antigen (circle) is depicted in conjunction with cell surface MHC class I or II molecules. Second signal for T_H cell activation may be provided by the CD28–B7 receptor–ligand interaction (not shown). Interference with these molecular interactions or with signals resulting from them can lead to tolerance induction.

been implicated. The intravenous route is the most effective for induction of tolerance due presumably, to the route of trafficking of antigen to the lymphoid tissue. Thus, in animals given a single allogeneic blood transfusion prior to organ transplantation from the same donor strain, it appears that the primary but not the necessary second signal for full and sustained T cell activation is provided. Presentation of donor MHC class I or II antigens on gene-transfected fibroblasts (non-professional APCs) before transplantation can also induce specific unresponsiveness.

In the absence of co-stimulatory factor activity, there is disruption of the interleukin 2 (IL-2) cytokine–cytokine receptor autocrine pathway. Direct interference with the IL-2 pathway using CsA or FK506 (both of which inhibit IL-2 gene transcription) or monoclonal antibodies directed against IL-2 or the IL-2 receptor (IL-2R) is highly effective in inducing immune suppression and has profound implications for the induction of peripheral tolerance (Figure 17.2). It appears that in animals made tolerant by infusion of allogeneic leukocytes, there is normal IL-2 gene induction but low IL-2R gene induction. This may reflect abnormal translational control of IL-2 production (although no such regulation has previously been described for the IL-2 gene). Alternatively, it is possible that an (as yet unidentified) IL-2 or IL-2R antagonist may exist in a fashion analogous to the IL-1 receptor antagonist.[47,48] It has been proposed (see, e.g., Jenkins[49]) that TCR occupancy leads to the production, through an active metabolic process, of negative regulators ('anergy proteins') that accumulate at later times and repress IL-2 gene transcription, possibly by antagonising the effects of positive cytokine gene transcription factors. In support of this hypothesis, T cell specific negative regulation of transcription of cytokine (IL-4) has recently been described. Moreover, a T cell-specific protein which can down regulate IL-4 promoter activity has been identified.[50] There is also evidence of cellular protein binding to the negative regulatory elements of the IL-2R 2-chain gene.[51] Within murine anergic T_{H1} cell clones, this effect on cytokine gene expression may relate only to IL-2, as other cytokines such as IL-3 or γ-interferon (IFN-γ) may be secreted, albeit at suboptimal levels.

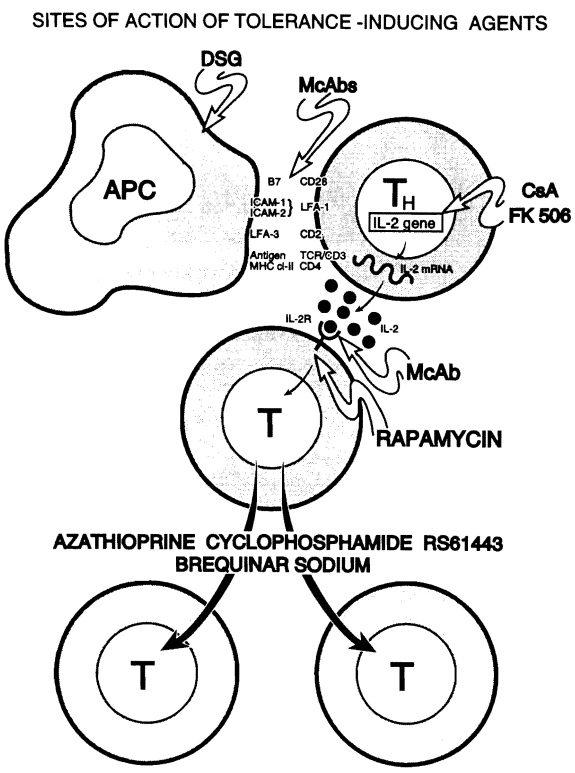

Fig. 17.2 Sites of action of immunosuppressive drugs and monoclonal antibodies (McAbs) which have the capacity to induce tolerance to organ allografts in experimental animals. Common to all of the drugs shown is the ability to (ultimately) inhibit T cell proliferation. Monoclonal antibodies which can induce tolerance in animals include non-T cell depleting anti-CD4 and anti-LFA-1 + anti-ICAM-1 antibodies. DSG = deoxyspergualin, APC = antigen-presenting cell, T_H = T helper cell, IL-2R = interleukin 2 receptor.

Under cover of potent immunosuppressive drugs which inhibit IL-2 production (CsA or FK506) and in the continuous presence of (graft) alloantigens, it is likely that the chronically stimulated T cell will continue to make negative regulators, thus reinforcing the state of anergy (see Figure 17.3). Anergic T cells remain viable and can proliferate in response to exogenous IL-2. It is also possible that anergy can be induced in IL-2-producing T cells that receive both TCR and co-stimulatory signals, but are prevented from responding to IL-2 or dividing by for example rapamycin (which blocks IL-2R-induced cell cycle S phase entry) or inhibitors of DNA synthesis, such as mycophenolate mofetil (RS-61443) or brequinar sodium, respectively (Figure 17.2). Antigen-specific anergy may ensue in a manner analogous to that observed in chronic microbial infection.[52] Significantly, Malkovsky and Medawar[53] showed that IL-2 administration to mice reversed neonatal tolerance, consistent with the

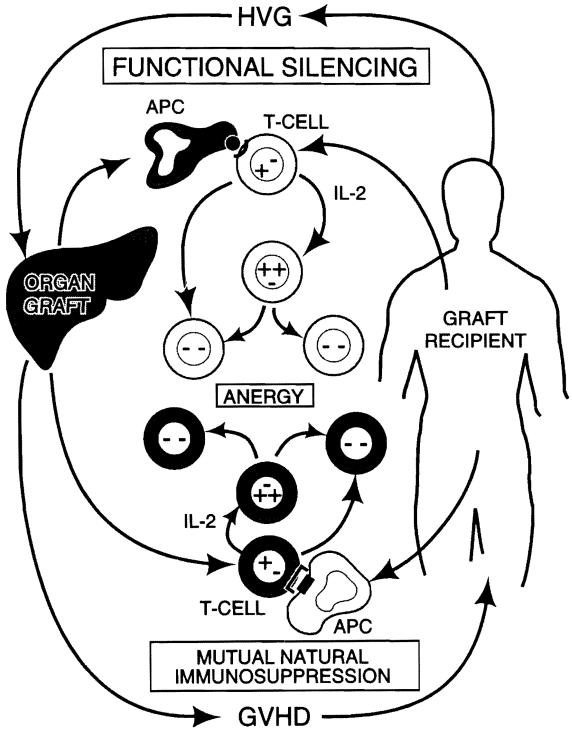

Fig. 17.3 Model of dendritic cell (APC)–T_{H1} cell interaction, showing the production within the nucleus of positive (+) and of negative (−) regulators (anergy proteins) of IL-2 gene transcription. In this model, anergy relates only to the IL-2 gene and other cytokines (e.g. IFN-γ) may be secreted, albeit at suboptimal levels. In the absence of persistent co-stimulatory signals (or under the umbrella of immunosuppressive drugs), cell division does not proceed and negative nuclear regulators accumulate, resulting in T cell anergy. In addition to the action of immunosuppressive agents, chronic antigen stimulation is also envisaged as promoting anergy. In some instances, tolerance can be broken, e.g. by administration of exogenous IL-2. HVG = host versus graft response (allograft rejection), GVHD = graft versus host disease.

view that a defect in signalling via a single cytokine might underlie and maintain the tolerant state. More recently, Dallman *et al*[54] reported that T cells infiltrating cardiac allografts of tolerant rats exhibited reduced cell surface expression of the IL-2Rα chain, and reduced IL-2Rα and IL-2Rβ mRNA expression. Although the IL-2 gene was induced, cells from the tolerant animals failed to make IL-2 in culture. Others have shown, using cell culture models of T cell clonal anergy, that IL-2 can reverse antigen-induced unresponsiveness in cloned T cells.[55]

There is clearly good evidence that peripheral T cell anergy can result after antigen presentation by APCs in the absence of the essential second signal. One of the principal candidates for the second signal is activation via the cell surface CD28 molecule (which requires primary signalling through the CD3–TCR or CD2 proteins) and which increases both the rate of transcription and mRNA stability of several cytokine genes, including IL-2.[56] Anti-CD28 monoclonal antibody or transfectants expressing B7, the ligand of CD28, provide the co-stimulatory function of APCs. Unlike CsA and FK506, which inhibit T cell activation via the TCR–CD3 pathway, and which fail to block activation via CD28, rapamycin is effective in inhibiting anti-CD28-induced proliferation.

Cytokines derived from T_H cells may also be involved in the induction of APC unresponsiveness. Thus, recent work distinguishing T_H cells as T_{H1} and T_{H2}[57] suggests a role for T_{H2} cells in suppression. By releasing IL-10 (cytokine synthesis inhibitory factor), T_{H2} cells are believed to inhibit the function of APC and thereby indirectly the production of cytokines (e.g. IL-2), which are important in T_{H1} cell responses.[58] Significantly, both FK506 and CsA have been shown to spare IL-10 mRNA expression by a murine TH_2 cell clone (see Chapter 7). This may, at least in part, contribute to their immunosuppressive and tolerance-inducing activities *in vivo*.

Tolerance in mature T cells can be induced by interference with accessory molecule expression using, for example, non-depleting anti-CD4 or anti-CD8 monoclonal antibodies which induce tolerance to skin and other grafts. Thus, in antibody-treated mouse Mls^a/Mls^b combinations, T cells are unable to proliferate in response to appropriate stimulator cells *in vitro*. These findings endorse the view that T cell immune recognition requires the contribution of the TCR as well as adhesion receptors, which promote the attachment of T cells to APCs and transduce regulatory signals for T cell activation. The LFA-1 and ICAM-1 adhesion molecules form one such heterophilic receptor–ligand pair. LFA-1, a candidate for the co-stimulatory signal, is required for a range of leukocyte functions, including lymphokine production by T cells in response to APCs and killer T cell-mediated target cell lysis. Activation of antigen receptors on T cells allows LFA-1 to bind its ligand with higher affinity. Also, the LFA-1:ICAM-1 interaction is required for optimising T cell function *in vitro*. Monoclonal antibodies to these molecules are important potential

agents for the prevention of graft rejection. The combination of anti-LFA-1 and anti-ICAM-1 monoclonal antibodies has been reported to lead to specific tolerance in a mouse heterotopic cardiac allograft model.[36]

Relation to other tolerance-inducing mechanisms

Recent reviews have emphasised the inadequacy of intrathymic clonal deletion to explain adult acquired transplantation tolerance and have focused on post-thymic mechanisms that include peripheral clonal deletion and anergy. The evidence for cell chimerism persisting in tissues of organ graft recipients as long as three decades after transplantation, is especially supportive of the concepts expounded by Coutinho and colleagues[59,60] and Cohen.[61] They have defined tolerance as a high (not anergic) level of sustained lymphocyte activity in complex communicating networks. Suppressor cells and/or veto cells could be epiphenomena of this activity. According to these views, if 'self' is positively defined by activated 'connected' lymphocytes, then alloreactive clones should be stimulated rather than suppressed if the recipient immune system is to consider the grafted tissues as self. On the other hand, if the immunological self of the donor is defined by its immune network, successful transfer of components of that network to graft recipients should assure donor-specific tolerance to the graft. An essential prerequisite for the transfer of these essential cellular components and for the establishment of cell chimerism would be an appropriate level of recipient systemic immune suppression, as can be achieved routinely using the variety of immunosuppressive agents considered above. It should now be possible, using drugs with known sites of molecular action, to pose specific questions about the relationship of drug-induced transplantation tolerance to the classical tolerance produced initially by Billingham, Brent and Medawar.[1,2]

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