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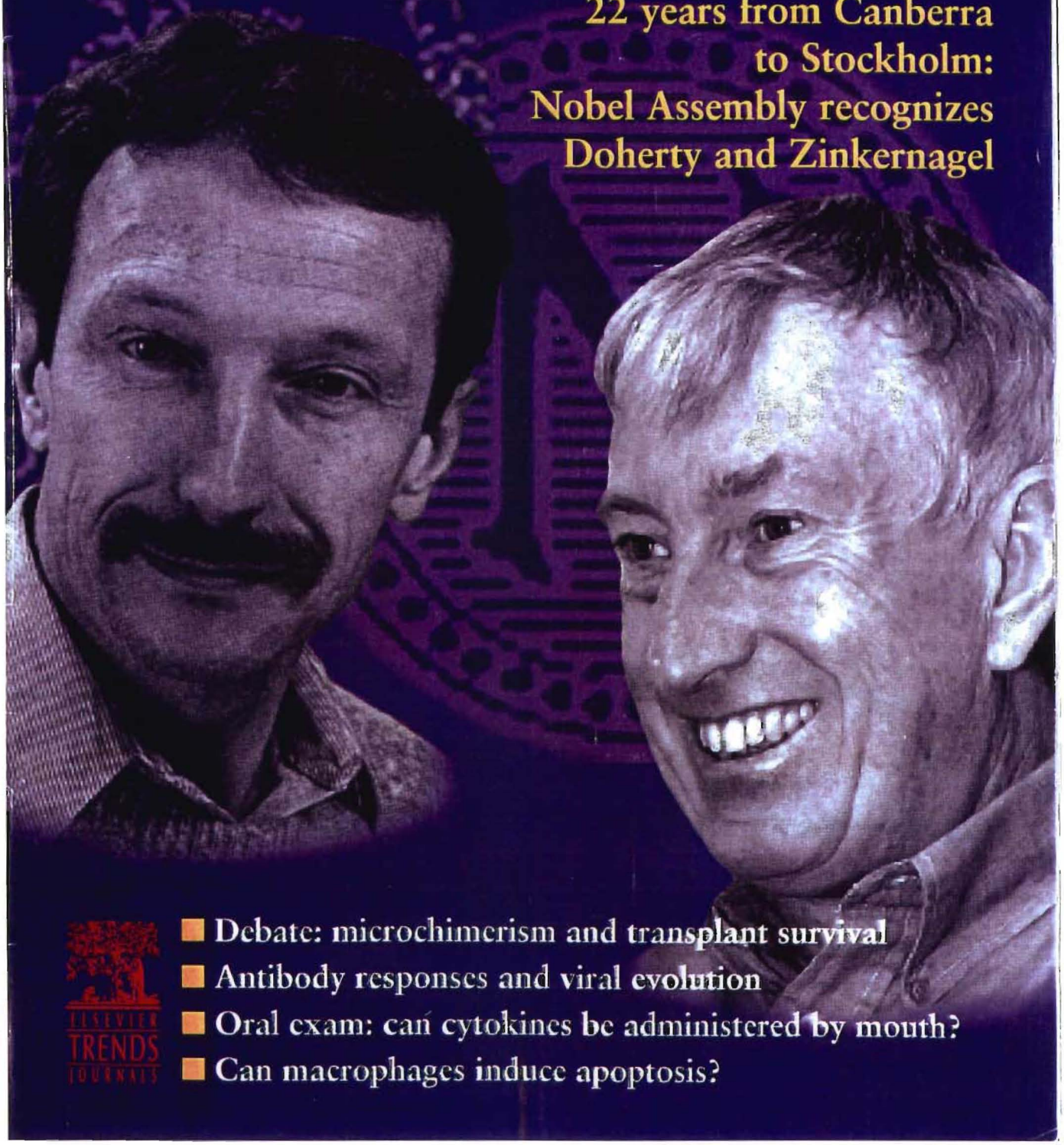
22 years from Canberra to Stockholm: Nobel Assembly recognizes Doherty and Zinkernagel



- Debate: microchimerism and transplant survival
- Antibody responses and viral evolution
- Oral exam: can cytokines be administered by mouth?
- Can macrophages induce apoptosis?

ELSEVIER TRENDS JOURNALS

The lost chord: microchimerism and allograft survival

Thomas E. Starzl, Anthony J. Demetris, Noriko Murase, Massimo Trucco, Angus W. Thomson and Abdul S. Rao

Recent evidence suggests that passenger leukocytes migrate after organ transplantation and produce persistent chimerism, which is essential for sustained survival of the allografts. Here, Thomas Starzl and colleagues argue that this hematolymphopoietic chimerism provides an important framework for the interpretation of basic and therapeutically oriented transplantation research.

Medawar's characterization of rejection[1] as a host-versus-graft (HVG) reaction (Fig. 1a) was the cornerstone of transplantation immunology. A decade later, this concept was transposed in the context of a graft-versus-host (GVH) reaction (Fig. 1b), in which histoincompatible hematolymphopoietic grafts rejected the immunologically defenseless recipients[2,3]. The resulting assumption that allograft acceptance or rejection could be understood by studying HVG or GVH immunologic responses in isolation led to prompt acceptance of the one-way *in vitro* tests of immune reactivity as 'minitransplant' surrogates. However, this assumption did not provide a blanket explanation for observations made in animal and human allograft recipients.

The one-way paradigm

Until 1959, preparatory donor leukocyte infusion into cytoablated organ recipients was an expected natural extension of the neonatal tolerance model of Billingham, Brent and Medawar[4] and its adult cytoablation analogues[5]. However, when long-term survival of human kidney allografts was accomplished in a few sublethally irradiated recipients without donor leukocyte infusion, and then regularly without cytoreduction under continuous pharmacologic immunosuppression, the need either for chimerism or host preconditioning lost favor.

The identification of 'passenger leukocytes' as the primary antigenic component of organs[6,7] led to the belief that their destruction by the host immune system was essential for organ engraftment. When these cells were found to be migratory[8], including dendritic cells (DCs)[9], their sensitization effects and presumed elimination at peripheral and intragraft sites was taken for granted.

Bone marrow transplantation

Major histocompatibility complex (MHC)-restricted models of acquired tolerance were widely considered to have validated Burnet's prediction that developing lymphocytes could be purged of self-reactive cells before they achieved functional maturity, even following bone marrow transplantation. The alternative possibility that donor and recipient immune-cell populations coexisted in neonatally tolerant animals in a mutually nonreactive state while retaining the ability to function collaboratively (e.g. in a joint immune response to infection) was abandoned when no direct experimental support could be found[10]. However, it has since been learned that the outcome in the neonatal tolerance model is highly variable and that a state approaching permanent clonal deletion is uncommon[11]. Recently, it has been shown that the ability of donor-derived leukocyte subsets to proliferate in response to a skin graft challenge was a more critical determinant of neonatal tolerance outcome than the baseline level of chimerism[12].

Organ transplantation

The conclusion that organ transplant acceptance was by different unidirectional mechanisms than those of bone marrow grafts was reinforced by the striking differences between the two varieties of procedures (Table 1). In addition, it was generally assumed that cytoablation (or cytoreduction) to 'make microenvironmental space' was a necessary condition for leukocyte engraftment and chimerism, in spite of early and recent evidence to the contrary (reviewed in Ref. 13).

The two-way paradigm

A link between bone marrow and organ transplantation was provided when microchimerism was detected with sensitive immunocytochemical and polymerase chain reaction (PCR) techniques in the tissues or blood of all 30 human kidney or liver recipients studied from 2.5–30 years postoperatively[14,15] (Fig. 1c). Many of the donor cells appeared to be DCs, potent antigen-presenting cells (APCs)[16]. Individual samples often do not contain the donor leukocytes, which wax and wane[17]. However, disseminated donor cells, including DCs, and/or donor DNA are consistently found if rodents bearing long-term grafts are thoroughly studied[18–20].

Along with peripheral migration of the donor cells from a successfully transplanted graft, there is an influx of host leukocytes that do not cause graft damage (Fig. 1c)[15]: both the allograft and recipient become genetic composites. A mirror image condition exists

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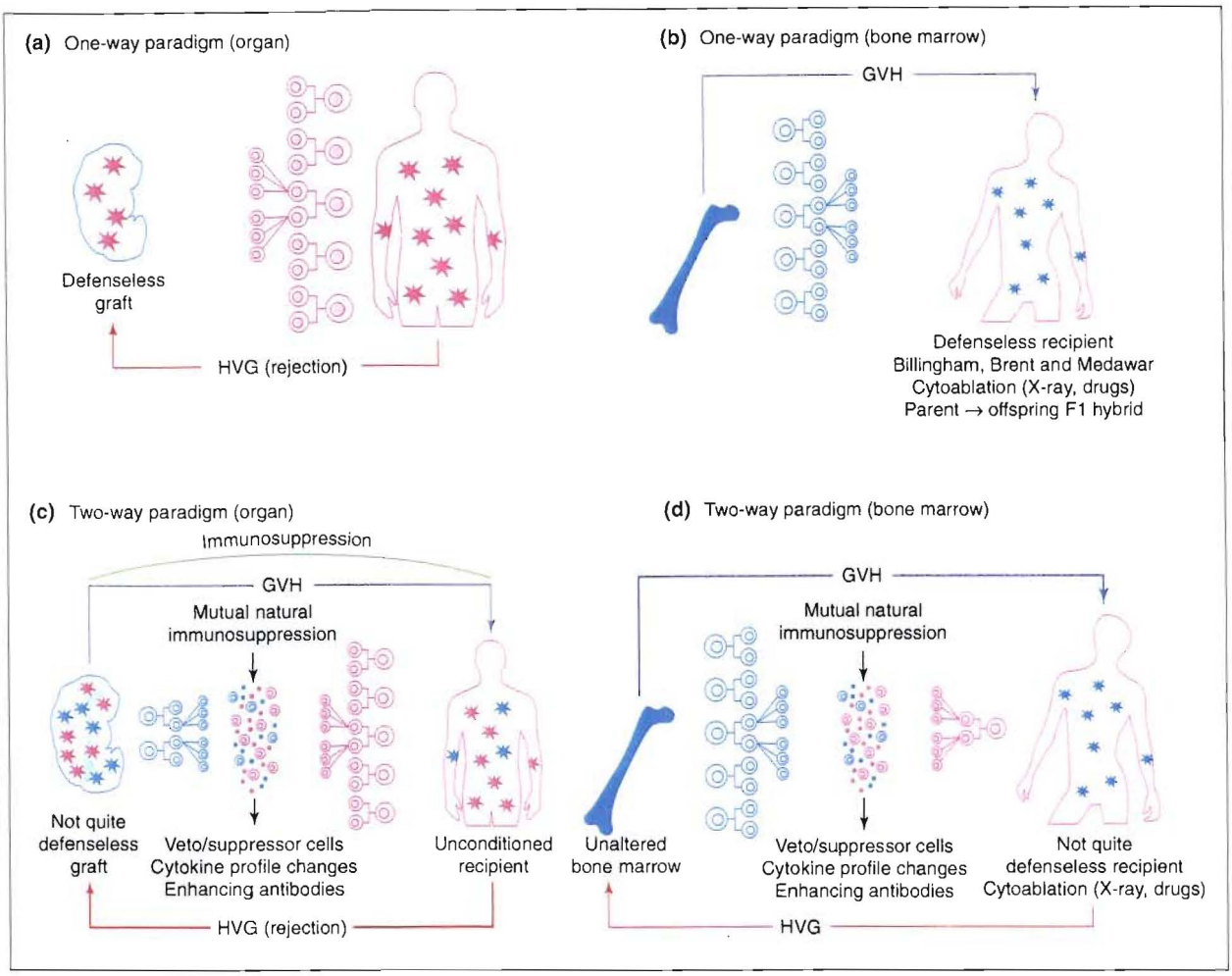

Fig. 1. *(Upper panels) One-way paradigm in which transplantation is conceived as involving a unidirectional immune reaction: (a) host-versus-graft (HVG) with whole organs and (b) graft-versus-host (GVH) with bone marrow or other lymphopoietic transplants. (Lower panels) Two-way paradigm in which transplantation is seen as a bidirectional and mutually cancelling immune reaction that is (c) predominantly HVG with whole organ grafts, and (d) predominantly GVH with bone marrow grafts.*

after bone marrow transplantation[21] (Fig. 1d), proved by demonstrating a trace residual population of host leukocytes in essentially all stable, human bone marrow recipients who previously were thought to have complete donor-cell chimerism[22].

Cause or effect?

In the one-way paradigm, which excludes a role for lymphoid cell microchimerism, it has become axiomatic that antigens of the parenchymal (or vascular endothelial) cells of transplanted organs permit or induce allograft acceptance[23] in various ways, e.g. via veto/suppressor cells, cytokine profile changes or enhancing antibodies. Furthermore, it has been argued that the microchimerism associated with successful transplantation, and conversely its disappearance with or just after irreversible rejection in experimental models[18,20], is epiphenomenal[24].

In a reassessment based on the discovery of microchimerism in organ recipients, we suggested that the donor leukocytes in organ recipients were components of antagonistic but reciprocally attenuated or abrogated HVG and GVH arms[14,15,21]. Deletion of the host arm by cytoablation prior to bone marrow but not organ transplantation altered the balance in this mutual antagonism and was thus responsible for the disparities in the two different kinds of transplantation (Table 1).

The microchimerism had consequences that could not be explained by the simple presence of antigen, as long as the balance was not disturbed and both cell populations were equally immunosuppressed. The dynamic 'nullification' effect of the two arms explained (1) the poor prognostic value of HLA matching for organ transplantation; (2) the rarity of GVH disease (GVHD) following the engraftment of immunologically active organs, such as the intestine and liver[14,15,21], and (3) the characteristic cycle of immunologic

Table 1. Differences between conventional bone marrow and organ transplantation

Bone Marrow	Feature	Organ
Yes	Recipient cytoablation[a]	No
Critical	MHC compatibility	Not critical
GVHD	Principal complication	Rejection
Common	Drug free state	Rare
Tolerance	Term for success	'Acceptance'[b]

Abbreviations: GVHD, graft-versus-host disease; MHC, major histocompatibility complex.
[a]All differences derive from this therapeutic step which in effect establishes an unopposed GVH reaction in the bone marrow recipient whose countervailing immune reaction is eliminated.
[b]Or 'operational tolerance'.

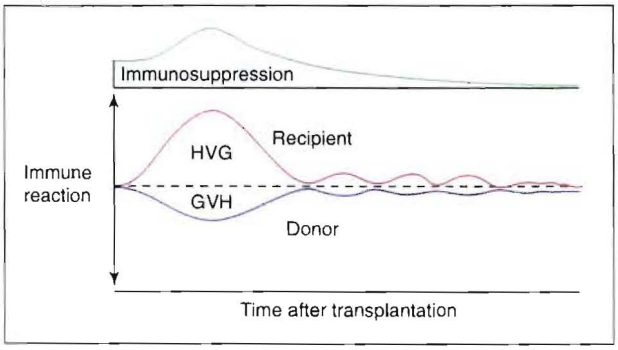

Fig. 2. *Simultaneous host-versus-graft (HVG) and graft-versus-host (GVH) reactions in the two-way paradigm of transplantation immunology. Following the initial interaction, the evolution of nonreactivity of each leukocyte population to the other is seen as a predominantly low-grade stimulatory state that may wax and wane, rather than a deletional one.*

crisis and resolution, first observed in kidney recipients[25], that was most-practically monitored by serial changes in organ allograft function (Fig. 2).

Finally, the discovery of chimerism cast new light on the B-cell lymphomas [post-transplant lymphoproliferative disorders (PTLDs)], that are usually of host origin in organ recipients and of donor origin after bone marrow transplantation. Except for their frequent Epstein–Barr virus association, these human malignancies are indistinguishable from those induced by Schwartz in a mouse chimerism model[26] three years before the PTLD complication was first recognized clinically[27] and explained by simple loss of surveillance[28]. By contrast, Schwartz ascribed the tumors to a lymphoproliferative response by the dominant immune apparatus to the persistent subclinical GVH counter-attack of the minority leukocyte population. The relevance of this conclusion, of 'Schwartz's rules' of pathogenesis, and of their therapeutic implications could not be appreciated until three decades later in the context of the two-way paradigm[29].

The role of immunosuppression

As in Schwartz's 'lymphoma-genic' experiments, immunosuppression is a temporary requirement for reliable induction of tolerance in numerous rodent organ allograft models. The same is true, but unpredictably, after liver[30] and, less commonly, kidney transplantation in outbred canines. Moreover, successful liver transplantation induces tolerance with no treatment at all in a significant percentage of outbred pigs as well as several rat[20,31] and virtually all mouse strain combinations[19]. Mouse heart and kidney allografts are also accepted spontaneously in a much more limited number of MHC disparate conditions (reviewed in Ref. 19). When a thorough search is made for microchimerism in the rodent models, it can always be found[19,20,32].

In all these species, the organs pass through an acute self-resolving rejection on the way to tolerance, which usually extends to subsequent transplantation of other donor-strain tissues and organs[33]. The tolerance is stable despite evidence from *in vitro* testing that anti-donor reactivity is retained (split tolerance)[19,20,31,34] or can be restored by the addition of appropriate cytokines.

The cumulative weight of the above observations does not support the possibility that microchimerism is a passive consequence of organ transplantation. Instead, an active role of the organ-associated chimerism can be identified in a continuum of classical tolerance models beginning with the original observations by Owen in Freemartin cattle (Fig. 3).

The stem cell question

The human chimerism studies suggested that hematopoietic stem and precursor cells were among the migratory cells from transplanted organs. In support of this contention, all lineages in supralethally irradiated mice can be reconstituted efficiently by the infusion of non-parenchymal cells with stem cell phenotype, isolated from syngeneic adult mouse livers[35]. In addition, irradiated rats can be reliably reconstituted with orthotopic liver transplantation rather than bone marrow[36].

Importantly, heterotopic heart transplantation also results in permanent hematopoietic reconstitution in occasional irradiated rats[36], a rescue that is increased to ≥70% by the post-cardiac transplant administration of lisofylline (N. Murase *et al.*, unpublished). Lisofylline is a phosphatidic acid inhibitor that facilitates bone marrow engraftment by suppressing hematopoiesis-inhibiting cytokines (e.g. tumor necrosis factor α, transforming growth factor β, macrophage inhibitory protein 1α and platelet factor 4) that are typically released in response to activation stimuli in the post-transplant period, while not altering levels or activities of the myeloid, progenitor-cell-promoting cytokines, granulocyte–macrophage colony-stimulating factor (GM-CSF) and G-CSF (Ref. 37).

Such experiments show that the chimerism produced with bone marrow infusion vs. conventional organ transplantation is

the same, with apparent differences that are largely determined by the radically divergent treatment regimens. Consistent with this, the chimerism following transplantation of the bone-marrow-containing hind limb to non-cytoablated recipients is much the same as after engraftment of parenchymal organs[36].

However, in practical terms the outcome (HVG, GVHD or both) is strongly influenced by the lineage profile of the mature immunocytes contained either in different vital organs (heart, kidney, liver and intestine) or in cell suspensions prepared from various primary or secondary lymphoid organs. Non-parenchymal cells of the liver (the most tolerogenic whole organ) resemble those of bone marrow (the lymphoid organ yielding the most tolerogenic cell suspension). Both include higher numbers of immature leukocytes and cells of myeloid origin than the lymphocyte-rich and GVHD-prone intestinal allograft and lymph node or spleen cell suspensions[20].

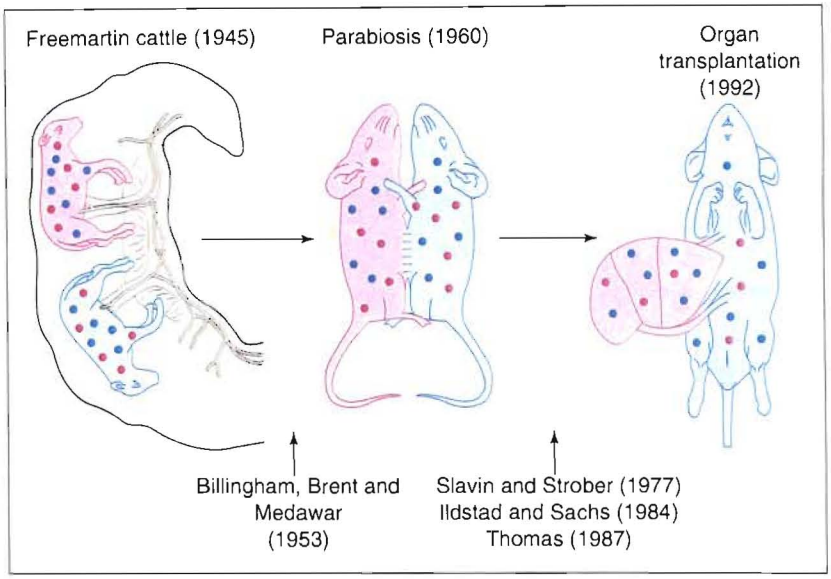

Fig. 3. *The continuum of chimerism from observations of R. Owen in Freemartin cattle, which was rejected as a mechanistic explanation of organ allograft acceptance from 1960 until the discovery in 1992 of microchimerism in organ recipients.*

Chimerism: level and duration

The implication of human and animal studies is that the threshold level of circulating donor leukocytes necessary for a tolerogenic effect has been set too high. Although treatment strategies that directly[18,19] or indirectly augment chimerism[37,39] in non-cytoablated experimental animals increase the reliability and completeness of tolerance, it is not at all clear that the process can be fundamentally hastened. One postulate is that the chimeric immune cells remain susceptible to further signals that reinforce specific nonreactivity in stages[40]. Rather than accelerating these steps, we have suggested that immunosuppressive agents, with diverse sites of action, merely permit them to develop (with variable success) by allowing the same underlying function of the immune system to be expressed as in models of spontaneous tolerance[41] (see earlier).

With liver transplantation in spontaneously tolerant and 'immunosuppression-assisted' rodent models, the cause (chimerism) and effect (tolerance) are induced almost simultaneously but these related events are usually separated by months or years in outbred animals and humans (Fig. 4). Many long-surviving human liver recipients have become immunosuppression-independent (most frequently because of treatment noncompliance) at highly variable postoperative times (Fig. 5). More-complete information was obtained in a prospective weaning trial of liver recipients who had at least five years of stable allograft function[42]. The majority of these patients were able to stop immunosuppression or are still in an uninterrupted weaning process[43]: 30% developed rejection, necessitating resumption of immunosuppression. No grafts were lost or had permanent impairment of function.

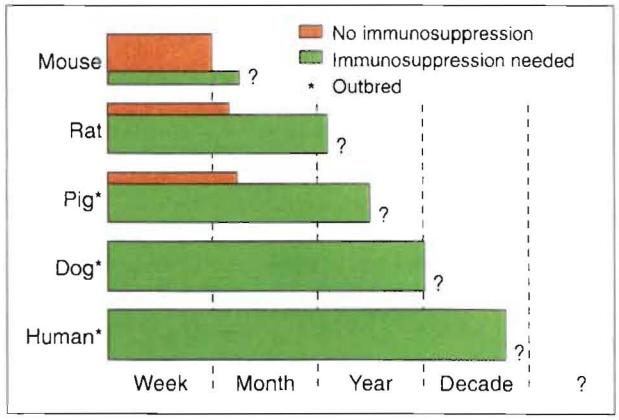

Fig. 4. *Time between cause (chimerism) and effect (donor specific tolerance) after liver allotransplantation in different species. Note that immunosuppression is not universally required in three of the five species shown.*

The desired drug-free state might never be reached in a proportion of human liver recipients, but the disseminated donor-derived leukocytes (and their companion organ) apparently can be maintained for a lifetime under immunosuppression. The same principle has been demonstrated in rat cardiac and renal recipients in which continued immunosuppression prevented the slow disappearance of chimerism and the onset of indolent chronic rejection[20].

As in animals, discontinuance of drug therapy in humans is thought to be more dangerous after transplantation of organs other than the liver. However, five of the ten longest-surviving patients bearing living-related kidney allografts have been completely off immunosuppression for between three and 30 years (Table 2).

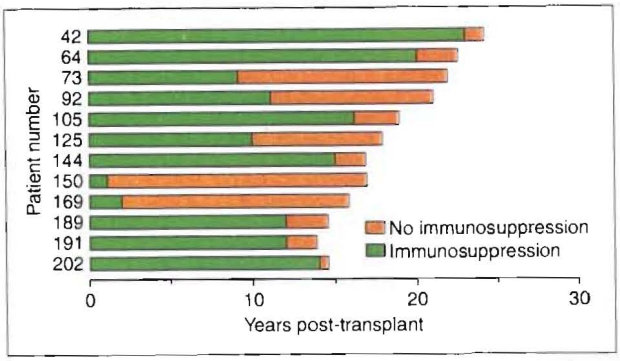

Fig. 5. *Time on (green) and off immunosuppression (orange) of 12 (28%) of our 42 longest-surviving liver recipients (15–26 years post-transplant) who are receiving no treatment as of December 1995. These drug-free patients remain well in September 1996.*

Patients 3 and 4, whose mixed lymphocyte response (MLR) tests to donor and third party targets were profoundly depressed prior to weaning[44], had gradual restoration of MLR to both in the drug-free state, but with no evidence of rejection.

There is no empirical method to determine the necessary duration of continued immunosuppression for maintenance of stable chimerism and allograft function in humans. Thus, quantitation of donor-derived leukocytes cannot be used to plan drug weaning protocols for patients. This must be done by cautious trial, with precautions to prevent irreversible error.

Genetic factors

Although the genetic basis for immune reactions is beyond question, the MHC effect is unambiguously evident only when the recipient is immunologically defenseless: i.e. in the neonatal tolerance model, recipient cytoablation in all species, or as the consequence of breeding (e.g. the F_1 hybrid preparations). When the recipient immune system is competent, organ transplantation outcomes have defied detailed genetic analyses, even in congenic mouse[19] and rat models[45,46]. A clear prognostic effect of MHC after organ transplantation in immunologically intact humans has been clearly identifiable only with a perfect or near perfect HLA match[47]. The lack of predictability can be explained by the interaction implicit with chimerism in which each population follows its own genetic program.

MHC did not evolve for immunologic segregation of transplant patients and their tissues but rather to meet the need of populations, not individuals, for immunologic flexibility: allograft rejection was an unforeseen byproduct of modern technology. Transplantation of surgically revascularized allografts was, in essence, no different than the induction and then the control of an organ-specific autoimmune disease. Thus, there were no hard genetic rules that prohibited chimerism or successful organ transplantation.

Cellular and molecular mechanisms

So-called 'parking' experiments, in which grafts are temporarily placed in a third-party recipient prior to retransplant into the intended host, have been put to good use in transplantation research[7,48,49]. However, we would argue that the presence of altered (nonreactive) leukocytes that repopulate an organ during residency in the intermediary allogeneic host make such retransplantation models inappropriate for the study of complex tolerance mechanisms. In addition, the leukocyte replacement during the parking period is incomplete. Even at one year of residence in a tolerant recipient, 10% of the non-parenchymal cells remain donor, a proportion that is essentially fixed from day 100 onward[18]. Not surprisingly, the results following retransplantation are hard to interpret[50,51].

In simpler experiments involving only the depletion of organ leukocytes by donor irradiation or other means, both the tolerogenicity and antigenicity of heart[39], liver[52] and free pancreas islet allografts[53] are abrogated or weakened. The tolerogenicity of liver can be restored by an infusion of donor-strain splenocytes into irradiated donors 24 hours before the organ is removed for transplantation[54]. The same is true of islets after adding back donor leukocytes.

In contrast to the interpretive artefacts introduced with the parking models, successful transplantation in the two-way paradigm is defined as persistent chimerism, whether or not it is immunosuppression-dependent. A failed transplantation connotes the therapeutically uncontrollable ascendency either of HVG or GVH (Refs 15, 41). Pathologic evidence of both processes is frequently found in failed cases, but the ultimate result is predominantly rejection or GVHD.

In this context, the vast literature addressing the basis of tolerance, and that preoccupied with rejection, can be brought to bear on problems of transplantation. Many experiments have been one-way paradigmatic, showing the effects of exogenous or transgenic antigen on T cells and other immune cell subpopulations. The interpretation of such data in transplantation must encompass the alterations in two cell populations, each of which can modulate the other. In addition to a mutual antigen stimulus, the two-way paradigm implies active protection of the coexisting arms (GVH or HVG), which is particularly important if one cell population is outnumbered or if there is severe MHC disparity. Such a reciprocal 'defensive' mechanism of graft enhancement has been the subject of investigation but only in connection with hematolymphopoietic reconstitution after recipient cytoablation[55–57].

Experimental manipulations under highly controlled conditions are usually directed at understanding T-cell tolerance. However, T cells are only one of a number of specialized immune regulatory leukocytes. For instance, Burlingham *et al.*[58] have isolated a circulating donor leukocyte, resembling the veto cell of Miller[59], in a tolerant human kidney recipient with such powerful function that a single cell could neutralize the *in vitro* activity of 10 000 recipient CTLs.

The possibility that transplantation tolerance is governed by APCs was raised by the invariably prominent presence of DCs in chimeric human[14,15] and animal organ recipients[18,19]. Using culture techniques adapted from Inaba *et al.*[60], donor-derived DC precursors have been propagated from disseminated locations in mouse recipients of spontaneously accepted liver allografts[61]: these are co-localized with recipient DCs that are undergoing the same

changes[61,62]. These immature DCs, which are phagocytic[63] and deficient in surface costimulatory molecule expression (B7 family)[64], have been shown to induce T-cell anergy *in vitro*[64] and to prolong organ allograft survival[65].

Such clues are intriguing, but it is unlikely that allograft acceptance can be fully understood from the results of studies of individual leukocyte lineages. Overall, the mechanisms of transplantation tolerance suggests learning adaptive immune functions of the whole system involved in self-integrity (i.e. cytokines, immunoregulatory cells, antibodies and other factors).

Table 2. Discontinuance of immunosuppression in long-term living related kidney recipients[a]

Patient	Years post-transplantation	Haplotype mismatch	Indication for weaning[b]	Years off drugs
1	33	0	nc	30
2[c]	32	1	comp	15
3	32	0	nc	29
4[c]	32	2	comp	0.5–3
5[c]	33	1	comp	3

[a]These are 5 of the 16 longest-functioning allografts in the world.
[b]comp, complications: skin cancer, warts, infection, hypertension, obesity and orthopedic problems. nc, non-compliant.
[c]These were children at the time of transplantation.

Transplant tolerance: central or peripheral

The role of the thymic vs. peripheral mechanisms in graft acceptance under both experimental and clinical circumstances has been controversial[66–68]. The prompt appearance of donor-derived leukocytes in the recipient thymus following organ transplantation[18] was of particular interest because of the strikingly tolerogenic effect in rodents of intrathymic inoculation of donor leukocytes[53]. However, thymectomy in adult rats does not influence either the chimerism or spontaneous tolerance induced by liver transplantation[69]. Dejbakhsh-Jones *et al.*[70] have shown that, after thymectomy and lethal irradiation, adult mice reconstituted with purified hemato-lymphopoietic stem cells developed similar levels of αβ T cells to those seen in control animals except for a reduced proportion in the spleen.

Between 1962 and 1965, 32 patients, including 24 who were part of a controlled randomized trial, underwent transthoracic thymectomy from 8 to 112 days (average 22) before renal transplantation either from living related or unrelated donors. Between 3.5 and 7 years later, no clinical differences were apparent between the thymectomized and control recipients, although there was a trend towards better histopathology in the thymectomized group[71]. In 1992, comprehensive *in vitro* immunologic studies of many of the remaining recipients and their donors did not reveal any distinguishing features of one cohort vs. the other (G. Shearer and A. Zeevi, unpublished). After 25 to 30 years, the thymectomized patients had no clinical advantage or disadvantage.

Therapeutic implications

In the context of the two-way paradigm, early efforts to improve transplantation results with donor-specific blood transfusion[72] and the donor bone marrow augmentation of organ recipients[73,74] were based on sound therapeutic principles involving the unrecognized augmentation of chimerism. Also in retrospect, it is obvious why whole organs are inherently tolerogenic as first convincingly demonstrated by Calne *et al.*[32].

Understanding the concept of a donor–recipient leukocyte dialogue should help predetermine what can (and cannot) be accomplished with various tolerance-inducing strategies, all of which are attempts to influence this interaction. Our first clinical premise was that the spontaneous microchimerism of organ transplantation could be greatly augmented by the co-administration of unmodified donor bone marrow cells without a significant risk of GVHD, providing the two immunocyte populations were initially competent and that immunosuppression was delivered to both equally. It was also predicted that the timing, severity and frequency of acute rejection would be approximately the same as in non-bone-marrow-augmented control patients[14,41,75].

These expectations have been fulfilled in 150 human organ recipients treated at the University of Pittsburgh[75,76]. The presence of donor DNA in the myeloid and erythroid colonies generated from recipient's peripheral blood mononuclear cells (PBMCs) as measured in standard[76] or innovative clonal hematopoietic progenitor cell assays[77] has provided unequivocal evidence of augmented stem cell chimerism. There were no examples of significant GVHD.

The hypotheses of therapeutic efficacy being tested were that the threat of delayed (acute or chronic) rejection could be reduced and that the frequency of ultimate drug independence would be increased by the higher persistent level of chimerism. An efficacy evaluation is expected to take 5–10 years[41], roughly the same time frame mapped out by clinical experience with MHC-incompatible liver and bone marrow transplantation (Figs 4 and 5).

Other chimerism-enhancing strategies (e.g. G-CSF, GM-CSF or lisofylline) should follow the same safety/efficacy rules. By contrast, procedures that alter only one of the interacting arms must be approached with caution, as exemplified by the historical experience with GVHD following cytoablation and bone marrow transplantation. When the converse tactic of leukocyte or T-cell-specific depletion of intestinal allografts was attempted as GVHD-prophylaxis in the 1980s, virtually every bowel recipient who survived the perioperative period developed lethal Epstein–Barr-virus-associated B-cell lymphomas[78].

In an experimental example of unbalance which has potential clinical relevance, prior induction of tolerance with bone marrow in briefly immunosuppressed rats followed by delayed liver transplantation resulted in GVHD (Ref. 19), a complication not seen after either bone marrow or liver transplantation, or both simultaneously. The results of the second stage transplantation resembled those in the parent to defenseless offspring F_1 models.

Conclusion

The assumption that stem cell driven hematolymphopoietic chimerism was irrelevant to successful whole organ transplantation, as currently practiced, has led to inadequate explanations of organ allograft acceptance and clouded the meaning of successful bone marrow transplantation, thus precluding the development of a central principle of transplantation. Incorporation of the chimerism factor into a two-way paradigm has allowed previous enigmas of organ and bone marrow engraftment to be explained and should allow key advances in basic immunology to be more meaningfully exploited in transplantation.

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Chimerism and transplantation tolerance: cause and effect

Kathryn Wood and David H. Sachs

Evidence for persistence of donor leukocytes in recipients of long-term organ allografts has prompted the hypothesis that such microchimerism is not only essential to graft survival but that donor and host cells both play active roles. Here, Kathryn Wood and David Sachs caution that the jury is still out on whether such microchimerism is the cause or merely the consequence of long-term allografting.

Donor cells or genetic material can often be detected in recipients following transplantation of a solid-organ allograft. Such recipients are described as exhibiting peripheral donor microchimerism and in some cases the donor material is detected for long periods after transplantation[1–5]. It has been suggested not only that peripheral donor microchimerism is associated with long-term acceptance of the organ graft but that it plays an active role in the induction and maintenance of unresponsiveness[1–3,6]. This hypothesis, first proposed by Starzl and colleagues in 1992 (Ref. 1), has stimulated a great deal of interest and activity in the transplant community. However, from the published reports to date, it remains difficult to determine whether such microchimerism is the cause or the consequence of long-term graft survival.

Microchimerism: observations

Solid-organ grafts contain passenger leukocytes[7,8], the number and lineage of which vary considerably among different organs. For example, the liver contains an abundant supply of passenger cells, whereas relatively few leukocytes are present within the heart[9]. In addition, the passenger leukocytes present in the liver of rodents contain a sufficient number of haematopoietic stem cells[10] to rescue a lethally irradiated recipient when a syngeneic liver graft is transplanted; rescue cannot be achieved reliably by the transplantation of a syngeneic cardiac allograft[11]. Passenger leukocytes present within solid organs have been isolated and shown to possess the potential to stimulate immune responses *in vitro*[12]. However, before acquiring full immunostimulatory potential, it appears that such cells must develop into a more mature form. This has been achieved *in vitro* by addition of growth factors,

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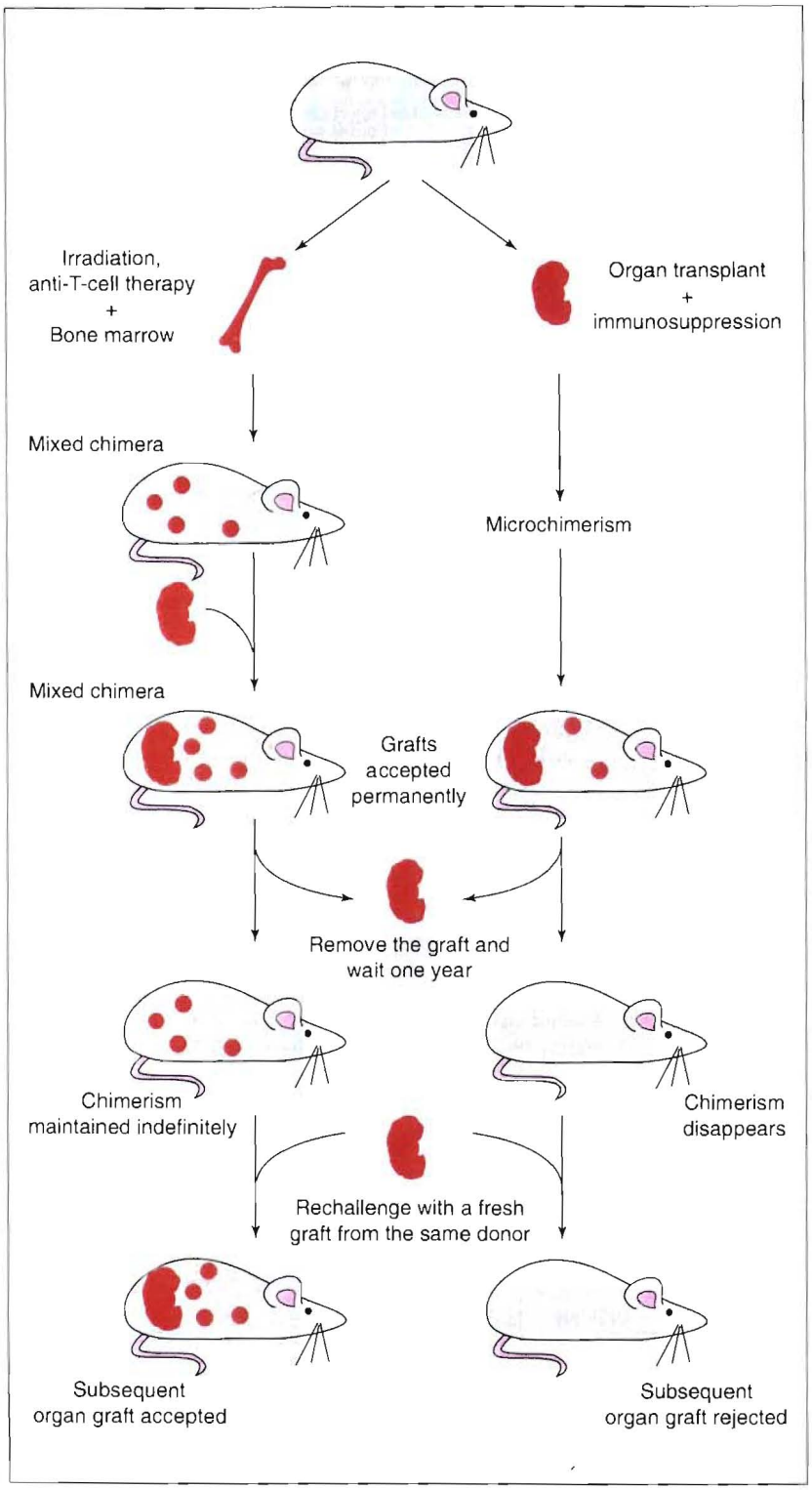

Fig. 1. *A comparison of the microchimerism detected following allogeneic organ transplantation using standard immunosuppression, and the chimerism established by intentional bone marrow transplantation prior to an organ transplant.*

such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and a period of culture[12,13]. On the basis of these observations it has been suggested that some passenger leukocytes are immature dendritic cells[14]. *In vivo*, passenger leukocytes present within an organ graft have been shown to migrate from the graft to recipient lymphoid tissue after transplantation[15,16]; donor derived, major histocompatibility complex (MHC) class II$^+$ leukocytes could be detected in the spleen within 48 hours of transplantation of a heart allograft into a naive mouse[16]. Within the spleen, the donor leukocytes were associated with CD4$^+$ T cells and it was suggested that this interaction is responsible for initiating the rejection response in non-immunosuppressed recipients[16]. These data support the conclusion drawn in earlier studies by Lechler and Batchelor that intragraft passenger leukocytes, most probably dendritic cells, provide the major stimulus for graft immunogenicity[17].

In support of this hypothesis, organs depleted of passenger leukocytes have been reported in some cases to enjoy prolonged survival without administration of exogenous immunosuppressive therapy[17–21]. In some of these studies, organs were depleted of passenger cells by 'parking' the graft in a primary recipient receiving immunosuppressive therapy[17,19,20]. Such 'parked' organs from long-term surviving primary recipients were then re-transplanted to fresh non-immunosuppressed syngeneic secondary recipients and the survival of the graft monitored. In some strain combinations, the passenger-cell-depleted kidneys survived indefinitely and in others they showed prolonged survival. Interestingly, in these studies, induction of chimerism by the administration of donor bone marrow to the secondary host before transplantation led to graft rejection, as did the administration of dendritic-cell-enriched leukocytes[17].

In primary graft recipients, donor leukocytes migrating from transplanted hearts in a mouse model were only detectable for a few days within the spleen unless the animals received immunosuppression, after which donor cells could be detected for longer periods following transplantation. In some human kidney transplant patients,

donor material has been detected in the periphery more than 25 years after transplantation[3]. Impressively, donor-derived cells have been detected in some patients with stable graft function who have stopped taking immunosuppressive drugs[22]. However, the detection of donor-derived material or cells in patients with long-term surviving organ grafts is not a consistent finding. For example, in a study carried out in Paris, donor microchimerism could only be detected in a third of patients who had exhibited long-term stable kidney graft function for more than 20 years[5]. Therefore, the role that donor leukocytes migrating from an organ graft to the peripheral tissues of the recipient play in either the induction or maintenance of unresponsiveness to the organ graft is unclear. No correlation between the state of microchimerism and the absence of acute or chronic rejection was found in heart transplant patients[4]. Moreover, donor-derived material was detected in a patient undergoing graft rejection eight years after liver transplantation[23]. If extended donor microchimerism plays a role in the development of unresponsiveness to the graft, it is surprising that it can be detected in this situation.

In contrast to donor microchimerism detectable after organ transplantation, haematolymphopoietic chimerism is achieved at readily detectable levels following administration of allogeneic bone marrow to appropriately conditioned recipients. In this situation, bone marrow engraftment generally requires the dual strategy of ablation of the host's haematopoietic system, in order to 'make room' for the donor marrow, along with additional immunosuppression to prevent rejection of the allogeneic cells: the former is usually achieved by irradiation or radiomimetic drugs, the latter by T-cell depletion or by immunosuppressive agents.

When bone marrow transplantation is performed to treat haematologic malignancies, complete ablation (e.g. lethal irradiation) is generally used, since 100% chimerism is desired to ensure elimination of leukaemia cells. By contrast, when chimerism is being used to induce transplantation tolerance, complete chimerism is neither necessary nor desirable. Instead, it is preferable to achieve a low but persistent level of donor lymphohaematopoietic chimerism, so that host-type immune-cell populations are available to provide immunocompetence peripherally, while donor-derived cells (probably dendritic cells) provide a persistent source of antigen in the thymus, capable of effecting negative selection[24]. A non-myeloablative preparative regimen, using sublethal irradiation and anti-T-cell monoclonal antibodies, has been demonstrated to achieve long-lasting mixed lymphohaematopoietic chimerism without the requirement for immunosuppressive therapy beyond the immediate post-transplant period[25,26]. Data from these studies showed that T-cell depletion and partial ablation of the recipient's immune system before bone marrow infusion were required to achieve the persistent level of chimerism necessary to induce tolerance.

Differences between the forms of chimerism detected in these situations

There is a fundamental difference between the microchimerism detected following allogeneic organ transplantation using standard immunosuppression, and the chimerism established by intentional bone marrow transplantation prior to an organ transplant (Fig. 1). In the former case, the chronic immunosuppressive regimen must be sufficient to suppress rejection of the graft, and as such it is undoubtedly also sufficient to suppress the elimination of donor cells which might escape from the transplanted organ. Therefore, detection of such cells elsewhere in the recipient might be considered as evidence of microchimerism, but it does not imply that the cells detected are the *cause* of the graft's acceptance. On the contrary, they may be the *result* of the graft's acceptance and of the immunosuppression required to maintain that acceptance.

By contrast, when chimerism is established by deliberate bone marrow infusion following T-cell depletion of the recipient, and partial or complete ablation of the recipient's lymphohaematopoietic system, this chimerism is clearly the cause of tolerance[25,27–29]. In this case, subsequent transplants of other tissues or organs from the same donor are uniformly accepted without the requirement for additional long-term immunosuppression. Moreover, the loss of tolerance following elimination of donor haematopoietic cells from the recipient demonstrates that chimerism is responsible for inducing tolerance[30].

This fundamental mechanistic difference is also evident in the behaviour of subsequent allografts from the same donor after removal of the original transplant. In the case of long-term graft acceptance induced by immunosuppressive agents, removal of the allograft leads to loss of the tolerant state over a period of weeks to months following explant[31–33]. Thus, although a second graft is usually accepted if transplanted immediately into such recipients, it is rejected if the animal is allowed to remain without a graft[32,33]. By contrast, when tolerance is induced by establishment of mixed chimerism, that tolerance is stable after graft removal. A second allograft from the same donor strain will be accepted without immunosuppression at any time thereafter, for the life of the recipient[25].

This is not to say that detection of chimerism is not important in both cases. Indeed, regardless of the mechanism responsible for the establishment of mixed chimerism, its detection might serve as a marker for graft acceptance, and thus might be useful diagnostically. Indeed, cells from the donor which escape to other sites might play a role in diminishing the immune response to the transplant[22]. However, since detectable microchimerism after organ transplantation is not a consistent finding in patients with short- or long-term stable graft function[4,5], and microchimerism has been shown to persist in patients during graft rejection[23], it might only be useful as a marker in conjunction with other parameters[3]. The detection or lack of detection of donor microchimerism after solid-organ transplantation alone may be misleading.

Clinical implications

Starzl and colleagues have proposed a paradigm in which the states of immunologic tolerance achieved either by bone marrow transplantation or by organ transplantation are linked by a common dependence on the presence of haematopoietic chimerism[1,6]. The description of this paradigm is important both because it provides a theoretical construct for understanding the complex interactions

between host and graft that occur following any transplant, and because it suggests experimental manipulations which increase the likelihood of tolerance induction and which are therefore worthy of further testing. However, given the likely differences in mechanism by which chimerism is achieved in these two distinct situations, it would be premature and potentially dangerous to discontinue immunosuppression in transplant patients solely on the basis of the detection of peripheral microchimerism. Immunosuppression generally diminishes T-cell responses by suppressing the activity of T cells capable of recognizing the transplanted tissue rather than by eliminating them. Therefore, when immunosuppression is stopped, T-cell reactivity to the transplant can be expected to return, unless some additional mechanism to delete or inhibit the activity of donor-reactive T cells is acquired in the interim.

Such mechanisms may exist and should be explored. For example, it might be possible to induce specific anergy among residual T cells during the period of immunosuppression[34]. Alternatively, if cells from the transplant migrate to sites capable of achieving negative selection of new T cells, e.g. the thymus, and if sufficient time passes during the period of immunosuppression for existing T cells to be replaced, then a deletional tolerance could result. However, some T cells are known to be extremely long-lived[35], thus the period necessary for such a result to be achieved could be long and variable. Clearly, additional laboratory studies directed towards elucidating the mechanisms by which the unresponsive state is maintained are essential before it will be safe to discontinue immunosuppression in clinical transplantation.

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Response to Starzl et al.

Starzl and colleagues have provided a comprehensive review of the arguments that they have previously raised in favour of their 'two-way paradigm' of tolerance. However, they fail to shed new light on the central question of whether the chimerism observed following a transplant performed with conventional immunosuppression is the cause of graft acceptance or the result of effective immunosuppression. In order to do so they will have to explain why, in the case of long-term graft acceptance induced by immunosuppressive drugs, removal of the allograft leads to the loss of the tolerant state, whereas tolerance induced by the establishment of mixed chimerism via bone marrow transplantation persists for the lifetime of the recipient, regardless of whether or not the allograft is removed.

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Response to Wood and Sachs

When the mathematician, Andrew Wiles, faced skeptics after proving Fermat's last theorem[1], he said in effect that either he had solved the problem or gone mad. A radical departure from an ossified dogma provokes such thoughts.

A new idea must be understood to be judged. The madness of equating chimerism with drug-free tolerance or of using the presence and level of chimerism to guide drug weaning is not part of, or derivative from, the two-way paradigm. While agreeing unreservedly with Wood and Sachs' caution not to recklessly stop immunosuppression, we point out that their advice will apply equally to clinical use of the mixed chimerism models that they describe (see pp. 584–587). Already, this advice is a dominant theme in all management protocols of conventional bone marrow transplantation.

In the latter context in which the two-way system is distorted by cytoablation, human recipients of major histocompatibility complex (MHC)-matched bone marrow routinely require many months of immunosuppression to avoid graft rejection and the converse problem of graft-versus-host disease (GVHD). Even with an HLA incompatibility of only one allele, the patient is committed to years of drug therapy to avoid these complications.

Suggestions contrary to the two-way paradigm have been attributed to us by inaccurate citations (summarized in Ref. 2), not limited to the reports of weaning complications used by Wood and Sachs to support their contention that the mechanisms of organ- and bone-marrow-induced tolerance are not the same. There is no purpose in restating the evidence that the mechanisms are the same, beyond emphasizing our agreement with Wood and Sachs that recipient cytoreduction enhances the ease and extent of donor leukocyte engraftment in proportion to its severity .

However, the penalty is proportionate weakening of the biologic safety device, both against GVHD and rejection, that is provided by the nullification mechanism of dual cell populations. With each further increment in cytoreduction, successful transplantation depends more on tissue matching. Consequently, the progressive restriction of the acceptable donor pool will be a particularly grave handicap if this strategy is applied to xenotransplantation.

We have concluded that failure to find microchimerism after successful human organ transplantation, or in clinically relevant surrogate animal models, implies an incomplete search. In our clinical studies in which sampling was from multiple sites, the yield from individual locations was comparable with yields reported by others. However, when the results were pooled from the different sites in individual patients, all 30 tested patients had microchimerism. In rat experiments where tissues could be retrieved without limit, the association of chimerism with avoidance of chronic rejection has been absolute in our hands.

The conceptual flaw of using classical 'parking models' to study tolerance mechanisms was discussed previously in this issue (pp. 577–584). We have left open the possibility that the organ parenchymal cells facilitate chimerism by contributing to a favorable microenvironment, most likely by regional secretion of granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF, other growth and anti-growth factors, soluble MHC class I antigens or as yet unidentified molecules (reviewed in Ref. 3).

It would be unjust not to point out how dependent the evolution of the two-way paradigm has been on the past and continuing research of Wood, Sachs and many others. To comprehend how the disorientation about transplantation tolerance occurred, it is important to read classical contributions to the literature of mixed chimerism[4–9] (see Fig. 3, p. 580). The historical observations can be fully understood only in the context of the two-way paradigm.

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