CHIRON AWARD

# The basis of allograft acceptance

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Further real growth of transplantation will depend on the use of animal organs, an elusive goal that depends on first understanding how allografts are accepted. For nearly 50 years, after Medawar recognised that rejection was an immune reaction (1), an organ allograft (or xenograft) was envisioned as a defenceless island, under siege in a hostile recipient sea.

## The mystery of allograft acceptance

When Billingham, Brent, and Medawar (2, 3) showed that neonatal tolerance could be induced by engrafting haematolymphopoietic donor cells into immunologically immature mice, the door to transplantation was pushed ajar. Simulation of the mouse defenceless state with recipient cytoablation (4) ultimately allowed clinical BM transplantation (5-8) which was long viewed as a replacement of the immune system (complete donor leucocyte chimerism). When histo-incompatible donor BM or spleen cells transplanted into mouse (9-11) and human recipients (5, 6, 8) rejected the immunologically incompetent recipients, it appeared to be the same process in reverse that destroyed organ allografts.

The resulting unidirectional paradigm of transplantation immunology seemingly accommodated the findings following BMT but it did not explain organ allograft acceptance. In 1962-63, it was found that organ rejection, which had previously been considered inexorable in non-cytoablated MHC-incompatible recipients, could be reversed (12). Of equal importance, subsequent immunosuppression requirements frequently declined (12). These 2 related events were promptly shown to be generic, no matter what the baseline drug or which organ (13-15). Their control is the practical basis of the clinical field of transplantation.

This pattern of convalescence (Figure 1) was delineated initially from experience with kidney transplantation under treatment with azathioprine and dose-manoeuverable prednisone (12), the first effective double drug cocktail. At the time of this first report, the donor-specific non-reactivity was relative and still drug-dependent. In some cases, however, the tolerance became complete. A third of a century later, 10 (22%) of the first 46 Colorado recipients of living related donor kidneys (all treated before 1964) still have function of their original allografts (16). Five of these 10 kidney recipients are currently drug-free, and have been for 3 to 30yr. The cumulative time of these pts without drugs equals the time on treatment (Figure 2). Two of the 5 allografts were from HLA identical donors (top and third bars). However, 2 were one-haplotype mismatched (2nd and bottom bars), and one patient received a double-haplotype incompatible kidney from a great aunt (second from bottom). Complete tolerance has also been observed repeatedly after HLA mismatched cadaveric liver transplantation (17). Amongst our 42 longest surviving liver recipients, now 15 to 27yr post-transplantation, 12 (28%) have been drug-free for as long as 16yr (16). Their cumulative time without immunosuppression is almost equal to the time under treatment (Figure 3). With more potent baseline drugs, survival of all organ grafts rose in 3 distinct leaps over a 33yr period using azathioprine, cyclosporine, and most recently tacrolimus-based immuno-

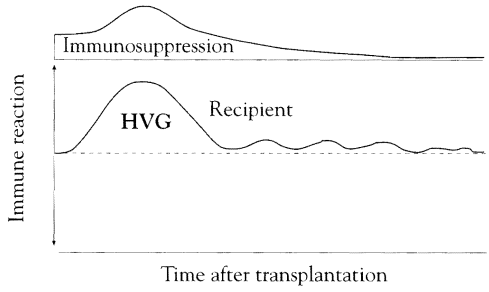


*Figure 1. Characteristic immunologic confrontation and resolution under immunosuppression that is the practical basis of organ transplantation*

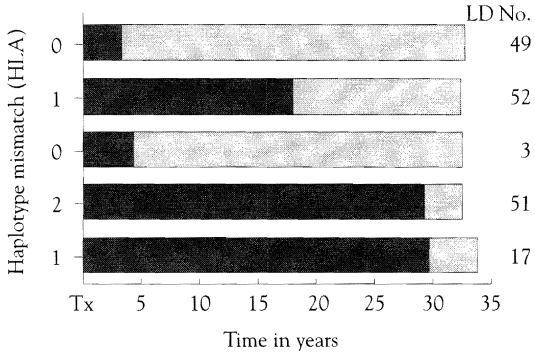


*Figure 2. Time of immunosuppressive treatment (dark shade) and time off drug therapy (light) in 5 non-twin living related kidney recipients whose allografts have functioned > a third of a century.* LD: *living donor*
■ *Drug;* □ *No drug*

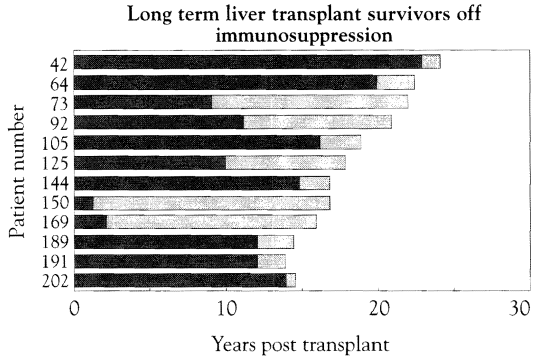


*Figure 3. Time on (black) and off immunosuppression (crosshatched) of 12 (28%) of our 42 longest surviving liver recipients (15-26yr post-transplant) who are receiving no treatment as of December, 1995.* *These drug-free pts remain well as of August 1996.*
■ *Immunosuppression;* □ *No immunosuppression*

suppression (18). However, the sequence and timing of immunologic confrontation and resolution did not change. It was merely better controlled.

## Disorienation 1962-63

There was, in fact, no explanation why organ allografts would ever survive, much less routinely. By 1963, donor leucocyte chimerism, the means to the end of Medawar's acquired tolerance and the raison d'etre of BMT, was eliminated by consensus as a factor in organ acceptance. It was the beginning of a long trek in the wilderness, without a compass, and in the wrong direction.

### Passenger leucocytes: The putative enemy

It was postulated 40yr ago by George Snell (19) and confirmed experimentally (20), that the highly antigenic passenger leucocytes of BM origin which are a component of tissue and organ allografts elicited rejection. Consequently, these donor leucocytes were viewed by transplanters as "the enemy" that had to be destroyed by the host immune system if organ transplantation was to succeed (Figure 4). This destruction could be envisioned at peripheral as well as intragraft sites when it was later learned by Nemlander and Hayry (21), Larsen et al. (22), Demetris and Murase (23), Qian et al (24), and others (14-17) that the donor leucocytes (including dendritic cells) promptly migrated in the blood to secondary lymphoid sites after organ revascularisation.

### The dichotomy of BM and organ transplantation

The remarkable disparities in treatment and outcome, ostensibly involving chimerism for

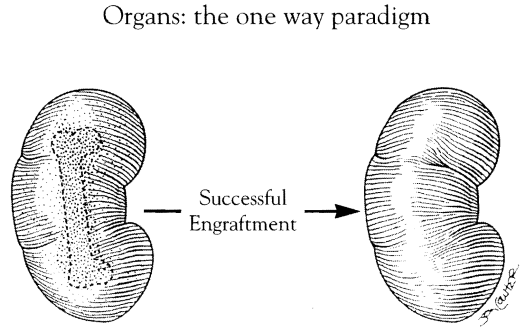


*Figure 4. Conventional view of a successfully transplanted allograft in which the nonparenchymal white cells (passenger leucocytes) were assumed to have been destroyed by the host immune system*
▨ *Non parenchymal;* ▭ *Parenchymal*

BM but not for organ transplantation, sustained the argument for 30yr that these 2 kinds of procedure were successful by divergent mechanisms. The differences (Table 1) were dependence (BM, left column) vs. independence on HLA-matching (organ, right column), risk vs. freedom from GVHD, the frequency with which the drug-free state could be achieved, and a semantic distinction between BM tolerance on the one hand and organ graft acceptance on the other. As it turned out, all of these dissimilarities were more or less dependent on a single treatment variable, recipient cytoablation for the BM but not for the organ recipient.

## An epiphany: 1992

What had happened after organ transplantation, was recognised in 1992, when donor leucocyte chimerism was detected in the peripheral tissues or blood of all 30 human kidney or liver recipients studied 2.5 to 30yr post-transplantation (14-17, 25). Sampling was from blood and multiple tissue sites. The sparse chimerism, in which dendritic cells were prominent, was demonstrated with donor HLA allele specific monoclonal antibodies. In addition, the presence of Y chromosomes in female recipients of male organs was documented with *in situ* hybridisation (26). Finally, donor alleles of chromosome 6 (HLA) and/or chromosome 2 (sex) were proven with PCR.

### The 2-way paradigm

With this information, we postulated that clinical organ transplantation under immunosuppression involved a double immune reaction which had host-versus-graft as well as graft-versus-host factors (Figure 5). The characteristic cycle of immunologic crisis and resolution that is the basis of all successful organ transplantations was the product of this bi-directional modulation. The reciprocal neutralisation of the 2 factors explained the blindfolding and thus the poor prognostic value of HLA-matching for organ transplantation (27). The cancellation effect also explained the rarity of GVHD, even with transplantation of lymphoid-rich organs like the liver and intestine (14-17, 25, 28). Because the cell-trafficking is bi-directional, both the allograft and recipient become genetic composites (Figure 6, upper panel). In essence, the passenger leucocytes contained in the organ allografts constituted a rapidly disseminated fragment of extramedullary donor BM (shown as a bone silhouette in Figure 6, upper panel) that contains pluripotent stem cells (29).

In the mirror image of successful BMT to cytoablated recipients (Figure 6, lower panel), a previously unsuspected trace population of host leucocytes can invariably be found (30, 31). With either organ or BMT, veto and suppressor cells, cytokine profile changes, and enhancing antibodies were viewed as derivative (and accessory) phenomena following the primary event of

*Table 1. The dichotomy between BM and organ transplantation*

| BM | | | | Organ |
|---|---|---|---|---|
| Critical | ← | MHC compatibility | → | Not critical |
| GVHD | ← | Principal complication | → | Rejection |
| Common | ← | Drug free state | → | Rare |
| Tolerance | ← | Term for success | → | Acceptance |
| Yes | ← | Recipient cytoablation | → | No |

*All differences derive from this therapeutic step*

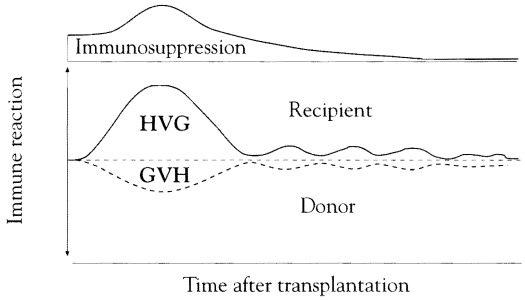


***Figure 5. Contemporary host- versus-graft (HVG) and graft-versus-host (GVH) reactions in the two-way paradigm of transplantation immunology.*** *Following the initial interaction, the evolution of non-reactivity of each leucocyte population to the other is seen as a predominantly low-grade stimulatory state that may wax and wane, rather than a deletional one*

mutual cell engagement (Figure 6).

Thus, the operational principle of organ allograft acceptance by chimerism (Figure 7) was the same as in the neonatal model (2, 3), cytoablation-dependent BMT (2, 3, 4-8), and "mixed chimerism" tolerance models. The last included the parabiosis models of Martinez and Good (32) and those of Slavin and Strober (33), Ildstad and Sachs (34), and Judy Thomas (35). The theme of chimerism had come full circle to the observations by Ray Owen 51yr ago of natural tolerance in Freemartin cattle (36).

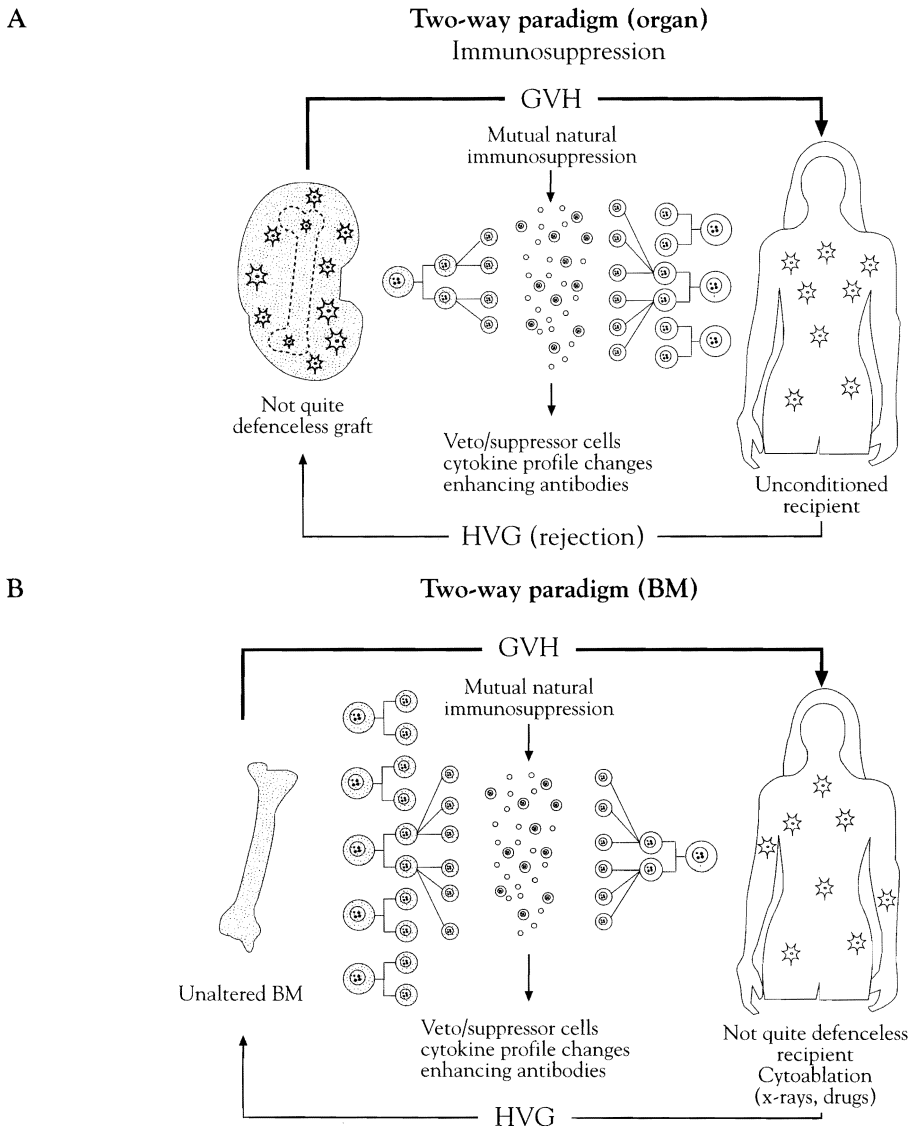


***Figure 6. Two-way paradigm with which transplantation is seen as a bi-directional and mutually cancelling immune reaction that is predominantly HVG with whole organ grafts, and predominantly GVH with BM grafts***

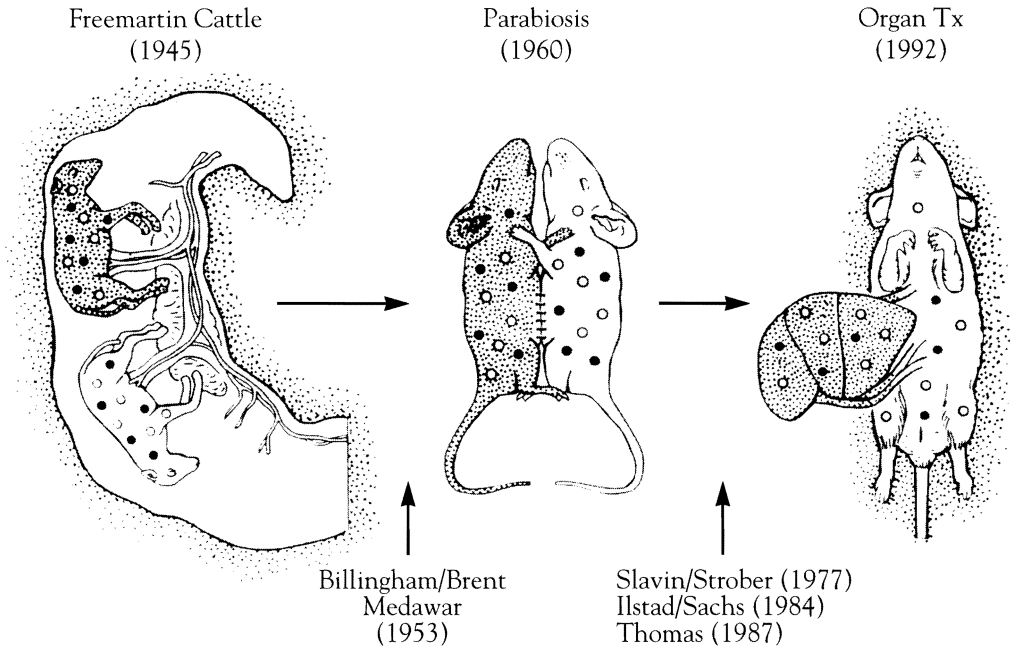


*Figure 7. Continuum of chimerism from observations of Ray Owen in Freemartin cattle to the discovery in 1992 of microchimerism in organ recipients*

**Transplant success and failure redefined**

Successful transplantation meant that chimerism had been introduced which might or might not be dependent on immunosuppression for stability. Failure connoted the therapeutically uncontrollable ascendancy of a HVG or GVH reaction. The explicit warning contained in this definition (14-16) was that quantitation of chimerism could not be used to guide drug-weaning decisions. This conclusion has sometimes gone unheeded, not been understood, or perhaps simply used as a "straw-man" for debating purposes.

**Level vs. duration of chimerism**

There is substantial reason to believe that the level of chimerism is less important than its duration (15, 16), best illustrated by experience with hepatic transplantation. In rodent liver-transplant models, the cause (chimerism) and effect (tolerance) are almost contemporary. In most mouse (24) and several rat strain combinations (37, 38), tolerance to liver allografts does not even require immunosuppression. The same observation had been made in the mid-1960's by Cordier et al. (39), Peacock and Terblanche (40) and Calne et al (41) in about 15% of outbred pigs (Figure 8). In contrast, chimerism and tolerance are separated by months or years despite immunosuppression in outbred dogs (13), and humans (15, 16). In some, the drug-free end-point may never be reached, necessitating a lifetime of immunosuppression to maintain hepatic allograft stability. One can only assume that the time to reach stable chimerism in an animal-to-human combination will be off the scale shown in Figure 8.

**Adjunct BM infusion**

All transplantation tolerance strategies are direct or indirect attempts to alter the donor/recipient leucocyte interaction. The infusion of unaltered donor BM in organ recipients (42, 43), a strategy long-advocated by Monaco (43, 44), is the most primitive example. Our clinical trials with adjunct BM for organ recipients (45, 46 and further reported in this issue) were based on the premise that persistent chimerism could be increased without affecting the rate of acute rejection and without increasing the risk of GVHD, providing immunosuppression was given to both immunocyte populations equally (15, 16, 45). These

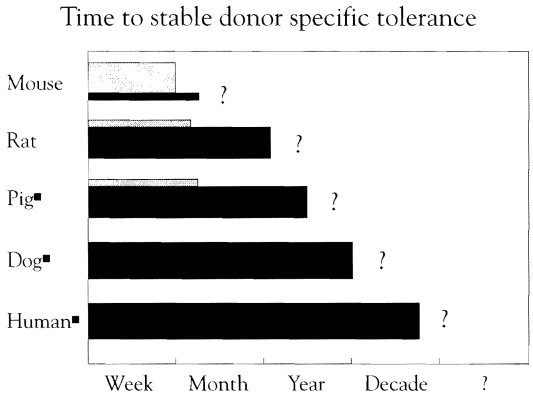


***Figure 8. Time between cause (chimerism) and effect (donor specific tolerance) after liver allo-transplantation in different species.*** *Note that immunosuppression is not universally required in 3 of the 5 species shown.*
☐ *No immunosuppression;* ■ *Immunosuppression needed;* ■*Outbred*

expectations have been verified in approximately 200 cases involving all of the major organs including the intestine.

These were in essence safety questions. The therapeutic hypothesis was quite a different matter. Here the premises were that the threat of delayed acute and chronic rejection would be reduced and that the frequency of ultimate drug- independence would be increased. Full efficacy evaluation is expected to take the same 5 to 10yr shown in Figure 8, the time frame already delineated by 3 decades of human experience with MHC incompatible liver and BMT (15, 16).

Procedures that selectively alter one of the interacting arms are potentially hazardous, exemplified by the historical BMT experience with GVHD after unloading the host immune system by cytoablation (Figure 9, lower panel). Delayed multiple BM infusions, currently being evaluated in Miami (47), could be a more subtle example in which the delayed uploading of a partially tolerant recipient with infused donor cells could have an increased GVHD potential. We will depend on the Miami team for accurate information about the dimensions of the risk of delayed BM infusions in human organ recipients.

Indications have come from rat models in which combined BM and liver transplantation done simultaneously under a short course of tacrolimus were well tolerated (23). However, when the transplants were staged, the second graft (even if it was the organ) always caused lethal GVHD (Figure 10). The naïve donor leucocytes delivered to the primed rats mimicked the outcome of a parent to defenceless offspring $F_1$ hybrid model (23). Persico and Remuzzi (48) have shown the GVHD potential with either simultaneous or staged rat BM and kidney allografts (Brown Norway Lewis) without any immunosuppression.

## Xenotransplantation

Xenotransplantation must inevitably follow guidelines imposed by the 2-way paradigm (49). The necessity for chimerism was recognised a dozen years ago by Ildstad and Sachs (34), based on evidence from the rat-mouse combination. The creation of transgenic animals is in essence an attempt to improve the cross-species tissue match, designed to reduce the acute barrier of humoral rejection. This principle, with emphasis on the transfection in pigs of human complement regulatory genes was first postulated by Platt and Bach (50) and verified by David White and Jeffrey Platt of Cambridge and Duke University, respectively (51-53).

Such procedures will not, however, resolve

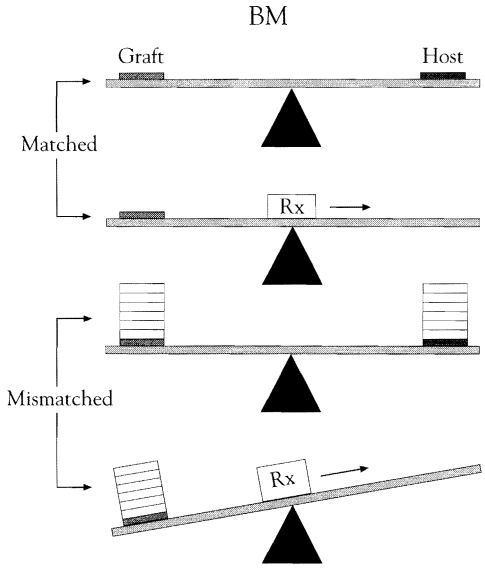


***Figure 9. Explanation of obligatory MHC matching for BMT in cytoablated recipients (second and fourth see-saws), vs. freedom from this restriction (first and third see-saws) if the host immune system is intact***

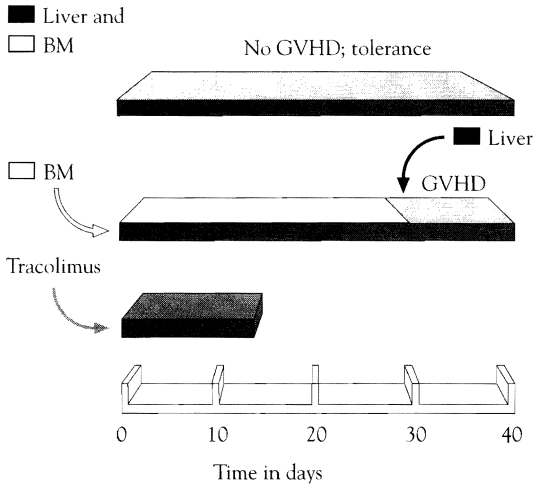


***Figure 10. Experimental models in rodents revealing the risk of GVHD with the delayed migration of naïve passenger leucocytes (see text) from subsequently transplanted organ.*** *Liver-BM experiments were reported by Demetris et al. (23). Similar observations with kidney-bone marrow have been made by Persico et al. (48)*

the problem of maintaining cohabitation of the animal and human immune systems for the predictably long period required for their stable merger (52, 53).

## The Zanjani sheep experiments

A potential crack in the xenotransplant wall has been suggested by the experiments of Zanjani et al (54, 55), using a modification of the Billingham-Brent-Medawar mouse model. At the 40 to 50d stage of the 4-5mo sheep gestational period, sheep embryos were inoculated intraperitoneally with leucocytes from human foetal livers, or with human stem cells purified from adult BM (Figure 11). A handful of the sheep foetuses completed their intrauterine life in a healthy state and have stable >5% human leucocyte chimerism 6 to 7yr later. The chimeric BM has been adoptively transferred by inoculation of other sheep foetuses (55).

## Xenogeneic chimerism in pigs

Is it necessary to go so far back in gestation for inoculation, or to use stem cell-rich preparations? One year ago, Dr Abdul Rao in our laboratory inoculated more than a dozen newborn pigs with $5x10^9$ unaltered intravenous baboon (or human) BM cells a few hours after birth, with no immunosuppression (n=2) or with subsequent tacrolimus only (n=5) or in combination with mycophenolate (n=5). The best results were without immunosuppression (Table 2). During the ensuing year, all of the 5 surviving animals, now weighing 350 to 410 pounds, have had low level blood (Figure 12) and/or BM chimerism.

In related experiments in which unaltered human BM was infused into cytoreduced adult baboons (750R total lymphoid irradiation), human colony forming units of all lineages have been grown (18mo post-transplantation) from the baboon BM (56). Our assumption is that interspecies cellular tolerance, if it develops at all (particularly in the human, neonatal pig model), will require protracted mutual exposure of the 2 cell populations. When the 5 pigs given human BM were tested at 11mo, there was evidence of donor-specific hypo-reactivity in 3 of the 5 animals (Figure 13). This had become more pronounced with time (Figure 14).

However, the critical question is if humoral immunity will be abrogated as has been reported by Aksentijevich et al.(57)after rat-mouse xeno-transplantation. In the serum of Zanjani's humanised sheep, anti-human endothelial antibodies were detectable with *in vitro* assays, even after 2yr of stable chimerism (58).

The decisive test of sheep-human transplantation could not be remotely considered without a preliminary parallel study of baboonised sheep. This experiment is underway in our laboratory after producing baboon chimerism in pigs rather than sheep.

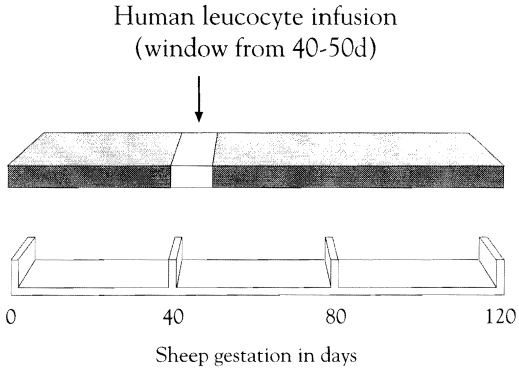


***Figure 11. Experiments of Zanjani showing the feasibility of inducing neonatal xenogeneic tolerance, by the intraperitoneal infusion of human leucocytes into sheep foetuses early in their development***

*Table 2. Recipient survival after human to pig BMT*

| Groups | n | Treatment | Survival (at 1yr) |
|---|---|---|---|
| I | 2 | Human BM | 2/2 |
| II | 5 | Human BM + FK 506 | 2/5 |
| III | 5 | Human BM + FK + MMF | 1/5 |

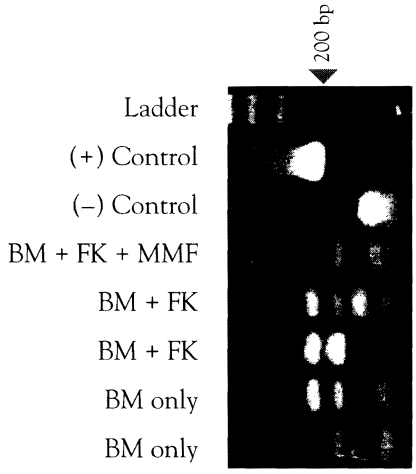


***Figure 12. Ethidium bromide-stained gel of electrophoresed products of double hot start PCR amplification of peripheral blood mononuclear cells (PBMC) obtained from human → pig BM recipients at 340d post-infusion. A primer for DR4 was used for detection of human cells; PBMC from untreated animals and DR4+ individuals were used as negative and positive controls, respectively. Note that human DNA is present in all except pig 1 (BM + FK + MMF) blood samples***

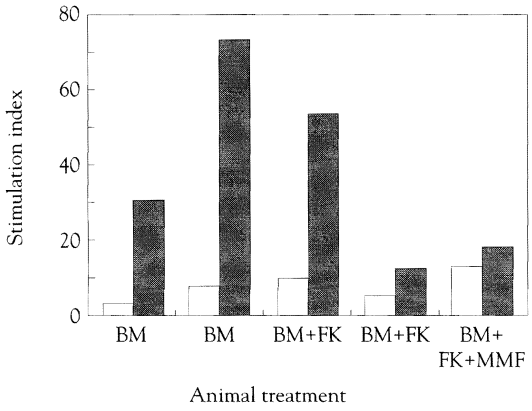


***Figure 13. Mixed lymphocyte reactivity in 5 human → pig BM transplant recipients at approximately 1yr post-infusion.*** *Pig PBMC were used as responders against either donor or third party splenocytes in a 6d proliferative assay; the wells were pulsed with (3H) thymidine (1μCi/well) and harvested 12-14h later for the determination of its incorporation. The results are expressed as stimulation index (experimental CPM/background CPM).*
*□ Donor; ■ Third party*

The ultimate pre-clinical test will be pig to baboon organ transplantation.

## Adoptive transfer of xenogeneic tolerance

Such experiments are difficult, expensive, and may take years. However, if a level of interspecies compatibility is achieved in the inoculated pigs, these "golden animals" could become a renewable resource that will permit colony expansion by transferring the pre-adapted BM to supralethally irradiated adult pigs or to newborn piglets. The eventual clinical objective would be to transfer the humanised pig BM to cyto-ablated pts in preparation for a subsequent transplantation of a chimeric organ obtained from the expanded colony (Figure 15).

## Transgenic and chimerism technologies combined

Aside from the observations by Rice et al. (58), there has been other evidence that chimerism alone will not ameliorate the hyperacute rejection that follows xenotransplantation between discordant species (49). Species restriction of complement activation

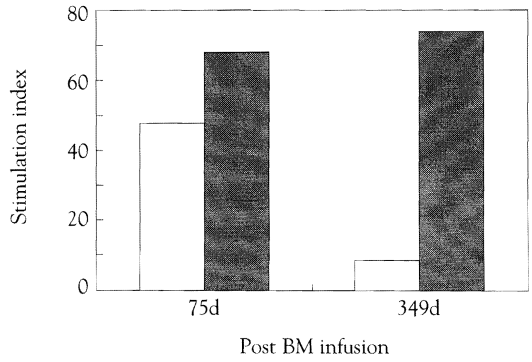


***Figure 14. Donor-specific mixed lymphocyte reactivity in a human → pig recipient of unmodified BM at 349d as compared to that of 75d post-infusion.*** *For methods, refer to the legend of Figure 13.*
*□ Donor; ■ Third party*

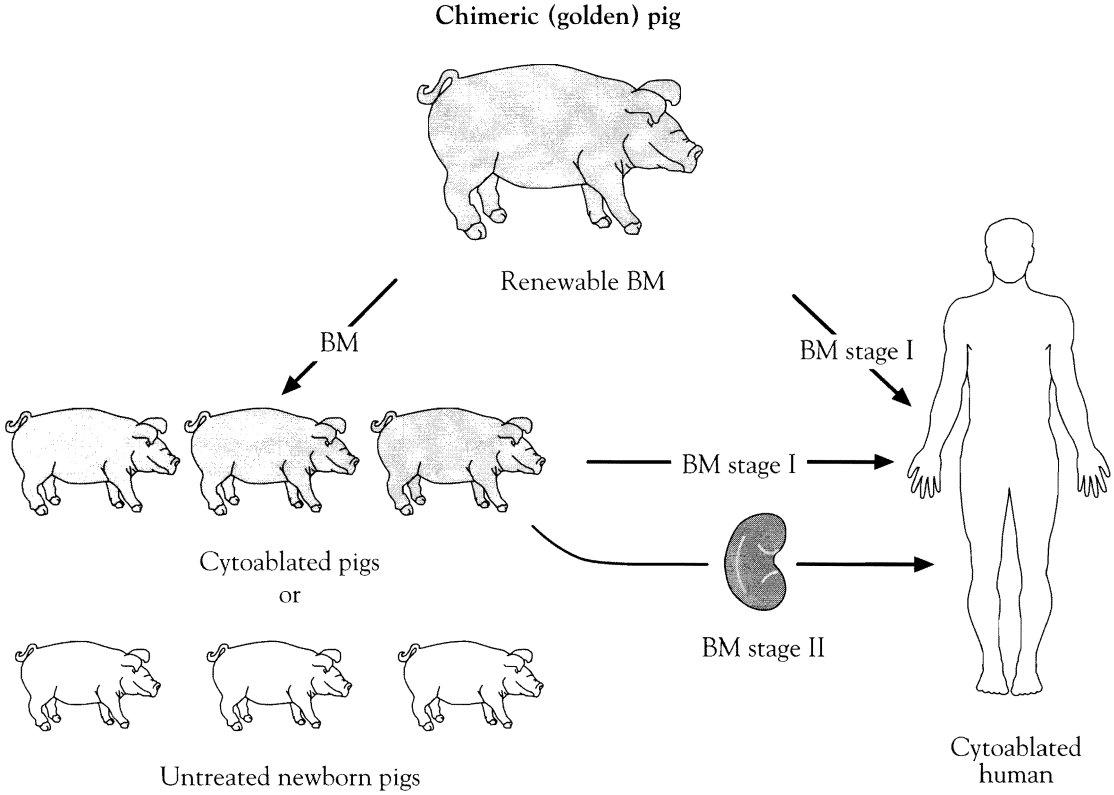


***Figure 15. Possible strategy in which humanised (chimeric) pigs are inoculated at birth with unaltered human BM.*** *The hypothesis is that the lengthy mutual exposure of the 2 leucocyte populations will lead to tolerance which can be transferred to cytoablated or untreated newborn pigs, or ultimately to cytoablated prospective human organ recipients (see text). If this procedure is performed on pigs with human complement regulatory genes, the chance of a practical solution to pig → human xenotransplantation should be improved*

has been described in earlier reports of Valdivia et al. (59, 60) and strongly reinforced by the recent observations of Rajasinghe et al. (61) in a rat-sheep variation of the original Zanjani model. In the latter experiments, sheep foetuses hyperacutely rejected rat cardiac xenografts in the absence of anti-rat antibodies (alternative pathway).

Because the liver is the primary source of complement synthesis (62, 63) it will not be surprising if the presence of leucocyte chimerism fails to reduce the complement activation that has been known for more than 30yr to be highly targeted to the vasculature of whole organ allografts (64-66) and xenografts (67, 68). By inducing chimerism in pigs who have human complement regulatory proteins in their organs at birth, the problems of complement activation and cellular tolerance can be jointly attacked with the strategy shown in Figure 15.

## Summary and conclusions

The assumption for the last third of a century that stem cell-driven haematolymphopoietic chimerism was irrelevant to successful conventional whole organ transplantation, has prompted alternative inadequate explanations of organ allograft acceptance. This assumption clouded the biological meaning of successful organ as well as BMT, and precluded the development of a cardinal principle that accommodated all facets of transplantation.

Recognition of this error and the incorporation of the chimerism factor into a 2-way paradigm has allowed previous enigmas of organ as well as BM engraftment to be explained. No credible evidence has emerged to interdict this interactive concept. If the 2-way paradigm is correct, it will allow the remarkable advances that have been made in basic immunology to be

more meaningfully exploited for transplantation, including that of xenografts.
Because of the important potential clinical implications of Dr. Abdul Rao's experiments in which baboon or human bone marrow was infused into the neonatal pigs, different probes have been used in an attempt to validate the presence of chimerism. Sustained chimerism in pigs given baboon bone marrow was thereby independently confirmed. However, Dr. Rao was not able to verify the presence in the pigs infused with human leukocytes. The reason for the discrepancy with the outcome after baboon and human infusions is under investigation.

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