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The Future of Transplantation: With Particular Reference to Chimerism and Xenotransplantation

Thomas E. Starzl, Anthony J. Demetris, Noriko Murase, Luis Valdivia, Angus W. Thomson, John Fung, and Abdul S. Rao

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[1] recognized rejection to be an immune reaction, an organ allograft (or xenograft) was envisioned as a defenseless island under siege in a hostile recipient sea.

THE MYSTERY OF ALLOGRAFT ACCEPTANCE

When Billingham et al[2,3] showed that neonatal tolerance could be induced by engrafting hematolymphopoietic donor cells into immunologically immature mice, the door to transplantation was pushed ajar. Simulation of the mouse defenseless state with recipient cytoablation[4] ultimately allowed clinical bone marrow transplantation[5–8] which was long viewed as a replacement of the immune system (complete donor leukocyte chimerism). When histo*in*compatible donor bone marrow 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 bone marrow transplantation, but it did not explain organ allograft acceptance. In 1962 to 1963, it was learned that organ rejection, which previously had been considered inexorable in noncytoablated MHC-incompatible recipients, could be reversed.[12] Of equal importance, subsequent immunosuppression requirements frequently declined.[12] These two related events were promptly shown to be generic, no matter what the baseline drug or what organ.[13–15] Their control is the practical basis of the clinical field of transplantation.

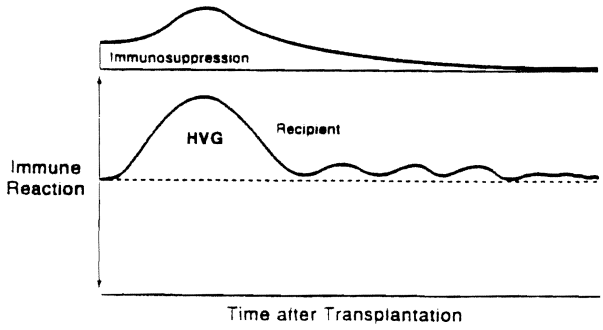

Fig 1. Characteristic immunologic confrontation and resolution under immunosuppression that is the practical basis of organ transplantation.

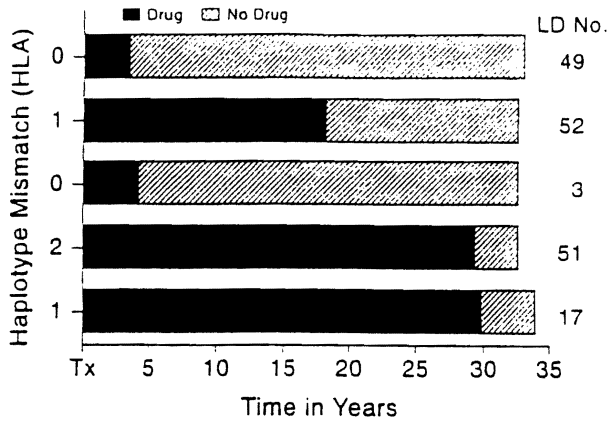

Fig 2. Time of immunosuppressive treatment (dark shade) and time off drug therapy (light) in five non-twin living-related kidney recipients whose allografts have functioned a third of a century or more. LD, living donor.

This pattern of convalescence (Fig 1) was delineated initially from experience with kidney transplantation under treatment with azathioprine and dose-maneuverable prednisone,[12] the first effective double-drug cocktail. At the time of this first report, the donor-specific nonreactivity 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

From the Transplantation Institute and the Departments of Surgery and Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania.

Supported by grant DK 29961 from the National Institutes of Health, Bethesda, Maryland.

Address reprint requests to Thomas E. Starzl, MD, PhD, 3601 Fifth Ave, 4C Falk Clinic, Pittsburgh, PA, 15213.

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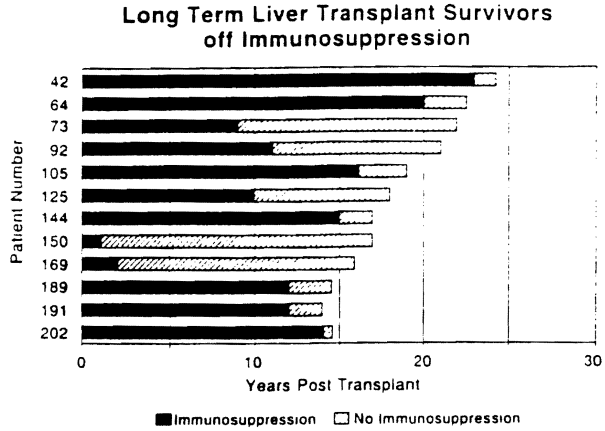

Fig 3. Time on (black) and off immunosuppression (cross-hatched) of 12 (28%) of our 42 longest-surviving liver recipients (15 to 26 years posttransplant) who have not received treatment since December 1995. These drug-free patients remain well in August 1996.

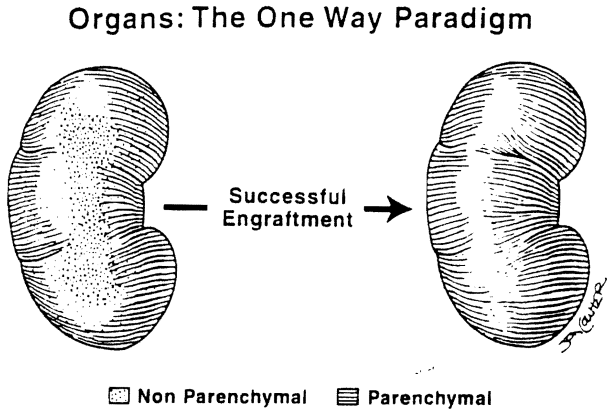

Fig 4. Conventional view of a successfully transplanted allograft in which the nonparenchymal white cells (passenger leukocytes) were assumed to have been destroyed by the host immune system.

donor kidneys (all treated before 1964) still have function of their original allografts.[16]

Five (one half) of these 10 kidney recipients are currently drug free, and have been for 3 to 30 years. The cumulative time of these patients off drugs equals the time on treatment (Fig 2). Two of the five allografts were from HLA-identical donors (top and third bars). However, two were one-haplotype mismatched (second and bottom bars), and one patient received a double-haplotype incompatible kidney from a great aunt (second from bottom).

Complete tolerance also has been observed repeatedly after HLA-mismatched cadaveric liver transplantation.[17] Among our 42 longest surviving liver recipients—now 15 to 27 years posttransplantation—12 (28%) have been drug free for as long as 16 years.[16] Their cumulative time off immunosuppression is almost equal to time treated (Fig 3).

With more potent baseline drugs, survival of all organ grafts rose in three distinct leaps over a 33-year period using azathioprine, cyclosporine (CyA), and most recently tacrolimus-based immunosuppression.[18] However, the sequence and timing of immunologic confrontation and resolution did not change. It was merely better controlled.

DISORIENTATION: 1962 TO 1963

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

Passenger Leukocytes: The Putative Enemy

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

The Dichotomy of Bone Marrow and Organ Transplantation

The remarkable disparities in treatment and outcome, ostensibly involving chimerism for bone marrow but not for organ transplantation, sustained the argument for 30 years that these two kinds of procedures were successful by divergent mechanisms. The differences (Table 1) were dependence (bone marrow, left column) vs independence on HLA matching (organ, right column), risk vs freedom from graft-vs-host disease (GVHD), the frequency with which the drug-free state could be achieved, and a semantic distinction between bone marrow tolerance on one hand and organ graft acceptance on the other. As it turned out, all of these dissimilarities were more or less dependent on

Table 1. The Dichotomy Between Bone Marrow and Organ Transplantation

Bone Marrow				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.

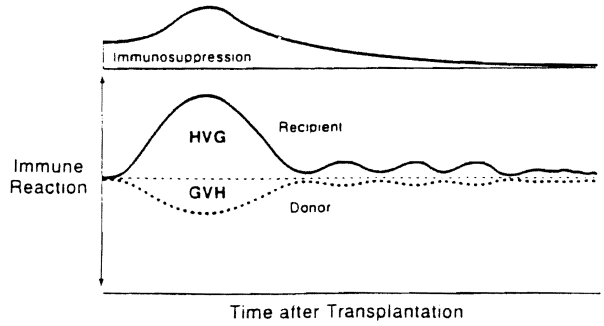

Fig 5. Contemporaneous HVG and GVH reactions in the two-way paradigm of transplantation immunology. Following the initial interaction, the evolution of nonreactivity of each leukocyte population to the other is seen as a predominantly low-grade stimulatory state that may wax and wane, rather than as a deletional one.

a single treatment variable—recipient cytoablation for the bone marrow, but not for the organ recipient.

AN EPIPHANY: 1992

What had happened after organ transplantation was recognized in 1992 when donor leukocyte chimerism was detected in the peripheral tissues or blood of all 30 human kidney or liver recipients studied 2-1/2 to 30 years posttransplantation.[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 hybridization.[26] Finally, donor alleles of chromosome 6 (HLA) and/or chromosome 2 (sex) were proved with polymerase chain reaction (PCR).

The Two-Way Paradigm

With this information, we postulated that clinical organ transplantation under immunosuppression involved a double immune reaction which had host-vs-graft (HVG) as well as graft-vs-host (GVH) arms (Fig 5). The characteristic cycle of immunologic crisis and resolution *that is the basis of all successful organ transplantations* was the product of this bidirectional modulation. The reciprocal neutralization of the two arms explained the blind folding 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 bidirectional, both the allograft and recipient become genetic composites (Fig 6, upper panel). In essence, the passenger leukocytes contained in the organ allografts constituted a rapidly disseminated fragment of extramedullary donor bone marrow (shown as a bone silhouette in

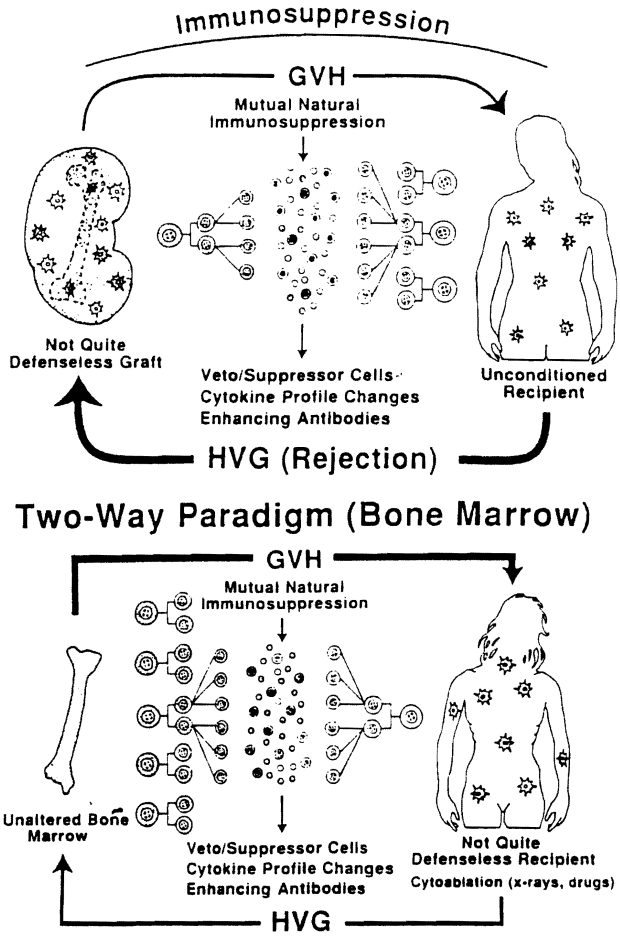

Fig 6. Two-way paradigm with which transplantation is seen as a bidirectional and mutually cancelling immune reaction that is predominantly HVG with whole organ grafts **(upper panel)** and predominantly GVH with bone marrow grafts **(lower panel).**

Figure 6, upper panel) that contains pluripotent stem cells.[29]

In the mirror image of successful bone marrow transplantation to cytoablated recipients (Fig 6, lower panel), a previously unsuspected trace population of *host* leukocytes invariably can be found.[30,31] With either organ or bone marrow transplantation, veto and suppressor cells, cytokine profile changes, and enhancing antibodies were viewed as derivative (and accessory) phenomena following the primary event of mutual cell engagement (Fig 6).

Thus, the operational principle of organ allograft acceptance by chimerism (Fig 7) was the same as in the neonatal model,[2,3] cytoablation-dependent bone marrow transplantation,[2,3,4-8] and mixed chimerism tolerance models. The last included the parabiosis models of Martinez et al[32] and those of Slavin et al,[33] Ildstad and Sachs,[34] and Thomas et al.[35] The theme of chimerism had come full circle to the observations by Ray Owen 51 years ago of natural tolerance in freemartin cattle.[36]

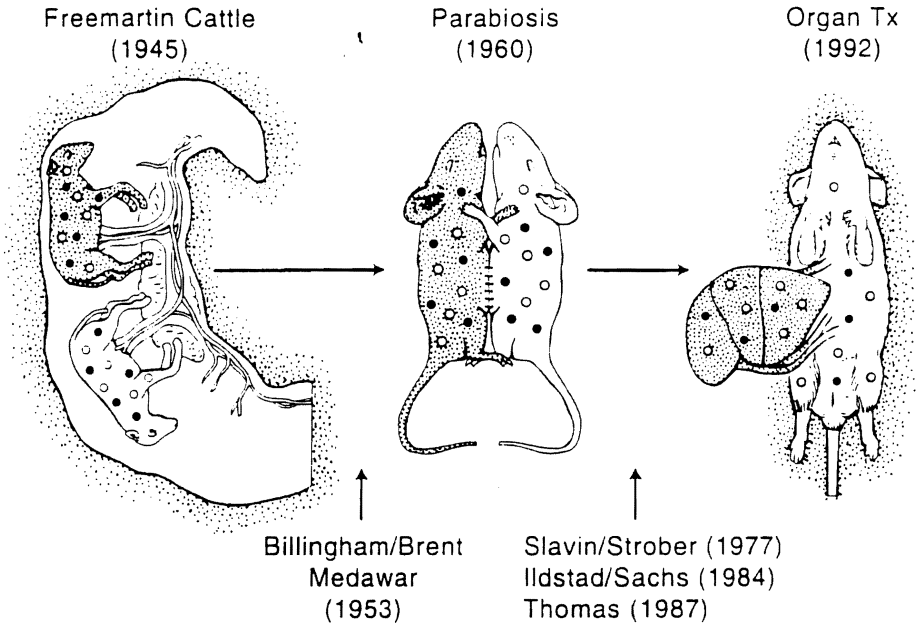

Fig 7. Continuum of chimerism from observations of Ray Owen in freemartin cattle to the discovery in 1992 of microchimerism in organ recipients.

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, has not been understood, or perhaps simply has been used as a strawman 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] which is best illustrated by experience with hepatic transplantation. In rodent liver transplant models, the cause (chimerism) and effect (tolerance) are almost contemporaneous. 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-1960s by Cordier et al.[39] Peacock and Terblanche,[40] and Calne et al[41] in about 15% of outbred pigs (Fig 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 Fig 8.

Adjunct Bone Marrow Infusion

All transplantation tolerance strategies are direct or indirect attempts to alter the donor/recipient leukocyte interaction. The infusion of unaltered donor bone marrow in organ recipients,[42,43] a strategy long advocated by Monaco and others,[43,44] is the most primitive example. Our clinical trials with adjunct bone marrow for organ recipients[45,46] (and further reported in this issue) were based on the

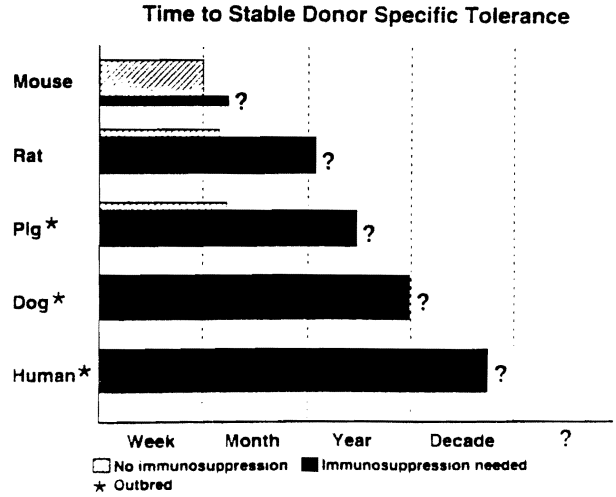

Fig 8. Time between cause (chimerism) and effect (donor-specific tolerance) after liver allotransplantation in different species. Note that immunosuppression is not universally required in three of the five species shown.

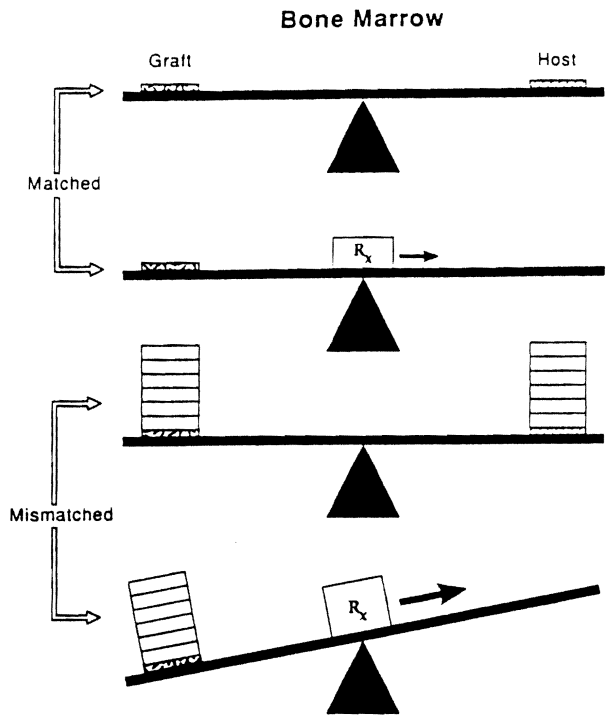

Fig 9. Explanation of obligatory MHC matching for bone marrow transplantation in cytoablated recipients (second and fourth teeter totters) vs freedom from this restriction (first and third teeter totters) if the host immune system is intact.

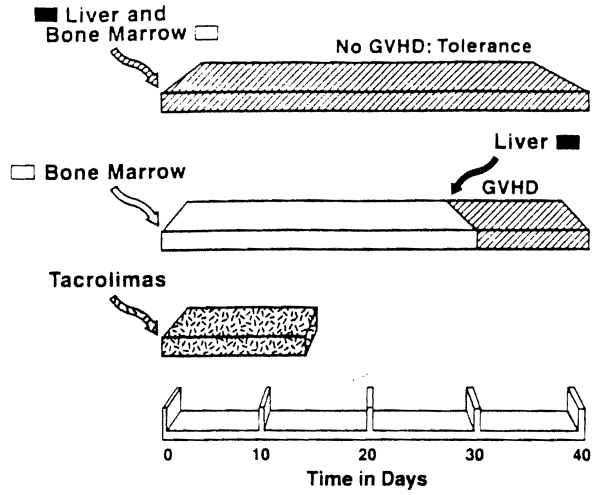

Fig 10. Experimental models in rodents revealing the risk of GVHD with the delayed migration of naive passenger leukocytes (see text) from subsequently transplanted organ. Liver-bone marrow experiments were reported by Demetris et al.[23] Similar observations with kidney-bone marrow have been made by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

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 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 10 years shown in Fig 8, the time frame already delineated by three decades of human experience with MHC-*in*compatible liver and bone marrow transplantation.[15,16]

Procedures that selectively alter one of the interacting arms are potentially hazardous, exemplified by the historical bone marrow transplant experience with GVHD after unloading the host immune system by cytoablation (Fig 9, lower panel). Delayed multiple bone marrow 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 bone marrow infusions in human organ recipients.

Warnings have come from rat models in which combined bone marrow and liver transplantation done simultaneously under a short course of tacrolimus was well tolerated.[23] However, when the transplants were staged, the second graft (even if it was the organ) always caused lethal GVHD (Fig 10). The naive donor leukocytes delivered to the primed rats mimicked the outcome of a parent-to-defenseless offspring F_1 hybrid model.[23] Persico et al[48] have shown the GVHD potential with either simultaneous or staged rat bone marrow and kidney allografts (Brown Norway → Lewis) without any immunosuppression.

XENOTRANSPLANTATION

Xenotransplantation inevitably must follow guidelines imposed by the two-way paradigm.[49] The necessity for chimerism was recognized 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 (summarized in refs. 51 to 53).

Such procedures will not, however, resolve the problem of maintaining cohabitation of the animal and human immune systems for the predictably long period required for their stable merger.[52,53]

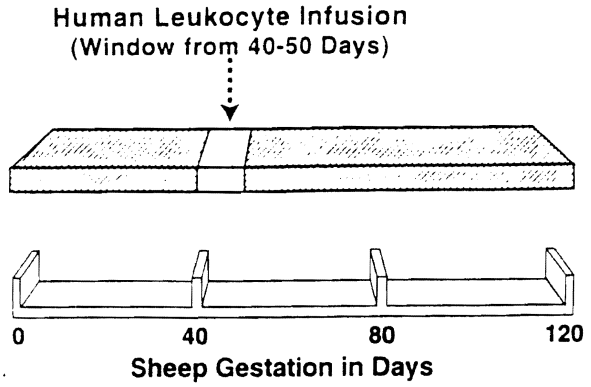

Fig 11. Experiments of Zanjani showing the feasibility of inducing neonatal xenogeneic tolerance, by the intraperitoneal infusion of human leukocytes into sheep fetuses early in their development.

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 50-day stage of the 4- to 5-month sheep gestational period, sheep embryos were inoculated intraperitoneally with leukocytes from human fetal livers, or with human stem cells purified from adult bone marrow (Fig 11). A handful of the sheep fetuses completed their intrauterine life in a healthy state and have a stable 5% or higher human leukocyte chimerism 6 to 7 years later. The chimeric bone marrow has been adoptively transferred by inoculation of other sheep fetuses.[55]

Xenogeneic Chimerism in Pigs

Is it necessary to go back so far in gestation for inoculation, or to use stem cell-rich preparations? One year ago, one of us (A.S.R.) inoculated 12 pigs with 5×10^9 unaltered IV human bone marrow cells a few hours after birth, with no immunosuppression (n = 2) or with subsequent tacrolimus only (n = 5) or in combination with mycophenolate (MMF) (n = 5). The best results were without immunosuppression (Table 2). During the ensuing year, all of the five surviving animals—now weighing 350 to 410 lb—have had low-level blood (Fig 12) and/or bone marrow chimerism.

In related experiments in which unaltered human bone marrow was infused into cytoreduced adult baboons (7.5 Gy total lymphoid irradiation), human colony-forming units of

Table 2. Recipient Survival After Human-to-Pig Bone Marrow Transplantation

Groups	n	Treatment	Survival (at 1 Y)
I	2	Human bone marrow	2/2
II	5	Human bone marrow + FK 506	2/5
III	5	Human bone marrow + FK 506 + MMF	1/5

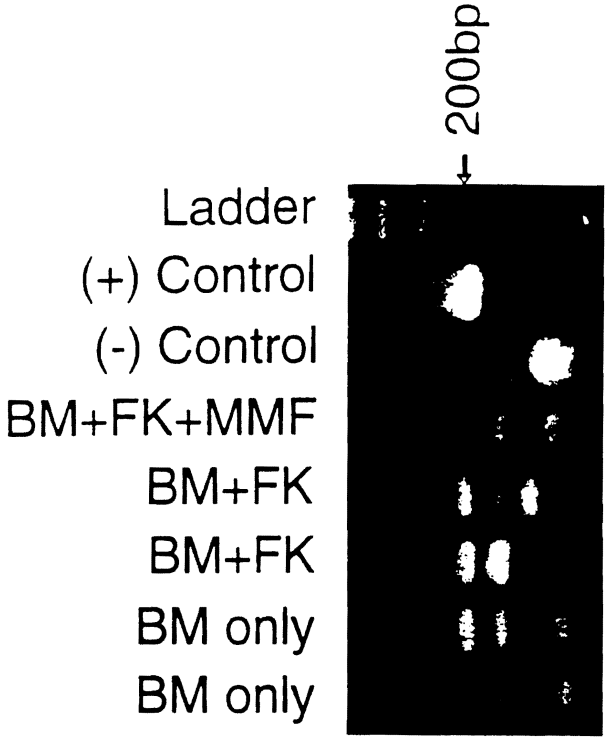

Fig 12. Ethidium biomide-stained gel of electrophoresed products of double hot start PCR amplification of peripheral blood mononuclear cells (PBMC) obtained from human → pig bone marrow recipients at 340 days postinfusion. A primer for DR4 was used for detection of human cells; PBMC from untreated animals and DR4$^+$ individuals were used as negative and positive controls, respectively. Note human DNA is present in all except pig 1 (bone marrow + FK 506 + MMF) blood samples.

all lineages have been grown (18 months posttransplantation) from the baboon bone marrow.[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 two cell populations. When the five pigs with human chimerism were tested at 11 months, there was evidence of donor-specific hyporeactivity in three of the five animals (Fig 13). This had become more pronounced with time (Fig 14).

However, the critical question is whether humoral immunity will be abrogated as has been reported by Aksentijevich et al[57] after rat → mouse xenotransplantation. In the serum of Zanjani's humanized sheep,[58] antihuman endothelial antibodies were detectable with in vitro assays, even after 2 years of stable chimerism.

The decisive test of sheep → human transplantation could not be remotely considered without preliminary parallel study of baboonized sheep. This experiment is underway in our laboratory after producing baboon chimerism in pigs rather than in sheep. The ultimate preclinical test will be pig-to-baboon organ transplantation.

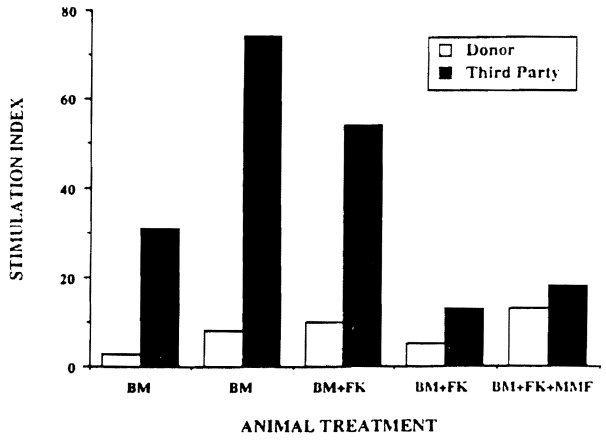

Fig 13. Mixed lymphocyte reactivity in five human → pig bone marrow transplant recipients at approximately 1 year postinfusion. Pig PBMC were used as responders against either donor or third-party splenocytes in a 6-day proliferative assay; the wells were pulsed with (^{3}H) thymidine (1 μCi/well) and harvested 12 to 14 hours later for the determination of its incorporation. The results are expressed as stimulation index (experimental CPM/background CPM).

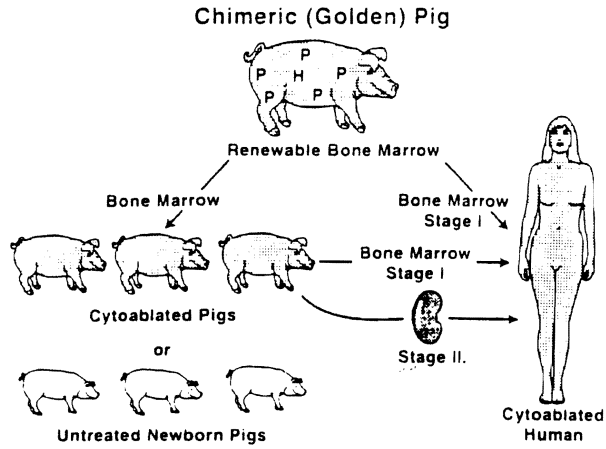

Fig 15. Possible strategy in which humanized (chimeric) pigs are inoculated at birth with unaltered human bone marrow. The hypothesis is that the lengthy mutual exposure of the two leukocyte populations will lead to tolerance which can be transferred to cytoablated or untreated newborn pigs, or ultimately 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.

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 preadapted bone marrow to supralethally irradiated adult pigs or to newborn piglets. The eventual clinical objective would be to transfer the humanized pig bone marrow to cytoablated patients in preparation for a subsequent transplantation of a chimeric organ obtained from the expanded colony (Fig 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 (summarized in ref. 49). Species restriction of complement activation has been described in earlier reports of Valdivia et al[59,60] and has been 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 fetuses hyperacutely rejected rat cardiac xenografts in the absence of antirat antibodies (alternative pathway).

Because the liver is the primary source of complement synthesis,[62,63] it will not be surprising if the presence of leukocyte chimerism fails to reduce the complement activation that has been known for more than 30 years to be highly targeted to the vasculature of whole organ allografts[64–66] and xenografts.[67,68] By inducing chimerism in pigs that have human complement regulatory proteins in

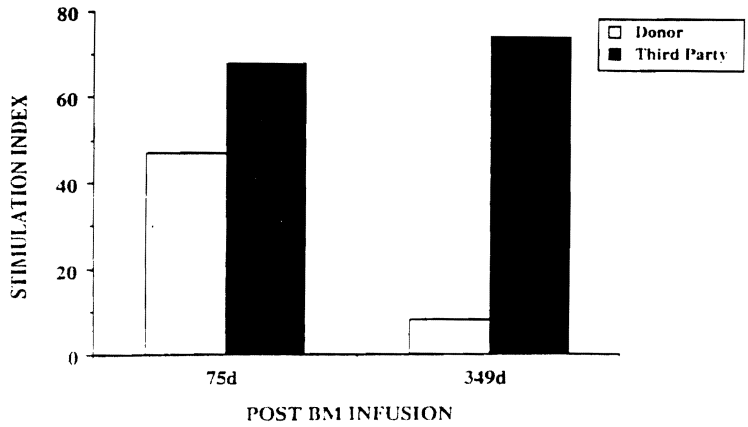

Fig 14. Donor-specific mixed lymphocyte reactivity in a human → pig recipient of unmodified bone marrow at 349 days as compared to that of 75 days postinfusion. For methods, refer to the legend of Fig 13. □, donor; ■, third party.

their organs at birth, the problems of complement activation and cellular tolerance can be jointly attacked with the strategy shown in Fig 15.

SUMMARY AND CONCLUSIONS

The assumption for the last third of a century that stem cell-driven hematolymphopoietic chimerism was irrelevant to successful conventional whole organ transplantation has prompted alternative inadequate explanations of organ allograft acceptance. This assumption clouded the biologic meaning of successful organ as well as bone marrow transplantation, 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 two-way paradigm have allowed previous enigmas of organ as well as bone marrow engraftment to be explained. No credible evidence has emerged to interdict this interactive concept. If the two-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.

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