Chapter 10

Migratory Nonparenchymal Cells After Organ Allotransplantation: With Particular Reference to Chimerism and the Liver

By THOMAS E. STARZL, ANTHONY J. DEMETRIS, ABDUL S. RAO, ANGUS W. THOMSON, MASSIMO TRUCCO, *and* NORIKO MURASE

We have proposed recently[1–7] that the exchange of migratory leukocytes between the transplant and the recipient, with consequent long-term chimerism (co-existence of donor and recipient cells) in both, is the basis for acceptance of whole organ allografts and xenografts (Fig 10–1). Although such chimerism was first shown only in the spring of 1992, the observations have increased our insight into transplantation immunology and have encouraged the development of alternative therapeutic strategies.

LOCAL (GRAFT) CHIMERISM OF THE LIVER AND OTHER ORGANS

It was shown with karyotyping techniques in 1969 that human liver allografts become genetic composites (local chimerism). In female recipients of livers obtained from male cadaveric donors, the hepatocytes as well as the endothelium of the major blood vessels of the grafts retained their donor sex, whereas within 100 days the entire macrophage system including the Kupffer cells was replaced with cells identified as female by their characteristic Barr bodies.[8, 9] For more than two decades, the composite genetic structure of the hepatic allograft was assumed to be a unique feature of this organ.

This illusion was dispelled in 1991 with the finding, first in rat intestinal allografts[10] and then in transplanted human bowel,[11] that the epithelium and vascular endothelium remained donor, whereas lymphoid, dendritic, and other leukocytes were replaced by recipient cells in the lamina propria, Peyer's patches, and mesenteric nodes. The same kind of transformation is now known to occur with all whole organ grafts.[4, 12–16]

DISCOVERY OF SYSTEMIC CHIMERISM

Indirect Evidence

Early circumstantial evidence that these cells (the donor leukocytes leaving the grafts) were still viable was largely ignored or misinterpreted. For example, it was shown in 1963 that delayed hypersensitivity reactions (tuberculin, histoplasmin, etc) present in kidney donors were transferred to previously negative recipients

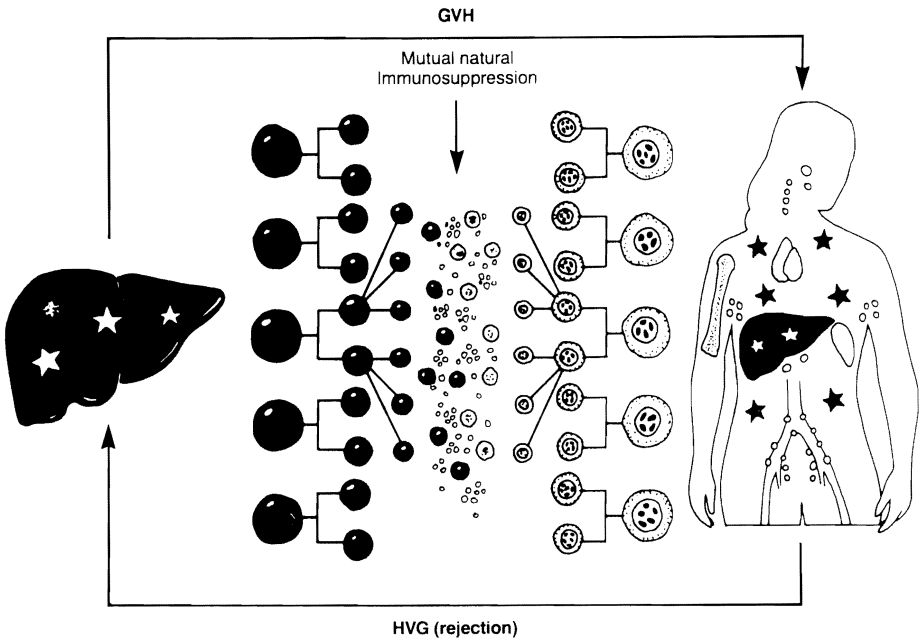

Figure 10–1. The mutual engagement of migratory immunocytes from the graft and the recipient after organ transplantation under potent pharmacological immunosuppression. GVH, graft-versus-host; HVG, host-versus-graft. (Reprinted with permission.[94])

with successful renal transplantation,[17] but not if the kidney was lost to rejection. The explanation that this represented "adoptive transfer of donor cellular immunity by leukocytes in the renal graft vasculature and hilar lymphoid tissue"[17] was considered implausible at the time because the kidney was construed to be a "leukocyte-poor" organ.

Clues were also overlooked in liver recipients. In these patients, new donor-specific immunoglobulin (IgG) types appeared and persisted in the blood.[9,18] Much later, donor-specific leukocytes were postulated to be the source of anti–red blood cell antibodies that developed in recipients of livers from donors who had ABO nonidentity.[19] Finally, Davies et al[20] reported the appearance of donor-specific soluble class I antigens in the blood of liver recipients that were thought to be synthesized by the graft hepatocytes. Because these molecules can also be produced by bone marrow–derived macrophages and/or dendritic cells,[21–23] a more plausible explanation is that they originated from donor chimeric cells in the same way as the additional IgG types and anti–red blood cell antibodies.

Direct Evidence of Chimerism

During the period of April through June 1992, we began a systematic search for ectopic donor leukocytes in rats[24–26] and in human recipients of kidneys, livers, and other organs whose successful transplants had been performed many months or years earlier. The search in patients was made feasible by the distinctive features of two chromosomes: Y chromosomes in females who had been given organs from male donors, and/or HLA alleles of chromosome 6 in all patients. In either

instance, one or the other of two technologies, and usually both, were exploited.[1–6] One was cytostaining, which allows the location and morphological characterization of phenotypically distinct donor and recipient cells. The cytostaining for the Y probe was with a fluorescence method after *in situ* hybridization. The immunostaining for the HLA markers was with indirect immunofluorescence and/or an avidin-biotin–complex immunoperoxidase method, using monoclonal antibodies to MHC class I and class II antigen specific for the donor but not the recipient.

The other technology was polymerase chain reaction (PCR), which permits the distinction of donor from recipient DNA.[1–6] In the PCR search for the Y chromosome, oligonucleotides specific for the satellite region of the Y chromosome centromere Y-A and for the sex-determining region of the Y chromosome were used as primers to determine the presence of male DNA in the female recipient tissues. The PCR tests for donor- and recipient-specific HLA alleles of chromosome 6 were performed by preliminary generic amplification of the DRB gene (encoding the beta chain of DR), followed by allele-specific amplification and testing.

The Human Kidney Recipients

Some of these patients, including the longest surviving kidney recipients in the world,[27] had participated in the skin test studies nearly three decades before (discussed previously). Of the five patients studied, one had stopped immunosuppression 12 years earlier, whereas the others were still taking azathioprine with or without prednisone. All five had received HLA-incompatible kidneys, which in two cases had come from donors of the opposite sex.

Low-level chimerism was found in the skin, lymph nodes, and often the blood of each of these kidney recipients. In addition, biopsies of the allografts showed that the cells departing the transplants had been largely replaced by similar cells from the host. Thus, both the recipients and their grafts were composed of cells with two different genomes.[4, 12]

The Human Liver Recipients

A much larger cohort of 25 liver recipients was studied 2 to 22 years after transplantation under azathioprine- or cyclosporine-based immunosuppression.[2, 6] Most were clinically well and fully immunocompetent by conventional *in vitro* testing. Donor cell chimerism was found with immunocytochemical or PCR techniques in all 25 in locations that included skin, lymph nodes, heart, lungs, spleen, intestine, kidneys, bone marrow, and thymus. Chimeric cells were in larger numbers at any given site than in the contemporaneously studied long-surviving kidney recipients, although the absolute numbers were still quite small.

CELL TRAFFIC AND SITES OF DONOR-RECIPIENT IMMUNOLOGIC INTERACTIONS

The early events leading to the chimeric state after liver transplantation have subsequently been studied in rats[7] and mice,[28] and the pathways of passenger

leukocyte dissemination have been well worked out. Within minutes or hours, these cells leave the liver and home to the spleen, lymph nodes, thymus, and bone marrow where they are destroyed by rejection in most untreated animals except mice or pigs, and some rat strains. However, under temporary immunosuppression in rats (2 weeks daily FK506 therapy), the donor mononuclear cells pause for about 2 weeks in the lymphoid organs, but then move on to all recipient tissues.[7] Presumably similar pathways of dissemination are also taken by donor bone marrow–derived cells after whole organ transplantation in humans (Fig 10–2). In several rat strain combinations (for example, Brown Norway [BN] to Lewis [LEW]), recipients treated in this manner survive indefinitely without further treatment and retain their graft and systemic chimerism.

The permanent survival of engrafted livers without any immunosuppression in some rat strain combinations (of which BN → LEW has been most completely studied[29]), and in most mouse strains has been poorly correlated with histocompatibility.[30] These "nonrejecting" liver recipients in either species and those whose liver acceptance is induced with immunosuppression can receive normally rejected skin, kidney, or heart from the original donor strain but from no other (thus, donor-specific nonreactivity).

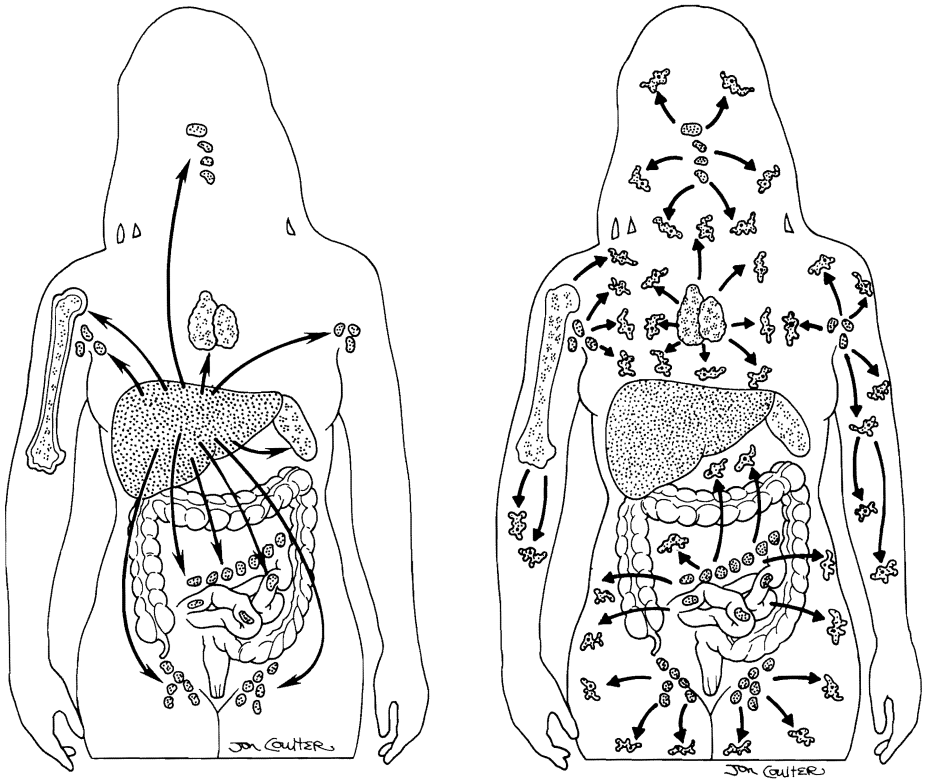

Figure 10–2. The dissemination of bone marrow–derived cells from the graft to the central lymphoid organs (left) and then after a brief pause ubiquitously to other recipient's tissues (right). The events are similar to those after successful bone marrow transplantation.

Hepatic Tolerogenicity

Although we believe that cell migration and repopulation is the central mechanism of acceptance of all whole organ grafts,[1–7] there are quantitative and qualitative differences between organs in the density of the potentially migratory dendritic cells, macrophages, and lymphoid population. The heavy endowment of the liver with cells of multilineage phenotypes (especially Kupffer cells) is a particularly striking feature that invites further speculation about the role of these cells in the well-known tolerogenicity of this organ. Here, tolerance is defined as the specific absence of an immune response to an antigen. This is usually an active process that is related either to deletion or anergy of antigen-reactive lymphocytes, or to a suppressor mechanism. The immunologic advantage of the liver relative to other organs includes a greater ease of inducing the acceptance of hepatic allografts or xenografts after a limited course of immunosuppression[28, 29, 31, 32] or in some swine[33–35] or rat[36, 37] and virtually all mouse[30] strain combinations with no treatment at all.

In addition, the transplanted liver is relatively resistant to the performed alloantigraft antibodies that cause hyperacute rejection of the kidney and heart.[38–41] Another quality is its unusual ability to induce a state of immunologic unresponsiveness to other tissues and organs transplanted concomitantly or subsequently from the donor or donor strain[36, 39, 42] and even shield these organs from the hyperacute rejection caused by performed allospecific (against antigens from another member of the same species)[41] or xenospecific (against antigens from different species)[43] antidonor antibodies. In all of these circumstances, the liver can quickly transform the recipient environment to one more favorable for all donor tissues including itself. All of these qualities of the liver are subject to analysis in the mouse.[30]

The foregoing observations have been attributed to "hepatic tolerogenicity," incorrectly we believe, because the term implies that the hepatocytes are responsible. We have proposed that the crucial variable distinguishing the tolerogenicity of one organ graft from another is its leukocyte, not its parenchymal component.[1–7, 30] This is a reversal of the immunogenic role described classically for the bone marrow–derived cells.[44–57] Thus, because of its dense constituency of these migratory leukocytes, the liver is high on the favorable tolerogenic list, with the lung and intestine a considerable distance behind, and the kidney and heart bringing up the rear. Experimental studies showing less striking tolerogenicity of the lymphoreticular-rich spleen,[58–60] intestine,[10] and lung[61, 62] are compatible with this generalization.

Tolerogenicity of Leukocyte-Poor Organs

The same kind of traffic but in the context of alloactivation (T-cell activation in response to alloantigen stimulation) and rejection rather than tolerization was well worked out earlier with the so-called lymphoid-poor organs such as the kidney, exemplified by the classical study in 1981 by Nemlander et al of untreated rat kidney recipients.[63] If Nemlander et al had administered one or two doses of cyclosporine in his experiments (which were with an "easy" strain combination) and had followed up his animals further, we believe that he would have uncovered

the events of cell migration and long-term chimerism that awaited another dozen years for exposure with the liver.

Such studies in untreated animals have shown that the alloreaction (the mutual interaction of host and donor immune cells) starts in two general sites, peripherally in the graft and centrally in the recipient lymphoid tissues, as was emphasized by Nemlander et al. Larsen et al[64] found that donor dendritic cells from heterotopic cardiac allografts were released into the circulation, where they eventually homed into the T-cell areas of the recipient spleen. In the spleen, the donor cells initiate proliferation of recipient cells, and vice versa.[63–67] This reaction might be thought of as an *in vivo* mixed lymphocyte response (MLR) (proliferation of alloreactive T cells in response to allogeneic accessory cells) in the course of central allosensitization. Failure to appreciate that there was a potential alternative outcome of tolerization was the missing link in understanding why whole organ allografts could be accepted.

Allosensitization (or tolerization) also occurs within the graft. Forbes et al[66] showed that clustering of recipient lymphocytes occurs around donor dendritic cells in the interstitium of cardiac grafts, within a few days after transplantation. The recipient lymphoid cells undergo blastogenesis and proliferate within these clusters. We have described analogous events in rejecting rat livers.[65] In human recipients of kidney grafts[68, 69] receiving cyclosporine-prednisone immunosuppression, Hayry and Willebrand noted what seemed to be a bidirectional MLR in needle aspiration biopsies. When studied with the *Staphylococcus aureus* assay and alloantibodies to nonshared donor and recipient allelic specificities, the majority of the isolated blast cells in some of their human cases were derived from the donor, whereas in others the cells were both of donor and recipient type, "resembling a bidirectional mixed lymphocyte reaction *in vitro*."[68]

With the various extrahepatic organs, the central or peripheral events seem to be only quantitatively different from those following transplantation of the more tolerogenic liver. With the smaller number of passenger leukocytes, and perhaps a lower representation of subpopulations of certain lineages, there is a greater tendency to allosensitization and less to tolerogenicity. Nevertheless, Corry et al[70] and Russell et al[71] showed that tolerance without drug therapy could be induced by heart and kidney transplantation in mice between weakly MHC-incompatible strains, reflected later by permanent acceptance of donor strain skin grafts (but not third party).

FUNCTIONAL CONSEQUENCES OF MICROCHIMERISM

Questions as to whether low-level chimerism in long-surviving patients and experimental animals is an irrelevant histopathologic curiosity seem naive in view of Russell's elegant formal proof of the association of chimerism with acquired tolerance as well as with runt disease (graft-versus-host disease [GVHD]).[72] However, the low numbers (microchimerism) of the chimeric donor cells in the recipient tissues requires explanation. The term microchimerism was introduced into the literature in 1974 by Liegeois et al[73] to describe a small proportion of chimeric cells in the recipient spleen of mice as long as 5 months after bone marrow transplantation. The cumulative effect of these microchimeric cells is substantial, especially after liver transplantation when they are most easily shown.

How Do Donor Cells Perpetuate?

A key question concerns how the chimeric cells survive and perpetuate within tissues of organ allograft recipients long after transplantation. In recent laboratory experiments, we have examined whether progeny of the chimeric cells present within various tissues of unmodified liver allograft recipients can be generated *in vitro* under appropriate culture conditions.[74–76] In freshly prepared cell suspensions from recipient's bone marrow, spleen, or thymus 14 or 150 days posttransplant, only very low levels of chimeric cells (donor MHC class I^+) can be shown. However, following stimulation of the cells with granulocyte-macrophage colony-stimulating factor for 10 days we have succeeded in propagating not only recipient cells but also large numbers of myeloid cells bearing donor phenotype (MHC class I^+ or class II^+). These observations suggest that progenitors have migrated from the allograft and seeded into different host tissues, and provide an explanation for the perpetuation of stable donor cell chimerism for decades after human organ transplantation.

Metabolic Effects

A small population of chimeric cells has been shown to affect total body metabolism in patients treated with liver transplantation for the enzyme deficiencies of type IV glycogen storage disease and Gaucher's disease. These disorders in which the consequences of the missing enzymes are widespread storage of amylopectin and glucocerebroside, respectively,[3] were previously thought to be treatable only by bone marrow transplantation. Yet, 2 to 8 years after liver replacement, there was a dramatic resorption of both kinds of storage material from host tissues[3] for type IV glycogen storage disease. As an explanation for the metabolic amelioration, chimeric donor cells were found ubiquitously in recipient tissues including heart, lymph nodes, bone marrow, intestine, and skin. There apparently had been a co-culture effect of a small number of enzyme-replete chimeric donor cells on the contiguous overwhelming numbers of enzyme-deficient recipient cells leading to resolution of abnormal amylopectin deposits.

The Immunologic Interface

The potential effect of cell-to-cell interaction and its role in immunologic rather than metabolic processes cannot be so easily measured. In an earlier section (How Do Donor Cells Perpetuate), the demonstration of dendritic cell precursors was described in mouse livers, blood, and bone marrow.[74–76] Under most circumstances, the progeny of these precursor cells would be expected to reach terminal differentiation unless there is a need for their continued proliferation, or else maintenance of a pool of precursor population. We have suggested that the survival and continued renewal of these cells depends on chronic mutual stimulation of the donor and recipient cells,[5, 7] highlighting not only the commonality between the cellular processes involved in tolerization and immunity,[77] but the changes that occur in these cells.

Changed Host and Graft Interactions

There are indirect ways to show that the coexisting immunocyte populations in successful cases (Fig 10–1) come to regard each other in a revised light. The evidence on one hand is the fading of the threat of clinical rejection concomitant

with development of donor-specific nonreactivity in spite of lightened treatment (or in some animal models with no treatment at all) and on the other, the waning specter of GVHD. In a human organ recipient, both cell populations are subject to the treatment conditions because both have the same protective umbrella of immunosuppression during the process of change.

The appreciation of the interactions between the donor and recipient cells and the overwhelming need to preserve this relationship by refraining from cytoablating one side or the other was the crucial advance that permitted the successful engraftment of leukocyte-rich organs such as the liver or intestine, both together, or all of the intraabdominal organs (multivisceral transplantation).[78] Once the cardinal principle was understood that low-level mixed allogeneic chimerism invariably was found after the successful transplantation of any whole organ, the reason seemed obvious why GVHD was not common in liver, intestinal, or multivisceral recipients. Mixed chimerism was being produced in the same way as had been documented in the classical GVHD free mouse bone marrow mixed chimerism models of Slavin et al[79] and Ildstad and Sachs,[80] although the number of donor cells was much lower.

The Critical Dendritic Cell

Generation of an immune response leading under normal circumstances to graft destruction and/or GVHD requires effective antigen presentation and recognition in its initial phase followed by a second costimulatory signal and the response of the naive T cells to the combined signal.[81] Both of these signals are normally delivered to T cells by professional antigen presenting cells (APCs). Although any cell that expresses an appropriate MHC class II molecule can present antigen to sensitized T cells, it is only dendritic cells that are thought to be most efficient in presenting antigens to naive T cells.[46–48] Dendritic cells, which are of bone marrow origin ($CD45^+$), are ubiquitously distributed throughout the body. They have an irregular shape, small round phase-dense mitochondria and sparse, rough endoplasmic reticulum. They are nonphagocytic in culture, and express very low levels of Fc and complement receptors. However, they constitutively express high levels of MHC class I and II antigens and can upregulate B7/BB1 molecule. Because the cell surface expression of these molecules can be modified, they play a major role in determining how antigen signals are heeded by T cells.[49] Furthermore, by morphological criteria, the most abundant chimeric cells in whole organ transplant recipients were dendritic cells.[1–7] Thus, the dendritic leukocyte is the prime candidate for mediator of tolerance induction even though other lineages (eg, B or even T cells) may also be essential for a successful outcome.

The "Blindfolding" of Tissue Matching

In both the directions of host-versus-graft (HVG, rejection) and GVH, cellular interactions resulting in "mutual natural immunosuppression" are envisioned as occurring on a sliding scale with each further level of histoincompatibility. With effective immunosuppression, it has been increasingly possible to orchestrate the outcome of donor and recipient cell engagement after transplantation in a manner that would allow the tolerogenic changes to occur and a compromise to be

reached between the coexisting immunocytes. The anticipated influence of histoincompatibility on both rejection and the severity of GVHD are then expected to dwindle. We have postulated[1, 4–6] that this explains the poor correlation of HLA matching with outcome after the cadaveric transplantation of whole organs including the kidney.[82–84] With liver transplantation, two large centers actually have reported an inverse relation between HLA matching and the clinical outcome.[85, 86] Furthermore, it has been proposed that HLA-DR matching increases the risk of cytomegalovirus (CMV) hepatitis in both primary and secondary CMV infections.[87]

RELATION OF CELL MIGRATION TO TOLERANCE

The inadequacy of thymic clonal deletion to explain acquired transplantation tolerance has been emphasized in recent reviews.[88] Although a discussion of the meaning of tolerance is beyond our intention, it should be noted that all of the mechanisms put forth to explain clonal "silencing" including peripheral (nonthymic) clonal deletion and anergy could mesh with the discovery of the enduring graft-host intimacy that is inherent with chimerism. The production of suppressor and/or veto cells (cells capable of inactivating or suppressing the activity of cytotoxic T lymphocytes) could be epiphenomenologic consequences. The evidence of long-term vitality and turnover of donor leukocytes in recipient tissues is particularly supportive of the opinions of Bandeira et al,[77] Coutinho[89] and Cohen[90] who have defined acquired tolerance as a high (not anergic) level of sustained immune activity in immunologic networks. These networks presumably interact in a more complex way than the idiotype systems originally postulated by Jerne.[91]

Apart from explaining why the events of convalescence follow the same pattern of vigorous immune resistance and then collapse after all transplantations, no matter what the organ,[28, 31, 92] the cell migration-chimerism concept also shows how donor-specific nonreactivity can be achieved with a common mechanism, irrespective of the site of action of the immunosuppressive drugs or, in some experimental models, without the need for drugs. It has been proposed from observations in drug-free models of tolerance induction that occupancy of T-cell receptor (TCR) leads to production of negative regulators of interleukin-2 (IL-2) production (anergy proteins).[81, 93] According to this hypothesis, during the course of a normal T-cell response (to alloantigens) these negative regulators of IL-2 production have an inconsequential effect because they are diluted out by vigorous cell replication driven by IL-2. However, these negative regulators would accumulate with consequent anergy if clonal expansion were prevented at any level (Fig 10–3): for instance, by the absence of a co-stimulatory signal in drug-free models.[81]

The same effect could be induced iatrogenically by pharmacological interdiction of IL-2 gene transcription (cyclosporine and FK506) or administration of a DNA synthesis inhibitor (azathioprine, cyclophosphamide, and numerous others). The use of non–T-cell–depleting monoclonal antibodies, such as those directed against the cell surface CD4 antigen or monoclonal antibodies against adhesion molecules including intracellular adhesion molecule-1 and lymphocyte function-associated antigen-1[94] can also be envisaged.

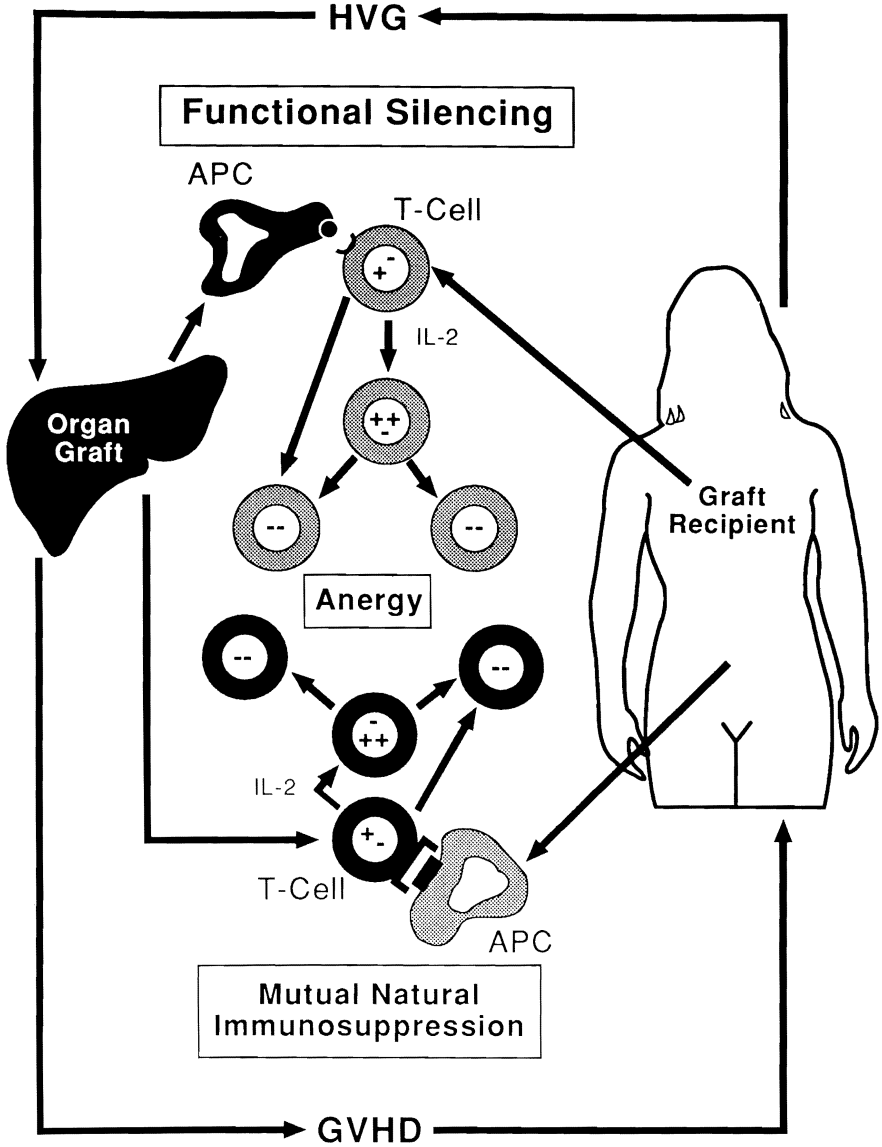

Figure 10–3. Model of dendritic cell (APC)-Th_1-cell interaction, showing the production within the nucleus of positive (+) and of negative (−) regulators (putative anergy proteins) of IL-2 gene transcription. It has been suggested[81] that TcR occupancy leads to the production, through an active metabolic process, of negative regulators (anergy proteins) that accumulate at later times and repress IL-2 gene transcription, possibly by antagonizing the effects of positive gene transcription factors. In the absence of persistent costimulatory signals (or under the umbrella of immunosuppressive drugs), cell division does not proceed and negative nuclear regulators accumulate, resulting in T-cell anergy. In addition to the action of immunosuppressive agents, chronic antigen stimulation is also envisaged as promoting anergy. In some instances, tolerance can be broken, eg, by administration of exogenous IL-2. HVG, host-versus-graft response (allograft rejection); GVHD, graft-versus-host disease. (Reprinted with permission.[94])

Whatever the mechanism, the reciprocal educational process of donor and recipient leukocytes and its perpetuation resembles in either the direction of HVG or GVHD (Fig 10–3) the "infectious" transplantation tolerance of Waldmann and Cobbold that can be passed on to naive lymphocytes and be self-sustaining in some circumstances.[95] It is postulated that in fully successful cases, the mini-immune system of the graft is incorporated into the existing recipient immunologic network,[5,7] compatible with the hypothesis of Coutinho.[89]

UNSTABLE MIXED CHIMERISM

Cell migration conceptually reunites bone marrow transplantation with transplantation of whole organs. Far from involving different mechanisms for successful engraftment, we believe that these two seemingly disparate clinical disciplines merely reflect contrasting treatment dogmas. For bone marrow transplantation, the conventional treatment strategy of recipient cytoablation eliminates mutual immunocyte engagement and thus necessitates heavy reliance on HLA matching to prevent GVHD in the unbalanced system. The treatment for solid organ transplantation encourages, or at least allows, these consequences of mutual cell engagement, thereby liberating the patient from the restrictions of HLA matching and an overwhelming threat of GVHD.

Failure of the chimeric donor and recipient immunocytes to reach an immunologic "truce" (Fig 10–1) leads to rejection of the transplanted whole organ on one hand and to GVHD on the other, or sometimes to both simultaneously. This has been particularly well studied after intestinal transplantation between certain rat strain combinations involving the Brown Norway (BN) strain.[24–26] In ACI, PVG, or LEW rats treated daily with variable doses of FK506 for the first 14 days after transplantation and weekly thereafter, successful intestinal transplantation from fully allogeneic BN donors was not complicated by either rejection or by fatal GVHD.[26] In contrast, when BN was the recipient, rejection of the ACI intestine was difficult to control, and when LEW or PVG intestine was transplanted, GVHD invariably developed once the daily treatment was stopped. Yet, the two-way lymphocyte traffic from graft to host lymphoid organs and vice versa was similar with either strain direction.[24,25] Saat et al[97] have described analogous findings of GVHD predisposition and rejection under cyclosporine after WAG to BN rat intestinal transplantation but not BN to WAG.

Further experiments in our laboratory have not clarified why the BN rat is an "easy" donor and a "difficult" recipient. At a clinical level, the unresolved practical question is how to identify and avoid bad donor-recipient combinations analogous to LEW, ACI, or PVG to BN rats, particularly when immunologically active organs such as the liver and intestine are engrafted.

With human liver transplantation, preoccupation with rejection long obscured the fact that the graft-versus-host reaction, which is an incipient process and in our opinion a requisite for sustained engraftment in every case, can evolve to serious or fatal syndromes[98–105] in the early postoperative period. Clinically recognizable GVHD is observed in our liver program in approximately 5% to 10% of cases, usually manifesting as trivial dermatitis.[6] In the past, this usually was

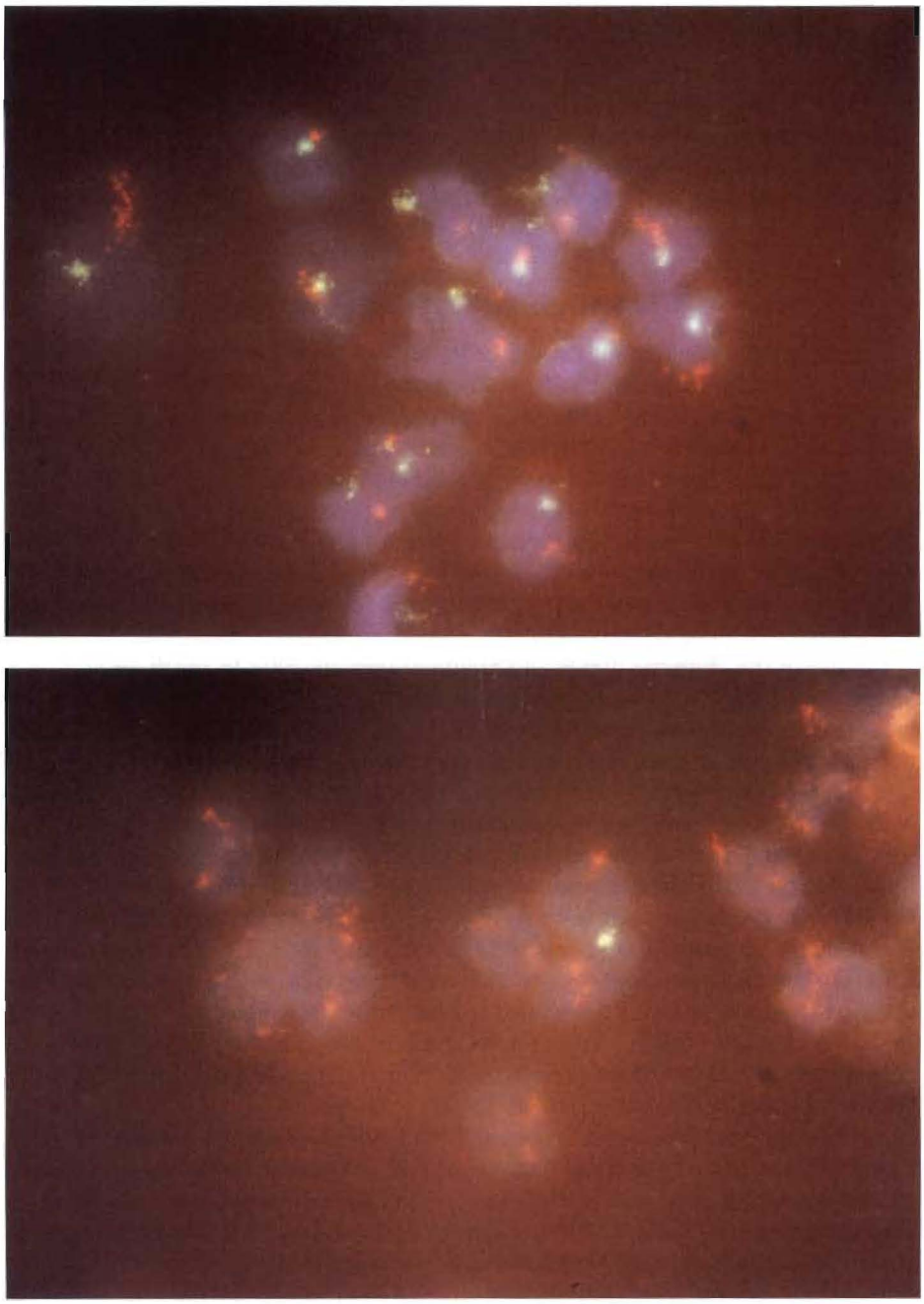

Figure 10–4. Fluorescent *in situ* hybridization for X [red] and Y [yellow] chromosomes in cytocentrifuge preparations of peripheral blood cells obtained from (A) normal male [positive control] and (B) a female recipient of male kidney and bone marrow 235 days after transplantation. Biotinylated Y-specific and digoxigenin-conjugated X-specific probes were used, which were visualized with fluorescein isothiocyanate-conjugated avidin and tetramethylrhodamine isothiocyanate-conjugated antidigoxigenin, respectively. Nuclei were counterstained with 4,6-diamino-2-phenylindone.

attributed to a self-limiting drug reaction or an allergic manifestation, but with techniques of donor cell identification, the true diagnosis can be readily made.

Although most of these patients can be treated successfully with increased immunosuppression (particularly prednisone) or occasionally by decreasing treatment, liver recipients with extensive skin involvement, gastrointestinal symptoms, and depression of the formed blood elements have a high mortality.[103] The chimerism that has been documented in such patients has differed only by being more extensive than that observed in patients who have a benign convalescence.

CLINICAL TRIALS OF BONE MARROW AUGMENTATION

Summary of Experience

The overview developed in this chapter is that the migration from organ allografts of donor leukocytes and their ubiquitous persistence in recipient tissues is the seminal explanation for allograft acceptance, and the first stage in the development of donor specific nonreactivity (tolerance). In a direct extension of this concept, 16 unconditioned patients were infused with donor bone marrow cells on the day of cadaveric renal (n = 9), liver (n = 6), and heart (n = 1) transplantation. The 16 patients, who were also treated with standard FK506-prednisone immunosuppression, included three diabetics who also received pancreatic islets and a liver recipient with a positive lymphocytotoxic crossmatch.

All 16 patients have good whole organ function 3.5 to 14 months later (mean serum bilirubin of liver recipients, 0.6 mg/dL; mean serum creatinine of kidney recipients, 1.5 to 1.7 mg/dL; good cardiac function in heart recipient); two of the three diabetics have detectable C-peptide activity. Using flow cytometry and qualitative or quantitative PCR techniques to detect donor HLA alleles and with study of Y chromosomes in four female recipients of male organs, persistent multilineage leukocyte chimerism was found in the blood of all recipients by Southern analysis of the Y chromosome–specific SRY gene (not shown), and as otherwise shown in Fig 10–4 and Table 10–1, except one patient whose complete HLA match and same-sex donor precluded study (Table 10–1). Rejection in 9 (56%) of the 16 patients and transient GVHD in 2 (12.5%) was diagnosed and successfully treated (Table 10–1). Sustained donor specific hyporeactivity as early as 40 or 50 days postoperatively was demonstrable with *in vitro* tests in the majority of recipients (Table 10–1).

The Old Paradigm and Its Fit With The New One

Tolerance induction with donor leukocytes is the most ancient therapeutic strategy of transplantation. It was introduced with the injection of spleen cells in fetal or perinatal mice by Billingham et al,[106] and extended by Main and Prehn to the production of radiation chimeras with bone marrow cells.[107] Hundreds of subsequent tolerance induction experiments and eventually clinical bone marrow transplantation depended on a similar natural or imposed defenseless recipient state. The first exception, reported by Mariani et al[108] was induction of tolerance with splenocytes to the sex-linked (Eichwald-Silmser) histocompatibility differ-

Table 10–1. In Vitro *Immune Status and the Detection of Donor Cells in the Combined Bone Marrow and Whole Organ Recipients*

Case No.	Allografts	HVG* (POD)	GVH† (POD)	MLR Response‡ % (POD)	Detection of Donor Cells		
					POD§	FACS (%)	PCR (cPCR)
1	Liver + islets	15,86	None	34 (85)	82	1.7	+
2	Liver	7,22	None	36 (108)	108	1.8	+
3	Liver	None	54	6 (120)	146‖	1.9	+ (1.0%)
4	Liver	None	21,74	87 (145)	167	<0.5	+
5	Liver	33	None	Low responder¶	175	5.0	+
6	Liver	24	None	15 (265)	265	NF#	+
7	Kidney	None	None	59 (48)	48	1.7	+
8	Kidney	None	None	5 (72)**	19	NF#	NF††
9	Kidney	None	None	70 (113)	120	1.9	+
10	Kidney + islets	41,66	None	5 (166)	133	1.4	+
11	Kidney + islets	16	None	50 (168)	171	3.0	+
12	Kidney	None	None	22 (225)	225	0.6	NF††
13	Kidney	16	None	18 (177)	232	NF#	+
14	Kidney	16	None	26 (68)**	315	<0.5	+
15	Kidney	None	None	NF‡‡	367	NF#	+ (0.5%)
16	Heart	12–40§§	None	130 (65)	68	1.5	+

*Host-versus-graft reaction [rejection].
†Graft-versus-host reaction.
‡Percentage of donor-specific MLR responses as compared to third party on the last sample tested.
§Last postoperative day (POD) tested.
‖The samples for PCR and cPCR were obtained on POD 128.
¶Cells did not respond to any stimulation *in vitro* for up to POD 134.
#Not feasible; cross-reactive antibodies.
**No change in donor-specific responses before and after transplant.
††Not feasible; no MHC class II mismatch.
‡‡Not feasible; no adequate donor spleen cells.
§§Single rejection episode, gradually resolving from grade 3A (multi focal moderate acute cellular rejection [ACR]) on POD 12 to grade 1B (diffuse mild ACR) on POD 40. All subsequent biopsies were negative.

ence in unconditioned adult syngeneic mice, and then in a limited number of allogeneic mouse strains by Brent and Gowland[109] and other investigators.

The discovery by Billingham and Brent[110] and Trentin[111] that GVHD was the penalty for preexisting or iatrogenic general immunologic nonreactivity forestalled for many years the clinical use of major histocompatibility complex (MHC)-mismatched bone marrow or other mature immunocytes to facilitate whole organ graft acceptance.[112, 113] The alternative of using killed donor cells was relatively ineffective as first reported in a clinical renal transplantation trial by Kelly et al[114] in 1967.

In contrast, donor blood seemed to be tolerogenic if it was transfused fresh as in the canine experiments of Halasz et al.[115] Explanations for the experimental effect, and later the seeming benefit in clinical kidney transplantation of donor-specific[116] or third-party transfusions,[117] were hampered by uncertainty about the timing of optimal treatment, the inability to quantitate cell dose, and variable policies of conserving (or deliberately eliminating) the leukocyte constituency of the blood. In addition, few investigators looked for persistent chimerism,

perhaps because of the evidence that allogeneic leukocytes had a transient life span.[118] However, based on circumstantial evidence, van Twuyver et al[119] postulated that the blood transfusion benefit was caused by persistent microchimerism, the term that had been introduced to the literature by Liegeois et al[73] in 1974.

Bone marrow also has been used as the leukocyte source with the explicit objective of establishing long-term chimerism. The concept that engrafted allogeneic (and xenogeneic) immune cells carried a low risk of GVHD if they coexisted with host cells (mixed chimerism) was based on observations in rat recipients conditioned preoperatively with total lymphoid irradiation (TLI) by Slavin et al[79] and in mice prepared for the marrow with total body irradiation in a sophisticated model developed by Ildstad and Sachs.[80] Clinical trials of bone marrow plus preconditioning with TLI were performed in kidney,[120] heart,[121] and liver[122] recipients. In spite of the expectation of GVHD freedom, one of two human liver–bone marrow recipients conditioned with 550-R TLI at the University of Pittsburgh developed severe GVHD and eventually died of multiple complications.[122] Although TLI is still sporadically used, its use with marrow augmentation has fallen into disfavor.

An alternative strategy that also results in preconditioning of the recipient before infusion of adjuvant donor bone marrow has been called the "Monaco model" following its orderly development in mouse,[123] dog,[124] and subhuman primate[125] models before application in a human case of cadaver renal transplantation in 1973.[126] In 1987–1989, Barber et al[127] used a similar regimen but with more effective drugs in 57 patients whose cadaver kidney transplants were required to be functioning at the end of 21 postoperative days for entry into the trial.

Immunosuppression during the provisional 3 weeks was with polyclonal antilymphocyte globulin and prednisone to which azathioprine and cyclosporine were added by day 6. Graft survival in the test series was significantly better than in contemporaneous controls, and there was other clinical evidence of benefit including a reduced need for immunosuppression. Chimerism in blood samples was detected by PCR long after transplantation in an unstipulated number of recipients,[127] an observation confounded by a significant incidence as well in the nonmarrow controls (A.G. Diethelm and W.H. Barber, personal communication, January 1993).

The latter unexpected finding subsequently explained the observation that is the central theme of this review—the detection of low-level leukocyte chimerism in the tissues of all long-surviving organ recipients and in the blood of many, most obviously in liver transplant recipients.[1, 2, 4, 6] We have postulated that the multiple immunobiological changes seen in organ recipients (eg, altered cytokine profiles, suppressor and veto cells, enhancing antibodies) are derived from the sustained two-way interactions between the coexisting donor and recipient immunocyte populations.[1, 5–7, 30, 75, 128]

Because the chimeric leukocytes dispersed from the allograft are of bone marrow origin, a corollary expectation was that acceptance of organs less tolerogenic than the liver such as the heart and kidney (or even the liver itself) would be facilitated by augmenting this natural process with the infusion of

unaltered donor bone marrow perioperatively.[1,4–6] The results thus far are consistent with this hypothesis. The ease with which detectable chimerism could be reliably produced and sustained without "making space" for the infused bone marrow by host cytoreduction and with no deviation from standard immunosuppressive management was surprising. All 15 testable recipients of kidneys, livers, and a heart have good transplant function and blood chimerism estimated to be 1,000-fold greater than in our earlier studies of naturally occurring chimerism. Care was taken not to overestimate the presence or the magnitude of the chimerism by the use of corroborating cytological and molecular techniques, blind reading of samples, and a new PCR method to quantitate donor DNA.

Almost invariably, an initial surge of donor cells diminished to a nadir at approximately 1 month as we[11] and other investigators have described after organ transplantation without marrow.[129,130] However, instead of disappearing, these cells secondarily increased to a stable equilibrium level (Figure 10–4) as was particularly well documented with a competitive PCR technique.[131] In spite of the sustained chimerism, the diagnosis of rejection in nine patients (56%) and minor GVHD in two (12.5%) underscores earlier warnings that chimerism is not synonymous with tolerance, but only a necessary condition for its achievement.[1,5,6,132] The pitfall of extrapolating the association of chimerism and rapid tolerance induction commonly seen in rodent experiments to the management of human recipients of HLA-mismatched organs could not be more clearly illustrated than with the clinical experience herein reported. The pace of drug weaning with the eventual goal of drug discontinuation will have to be determined in each of our patients individually, with guidance from serial tests of *in vitro* immune reactivity. Although the majority of our 16 patients have evidence of evolving donor-specific nonreactivity by *in vitro* testing, no one has yet had their therapy stopped.

Thus, what we have shown so far is the ability to systematically produce persistent and readily detectable chimerism with the expectation that it will confer an advantage. Whether the chimerism will evolve to a state no longer requiring drugs, and with what frequency, remains to be seen. Beyond its adjuvant role for whole organ transplantation, it will be important to determine if MHC-mismatched bone marrow engrafted under this management regimen can be used without an accompanying organ in patients whose disease can be corrected with a mixed chimeric state. The potential list of such indications is exhaustive,[133] exemplified by the lysosomal enzyme deficiencies.[3] In addition, the new insight obtained about appropriate timing should be applicable to donor-specific blood transfusion with which the white blood cell effect presumably is comparable to that of a small dose of bone marrow.

Whatever the source of leukocytes, studies of the interactions between the coexisting donor and recipient cell populations, the key lineages governing the outcome, and the cellular and molecular mechanisms involved may show ways to expand the perioperative window of opportunity shown by our observations. However, pretransplant cell infusion carries a known risk of sensitization,[116] and delayed administration of donor immunocytes can cause rejection.[124] In addition, it was recently shown in rat experiments that the engraftment of bone marrow, followed by second-stage transplantation of a liver allograft with its high content

of naive donor leukocytes, frequently resulted in GVHD resembling the parent to F_1 hybrid defenseless host syndrome.[7]

SUMMARY

Evidence has been summarized that the migration from organ allografts of donor leukocytes of bone marrow origin and their ubiquitous persistence in recipient tissues is the previous unrecognized seminal explanation for allograft acceptance, and the first stage in the development of donor-specific nonreactivity (tolerance). The unusual immunologic privilege of the liver (called hepatic tolerogenicity) has been explained by its heavy content of leukocytes and its diverse lineage profile that includes precursor dendritic cells. In a direct extension of this new and generically applicable paradigm of transplantation immunology, unconditioned patients have been infused with donor bone marrow cells on the day of cadaveric liver, renal, and heart transplantation and treated otherwise with standard FK506-prednisone immunosuppression. All of the first 16 patients on this protocol have good whole organ function 2.5 to 13 months later. Using flow cytometry and qualitative or quantitative PCR techniques to detect donor HLA alleles, and with study of Y chromosomes in female recipients of male organs, persistent multilineage leukocyte chimerism was regularly found in the blood of these recipients. Rejection was diagnosed and successfully treated in 9 (56%) of these first 16 patients and transient GVHD in 2 (12.5%). Sustained donor-specific hyporeactivity as early as 40 days postoperatively was demonstrable with *in vitro* tests in the majority of these recipients.

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