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IMMUNE STATUS OF RECIPIENTS FOLLOWING BONE MARROW-AUGMENTED SOLID ORGAN TRANSPLANTATION[1,2]

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It has been postulated that the resident "passenger" leukocytes of hematolymphoid origin that migrate from whole organ grafts and subsequently establish systemic chimerism are essential for graft acceptance and the induction of donor-specific nonreactivity. This phenomenon was augmented by infusing 3×10^8 unmodified donor bone-marrow cells into 40 patients at the time of organ transplantation. Fifteen of the first 18 analyzable patients had sequential immunological evaluation over postoperative intervals of 5 to 17 months, (which included 7 kidney (two with islets), 7 liver (one with islets), and one heart recipient). The evolution of changes was compared with that in 16 kidney and liver nonmarrow controls followed for 4 to 5 months. The generic immune reactivity of peripheral blood mononuclear cells (PBMC) was determined by their proliferative responses to mitogens (PHA, ConA). Alloreactivity was measured by the recipient mixed lymphocyte reaction (MLR) to donor and HLA-mismatched third-party panel cells. Based on all 3 tests, the recipients were classified as donor-specific hyporeactive, intermediate, and responsive; patients who were globally suppressed made up a fourth category. Eight (53%) of the 15 marrow-treated recipients exhibited progressive modulation of donor-specific reactivity (3 hyporeactive and 5 intermediate) while 7 remained antidonor-responsive. In the nonmarrow controls, 2 (12.5%) of the 16 patients showed donor-specific hyporeactivity, 10 (62.5%) were reactive, and 4 (25%) studied during a CMV infection had global suppression of responsiveness to all stimuli.

We have proposed that the donor bone marrow–derived cells that migrate from transplanted organs and persist in the tissues of long-surviving recipients are essential for graft acceptance and the evolution of donor-specific nonreactivity (*1, 2*). This spontaneous chimerism has been augmented in 40 whole organ recipients by the perioperative infusion of 3×10^8/kg unaltered donor bone marrow cells (*3*). We report here the sequential immunological evaluation of 15 of the first 18 analyzable recipients. In addition, 16 nonmarrow control recipients of livers and kidneys were studied. To determine the development of donor specific hyporeactivity in both cohorts, in vitro tests were performed monthly with the recipient's peripheral blood mononuclear calls (PBMC),* and the results were correlated with the clinical findings.

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* Abbreviations: IL-2, interleukin 2; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Case material. Bone marrow group: All 40 patients given bone marrow augmentation are well. The present study was of 15 of the first 18, all adults, whose cases have been reported elsewhere (*3*), including details of marrow preparation. The dose of 3×10^8/cell/kg unaltered bone marrow was given a few hours after organ revascularization (*3*). Three of the first 18 recipients could not be evaluated, either because they had good MHC matches and consequent donor-specific nonreactivity to begin with (n=2) or because donor cells were not available (n=1). No recipient conditioning was carried out, and postoperative immunosuppression with FK506 and prednisone did not differ from that used routinely (*3*). The 15 evaluated patients were recipients of 7 kidneys (2 with pancreatic islets), 7 livers (one with islets), and one heart. Using HLA alleles of chromosomes 6 or the Y chromosome (after male-to-female transplants) as markers, all had readily detectable chimerism with FACS, cytospin, or polymerase chain reaction (PCR) almost invariably with multiple technologies. This was estimated to be at least 1000 times greater than the microchimerism that occurs spontaneously (*3*).

Nonmarrow controls: These 16 adults (5 kidney and 11 liver recipients) were from a consecutive series of 19 patients for whom donor bone marrow could not be obtained. The 3 exclusions from the analysis were for the same reasons of preexisting nonreactivity or unavailability of samples as in the marrow group. Immunosuppression and other details of treatment were not different than in the study group. Blood microchimerism was present in 56% of the patients with PCR, but never as in the bone marrow cohort to the level detectable with FACS.

In vitro immune monitoring. Mitogen response assays: PBMC were isolated from the recipient's heparinized blood by Ficoll-Hypaque density centrifugation and resuspended at 10^6 cells/ml in tissue culture medium (RPMI 1640) supplemented with 5% pooled normal human serum (*4*). They were then incubated (10^5 cells/well) for 72 hr at 37°C in the presence of medium alone (background), concanavalin A (Con A) (4 μg/ml), or phytohemagglutinin (PHA) (10 μg/ml) in a final volume of 200 μl. For the last 18 hr of incubation, each well was pulsed with 1 μCi of ^{3}H-thymidine (ICN Radiochemicals, Cosa Mesa, CA), and the uptake of radioactivity was measured by liquid scintillation counting. The results are expressed as mean ± SD of counts per minute.

Mixed lymphocyte response (MLR): To monitor the development of donor-specific hyporeactivity, MLR assay was used with freshly isolated recipient cells as responders and the donor splenocytes and control third-party HLA-unrelated panel cells as stimulators (*5*). To determine the experimental variability and to assess the stimulatory capacity of the donor and other panel cells, each assay included PBMC obtained from normal individuals as control responders. Recipients' PBMC were also used as stimulators for self and for normal PBMC responders. The MLR proliferative assay was set up for 6 days with responders (10^5 cells/well) and γ-irradiated (2000 rads) stimulators (10^5 cells/well) in tissue culture medium supplemented with 5% human serum in a final volume of 200 μl. Proliferation was assessed as described above for the mitogen assays.

Recipients' donor-specific MLR responses at various times posttransplantation were compared with the recipients' pretransplant (posttransplant:pretransplant donor MLR) as well as with the responses of control third-party (donor MLR:third-party MLR [D/TP]) cells. Donor-specific hyporeactivity (category I) was defined as at least a 70% decrease in posttransplant vs. pretransplant donor-specific MLR responses, while maintaining reactivity to both control third-party stimulator cells (D/TP ratio <40%) and to mitogens (>50% of pretransplant responses). Donor-specific intermediate reactivity (category II) was designated when there was a 40–70% inhibition of antidonor reactivity with retention of third-party responsiveness whereas reactive (category III) meant that there was minimal or no decline in donor-specific nonreactivity. Suppression (category IV) connoted a nonspecific diminished proliferative response to mitogens as well as to alloantigens. Representative examples of each of these 4 categories as measured at 3 postoperative months are shown in Table 1.

Limiting dilution analysis (LDA) of donor-specific helper T lymphocytes (HTL): The frequency of donor-specific HTL was determined according to a previously described method (*7*). Briefly, various dilutions of irradiated recipients' PBMC (250R) were added as responders to round-bottomed microtiter wells that contained 5×10^4 irradiated donor cells (2000R) in RPMI supplemented with 5% pooled human serum. After 48 hr of incubation at 37°C, an interleukin 2 (IL-2)-dependent T cell line (10^3 cells; CTLL-20) was added to each well for an additional 24 hr. Proliferation was measured by pulsing the cultures for 6 hr with ^{3}H-thymidine. Cultures were considered positive for IL-2 release if cpm exceeded the mean plus 3 SD of ^{3}H-thymidine incorporation in 20 background control wells (microtiter wells that lack the responder cell). Minimal frequency of HTL was calculated using a computer program, the use of which has been described previously (*8*), and the results were expressed as HTL/million cells.

Propagation of graft-infiltrating cells: Small fragments of kidney core biopsies were used to propagate alloreactive T cells in culture (*9*). The biopsies were teased into 4–5 small fragments and placed in individual wells of a 96-well round-bottomed microtiter plate in the presence of tissue culture medium supplemented with 5% human serum and 20 U/ml IL-2. Wells were regularly observed for cellular proliferation from the biopsy and fed every fourth day with IL-2.

RESULTS

Variability of donor-specific reactivity. Representative examples of recipients' pre- and posttransplant proliferative responses, classified according to their in vitro reactivity are illustrated in Table 1. Recipient 1 (BM + kidney) exhibited category I "donor-specific hyporeactivity" by three months posttransplant, at which time the MLR response to donor

TABLE 1. In vitro analysis of immune reactivity

Patient	POD (month)	Proliferative responses (×10^4 cpm)				Donor reactivity pattern
		Con A	Auto	Donor	Third-Party	
1	Pre-Tx	108	1	74	126	Hyporeactive
	3	79 (73)[a]	0.3	9 (12)[a]	53 (17)[b]	
2	Pre-Tx	56	2	90	56	Intermediate
	3	38 (68)[a]	0.7	41 (45)[a]	52 (79)[b]	
3	Pre-Tx	50	0.5	30	36	Reactive
	3	39 (78)[a]	0.8	39 (130)[a]	49 (80)[b]	
4	Pre-Tx	36	0.7	30	37	Suppressed
	3	18 (50)[a]	0.3	1 (3)[a]	5 (20)[b]	

[a] % relative response—posttransplant: pretransplant.
[b] % relative response—donor MLR: third party MLR.

TABLE 2. Donor-specific reactivity in bone marrow–augmented and control transplant recipients

Donor-specific MLR patterns	Bone marrow–augmented[a]	Control[a]
Hyporeactive POD (mean ± SD)[b]	3 (1K, 2L) 145±52	2 (1K, 1L) 112±10
Intermediate POD (mean ± SD)[b]	5 (2K, 3L) 154±70	0
Reactive	7 (4K, 2L, 1H)	10 (2K, 8L)
Suppressed	0	4 (2K, 2L)

[a] Number of transplant recipients: (K) kidney, (L) liver, (H) heart.
[b] Postoperative day when change in donor MLR reactivity was observed.

TABLE 3. Immunosuppression and donor-specific reactivity in kidney transplant recipients—patterns of donor-specific MLR responses

A. Kidney with BM

	Hyporeactive	Intermediate	Reactive
Patients (n)	1	2	4
FK dose (mg/day)	4	20±3	21±14
FK level (ng/ml)	0.8	0.9±0.05	0.8±0.3
Steroids (mg/day)	0	0, 2.5	11±3
Azathioprine (mg/day)	0	75 (1)[a]	75 (1)[a]
Incidence of rejection	0	0	75%
HLA mismatches	3	2, 5	1, 5, 5, 6

B. Kidney without BM

	Hyporeactive	Suppressed	Reactive
Patients (n)	1	2	2
FK dose (mg/day)	14	18±16	14±8
FK level (ng/ml)	0.2	0.7±0.3	1.2±0
Steroids (mg/day)	7.5	7.5, 25	7.5±3.5
Azathioprine (mg/day)	125	100 (1)[b]	0
Incidence of rejection	100%	100%	100%
HLA mismatches	6	4, 5	3, 3

[a] The two kidney-islets recipients who received azathioprine.
[b] Number of patients.

cells was significantly lower than that pretransplant (relative response 13%), whereas the proliferative responses to third-party and mitogens were not suppressed. In contrast, recipient 2 (BM + kidney) was considered a category II "donor-specific intermediate" responder since the donor-specific MLR, though lower, was still 45% of pretransplant and the ratio of donor to third-party MLR was greater than 40%. The Category III example ("reactivity") is exemplified by BM + kidney recipient 3, in whom PBMC responses against the donor or third-party cells remained essentially unchanged. Liver control recipient 4 qualified for category IV (suppressed) because proliferative responses to all stimuli were significantly inhibited (this was characteristic during CMV infection).

Eight (53%) of the 15 bone marrow–augmented patients exhibited progressive modulation of donor-specific reactivity, 3 (1 kidney and 2 livers) with hyporeactivity and 5 (2 kidneys and 3 livers) with intermediate antidonor responses. The other 7, including the heart recipient, remained reactive u to the last date tested (4–6 months postsurgery) (Table 2)

In the nonmarrow control group, 2 (13%—1 kidney and liver) of the patients showed donor-specific hyporeactivity, who were studied during or shortly after CMV infection wer suppressed globally, and 10 remained vigorously reactive t donor cells (Table 2).

Immune reactivity versus immunosuppression and graf function. Kidney recipients: The 3 (43%) of the 7 marrow treated recipients showing donor-specific hypo- (or interme diate) reactivity had no evidence of rejection; 2 recipients ar off steroids and the third receives 2.5 mg/day prednison (Table 3). In contrast, 3 (75%) of the 4 recipients who main tained vigorous antidonor reactivity underwent acute rejec tion episodes and are currently receiving 7.5–12.5 mg/day o prednisone. The two pancreatic islet recipients (one exhibit ing intermediate and the other reactive responses against the donor) are additionally receiving 75 mg/day of azathio-prine. Both have evidence of plasma C-peptide activity but levels (0.44 and 0.11 pmol/ml) are not high enough to permit an insulin-free existence.

All 5 of the nonmarrow control recipients experienced transient rejection episodes. The only one classified as donor-specific hyporeactive is being maintained on FK506 (14 mg/day), prednisone (7.5 mg/day), and azathioprine (125 mg/day) (Table 3). The higher drug requirement and persistently reactive immune status of the patients in the control group may reflect in part their shorter period of follow-up (137±24 days) as compared with the patients in the study group (282±113 days). However, the same general patterns were already identifiable within 90–120 days.

The mean creatinine at the 4-month milestone and at the most recent follow-up was superior in patients in the marrow treated group compared with the controls (1.6±0.5 vs. 2.4+1 mg/dl). Both groups had similar rates of rejection in the first 4 months (50% vs. 62%) and infection (20% vs. 25%). The

TABLE 4. Immunosuppression and donor-specific reactivity in liver transplant recipients—patterns of donor-specific MLR reactivity

A. Liver with BM

	Hyporeactive	Intermediate	Reactive
Patients (n)	2	3	2
FK dose (mg/day)	2.0±0	6.6±1	20±4
FK level (ng/ml)	0.2±0.1	0.33±0.2	0.5±0.4
Steroids (mg/day)	0	5 (1)[a]	5 (1)[a]
Incidence of rejection	100%	33%	50%

B. Liver without BM

	Hyporeactive	Suppressed	Reactive
Patients (n)	1	2	8
FK dose (mg/day)	10	10±8	11±4
FK level (ng/ml)	0.4	0.65±0.07	0.6±0.3
Steroids (mg/day)	0	0	10±4 (4)[a]
Azathioprine (mg/day)	0	50 (1)[a]	0
Incidence of rejection	0%	100%	63%

[a] Number of patients.

degree of HLA matching did not influence graft function or the frequency of donor-specific hyporeactivity (Table 3).

As reported elsewhere (*3*), the chimerism was many-fold more dense, with an incidence of 100% in the bone marrow group (one patient who shared 1B and 2 DR loci and same sex with the donor could not be followed), compared with low-level chimerism in 60% of patients in the control group.

Liver recipients: A similar pattern was noted in the marrow-augmented liver recipients compared with controls. The bone marrow cohort had a higher rate of hypo- or intermediate antidonor reactivity (71% vs. 27%), lower doses of immunosuppression, and a similar incidence of rejection and infection (Table 4). Liver function was equivalent in both groups. Interestingly, as in the kidney series, there were no examples in the bone marrow–treated patients of the global suppression noted in 25% of the controls.

Two BM-augmented liver transplant recipients developed transient skin rashes that were diagnosed as GVHD by histopathology. One was treated with an increased dose of steroids (from 7.5 to 15 mg/day), whereas the other resolved spontaneously. The initial C-peptide activity of 0.83 pmol/ml in the liver recipient also given pancreatic islets diminished to 0.02 pmol/ml after an episode of acute cellular rejection precipitated by withdrawal of immunosuppression to treat colonic posttransplant lymphoproliferative disease (PTLD).

The continued presence of donor DNA in the PBMC of liver recipients was documented by PCR in all 7 patients in the study group, whereas donor chimerism was detected in only 54% (6/11) of patients in the control group (*3*).

Instability of donor-specific nonreactivity. Three bone marrow–augmented patients who developed unequivocal early evidence of donor specific hypo- (D/TP ratio 0.04 and 0.07) or intermediate (D/TP ratio 30) reactivity lost this quality farther on in their course and became donor-reactive. The modulation of donor-specific MLR was significant in all three recipients (2614 vs. 33,933 cpm, 2986 vs. 20,619, and 15,087 vs. 45,755 cpm, respectively) while their third-party MLR responses remained similar (mean reactivity ± SD: 53,749±4545 cpm, 71,362±9036 cpm, and 40,726±4 cpm, respectively). All three patients underwent easily controlled late rejection: a liver-islet recipient (day 90), kidney-islet (day 232), and kidney (day 261).

The events were similar in all 3 cases, epitomized by the course of a 25-year-old female recipient of a 5-HLA-matched male kidney (Fig. 1) in whom the presence of the Y chromosome allowed tracking of chimerism. After a bout of steroid-treated rejection on day 16, her recovery was uncomplicated. After 4 months, she had developed donor-specific hyporeactivity. Immunosuppression was reduced on day 175, and prednisone was eventually stopped. On POD 263, the serum creatinine increased, and a biopsy-proven rejection was successfully treated with a steroid bolus and a transient increase in the FK506 dosage (Fig. 1). The infiltrating cells propagated from the graft biopsy exhibited a 25-fold higher donor-specific alloreactivity than that of the background controls. Two days preceding these events, a blood sample obtained on POD 261 showed an ~6-fold increase in donor-specific reactivity. The donor-specific helper T cells (HTL) were found to have increased three-fold from 15/10^6 cells (1 in 65,000 POD 164) to 45/10^6 cells (1 in 20,000 POD 232) one month prior to a rejection episode. She still maintains antidonor reactivity at the last date tested (POD 292). However, after successful treatment of rejection, a lymphocyte culture could not be propagated from a follow-up kidney biopsy. Throughout the course, the Y chromosome persisted in the patient's blood samples.

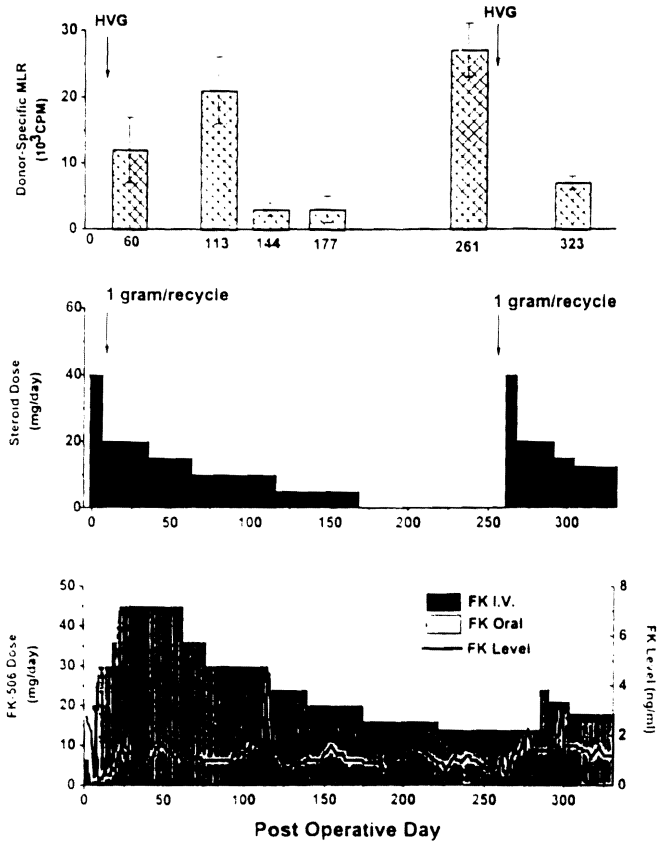

FIGURE 1. Immunosuppression profile, rejection episodes (host-versus-graft rejection: HGV), and donor-specific MLR response during the first 12 postoperative months in a bone marrow and kidney transplant recipient.

DISCUSSION

The premise upon which this study was based is that donor leukocyte chimerism is the essential basis for organ graft acceptance and the evolution of the donor-specific nonreactivity that follows a characteristic cycle of immunologic crisis and resolution (*1, 2*). With the demonstration that this spontaneous chimerism can be reliably and safely increased many-fold by bone marrow augmentation (*3*), it is possible to envision for the first time a future population of recipients of MHC-incompatible organs who can aspire to a drug-free state.

However, it is equally clear that the enigmatic process leading to donor-specific nonreactivity is very gradual. One of the principal questions asked in this study is if the in vitro immune reactivity profile of the bone marrow treated patients was different, during the first few months, from that resulting from organ transplantation alone. According to the chimerism concept, the same leukocyte-dependent mechanisms of graft acceptance are involved with or without adjuvant bone marrow.

Consequently, it was no surprise that the changes observed in our marrow cohort were merely magnified and

more frequent versions of those also seen in the control population, with no significant differences among recipients of various organs. Similar findings were noted often by others in conventionally treated recipients of various organs (*10–21*), most frequently the liver (*22*). By themselves, the significance of the more pronounced and more frequent early changes in the bone marrow–augmented recipients would be difficult to assess. However, the remarkable benignancy of recovery in these patients and the accrual of 40 consecutive cases (kidneys, livers, and hearts) with no mortality compared with less-satisfactory results in contemporaneous controls (*3*) has added to our impression that the marrow enriched recipients' have gained a profound advantage without paying a commensurate penalty. Of special note was the fact that none of the 15 marrow-augmented patients treated with conventional immunosuppression had residual global suppression (category IV) by the time of last testing.

The second principal question is whether immunologic monitoring can guide the management of these patients, and provide signals about when it has become safe to wean drugs or stop them. Previous reports have emphasized the poor prediction index of in vitro tests for identification of stable tolerance in animal models (*23, 24*) or human organ recipients (*25*). The limitations of such analyses were illustrated by the 3 patients in the present study whose hyporesponsiveness was reversed after premature reductions of immunosuppression, with the onset of rejection. Because of the variability of the outbred human population, it is hard to escape the conclusion that an element of trial and error will always be involved no matter how exhaustive the immune monitoring.

Nevertheless, a role for immune monitoring is evident in these trials, if only because of the early warning that it can provide and for its use to assess the response to therapeutic course correction. It goes without saying that these examinations are optimally useful only when they are done serially. Expansion of the test panel to include other methods may prove to be of practical value—and, in addition, these may provide more complete insight about the changes that occur at the interface between the donor and recipient cell populations, both of which retain immunologic reactivity.

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