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Frequent Achievement of a Drug-Free State After Orthotopic Liver Transplantation

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THE recently discovered phenomena of bidirectional cell migration and consequent systemic as well as graft chimerism have been proposed to be essential for the long-term acceptance of any kind of whole organ graft.[1,2] Under effective immunosuppression, donor passenger cells that are of monocyte/macrophage and other lineages of bone marrow origin pass from the graft into ubiquitous recipient tissues while similar leukocytes from the recipient replace them in the transplant.[3–5] We have postulated that the bodywide engagement results in initial mutual activation of these coexisting immunocyte populations, leading in successful cases to variable donor and recipient-specific nonreactivity.[1,2] This implies the eventual possibility in some patients of achieving immunologic tolerance and a drug-free state. Because these migratory cells are more densely represented in the liver we predicted that this desirable end point would be achieved most frequently with this organ. We report some clinical and immunologic observations that are congruent with this hypothesis.

METHODS

Case Material

Group I, Noncompliance. Eight patients stopped their medications 0.5 to 11 years after transplantation; seven have remained drug free for 1 to 14.3 years subsequently. One of these patients underwent a second successful transplantation in April 1992, 12 years after the first grafting, but had been off immunosuppression for 7.7 years. The liver had been destroyed by chronic viral hepatitis (hepatitis C virus [HCV]) and contained no evidence of rejection (Case 6, Table 1).

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Table 1. Noncompliance and Physician-Directed Drug Weaning

Patient No.	Diagnosis	Age at Tx (y)	F/U Post-Tx (y)	Previous immunosuppression	Time Post-Tx off Medication (y)	Total Time off (y)	Liver Function
Group I (noncompliant)							
1	Biliary atresia	4	19.7	Azathioprine/prednisone	9	10.7	Normal
2	Chronic active hepatitis	11	18.6	Azathioprine/prednisone	11	7.6	Normal
3	Biliary atresia	0.8	15	Azathioprine/prednisone	10	5	Normal
4	Secondary biliary cirrhosis	15	14.8	Azathioprine/prednisone	0.5	14.3	Normal
5	Wilson's disease	21	13.5	Azathioprine/prednisone	2	11.5	Normal
6*	Chronic active hepatitis	28	12.7	Cyclosporine/prednisone	5	7.7	Failed re-Tx, well
7	Alagille's syndrome	2	9.8	Cyclosporine/azathioprine/prednisone	8	1.8	Normal
8	Chronic active hepatitis	9	8.7	Cyclosporine/prednisone	7.7	1	Normal
Group II (physician wean)							
9	Biliary atresia	3	20.3	Azathioprine/prednisone	20	0.3	Normal
10	Primary biliary cirrhosis	30	17.3	Azathioprine/prednisone	16.8	0.5	Normal
11†	Primary biliary cirrhosis	35	13.7	Azathioprine/prednisone	13.4	0.3	Normal restart
12	Biliary atresia	4	9.4	Cyclosporine/prednisone	8.7	0.7	Normal
13	Alagille's syndrome	6	10.2	Azathioprine/prednisone	9.6	0.6	Normal

*Required retransplantation for chronic hepatitis C 7 years after primary Tx. Presently on immunosuppression.
†Developed enzyme elevation requiring reinstitution of immunosuppression.

Table 2. Group III Patients: Treatment Stopped Because of Complications

Patient No.	Diagnosis	Age of Tx (y)	Previous immunosuppression	Time Post-Tx Taken off Medication (y)	Total Time off (y)	Type of Infection	Liver Function
14	Biliary atresia	3	Cyclosporine/prednisone	6	3.6	HIV	Normal
15	Polycystic liver disease	15	Cyclosporine/prednisone	8	0.5	PTLD	Normal
16*	Biliary atresia	5	Cyclosporine/prednisone Azathioprine/ATG/OKT3	7	2.8	HIV	Failing
17	Biliary atresia	0.6	FK 506/prednisone	1.2	2.6	PTLD	Normal
18	Neonatal hepatitis	0.3	FK 506/prednisone	0.8	1.9	PTLD	Normal
19	Fulminant hepatitis C	1	FK 506/prednisone	0.5	2.6	HCV	Normal
20	Biliary atresia	0.6	FK 506/prednisone	0.8	2.3	PTLD	Normal
21	Biliary atresia	1	FK 506/prednisone	1.3	1.1	PTLD	Normal
22	Biliary atresia	1.3	FK 506/prednisone	2	0.9	HCV	Normal
23†	Cats eye syndrome	1.5	FK 506/prednisone	1	1.3	PTLD	Re-Tx
						HCV	Dead

*Liver dysfunction associated with terminal AIDS.
†Died after retransplantation for chronic hepatitis C infection.

Of the 8 patients, the first 6 (Table 1) were among 44 who were alive in June 1992 after having survived for 10.5 to 22.5 years. The high rate of noncompliance (6 of 44, 14%) reflected the word of mouth news within this closely knit group that drugs were being successfully stopped by others. This was coupled with the information that immunosuppression-related complications had been the most common cause of late death of seven other original members of their one decade survivor club after 11.1 to 18.4 postoperative years. None of these late deaths had been caused by rejection.[4]

In addition, two pediatric recipients who were taken off their medication by their parents 7 to 8 years posttransplantation have been drug free for 1 and 1.8 years (Table 1).

Group II, Physician Wean. Physician-directed drug weaning was carried out in five other long-surviving recipients who had been rejection free for at least 5 years (Table 1). With total posttransplantation follow-up of 9 to 20 years, four have been drug free for 4 to 8 months. Baseline immunosuppression was reinstituted in one patient (Table 1).

Group III, Infectious Complications. Ten pediatric recipients had immunosuppression stopped 6 months to 8 years after transplantation because of infectious complications: posttransplant lymphoproliferative disease (n = 6), HCV (n = 2), and human immunodeficiency virus (HIV) (n = 2) (Table 2). One of the children underwent unsuccessful retransplantation because of progressive chronic viral hepatitis. The other 8 patients have been off immunosuppression for 6 months to 3.6 years (Table 2).

Weaning Regimen

Most of the noncompliant patients stopped their medications sporadically at first, and then completely when nothing happened. Physician-directed weaning was performed by the systematic decrease in the baseline immunosuppression, starting with drugs having a specific side effect such as renal dysfunction, neurotoxicity, hypertension (cyclosporine [CyA] or FK 506). Steroids and CyA were weaned more aggressively in the presence of facial brutalization and growth retardation. Liver function was assessed biweekly by routine liver function tests (bilirubin, alanine transaminase [ALT], aspartate transaminase [AST], gamma-glutamyl transpeptidase [GGT]). In the event of abnormal results, repeat sampling was requested and followed by a percutaneous liver biopsy if indicated. Rejection was treated in the standard way.

Chimerism Studies

Chimeric cells in whole organ recipients were identified by the distinctive features of chromosomes 2 (sex) and 6. In females who had been given an organ from a male donor, the presence in recipient tissues (or blood) of cells with the Y chromosome was considered unequivocal evidence of systemic chimerism. Alternatively, probes were used that detected HLA alleles of chromosome 6. For study of either the Y chromosome or chromosome 6, one or the other of two technologies, and usually both, were exploited: (1) immunostaining, which allows the location and morphologic characterization of phenotypically distinct donor and recipient cells; and (2) polymerase chain reaction (PCR), which distinguishes donor from recipient DNA. These techniques have been described in detail elsewhere.[3–5]

In Vitro Immunologic Studies

In vitro studies were performed to assess different T-helper subsets that are activated during Epstein-Barr virus (EBV) infections, assessing the release of various cytokines (TH1 vs TH2) that may influence (augment or suppress) the immune response of the host to the allograft. Studies were also performed to assess proliferation to third party alloantigens.

Mitogen-Induced Proliferation. Mononuclear cells were isolated by Ficoll Hypaque gradient from heparinized peripheral blood samples of the pediatric liver transplant recipients. Lymphocytes (5×10^4 cells/well) were cultured in tissue culture media supplemented with 5% pooled human serum, for 72 hours in 37°C in the presence of either concanavalin A (ConA, 4 μm/mL) or phytohemagglutinin (PHA, 10 μm/mL). Proliferation was assessed by ^{3}H-thymidine uptake for the last 20 hours of incubation.

Mixed Lymphocyte Reaction. Unidirectional MLR cultures were set up with 5×10^4 cells/well of irradiated normal unrelated lymphocytes (third party cells) in tissue culture medium for 6 days. Proliferation was assessed by ^{3}H-thymidine uptake for the last 20 hours of incubation.

Homozygous Typing Assay. This assay was set up using patients' peripheral blood lymphocytes (PBL) as responders and homozygous typing cells (HTC) as stimulators following the standard protocol of MLR as described above. HTCs for seven different Dw specifics were tested with two to three HTCs per specificity.

Detection of Cytokine Gene Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from 2×10^6 pelleted PBL using RNAzol B. Quantitation of RNA was performed spectrophotometrically. A known aliquot of total RNA was used as a template for cDNA synthesis. Subsequently, this first strand cDNA was used as a template for PCR amplification carried out for 30 cycles using custom made oligonucleotide amplimers. The PCR products were analyzed by gel electrophoresis on 2% agarose gel stained with ethidium bromide. Both positive and negative control samples for each primer were included in each analysis and B-actin served as positive control for PCR reaction. We tested the presence of IL-2, IL-4, IL-6, and IL-10 mRNA in patients' PBL samples before and after PHA stimulation.

RESULTS

Chimerism

The first six patients in Group I were all shown to have systemic chimerism with both cytostaining (immunocytochemical or Y chromosomes or both) and PCR. These results have been reported case by case elsewhere.[3,4] With the same techniques, chimerism was demonstrated in Patients 9 to 11 of Group II and Patient 17 of Group III. Biopsies were not performed on the others because all chronically surviving liver recipients studied during the last year ($n > 50$) had chimerism. Thus, multiple biopsies without an explicit purpose (such as study of graft-vs-host disease [GVHD]) are no longer performed routinely in liver recipients.

Clinical Outcome

These data are summarized in Table 1 for Groups I and II and in Table 2 for Group III. The ability to stop treatment was not specific to any of the azathioprine, CyA, or FK 506-based regimens used throughout the more than two decades of case accrual.

The absence of rejection after drug stoppage was striking. Patient 11, whose original disease was primary biliary cirrhosis (PBC), was returned to therapy 3 months after its discontinuance when transaminases rose 10-fold from baseline (highest 120 IU) without jaundice. After treatment with steroids and reinstitution of azathioprine, liver function became normal.

The loss of the liver in Case 6 (an adult) was unequivocally due to recurrent viral chronic active hepatitis as reported in detail elsewhere.[4] The same disease was responsible for the graft loss in Patient 23 who also had PTLD, which regressed after stopping immunosuppression; he died after retransplantation. Patient 16 (a child) who is dying of acquired immunodeficiency syndrome (AIDS), has had this diagnosis with slowly worsening condition for almost 10 years, since the early postoperative period. His AIDS complications are cryptosporidium gastroenteritis and toxoplasma meningoencephalitis.

Table 3. Proliferative Responses of PBL From Pediatric Liver Transplant Recipients off Immunosuppression (Group II)

		Proliferative Responses (cpm)		
Patient No.	Background	CONA	PHA	MLR
17	1,052	12,491	71,502	88,111
20	641	31,228	161,167	23,035
21	1,014	nt	188,434	72,061
22	288	41,979	131,683	47,158
23	765	18,451	58,385	53,519

The course of Patient 15 who received a kidney and liver from the same donor on November 18, 1984 was instructive. This child with PTLD associated with EBV infection was initially treated for this complication with a reduction in immunosuppression and IV acyclovir. Because of bouts of recurrent PTLD in the following 12 months, immunosuppression was stopped. Two months later, she presented with elevations of the ALT + AST (10 times normal). Rejection was diagnosed on a liver biopsy that revealed portal tracts infiltrated with lymphocytes and eosinophils. Although endothelitis and bile duct damage also were seen on the biopsy, immunosuppression was not restarted because of the PTLD threat. The liver enzyme profile became normal after 4 weeks and has remained so.

Immunologic Studies

It was previously shown that adult liver recipients are immunocompetent with or without maintenance immunosuppression.[4] Five pediatric patients who had immunosuppression withdrawn from 0.9 to 2.6 years earlier because of infectious complications all had normal mitogen (ConA and PHA)-induced proliferation of PBLs, and normal MLR response to third party lymphocytes (Table 3).

Donor cells were not available for MLR testing, however, we could demonstrate donor-specific hyporeactivity in two of these recipients by using the HTC assay. The proliferative responses to HTCs that express donor HLA-DR antigen were low and in the same range as the responses observed towards stimulators that present self HLA-DR antigens. Both self and donor-specific responses were less than 50% of the proliferative responses towards unrelated third party stimulator (Table 4).

Cytokine mRNA profile of PBL in three of four patients showed the presence of IL-4, IL-10, and IL-6 mRNA prior to PHA stimulation. Following activation, all PBL samples showed IL-2, IL-4, IL-6, and IL-10 mRNA (Table 5).

Table 4. Homozygous Typing Assay to Detect Donor-Specific Hyporeactivity of PBL From Pediatric Liver Transplant Recipients (Group III)

Responder	Self Antigen*	Donor Antigen*	Third Party Antigen*
17	10,765 ± 167 (n = 3)	4,229 ± 811 (n = 3)	21,159 ± 1,740 (n = 6)
20	4,483 ± 1,047 (n = 4)	5,017 ± 544 (n = 6)	15,376 ± 1,874 (n = 6)

Note: Proliferative responses (mean ± SE cpm).
*Stimulator cells (n) were used in MLR that express self DR antigens, donor-specific DR antigens, or unrelated third party DR antigens.

DISCUSSION

The hypothesis that cell migration and chimerism explain graft acceptance and constitute the seminal step toward donor-specific nonreactivity and immunologic tolerance[1–5] is consistent with the results of in vitro tests in the pediatric liver recipients in this case collection, and in a larger series of adults reported elsewhere.[4] Both the adults and children appeared to be fully immunocompetent, and in the adult studies, long-surviving recipients were not distinguishable according to whether drug therapy had been stopped. This implied donor-specific nonreactivity just as in the children herein reported, but the unavailability of donor cells precluded direct testing this with MLR. However, in two of the children, the results of HTC assay appeared to be confirmatory of donor-specific nonreactivity.

Of interest also was the cytokine profile of four children whose immunosuppression was stopped 6 to 15 months after transplantation because of PTLD, and not started again. One to 2.6 years later (long after clinical recovery), the resting PBL cytokine pattern of a negative IL-2 with positive IL-4, 6, and 10 suggested the TH-2 immune response that has been associated with systemic or local donor-specific nonresponsiveness and graft acceptance.[6,7] The inherent responsiveness of the PBL was demonstrated with PHA stimulation. These observations were consistent with our contention that allograft tolerance is an active process that requires continuous low grade mutual stimulation bodywide by the coexisting donor and recipient cell populations,[2] the process involving the release of differing groups of cytokines (TH_1 vs TH_2).

Table 5. Cytokine mRNA Profile of PBL Samples From Pediatric Liver Transplant Recipients off Immunosuppressive Drugs (Group III)

Patient No.	Sample*	Actin	Cytokine mRNA IL-2	IL-4	IL-6	IL-10
17	PBL	+	−	+	−	+
	PBL-PHA	+	+	+	++	++
18	PBL	+	−	++	++	++
	PBL-PHA	+	−	+	−	−
19	PBL	+	−	−	++	−
	PBL-PHA	+	+	+	++	++
20	PBL	+	−	++	++	++
	PBL-PHA	+	+	+	++	+

*2×10^6 mononuclear cells obtained by Ficoll Hypaque centrifugation from PBLs were tested for cytokine mRNA before and after 2 hours PHA stimulation.

The observations in the total 23 cases herein reported are reminiscent of those reported in dogs nearly 25 years ago after orthotopic canine transplantation. The animals that survived for 4 months under azathioprine therapy could almost always be maintained for long nonrejecting periods thereafter (eventually out to 12 years) without further treatment.[8] This was in contrast to canine renal recipients that rejected their kidneys early and far more frequently. As the result of seeing the same thing in patients, we suspect that the majority of human liver recipients who have had a benign postoperative course do not need immunosuppression if they are significantly beyond 5 years.

Because immunosuppression has liability, particularly in children who have a high cumulative incidence of PTLD, we have begun a prospective trial of drug weaning in 50 long-surviving liver recipients. The safety of such a trial has been illustrated by the ease with which signs of presumed rejection could be controlled as in the one patient suspected to have this diagnosis in the currently reported series. In this patient whose immunosuppression was restarted with a prompt response, the original disease that destroyed her native liver was PBC. An occult danger of stopping immunosuppression in patients with autoimmune hepatic disorders could be the development of these diseases (PBC, autoimmune hepatitis, and others) in the allograft, a possibility that will have to be carefully looked for in future cases.

It is possible that an apparent flare up of rejection after drug discontinuance can be managed in some patients (as in Patient 15) without resuming immunosuppression. A "rebound cellular response" and spontaneous subsequent remission have been reported in experimental animals when immunosuppression was stopped.[9] The decision to continue withholding therapy for Patient 15 was crucial because of her recurrent PTLD, and was rewarded by normalization of her liver function with no further lymphoma recurrence.

The duration of follow-up before drugs can be stopped with a high expectation of success is not known, nor is it predictable with any currently available method of screening. In one extraordinary case, a noncompliant 15-year-old girl who did not appear to be overimmunosuppressed stopped all treatment at 6 months, and has a perfect result 14.3 years later. Those experienced in liver recipient management would recoil at considering such a dangerous step, and no patient in our prospective program would be a candidate for weaning before at least 5 years of uninterrupted therapy.

However, discontinuance much earlier than this was mandated in the 10 patients who had life-threatening complications, of which the most common was PTLD. The

ability to stop treatment so early cannot be construed as a guideline in patients without such evidence of overimmunosuppression, particularly if all other prognostic factors are favorable. Apart from iatrogenic immunosuppression, an augmenting immunosuppressive factor could be the PTLD itself. Studies on adult liver recipients with PTLD using snap frozen liver biopsy tissues were analyzed by PCR and found to be positive for EBV, IL-4, and IL-10 mRNA and negative for IL-2 and IFN-gamma (Dr Michael Nalesnik, unpublished observations). Thus, the tolerogenic cytokine pattern with TH_2 dominance could be facilitated by PTLD and other viral infections.

Although there is evidence that the same mechanisms apply generically to all whole organ allografts, the migratory cell constituency of other kinds of grafts is smaller than the liver, explaining on one hand the high tolerogenic potential of the liver, and emphasizing on the other the much greater risk of drug weaning should this be unwisely attempted in recipients of leukocyte-poor organs such as the kidney and heart. The strategy of augmenting the natural migratory traffic in these organs with bone marrow has been discussed elsewhere.[1,5]

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