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## Early Tolerance in Pediatric Liver Allograft Recipients

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● The authors report on six pediatric liver transplant recipients for whom allograft tolerance occurred shortly after transplantation (ie, less than 1.5 years). All the patients had associated life-threatening viral complications. They are currently immunocompetent. The tolerant state may be related to the development of a TH<sub>2</sub> cytokine pattern.  
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**INDEX WORDS:** Liver transplantation, allograft.

**D**EVELOPMENT of a drug-free state has long been an aspiration in transplantation. It is particularly desirable in children because they are subject to complications secondary to the lifelong administration of immunosuppressive agents. We report on a small group of pediatric patients who, in the presence of life-threatening complications, had allograft tolerance shortly after transplantation. The immune unresponsiveness appeared to be donor-specific. They were shown to be otherwise immunocompetent.

### MATERIALS AND METHODS

#### Case Material

Among our pediatric transplant population (liver, kidney, intestinal, and pancreatic islet recipients), there were six patients (Table 1) who had life-threatening viral infections not long after transplantation but also had tolerance to their grafts. All the patients were liver transplant recipients, treated with FK-506-based immunosuppression.<sup>1</sup> The age range at the time of transplantation was 0.3 to 1.5 years. The primary disease was biliary atresia (3), neonatal hepatitis (1), fulminant hepatitis C (1), or cat's eye syndrome (1). Posttransplant, three of them had one or more episodes of acute rejection that required treatment with steroids, and three had no rejection at all.

The complications that necessitated discontinuation of the immunosuppression were lymphoproliferative disease (PTLD)

(n = 5) and severe hepatitis C (n = 2). Patient no. 4 had both complications. Hepatitis was recurrent in one case and acquired after transplantation in the other. Immunosuppression was discontinued 0.5 to 1.3 years posttransplantation, and, to date (1.3 to 2.8 years later), it has not been resumed.

One patient died after retransplantation for hepatitis C. All others are alive with the same grafts. PTLD and hepatitis C have been controlled successfully in all surviving patients. There have been no other severe infections. All patients are rejection-free; this was demonstrated clinically, biochemically, and histologically.

Another patient (no. 7, Table 1), a 1-year-old girl who received a liver allograft under FK-506 for biliary atresia, had severe hepatitis C 2 years after transplantation, and the immunosuppression was withdrawn. She remained rejection-free for 0.9 years. Rejection then developed, which required resumption of FK-506. The patient still has evidence of active hepatitis C.

#### In Vitro Immunologic Studies

These tests were done to evaluate immune competence. Functional evaluation of T helper cells was performed by measuring the ability of peripheral blood lymphocytes (PBL) to proliferate in response to nonspecific plant lectins: phytohemagglutinin (PHA), concanavalin A (Con A), and alloantigens. Although the "normal" range for each test may vary, the response is considered significant if the test value is at least 5- to 10-fold higher than the background value obtained in the absence of any stimuli. Patients who are immunocompromised and at high risk for frequent opportunistic infections exhibit very low responses to mitogens and alloantigen. These low responders may include transplant recipients on high doses of immunosuppressive drugs or patients with the human immunodeficiency virus.<sup>2,3</sup>

The specific response of recipient PBL to donor alloantigens can be tested with one-way mixed lymphocyte reaction (MLR), whereby equal numbers of recipient PBL and irradiated donor cells are mixed, and proliferation is assessed by the incorporation of <sup>3</sup>H-thymidine at day 6 of coculture. Donor-specific hyporeactivity is considered when the patient's MLR response to the donor decreases in comparison to the pretransplant values, while the proliferative responses to other HLA-incompatible donors remain high.

When donor cells are not available, we use a panel of stimulators in the MLR, which are homozygous for class II HLAs. These homozygous typing cells (HTC) are selected to represent cells that express "self" HLAs, "donor" HLAs, and other HLAs that are unrelated to recipient or donor antigens. Donor-specific hyporeactivity occurs when the proliferative responses of patient PBL to self and donor HTC are similar, and both responses are significantly lower than those observed toward unrelated HTCs.<sup>4</sup>

Helper T cells respond to stimuli in vitro and in vivo by producing various cytokines. These mediators have an important role in the regulation of cell-mediated and humoral immunity. T helper cells can be categorized according to the cytokine profile they secrete, ie, TH<sub>1</sub> or TH<sub>2</sub>.<sup>5</sup> Cytokines produced by the TH<sub>1</sub> subset are involved in allograft rejection and cellular responses to viral infections, whereas those secreted by TH<sub>2</sub> cells are mainly elevated in allergic responses and helminth infections. Cytokines produced by one subset may modulate and antagonize the effector function of the other subset. For instance, TH<sub>2</sub>-like cytokines (ie,

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Table 1. Liver Transplant Recipients Who Had Developed Early Tolerance to Grafts

Patient No.	Sex	Primary Diagnosis	Age at Transplantation	Immunosuppression	Posttransplant ACR	Time Posttransplant Until Off Medication (yr)	Total Time Off Medication (yr)	Complication	Current Biopsy
1	M	Biliary atresia	0.6	FK-506, prednisone	None	1.2	2.8	PTLD	No ACR
2	F	Neonatal hepatitis	0.3	FK-506, prednisone	Mild	0.8	2.1	PTLD	No ACR
3	F	Biliary atresia	0.6	FK-506, prednisone	ACR X2	0.8	2.5	PTLD	No ACR
4	M	Cat's eye syndrome	1.5	FK-506, prednisone	Multiple	1	1.3	PTLD, HCV	HCV*
5	M	Biliary atresia	1	FK-506, prednisone	None	1.3	1.3	PTLD	No ACR
6	F	Fulminant hepatitis		FK-506, prednisone	None	0.5	2.8	HCV	No ACR, no HCV
7	F	Biliary atresia	1.3	FK-506, prednisone	ACR X2	2	0.9	HCV	Mild ACR, HCV*

Abbreviations: ACR, acute cellular rejection; HCV, hepatitis C virus.

\*Died after retransplantation for hepatitis C.

elevated levels of interleukin [IL] 4 or 10) may downregulate the TH<sub>1</sub> function (secretion of interferon and activation of macrophages).<sup>6</sup>

Circulating PBL may already express elevated levels of cytokine genes, which could be detected by using sensitive molecular probes. In addition, PBL respond to in vitro stimuli by a transient upregulation of cytokine genes. We evaluated the T helper phenotype (TH<sub>1</sub> v TH<sub>2</sub>) in pediatric liver transplant recipients by testing cytokine mRNA expression before PHA stimulation (spontaneous) and after 2 hours' exposure to PHA. The studies performed in our patients were as follows.

**Mitogen-induced proliferation.** Mononuclear peripheral blood cells from these patients were isolated with Ficoll gradient and cultured in tissue-culture media in the presence of either Con A or PHA. Proliferation was measured by <sup>3</sup>H-thymidine uptake.

**Mixed lymphocyte reaction (MLR).** Unidirectional MLR cultures were set up with normal, unrelated, irradiated lymphocytes in a tissue-culture medium. Proliferation was assessed with <sup>3</sup>H-thymidine.

**Homozygous typing assay.** In the absence of donor lymphocytes, HTC were used as stimulators of the patients' PBL, using the MLR protocol described above. Seven different Dw specificities were tested with 2 to 3 HTC, for specificity.<sup>4</sup>

**Detection of cytokine gene expression by reverse transcriptase polymerase chain reaction (RT-PCR).** Total RNA was extracted from PBL. A known quantity of total RNA was used for cDNA synthesis. The latter was used as a template for PCR amplification. The PCR products were analyzed by gel electrophoresis. The presence of IL-2, 4, 6, and 10 mRNA in the patients' PBL was tested before and 2 hours after PHA stimulation.

## RESULTS

Five of the tested patients (nos. 1, 3, 4, 5, and 7) had normal Con A- (12.491 to 41.979 cpm) and PHA-induced (58.385 to 188.434 cpm) proliferation of PBL 0.9 to 2.6 years after discontinuation of immunosuppression. They also had normal ratio of MLR to third-party lymphocytes (23.035:88.111 cpm). These results suggest that pediatric liver transplant recipients who are off immunosuppression are immunocompetent.

In two patients (nos. 1 and 3) in whom HTC assay could be performed, the proliferative response to

HTC that presents donor antigens was in the same range as those towards stimulators that present self HLA-DR antigens. Both self and donor-specific responses were less than 50% of the responses toward an unrelated third-party stimulator. These results suggest that donor-specific hyporeactivity was achieved in these two patients.

Three of four tested patients (nos. 2, 3, and 6) showed the presence of IL-4, IL-10 and IL-6 mRNA before PHA stimulation. After activation, all PBL samples (from patients 1, 2, 3, and 6) showed IL-2, 4, 6, and 10 mRNA. The spontaneous cytokine profile is of the TH<sub>2</sub> phenotype. An increase of IL-2 mRNA in the stimulated samples confirmed the patients' immunocompetence.

## DISCUSSION

The description of the two-way cell traffic between donor and recipient and the development of mixed chimerism<sup>7,8</sup> have exposed previously unrecognized mechanisms of tolerance induction in human recipients of whole organs, particularly of livers. It also led to a controlled attempt at reduction of immunosuppression at our center, with total withdrawal as the goal.<sup>9</sup> Long-term survivors have been selected for this trial.

To date, three kidney recipients (of living-related 1-HLA haplotype-matched grafts) and 52 liver recipients (mean survival time after transplantation, 8 years) have been enrolled in this study.<sup>10</sup> Fifteen liver (29%) and two kidney recipients are currently off immunosuppression. The remaining patients are gradually being weaned. All patients are alive, and there have been no graft losses. Eleven patients (21%), all liver recipients, have sustained one episode of rejection, which responded to resumption of immunosuppression. In three patients, mild rejection was not treated and subsided spontaneously.

The fact that immunosuppression can be reduced<sup>1,11</sup> or stopped<sup>8</sup> after transplantation is best exemplified in pediatric patients. In them, immunosuppression is automatically down-regulated because no increase is made to compensate for growth.

Discontinuation of immunosuppression has been practiced in the treatment of life-threatening complications after transplantation.<sup>12</sup> The treatment usually is resumed because of the fear or occurrence of rejection. All but one of our seven patients (no. 7) exhibited graft tolerance and were immunocompetent. The potential drug independence of liver recipients has been noted previously,<sup>8</sup> but it did not occur as after transplantation; in our study, the average time until drug independence was 0.93 years. The early timing of tolerance and the association with life-threatening viral infections differentiate these patients from those who had the immunosuppression withdrawn systematically.

All the patients were treated with FK-506. Although in experimental animals there was a higher incidence of drug-free tolerance induction with FK-506 than with conventional immunosuppression including cyclosporine,<sup>13,14</sup> the end result was not drug-specific;<sup>15,16</sup> in humans, tolerances has been achieved with azathioprine or cyclosporine-based cocktail regimens.<sup>8</sup> We are currently observing a patient treated with cyclosporine who is tolerant to her combined liver-kidney graft 8 years after transplantation.

All patients in the present series were solitary liver recipients. Although the liver is known to be more tolerogenic than the other solid organs,<sup>8,16-18</sup> rejection remains a threat, as exemplified by case 7. If drug weaning is undertaken, the need for careful supervision and long-term follow-up cannot be overemphasized.

The resting PBL cytokine pattern was negative for IL-2 mRNA and positive for IL-4, 6, and 10, suggesting a TH<sub>2</sub> cytokine pattern. This dominant TH<sub>2</sub>-like immune response may have facilitated donor-specific hyporeactivity and graft acceptance in a drug-free environment.<sup>19</sup> A similar TH<sub>2</sub> cytokine pattern has been seen in liver biopsy specimens from patients with PTLD.<sup>20</sup>

The emergence of T helper phenotype 2 in liver transplant recipients after PTLD or other infections requires further consideration. TH<sub>2</sub>-like cytokines (IL-4 and IL-10) may produce a noninflammatory environment by down-regulating the immune response to the allograft, promoting anergy and accelerating graft acceptance. In these cases, tolerance can be maintained in a drug-free environment.

We conclude that resumption of immunosuppression is not needed in some cases after life-threatening PTLD and HCV. This phenomenon may be more common in FK-506-treated liver recipients. The development of a tolerant state may be related to an alteration of cytokine patterns.

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