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Persistent donor-specific alloreactivity may portend delayed liver rejection during drug minimization in children

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1. ABSTRACT

Immunoreactivity, immunosuppression requirement and liver graft function was assessed serially for its relationship to delayed/recurrent acute cellular rejection (ACR) after the first 60 days in 36 pediatric primary liver transplant (LTx) recipients. Subjects were classified as rejectors (n=20) or Non-Rejectors (n=16) based on the presence/absence of biopsy-proven ACR in the first 60 days. All children recieved anti-lymphocyte induction and steroid-free Tacrolimus or Sirolimus monotherapy, as reported previously. Median age was 4 years (0.45-18) and follow-up was 570 days (106-1144). Compared with non-rejectors, rejectors 1. took significantly longer to achieve reduced donor-specific alloreactivity by MLR (p=0.049), and "low" TAC/SRL whole blood requirements defined as TAC levels $\leq$8 ng/ml (p=0.0048), 2. experienced significantly greater variation in time to achieve reduced donor-specific immunoreactivity (SEM 0.8 vs 3.85, p=0.0048), and 3. experienced greater ACR incidence during minimization of immunosuppression (35% versus 6%, p=0.032). Serial monitoring of immunoreactivity may increase the safety with which immunosuppression is minimized in pediatric LTx.

2. INTRODUCTION

The greatest risk of acute cellular rejection (ACR) occurs in the first few months after liver transplantation (LTx) in children (1). Thereafter, this risk and the requirement for immunosuppression are thought to be lower. ACR occurring after the first few months is related to minimization of maintenance agents. This minimization of immunosuppression is guided by the presence of normal liver function tests, and is aimed at reducing the risk of infectious mortality, renal dysfunction and post-transplant lymphoproliferative disorder (2, 3). Because these decisions are based on clinical judgement rather than an objective understanding of the host-graft interaction, or immunoreactivity, one-fifth to one-third of all subjects may experience recurrent or delayed ACR (4).

Recently, in children induced with rabbit antihuman thymocyte globulin (rATG) and steroid-free Tacrolimus (TAC) or Sirolimus (SRL) based immunosuppression, we demonstrated that patients with early ACR demonstrated enhanced donor-specific alloreactivity, compared with those children who were rejection-free early after LTx (5). No differences were seen

Table 1. Indications for liver transplantation among rejectors and non-rejectors

Indication for Liver transplant	Rejectors (n=20)	Non-rejectors (n=16)
Biliary atresia	5	4
Autoimmune hepatitis	3	
Fulminant liver failure	2	
Maple syrup urine disease	2	3
Paucity of Bile ducts	1	
Ornithine transcarbamylase deficiency	1	1
Glycogen storage disease	1	
Progressive Familial cholestasis	2	1
Sclerosing cholangitis	1	
Alagille's syndrome	1	
Wilson's disease		2
Congenital hepatic fibrosis	1	
Hepatoblastoma		1
Hepatocellular carcinoma		2
Langerhan's cell histiocytosis		1
Alpha-1 antitrypsin deficiency		1

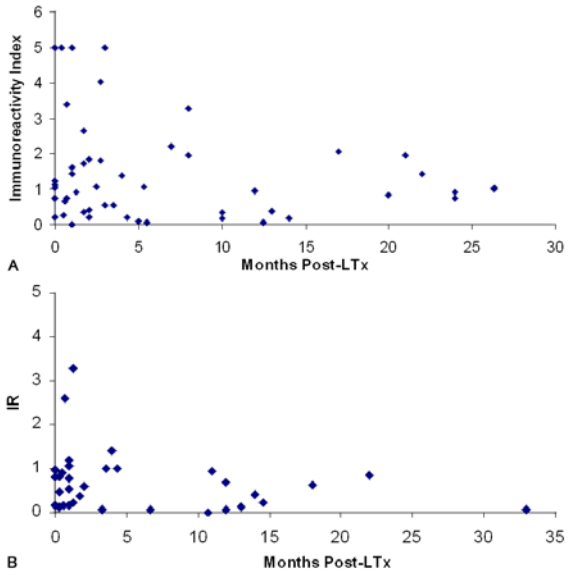

Figure 1. Post-LTx changes in immunoreactivity Index. Changes in immunoreactivity index in Rejectors (A, n=20) and Non-rejectors (B, n=16) are shown for several months after LTx. Median±SEM time to decreased rejection risk defined by IR<1 was significantly longer in Rejectors than in Non-Rejectors (10±3.8 vs 1.7±0.8, p=0.0352).

between these groups in the response to third-party alloantigen as assessed in mixed lymphocyte reactions (MLR), and in whole blood concentrations of maintenance agents. This led us to formulate and examine the hypothesis that differences in the risk of delayed or recurrent ACR between these groups could be related to persistence of 1) enhanced donor-specific alloreactivity relative to third-party alloreactivity. This was defined as the immunoreactivity index (IR), or the ratio of donor: third-party-induced proliferation of recipient lymphocytes in MLR. IR>1 implied enhanced risk of ACR, while IR<1 implied decreased risk. 2) "Increased" TAC requirement. This was defined as TAC or SRL whole blood concentrations > 8 ng/ml, and 3) liver function tests (LFT) above the normal range. This was defined as aspartate alanine transaminase (ALT) and aspartate glutamine transaminase (AST) >40 international units (IU) per liter, and glutamyl galactosyl transaminase >65 IU/l. Our assumption was tested in extended follow-up, median 570 days (106-1144), in the abovementioned cohort of pediatric LTx, using time-to-event analysis. The incidence of delayed or recurrent ACR was also recorded within groups.

3. METHODS

All studies were performed after approval by the Institutional Review Board of the University of Pittsburgh from 2001 to 2004. Sixteen subjects were rejection-free during the first 60 days post-LTx and were termed Non-Rejectors. Twenty subjects experienced biopsy-proven ACR during this time period and were termed Rejectors. Eighty six total measurements of IR were available for an average of 2.4 sequential measurements in each of 37 total study subjects. The technique for MLR has been described previously (5). Briefly, after 5-day co culture with irradiated HLA-DR-matched (donor) or mismatched (third-party) splenocytes, recipient lymphocyte proliferation was measured by uptake of ^{3}H-thymidine. TAC/SRL levels were measured by immunoassay (IMx, Abbot Labs, Illinois) and HPLC method, and LFT by colorimetric methods (Vitros Analyzers 950, Johnson and Johnson, Rochester, NY) at least weekly in the first month, bimonthly until the third month and monthly thereafter.

4. RESULTS

Indications for LTx are shown for each group in Table 1. No differences were observed between Rejectors and Non-Rejectors in mean values for age (6.1±5.8 vs 8.5±6 years respectively, p=NS) gender distribution (M: F ratio 8:12 vs 7:10 respectively, p=NS) and follow-up (580±238 vs 503±320 days respectively, p=NS). Table 2 summarizes the results of time-to-event analyses using the Kaplan-Meier (Breslow) method. Compared with Non-Rejectors experiencing delayed ACR, a significantly greater number of Rejectors experienced recurrent ACR after the early post-LTx period (6% versus 35%, p=0.032). Median pre-LTx immunoreactivity index (IR) in Rejectors and Non-Rejectors was 1.12 (range 0.2-25.7) and 0.8 (range 0.15-0.97), respectively, (p=0.158) (Fig.1). Median peak post-LTx IR in Rejectors and Non-Rejectors was 1.22 (range 0.21-477) and 0.85 (range 0.1-2.6), respectively, as shown in Figure 1 (p=0.154). The median time to achieve resolution of enhanced donor-specific alloreactivity and "decreased" TAC requirements was also significantly longer in Rejectors, compared with Non-Rejectors (p=0.0492 and 0.0048 respectively), as shown in Table 2. Rejectors also demonstrated four-fold greater variation in the time required to achieve decreased donor-specific alloreactivity (IR<1), compared with Non-Rejectors (SEM values 3.85 versus 0.81). This is evident in the graphic representation for changes in IR values with time in the two patient groups in Figure 1. The groups did not differ in the time to normalization of LFT as shown in Table 2.

4.1. Clinical correlation: Recurrent/delayed rejection

Eight subjects received steroids for recurrent/delayed ACR after a steroid-free period of at least

Table 2. Time in months (Median±SEM) required to achieve outcome parameters.

Outcome Parameter	Unit	Rejectors (n=20)	Non-Rejector (n=17)
Immunoreactivity	IR < 1	10±3.85	1.7±0.81, p=0.0492
Immunosuppression requirement	TAC/SRL levels ≤ 8 ng/ml	12±4	3±3, p=0.0048
Graft Function	SGOT<40 IU/ml	12±2	12±6, p=0.8006, NS
	SGPT<40 IU/ml	12±2	12±3, p=0.5199, NS
	GGT<65 IU/ml	3±1	3±1, p=0.8674, NS
Recurrent/delayed Rejection		7 (35%)	1 (6%), p=0.032

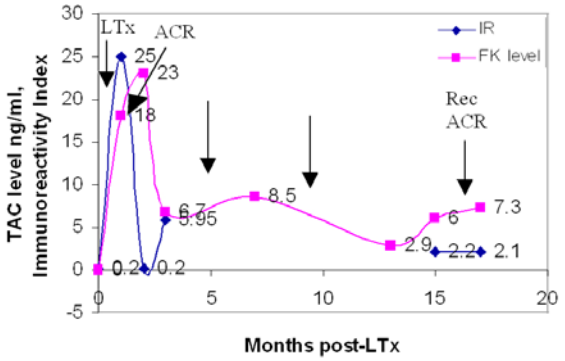

Figure 2. Changes in immunoreactivity index (black line) and TAC (FK) levels in a Rejector who went on to experience recurrent rejection. This 17-year old recipient who received LTx for acute liver failure experienced acute cellular rejection on post-operative day 7. Peak immunoreactivity characterized by an IR>25 (actual 477) was measured on POD 28. It subsided thereafter as TAC levels increased, reflecting ongoing treatment of rejection (peak 23 ng/ml). With reduction of TAC doses to achieve levels <8 ng/ml between post-LTx months 10 and 17, biopsy-proven ACR occurred again in month 17. This was preceded by TAC levels 2.9-7.3 in the three-month period between months 14-17 due to non-compliance. IR during these months was >2 suggesting the persistence of enhanced donor-specific alloreactivity, and persistent ACR-risk.

7 days (median 24 days, range 7-505). These episodes occurred at a median interval of 156 days (range 68-512) after primary LTx. Biopsy-proven ACR was documented in the only Non-Rejector who experienced delayed ACR. A viral syndrome prompted a decrease in SRL dose. Whole blood concentration of SRL was 5 ng/ml at the time of ACR. Except in 2 subjects in whom recurrent ACR was inferred because elevated transaminases were not accompanied by evidence of biliary dilatation or vascular compromise on ultrasound testing, all the children with graft dysfunction had biopsy proven rejection. Routine clinical reduction of immunosuppression in five subjects and non-compliance in two was associated with median TAC/SRL whole blood concentrations of 4±0.6 ng/ml at the time of recurrent ACR. Graft dysfunction resolved in all but one subject after steroids were resumed.

The time course of immunoreactivity is detailed for a 17-year old girl who experienced steroid-resistant recurrent ACR in Figure 2. This patient first received steroids during post-LTx days 8 to 67 for the first episode of ACR. Thereafter, she was maintained on TAC monotherapy titrated to whole blood concentrations < 8 ng/ml at the end of the first year. TAC doses were stable during this period at 5 mg per day. Whole blood concentrations measured thrice during post-LTx months 14-17 were 2.9, 6, and 7.3 ng/ml as shown in Figure 2. She experienced recurrent ACR in month 17, presenting as elevated AST 290 IU/ml and ALT 358 IU/ml. Bolus methyprednisolone was given followed by steroid taper. Despite initial response, her liver enzymes became elevated once again. Repeat steroid bolus and taper were given along with rituximab, and LFTs normalized. Throughout her post-LTx course, donor-specific alloreactivity remained enhanced relative to third party alloreactivity, as suggested by IR=2.1.

5. DISCUSSION

Data presented here validate several clinical observations and characterize them further. Early Rejectors are often ones felt to be at enhanced risk of ACR during drug minimization, and are therefore maintained on higher doses of maintenance immunosuppressive agents. This is seen in our Rejector study group. Over a third experienced recurrent ACR, and TAC whole blood concentrations remained elevated for 10 months in the majority, above the threshold of 8 ng/ml. This increased risk can be attributed to the highly variable time period during which donor-specific alloreactivity remains greater than third-party alloreactivity among Rejectors. Clinically, this may explain why the outcome of weaning of immunosuppression by "protocol" may be less predictable in Rejectors than in Non-Rejectors. Therefore, patients who experience early ACR and are more likely to develop recurrent rejection would benefit from the availability of a predictive index. Not surprisingly, the time required for normalization of LFT did not differentiate Rejectors from Non-Rejectors. For long, clinicians have intuitively articulated the need for a test that allows preemptive intervention, rather than intervention after the fact, when LFT have become elevated.

This has several limitations. The MLR is considered to have a variable output and can also be influenced by the amount of immunosuppressant. To minimize this, the same "donor" and "third-party" stimulators have been used in all measurements from a given patient. Further, donor-induced proliferation is referenced to third-party-induced proliferation measured simultaneously, and expressed as the immunoreactivity index. Another limitation is the availability of an average of two IR measurements per subject. This gives our study a cross-sectional rather than a longitudinal character. This was an unavoidable consequence of subject compliance with study visits in a referral patient population which is not local, and the inconsistent availability of adequate numbers of donor cells. The numbers of pediatric subjects

evaluated (n=36) in this study during the course of a controlled immunosuppressive regimen by the same investigators at a single center, may negate to some extent, these valid criticisms.

The association of immunoreactivity with the risk of rejection in pediatric liver recipients appears analogous to that seen in renal transplant recipients with more sophisticated derivatives of the MLR such as the ELISPOT assay (6). Both approaches allow non-parametric clinical endpoints such as rejection and rejection-free survival to be represented on a continuous scale. In the current study, the risk of rejection is defined by the immunoreactivity index, which has a dynamic range of 0-10 in the majority of subjects in our study. This allowed us to distinguish at a statistical level of significance, the response of two outcomes groups to a controlled regimen using a relatively modest patient sample. Such an approach may prove useful in the evaluation of immunosuppressive regimens within "rare" patient populations. This concept is under investigation in our laboratory.

6. ACKNOWLEDGEMENTS

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