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ALLOREACTIVE T LYMPHOCYTES CULTURED FROM LIVER TRANSPLANT BIOPSIES: ASSOCIATIONS OF HLA SPECIFICITY WITH CLINICOPATHOLOGICAL FINDINGS

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Supported by NIH grants AI 23467 and HL 36416.

[2] Recipient of Fellowship Grant from the Deutsche Forschungsgemeinschaft

Running Title: T-cells grown from hepatic allografts

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Clin Transplant

ABSTRACT

Lymphocyte cultures grown from liver allograft biopsies were shown to exhibit alloreactivity towards donor cells as measured by primed lymphocyte testing (PLT). The PLT specificity was determined in assays using HLA typed panel cells and/or by inhibition testing with HLA specific monoclonal antibodies. Certain cultures exhibited PLT specificity towards class I HLA antigens of the donor, whereas others were specific for class II HLA antigens or recognized mixtures of class I and II antigens. These PLT specificity patterns were compared with clinical, histological and laboratory findings on the liver transplant patients at the time of the biopsy. Biopsies yielding class I specific PLT cells were taken generally during the earlier posttransplant period, whereas class II specific cells were grown from later biopsies. There was no significant correlation of the PLT specificity towards class I vs II antigens with the levels of total or direct bilirubin, serum glutamate oxaloacetic transaminase (SGOT), and serum

glutamate pyruvate transaminase (SGPT), although a trend towards higher values was noted for biopsies presenting with a class II specific infiltrate. However, the levels of gamma glutamyl transpeptidase (GGTP) and alkaline phosphatase (AP) were significantly increased when biopsies yielded class II specific rather than class I specific PLT cells. Biopsy histology showed more damage to bile duct epithelium in association with class II PLT specificity whereas intense but often reversible infiltrates were found in biopsies yielding class I specific cells. The elevated GGTP and AP levels are probably related to the interaction of class II specific T cells with bile duct epithelium, which has been shown to express induced class II HLA antigens on their cell surface.

Keywords: T-lymphocytes, liver transplants, donor HLA alloreactivity, liver function tests, histopathology, bile duct damage

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INTRODUCTION

While liver transplantation has become an accepted form of treatment for a variety of endstage liver diseases, the understanding of the immunobiology of liver transplant rejection is still incomplete. Various findings suggest that the immunologic course of liver transplants differs substantially from that of other solid organ transplants (1-8). T-lymphocytes are known to play a major role in cell mediated allograft rejection (9-10). In cellular infiltrates of transplants it is possible to demonstrate activated T cells expressing Interleukin-2 (IL-2) receptors on their cell surface. These cells are capable of undergoing further proliferation upon exposure to IL-2 (11-12).

Previous studies have shown that activated T-cells can be propagated in vitro from liver allograft biopsies in the presence of exogenous recombinant IL-2 (13,14). Further analysis of these biopsy grown lymphocytes has revealed the presence of alloreactive T-cells specific for donor HLA antigens. Often enough, these cell cultures exhibit restricted specificity patterns against one or few of the donor HLA antigens. This alloreactivity could also be blocked with monoclonal antibodies (moAb) against appropriate HLA determinants. Similar observations have been made with lymphocyte cultures grown from heart transplant biopsies (15). In certain patients, there appeared a

sequential infiltration of the allograft by class I specific lymphocytes in earlier biopsies followed by class II or mixed class I/II specific lymphocytes in later biopsies (16).

The aim of this study was to evaluate the association of the HLA class I and class II allospecificity of lymphocytes grown from liver allograft biopsies with clinical, biochemical and histopathologic findings.

MATERIALS AND METHODS

All liver transplant recipients received cyclosporine and steroids as immunosuppressive drugs. As of December 1984 OKT3 monoclonal antibody therapy has been added to treat acute rejection episodes. Samples of hepatic allografts were obtained from percutaneous liver biopsies or removed allografts. Indications for sampling were derangements in liver function tests and bile composition. It is important to emphasize, that all biopsies were obtained when problems occurred and that these were no protocol biopsies. All samples were taken in a sterile manner for propagation of infiltrating cells and histologic evaluation. No attempt for HLA matching of liver transplants was made, because of the very limited time available for all the events related to the harvesting and transplant procedure.

Liver biopsies were divided into smaller segments and cultured in microculture wells with 100 ul of recombinant IL-2 and 100 ul of tissue culture medium as described previously (13). The cultures were observed daily on an inverted stage microscope and supplemented with IL-2 at 2-3 day intervals. After approximately 2 weeks, sufficient cells ($0.5\text{-}1.0 \times 10^6$) were obtained for primed lymphocyte testing (PLT) assays. The cultured lymphocytes were tested against cryopreserved donor splenocytes, exogenous IL-2 and an informative panel of unrelated lymphocytes with known HLA type. In blocking studies different anti-class I and anti-class II moAb were tested for their inhibitory effect on the PLT response of these cultured T cells. One or more of the following moAb, with their corresponding HLA molecular specificity, were selected: SG157 (anti-DR) and SG465 (broad anti-class II) (S. Goyert and J.Silver [17]); L243 (anti-DR) and Leu 10 (anti-DQw1 + w3)(Becton Dickinson, Mountain View, CA [18-20]); PA2.6 (anti-HLA-A,B,C)(P.Parham[21,22]); or w6/32 (anti HLA - A, B, C)(Pel-Freez, Rogers, Arkansas [23-24]).

Information about the clinical and biochemical data prior to taking a biopsy was obtained from a review of the medical charts with special attention to the time interval after the liver transplant procedure, total and direct bilirubin (T-BIL, D-BIL), serum glutamate oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase

(SGPT), alkaline phosphatase (AP) and gamma glutamyl transpeptidase (GGTP). All values were recorded from the day before obtaining the biopsies.

All liver tissue submitted for histopathologic review was taken from the same biopsy core or from the same region of the liver (in failed allografts) as samples utilized for lymphocyte cultures. The tissues were fixed in neutral buffered formalin, sectioned at 6 microns and routinely stained with hematoxylin, eosin, and trichrome stains. Histologic sections were examined in a blind manner by one of us (A.J.D.). Each biopsy specimen was reviewed according to a defined protocol developed for the National Liver Transplant Data Base (25). This protocol histopathologic examination includes thirty criteria with special emphasis on findings considered to be important in hepatic rejection, namely the presence of a mononuclear portal inflammatory infiltrate associated with ductal epithelial and venular endothelial damage (26).

Statistical analysis of relevant data was performed by the nonparametric statistical tests of Mann-Whitney and Kruskal-Wallis using the SPSSPC software package (27).

RESULTS

For 35 out of 67 lymphocyte cultures established from liver allograft biopsies we could assess a specificity towards HLA class I (n=8), mixed HLA class I and II (n=15) or HLA class II antigens (n=12). Clinicopathologic data including biochemical parameters and histopathological findings are shown in Table 1. It may be seen, that there was considerable heterogeneity in the laboratory values of the total population and often enough in the various subgroups of transplant recipients. Nevertheless, this database proved useful in analyzing differences between biopsies yielding class I versus class II specific T cells after in vitro propagation and expansion.

It was noted, that class I specific cells were generated from biopsies obtained earlier during the post-transplant period than those yielding class II specific cells. The median post transplant periods were 8.5 days (range from 4 to 48) for HLA class I, 17 days (range from 2 to 237) for HLA class I/II and 29 days (range from 5 to 753) for HLA class II specific T-cell cultures (class I vs. class II, $p=0.003$) (Table 2).

No statistically significant differences were found in the bilirubin, SGOT and SGPT values for the different groups, although a trend towards higher values for biopsies

exhibiting class II specific T-cells was noted (Table 2). However we observed statistically significant elevations of GGTP and AP values associated with HLA class II specific cells in the biopsy derived lymphocyte cultures (class I vs class II: for GGTP $p=0.017$, for AP $p=0.014$) (Table 2). A trend versus higher values of bile duct damage was noted in biopsies yielding class II specific lymphocyte cultures. This elevation was statistically significant when the both biopsies presenting only with harvesting injury were excluded from the analysis ($p=0.038$).

Histopathologic examination showed that 5 of 8 biopsy specimens yielding class I HLA specific lymphocytes, had undergone mild to severe portal tract inflammation. The cellular infiltrate consisted of an admixture of cells typical of that described for acute cellular rejection (26). Two patients in the class I specific group showed histological signs of recurrent hepatitis. The pretransplant diagnosis of patient 942 was chronic liver failure due to non A, non B hepatitis and patient 914 originally had endstage liver disease due to chronic active hepatitis B. The percentage of damaged biliary ducts was in all cases equal to or less than 60%.

Biopsies from specimens with HLA class II specificity only, generally showed mild to moderate portal tract infiltration. In no instance was the portal inflammation

scored as "severe". Two biopsies (patients no. 499, biopsy from day 5; 647, biopsy from day 6) in the class II only group were obtained during the first week post transplant. Both were diagnosed as harvesting injury. Based on the histologic evaluation, rejection was not thought to be the cause of graft dysfunction in these two instances, although biopsy grown lymphocytes showed donor specific alloreactivity.

The remaining allograft biopsy specimens yielding class II only specific lymphocytes showed a mild (n=5) or moderate (n=5) portal infiltrate. In these ten biopsies, the percent of bile duct damage in patients with primary rejection was higher than in both other groups. These and subsequent biopsy samples from four patients (patients no. 339, 346, 353, 472) showed indolent, ongoing chronic rejection with prominent duct damage. Three of these grafts were later lost because of a paucity of bile ducts and obliterative vasculopathy.

Biopsies yielding both HLA class I and II reactive lymphocytes displayed histopathologic findings intermittent between the two groups. The diagnoses varied from preservation injury to severe rejection. Notably, one of the patients with both class I and II donor specific reactivity (patient no. 673) experienced progressive graft dysfunction resulting in later graft loss from chronic rejection.

In four patients two biopsies were taken from the same graft at various times post transplant (patients no. 673 first graft, 673 second graft, 490, 493). In three of these cases serial biopsies showed the same specificity for the infiltrates. In one case (patient no. 490) the first biopsy showed a class I specific infiltrate and a biopsy performed four days later showed a mixed class I and II specific infiltrate. For patient 673 both biopsies of the first liver allograft rendered a class I specific infiltrate, while biopsies of the retransplant showed very early a mixed class I and II specific infiltrate.

DISCUSSION

Immunocytochemical analysis has previously shown a selective destruction of bile ducts in liver allografts undergoing rejection as demonstrated by striking elevations of GGTP activity (28). It has been postulated that the induced expression of DR antigens on structures targeted for immune destruction may be an important event in the pathogenesis of liver allograft rejection. The current analysis suggests that the increased destruction of bile duct epithelium might be associated with HLA class II specific lymphocytes invading the allograft tissue and that this damage might be selectively restricted to HLA class II bearing target structures, namely the biliary epithelium. In our analysis infiltration with class II specific cells was

associated with a statistically significant elevation of mean GGTP, AP, and a higher degree of bile duct damage. GGTP is specifically released at times of biliary epithelial cell (BEC) damage. In addition BEC are the main structural element being capable of expressing HLA class II antigens in the liver allograft and are a preferential target for invading lymphocytes (28). These events might not be easily revealed at histologic examination, since reports from heart biopsies presenting with a histological grade 0 according to Billingham (no lymphocytic infiltrate) have shown, that still from about 30% of these biopsies activated lymphocyte cultures can be established (29).

Even at later days post-transplant two biopsy specimens presented with a class I infiltrate (patients no. 914, 942). Both rendered a histological primary diagnosis of recurrent hepatitis. Hepatocytes are believed to express mainly class I antigens (30, 31 submitted) and viral infections, restricted to hepatocytes are shown to increase class I antigen expression (32). This might explain an association of class I specific T cell infiltrates with infectious process in the liver allograft restricted to hepatocytes.

Previous studies have shown that biopsy grown lymphocytes from certain heart allografts exhibited a trend towards HLA class I specific cells being more predominant in earlier biopsies, followed by mixed HLA class I/II or class

II specific cells in later biopsies (16). The same trend was noted here in lymphocytes grown from liver allografts. Class I specific lymphocytes might play a role in inducing the primary steps of the rejection cascade. On vascular endothelial cells class I antigens rather than class II are present early after transplantation. These class I antigens can serve as specific recognition structures, thereby facilitating the preferential adherence of class I specific lymphocytes to vascular endothelium and their subsequent migration into the surrounding tissues (29, 33 submitted).

Through the release of lymphokines, specifically gamma-Interferon (Y-IFN), these invading class I specific lymphocytes can promote additional HLA class II antigen expression on the vascular endothelial cells (34 submitted) with subsequent class II specific lymphocyte adherence and invasion (33 submitted). These class II specific lymphocytes might then recognize class II antigens on BEC, leading to subsequent BEC damage as shown by elevated serum levels of GGTP and AP.

In addition, various other factors such as ischemia and viral infections might serve as class II antigen promotors on vascular endothelial cells and BEC, thereby facilitating class II specific infiltrates and promoting class II specific intragraft effector mechanisms. Interestingly, harvesting injury was determined to be the major cause of

liver dysfunction in both cases in which biopsies were taken during the first week after transplantation and the subsequent lymphocyte culture displayed HLA class II specificity.

The limited experience does not allow firm conclusions, but it seems interesting to note, that class I specific infiltrates even when showing more severe signs of acute rejection were associated with a favorable response to the currently used rejection treatment with OKT3, steroids, adjustment of Cyclosporine and in some cases additional Imuran. On the other hand biopsies from 4 allografts showed at histologic examination chronic rejection with a very high degree of bile duct loss. All these specimens rendered a class II specific infiltrate. There was no biopsy showing chronic rejection in the class I or the mixed class I/II specific groups.

The histopathologic findings described in this report although limited in number, offer some interesting avenues for further investigation. However, more evaluation is needed in terms of defining the type of donor specificity (i.e. proliferative vs cytotoxic response), examining any lymphocyte activity against viral or autoimmune antigens, the addition of greater numbers to confirm these observations, and the sequential culturing of samples from the same patient.

ACKNOWLEDGEMENTS

The authors would like to thank the Pittsburgh Transplant Foundation for their invaluable aid in donor sample procurement. We also would like to thank Carlos Esquivel, Shunzaburo Iwatsuki, Leonard Makowka, Robert Gordon, Wallis Marsh, and Andrei Stieber for their cooperation.

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Table 1: Clinicopathologic data associated to liver allograft biopsies[a]

PATIENT NO.	GRAFT NO.	DAYS BIOPSY POST LTX	BROAD HLA SPECIFICITY	SPECIMEN TYPE[b]	BILIRUBIN D mg/dl	BILIRUBIN T mg/dl	SGPT U/ml	SGOT U/ml	GGTP IU/L	AP IU/L	PORTAL TRACT INFLAMMATION[c]	PERCENT BILE DUCT DAMAGE	HISTOPATHOLOGIC DIAGNOSIS[d]
237	2	7	I	LB	1.7	2.2	30	9	126	108	++	60	moderate ACR
490	1	9	I	LB	10.0	14.9	322	190	97	18	++	20	moderate ACR
493	1	8	I	FG	9.1	12.6	1472	673	98	155	+++	40	moderate ACR
670	1	8	I	LB	3.0	5.2	26	35	92	106	+	0	non specific changes
673	1	4	I	LB	4.8	7.8	208	94	251	249	+	30	mild ACR
673	1	18	I	LB	7.3	10.7	6	4	182	185	+++	60	severe ACR
914	2	48	I	LB	1.6	2.3	62	114	397	277	++	20	recurrent hepatitis, mild ACR
942	1	29	I	LB	3.8	5.7	12	36	158	201	+	20	moderate recurrent hepatitis
339	2	753	II	FG	10.7	15.5	1289	2478	249	491	++	90	chronic rejection
346	2	54	II	FG	10.8	16.4	666	477	1026	516	++	90	chronic rejection, PVT
353	1	665	II	LB	1.1	1.8	354	270	848	226	+	60	chronic rejection
472	1	73	II	LB	1.2	1.7	183	128	730	391	++	90	chronic rejection
473	1	34	II	LB	2.9	5.1	119	125	383	344	+	60	moderate ACR
499	1	5	II	FG	13.4	22.6	5449	3228	216	232	+	0	harvest. injury
499	2	25	II	FG	7.8	12.5	217	106	358	320	+	20	mild ACR
629	1	17	II	FG	14.0	19.7	28	28	238	153	+	25	mild ACR, ischemic injury
647	2	6	II	LB	11.7	16.6	1026	1007	94	100	-	0	harvest. injury
752	1	28	II	LB	1.9	2.5	29	37	371	400	++	30	mild ACR, CMV hepatitis
987	1	21	II	LB	5.0	7.9	26	28	359	390	++	50	moderate ACR, harvest. injury
1029	1	30	II	LB	3.6	4.6	60	63	1031	300	+	70	mild ACR, steatosis
490	1	13	I and II	FG	27.6	47.5	2252	978	167	97	++	20	moderate ACR
493	2	12	I and II	LB	7.1	11.0	99	54	670	262	+++	40	moderate ACR, ischemic injury
493	2	39	I and II	LB	2.9	4.2	45	49	465	765	+++	40	moderate ACR, ischemic injury
497	1	4	I and II	LB	9.1	14.8	552	448	222	306	+	40	mild ACR
645	1	17	I and II	FG	1.0	1.4	38	16	143	203	++	35	moderate ACR
673	2	2	I and II	LB	5.2	8.4	298	405	178	370	+	30	mild ACR
673	2	6	I and II	LB	3.8	6.0	194	188	162	279	-	0	non specific changes
801	1	75	I and II	LB	1.5	2.5	26	16	122	220	-	0	non specific changes
838	1	13	I and II	LB	13.6	17.5	24	11	226	535	++	75	moderate ACR
905	1	237	I and II	LB	0.2	0.9	252	146	206	186	++	60	moderate ACR
952	1	26	I and II	LB	1.3	2.2	11	17	47	64	+	65	mild ACR
994	1	42	I and II	LB	1.1	1.6	22	18	773	1563	++	55	ACR treated
1052	1	9	I and II	LB	10.8	15.1	91	83	127	82	+	60	ischemic injury, ACR treated
1052	2	33	I and II	FG	24.1	34.6	58	101	2936	1053	+	90	moderate ACR, VBDS
1079	1	20	I and II	FG	13.2	20.4	13	29	73	83	++	40	moderate ACR, focal infarcts

[a] all biochemical data recorded from the day before obtaining the allograft biopsy
[b] LB = Percutaneous liver biopsy, FG = Failed and removed graft
[c] +++ = severe, ++ = moderate, + = mild, - = none
[d] ACR = acute cellular rejection, PVT = portal vein thrombosis, VBDS = vanishing bile duct syndrome, CMV = Cytomegalovirus

Table 2: Analysis of clinicopathologic data associated to HLA specificity of liver allograft infiltrating lymphocytes

CLASS	BIOPSIES	MEDIAN (RANGE) DAYS TO LTX	BILIRUBIN D mg/dl	BILIRUBIN T mg/dl	SGPT U/ml	SGOT U/ml	GGTP IU/ml	AP IU/ml	BILE DUCT LOSS
I	8	8.5 (4-48)	4.3 (1.6-10.0)	6.7 (2.2-14.9)	46.0 (6-1472)	65.0 (4-673)	142.0 (92-397)	170.0 (18-277)	25.0 (0-60)
I/II	15	17.0 (2-237)	5.2 (0.2-27.6)	8.4 (0.9-47.5)	58.0 (11-2252)	54.0 (11-978)	178.0 (47-2936)	262.0 (64-1563)	40.0 (0-90)
II	12	29.0 (5-753)	6.4 (1.1-14.0)	10.2 (1.7-22.6)	200.0 (26-5449)	126.5 (28-3228)	365.0 (94-1031)	332.0 (100-516)	55.0 (0-90)
P-values for class I vs. class II:									
all biopsies:		0.076	0.440	0.487	0.203	0.142	0.017*	0.014*	0.212
only biopsies with histologic evidence of rejection:		0.003*	0.806	0.806	0.902	0.540	0.005*	0.010*	0.038+*

* statistically significant

\+ only two biopsies showing harvesting injury were excluded from the analysis