

731

Analysis of T Lymphocytes Infiltrating Human Hepatic Allografts

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LIVER TRANSPLANTATION has become an accepted form of treatment for a variety of end-stage liver diseases. In addition to improved surgical techniques, important advances in the immunosuppressive treatment of hepatic allograft recipients have been made through the introduction of cyclosporine (Cs) and, more recently, the addition of a T cell-specific murine monoclonal antibody (MoAb) OKT₃ to treat acute rejection episodes.^{1,2} In spite of these achievements, allograft rejection remains a major problem in liver transplantation.^{3,4}

T lymphocytes play a major role in cell-mediated allograft rejection. During the course of alloactivation, T cells are primed against donor antigens. Alloactivated T cells then express Interleukin-2 (IL-2) receptors on their cell surface, and IL-2 is required for their further propagation. Based on this rationale, IL-2 has been used to propagate alloactivated T cells in vitro from liver allograft biopsies. Earlier studies have demonstrated the feasibility of these methodologies using IL-2 as a sole source of growth require-

ment. Further analysis revealed proliferative as well as cytotoxic responses of these cultured cell lines to donor HLA antigens present on donor splenocytes.⁵ We report results of our extended study which now includes 36 biopsies from 31 liver transplant recipients.

MATERIALS AND METHODS

All liver transplant recipients received Cs and steroids as immunosuppressive drugs. Samples of hepatic allografts were obtained from percutaneous liver biopsies, intraoperative liver biopsies, or removed allografts. Indications for sampling were derangements in liver function tests and bile composition. At times of allograft hepatectomies, more extensive tissue samples were obtained, as were lymph node samples from the hilar region of the removed allograft.⁶ All samples were taken in a sterile manner for propagation of infiltrating cells and histologic evaluation. Liver biopsies were divided into smaller segments and cultured in microculture wells with 100 μ L of IL-2 and 100 μ L of tissue culture medium. The cultures were observed daily on an inverted-stage microscope and supplemented with IL-2 at 2- to 3-day intervals. After ~2 weeks, sufficient cells (0.5 to 1.0×10^6) were obtained for further studies. Cell surface phenotypic analysis of biopsy-grown T cells was achieved using a modification of the avidin-biotin-immunoperoxidase technique. Immunostaining was also done on lymphocyte suspensions extracted from rejected liver allografts. Primed lymphocyte testing (PLT) and cell-mediated lympholysis (CML) assays were performed. In blocking studies, different anti-class I and anti-class II MoAb were tested for their inhibitory effect on the PLT response of cultured biopsy T cells using previously described methods.⁵

RESULTS

Patient Profile

Thirty-one liver allograft recipients provided 36 transplant biopsies at various time intervals after transplantation, ranging from 3 days to 330 days. Samples were taken on 27 occasions from first allografts and on nine occasions from second allografts. The population of liver allograft recipients consisted of patients with end-stage liver failure due to a

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variety of causes as well as patients with inborn errors of metabolism and hepatic malignancy.

Phenotype Analysis

Biopsy-grown lymphocytes were usually analyzed for cell surface phenotype markers after 2 to 4 weeks in culture. Analysis of five culture lines revealed a predominance of CD3-positive T lymphocytes, many of which also expressed DR antigens as an indication of activation. A mixture of CD4 and CD8 positive cells was found in most cultures, with a trend toward CD4 predominance. Two cell preparations extracted by mechanical disruption from rejected liver allografts showed phenotype profiles similar to those of biopsy-grown cells.

Allospecificity Determined in Secondary Proliferation Assays

All tested cell lines exhibited low background thymidine incorporation, but a high proliferative response to IL-2, suggesting the presence of activated T cells. For 21 biopsy cultures, we had available cryopreserved splenocytes from the original transplant donor. The majority of these biopsy-derived T cell culture lines showed a high proliferative response to original donor antigens present on splenocytes and informative panel cells of known HLA types. Table 1 shows representative examples of IL-2-induced and donor cell-induced proliferation of cultured biopsy cells. Their specificity was determined against a panel of unrelated cells selected to share HLA antigens with the original transplant donor. In

most cases, the PLT specificity of biopsy-grown cells corresponded to one or more class I and/or class II HLA antigens of the donor.

Patient M.R. (Fig 1A) was HLA-typed as A2,-;B35,-;DR4,7, and the corresponding donor was A25,32; B18,60;DR2,4. Lymphocytes grown from a biopsy 17 days posttransplant appeared to be specific against DR2 and DR4 antigens of the donor. Despite the fact that both recipient and donor carried the DR4 antigen, the DR4 specificity can be explained by the complexity of the DR4 antigen. Patient C.L. (Fig 1B) typed as A1,-;B8,62;DR2,3. The allograft from the first donor (A2,31;B38,41;DR5,6) was rejected after 18 days. The second transplant came from a donor who typed as A2,3;B14,44;DR4,7. Lymphocytes grown from a biopsy 6 days after retransplantation showed a high PLT response related to DR4 and DR7 antigens shared by the second donor. No significant proliferation was detected using spleen lymphocytes from the first donor as stimulators.

Patient L.W. (A2,-;B35,51;DR4,6) received a second liver allograft from a donor typed A3,11;B7,40;DR2,5. This transplant was rejected 25 months later, and lymphocytes grown from a biopsy obtained from the removed allograft showed a high proliferative response to DR2 and DR5 antigens shared by the second donor (data not shown). There was also strong proliferation of lymphocytes from this biopsy toward splenic cells from the donor (A2,28;B44,70;DR1,11) of the third liver transplant. Histologic examination of biopsies from this third allograft showed on day 10 acute and day 28 partially treated rejection.

Table 1. Proliferative Responses and Allospecificities of Liver Biopsy-Grown Lymphocytes

Liver Biopsy	Proliferative Responses (cpm \pm SD)			PLT Alloreactivity
	Background	Exogenous IL-2	Donor-Stimulated	
1	585 \pm 105	12,815 \pm 9	4,344 \pm 1,385	DR7, DR4
2	1,272 \pm 585	4,152 \pm 170	44,802 \pm 1,280	A11, DR6
3	692 \pm 127	49,797 \pm 1,085	32,337 \pm 943	DR2, DR4
4	1,612 \pm 490	35,691 \pm 2,097	51,620 \pm 2,005	Undetermined
5	335 \pm 38	6,936 \pm 380	9,397 \pm 1,395	B53, B44

Abbreviations: IL-2, Interleukin-2; PLT, primed lymphocyte testing.

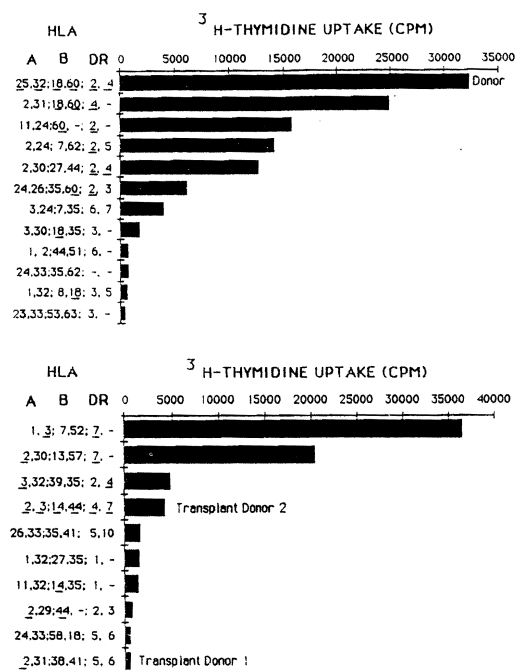


Fig 1. Primed lymphocyte testing (PLT) specificities of T lymphocytes grown from liver allograft biopsies. Tritiated thymidine uptake was determined in 3-day proliferation assays. HLA antigens of the panel cells are listed; those shared with the donor are underlined. **A:** Results from lymphocytes obtained from a biopsy of a removed allograft 17 days posttransplant from patient M.R. Background proliferation in 10% AB serum was 692 ± 127 . **B:** PLT results from lymphocytes obtained from a biopsy 6 days after retransplantation from patient C.L. Splenocytes from both the second transplant donor and the first donor were used as stimulators. Background proliferation in 10% AB serum was 928 ± 245 .

Finally, this graft had to be removed with additional signs of ischemic injury.

Serial Biopsies

Serial liver biopsies were obtained from four liver allograft recipients. One patient had allograft biopsies on day 9 and day 13 after transplant. PLT specificity of cells grown from the early biopsy corresponded to class I antigens, whereas cells from the later biopsy appeared to be specific for class I and class II antigens. Two T cell lines grown from biopsies of two consecutive allografts from a second

patient both showed class II antigen specificity. Another patient received two consecutive allografts. T cells from the biopsy of the first allograft appeared to be specific for donor class I antigens, whereas T cells from two subsequent biopsies of the second allograft both showed PLT specificity against a mixture of class I and class II antigens. In one patient, an OKT₃ MoAb treatment was started 4 days after transplantation of the hepatic allograft. T cell lines from day 3 posttransplant and day 17 (14 days from start of OKT₃ treatment) both showed class I PLT reactivity, with a possible weak class II effect for the later one. The class I reactivity was confirmed by MoAb inhibition studies with 41.5% and 57.7% inhibition of class I activity, respectively. After 2 weeks of OKT₃ administration, the treatment was not yet successful and was subsequently extended for another 7 days. The patient then responded well to the therapy.

DISCUSSION

These studies further extend our previous observations that alloactivated T cells can be readily propagated from liver transplant biopsies.^{5,7,8} They demonstrated that it is possible to culture these cells in IL-2 without the requirement of any type of exogenous feeder cells, which means that the current methodology minimizes the risk of de novo activation of cultured intra-graft T lymphocytes. The IL-2 responsiveness and HLA-specific PLT reactivity demonstrate the presence of donor-specific alloactivated T cells in lymphocyte cultures grown from the liver transplant biopsies.

Phenotypic analysis revealed that both CD4- and CD8-positive lymphocytes can be propagated from liver allograft biopsies. There did not appear to be a consistent pattern of either CD4 or CD8 in association to class I or class II allospecificities. A trend toward predominance of CD4-bearing lymphocytes was noted. On two occasions, we were able to compare phenotypic analysis of biopsy-grown

lymphocytes with *in situ* lymphocytes obtained from allograft tissues at times of allograft removal. Both showed similar patterns of CD4 and CD8 cell surface markers.

Both class I and class II antigens on donor lymphoid cells can serve as recognition antigens in proliferation and cytotoxicity assays for biopsy-derived T cells. Panel cell analysis indicates that the PLT response to donor alloantigens may often be restricted to a few HLA antigens. We have preliminary evidence for a time-related pattern showing that during the early posttransplant period generally more class I-specific T cells can be grown from the allograft. Later, the biopsy cultures tend to contain mixtures of class I- and class II-specific cells or, often, predominantly class II-specific cells. When serial biopsies from allograft recipients were obtained, a shift from a class I PLT activity pattern to a mixture of class I and class II specificities was often observed. This is in accordance with findings in the heart allograft in which a sequential infiltration pattern has been described.⁸ Exceptions may occur, however, whereby early biopsies generate only class II-specific T cells.

Ongoing studies on limiting dilution analysis of biopsy-derived T cell cultures have demonstrated clones specific for donor antigens in

concordance to specificities of bulk culture lines as defined in PLT assays with panel cells. A small number of clones exhibit no PLT response toward donor lymphoid cells although they proliferate well in the presence of IL-2. It is not known whether these lymphocytes have been sensitized to other non-HLA antigens (eg, vascular endothelial antigens) or whether they have different functions.

We have previously shown that the PLT response of cultured-biopsy lymphocytes can be inhibited by various defined anti-HLA MoAbs.⁵ This further confirms the class I- or class II-associated specificity patterns found in secondary proliferation assays using panel cells. Several biopsy-derived lymphocytes have also shown cytolytic activity against donor targets. Using PHA-transformed T lymphoblasts (class I targets) and Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines (class I and class II targets), we were able to demonstrate that cytolytic activity of certain biopsy-grown cells was specific for class I antigens whereas others recognized class II antigens or both.^{5,9}

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