Transpl Int (1998) 11 [Suppl 1]: S239–S241

A.P. van den Berg
A. de Haan
B.G. Hepkema
E. van Dijk
E. van Twuyver
I.J. Klompmaker
L.H.F.M. de Leij
L.P. de Waal
M.J.H. Slooff
T.H. The

Donor-specific immunological non-responsiveness after liver transplantation

A.P. van den Berg (✉) · I.J. Klompmaker
Department of Gastroenterology and Hepatology, University Hospital, PO Box 30.001, 9700 RB Groningen, The Netherlands
Fax +31 50 361 31 51; e-mail A.P. van.den.Berg @interne.AZG.NL

M.J.H. Slooff
Department of Surgery, University Hospital, Groningen, The Netherlands

A. de Haan
Department of Cardiopulmonary Surgery, University Hospital, Groningen, The Netherlands

B.G. Hepkema
Laboratory for Transplantation Immunology, University Hospital, Groningen, The Netherlands

E. van Dijk · L.H.F.M. de Leij · T.H. The
Department of Clinical Immunology, University Hospital, Groningen, The Netherlands

E. van Twuyver · L.P. de Waal
Department of Transplantation Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

Abstract Since the rate of immunological losses of liver allograft after the immediate posttransplant period is much lower than in other organs, we studied the immune responses against donor HLA antigens in 18 patients with a good long-term outcome to determine whether the development of a state of immunological non-responsiveness to donor antigens might account for this favorable outcome. The reactivity against donor spleen cells was measured before and 2 years after transplantation. The reactivity in mixed lymphocyte culture (MLC) and the frequencies of cytotoxic T cell precursors (CTLp) were determined. Responses against third-party spleen cells were determined concurrently to exclude a generalized reduction of immunocompetence due to chronic immunosuppressive treatment. Before orthotopic liver transplantation, the majority of patients had normal T cell responses against donor antigens that were comparable to those against third-party antigens. Two years after transplantation, donor-specific MLC non-reactivity had developed in 10 of the 18 (56%) patients. In addition, 15 of 18 (83%) patients had developed donor-specific cytotoxic T cell (CTL) non-responsiveness; 2 had reduced numbers of CTLp against both donor and third-party cells, while the remaining patient had maintained reactivity against donor antigens. In conclusion, donor-specific non-responsiveness is present in the majority of patients 2 years after successful liver transplantation, but occurs predominantly at the CTL level.

Key words Donor-specific immunological non-responsiveness · Cytotoxic T cells · Mixed lymphocyte culture · Liver transplantation

Introduction

Liver allografts appear to be immunologically privileged because the incidence of graft loss due to chronic rejection is low, and HLA matching is not required. We hypothesized that this might be the result of the development of a state of immunological non-responsiveness. Indeed, eight of nine patients had developed donor-specific cytotoxic T cell (CTL) non-responsiveness 2 years after successful liver transplantation, whereas a comparable degree of donor-specific non-responsiveness occurs only infrequently in kidney transplant patients [6]. Matthew et al. reported previously that CTL against donor antigens had disappeared from the peripheral blood in five of six liver transplant patients [3], but no correlation between changes in frequencies of donor-directed

CTL were found by Eberspacher et al. [2]. All these studies involved only a small number of patients. The results of our pilot study may also have been influenced by the fact that only patients with primary biliary cirrosis (PBC) were included [6]. We, therefore, have extended our observations and have also started to analyze the reactivity against donor antigens in patients transplanted because of other underlying diseases.

Materials and methods

Patients

A total of 18 recipients of a first liver transplant with good graft function after 2 years were studied. Underlying diagnoses were PBC ($n = 13$), primary sclerosing cholangitis ($n = 1$), alcoholic cirrosis ($n = 1$), cryptogenic cirrosis ($n = 1$), and congenital fibrosis of the liver ($n = 1$). Results of nine of the PBC patients have been published before [5]. Immunosuppression consisted of cyclosporine A (CSA), azathioprine, and prednisolone, with a 1-week induction course of cyclophosphamide as reported previously [1]; at 2 years, all but one patient received CSA (trough levels, as determined by HPLC, between 75 and 125 ng/ml), 10 mg of prednisolone, and 125 mg of azathioprine daily; the remaining patient was on prednisolone with azathioprine. Eight patients had experienced an episode of acute rejection, which was confirmed by needle biopsy in all but one patient. Rejection treatment consisted of methylprednisolone pulses ($n = 4$), a steroid recycle ($n = 3$), or both ($n = 1$); 2 patients with steroid-resistant rejection received rabbit anti-thymocyte globulin, as previously described [1].

Mixed lymphocyte culture (MLC)

Cryopreserved recipient peripheral blood cells (5×10^4) were cocultured for 5 days with 5×10^4 20-Gy-irradiated donor or third-party spleen cells. Cells were cultured in RPMI 1640 (Gibco) supplemented with 20% fetal calf serum in flat-bottomed microtiter plates (Costar) in a total volume of 200 µl per well. Proliferation was determined by measuring ^{3}H thymidine incorporation.

Determination of the frequency of cytotoxic T cell precursors (CTLp)

Details on CTLp frequency determination in the first nine patients has been described [6]. In the next nine patients the protocol was slightly modified. Limiting dilution assay cultures were set up in 96-well round-bottomed microculture plates (Costar) in RPMI 1640 medium, supplemented with L-glutamine (Gibco), gentamycin (Gibco; 0.06 mg/ml), 15% pooled human AB serum (hereafter referred to as RPMI medium). Graded numbers of recipient responder cells (5×10^4–1.25×10^3 cells per well in RPMI medium; 24 replicate cultures per responder concentration) were cultured with 5×10^4 50-Gy-irradiated stimulator cells (donor or third-party spleen cells) in RPMI medium. After 3 days of culture, a 50 µl sample of culture fluid was transferred to round-bottomed 96-well plates for future assessment of IL-2 production. Fresh RPMI medium supplemented with 5 IU IL-2 (Cetus) per ml was added which was repeated on day 6. The CTLp frequencies were determined on day 10 by assessing the capacity of the cultured cells to kill ^{51}Cr-labeled target cells. Target cells were donor or third-party-derived spleen cells stimulated for 2 days with PHA (10 µg/ml) and, subsequently, for an additional 3 days with Il-2 (5 IU IL-2/ml) prior to labeling. Labeling was done with 100 µCi ^{51}Cr (Amersham) for 1 h followed by extensive washing. After removal of 100 µl of culture fluid, 3×10^3 ^{51}Cr-labeled target cells per well were added. After 4 h, supernatants were harvested and the released ^{51}Cr was measured. Wells were scored positive if readings were higher than the mean counts, plus 3 SD of the control wells containing stimulator cells only. Target cells with HLA typing unrelated to donor or third party were not lysed in these assays, indicating that donor or third-party cell lysis was HLA antigen specific.

Frequencies and statistical analysis

Frequencies of donor or third-party allotype specific T cells were determined by the chi-squared minimization procedure using a computer program based on statistical methods described by Taswell [5]. Frequencies were considered reliable only if probability values were indicative of single hit kinetics. P values for differences between MLC reactivity and precursor frequencies before and after orthotopic liver transplantation (OLT) were determined using the Wilcoxon test for paired observations. Values below 0.05 were considered to indicate statistical significance.

Results

MLC reactivity before and after transplantation

The median stimulation index of the response against donor spleen cells decreased from 28.5 (range 10–330) before OLT to 8 (1–160) at 2 years ($P = 0.018990$), whereas the response against third-party cells did not change significantly [from 19.5 (5–370) to 20.5 (1–360), $P = 0.899890$]. Ten of the 18 (56%) patients developed a state of donor-antigen-specific non-responsiveness, but 6 patients (33%) maintained reactivity against donor antigens.

CTLp frequencies before and 2 years after transplantation

Before transplantation most patients showed high numbers of CTLp both against donor and third-party antigens. Two years after OLT, numbers of anti-donor CTLp had decreased strongly from a median 358 (range 34–942) per 10^6 to 5 (range 1–623) per 10^6 ($P = 0.000196$), whereas the reactivity against third-party antigens had not changed significantly, from 211 (range 6–1432) per 10^6 to 240 (range 5–1190) per 10^6 ($P = 0.447788$) (Fig. 1). Donor-specific non- or hyporesponsiveness, as defined by a significant decrease in the frequency of CTLp against donor antigens in the presence of maintained anti-third-party reactivity, occurred in 15 of the 18 patients (83%). In 2 patients, CTLp both against donor and third-party antigens had de-

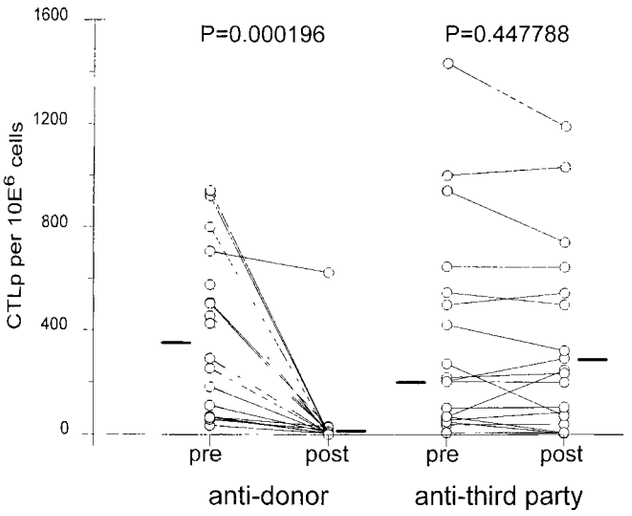

Fig. 1 Cytotoxic T cell (CTL) reactivity, represented as the numbers of CTL precursors *(CTLp)* per 10^6 peripheral blood mononuclear cells, against donor and third-party spleen cells before and 2 years after orthotopic liver transplantation. Median values are indicated by *horizontal lines*

creased, whereas no decrease in the number of CTLp against donor antigens occurred in the remaining patient. Twelve of the 13 patients with PBC developed donor-specific non-responsiveness, compared with 2 of the 4 patients transplanted because of other diagnoses; donor and third-party non-reactivity was present at 2 years in the other 2 non-PBC patients.

Discussion

These data confirm and extend our previous observations on the immunoreactivity against donor antigens after liver transplantation [6]. Donor-specific CTL non-responsiveness developed in 83% of patients during the first 2 years after transplantation. An additional 11% had low numbers of CTLp both against donor and third-party splenocytes, suggesting a more general reduction of immune reactivity. Donor-specific non-reactivity occurred both in patients transplanted because of PBC as well as those transplanted for other reasons. Helper T cell responses, as suggested by the MLC results, also decreased after OLT, but just over 50% of the patients became donor-antigen non-responsive in this test system. This is lower than reported in our pilot series [6], but in agreement with observations by Reinsmoen et al. who could demonstrate donor-antigen-specific hyporesponsiveness in 10 of 25 (40%) liver transplant recipients [4].

Our results differ from those of Eberspacher et al., who found a biologically significant decrease in the number of donor-directed CTLp in only 3 of 13 (23%) patients. This may be due to differences in the immunosuppressive regimen: all but 1 of our patients received triple therapy, compared with only 6 in the study of Eberspacher, 4 of which had decreased reactivity against the donor. In addition, we studied patients 2 years after OLT, whereas the longest observation time in the study of Eberspacher was 22 months, with only 7 of the 13 patients studied after 12 months. Although we do not yet exactly know the time frame for development of non-responsiveness, 1 year may be too short. Additional studies are in progress to answer this question, and to elucidate the mechanism underlying the development of CTL non-responsiveness. It is tempting to speculate that CTL non-reactivity against donor antigens, because of its high incidence, is just an epiphenomenon of a successful liver transplant. Our MLC data suggest that non-responsiveness occurs not nearly as frequently at the helper T cell level and, therefore, may be a more relevant marker for tolerance and the requirement for less immunosuppression.

Acknowledgement This work was supported by De Nederlandse Maag Lever Darm Stichting.

References

1. Bijleveld CGE, Klompmaker IJ, Berg AP van den, Hepkema BG, Gouw ASH, Haagsma EB, Lems SPM, Verwer R, Slooff MJH (1996) Incidence, risk factors, and outcome of anti-thymocyte globulin treatment of steroid-resistant rejection after liver transplantation. Transpl Int 19: 570–577
2. Eberspacher MLL, Otto G, Herfarth C, Kabelitz D (1994) Frequency analysis of donor-reactive cytotoxic T lymphocyte precursors in liver allograft recipients. Transplantation 12: 1746–1752
3. Matthew JM, Marsh JW, Susskind B, Mohanakumar T (1993) Analysis of T cell responses in liver allograft recipients. Evidence for deletion of donor-specific cytotoxic T cells in the peripheral circulation. J Clin Invest 91: 900–906
4. Reinsmoen NL, Jackson A, McSherry C, Ninova D, Wiesner RH, Kondo M, Krom RAF, Hertz MI, Bolman RM III, Matas AJ (1995) Organ-specific patterns of donor antigen-specific hyporeactivity and peripheral blood allogeneic microchimerism in lung, kidney, and liver transplant recipients. Transplantation 60: 1546–1554
5. Taswell C (1981) Limiting dilution assays for the determination of immunocomponent cell frequencies. I. Data analysis. J Immunol 126: 1614
6. Twuyver E van, Hoop J de, Berge RJ ten, Wilmink JM, Lems SP, Berg AP van den, Slooff MJH, Waal LP de (1996) Comparison of T cell responses in patients with a long-term surviving renal allograft versus a long-term surviving liver allograft. Transplantation 61: 1392–1397