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Characterization, quantification, and localization of passenger T lymphocytes and NK cells in human liver before transplantation

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Abstract Quantification and localization of the main lymphocyte populations were studied in the livers of normal ($n = 8$) and brain dead ($n = 8$) subjects. Cytometric analysis performed on mononuclear cell suspensions obtained from liver biopsies was compared to an automatic image analysis of immunostained sections. The overall number of liver associated lymphocytes was in the usual range of peripheral blood content (2 to $9 \cdot 10^9$ cells). Phenotypic analysis showed predominant NK and CD8+ cells that highly expressed class II antigen and CD25 and CD69 activation markers. Quantitative mapping of these activated lymphocytes revealed their preferential localization in the portal tract and the perisinusoidal area as compared to the pericentrolobular zone, especially in donor livers. This strategic localization could suggest a possible early cooperation between donor lymphocytes and initial infiltrating cells from the recipient and could explain the special immunological status of allografted livers.

Key words Liver transplantation, T lymphocytes, NK cells · T lymphocytes, NK cells, liver transplantation · NK cells, T lymphocytes, liver transplantation

Introduction

T-cell populations infiltrating a liver allograft have been studied extensively [3, 10]. Interaction between graft biliary or vascular epithelium and lymphocytes of recipient origin is one of the main features of acute rejection, but the consequences of the initial infiltration of the graft by recipient cells remain controversial: no pattern specific to rejection could be described among the subsets of infiltrating lymphocytes [4]. In the absence of antilymphocyte prophylaxis, which is a rule in liver transplantation but not in the transplantation of other organs, constitutive cellular components of the liver might account for the moderate intensity of acute rejection and the relative tolerance of liver allografts. One of the main reasons proposed is that the liver contains resident lymphocytes and Kupffer cells that do not react against recipient cells [9].

Quantification of transmitted donor leukocytes has been achieved [19] but the functional relevance of donor T cells and their traffic in the recipient remains obscure. The major role of passenger leukocytes could be the sensitization of the recipient immune system either in the peripheral blood or lymphoid tissues of the host, as donor lymphocytes express MHC HLA-antigens, or in the graft itself, as in situ T lymphocytes produce cyto-

kines that can upregulate DR expression and induce cell-adhesion molecules on graft endothelial cells [9]. Moreover, cooperation between resident T-lymphocyte populations contained in liver grafts and recipient immunocompetent cells recruited in situ may exert an immunomodulatory role and modify the immune response against graft alloantigens [6].

Just how liver-associated T lymphocytes interfere with the recipient immune system may depend on their subset composition, their localization, and their state of preactivation in the donor liver. Liver-associated lymphocytes (LAL) have been investigated extensively in graft rejection, but few studies have been made in the normal liver. As shown by Hata et al. [11] and others [16], propagation of lymphocytes from liver biopsies, usually using clonal expansion by exogenous rIL2 or feeder cells [22], may not be necessary to characterize lymphocytes. Mononuclear cell suspension can be obtained by the mechanical disruption of liver biopsies and analyzed by flow cytometry (FCM), thus avoiding de novo activation or selection of committed T cells. Alternatively, the expression and distribution of lymphocyte markers can be assessed by immunohistochemical studies from liver biopsies [16]. In this study we have phenotypically analyzed and quantified the major T-cell subpopulations in human liver specimens collected before transplantation and tried to correlate the activation status of these cells and their distribution within the liver with their possible biological role after transplantation.

Patients and methods

Sixteen liver specimens were analyzed using a standard technical procedure. The specimens were obtained either from normal subjects (NL) during laparotomy performed for extrahepatic disease, cholecystectomy, or esophageal reflux ($n = 8$) or from donor livers (DL) at liver procurement in brain dead subjects ($n = 8$). Peripheral blood samples were collected simultaneously for flow cytometric analysis. Liver biopsies were performed after approval by the ethics committee of the University Hospital at Lille and, in normal subjects, after informed consent.

Biopsies

Needle biopsies were taken from each liver using Menghini type 1.8-mm diameter needles. Biopsy material was divided up immediately and handled in one of three ways:

1. fixed in Bouin's medium, embedded in paraffin, and stained by haematoxylin-eosin, sirius red, and Masson's trichrome for conventional microscopic examination,
2. snap-frozen in liquid nitrogen for immunohistological analysis, or
3. placed in cell culture medium (RPMI 1640) for flow cytometric analysis (FCM).

Biopsies were taken from donors before perfusion of the liver. In both normal and donor livers, resuspension of biopsy material in RPMI had a "washing-out" effect, eliminating blood lymphocytes, especially those weakly attached to endothelial cells in the lumen, as described by others [19].

Antibodies

Anti-CD4 (T4), anti-CD8 (T8), and anti-CD56 (NKH-1) monoclonal antibodies were purchased from Coulter (Hialeah, Fla, USA). Anti-CD3 (Leu-4), anti-CD69, and anti-HLA-DR antibodies were purchased from Becton-Dickinson (Mountain View, Calif., USA). Irrelevant mouse IgG1 (MsIgG1, Coulter) was used as a negative control.

FCM analysis

Biopsy cylinders of definite weight – between 50 and 100 mg – were washed in RPMI, teased, and minced gently before fragmentation using a loose Poter pestle, then ground on a nylon mesh (50 μm), and filtered. The cell suspension was then washed twice in PBS and vortexed 5 mm at 37 °C. The cell suspension obtained contained mononuclear cells and parenchymal cells. After centrifugation at 400 *g*, the pellet containing lymphocytes and other liver cells was resuspended in 1 ml of PBS and incubated for 20 mm with FITC- or phycoerythrin-labeled antibodies at the manufacturer's recommended optimal concentration. For two color immunofluorescence analysis, both monoclonal antibodies were added simultaneously. FCM analysis was performed on an Epics Profile (Coulter). The lymphocytes were gated on the basis of their forward and right angle light scatter that discriminated them from other liver cells. The absolute number of lymphocytes in liver tissue was estimated by averaging the cell count from the lymphocyte gate. Results are reported as mean percentage of positive lymphocytes + standard error of the mean (SEM).

Immunohistochemistry

Tissue sections were stained following an indirect immunoperoxydase method. Cryostat sections were fixed in acetone, then washed twice in PBS. Overnight incubation in a humidity chamber at room temperature was done with 20 % rabbit serum to diminish background staining. Sections were then incubated with murine monoclonal antibodies and washed with albumin-supplemented PBS and biotine. Peroxydase activity was revealed using diaminobenzidine. Sections were counterstained with hematoxylin for 1.5 min and dehydrated with alcohol.

Quantitative analysis in liver sections

Conventional immunohistochemistry provides a semiquantitative evaluation of liver-associated T cells that allows only an approximate comparison between the different liver territories. Therefore, lymphocytes were counted in the portal tract, the perisinusoidal zone, and around the centrolobular vein using automatic image analysis and a Biocom 200 analyzer composed of compac Deskpro 386/2 compatible PC – AT with histobiocom software for treatment and storage of data, a video camera (Panasonic VW – CD 52), an image monitor, and an orthoplan (Leitz) microscope. Each biopsy was analyzed at a 25-fold magnification. Two sections were studied for each biopsy. The visual field was approximately 40,000 mm^2 and, depending on the size of the biopsy specimen, a mean of two to five consecutive fields were studied. Identification

Table 1 Comparison of phenotypic characteristics of T lymphocytes in blood and in liver tissue between normal and donor subjects. Results are expressed as mean percentage of positive lymphocytes + SEM by FCM analysis. * $P < 0.05$ between normal and donor liver

	CD4		CD8		CD8DR		CD56		CD69	
	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood	Liver
Normal liver	30.4 (4.77)	14 (7.9)	20 (8.0)	42.4 (17.4)	1.92 (1.1)	7.34* (3.9)	16.8 (9.3)	38.0 (8.6)	1.8 (1.0)	23.2 (16.3)
Donor liver	41.4 (3.2)	15.4 (3.05)	24 (9.2)	39.6 (13.1)	1.0 (0.5)	2.98* (1.6)	7.66 (1.0)	31.6 (21.2)	0.72 (0.6)	26.3 (14)

Table 2 Comparison of phenotypic characteristics of T lymphocytes in liver tissue between normal and donor subjects. Results are expressed as absolute number of positive lymphocytes per surface unit + SEM by automatic image analysis

	CD4	CD8	CD8DR	CD56	CD69
Normal liver	593 (151)	1137 (302)	16 (4)	11 (4)	71 (45)
Donor liver	619 (110)	1131 (350)	25 (8)	164 (105)	131 (62)

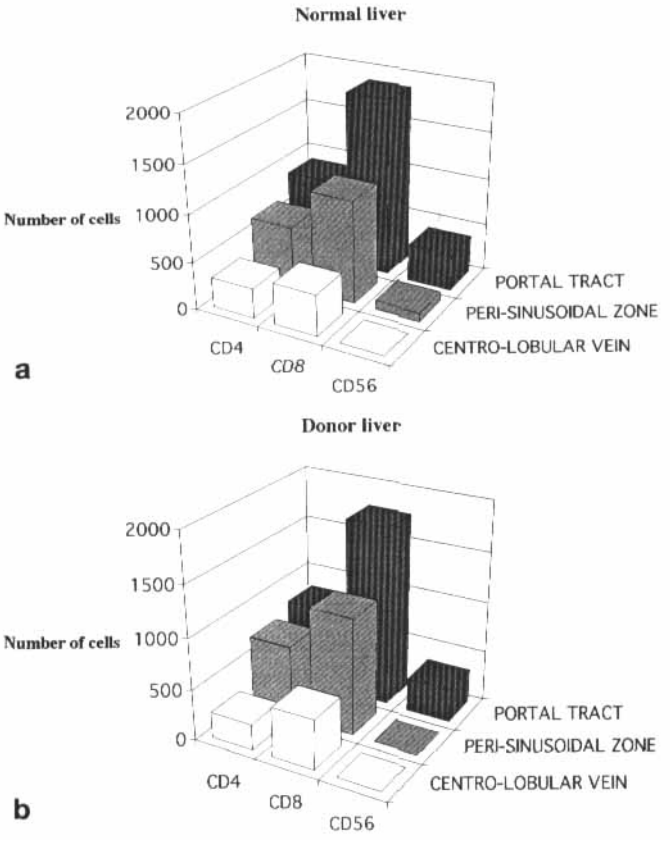

Fig. 1a,b Quantification of lymphocyte subset distribution according to the zones of the liver via automatic image analysis. Given are the mean absolute numbers of immunostained cells by visual field. For each marker, two to five fields were analyzed in eight subjects with **a** normal and **b** donor livers

of immunolabeled lymphocytes was made on the basis of complete morphological characteristics, differentiating them from others leukocytes, and stained fragments of cell membranes were eliminated. The number of immunostained cells by surface unit, reflecting the absolute number of lymphocytes bearing each marker, and the immunostaining density by cell (the ratio immunostained surface to number of cells in the total field surface), reflecting the intensity of expression for each marker, were calculated. In comparison with the FCM analysis, the absolute number of immunostained lymphocytes determined by automatic analysis reflected the contents of the two sections studied, not of the whole biopsy.

Statistical analysis

Differences between samples were tested using the nonparametrical Wilcoxon and Kruskall-Wallis rank tests.

Results

FCM analysis of liver-associated lymphocytes

The absolute numbers of lymphocytes were $0.5–3 \times 10^6$/g liver tissue with a slightly higher cellularity in normal liver than in donor liver (2500 cells vs 2200 cells per lymphocyte gate; P = NS). Immunophenotypic characteristics of T lymphocytes were compared in peripheral blood and liver (Table 1). The lymphocyte subset distribution in peripheral blood showed no statistically significant difference between normal and donor livers. T cells found in the liver were predominantly CD8+ and a significant proportion of these cells coexpressed the activation marker HLA-DR (CD8+ HLA DR+/CD8+: 17 % in normal liver vs 7 % in donor liver), whereas CD25 (IL-2 receptor α chain) was detected on only a few cells. Expression of CD69, also known as activation-induced molecule, was 10–30 times higher on liver-associated lymphocytes than on peripheral blood T lymphocytes, comprising around 25 % of liver-associ-

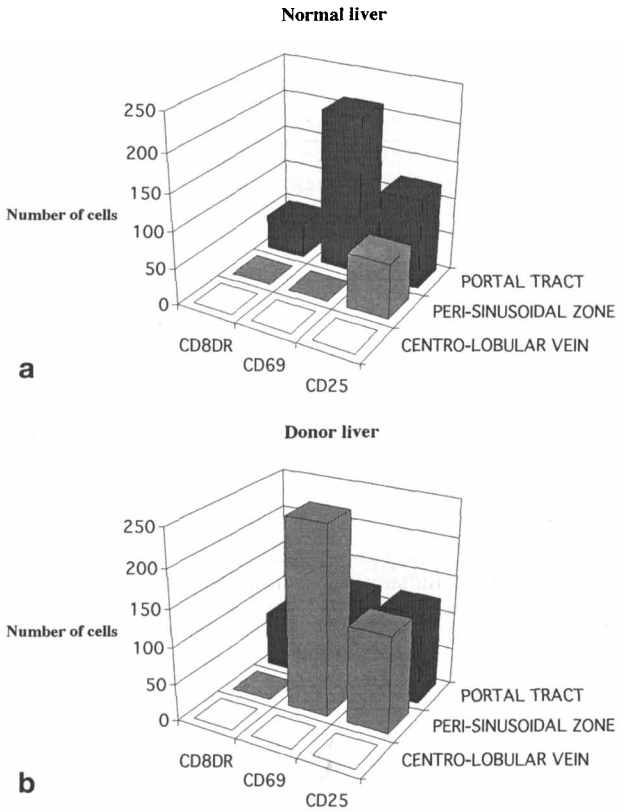

Fig. 2a,b Quantification of activation marker distribution according to the zones of the liver via automatic image analysis. Given are the mean absolute numbers of immunostained cells by visual field. For each marker, two to five fields were analyzed in eight subjects with **a** normal and **b** donor livers

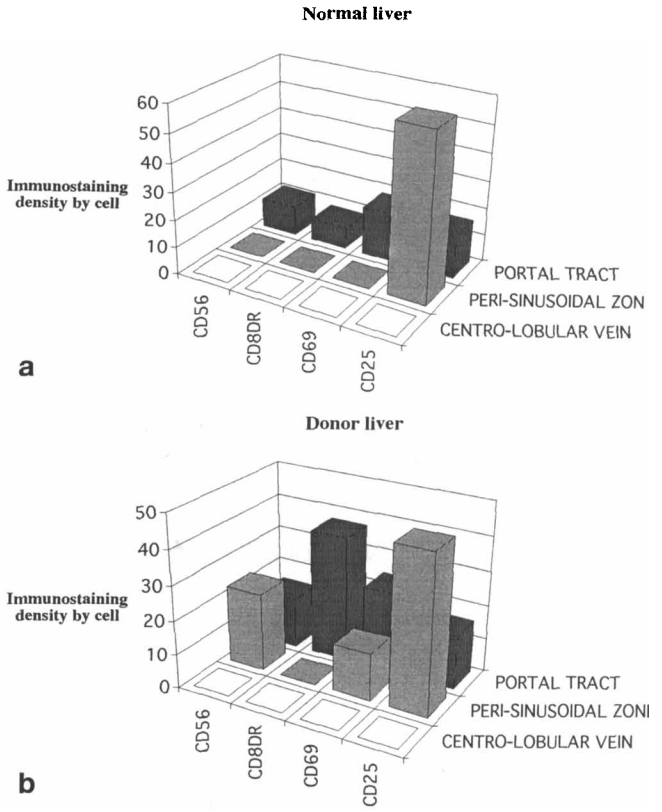

Fig. 3a,b Mean fluorescence intensity by immunostained cell according to the zones of the liver via automatic image analysis. For each marker, data were pooled from two to five analyses performed on eight subjects with **a** normal and **b** donor livers

ated lymphocytes. Cells expressing CD56, a NK cell marker, were the second predominant lymphocyte population (31 % in normal liver vs 38 % in donor liver) and were increased twofold compared to peripheral blood lymphocytes. In the only steatosic donor liver, CD69+ and CD56+ cell counts were highest (three times the mean value).

Histochemical analysis of liver-associated lymphocytes

Morphometric and phenotypic characteristics of lymphocytes determined in whole liver biopsy by automatic image analysis showed a pattern of lymphocyte subsets similar to that revealed by FCM (Table 2). CD8+ cells were the main population, but the CD56+ cell counts were smaller than those measured by FCM, presumably by CD56 marker shedding off the cell surface by air-drying for the immunohistological technique in comparison with "in suspension" staining for FCM [11]. Moreover, HLA-DR expression on CD8+ cells was lower than by FCM due to possible technical limits of double immunolabelling and detection, such as color threshold for slight differences in fluorescence intensity levels between simple and double immunolabelling [16].

Localization of liver-associated lymphocytes

CD4+ and CD8+ lymphocyte distributions were similar in both normal and donor livers (Fig. 1), and their numbers were two to ten times greater in the portal tract than in the perisinusoidal areas or in the centrolobular vein zone, respectively. CD8+ DR+ cells belonged only to the portal tract.

In normal livers (Fig. 2a), activated lymphocytes (CD25+ cells and CD69+) were localized in the portal tract only, contrary to donor livers where CD25+ cells and CD69+ cells were also present in the perisinusoidal area (Fig. 2b). No such activated cells were detected in the pericentrolobular zone, either in normal or in donor livers. CD56+ cells were mainly encountered in the portal tract in normal livers but also in significant proportions in the perisinusoidal areas of donor livers, and

their immunostaining density was significantly higher in these areas.

The staining intensity by cell for HLA-DR in CD8+ lymphocytes and for CD25 and CD69 (reflecting the density of expression of activation markers by lymphocytes) was higher in the perisinusoidal areas than in the portal tract, possibly signifying a different degree of activation according to the liver compartments (Fig. 3).

Discussion

Quantitative analysis of lymphocytes contained in potential liver grafts reveals numbers of cells as high as the usual overall count of peripheral blood T lymphocytes in normal subjects. Our data show that these counts can vary, according to liver weight disparity, from 2 to $9 \cdot 10^9$ cells. These findings are in agreement with the Hannover group's results [19]. They also confirm the validity of analysis of the mononuclear cell suspension from liver biopsies by FCM to characterize in situ lymphocyte subsets, as already described in rat livers or in diseased human livers [2, 14, 17]. Similar absolute numbers of lymphocytes were recovered in donor and normal livers, indicating that such cells represent a constitutive population of the liver and not a specific recruitment into the livers of brain-dead subjects. Accordingly, the large numbers of immunocompetent cells transferred with donor livers support the recent findings that grafted livers react against the recipient organism or interfere with its immune response to alloantigens [8, 23, 24], in contrast with the low levels of tissue-associated lymphocytes in lung, kidney, or heart pretransplant grafts [21].

The phenotypic analysis of liver-associated lymphocytes showed a predominant CD8+ well population exceeding CD4+ cells, which represented a minor subset. This is in contrast to the lymphocyte distribution in peripheral blood, which seems to be altered for a long time only in infected immunocompromised hosts [13]. Predominance of CD8+ lymphocytes is usually encountered within cell populations living in organs. Most of these lymphocytes are memory T cells (CD45RO+/CD45RA−), as previously reported by others [20]. Our data show that CD56+ cells were the second major lymphocyte subset within the liver. Unfortunately, CD11 and CD16 markers, to accurately determine NK subsets, were not studied. According to biopsy conditioning and tissue section analysis, these CD56+ cells were not endothelium-attached lymphocytes (pit cells), which are numerous inside the lumen of liver sinusoidal vessels [5], but tissue cells. However, the question arises as to whether these cells are of extrahepatic origin, mainly from blood, or whether they are derived from an immature population of hepatic origin. Similarities with pit cells from the rat liver [1] or large granular lymphocytes described from the human liver [27] suggest that these CD8+ and CD56+ cells are recruited from the vascular compartment after their adhesion to endothelial lining cells. This phenomenon predominates in liver sinusoids where endothelial cells are known to play an active role in lymphocyte trafficking. It has been recently demonstrated that this adhesion process is associated with a spontaneously activated state, as suggested by high levels of lymphokine-activated killer activity [5].

The origin quantitation by automatic image analysis in this study made it possible to evaluate the immunological relevance of the different sites within the liver. Strategic localization of CD56+ and CD8+ cells in the portal tract and around the perisinusoidal veins suggests that these sites are a major trafficking route between recipient and donor.

In the perisinusoidal zone, CD25+ cells were few in number but expressed the receptor for IL2 at a high level, signifiying recent activation consistent with recruitment. The expression of CD69 was higher in donor than in normal livers and could be attributed to activated NK cells. CD69 is known to be an early activation-induced molecule, the expression of which precedes CD25 and HLA class II during T-cell activation [25]. CD69 is absent from peripheral blood-resting lymphocytes but is expressed by in vivo activated lymphocytes infiltrating sites of local inflammation [18]. A higher state of activation of donor liver lymphocytes presumably results from local (hepatocytes, endothelial cells) or general aggression of the donor on the period of organ procurement. These primed T cells could respond rapidly to a secondary stimulation. In liver allografts, such a stimulation could result from early infiltration by host lymphocytes and macrophages or from Kupffer cell activation following the surgical procedure of transplantation [12]. The portal tract was also rich in lymphocytes, and a significant proportion of these cells were in an activated state, as indicated by high levels of HLA class II (DR) expression, especially on CD8+ cells. This pattern is consistently different from recirculating cells, which bear low levels of class II antigens and can be associated with tissue-selective migrating properties [5].

Centrolobular vein areas, in contrast, had a poor lymphocyte content and these lymphocytes did not express any activation markers. This is in correlation with the moderate endothelitis of the centrolobular vein usually seen in initial rejecting and nonrejecting liver allograft biopsies. Thus, the question arises as to whether these cells could be resident lymphocytes constitutively present in the liver, locally differentiated from stem cells, rather than migrated cells from blood.

Host lymphocyte migration into the graft starts through the portal epithelium and endothelial venules of the perisinusoidal system [4, 14] where many of the

activated donor lymphocytes associated with the liver were found. Such trafficking can only occur in the early course of transplantation as the donor T cells decrease rapidly and tend to be undetectable in the recipient a few days after transplantation [19]. In can be postulated that intragraft proliferation of recipient lymphocytes observed shortly after transplantation signifies, in the absence of clinical rejection, that donor cells exert in situ immunomodulatory properties that inhibit the recipient alloresponse [15]. Donor CD8+ cells might modify the recipient immune response towards the alloantigens expressed on their own surface, especially in immunosuppressive conditions [7], and could either induce a state of clonal anergy or acts as "veto cells". Similarities with selected CD8+ cells from transfused autologous donor bone marrow, whose persistence in the long-term recipient seem to be associated with the induction of allospecific unresponsiveness [26], require further investigation.

Netherveless, the present quantitative and topographic analysis of lymphocytes contained in liver grafts establishes the presence of activated CD8+ donor cells and NK donor cells in the portal tract and perisinusoidal zone of the liver tissue, something which might initiate an in situ cooperation between donor and recipient lymphocytes in the early course of liver transplantation.

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