or anti-CD8 mAb together with anti-CD16/32 mAb for 30 minutes at 4°C. After cell surface antigen staining, the cells were stained with annexin V for gating out either dead or apoptotic cells as follows. The cells were resuspended in 100 μl of annexin V binding buffer (BD Pharmingen) after washing with plain PBS and then stained with allophycocyanin (APC)-conjugated annexin V for 15 minutes at a room temperature. Following cell surface antigen and annexin V staining, the cells were washed with PBS, and the washing was followed by fixing, permeabilizing and intracellular cytokine immunofluorescence staining (ICIS) according to the manufacturer's instructions. Briefly, one million cells were fixed and permeabilized using 200 μl of Cytofix/Cytoperm™ solution (containing 4% of paraformaldehyde and saponin) (BD Pharmingen) for 20 minutes at room temperature, and washed twice with Perm/Wash™ buffer (containing FBS and saponin) (BD Pharmingen). For intracellular cytokine staining, phycoerythoin (PE)-conjugated anti-cytokine mAbs, i.e., anti-IL-2 (JES6-1A12), IFN-γ (XMG1.2) or and rat IgG1 (an isotype matched control), were used. One million of the fixed and permeabilized cells were incubated with 20 μl of anti-cytokine mAbs in 100 μl of Perm/Wash™ buffer for 20 minutes at room temperature, and were washed and resuspended in 200 μl of Pharmingen Stain Buffer (containing 2% FBS and 0.09% sodium azide) (BD Pharmingen) for use of FCM analysis. Four-color FCM was performed on a FACSCalibur® dual-laser cytometer (Becton Dickinson, Mountain View, CA) using standard Cell Quest™ acquisition/analysis, and fluorescence compensation was achieved using an appropriate single fluorochrome-labeled sample. For compensation in multiparameter FCM, CFSE intensity of undivided cells was set at between 10^3 and 10^4, and an equilibrium between the FL-1 (CFSE) and FL-2 (PE) was sought, by adjusting the FL-1 and FL-2 parameters, to properly scale both FL-1 and FL-2 in a dot plot. Then, the FL-2 and FL-3 (PerCP-Cy5.5) signals and the FL-3 and FL-4 (APC) signals were compensated in turn. Typical compensation values were as follows: 1% for FL-1–FL-2, 30% for FL-2–FL-1, 5% for FL-2–FL-3, 15% for FL-3–FL-2, 5% for FL-3–FL-4, and 10% for FL-4–FL-3. Between 5 and 10×10^5 total events were collected yielding 1×10^5 annexin V-negative events.

Quantitative Estimation of T Cell Proliferation in Response to Allo-stimulation by MLR Assay Using a CFSE-Labeling Technique

On CFSE fluorescence histograms, $CD4^+$ and $CD8^+$ T cells were selected by gating and analyzed for CFSE fluorescence. Divisions of alloreactive T cells, which were identified and determined by their CFSE intensities, were labeled from 0 to n as division round. From the experimentally determined values of percentage of either $CD4^+$ or $CD8^+$ T cell events in each division n (a) and total number of living $CD4^+$ or $CD8^+$ T cell events at the time of harvest (b), the total number of daughter T cells that had divided n times was calculated (c). The total number of original or precursor T cells required to have generated these daughters was extrapolated by dividing the number of daughters at n divisions by 2^n (d). The total number of proliferation events was determined by subtracting the number of division precursors from the number of daughters generated by each precursor population (c minus e is f). Precursor frequency was calculated by dividing the sum of alloreactive precursor T cells that had divided once or more by the sum of all precursor T cells, including the precursor T cells that had not divided (c divided by d is e). Because the value of a proliferation event is

influenced by the sum of T cells subjected to FCM analysis, the proliferation index was extrapolated by dividing the sum of proliferation events by the sum of all precursor T cells (h divided by g).

Cytokine Levels in MLR Culture Supernatants

Interleukin-2 (IL-2) and interferon-γ (IFN-γ) levels in the MLR culture supernatants were measured by FCM using a BD Mouse Th1/Th2 Cytokine Cytometric Bead Array (CBA) Kit® according to the manufacturer's instructions. The recently developed CBA assay is based on the principle of the sandwich immunoassay using uniform-sized microparticles coupled to multiple mAbs against cytokines.

Statistics

The results were statistically analyzed by the unpaired or paired Student's T test or the linear regression analysis when appropriate. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Exclusion of Non-viable Cells from Analysis by Staining with Annexin V Prior to Fixation and Permeabilization

Since intracellular esterases hydrolyze CFSE into a fluorescent dye that binds covalently to cytoplasmic protein residues such as lysine, only viable cells are labeled. However, labeled cells that die during the culture period remain detectable until they disintegrate. These apoptotic/necrotic cells and cell fragments can cause an overall increased, less-defined, CFSE signal that may artifactually affect results. In addition, dead or dying cells may nonspecifically bind mAbs used for immunophenotyping or compromise analysis due to autofluorescence. In order to accurately detect the proliferative response of CFSE-labeled lymphocytes by FCM analysis, therefore, viable cells should be discriminated from apoptotic or dead cells. For that purpose, in the present study, the MLR cultured cells were stained with APC-conjugated annexin V, a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS), prior to fixation and permeabilization for the following ICIS. Since the membrane PS is translocated from the inner leaflet to the outer leaflet of the plasma membrane in apoptotic cells, cells in the early stage of apoptosis as well as cells that are in the late stage of apoptosis or already dead are stained with annexin V. In the present experiments, cell fixation and permeabilization did not interfere with annexin V-staining. As shown in Fig. 1, from the analysis by forward-scatter and annexin V-staining, non-viable cells could be excluded from further analysis, making the proliferated cells visible as cells that exhibited a decrease in CFSE intensity. The stimulator cells could be excluded from the analysis by use of this method. In our preliminary experiments, all of the annexin V-negative cells that had been harvested after the MLR were stained with anti-responder-type major histocompatibility complex class I (data not shown). The percentage of live cells (annexin V-negative cells) after

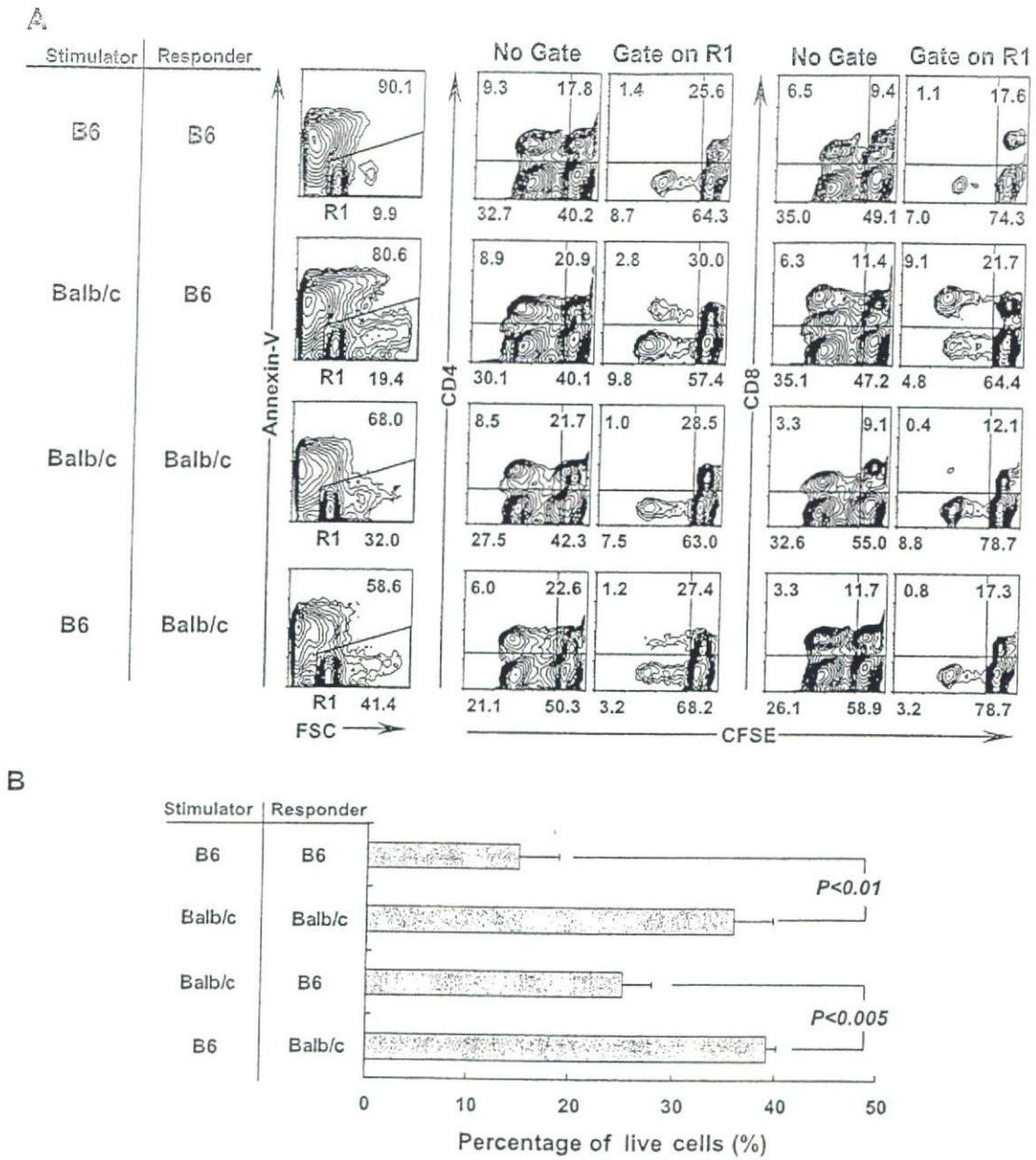


Figure 1. Exclusion of non-viable cells from analysis by staining with annexin V. The MLR cultured cells were stained with APC-conjugated annexin V prior to fixation and permeabilization for the following ICIS. Cells in the early stage of apoptosis as well as cells in the late stage of apoptosis or already dead are stained with annexin V. By setting a gate on annexin V-negative cells (R1), non-viable cells could be excluded from further analysis. A) FCM profiles of the MLR cultured cells with and without setting a gate on annexin V-negative cells are shown. Percentages given are of total cells in each scan. To ensure statistical significance, 150,000 –300,000 events were collected for each sample. The result was consistent in four repeated experiments. B) Percentages of live cells (annexin V-negative cells) after MLR are shown. Average values ± SEM for four independent experiments are shown ($P < 0.05$, analyzed by Student's t test).

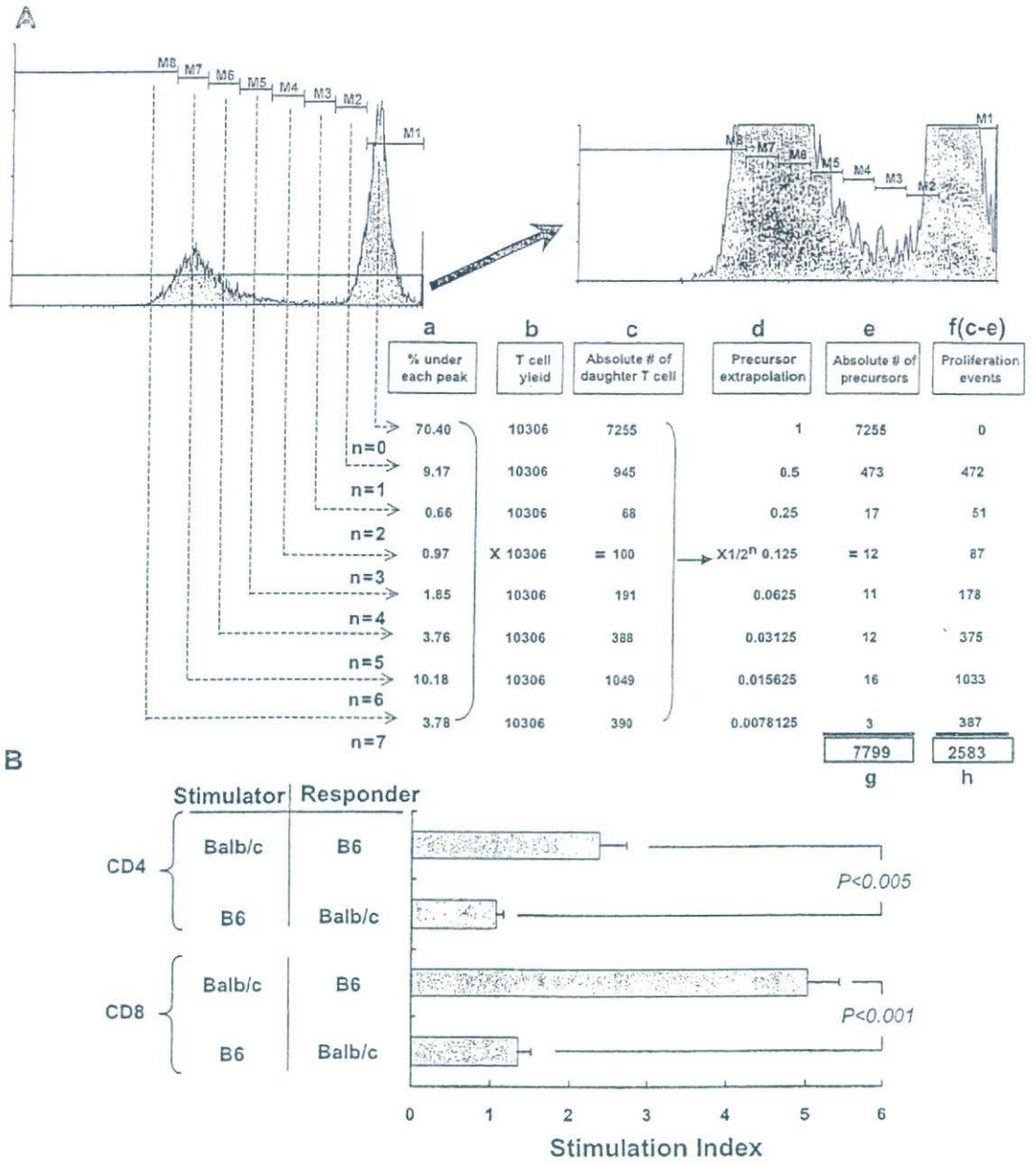


Figure 2. Quantitative estimation of T cell proliferation in response to allo-stimulation by MLR assay using a CFSE-labeling technique. Irradiated stimulator splenocytes were cultured with CFSE-labeled responder splenocytes. **A:** CFSE fluorescence histograms of $CD4^+$ and $CD8^+$ T cells show their proliferative history in response to allostimulation. $CD4^+$ and $CD8^+$ T cells were selected by gating and analyzed for CFSE fluorescence. Divisions of alloreactive T cells, which were identified and determined by their CFSE intensities, were labeled from 0 to n as division round. The limit of detection is seven or eight division cycles caused by compression of peaks as the CFSE intensity approaches autofluorescent levels. Thus, divisions beyond six cycles are indistinguishable and are collectively referred to as division 7+ . The method used for calculating precursor frequency and the proliferation index is described in the Materials and Methods. **B:** Stimulation indexes were calculated by dividing proliferation index of allogeneic combinations by those of control syngeneic combinations. Average values ± SEM for four independent experiments are shown ($P < 0.05$, analyzed by Student's t test).

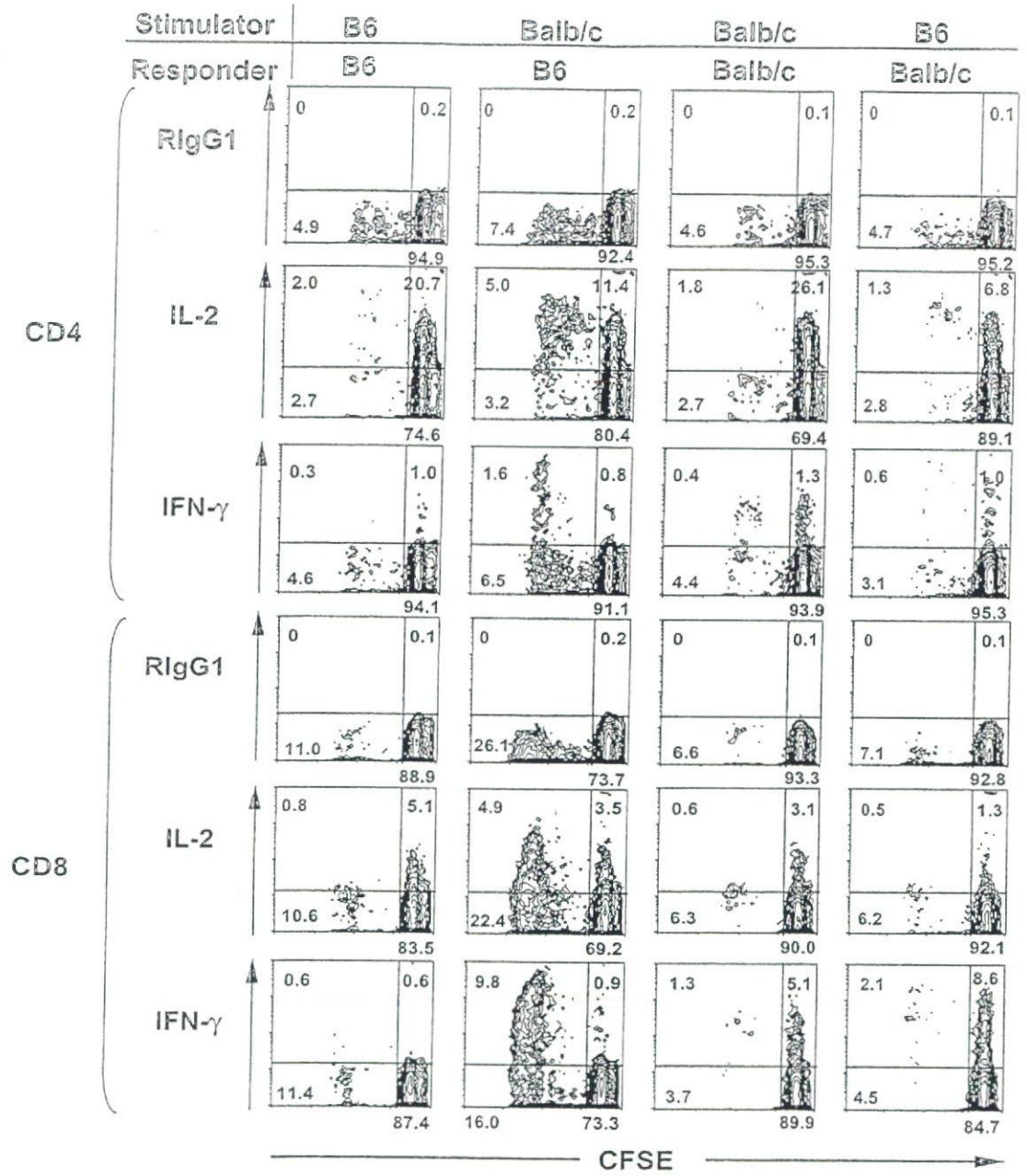


Figure 3. Visualization of proliferation and cytokine production of $CD4^+$ and $CD8^+$ T cells in response to allo-stimulation by multiparameter FCM. B6 mice and Balb/c mice were used. CFSE-labeled responder splenocytes were cultured with irradiated stimulator splenocytes. The MLR cultured cells were stained with PerCP-Cy5.5-conjugated anti-CD4 and anti-CD8a, followed by staining with APC-conjugated annexin V. Then, the cells were fixed, permeabilized and stained with PE-conjugated mAbs against various cytokines (IL-2 and IFN-γ) or isotype matched control Ab (IgG1). Four-color FCM was performed for determination of proliferation and cytokine-secreting activity in the MLR. Either $CD4^+$ T cells or $CD8^+$ T cells were selected by gating and analyzed for cytokine (IL-2 and IFN-γ)-secreting activity. FCM profiles shown are representative of four independent experiments. The number refers to the percentage of total cells in each quadrant.

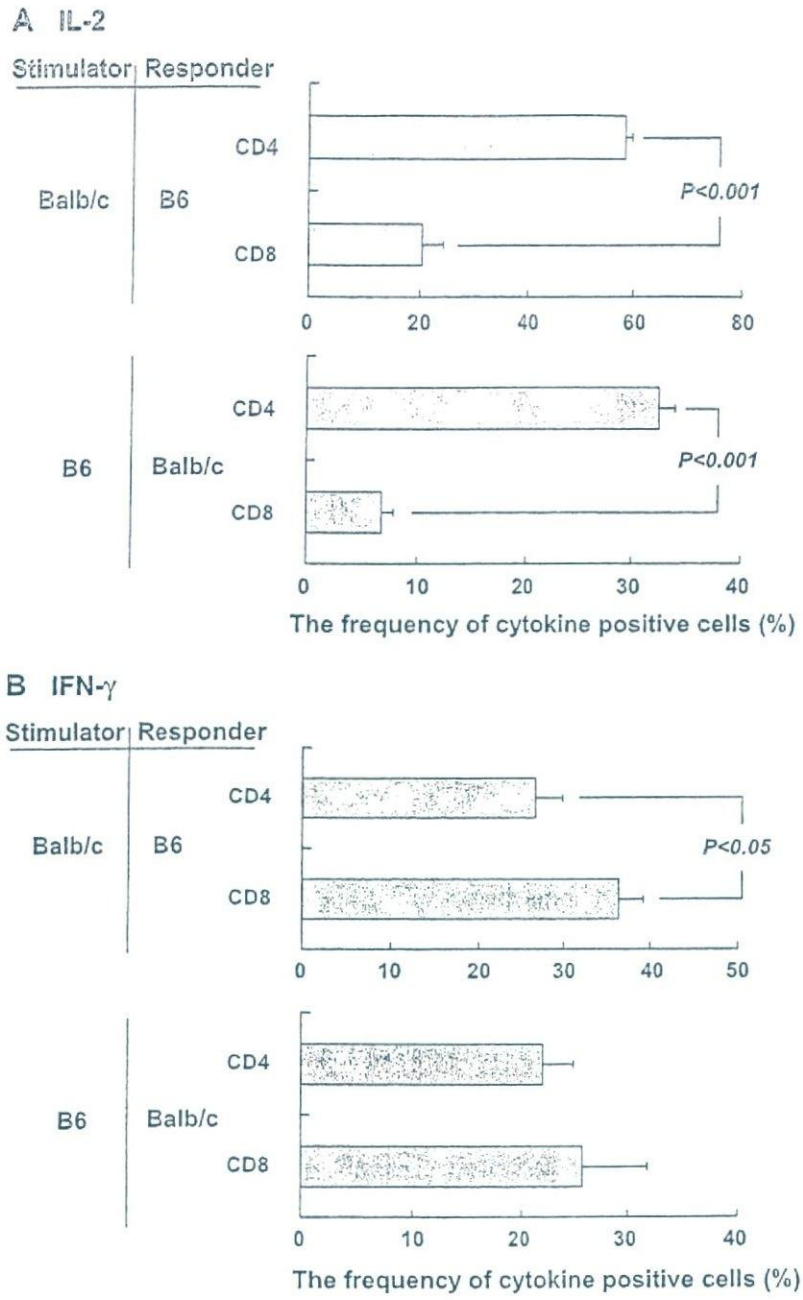
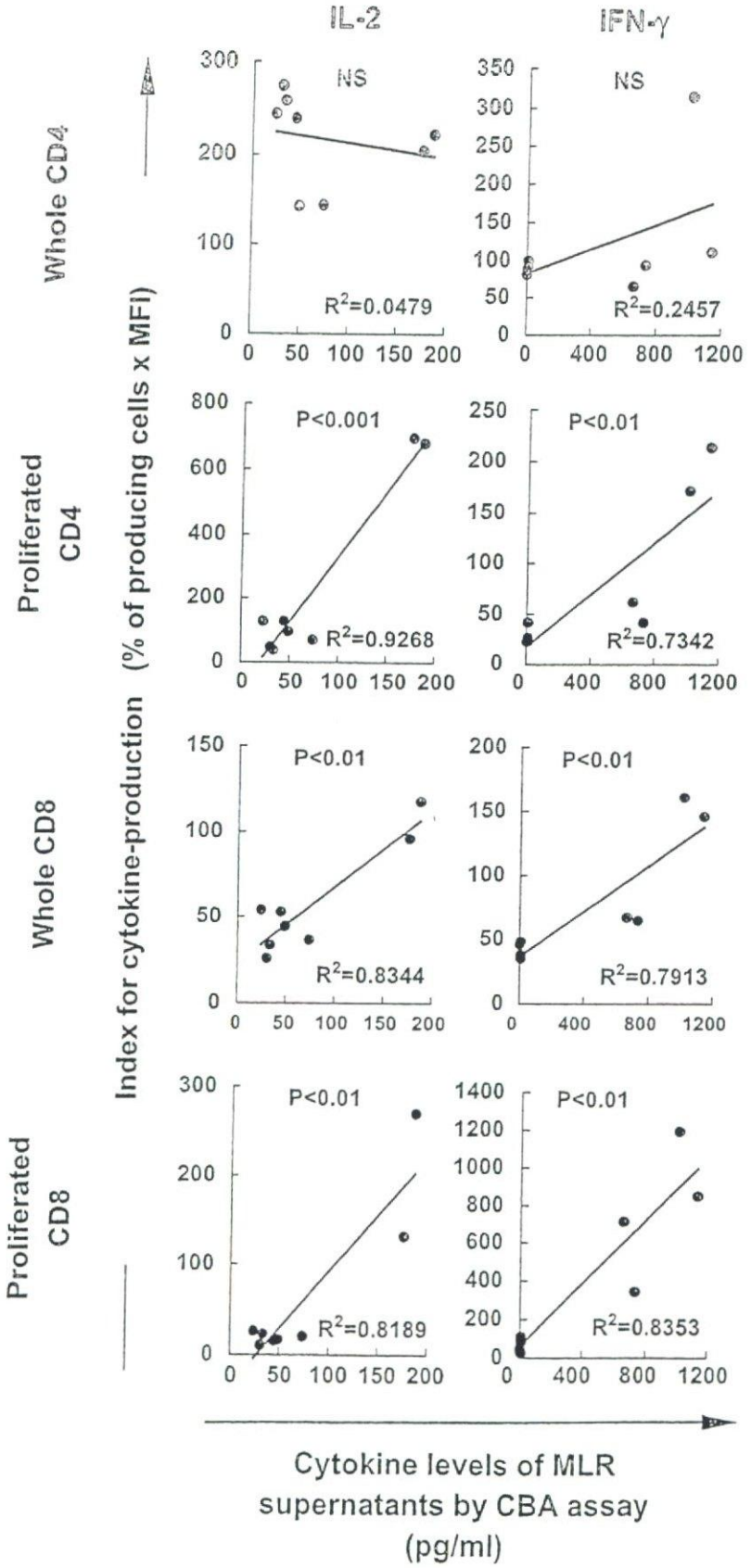


Figure 4. The frequency of IL-2 and IFN-γ-producing cells in the proliferated $CD4^+$ or $CD8^+$ T cell fractions. The frequency (%) was calculated using the quadrant data of the FCM contour plots in Fig. 3 by the following formula: percentage of the upper left/(percentage of the upper left + percentage of the lower left) × 100. Average values ± SEM for four independent experiments are shown ($P < 0.05$, analyzed by Student's t test).


IL-2
IFN-γ
Whole CD4
Proliferated CD4
Whole CD8
Proliferated CD8
Index for cytokine-production (% of producing cells x MFI)
NS
NS
R²=0.0479
R²=0.2457
P<0.001
P<0.01
R²=0.9268
R²=0.7342
P<0.01
P<0.01
R²=0.8344
R²=0.7913
P<0.01
P<0.01
R²=0.8189
R²=0.8353
Cytokine levels of MLR supernatants by CBA assay (pg/ml)

MLR approximately ranged from 15% to 40%. Balb/c responder cells survived after the MLR, either in syngeneic or allogeneic combination, at the significantly higher rate than B6 responder cells, probably reflecting strain-associated peculiarity (Fig. 1B).

Visualizing Proliferation of $CD4^+$ and $CD8^+$ T Cells in Response to Allo-stimulation by Multiparameter FCM

Fixation and permeabilization did not influence fluorescent signals in the CFSE-labeled cells, thus enabling simultaneous determination of cell proliferation and identification of internal proteins linked to cell division. In both the Balb/c stimulator-versus-B6 responder (hereafter referred to as Balb/c-vs.-B6) (high-responsive combination) and the B6-vs.-Balb/c (low-responsive combination) allogeneic combinations, proliferation of $CD4^+$ and $CD8^+$ T cells was detectable, as seen by a decrease in the fluorescence intensity of CFSE-positive cells and an increase in the total percentage of cells that exhibited a decrease in CFSE intensity (Fig. 1). Such T cell proliferation was more easily detectable in the high-responsive Balb/c-vs.-B6 combination. In the B6-vs.-B6 and Balb/c-vs.-Balb/c syngeneic combination, on the other hand, the proliferation of $CD4^+$ and $CD8^+$ T cells was almost undetectable.

Quantifying Proliferation of $CD4^+$ and $CD8^+$ T Cells in Response to Allo-stimulation by Multiparameter FCM

Precursor frequency (PF), proliferation index (PI) and stimulation index (SI) were quantitatively estimated using the method described previously, as shown in Fig. 2A (Wells et al., 1997). The CFSE fluorescence intensity of the peak of cell division, which was divided once, shows a half value of CFSE fluorescence intensity of the peak of nonreactive cell division. Divisions of reactive cells, which were identified and determined by their CFSE intensities, were labeled from 0 to n as dividing time. A single cell dividing n times will generate 2^n daughter cells. Using this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division and from proliferation events and PF in $CD4^+$ and $CD8^+$ T cell subsets. Using these values, proliferation events and PI were calculated. SI was calculated by dividing PI of allogeneic combinations by those of self-control. The PF of Balb/c T cells reacting to B6 stimulator cells was 3.64 ± 1.10 (mean ± SD)% for CD4

Figure 5. The relationship between the values of cytokines in MLR culture supernatants determined by cytometric bead array (CBA) assay and the index for cytokine-production (CPI) determined by the combined CFSE–MLR and ICIS. The levels of IL-2 and IFN-γ in the culture supernatants of the MLR in both the syngeneic (B6-vs.-B6 and Balb/c-vs.-Balb/c) and allogeneic (Balb/c-vs.-B6 and B6-vs.-Balb/c) combinations determined by the CBA assay. In the CBA assay, the standard curve for each cytokine covers a defined set of concentrations from 20–5000 pg/ml. CPI was calculated by multiplying the percentage of cytokine-producing cells in each T cell fraction (whole $CD4^+$ or $CD8^+$ T cell fraction, or proliferated $CD4^+$ or $CD8^+$ T cell fraction) and mean fluorescence intensity of cytokine-staining in the T cell fraction. The data were obtained from two independent experiments ($P< 0.05$, analyzed by linear regression analysis).

T cells and 5.23 ± 1.94% for CD8 T cells (n = 4). The PF of B6 T cells reacting to Balb/c stimulator cells was 1.70 ± 0.70% for CD4 T cells and 4.78 ± 2.06% for CD8 T cells (n = 4). These data are consistent with previously retorted data, i.e., the frequency of Balb/c splenocytes reacting B6 stimulator cells was 4.61 ± 2.22% for all T cells and 1.82 ± 0.95% for CD4 T cells (Suchin et al., 2001). As shown in Fig. 2B, the SI for both CD4 and CD8 T cells in Balb/c-vs.-B6 combination were significantly higher than that in the B6-vs.-Balb/c combination, accurately reflecting their inherited characteristic susceptibility to allostimulation. Stimulation with PMA and ionomycin shortly before harvesting did not influence T cell proliferation, indicated by the consistent results of the SI, regardless of the presence or the absence of PMA and ionomycin stimulation (data not shown).

Cytokine-Secreting Cells were Simultaneously Detectable in Both Proliferating $CD4^+$ and $CD8^+$ T Cells

Without any additional stimulation, IL-2- and IFN-γ-secreting cells were barely detectable after allogeneic MLR (not shown). PMA and ionomycin stimulation at the end of the MLR remarkably enhanced the intracellular intensity of those cytokines but did not increase non-specific T cell proliferation, as described above. The levels of both IL-2- and IFN-γ-production were affected by division number, with higher production from the most extensively divided cells. In both of the Balb/c-vs.-B6 and the B6-vs.-Balb/c allogeneic combinations, IL-2-secreting cells were identified predominantly in proliferating $CD4^+$ T cell fraction (Fig. 3 and 4). The frequency of IL-2-producing cells was significantly higher in the proliferated $CD4^+$ T cell fraction than in the proliferated $CD8^+$ T cell fraction (Fig. 4). In contrast, the frequency of IFN-γ-producing cells was higher in the proliferated $CD8^+$ T cell fraction than in the proliferated $CD4^+$ T cell fraction. Since factors for a general capacity of cytokine production include the frequency of cytokine-producing cells and cytokine-producing activity per cell, we have provided an index for cytokine-production (CPI) by multiplying the percentage of cytokine-producing cells in T cells and mean fluorescence intensity of cytokine-staining. Between the values of cytokines in culture supernatants determined by CBA assay and CPI determined by the combined CFSE–MLR and ICIS, there was a statistically significant correlation (Fig. 5). The supernatant levels of IL-2 were most closely correlated to the CPI for IL-2 in the proliferating CD4 fraction. The supernatant levels of IFN-γ were most closely correlated to the CPI for IFN-γ in the proliferating CD8 fraction. Thus, IL-2 and IFN-γ were secreted predominantly by the proliferating $CD4^+$ and $CD8^+$ T cell fraction in allogeneic MLR, respectively.

DISCUSSION

The discovery that cells labeled with the CFSE equally apportion fluorescence between daughter cells upon proliferation made it possible for the division history of a cell population to be monitored by FCM (Lyons and Parish, 1994; Paramore et al., 1992). Importantly, cellular differentiation turned out to be unaffected by the dye, because labeled lymphocytes were shown to switch isotype, develop cytokine secreting potential and alter their cell surface phenotype as normally (Gett and Hodgkin, 1998;

Hasbold et al. (1998,1999). In addition, cell fixation and permeabilization conditions could be used that preserve the CFSE profile and allow simultaneous intracellular labeling with mAbs against various cytokines (Bird et al., 1998; Fazekas de St Groth et al., 1999). In this study, we applied such a system to an allogeneic MLR assay. By combining MLR using the CFSE-labeling technique and ICIS, proliferation, phenotypic characterization and cytokine-secreting activity in T cells responding to an allo-stimulation could be simultaneously determined through FCM analyses.

By applying a CFSE-based method, the proliferation of $CD4^+$ and $CD8^+$ T cells in response to allo-stimulation could be quantified using multiparameter FCM. Since allo-stimulation polyclonally activates the corresponding T cells, there was marked asynchrony in the kinetics of cell division, with some T cells having divided numerous cycles within 5 days, while other T cells remained undivided throughout this time period. In both $CD4^+$ and $CD8^+$ T cells, two remarkable peaks were observed on CFSE fluorescence as shown in Fig. 2A. Undivided T cells can be seen in the rightmost peak (brightest peak), and the T cells that have divided 6 times can be seen in the leftmost peak. Between those two peaks, distinct peaks were not obvious from the FCM data on CFSE fluorescence. Such a biased distribution in division is probably due to a different tempo of proliferation among divisions. It has been previously demonstrated that cells that have divided numerous times (5 to 8) do not, on average, proliferate more rapidly than those that have divided 2–4 times by CFSE profiles and BrdU labeling of dividing T cells (Gett and Hodgkin, 1998). Although the determination of divisions was not feasible by identifying each division peak in this study, the numbers of divisions of alloreactive T cells could be mathematically determined by the CFSE intensities, based on the theory that the CFSE fluorescence intensity of cells that have divided once shows a half value of CFSE fluorescence intensity of non-divided cells.

The combined MLR using the CFSE-labeling technique and ICIS offers simultaneous assessment of division-linked changes in cytokine production and the frequency of cytokine-secreting cells, that might be advantageous over conventional methods. In this study, we have proven the suitability of this method by demonstrating a close relationship between the values of cytokines in culture supernatants determined by CBA assay and CPI (obtained by multiplying the percentage of cytokine-producing cells in T cells and mean fluorescence intensity of cytokine-staining) determined by the combined CFSE–MLR and ICIS (Fig. 5). The values of cytokines in MLR culture supernatants reflect a general capacity of cytokine production predominantly from responding T cells during the MLR, and probably partly from stimulator cells. The supernatant levels of IL-2 were closely correlated to the CPI for IL-2 in the proliferating $CD4^+$ fraction, but not to those in whole $CD4^+$ T cells. The extensively divided $CD4^+$ cells showed higher intracellular intensity of IL-2 in allogeneic combinations, but non-divided $CD4^+$ cells also showed considerable level of IL-2-intracellular intensity even in syngeneic combinations, in which IL-2 levels in MLR culture supernatants were barely detectable. A similar trend was observed in $CD8^+$ T cells with the intracellular intensity of IFN-γ. It could be possible that non-divided T cells with intracellular intensity of IL-2 or IFN-γ are not actively producing those cytokines, but develop production activity in a division-dependent manner. To address this possibility, further studies are needed.

Although the majority of transplant recipients respond immunologically to the grafted organ despite immunosuppression treatment, some patients never seem to

generate any rejection activity, and the transplanted organ can be maintained with small doses of immunosuppressive drugs in such patients. In some cases, the transplanted organ has in fact been maintained for years without rejection in patients who stopped all of their medications. On the other hand, some patients seem to require high doses of exogenous immunosuppression. Since the amount of immunosuppression is not the same for every individual, a reliable method for monitoring anti-donor alloreactivity is needed. The different sensitivities to immunosuppressive therapy might be explained at least in part by Th cell function (Barbara et al., 2000; Exley et al., 2001; Jordan and Ritter, 2002). It has been recently reported that IL-2-producing helper T cells precursor frequency, which is determined by limiting dilution analysis using the peripheral blood of recipients and donor splenocytes, precedes the histological detection of rejection in clinical cardiac transplantation (Weston et al., 2000). Since CFSE–MLR analysis combined with ICIS can provide simultaneous assessment of division-linked cytokine production and the phenotypic property of cytokine-producing cells in allogeneic MLR, which cannot be done by the limiting dilution analysis, the method presented here might be useful for monitoring alloreactivity in order to tailor immunosuppressive therapy for the individual patient.

ABBREVIATIONS

MLR	mixed lymphocyte reaction
CFSE	carboxyfluorescein diacetate succinimidyl ester
ICIS	intracellular cytokine immunofluorescence staining
IL	interleukin
IFN	interferon
FCM	flow cytometry
PBS	phosphate-bufferd saline
FBS	fetal bovine serum
PMA	phorbol 12-myristate 13-acetate
mAbs	monoclonal antibodies

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Low Incidence of Acute Rejection after Living-Donor Liver Transplantation: Immunologic Analyses by Mixed Lymphocyte Reaction using a Carboxyfluorescein Diacetate Succinimidyl Ester Labeling Technique

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Background. To monitor antidonor alloreactivity for accurate diagnosis of acute rejection after living-donor liver transplantation (LDLT), we used a mixed lymphocyte reaction (MLR) assay using an intracellular fluorescent dye carboxyfluorescein diacetate succimidyl ester (CFSE)-labeling technique (CFSE-MLR) in 29 consecutive patients who underwent adult-to-adult LDLT.

Methods. For patients who developed moderate or severe disorders in liver function, CFSE-MLR was performed together with needle biopsy of the liver allografts immediately after liver dysfunction had occurred. CFSE-labeled peripheral blood mononuclear cells (PBMC) from recipients and irradiated autologous, donor, or third-party PBMC were cultured, and then proliferation and CD25 expression in each of the $CD4^+$ and $CD8^+$ T cell subsets were analyzed by flow cytometry.

Results. Twelve (41.4%) of the 29 patients developed moderate or severe disorders in liver function within 6 months after LDLT. Eight of the 12 patients (overall incidence of 27.6%) suffering from liver function disorder were diagnosed on the basis of liver biopsy results as having mild or moderate acute rejection. However, only 4 of the 12 patients (overall incidence of 13.8%) showed remarkable proliferation of $CD8^+$ T cells in association with CD25 expression on antidonor CFSE-MLR. The other eight patients were eventually diagnosed as having recurrence of original hepatitis, drug-induced hepatotoxicity, or congestion of the anterior segment of the liver allograft by further extensive examinations or in retrospect.

Conclusions. The results of CFSE-MLR assays, which could be used for rigorously monitoring rejection, provided evidence of low incidence of acute rejection after LDLT.

Keywords: Living-donor liver transplantation, Flow cytometry, Mixed lymphocyte reaction, Acute rejection, Immune monitoring.

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The incidence of acute cellular rejection (ACR) after liver transplantation has been reported to be approximately 30% to 60% (*1–3*). Such a wide range might be caused by the difficulty in differential diagnosis from rejection, recurrence of original disease (such as viral hepatitis), and drug-induced hepatotoxicity even by pathologic examinations (*4*). More accurate diagnosis of ACR would reduce risks of morbidity and mortality caused by inappropriate immunosuppressive therapy. To practice a necessary and sufficient immunosuppressive therapy in clinical transplantation, the development of a reliable assay for monitoring immune response is needed. The mixed lymphocyte reaction (MLR) is a widely used method for evaluating immune response to alloantigens in both experimental and clinical transplantation. The MLR assay was initially used to determine the proliferation of host (responder) T cells in response to antigens expressed on leukocytes obtained from the donor (*5, 6*). Later, it was shown that host cytotoxic T cells against antigens of the donor could be generated in MLR (*7*). In addition to constituting the majority of the proliferating cells in allogeneic MLR, the $CD4^+$ T-helper cells secreted cytokines that enabled the killer T cells to undergo functional maturation to possess killer activity (i.e., collaboration between $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells). However, traditional MLR using tritiated thymidine incorporation for quantifying cell division does not enable phenotypic or functional analysis of proliferating cells in such heterogeneous MLR. Flow cytometric (FCM) analysis of lymphocyte division by serial halving of the fluorescence intensity of the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) has recently been used instead of the tritiated thymidine method in the MLR (*8–10*). CFSE stably stains intracellular proteins

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without toxicity, and the fluorescence of each stained cell segregates equally to daughter cells upon cell division, resulting in sequential halving of cellular fluorescence intensity with each successive generation (*11*). When analyzed by FCM, this sequential halving of fluorescence is visualized as distinct peaks or populations of cells and can be used to track cell division in populations of proliferating cells. This, then, allows phenotypic analysis of proliferating cells in addition to determining the number of cells produced in each generation by multicolor FCM analysis. To monitor antidonor alloreactivity for more accurate diagnosis, we have used an MLR assay using intracellular a CFSE-labeling technique (hereafter referred to as CFSE-MLR assay) in patients who underwent adult-to-adult living-donor liver transplantation (LDLT).

PATIENTS AND METHODS

Patient Population

Twenty-nine consecutive patients who underwent adult-to-adult LDLT at Hiroshima University Hospital were enrolled in this study. The 29 patients included 17 males and 12 females, ranging in age from 28 to 68 (mean±SD 52.3±7.2) years. Original diseases of the patients are shown in Table 1. The graft donors were 18 children, 4 siblings, 4 spouses, 2 parents, and 1 other relative, with ages ranging from 18 to 61 (mean 36.3±6.0) years.

Immunosuppressive Protocol

The basic immunosuppressive regimen after LDLT consisted of tacrolimus/cyclosporine and methylprednisolone, with doses gradually being tapered off. In patients with hepatitis B or C virus (HCV), the dose of methylprednisolone was rapidly tapered off, and administration was stopped within 1 month after LDLT, which would be beneficial for preventing enhanced viral replication. Instead, basiliximab was usually administered (20 mg on days 0 and 4 after LDLT) to those patients. When patients developed significant disorders in liver function as determined by laboratory tests after LDLT, CFSE-MLR was performed together with needle biopsy of the liver allografts immediately after liver dysfunction had occurred. Unless graft liver dysfunction was progressive, immunosuppressive treatment usually was not intensified. However, some patients in whom liver function

TABLE 1. Patient characteristics and compatibility

Patient no.	Age at LTx (years)	Sex	Original diagnosis	Viral hepatitis	Donor	HLA mismatches, A-B-C-DR	Liver allograft dysfunction
1	63	M	Liver cirrhosis with HCC	HCV	Offspring	0-1-0-1	−
2	50	M	Liver cirrhosis with HCC	HCV	Spouse	1-1-1-1	−
3	66	F	Liver cirrhosis with HCC	HCV	Offspring	1-1-0-2	−
4	62	M	Liver cirrhosis with HCC	HBV	Offspring	1-1-0-1	−
5	40	M	Fluminant hepatitis	—	Sibling	0-1-0-0	−
6	48	F	Autoimmune hepatitis	—	Sibling	2-2-1-1	+
7	52	M	Liver cirrhosis	HCV	Offspring	0-1-0-1	−
8	57	M	Liver cirrhosis with HCC	HBV	Offspring	1-1-0-1	+
9	58	M	Liver cirrhosis (Alcoholic)	—	Offspring	1-1-1-0	+
10	46	F	Liver cirrhosis (Alcoholic)	—	Spouse	2-1-0-2	−
11	66	F	Liver cirrhosis with HCC	HCV	Offspring	1-0-0-1	−
12	56	M	Fluminant hepatitis	HBV	Offspring	1-1-0-1	−
13	59	F	Liver cirrhosis	HCV	Offspring	1-1-0-1	+
14	56	F	Liver cirrhosis with HCC	HCV	Offspring	1-1-1-1	−
15	49	M	Liver cirrhosis with HCC	HCV	Offspring	0-1-1-1	+
16	60	M	Liver cirrhosis with HCC	HCV	Offspring	1-1-0-ND	−
17	54	M	Liver cirrhosis with HCC	HBV	Offspring	1-1-1-ND	−
18	55	F	Liver cirrhosis with HCC	HCV	Sibling	0-0-0-0	−
19	49	M	Liver cirrhosis with HCC	HBV	Offspring	0-1-0-2	−
20	28	M	Liver cirrhosis	HCV	Parent	1-0-0-ND	−
21	47	M	Liver cirrhosis with HCC	HCV	Offspring	1-1-1-1	+
22	51	F	Secondary biliary chirosis	—	Other relative	0-0-0-0	+
23	43	M	Liver cirrhosis with HCC	HBV	Spouse	1-1-1-2	+
24	28	M	Insulinoma (Liver metastasis)	—	Parent	0-0-0-1	+
25	57	F	Liver cirrhosis	HCV	Spouse	2-1-1-1	+
26	58	M	Liver cirrhosis	HBV	Offspring	1-1-1-1	−
27	44	F	Autoimmune hepatitis	—	Sibling	0-0-0-1	+
28	46	F	Liver cirrhosis with HCC	HBV	Offspring	1-1-0-1	−
29	68	F	Liver cirrhosis with HCC	HCV	Offspring	1-1-0-1	+

LTx, liver transplantation; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; ND, not done; HLA, human leukocyte antigen.

progressively worsened, received low-dose steroid-pulse therapy (125–250 mg/day of methylprednisolone for 2–3 days). Patients who were eventually diagnosed as having acute rejection by liver-allograft biopsy and CFSE-MLR assay received additional steroid-pulse or OKT 3 therapy if necessary. However, when antidonor MLR revealed a hypo-response, the patient did not receive further antirejection therapy.

CFSE Labeling

Peripheral blood mononuclear cells (PBMC, 1×10^7 cells/mL) were resuspended in phosphate-buffered saline (PBS). 5-(and 6)-CFSE (Molecular Probes, Inc., Eugene, OR) was added to make a final concentration of 5 μM, and the cells were gently mixed and incubated for 15 minutes at 37 °C in a CO_2 incubator protected from light. Labeling of cells was stopped by adding cold PBS with 2% fetal bovine serum (Sanko, Tokyo, Japan), and the cells were then washed and resuspended in MLR medium, that is, RPMI culture medium containing 15% controlled process serum replacement-type 3 (Sigma, St. Louis, MO), 50 μM 2-mercaptoethanol (Katayama, Osaka, Japan), 1% HEPES buffer (Gibco, NY, NY), and 100 IU/mL penicillin-100 μg/mL streptomycin (Gibco, NY, NY).

MLR Assay

The PBMC prepared from recipients (autologous control), donors, and healthy volunteers (third-party control) as stimulator cells were irradiated with 30 Gy, and those as responder cells from recipients were labeled with CFSE, as described above. Both the stimulator and responder cells in the MLR medium were adjusted to 2×10^6 cells/mL of medium and cocultured in a total volume of 2 mL of medium in 24-well flat-bottom plates (BD Labware, Franklin Lakes, NJ) at 37°C in a 5% CO_2 incubator in the dark for 5 days. After MLR culture, nonadherent cells were harvested and stained with either phycoerythrin-conjugated CD4 or CD8 monoclonal antibodies (mAbs; BD Farmingen, San Diego, CA) together with allophycocyanin-conjugated CD25 mAb (BD Farmingen). Four-color FCM was performed on a FACSCalibur dual-laser cytometer (Becton Dickinson, Mountain View, CA) using standard Cell Quest acquisition/analysis, and fluorescence compensation was achieved using an appropriate single fluorochrome-labeled sample. Dead cells were excluded from the analysis by light-scatter or propidium iodide.

Quantifying Proliferation of $CD4^+$ and $CD8^+$ T Cells

Precursor frequency (PF), proliferation index (PI), and stimulation index (SI) were quantitatively estimated using a method described previously (*10, 12*). The CFSE fluorescence intensity of the peak of cell division, which was divided once, shows a half value of CFSE-fluorescence intensity of the peak of nonreactive cell division. Divisions of reactive cells, which were identified and determined by their CFSE intensities, were labeled from 0 to n as dividing time. A single cell dividing n times will generate 2^n daughter cells. With use of this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division and from proliferation events and PF in $CD4^+$ and $CD8^+$ T-cell subsets. With use of these values, proliferation events and PI were calculated. SI was calculated by dividing PI of allogeneic combinations by those of self-control.

RESULTS

Clinical Characteristics

The target blood levels of calcineurin inhibitors were achieved in all 29 recipients in this series (i.e., trough whole-blood levels of tacrolimus were maintained between 8 and 15 ng/mL in the first few postoperative weeks and thereafter between 5 and 10 ng/mL, and those of cyclosporine were maintained between 100 and 200 ng/mL in the first few postoperative weeks and thereafter between 100 and 150 ng/mL). Twelve (41.4%) of the 29 patients developed significant disorders in liver function (levels of serum bilirubin, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were routinely measured as indexes of liver function) within 6 months after LDLT (Table 2). In 6 of those 12 patients, immunosuppressive treatment was not intensified because liver dysfunction was not progressive. However, the other six patients (including 4 patients who were eventually diagnosed by CFSE-MLR as having ACR) in whom liver function progressively worsened received low-dose steroid-pulse therapy (125–250 mg/day of methylprednisolone for 2–3 days) after CFSE-MLR, and needle biopsy had been performed but before their results had been obtained. Eight of the 12 patients suffering from liver function disorder were diagnosed as having mild or moderate ACR by liver-allograft biopsy (overall incidence of 27.6%). The other four patients in whom ACR was ruled out by results of liver biopsy were retrospectively diagnosed as having either drug-induced hepatotoxicity (in 2 patients) or congestion of the anterior segment of the liver allograft (in 2 patients). In contrast, only 4 of the 12 patients suffering from liver-function disorder showed remarkable proliferation of $CD8^+$ T cells in association with CD25 expression on anti-donor CFSE-MLR (overall incidence of 13.8%). In those four patients, antirejection therapy consisting of steroid pulse (250 mg/day for 3–5 days) with OKT 3 therapy (in 2 patients) or without OKT3 therapy (in 2 patients) resulted in remarkable improvement. Four patients who were diagnosed as having ACR but did not show significant antidonor responses of CD8 T cells in the CFSE-MLR were eventually diagnosed as having recurrence of original hepatitis (i.e., HCV in 2 patients and autoimmune hepatitis [AIH] in 1 patient) or drug-induced hepatotoxicity (in 1 patient) by results of further extensive examinations (i.e., detection of elevation of HCV RNA levels or autoantibodies/globulins in peripheral blood). In the patients diagnosed as having HCV recurrence, immunosuppressive treatment was not intensified. In contrast, in the patients diagnosed as having AIH recurrence, mycophenolate mofetil was administered (750–1,500 mg/day) together with tacrolimus and methylprednisolone.

Histology

Discrepancy between liver-biopsy and MLR results is likely to be caused by the difficulty of differential diagnosis between ACR and recurrence of original hepatitis (HCV and AIH) (*4*). In general, histologic characteristics of ACR are portal inflammatory infiltrates and nonsuppurative cholangitis with or without endotheliitis (Fig. 1, A and B). In con-

TABLE 2. Results of liver allograft biopsy and CFSE-MLR

Patient no.	Timing of biopsy and MLR (postLTx days)	Histopathologic diagnosis	CFSE-MLR stimulation index				$CD25^+$ cells among proliferating $CD8^+$ cells (%)	
			CD4		CD8			
			Donor	Third	Donor	Third	Donor	Third
6	12	Acute rejection (mild)	1.4	2.0	1.6	2.4	41.7	69.1
8	170	Acute rejection (mild)	0.4	0.4	1.1	0.9	19.9	17.1
9	68	Focal necrosis and bile stasis	0.6	2.6	0.3	0.4	ND	ND
13	59	Acute rejection (mild)	1.2	2.4	3.5[a]	1.4	69.7	30.1
15	29	Acute rejection (mild)	0.7	1.5	1.2	1.3	27.5	20.2
21	58	Acute rejection (mild)	0.4	1.1	0.9	1.3	9.0	43.6
22	26	Bile stasis	1.8	1.8	1.0	3.8	33.3	85.5
23	15	Acute rejection (mild)	14.5	5.2	68.3[a]	14.7	76.6	82.8
24	21	Centilobular hepatocellular degeneration	4.4	4.8	1.5	4.0	27.0	64.6
25	14	Acute rejection (mild)	3.3	1.5	3.6[a]	1.2	67.0	7.6
27	21	Acute rejection (moderate)	5.1	12.8	16.3[a]	9.3	80.8	82.3
29	30	Focal necrosis and bile stasis	4.5	2.5	2.4	2.1	1.4	5.7

[a] Data showing significant proliferation of CD8− T cells in anti-donor MLR.
ND, not done; MLR, mixed lymphocyte reaction; CFSE, carboxyfluorescein diacetate succinimidyl ester; LTx, liver transplantation.

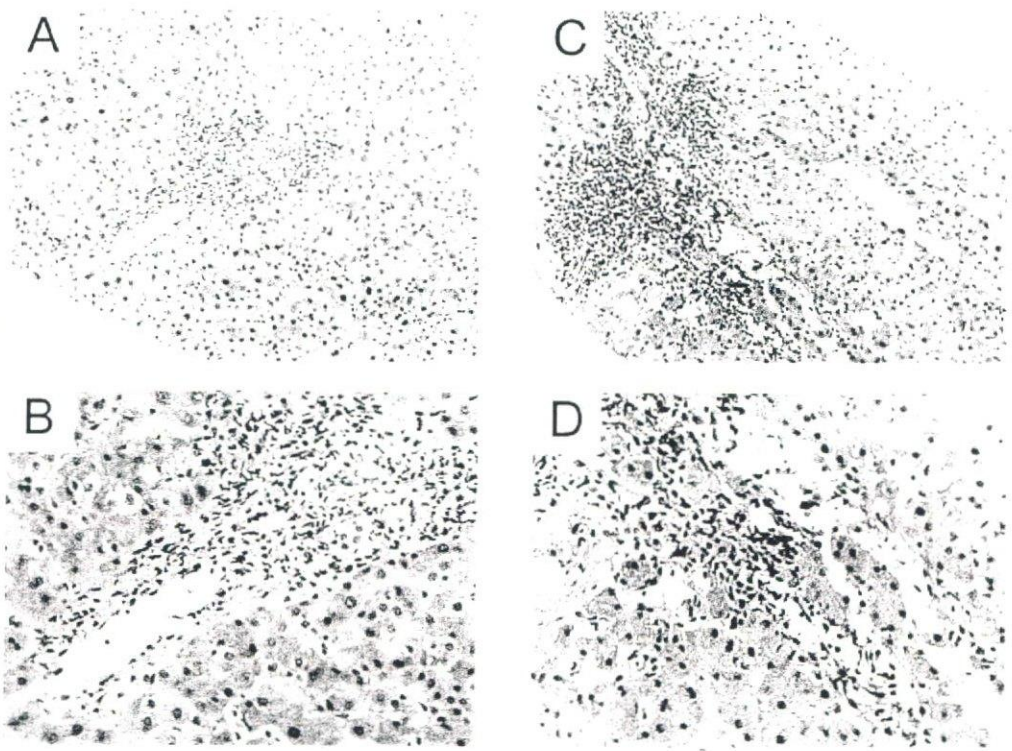

FIGURE 1. Representative histopathologic findings of liver allograft biopsies. (A) (magnification, ×100) and (B) (×200): portal inflammatory infiltrates and nonsuppurative cholangitis with endotheliitis were observed, leading to the histologic diagnosis of acute rejection (patient 23). (C) (×100) and (D) (×200): mild periportal hepatitis with lymphoid aggregates, the most common biopsy presentation of recurrent hepatitis C virus (HCV), was observed (patient 21). Because lymphoid cholangitis and endotheliitis were also found, recurrent HCV was difficult to distinguish from acute rejection. This patient was eventually diagnosed as having HCV recurrence.

trast, mild periportal hepatitis with lymphoid aggregates, with or without fatty change, is the most common biopsy presentation of recurrent HCV. It has been well noticed that lymphoid cholangitis and endotheliitis also are found, and in these instances, recurrent HCV is difficult to distinguish from acute rejection (Fig. 1, C and D). This was true of the two patients in this series who were diagnosed as having ACR by liver biopsy but were eventually diagnosed as having HCV recurrence. Recurrence of AIH is usually defined by the presence of autoantibodies and elevated globulins in association with periportal hepatitis in the absence of viral infection or rejection. The presence of periportal hepatitis with lymphoplasmacytic infiltrates, plasma cells, piecemeal necrosis, and bridging fibrosis in liver-biopsy samples is consistent with recurrent disease of AIH. However, the features of histopathology at the early phase of AIH recurrence were similar to

those for ACR in this series, making it difficult to distinguish between them.

Immune Monitoring by CFSE-MLR Assay

A representative FCM profile in a patient showing hyper-response in antidonor MLR is shown in Figure 2A. When compared with anti–third-party MLR, higher levels of CD4 and CD8 T-cell proliferation were observed. A comparable or even higher level of CD25 expression on the proliferating CD8 T cells in antidonor MLR suggested cytotoxicity activity against donor cells in this patient. A representative FCM profile in a patient showing hypo-response in antidonor MLR is shown in Figure 2B. When compared with anti–third-party MLR, limited levels of CD4 and CD8 T-cell proliferation were observed. Absence of CD25 expression on the proliferating CD8 T cells in antidonor MLR but the presence of that in anti–third-party MLR suggested a lack of cytotoxicity against donor cells in this patient (we have confirmed that only $CD25^+$ proliferating $CD8^+$ T cells have cytotoxic activity against donor cells in our preliminary studies). The SIs in each alloreactive $CD4^+$ and $CD8^+$ T cells in response to antidonor and anti–third-party MLR are shown in Table 2. CFSE-MLR could be a useful tool for precise diagnosis even when differential diagnosis between rejection and recurrence of viral hepatitis is difficult by pathologic examinations. The higher SI in both CD4 and CD8 T cells in antidonor MLR than those in anti–third-party MLR reflects strong antidonor reactivity, confirming the accuracy of diagnosis of ACR.

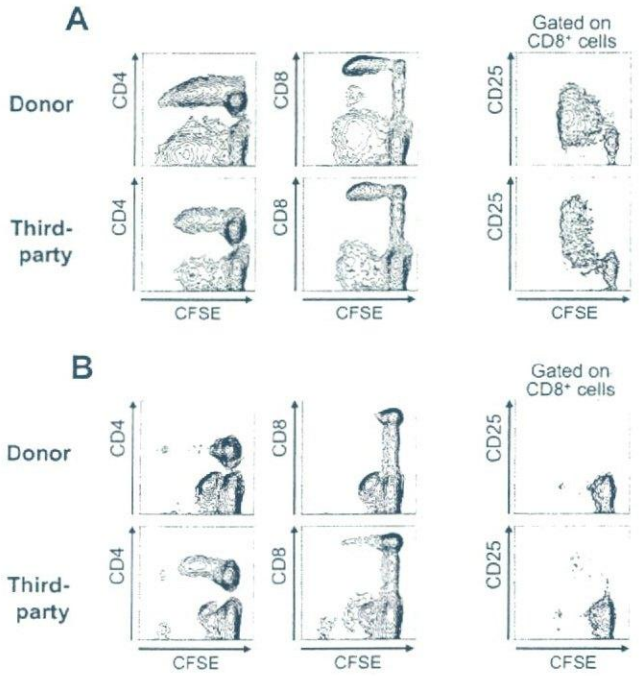

FIGURE 2. (A) Flow cytometry (FCM) profiles in the patient whose histologic appearance of liver allograft biopsy is shown in Figure 1, A and B (patient 23). When compared with anti–third-party mixed lymphocyte reaction (MLR), high levels of CD4 and CD8 T-cell proliferation were observed. A comparable or even higher level of CD25 expression on early proliferating CD8 T cells in both antidonor MLRs was observed. (B) FCM profiles in the patient whose histologic appearance of liver allograft biopsy is shown in Figure 1, C and D (patient 21). When compared with anti–third-party MLR, limited levels of CD4 and CD8 T-cell proliferation were observed. CD25 expression on proliferating CD8 T cells was undetectable in the anti-donor MLR. CFSE, carboxyfluorescein diacetate succinimidyl ester.

DISCUSSION

Antidonor alloreactivity, defined as the number and phenotype of alloreactive precursors in the recipient, can be used to monitor rejection or reduction/withdrawal of immunosuppression. Monitoring such alloreactivity using PBMCs in recipients of transplants does not necessarily mirror what will occur in the allograft tissue because allografts are regulated by infiltrating lymphocytes. However, it has been reported that the frequencies of donor-reactive T cells in PBMCs are closely linked with those in lymphocytes infiltrating allografts (*13*), indicating the validity of evaluation of T-cell responses to allogeneic stimulation using PBMCs for monitoring alloreactivity in transplant recipients. MLR using PBMCs is a widely used method for evaluating T-cell responses to allogeneic stimulation in both experimental and clinical transplantation. However, in conventional forms using tritiated thymidine incorporation, proliferative MLR bulk cultures have very little predictive value because of its low level of reproducibility in the context of transplantation (*14*). The low level of reproducibility of conventional MLR might be caused at least in part by the presence of nonviable cells (which might include unexpectedly surviving stimulator cells) that still have the ability to incorporate tritiated thymidine. By applying a CFSE-based method, the proliferation of viable $CD4^+$ and $CD8^+$ responder T cells in response to allostimulation could be separately quantified using multiparameter FCM. The lack of proliferation of both $CD4^+$ and $CD8^+$ T cells in antidonor MLR would reflect suppression of antidonor response. When remarkable proliferation was observed in $CD4^+$ T cells but not in $CD8^+$ T cells, we did not observe cytotoxic activity against donor cells in the subsequent CML assay in our preliminary studies (data not shown). In contrast, remarkable proliferation of $CD8^+$ T cells would reflect strong antidonor response. We further examined CD25 expression on the proliferating $CD8^+$ T cells by multicolor FCM. The remarkable elevation of CD25 expression on proliferating $CD8^+$ T cells might reflect their cytotoxic activity toward donor cells. In our preliminary studies, the proliferative activity levels of $CD4^+$ and $CD8^+$ T cells were generally higher in MLRs using PBMCs from the spouse than in MLRs using PBMCs from offspring or parents, even before transplantation. This may reflect the greater susceptibility to ACR in recipients of liver allografts from unrelated donors than in recipients of liver allografts from related donors. Consistent with this speculations, MLR-proven ACR occurred in 2 (50%) of the 4 patients receiving LDLT from their spouse but in only 2 (4%) of the 25 patients receiving LDLT from their offspring or parents in the present study. Although the usefulness of CFSE-MLR for the prediction of ACR remains to be elucidated, the results obtained by using this method provided evidence of low incidence of ACR after LDLT compared with previously reported results.

Possible alternative methodologies to diagnose ACR using laboratory-based immunologic modalities might include limiting dilution assay (LDA) and enzyme-linked immunospot (ELISPOT). LDAs provide precise quantification of immunity to a given stimulus and allow the estimation of frequencies of antigen-specific cells participating in an immune response (*15*). Although LDAs have been shown to be specific and reproducible as a measurement of alloreactivity (*16*), conflicting data regarding the usefulness of measurement of cytotoxic T-cell precursors for diagnosis/prediction of rejection in the context of solid-organ transplantation have been reported (*17, 18*). The ELISPOT assay is based on the detection of a cytokine produced by single cells after stimulation with mitogens or antigens (*19*). It has been used to identify the presence of donor-specific T cells in patients before surgery (*20*). However, data indicating the usefulness of this method for diagnosing ACR are less abundant at present.

One of the most difficult challenges in the care of HCV-positive liver-transplant recipients is the differentiation between ACR and HCV recurrence, which can have considerable histologic overlap (*4*). Although polymerase chain reaction allows identification of HCV RNA in biopsy tissue in such difficult cases (*21*), this method could not distinguish between HCV infection alone versus HCV infection complicated by ACR. Attempts to distinguish between these at the intrahepatic gene response level have been made in two studies. The first study analyzed ACR and HCV infection versus HCV infection alone by using gene array analysis (*22*). It has been found that ACR and recurrence of HCV are associated with distinct mRNA expression pattern (i.e., ACR is most notably associated with the relative over-expression of immune activation genes such as major histocompatibility complex classes I and II, tumor necrosis factor [TNF]-α, granzyme B, and complement components). Zekry et al. (*23*) concentrated on Th1 versus Th2-like gene expression and found that ACR in the setting of HCV infection was more like ACR in non–HCV-infected patients and was associated with increase in interleukin (IL)-10 and IL-4 gene expression rather than the IL-2/interferon-γ/TNF-α response seen more in chronic HCV alone. Higher average daily steroid dose and use of OKT 3 have both been associated with more severe recurrence of HCV, presumably through enhancing viral replication or attenuating viral clearance (*24–26*). Minimizing exposure to immunosuppressants for HCV-infected liver-transplant recipients thus requires accurate distinction of recurrence of HCV from ACR. Diagnosis of ACR is based on the detection of biochemical evidence of graft dysfunction and the presence of suggestive allograft histology, including distinct lymphocytic infiltrate patterns. The presence of a modest cellular infiltrate and biochemical abnormalities, however, are not specific to ACR. In addition to the above-described methods, CFSE-MLR also has potential as a tool for diagnosing ACR in HCV-reinfected patients. Additional studies with larger sample sizes are required to confirm this possibility.

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Original Paper

Wall Shear Stress and Intrahepatic Leukocytes of Graft in Living Related Donor Liver Transplantation

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ABSTRACT

Background/Aims: We investigated the influence of HTK solution against natural killer T cells and thymic T cells in liver graft before and after perfusion in adult living related donor liver transplantation.

Methodology: Graft samples were obtained before liver resection, after perfusion, and one hour after liver transplantation. Flowcytometry analysis was conducted using several human natural killer markers; CD16, CD56, CD57, and CD161.

Results: Natural killer T cells existed prominently in the liver leukocytes compared with their presence in peripheral blood lymphocytes, and the difference was significant. CD56+T and CD161+T cells, in comparison with CD16+T cells and CD57+T cells, were especially numerous in the liver. The proportion of CD56+T and CD161+T cells increased in the graft immediately after perfusion with HTK solution. However, CD16+T cells and CD57+T cells decreased in the graft immediately after perfusion and reperfusion of portal blood flow. Thymus-derived cells also decreased significantly after perfusion. The proportion of CD56+T cells among CD3+ cells showed a significant increase immediately after perfusion. All types of natural killer cells in the graft immediately increased after perfusion by HTK solution and reperfusion of portal blood flow. Compared with CD57+NKT cells, CD56+NKT cells showed a significant tendency to stay in the liver graft against the perfusion. CD57+NKT cells tended to wash out from the liver into the systemic circulation. Moreover, thymus-derived T cells showed the strongest tendency to wash out from the liver graft.

Conclusions: CD56+NKT cells and natural killer cells are more involved in local immunity, whereas thymus-derived cells and CD57+NKT cells are involved in regulation of systemic immunity. Allo-immunity between local and systemic systems may be affected by the dynamic changes in hepatic circulation associated with living related donor liver transplantation.

KEY WORDS:
Shear stress; Immunology; Intrahepatic leukocytes; NKT cell; Liver transplantation

ABBREVIATIONS:
Living Related Donor Liver Transplantation (LRDLT); Mononuclear Cell (MNC); Peripheral Blood Lymphocytes (PBL); Sinusoidal Endothelial Cell (SEC); Natural Killer (NK); Auxiliary Orthotopic Partial Liver Transplantation (APOLT); Extra-Hepatic Portal Venous Obstruction (EHO)

INTRODUCTION

The systemic and local immune systems are strongly interrelated, and dynamic immunological changes in the remnant liver and extra-liver site are observed following partial hepatectomy (1-3). The wall shear stress, a simple hemodynamic force caused by venous flow directed against vessel walls (4,5), is the most important factor in the link between the systemic and intrahepatic immune systems following partial hepatectomy (3). We reported that there are two types of leukocytes in the liver: resident leukocytes, such as extrathymic T cells, which tend to stay in the liver against shear stress, and passenger leukocytes, such as thymic T cells, which are washed by the increased portal flow out of the liver and recruited into the systemic circulation (6,7). We confirmed this hypothesis in an experiment on perfused liver in mice (8). Intermediate TcR cells and NK1.1T cells tended to stay in the liver against perfused solution. Conversely, thymic T cells, compared with natural killer T (NKT) cells, increased in the irrigated solution. In nude mice, these phenomena were more prominent (8). These dynamic immunological changes may influence the allo-immune reaction in liver transplantation. Therefore, we investigated the changes in proportion of NKT cells and thymic T cells in the liver graft before and after perfusion by HTK solution in adult living related donor liver transplantation (LRDLT).

METHODOLOGY

Patients

Adult patients (n=7, 17 to 55 years old) underwent LRDLT between January and December 2000. The primary diseases included two cases of liver cirrhosis, one related to hepatitis B and the other to hepatitis C, one case of primary biliary cirrhosis, one case of primary hepatic amyloidosis, one case of alcoholic liver cirrhosis, one case of neurological Wilson's disease, and

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