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Abstract

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KEYWORDS: renal transplantation, long-term stable recipients, specific CTL unresponsiveness, interleukin-2, interleukin-2 receptors

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Original Article

Analysis of the Immune Status in the Recipients with Long-Term Well-Functioning Kidneys Allografts

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Key words: renal transplantation, long-term stable recipients, specific CTL unresponsiveness, interleukin-2, interleukin-2 receptors

Patients who receive allografts are usually required to take immunosuppressants for their entire lifetimes. Long-term medication with immunosuppressants increases the risk of morbidity and mortality of organ recipients; a wide range of associated adverse effects are included among these risks. It is desirable to attempt to reduce or discontinue the dosage of immunosuppressants

in the selected patients with stable allografts.

In vitro assays that test a patient's immune status have been extensively performed to determine the reactivity of organ transplant recipients against donor alloantigens with a view to modifying immunosuppressive therapy. Mixed lymphocyte reactions (MLR) [1-5] and cell-mediated lympholysis (CML) assays [6-10] have been used to evaluate immune responsiveness against donor alloantigens. Although several researchers, including ours [11], have reported the development of donor-specific immune hyporesponsiveness in recipients with well-

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functioning allografts, there is still controversy concerning the prognostic value of these immunological studies of clinical transplantation [13-15].

This study was undertaken in order to analyze the more detailed mechanisms mediating the long-term acceptance of renal grafts under a low dosage of immunosuppressants. In the early phase of this study, changes in MLR and CML assays were examined using peripheral blood lymphocytes (PBL) freshly isolated from recipients and cryopreserved PBL at pretransplantation. To explore in more detail the mechanism(s) mediating donor-specific cytotoxic T lymphocyte (CTL) unresponsiveness, the following immunological assays were also performed: an interleukin-2 (IL-2) production assay in mixed lymphocytic culture (MLC) and a flow cytometric analysis of IL-2 receptor (IL-2 R) expression in MLC cells. The suppression of monoclonal antibodies (mAb) for IL-2 R on MLRs was compared in recipient cells, donor cells, and third party cells. A comparison of third party and donor cells was performed, in addition to the MLR and CML assays. We will discuss the immunological mechanisms mediating the donor specific CTL unresponsiveness below.

Material and Methods

Patients. Thirteen living and related renal transplant recipients were selected at random for this study based upon their long-term stable renal function and donor cell availability. All patients received a parent's kidney (HLA, one-haplotype-mismatched donor) and maintained well-functioning allografts following transplantation at the time of the study (3 years and 7 months to 11 years and 4 months; mean \pm SD; 8 years and 3 months \pm 2 years and 6 months). Informed consents for this study were obtained from the recipients, donors, and healthy volunteers who served as controls.

All patients received either dual immunosuppression therapy (methylprednisolone (MP) and azathioprine (AZP), 8 patients) or triple therapy (MP, AZP and cyclosporine A (CsA), 5 patients). In one patient, MP was withdrawn and in another patient undergoing triple therapy, mizorbine (MB) was administered instead of AZP. The mean level of serum creatinine at the study was 1.3 ± 0.3 mg/dl. The recipients' characteristics are listed in Table 1.

PBL. PBL were obtained from the blood of recipients, donors and healthy volunteers. PBL were

isolated by density gradient centrifugation over Ficoll-Hypaque. PBL of healthy volunteers were usually used as controls. Other donor cells were used in some cases based upon HLA disparity. When cryopreserved recipients' PBL were available prior to transplantation, they were subjected to the assays. We repeatedly confirmed the reactivity of cryopreserved cells; this reactivity was comparable to that of freshly isolated cells. The culture medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 25 mM HEPES buffer, penicillin, streptomycin, and 10% fetal calf serum.

MLR. The MLR culture was established in 96-well, round-bottomed microplates for a final volume of 200 μ l complete medium. Fifty thousand responder cells were cocultured with the equal numbers of mitomycin C (MMC)-treated stimulator cells for the indicated number of days (3-9 days). All cultures were pulsed 18 h before they were harvested with 1 μ Ci of 3 H-thymidine; and then counts per minute (cpm) of incorporated 3 H-thymidine were determined. The stimulation index (SI) and relative response (RR) were calculated using the following formula:

$$SI = \frac{\text{donor or third party cpm}}{\text{autologous cpm}}$$

$$RR = \frac{(\text{donor cpm} - \text{autologous cpm})}{(\text{third party cpm} - \text{autologous cpm})} \times 100$$

Table 1 Recipient characteristics

No.	Age	Sex	Period after Tx	DST	Immunosuppressants	CRTN (mg/dl)
1	37	m	11y4m	-	MP, AZP	1.5
2	40	m	10y9m	+	MP, AZP	1.1
3	32	f	10y9m	+	MP, AZP	1.1
4	43	m	10y5m	+	MP, AZP	1.9
5	30	f	9y6m	-	MP, AZP	1.5
6	43	m	9y3m	+	MP, AZP	1.2
7	34	f	8y10m	+	MP, AZP	0.8
8	38	m	8y10m	+	MP, AZP	1.2
9	38	m	7y8m	+	CsA, MP, AZP	2
10	25	f	7y7m	+	CsA, MP, AZP	1.2
11	23	m	4y5m	-	CsA, MP, AZP	1
12	28	m	4y2m	+	CsA, MP	1
13	26	m	3y7m	-	CsA, MP, MB	1.1
Mean	33.6					1.3
SD	6.6					0.3

AZP, azathioprine; CsA, cyclosporine A; DST, donor-specific transfusions; MB, mizorbine; MP, methylprednisolone; Tx, transplantation.

CML assays. Equal numbers (5×10^6) of responder and MMC-treated stimulator cells were cocultured for 7 days in a total volume of 10ml culture medium in a 25 cm³ culture flask. Upon the completion of incubation, the cells were harvested, washed, and resuspended as effector cells. The 5×10^6 stimulator and third party cells were cultured separately in 10ml of complete medium in the presence of 10 μ g/ml phytohemagglutinin-P (PHA-P). On the third day, additional 10 μ g/ml PHA-P was added and incubated for 4 more days. After 7 days of incubation, 1×10^6 PHA-P-stimulated target cells were labeled with 100 μ Ci ⁵¹Cr, incubated for 1 h, and then were washed and resuspended in culture medium. Five thousand ⁵¹Cr-labeled target cells (100 μ l) were added to graded amounts of effectors (50:1 to 6.25:1) in triplicate, in a 96-well, round-bottomed microplate for a total volume of 200 μ l culture medium. Following a 6 h incubation, the supernatant was removed and counted in a gamma counter. Spontaneous release was determined by incubating target cells in medium alone, while maximum release was determined from target cells exposed to 1N NaOH. The percentage of specific lysis was defined according to the following formula:

$$\% \text{Cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

% Cytotoxicity was represented at effector: target ratio of 50:1.

IL-2 production assay. IL-2 production by the responder cells was determined by the bioassay using an IL-2-dependent cell line, CTLL-2. Five thousand CTLL-2 were seeded to a 96-well, flat-bottomed microplate in 100 μ l complete medium. One hundred μ l of the MLC supernatant were harvested on day 3 under the same conditions as used for the MLR. Supernatant was added at various dilutions to microplates conditioned with CTLL-2 (final volume, 200 μ l). Microcultures were incubated for 24 h including a 4-hour terminal pulse with 1 μ Ci ³H-thymidine. Cultures were harvested and ³H-thymidine incorporation was determined. Cpm was converted to U/ml IL-2 using the standard curve generated with recombinant human IL-2 (rIL-2, Imunace[®] 35, Shionogi & Co., Ltd, Osaka, Japan).

Flow cytometric analysis of IL-2R expression. The following mAbs were used: fluorescein isothiocyanate (FITC)-conjugated CD25 (IL-2R α chain), phycoerythrin-conjugated Leu 3_a (CD4), Leu 2_a (CD8) (Becton Dickinson, Mountain View, CA, USA) and

biotinylated TU-25 (the mAb specific for IL-2 p75, β chain; a generous gift from Professor Sugamura of Tohoku University, Sendai, Japan). The cells were harvested from MLC on day 3 under the same conditions as those used for the MLR were stained simultaneously with FITC-conjugated CD25 or biotinylated TU-25 and PE-conjugated CD4 or CD8. When stained with biotinylated TU-25, the samples were washed, combined with FITC-avidin, and incubated for 30 additional min at 4 °C. After the completion of staining with the mAbs, the cell samples were analyzed using a FACScan (Becton Dickinson). The minimum fluorescence intensity considered positive for staining was established by labeling with control FITC and PE-conjugated mouse immunoglobulin. The percentage of IL-2 R α and β chains on CD4 or CD8 subsets were determined from two-color FACS counter plots.

Inhibition of the mAbs against IL-2 R on the MLR. The H-31 and TU-25 were IgG₁ mAb directed against IL-2 R α and β chains, respectively, and were kindly donated by Professor Sugamura. Both H-31 (0.5 μ g/ml) and TU-25 (2.5 μ g/ml) were added to the 3 combinations of the MLRs (recipient versus donor, recipient versus third party, and third party versus donor) from the beginning of the culture. The percentage of suppression was defined as:

$$\% \text{suppression} = (1 - \text{cpm in the presence of mAbs} / \text{cpm in the absence of mAbs}) \times 100$$

Statistical analysis. Where appropriate, paired *t*-tests were used for the comparison between the 2 groups and Tukey's test was used for the multiple comparison among the 3 groups. We did not perform a statistical analysis when one group had data for less than 5. A value of less than 0.05 was considered significant.

Results

MLR. The post transplant SI of anti-donor MLR increased, compared with that of pretransplantation SI (2.0 ± 0.3 versus 8.0 ± 7.4). The RR had slightly decreased, because the increase in anti-third party SI post-transplantation was greater than that of anti-donor SI (Table 2). In posttransplant MLR, anti-donor SI ≤ 4.0 was observed in only 4 out of 13 patients and the anti-third party SI was ≤ 4.0 in 2 of the 4 patients with anti-donor SI ≤ 4.0 .

In the kinetics study of posttransplant MLR from 11 patients, a peak response against donor or third party cells was seen on the same day, namely, on day 7 (C and

D in Fig. 1). In one patient, the day of the peak response of anti-donor and third party cells was the same, namely, on day 9, which was 2 days later than that of the majority (A in Fig. 1). Fig. 1 B, depicts the peak MLR response of one patient's cells against donor cells, this peak was seen later than that against third party cells. No early proliferation was observed after the donor stimulation had

been carried out.

CML assays. CTL against donor cells were not generated in any of the recipients tested except for in 2 of the recipients, as observed in posttransplant CML assays ($n = 10$). The percentage of cytotoxicity against donor cells in these 2 patients was less than 10%. Moreover, the addition of 50 U/ml rIL-2 at the initiation of the culture failed to restore the donor-specific CTL in 7 cases. Anti-donor CTL was detected in only one patient, in the presence of rIL-2 with a % cytotoxicity of more than 10% (17.1%). The percentage of cytotoxicity against donor cells in another 2 patients was very low (7.4% and 6.9%, respectively) even with the rIL-2 addition. In the pretransplant CML assays, anti-donor CTL were detected with a mean % cytotoxicity of 32.9 ± 18.9 in 4 of the recipients examined.

On the other hand, the levels of cytotoxicity against third-party cells detected in pre- and posttransplant CML assays were 31.6 ± 16.3 and 21.3 ± 13.9 , respectively.

Table 2 Results of the MLR assay

	pre-Tx ($n = 4$)	post-Tx ($n = 13$)
Stimulation index		
Against donor cells	2.0 ± 0.3	8.0 ± 7.4
Against third-party cells	2.8 ± 0.8	$18.1 \pm 16.7^*$
Relative response	63.4 ± 29.6	50.3 ± 23.1

Tx, transplantation.

*, $P < 0.05$ difference from the corresponding value against donor cells.

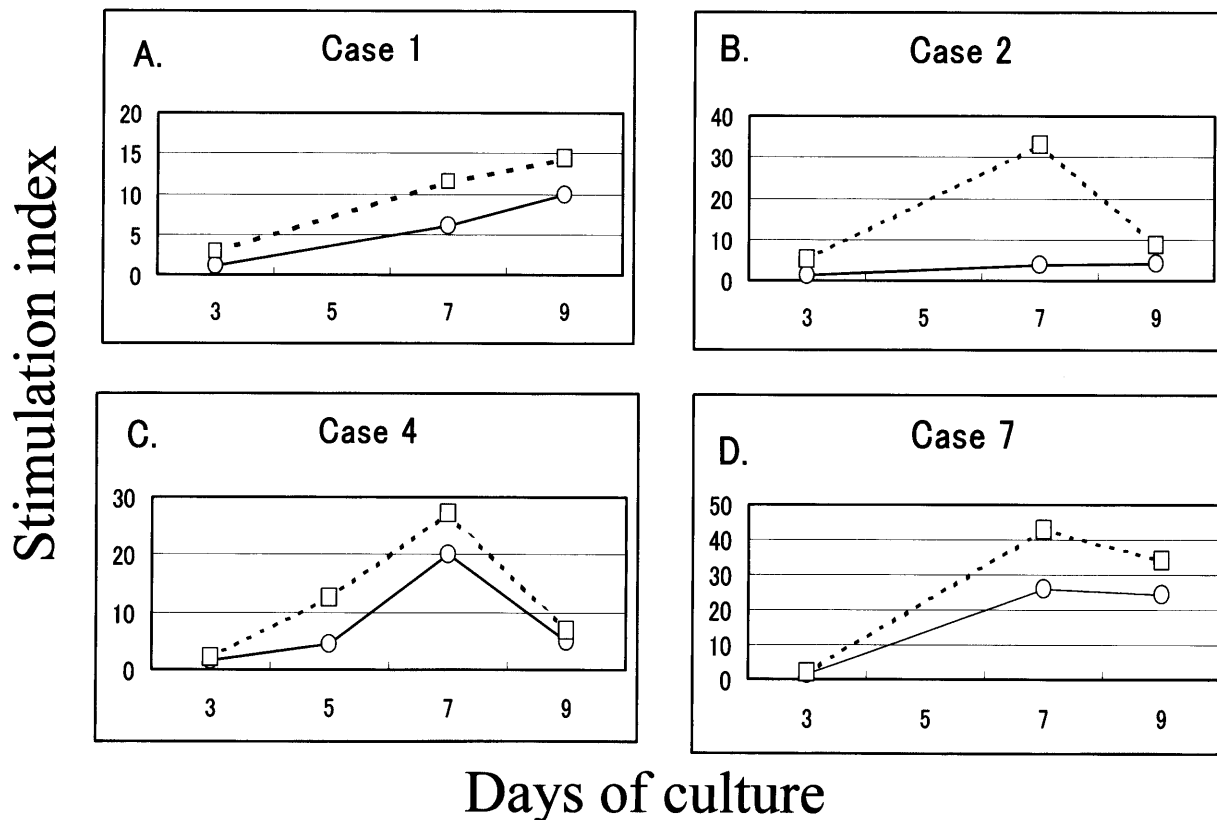


Fig. 1 The kinetics of posttransplant MLR. The stimulation index was calculated by the formula described in Materials and Methods. ○—○, anti-donor MLR; □—□, anti-third-party MLR.

The addition of rIL-2 to the effector induction culture slightly increased mean % cytotoxicity to 32.5 ± 13.9 , which was comparable to the pretransplant value (Table 3).

IL-2 production in MLC. The IL-2 production assays were performed using only posttransplant recipients' PBL due to the lack of available pretransplant cryopreserved cells. The MLC responder cells stimulated by donor cells made measurable amounts of IL-2 after 3 days of culture (13.0 ± 8.5 U/ml), these amounts were higher than those produced by the responder cells cultured with autologous cells (0.9 ± 0.7 U/ml). In a simultaneous comparison of the 3 groups, it was found that the responder cells stimulated by third-party cells produced the highest amounts of IL-2 (60.6 ± 38.6 U/ml) and there was a significant difference between the IL-2 production of the responder cells stimulated by third-party cells and those stimulated by autologous cells (Table 4).

IL-2 R expression on MLC cells. We

examined the IL-2 R expression in MLC cells to explore the mechanisms mediating donor-specific CTL unresponsiveness, even with the addition of rIL-2 in these long-term stable recipients. IL-2 R α and β chains expression was analyzed flowcytometrically on CD4⁺ or CD8⁺ subsets of MLC cells stimulated by donor or third party cells after 3 days of culture. Fig. 2 shows the representative results of these studies. As demonstrated in Fig. 2, CD4⁺ and CD8⁺ cells similarly expressed IL-2 R α and β chains on their surfaces after donor or third-party stimulation, although the expression of the IL-2 R α chain was relatively low in CD8⁺ cells. Similar results were observed in the cells of other recipients (Table 4).

Inhibition of the mAbs against IL-2 R on the MLRs. We previously reported a synergistic inhibitory effect of the mAb against the IL-2 R β chain, TU-25, on both MLR and CML in combination with the mAb against the IL-2 R α chain, H-31 [16]. The anti-donor CTL could not be induced even with the

Table 3 Results of the CML assay

	pre-Tx (n=4)	post-Tx (n=10)	post-Tx (n=10) IL-2 (50u/ml) additon
Against donor cells	32.9 ± 18.9	1.1 ± 3.0	3.1 ± 5.4
Against third-party cells	31.6 ± 16.3	21.3 ± 13.9 ^a	32.5 ± 13.9 ^b

Tx, transplantation.

Percent cytotoxicity is represented with that at an effector-to-target ratio of 50:1.

^a, $P < 0.01$ difference from the corresponding value against donor cells; ^b, $P < 0.0001$ difference from the corresponding value against donor cells.

Table 4 IL-2 production assay and IL-2 receptor expression analysis of MLC cells

Stimulator cells	IL-2 production ^a (U/ml)	IL-2 receptor expression ^b (%)			
		CD4 positive cells		CD8 positive cells	
		α chain	β chain	α chain	β chain
Autologous cells	0.9 ± 0.7 (n=5)				
Donor cells	13.0 ± 8.5 (n=9)	13.2 ± 3.2 (n=7)	47.3 ± 15.2 (n=3)	1.9 ± 1.1 (n=7)	46.6 ± 23.3 (n=3)
Third-party cells	60.6 ± 38.6* (n=9)	13.5 ± 3.1 (n=7)	44.2 ± 30.1 (n=3)	3.0 ± 1.6 (n=7)	40.9 ± 19.4 (n=3)

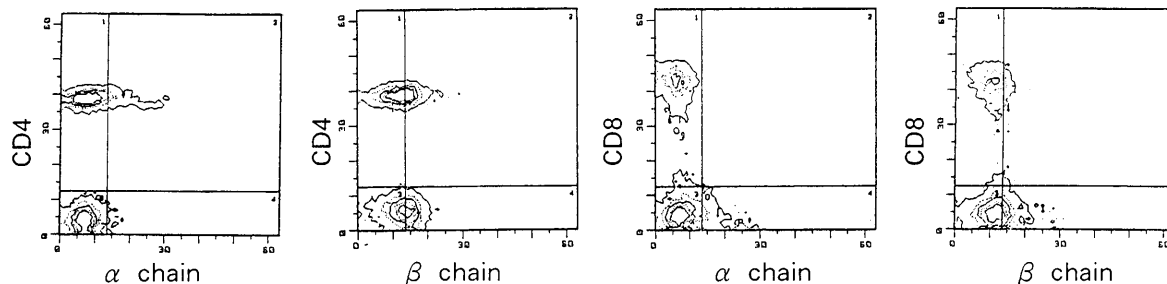
The number of patients is given in parentheses.

^a, supernatants of MLC cells on day 3 were tested for their IL-2 activity by a bioassay using the CTLL-2 cell line.

^b, the cells harvested on day 3 from MLC were stained with FITC or PE-conjugated monoclonal antibodies and then were analyzed using a FACScan.

*, $P < 0.05$ difference from the corresponding value against autologous cells.

A. Donor Cells



B. Third Party Cells

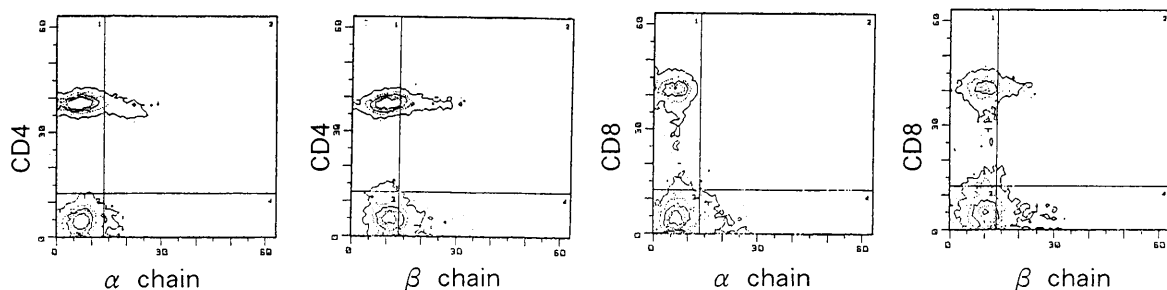


Fig. 2 Flow cytometric analysis of IL-2 R α and β chain expression on CD4⁺ and CD8⁺ subsets of MLC cells. The cells harvested on day 3 from MLC against donor cells (A) and against third-party cells (B) were stained with FITC or PE-conjugated mAbs and then analyzed using a FACScan.

addition of exogenous rIL-2, in spite of the same level expression of IL-2 R on MLC cells after stimulation with donor or third-party cells that was achieved in most cases. The inhibitory effects of the mAbs against IL-2 R were then tested on the MLRs with the stimulator from donor or third-party cells. The “H-31” (0.5 μ g/ml) and “TU-25” (2.5 μ g/ml) were simultaneously added to the 3 groups of MLRs (responder versus stimulator; recipient versus donor, recipient versus third-party and third-party versus donor). The level of mean % suppression on each MLR was as follows: 62.2 \pm 11.4% in the MLR, recipient versus donor group, 61.2 \pm 10.4% in the recipient versus third-party group, and 68.3 \pm 8.9% in the third-party versus donor group, respectively. There was no significant difference among them.

Discussion

Three striking findings emerged from the present study examining the immune status of long-term stable renal transplant recipients:

1. In most of the recipients, donor-specific CTL were not generated in the posttransplant CML assays, even with the addition of rIL-2; however, anti-donor CTL were detected at pretransplantation.
2. The MLC cells stimulated with donor cells expressed IL-2 R on their surfaces to the same degree as those stimulated with third-party cells. In addition, the mAbs against IL-2 R similarly inhibited the proliferative responses of the recipients' PBL against donor or third-party cells.
3. The SI of the MLR and IL-2 production against donor cells were lower than those against third-party cells, but the anti-donor SI was greater than that at pretransplantation.

The developments of donor-specific hyporesponsiveness or unresponsiveness in organ transplant recipients with well-functioning grafts have been documented by numerous other researchers [1-3, 8-11, 17, 18]. However, it remains unclear whether or not there is a correlation between the results of these immunological tests and their clinical prognostic value [12-15]. Such monitoring

efforts seem to be limited by the wide diversity of mechanisms involved in the rejection process, rendering the interpretation of a single examination very complicated.

Here, we discuss the results of various immunological tests performed on 13 renal recipients. In MLR, the mean anti-donor SI post transplantation value was higher than that observed at pretransplantation. Donor-specific hyporesponsiveness (anti-donor SI ≤ 4 and anti-third party SI > 4) in MLR was observed in only 2 patients. Nonspecific hyporesponsiveness (both anti-donor and third party SI ≤ 4) was also observed in 2 patients. In the other 9 patients, both anti-donor and third party SI were greater than 4. In the CML assays, the anti-donor CTL were not induced (the cytotoxicity percentage was > 10), however, anti-third party CTL were generated with a mean % cytotoxicity of 21.3. The addition of 50 U/ml rIL-2 to the effector-cell induction culture failed the donor-specific CTL in all but one recipient tested. Despite the lower amounts of IL-2 produced by the MLC cells stimulated with donor cells compared with those stimulated with third party cells (approximately 21.5%), CD4 and CD8 MLC cells similarly expressed IL-2 R after donor or third-party cell stimulation. We have reported that the mAb against the IL-2 R β chain inhibited both MLR and CTL generation synergistically with the mAb against the IL-2 R α chain [16]. Since anti-donor CTL could not be induced in posttransplant CML, we examined the inhibitory effects of anti-IL-2 R mAbs using MLRs. The mAbs specific for IL-2 R α and β chains equally inhibited the anti-donor and anti-third-party MLRs. We did not perform IL-2 production assays or IL-2 R expression analysis of pretransplant MLC due to the lack of available recipient PBL. Donor-specific hyporesponsiveness of MLR at posttransplantation was observed only in a limited number of recipients, namely, in 2 of 13 recipients tested, whereas in most cases, the unresponsiveness of anti-donor CTL in posttransplant CML was resistant to the addition of exogenous IL-2. These results indicate that the donor-specific CTL unresponsiveness might be mediated by mechanisms other than the interruption of IL-2 and IL-2 R systems in alloantigen-induced immune responses.

There was no definitive difference among the results of the above immunological examinations between the recipients preconditioned with the donor specific transfusions (DST) and those without DST, or between the patients treated with immunosuppressants including CsA and

patients treated without CsA. However, it should be noted that only a small number of patients belonged to each group in this study.

Transplantation tolerance, defined functionally as long-term allograft survival without immunosuppression, is rarely achieved clinically [19, 20], whereas the immunological tolerance to alloantigens has been successfully induced in laboratory animals, especially rats, by various protocols [21-23]. Three main mechanisms to explain transplantation tolerance have been proposed, namely, 1) clonal deletion, 2) clonal anergy, and 3) active regulation (reviewed in reference 24). The donor-specific unresponsiveness of CTL generation observed in the present study might be explained by any of these three mechanisms or by a combination thereof.

If the anti-donor CTL precursors (CTL_p) were selectively deleted in these recipients, it would account for the findings in this study. The data for the limiting dilution analysis (LDA) assay, which examined the frequency of donor specific CTL_p in renal transplant recipients, varied between investigators. Lantz *et al.* reported that there was no strong decrease in the frequency of donor-reactive CTL_p compared with that of third-party CTL_p in cadaveric renal transplant recipients [25], while Hardy GA *et al.* illustrated that 5/14 long term recipients preconditioned with DST exhibited a complete absence of anti-donor CTL_p in the LDA assay [26]. The LDA assay examines the frequency of CTL_p at the oligoclonal level and, because of the small number of responder cells in the MLC, it requires a T cell growth factor such as IL-2. It may be difficult use the LDA assay to demonstrate the clonal deletion of specific CTL_p in long-term stable recipients if the donor specific CTL unresponsiveness is resistant to the exogenous addition of IL-2, as observed in the present study. Vaderkerhove BA *et al.* demonstrated a low frequency of anti-donor CTL_p in a long-term renal transplant recipient was restored with an initial proliferation induced by PHA and a subsequent culture which involved the addition of IL-2 [27].

The anergy phenomenon was originally proposed by Schwartz using a CD4⁺ class II restricted T cell clone model [28]: antigenic stimulation without costimulatory signals leads to an absence of response and to a long-lasting subsequent state of anergy without an immunological response after optimal antigenic stimulation. Alard *et al.* studied 8 cadaveric renal recipients and reported donor-specific hyporeactivity on IL-2 production, and in the MLR and CML assays, as was observed in

our previous study [29]. They illustrated that the percentage of CD 25(IL-2 R α chain)⁺ cells and the increase in cell size were similar after donor or third-party stimulation, thus suggesting the possible involvement of clonal anergy in long-term graft acceptance. However, there remains substantial controversy concerning the definition of the anergy phenomenon *in vivo* and a detailed characterization of anergized cells would be helpful, especially in clarifying the relationship between anergy and IL2 R expression [30].

Active regulation by suppressor cells is also a compelling explanation of long-term allograft survival; this notion is derived from the results of cell mixing experiments [9, 31] and from the V-shaped curve obtained in LDA assays [32]. In the present study, although the RR of MLR at pre- and posttransplantation were comparable, the IL-2 production in anti-donor MLC were lower and anti-donor CTL were not induced at posttransplantation. Therefore, it is possible that potent suppressor cells proliferate in anti-donor MLC, and then inhibit IL-2 production and interrupt CTL generation. However, none of the present findings indicate that the suppressor cell system mediates specific unresponsiveness.

The patients examined in this study had been administered several types immunosuppressant; thus they did not tolerate kidney allografts. However, donor-specific CTL unresponsiveness seems to play an important role in maintaining allografts in these recipients, if they are on a low dose of immunosuppressants. If one could define anergy as the persistence of antigen-specific cells after antigenic stimulation and without T cell function (IL-2 secretion or CTL induction), we believe that the among 3 mechanisms proposed for organ transplantation tolerance clonal anergy phenomenon would be the primary underlying mechanism for specific CTL unresponsiveness in these recipients. Because PBL from all recipients tested retained a proliferative response against donor cells and expressed IL-2 R α and β chains similarly after donor or third-party cell stimulation. However, none of the available evidence definitively excludes the 2 other mechanisms of specific CTL unresponsiveness.

Recently, papers discussing the molecules mediating costimulatory signals and the subsequent intracellular events in T cell activation have been rapidly accumulating [34, 35]. Further studies, including molecular analyses of the alloantigen-induced immune response in long-term stable allograft recipients, are required to fully understand the mechanisms mediating allograft acceptance. We

believe such approaches are of critical importance in order to achieve a better clinical transplantation outcomes that lead to the final goal of clinical transplant tolerance.

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