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ANALYSIS OF THE DONOR-SPECIFIC CYTOTOXIC T LYMPHOCYTE REPERTOIRE IN A PATIENT WITH A LONG TERM SURVIVING ALLOGRAFT

Frequency, Specificity, and Phenotype of Donor-Reactive T Cell Receptor (TCR)- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ Clones¹

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In the present study the transplant specific CTL repertoire of a patient (HLA:A1,3, B8,18, Cw5,7 DR3, DQw2, DPw3) with a long term surviving HLA mismatched kidney graft (HLA: A1,24 B8,27 Cw2,7, DR3,w13 DQw2,6 DPw1,3) has been investigated. This patient was unable to generate specific cytolytic activity against donor-derived PHA-blasts in the MLC in which donor spleen cells or B lymphoblastoid cell line were used as stimulator cells. In addition, the CTL precursor frequencies against donor alloantigens were very low (1/67,000). The patient had otherwise normal immune responses *in vivo* and *in vitro* and no signs of transplant rejection. Transplant specific CTL clones were generated in high frequencies (1/195) from T cell bulk cultures activated by PHA in the absence of any sensitization by donor Ag *in vitro*. The repertoire of 14 donor-reactive CTL clones (12 TCR- $\alpha\beta^+$ and 2 TCR- $\gamma\delta^+$) was analyzed. Two TCR- $\alpha\beta^+$ CD8⁺ clones were specific for B27. Ten TCR- $\alpha\beta^+$ CTL clones directed against class II HLA Ag were isolated. Seven of these were CD4⁺ and recognized DRw13 (3), DQw6 (3), and DPw1 (1), whereas three of these clones were CD4-CD8⁺ recognizing DRw13 (1) and DQw6 (2). In addition, two donor-specific TCR- $\gamma\delta^+$ CTL clones were obtained recognizing HLA-A9(23,24) and DQw6. Our data indicate that the precursors of CTL clones specifically directed against donor class I or II HLA Ag are not deleted from the repertoire and that part of this reactivity resides in the TCR- $\gamma\delta^+$ fraction.

Animal studies have shown that transplantation can induce tolerance to the alloantigens expressed on the transplant. Administration of cyclosporin (1), antithymocyte globulin (2), previous donor-specific transfusions

(3) or infusion of donor-specific enhancing antibodies (4) in combination with allografting can result in some strain combinations in permanent acceptance of the grafts without further conditioning of the recipient. Although graft adaptation might occur in this process, tolerization of the recipient is also of importance, since these animals accept a subsequent graft of the same donor (5). This tolerance is donor-specific and in some experimental models tolerance can be transferred by donor-specific T cells (6).

Induction of transplantation tolerance in man is less well understood and the present knowledge is almost completely derived from *in vitro* studies and case reports. Such studies have shown that cessation of immunosuppressive therapy does not result in adverse effects in a proportion of the patients (7, 8). Furthermore, in about 70% of the patients no cytolytic activity against donor derived PHA-blasts can be generated in MLC 6 mo after transplantation (9, 10). This nonresponsiveness is specific for donor antigens. Other studies showed a decrease in the frequency of CTLp⁴ against donor transplantation antigens after successful transplantation (11). Finally, some evidence for the involvement of a suppressor mechanism has been presented by Goulmy et al. (12) who showed that HLA-B locus Ag of the donor can down-regulate the lytic activity against any HLA-A Ag coexpressed on the stimulator cells.

To investigate the nature of this nonresponsiveness in man, one kidney transplant patient was studied in more detail. This patient was selected because she was in apparent good health nine years after transplantation of a poorly HLA-matched kidney graft and because she developed cell-mediated lympholysis nonresponsiveness in the years after transplantation. In the present study, we describe the isolation of a series of TCR- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ CTL clones specific for donor class I or II Ag. In addition, limiting dilution experiments of PHA-activated PBMC indicated that precursors of these donor reactive CTL clones are present in high frequencies in this patient.

MATERIALS AND METHODS

Patient. Patient JFV is a 38-yr-old woman (HLA:A1,3 B8,18, Cw5,7 DR3, DQw2, DPw3) who received a cadaveric renal allograft

⁴ Abbreviations used in this paper: CTLp, CTL precursor; B-LCL: B lymphoblastoid cell line; FMF, flow microfluorimetry.

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of donor SP (HLA: A1,24, B8,27 Cw2,7, DR3,w13 DQw2,6 DPw1,3) in June 1979. Eight days after transplantation, a histologically proven rejection occurred which was treated successfully. There were no further complications except for multiple spinaliomas, which is the reason her medication was switched from azathioprin to oral cyclosporin (250 mg/day) in September 1987. At present, the patient is healthy, has a creatinin clearance of 96 ml/min, and no hypertension, proteinuria, or urine sediment abnormalities.

Generation of B-LCL. The EBV-transformed cell lines of the patient JFV, donor SP, and the panel members originated from infection of fresh PBMC or spleen cells (of the donor) with EBV obtained from the marmoset cell line B95-8. All cell lines were cultured in Yssel's medium supplemented with 1% pooled human AB⁺ heat-inactivated serum designated culture medium.

mAb. The following mAb were used for blocking studies or FMF analysis: W6/32, B9.12.1 (13) and B1.23.2 (14), all directed against monomorphic HLA class I structures, B1.1G6 (15) anti- β_2 -microglobulin, PdV5.2 (16) against a monomorphic epitope shared by DR, DP and most of the DQ alleles, IIB3 (17) anti-DQw1,8,9, B8.11.2 (18) anti-DR backbone, SPV-L3 (19) anti-DQ backbone, B7/21 (obtained from the Xth International Histocompatibility Workshop) anti-DP backbone, WT32 (anti-CD3), RIV6 (anti-CD4), FK18 (anti-CD8), WT31 (anti-TCR- $\alpha\beta$, gift of Dr. W. Tax, Radboudziekenhuis, Nijmegen), 11F2 (anti- $\gamma\delta$ TCR, gift of Dr. J. Borst, Netherlands Cancer Institute, Amsterdam) (20), δ TCS1 (T Cell Sciences, Cambridge, MA), BB3 (a gift of Dr. L. Moretta, National Cancer Institute, Genova) (21) and T1 γ A (gift of Dr. T. Hercend, Institut Gustave-Roussy, Villejuif) (22). All these mAb were used as diluted ascites, FITC or phycoerythrin-labeled Leu-2 (anti-CD8), Leu-3 (anti-CD4), Leu-4 (anti-CD3), Leu-16 (anti-CD20), anti-IL-2R (anti-CD25) and anti-TCR1 (WT31) were purchased from Becton Dickinson, Mountain View, CA.

Mixed lymphocyte reaction. Irradiated stimulator cells ($5 \cdot 10^4$ spleen cells or 10^4 B-LCL in 50 μ l culture medium) were incubated in microwells for 5 days with $5 \cdot 10^4$ responder cells in an equal volume in a humidified incubator of 5% CO₂. During the last 16 h 1 μ Ci of tritiated thymidine was added. Subsequently the cultures were harvested onto fiber glass filters and tritiated thymidine incorporation was determined by liquid scintillation spectroscopy.

Cytotoxic assay. Appropriate numbers of effector cells (cloned T cells or T cell bulk cultures) were mixed with $2 \cdot 10^3$ ⁵¹Cr-labeled target cells (PHA-blasts or B-LCL cell lines) in 0.2 ml of culture medium in U-shaped microtiter wells. The plates were centrifuged (600 \times g, 1 min) and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO₂. The supernatants were harvested with a Skatron harvesting system and counted in a gamma counter. Percentage of lysis was calculated according to the formula:

$$\text{Percent lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \cdot 100.$$

Maximal release was determined from a saponine lysate of the target cells. Spontaneous release was determined from at least eight cultures containing target cells only.

Blocking of cytotoxic activity with mAb was carried out as follows: a hundredfold dilution of the mAb containing ascites was preincubated with the target cells or effector cells in 0.1 ml at 37°C. After 30 min effector cells or target cells were added without prior washing.

Limiting dilution analysis. The analysis of CTLp frequencies in a limiting dilution culture was performed as described previously (23). Briefly, graded numbers of PBMC from the recipient were cultured in the presence of $5 \cdot 10^4$ irradiated spleen cells or PBMC (3000 rad) as stimulator cells in a total volume of 0.2 ml of culture medium. Twenty-four wells were analyzed for each responder cell concentration. rIL-2 was added as a growth factor. After 7 days of culture each microtiter well was tested for cytolytic activity by replacing 0.1 ml culture medium by 5000 ⁵¹Cr-labeled PHA target cells in the same volume. After mixing and centrifugation the cultures were treated as described above. Cultures were considered to be positive when the ⁵¹Cr release exceeded the spontaneous release (mean of 24 cultures) plus 3 SD. Frequencies of CTLp. 95% confidence interval and *p* value were calculated as described (23).

Generation of donor-specific CTL clones from PHA-blasts. PBMC (10^6 /ml) were stimulated with PHA (0.1 μ g/ml) for 4 days. The activated cells were layered on a Ficoll gradient and centrifuged (15 min, 600 \times g). Living cells were collected from the interface, washed three times, and cloned by limiting dilution at 1 cell in every three wells in 96-well U-shaped microtiter plates in volumes of 0.1 ml in the presence a feeder cell mixture consisting of 10^6 irradiated PBMC (4000 rad) and 10^5 irradiated B-LCL (5000 rad) and 0.1 μ g PHA/ml. HLA-typed feeder cells were used in order to ensure that the feeder cell mixture did not share any HLA Ag with the donor. In order to enhance specific cytotoxic activity and to reduce simultaneously nonspecific cytotoxicity (24, 25), IL-2 (20 IU)

and IL-4 (100 U) were added as growth factors. After 14 days of culture in a humidified incubator of 5% CO₂ at 37°C, growing cultures were divided into three equal parts in U-shaped microwells. Two of the three wells were assayed for cytotoxic activity against the donor B-LCL. Cultures that scored positive (mean of the duplo determination above the spontaneous ⁵¹Cr release plus 3 SD) in the cell-mediated lympholysis assay were transferred to a volume of 1 ml in a 24-well Costar plate and restimulated with a feeder cell mixture in the presence of IL-2 and IL-4.

Generation of donor-specific CTL clones from PBMC. PBMC of the patient were seeded by limiting dilution at 500 cells/well in 96-well U-shaped microtiter plates. To each well 10^4 irradiated donor B-LCL (5000 rad) were added in a final volume of 0.2 ml. One hundred U of IL-4 was added as a growth factor. After 7 days, 0.1 ml of medium was replaced by fresh medium, containing in addition to IL-4, 20 IU of IL-2. After an additional week at 37°C, growing cultures were tested for cytotoxic activity against the donor PHA-blasts as described above and subcloned at one cell in every three wells. Specific CTL clones were transferred to a 24-well Costar plate in volumes of 1 ml and restimulated with the feeder cell mixture in the presence of IL-2 and IL-4.

Proliferation assay. Between 9 and 14 days after the last restimulation, clones were tested for proliferation as follows: $2 \cdot 10^4$ clone cells were incubated with an equal number of the relevant irradiated B-LCL (5000 rad) in U-shaped microtiter wells. The cultures were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO₂. During the last 16 hours, one μ Ci of tritiated thymidine was added. Subsequently the cultures were harvested onto fiber glass filters and tritiated thymidine incorporation was determined by liquid scintillation spectroscopy.

FMF analysis. One hundred thousand cells were labeled with mAb and FITC-labeled goat anti-mouse (Becton Dickinson) according to the standard procedure described previously (26). For double labeling, cells were incubated with the appropriate mAb, one FITC-labeled and the other phycoerythrin-labeled, in PBS with 0.1% BSA and 0.1% sodium azide for 30 min. The samples were analyzed on a FACScan (Becton Dickinson).

RESULTS

Immunologic status of the patient. The patient had normal lymphocyte ($1800/\text{mm}^3$) and monocyte ($500/\text{mm}^3$) counts. FMF analysis of the PBMC showed that 77% were CD3⁺ T cells, 74% TCR- $\alpha\beta$ ⁺ and 2.4% TCR- $\gamma\delta$ ⁺ T cells. The patient had a normal CD4⁺/CD8⁺ ratio of 2.0 (44% vs 22%). There were no signs of T lymphocyte activation in vivo: 1.3% CD3⁺DR⁺ and 0.5% CD3⁺CD25⁺ circulating cells were observed. In addition, the spontaneous thymidine incorporation by PBMC of the patient was comparable to that of PBMC from healthy control donors. Furthermore, the proliferative responses to PHA and anti-CD3 mAb were in the normal range (not shown).

Proliferative and cytotoxic activity against donor alloantigens. To determine the proliferative and cytotoxic capacity of PBMC of the patient, MLR were carried out in which irradiated spleen mononuclear cells or B-LCL of donor SP were used as stimulator cells. In Table 1 it is shown that the PBMC of the patient proliferated in response to both the B-LCL and the spleen MNC of donor SP. The proliferative responses were in the normal range, as compared with 3rd party stimulator cells tested in

TABLE 1
MLR responses in the presence or absence of IL-2 and/or IL-4

Lymphokine Added ^a	Stimulator Cells: ³ H]TdR Incorporation (cpm.10 ⁻³) with		
	SP-B-LCL cells	SP-Spleen cells	3rd party B-LCL cells
IL-4	45 ^b	12	40-121
	91	27	85-119
IL-2	91	41	70-128
IL-2 + 4	93	ND	86-106

^a IL-2 (20 IU/ml) and/or IL-4 (100 U/ml) were added at the onset of the culture.

^b Spontaneous thymidine incorporation of irradiated stimulator cells or responder cells were always lower than 1000 cpm.

parallel and were enhanced by IL-2, IL-4, or combinations of IL-2 and IL-4.

The cytolytic activity against PHA-blasts of SP before transplantation varied between 25 and 40%, but decreased gradually and was negative 2 yr after transplantation (Table II). This donor-specific nonresponsiveness was still present at the time of this study (>9 yr after transplantation). In contrast, cytolytic activity induced by third party stimulator cells resulted in low levels of specific CTL-activity before and shortly after transplantation and higher levels thereafter, probably reflecting the improving general condition of the patient after transplantation. In Table III, it is shown that when B-LCL are used as stimulator cells the cytotoxic response remains negative, however, a low degree of donor-specific CTL activity against PHA-blasts of SP was measured after secondary MLC stimulation. The specific CTL activity was not enhanced when MLC were carried out in the presence of IL-2, IL-4 or a combination of IL-2 and IL-4 (not shown).

Frequency of donor-specific CTLp. To determine the frequency of donor-specific CTLp, limiting dilution analyses were carried out. Various numbers of PBMC of the patient were cultured for 7 days as indicated in materials and methods and tested against the specific target cell. The CTLp frequencies as measured against donor PHA-blasts were low: 1/67000, while CTLp frequencies against a 3th party control were within the normal range: 1/5500 (Fig. 1).

To exclude the possibility that the frequency determinations were influenced by suppressive mechanisms, CTLp frequencies against donor antigens were also determined at the clonal level. PBMC of the patient, activated for 4 days by PHA and cloned by limiting dilution at one cell in every three wells, resulted in 1167 growing cloned T cell lines. Six of these 1167 T cell lines were specifically cytotoxic for the SP PHA-blasts, accounting

TABLE II

Loss of donor-specific cytotoxic activity after transplantation

Time Point Relative to Transplantation	Induction Culture (Stimulator-Responder) ^a		
	SP-JFV	3rd party-JFV	SP-3rd party
-256 days	40 ^b	19	ND
0 days	25	14	ND
14 days	17	6	ND
1 yr	11	48	30
2 yr	-1	80	52
9 yr	-1	30	18

^a The induction cultures were set up with spleen cells of the donor or PBMC of 3rd party controls as stimulator cells. After 7 days they were assayed for cytotoxic activity against PHA-blasts of the stimulator cell used in the induction culture.

^b Percent lysis at a 50 to 1 E:T ratio.

TABLE III

Donor(SP)-specific lysis by primary and secondary MLR cultures

Induction Culture			Percent Lysis of Target Cells	
SP cells used	Responder	1st/2nd stimulation	SP PHA blasts	Autologous PHA blasts ^a
BLCL	JFV	1	1	-2
BLCL	3rd party ^b	1	18	0
Spleen	JFV	1	-1	-2
Spleen	3rd party	1	18	0
BLCL	JFV	2	12	0
Spleen	JFV	2	10	-1
Spleen	3rd party	2	65	2

^a Autologous to the responder of the induction culture.

^b 3d party responder: PBMC of a healthy individual sharing no HLA Ag with the donor cells.

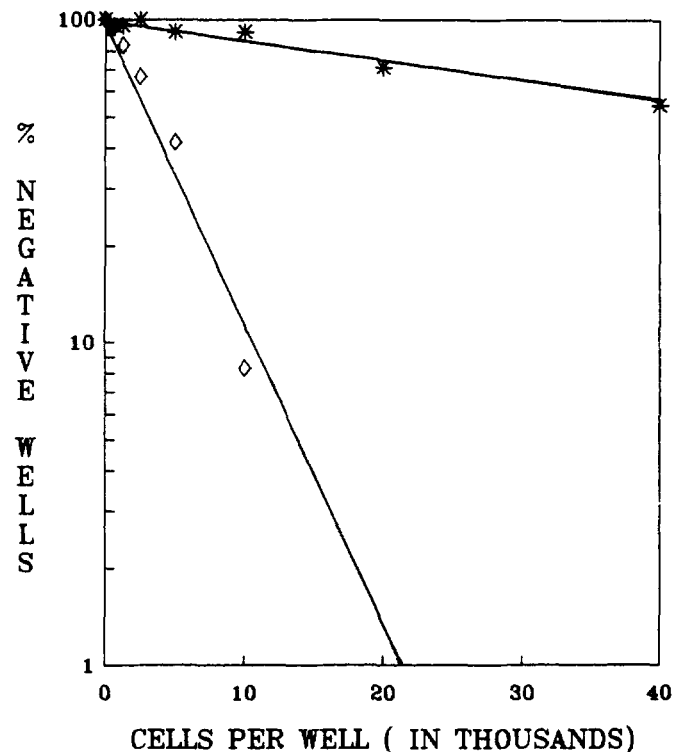


Figure 1. CTLp frequencies in the blood of patient JFV against the donor SP (*) and against a third party stimulator cell (◇). The *p* value for single hit kinetics was always >0.05.

for a precursor frequency of 1/195. The CTLp frequency determinations at the clonal level were carried out with typed feeder cells not sharing any HLA Ag with the donor. Therefore, these results demonstrate that donor-reactive CTLp are present in the circulation of this patient at relatively high frequencies.

Isolation of CTL clones specifically recognizing kidney donor MHC Ag. In a second series of experiments donor-specific CTL clones were generated by seeding unstimulated PBMC of the patient at 500 cells per well in the presence of the SP B-LCL and IL-4. Eight proliferating cultures that contained lytic activity specifically directed against SP were obtained and further subcloned at one cell in every three wells. The phenotype, proliferative and lytic properties of these eight CTL clones together with the six clones established after activation by PHA as described above are shown in Table IV. None of these clones lysed autologous patient derived B-LCL cells. Seven CTL clones were TCR- $\alpha\beta^+$ CD4⁺. In addition to their specific cytotoxic activities these clones also proliferated specifically in response to donor antigens. The cytotoxic activity against SP-BLCL of the CD4⁺ clone, X13, was weak and varied upon repetitive testing, but clone X13 was consistently found to be donor-specific as determined in proliferation assays (Table V). In contrast to the CD4⁺ CTL clones, the TCR- $\alpha\beta^+$ CD8⁺CD4⁻ CTL clones generally showed no or only weak proliferative responses to donor Ag (Table IV). Two donor-specific TCR- $\gamma\delta^+$ CTL clones (21 and 40) were obtained. Phenotypic analysis demonstrated that both clones were CD2⁺, CD3⁺, CD4⁻, CD8 was expressed on 30 to 60% of the cloned 40 cells and was weak or absent on clone 21. This CD8 expression, as the CD8 expression on the TCR- $\alpha\beta^+$ CD4⁺ clones, was induced by IL-4 present in the cultures (27) and was lost when the clones were cultured

TABLE IV
Phenotype, proliferative and cytotoxic responses of donor-specific clones

Clone ^a	Phenotype			Proliferation ^b (cpm · 10 ⁻³)		Percent lysis (10:1)	
	TCR	CD4	CD8	SP	JFV	SP	JFV
1. P25	αβ	1	100	2.1	1.5	88	2
2. X7	αβ	0	95	11.1	0.9	37	1
3. X212	αβ	2	100	7.5	0.7	38	0
4. X211	αβ	97	0	12.5	0.8	27	4
5. X13	αβ	100	50	12.2	0.9	15	-1
6. T7	αβ	100	39	68.1	1.2	28	-2
7. 33	αβ	0	95	2.0	1.3	65	1
8. 15	αβ	0	100	4.9	1.0	80	3
9. 31	αβ	100	1	60.1	0.6	59	-2
10. 36	αβ	100	24	50.5	0.8	63	0
11. 26	αβ	100	38	35.5	0.8	65	-1
12. 3	αβ	100	27	86.4	1.2	25	0
13. 21	γδ	0	11	1.5	1.5	26	0
14. 40	γδ	0	58	3.5	1.7	41	0

^a Clones 1 to 6 are the PHA derived clones, clones 7 to 14 were obtained by allostimulation of PBMC.

^b Spontaneous thymidine incorporation of the clones was always lower than 1000 cpm and the spontaneous incorporation of the stimulator cells was always lower than 3000 cpm.

TABLE V
Specificity of proliferation of X13

Code	Stimulator		Proliferation ([³ H]TdR cpm · 10 ³) of X13 in Presence of (mAb):			
	Shared HLA Ag	Background proliferation	None	SPV-L3 (anti-DQ)	B7/21 (anti-DP)	B8.11.2 (anti-DR)
SP		2, 4	12, 2	2, 8	17, 6	33, 2
JFV	None	0, 5	0, 7	1, 1	0, 7	0, 7
MAST	DQw6	0, 9	7, 3	1, 1	7, 2	9, 7
MVL	B27, Cw2	0, 6	1, 5			
DKB	A24	2, 9	3, 5			

in IL-2 in the absence of IL-4 (not shown). Furthermore, both clones expressed a TCR-γδ that is δTCS-1⁺, TiyA⁻ and BB3⁻, indicating the use of the Vδ₁Jδ₁ in these receptors (28). (F. Koning, unpublished results). SDS-PAGE analysis of the receptor complex of clone 40 after immunoprecipitation showed the lack of a disulfide bond between the γ and δ chains (approximate m.w.: γ: 44,000, δ:41,000), indicating the use of a Cγ2 gene segment in this receptor (not shown).

Specificity of the donor-specific TCR-αβ⁺ CTL clones. In order to determine the specificity of the TCR-αβ⁺ CTL clones, blocking studies with mAb against class I and II MHC Ag (Tables V and VI) and limited panel studies (Tables V and VII) were carried out. All seven CD4⁺ CTL clones recognized class II HLA Ag. One CTL clone was specific for DPw1, three CTL clones recognized DRw13, and three CTL clones reacted specifically with DQw6. Interestingly, three CD8⁺ CTL clones had the

“wrong” phenotype, because they were specific for HLA class II Ag: one CD8⁺ CTL clone (15) recognized DRw13 and the other two CD8⁺ CTL clones (X7,33) recognized DQw6. The DQw6-specific CTL clones did not lyse every DQw6⁺ target cell in the panels and are probably directed to a subtype of DQw6 as yet not detected by serology. This notion is supported by the observation that only DQw6⁺ target cells were lysed and that this reactivity was completely blocked by the anti-DQw1(w6) mAb IIB3. The remaining two CD8⁺ CTL clones recognized B27.

Specificity of the donor-specific TCR-γδ⁺ clones. Panel studies carried out with clone 40 indicated that 12 out of 13 HLA-A24⁺ B-LCL were lysed (Table VIII). One A23⁺ (which constitutes together with the more frequent A24 allele the HLA-A9 specificity) B-LCL was also recognized. In contrast, none of the 12 A9⁻ B-LCL cells were lysed, strongly suggesting that the clone recognized the HLA-A9 specificity or the product of a closely linked gene. However, the cytotoxic activity could not be blocked by three different anti-HLA class I specific mAb: W6/32, B9.12.1 and B1.23.2 (Fig. 2A). Similarly, an anti-β₂-microglobulin mAb, B1.1G6, which should inhibit all class I and class I-like specific lysis, was also unable to inhibit cytotoxicity. Instead, an enhanced lysis in the presence of these mAb was observed. The enhanced lysis was not due to antibody-dependent cellular cytotoxicity, since A9⁻ targets, coated with anti-class I MHC mAb, were never lysed (not shown). The lack of inhibition was observed using various concentrations of mAb, various numbers of cloned effector cells and various types of A9⁺ target cells (B-LCL or PHA-blasts of three different panel members). Only when mAb B1.1G6 and W6/32 were added together, a low degree of inhibition was measured (35%, *p* < 0.01). Furthermore, anti-class II, -DR, -DQ, -DP mAb did not block clone 40, excluding the possibility that A9 peptides were recognized in the context of an HLA class II molecule. Complete blocking was obtained with the anti-TCR-γδ⁺ and the anti-CD3 mAb. The IL-4-induced CD8 expression did not contribute to the specificity or affinity of this clone, since the anti-CD8 mAb, FK18, was unable to prevent lysis by this clone. Furthermore, the CD8⁺ and CD8⁻ fractions, separated by FMF, lysed the donor B-LCL cells equally well (data not shown).

Blocking studies showed that the reactivity of clone 21 was strongly inhibited by the anti-class II HLA backbone and HLA-DQ mAb, whereas mAb against HLA-DR and HLA-DP were ineffective. The specific reactivity of clone 21 was also inhibited by mAb directed against the TCR-γδ⁺ (50%) and CD3 complex (Fig. 2B). Limited panel stud-

TABLE VI
Inhibition of donor-specific cytotoxic activity of the TCR-αβ⁺ clones by anti-HLA mAb^a

mAb		Clones										
Code	Specificity	P25	X7	X212	X211	T7	33	15	31	36	26	3
B9.12.1	Class I	100	ND	100	0	ND	0	0	0	ND	0	ND
B1.23.2	Class I	ND	ND	ND	ND	ND	0	0	0	ND	0	ND
B1.1G6	β ₂ -Microglobulin	75	0	100	0	0	0	0	0	0	0	0
Pdv5.2	Class II	0	100	0	70	70	100	100	100	100	20	100
B8.12.1	DR	0	0	0	100	0	0	100	0	100	0	100
SPV-L3	DQ	0	100	0	0	0	100	0	100	0	100	0
IIB3	DQw1, 8, 9	ND	100	0	0	ND	100	0	100	ND	75	ND
B7/21	DP	0	0	0	0	70	0	0	0	0	0	0
Specificity:		ABC	DQ	ABC	DR	DP	DQ	DR	DQ	DR	DQ	DR

^a Results are expressed as percent inhibition. A percentage of inhibition between <0 and 10% and between 90 and 100% was depicted as 0 and 100%, respectively, for ease of survey.

TABLE VII
Specificity of the donor-reactive TCR- $\alpha\beta^+$ CTL clones as determined by panel studies^a

Targets		Clones										
Code	Shared Ag	P25	X7	X212	X211	T7	33	15	31	36	26	3
Donor	A24, B27, Cw2 DRw13, DQw6, DPw1	88	37	38	27	28	65	80	59	63	65	15
Mast	DQw6	0	0	0	0	0	2	0	39	-2	81	-1
ZUUR	A24, DQw6	0	18	ND	ND	ND	0	0	0	0	61	0
BROE	DRw13, DQw6	0	62	2	80	4	52	83	65	68	87	24
MVL	B27, Cw2	81	ND	24	-1	ND	0	0	0	0	0	0
PESA	B27, DRw13, DQw6	59	42	22	38	ND	0	19	13	46	63	25
HHK	DRw13, DQw6	0	36	ND	ND	2	38	ND	49	ND	32	ND
DKB	A24	0	2	0	-5	4	2	ND	0	ND	0	ND
PLIC	DPw1	ND	ND	3	-1	25	ND	ND	ND	ND	ND	ND
HAAN	DQw6	ND	ND	4	2	-1	ND	ND	ND	ND	ND	ND
BAKK	DQw6	ND	ND	0	3	2	ND	ND	ND	ND	ND	ND
ABEL	DPw1, DQw6	ND	ND	0	0	24	ND	ND	ND	ND	ND	ND
Specificity:		B27	DQw6?	B27	DRw13	DPw1	DQw6?	DRw13	DQw6?	DRw13	DQw6	DRw13
							DRw13?					

^a Results are expressed as percent lysis at a 10:1 E:T ratio.

TABLE VIII
Specificity of the TCR- $\gamma\delta^+$ clones 40 and 21 as determined by panel studies^a

HLA Typings of Targets							Percent Lysis by Clone			
A	B	Cw	Dw	DR	DQw	DPw	40.1		21.1	
							B-LCL	PHA	B-LCL	PHA
1, 24	8, 27	2, 7		3, 13	2, 6	1, 3	42	18	26	10
1	8	7	3, 24	3	2	4	0	0	1	
24	51		25	11	7	4	-5			
2, 24	27, 62	3		4, 6			27			
2, 24	27, 62	3		4, 6			39			
24	51, 63		5, 25	11	7	2	18			
28, 30	18, 60	3, 5	18	3, 13	6, 2		0		15	
2	62	3	4	4	3	2	4			
2, 24	35	3, 4		2, 4	2, 3		45			
24, 28	35, 42	3		3, 4	3		34			
24	60	10	23	9	9	4	50	12	-1	
3, 24	7, 55	3, 7	19	13, 8	6	2, 4	57			
3	7	7	18, 24	13	6	4	0			
1	35	4	5, 25	11	7		0			
24	51		1	1	5		15			
24	38, 35		18	2, 13	6		16			
2	51		8, 3	8	7	1, 4	2			
1, 3	37	6		2, 10	5, 6	3, 4	-1	0	16	
32	27	2	1	1	5	2	2			
3, 11	12, 16			2	2		-1			
1, 26	55, 27	1, 3	18	1, 13	6	2, 4	0		48	
24	7	7	2	2	6	2	59	15		
1, 2	8, 60	3, 7		3, 4	2, 3	2, 4	-4			
23	7	7		2	6	2	49	18		
1, 24	7, 8	7		2, 3	6, 2	1, 4	18			
1, 3	8, 18	5, 7		3	2		0	-1	0	-1

^a The lysis of A9 positive B-LCL is boxed for clone 40 and of DQw6 positive B-LCL for clone 21.

ies carried out with B-LCL targets indicated that clone 21 recognized DQw6 (Table VIII).

DISCUSSION

In the present study we demonstrated that CTL clones specific for donor class I or II HLA Ag can be isolated at relatively high frequencies from a patient who had been successfully transplanted with a kidney more than 9 yr ago. This patient was selected because she was in good health with an excellent graft function and no signs of graft rejection, in spite of a poor HLA-match. Furthermore, it was established that her PBMC taken before transplantation, had normal proliferative and cytotoxic reactivities against donor transplantation antigens, whereas donor-specific cytotoxicity disappeared gradually after transplantation. No donor-specific cytotoxicity

could be measured after 2 yr. The proliferative capacity toward donor derived stimulator cells remained intact. Similar data have been obtained in experimental animals with long term surviving organ allografts. Also in these models the donor-specific proliferative responses remained positive, whereas donor-specific cytotoxic activity was absent (6, 29).

In addition, the CTLp frequencies in our patient were specifically low against donor antigens, whereas the CTLp frequencies against 3rd party stimulator cells were in the normal range (which is between 1/2000 and 1/15000, depending on the individual tested) (23), suggesting that only the donor-specific CTLp frequencies decreased in this patient after transplantation. This is in agreement with the results of Herzog et al. (11), who reported decreased donor-specific CTLp frequencies after

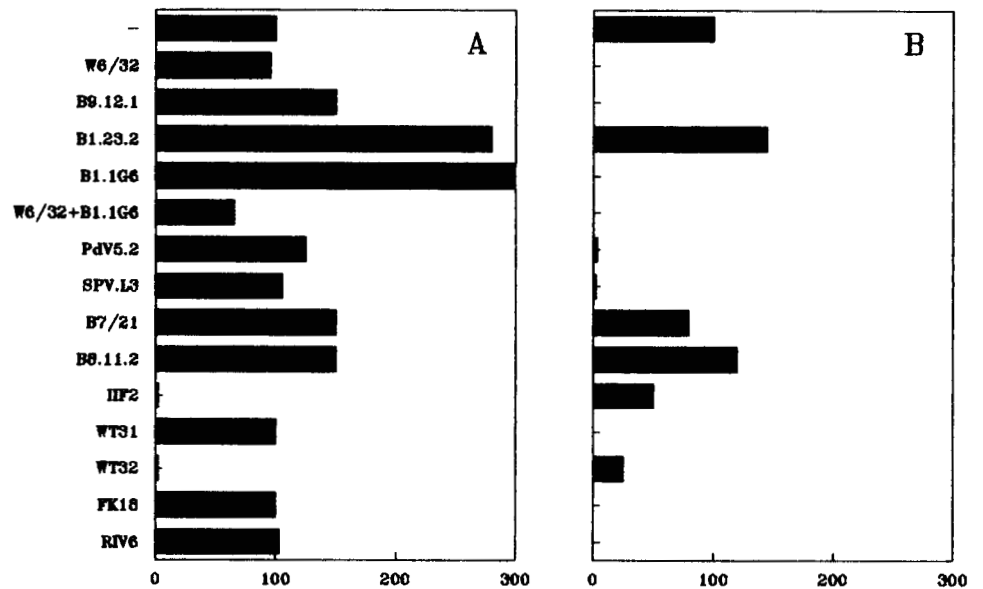


Figure 2. Effect of various mAb on the cytolytic activity of clone 40 (A) and clone 21 (B). These are the results of two representative experiments in which the 100% value for clone 40 was 32 and 48%, for clone 21, 41% and 26%.

transplantation in six kidney patients in whom rejection did not occur. It has to be noted, however, that a low degree of cytotoxic activity could be generated in secondary MLC.

In contrast, donor-specific CTL clones were obtained at high frequencies after activation by PHA and limiting dilution in the presence of HLA-typed feeder cells not sharing any HLA antigens with the kidney donor, indicating that such CTL clones could be obtained without sensitization by donor alloantigen *in vitro*. These results demonstrate unambiguously that CTLp are not deleted from the T cell repertoire. In this respect, our results are analogous to those obtained in experimental neonatal tolerance models, in which it has been shown that CTLp frequencies against tolerated alloantigens are dramatically reduced when measured by classical limiting dilution analysis. However, in the latter system (as in our patient), the donor-specific CTLp frequencies measured after culturing of limiting numbers of mitogen activated responder cells were high (1/100), comparable to or even higher than the frequencies measured in naive non-tolerant animals (30, 31). The finding that donor-specific CTLp frequencies as measured by classical limiting dilution assays were very low, whereas donor-specific CTLp frequencies as measured after activation by PHA as well as CTLp frequencies against 3rd party antigens were in the normal range, strongly suggests the presence of suppressor activities only affecting the generation of donor-specific cytolytic activity. An alternative explanation could be, that the transplant specific CTLp have special growth requirements which were provided by PHA stimulation *in vitro*, but which are absent upon activation by donor alloantigens *in vitro* or in the microenvironment *in vivo*.

The specificity, phenotype and functional properties of 14 donor-specific CTL clones were analysed. In addition to the TCR- $\alpha\beta^+$ CD4 $^+$ CTL clones recognizing the donor class II HLA Ag DQw6, DRw13, and DPw1, one TCR- $\gamma\delta^+$ clone and a relatively high number of TCR- $\alpha\beta^+$ CD8 $^+$ CTL clones were isolated, which also recognized HLA class II Ag of the donor. This class II specificity of the CTL clones lacking CD4 indicates that CD4 is not absolutely required for effective interaction with class II HLA Ag and suggests

the presence of high affinity TCR (13, 32), which is thought to be a specific property of CTL clones activated *in vivo* (33, 34). Although, some of the CD4 $^+$ donor-reactive CTL clones expressed CD8 which was—as we have shown previously (27)—induced by IL-4, no double positive clones were found that were specific for class I HLA Ag. IL-4 induced CD8 was also not involved in the specific recognition of A9 by the A9 specific TCR- $\gamma\delta^+$ clone. In addition, two TCR- $\alpha\beta^+$ CD8 $^+$ CTL clones recognizing the donor class I HLA Ag B27 were obtained.

Donor-specific clones were found against all mismatched HLA Ag, except HLA-C: one CTL clone was directed against HLA-A9, 2 clones were specific for B27, 4 clones were specific for DRw13, 6 reacted with DQw6, and 1 CTL clone was specific for DPw1. This pattern of reactivity is very similar to the donor-reactive repertoire of graft infiltrating cells at the time of an acute irreversible rejection, described by Bonneville et al. (34), indicating that the donor-specific CTL repertoire in our "tolerant" patient is not changed in specificity. Changes in the specificity of the repertoire after neonatal tolerization (shift from H-2K to H-2D) have been described in laboratory animals by Wood et al. (35). Furthermore, these authors also found that a relatively large proportion of the graft infiltrating donor class II reactive cells was CD8 $^+$ (2/14, compared with 3/12 in our series). However, in contrast to their findings, we found that 2/14 donor reactive clones were TCR- $\gamma\delta^+$. These clones were obtained without prior depletion for TCR- $\alpha\beta^+$ cells, indicating that part of the donor reactivity resides in the TCR- $\gamma\delta^+$ fraction.

Although alloreactive TCR- $\gamma\delta^+$ cells have been described in mice (36) and man (37), naive TCR- $\gamma\delta^+$ cells seems unable to mount an alloreactive response *in vivo*, as was demonstrated in nude mice. However, prolonged contact with alloantigens *in vivo* may upregulate the frequency of alloreactive TCR- $\gamma\delta^+$ cells (36). This may be the case in our patient. This notion is also supported by our finding that T cell cultures established from cardiac allograft biopsies taken 2 to 3 yr after transplantation contained in some patients a relatively high percentage of TCR- $\gamma\delta^+$ cells. TCR- $\gamma\delta^+$ cells are never found in the early posttransplantation period (N. Jutte et al. manu-

script in preparation).

The present data indicate that both TCR- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ CTL clones specific for class I or class II HLA Ag expressed on the kidney can be isolated directly from the peripheral blood of the patient. Despite the relative high frequencies of CTL clones specific for HLA Ag expressed on the transplanted kidney, no signs of graft rejection were observed in this patient, indicating that these transplant-specific CTL clones seem not to be operational in vivo. This may be due to the maintenance immunosuppressive therapy, initially imuran and since 1987 cyclosporin A, which may prevent the maturation and expansion of any CTLp to a functionally mature CTL. On the other hand, specific suppressor mechanisms not affecting normal immune responsiveness, may account for the absence of transplant specific activity in vivo, which is probably also the reason why some transplanted patients continue to do well without rejection of the transplant after immunosuppressive therapy was stopped (7, 8). The latter possibility is supported by the finding that the patient described here had strongly reduced donor-specific reactivity whereas normal proliferative and cytotoxic activity against 3rd party alloantigens was found. This is difficult to explain by the action of cyclosporin alone.

A final answer to the question as to whether specific suppressor mechanisms are operational in patients with long term surviving HLA-mismatched transplants has to come from similar studies as described here in patients who continue to do well after immunosuppressive therapy is discontinued.

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