*Kidney International, Vol. 51 (1997), pp. 1876–1884*

# Predominant Th1 cell infiltration in acute rejection episodes of human kidney grafts

MARIO M. D'ELIOS, RÉGIS JOSIEN, MARTA MANGHETTI, AMEDEO AMEDEI, MARCO DE CARLI, MARIA CRISTINA CUTURI, GILLES BLANCHO, FRANÇOISE BUZELIN, GIANFRANCO DEL PRETE, and JEAN-PAUL SOULILLOU

*Institute of Internal Medicine and Immunoallergology, University of Florence, Florence, Italy; INSERM U437 and ITERT "Institut de Transplantation et de Recherche en Transplantation," and Service d'Anatomie Pathologique, Centre Hospitalier Universitaire de Nantes, Immeuble Jean Monnet, Nantes, France*

**Predominant Th1 cell infiltration in acute rejection episodes of human kidney grafts.** T-cells and their cytokines are thought to play a major role in the genesis of cellular infiltration and rejection in human kidney allografts. Production of Th1 (IFN-γ) and Th2-type (IL-4 and IL-5) cytokines was assessed in a large series of T-cell clones, derived from core biopsies of kidney grafts in 10 patients with acute interstitial grade I/II rejection (AIR), 6 patients with a histology of "borderline rejection" (BLR) and 3 with cyclosporine A (CsA) toxicity, all receiving standard maintenance immunosuppression. Biopsies were pre-cultured in IL-2 in order to preferentially expand T-cells activated *in vivo*, and T-cell blasts were cloned with phytohemagglutinin (PHA) and IL-2 using a highly efficient (23 to 98%) cloning technique. A total of 483 T-cell clones obtained from AIR episodes were compared with 346 and 132 clones derived from patients with BLR episodes and CsA toxicity, respectively. In two series of 22 AIR and 77 BLR T-cell clones, alloreactivity against donor cells was shown by 25 and 14% of $CD8^+$ and 21 and 4% of $CD4^+$ clones, respectively. When stimulated by donor-derived EBV B-cells, all these alloreactive clones produced IFN-γ, but not IL-4 or IL-5 (Th1 clones). Upon stimulation with PHA, the principal qualitative and quantitative differences between AIR- and BLR-derived T-cell clones were that cells derived from AIR patients: (i) showed significantly higher proportions (80 ± 15 vs. 55 ± 13%) of Th1 clones in their progeny; (ii) included smaller proportions (3 ± 4 vs. 20 ± 17%) of clones incapable of producing IFN-γ, IL-4 or IL-5 ('null' clones); and (iii) produced significantly higher quantities of IFN-γ (100 ± 50 vs. 36 ± 7 U/$10^6$ cells/ml), these quantities also being significantly correlated ($r = 0.83$) with the degree of interstitial graft infiltration (item 'i' in the Banff histological grading). The clones derived from CsA toxicity biopsies exhibited a pattern very similar to that found in BLR cases. These data lead us to conclude that the powerful inflammatory response elicited in acute rejection of a kidney graft recruits and activates both allospecific and non-specific Th1 effector cells, which are primed to high IFN-γ production. Our results also suggest that IFN-γ could contribute, at least in part, to the degree of graft infiltration and to the severity of the rejection episode.

Allograft rejection results from the coordinated activation of the recipient's alloreactive T-cells, which recognize donor alloantigens either directly as unmodified molecules on the surface of donor antigen-presenting cells (APC) or indirectly as processed allogeneic peptides presented by recipient APCs [1]. Alloreactive T-cells include both MHC class II-restricted $CD4^+$ T-cells and MHC class I-restricted $CD8^+$ T-cells. It has been suggested that some Th1-dependent effector mechanisms, mainly related to delayed-type hypersensitivity and cytotoxic T lymphocyte (CTL) activity, play a central role in acute allograft rejection [2, 3]. In human kidney allografts, infiltration by mononuclear cells is the histological hallmark of episodes of acute interstitial rejection (AIR), and the extent to which cultured T lymphocytes from biopsy growth *in vitro* have been correlated with rejection [4, 5]. It has also been shown that rejected kidney grafts harbor T-lymphocytes whose clonal progeny include alloreactive T-cells expressing cytotoxicity against donor-derived EBV-transformed B-cell lines [6] or parenchymatous kidney cells [5] even after several weeks of culture.

Cytokines are probably involved in the genesis of the lesions characteristic of allograft rejection. It has been reported that during acute rejection episodes serum levels of TNF-α, IL-1 and IL-6 are increased, and that within the graft these cytokines are overexpressed [7–9]. Consonant results have not yet been obtained, however, in studies of the transcription of cytokine mRNA carried out using *in situ* hybridization or RT-PCR with mRNA extracted from core biopsies or fine needle aspirates. Of the Th1-type cytokines, mRNA for IFN-γ is consistently expressed in human renal allografts prior to or during rejection [10–12]. IL-2 mRNA is found in similar circumstances, if somewhat less consistently [13–15], but mRNA for IL-12 (a key cytokine in the polarization of Th1 responses [16, 17]) is nevertheless not associated with rejection [18]. On the other hand, mRNA for IL-4 were very rarely detected in renal allografts undergoing rejection [14, 15, 19]. These discrepancies may be related to differences in methodology, in drug therapy, and in clinical definitions of rejection.

Accumulation of IL-2 and IFN-γ mRNA during rejection has been observed most consistently in experimental transplantation, where no immunosuppression is given [3, 20]. Conversely, *in vivo* studies of cytokine expression patterns during the induction of tolerance have consistently shown a dramatic decrease in the expression of IL-2 and IFN-γ, and in some models an increase in IL-4 and IL-10 transcripts, for example, when anti-CD4 was used [21–24]. Although it remains to be proven, the Th1/Th2 paradigm

Received for publication November 8, 1996
and in revised form January 17, 1997
Accepted for publication January 20, 1997

may thus represent a basis for understanding at least some of the mechanisms of rejection and tolerance in transplantation [2, 25]. Other findings, however, do not fit an explanation along such lines. For instance, very low levels of transcripts for both Th1- (IL-2, IFN-γ) and Th2- (IL-4, IL-10 and IL-13) related cytokines, or even decreased IL-4 levels, have been found within heart or liver allografts in tolerant animals [20, 26, 27].

In humans, the Th1/Th2 cell balance in rejected or "tolerated" (that is, accepted) grafts has not yet been explored at the clonal level. Indeed, in patients on immunosuppressive treatment, well accepted grafts have low levels of infiltration and do not require histological inspection, making any possible imbalance in cytokine production difficult to assess. Nor has any attempt yet been made to correlate cytokine production in the graft with the degree of rejection, even in patients with a histological pattern of "borderline" rejection in whom the decision to treat has not been firmly established.

Th0, Th1 and Th2 clones have been defined in rodents, and the concept has been also more recently validated in humans [28]. The aim of this study was to assess the production of Th1 and Th2 type cytokines (IFN-γ, IL-4 and IL-5) in a large series of T-cell clones derived from the core biopsy of kidney allografts in patients undergoing interstitial rejection with a histology of either clear acute grade I/II rejection (AIR) or a pattern of "borderline" rejection (BLR), or in three additional cases, no rejection (CsA toxicity). The principal qualitative and quantitative differences between BLR and AIR revealed in this study were that patients with AIR showed: (i) significantly higher proportions of Th1 effectors in the clonal progeny of graft-infiltrating T-cells, and (ii) significantly higher IFN-γ production at clonal level, which appears to be closely correlated with the degree of interstitial graft infiltration as assessed by Banff histological grading.

## Methods

### *Patients and kidney graft biopsies*

Nineteen biopsies in 19 kidney allograft recipients were studied. Table 1 shows the principal clinical parameters of the recipients as well as the Banff grading of each biopsy from grade I/II acute interstitial rejection (AIR) and borderline rejection (BLR) episodes.

Ten samples were classified as showing an episode of grade I ($N = 7$) or grade II ($N = 3$) acute interstitial rejection (AIR). The mean number ± SD of HLA mismatches in this group of patients was 4.8 ± 1, with one full mismatch (patient #1). Mean serum creatinine level at the time of biopsy was 331.2 ± 162.8 μM with a percentage increment from the pre-rejection nadir of 87.5 ± 52.2%. All rejection episodes were treated with boluses of corticosteroids. Three patients had rescue treatment (anti-CD3 mAb in patient #11, anti-thymocyte globulin in patient #24) and one (patient #8) presented with an episode of rejection relapse on day 47. Seven patients' serum creatinine returned to pre-rejection nadir levels within three months of the acute rejection episode, whereas three were subject to long lasting renal dysfunction.

Six biopsies showed histology described as "borderline" (BLR) in terms of the Banff classification [29]. The characteristics of these patients are also set out in Table 1. The number of HLA mismatches in this group (3.5 ± 1.7) did not differ significantly from that observed in the AIR group. As is often the case with "borderline" histology, treatment and outcome were less homogeneous than in AIR. In the three patients without delayed graft function (DGF), the mean creatinine level at the time of biopsy was 294.3 ± 82.9 μM. Two patients with DGF were still under dialysis when biopsied, and another patient was biopsied for a persistently altered graft function with a plateau in serum creatinine level at about 300 μM. In two patients, the decision not to treat was maintained and was followed by a spontaneously favorable outcome. Three patients were treated (with corticosteroid boluses), leading to a good outcome in two, while in the third (patient #3), treatment brought about no further change in his persistently altered graft function. Finally, only one patient presented with CMV disease three weeks before biopsy (case number 2) and was treated with ganciclovir.

Three cases of typical CsA toxicity (patients #20, #26 and #30), with one associated with a weak ATN (patient #26) were included as additional controls. One last patient had a biopsy considered almost normal (systematic biopsy at 4 years, performed as part of a specific protocol to assess the long-term effect of a double therapy regimen) with the following Banff grading: g0, i0, t0, ah1, i0.

### *Reagents*

Recombinant human IL-2 was kindly provided by Eurocetus (Milan, Italy). Recombinant human IL-5 was purchased from Amgen Biologicals (Thousand Oaks, CA, USA). PHA was purchased from Gibco Laboratories (Grand Island, NY, USA) and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO, USA). Staphylococcal enterotoxins SEA, SEB, SED and SEE were purchased from Serva (Heildelberg, Germany). Anti-CD3, anti-CD4 and anti-CD8 mAbs were purchased from Ortho Pharmaceuticals (Raritan, NJ, USA).

### *Generation of T-cell clones*

Each kidney graft biopsy was cultured for a maximum of nine days in 10 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, $2 \times 10^{-5}$ M 2-ME 10%, heat-inactivated fetal calf serum (complete medium), and human recombinant IL-2 (50 U/ml) in order to expand preferentially T-cells activated *in vivo*. Biopsy specimens were then disrupted and T-cell blasts were cloned under limiting dilution conditions (0.3 cells/well) in round-bottomed microwell plates containing $10^5$ irradiated allogeneic PBMC (as feeder cells), PHA (0.5% vol/vol) and IL-2 (20 U/ml), as reported elsewhere in detail [30]. Surface marker analysis of T-cell clones was performed on a Cytoron Absolute cytofluorometer (Ortho Pharmaceuticals).

### *Screening assay for graft-infiltrating T-cell clone responsiveness to staphylococcal enterotoxins*

T-cell blasts ($5 \times 10^4$) from all $CD4^+$ and $CD8^+$ clones derived from seven biopsies were screened in triplicate cultures for their responsiveness to staphylococcal enterotoxins SEA, SEB, SED and SEE (5 ng/ml) in the presence of $5 \times 10^4$ irradiated allogeneic APCs by measuring $^3$H-TdR uptake after 60 hours of stimulation. A mitogenic index (the ratio of mean cpm in stimulated to unstimulated cultures) greater than 5 was considered positive.

**Table 1.** Clinical parameters of recipients and histological findings

| Case nb.[a] | Immunosuppression regimen | | Day of biopsy[d] | Histological diagnosis | Banff classification | Creatinine Nadir | Creatinine Peak | CsA level |
|---|---|---|---|---|---|---|---|---|
| | Induc.[b] | Maint.[c] | | | | | | |
| Grade I/II acute rejection | | | | | | | | |
| 1 | TT | TT | d + 36 | grade I AIR | g1, i2, t2, v0, ah0 | 180 | 393 | 315 |
| 7 | TT | TT | d + 28 | grade II AIR | g1, i2, t3, v0, ah0 | 132 | 340 | 205 |
| 8 | TT | TT | d + 19 | grade I AIR | g1, i3, t1, v0, ah0 | 150 | 440 | 210 |
| 11 | TT | TT | d + 25 | grade I AIR | g0, i3, t0, v0, ah0 | 120 | 231 | 300 |
| 13 | ALS | TT | d + 83 | grade II AIR | g0, i2, t3, v0, ah0 | 103 | 174 | 188 |
| 16 | TT | TT | d + 130 | grade I AIR | g0, i1, t2, v0, ah0 | 106 | 139 | 77 |
| 24 | ALS | TT | d + 7 | grade I AIR | g1, i3, t1, v0, ah1 | 161 | 252 | 267 |
| 32 | ALS | TT | d + 26 | grade II AIR | g1, i2, t1, v1, ah1 | 161 | 480 | 129 |
| 33 | ALS | TT | d + 40 | grade I AIR | g1, i2, t2, ah1 | 166 | 205 | 158 |
| 34 | TT | TT | d + 6 | grade I AIR | g0, i2, t1, ah0 | 343 | 658 | 143 |
| Borderline | | | | | | | | |
| 2 | ALS | TT | d + 67 | BLR | g0, i1, t1, v0, ah0 | 155 | 247 | 216 |
| 3 | TT | TT | d + 16 | BLR | g1, i1, t1, a2, ah0 | 282 | – | 149 |
| 4 | TT | TT | d + 48 | BLR | g0, i1, t1, v0, ah0 | 128 | 390 | 255 |
| 5 | TT | CsA + Aza | d + 23 | BLR | g0, i1, t1, v0, ah0 | 138 | 246 | 155 |
| 15 | TT | TT | d + 9 | BLR | g0, i1, t1, v0, ah0 | DGF | | 163 |
| 28 | TT | CsA + Cs | d + 27 | BLR | g0, i2, t1, v0, | DGF | | 187 |

[a] All patients first received a kidney transplantation, except patients #11 and #3, who first received a combined pancreas-kidney transplantation. The number of HLA mismatches (4.8 ± 1 vs. 3.5 ± 1.5), and the pregraft immunological status (% reactivity against PRA, number of blood transfusion) were not statistically different between AIR and BLR groups, respectively. Three additional patients (#20, #26 and 30) with a typical CsA toxicity were also included in the study; all of them received a TT at the time of biopsy.

[b] Induction treatment [TT, triple therapy (cyclosporine A, corticosteroids, azathioprine); ALS, anti-lymphocyte serum]

[c] Maintenance treatment (TT, triple therapy; CsA, cyclosporine A; Aza, azathioprine; Cs, corticosteroids)

[d] d0 = day of transplantation

*Preparation of EBV-transformed donor B-cell lines and screening assay for alloreactivity in clones of graft-infiltrating T-cells*

Lymph node cells from the donors of kidney allografts to patients #3 and #16 were incubated for two hours at 37°C with EBV-containing culture supernatant from the B95-8 marmoset cell line. Cells were then cultured in the presence of 1 μg/ml cyclosporine A for three weeks before being expanded in long-term culture.

In order to assess alloreactivity, T-cell blasts ($5 \times 10^4$) from all $CD4^+$ and $CD8^+$ clones derived from patients #3 and #16 were extensively washed, cocultured for 60 hours with $5 \times 10^4$ irradiated EBV B-cell lines derived from either the related or the unrelated donor, and $^3$H-TdR uptake was measured. A mitogenic index greater than 5 was considered positive.

*Characterization of the cytokine profile of T-cell clones*

To induce cytokine production in T-cell clones, $10^6$ T-cell blasts were stimulated for 36 hours with PHA (1% vol/vol) in 1 ml complete medium, as detailed elsewhere [31]. In order to assess antigen-induced cytokine production, T-cell blasts ($5 \times 10^5$) from alloreactive $CD4^+$ and $CD8^+$ clones derived from the two patients (#3 and #16) were each cocultured for 48 hours in 0.5 ml with $5 \times 10^5$ irradiated EBV B-cells derived from either of the two donors, related or unrelated. Culture supernatants were assayed for IFN-γ, IL-4, and IL-5 content. Quantitative determinations of IFN-γ or IL-4 were performed using commercial assays (BioSource International Inc., Camarillo, CA, or Quantikine, R & D Systems, Minneapolis, MN, USA, respectively). For the measurement of IL-5, the murine LyH7.B13 cell line was used as a source of indicator cells, as detailed elsewhere [31]. Supernatants showing IFN-γ, IL-4 or IL-5 levels 5 SD over the mean level in control supernatants derived from irradiated feeder cells alone were regarded as positive. T-cell clones able to produce IFN-γ, but not IL-4 or IL-5, were categorized as Th1; clones able to produce IL-4 and/or IL-5, but not IFN-γ, were categorized as Th2, and clones producing both IFN-γ and IL-4 or IL-5 were categorized as Th0.

*Preparation of mRNA, cDNA and polymerase chain reaction*

In 36 randomly selected T-cell clones apparently unable to produce IFN-γ, IL-4 or IL-5, ('null' clones) cytokine mRNA expression was assessed by 18 hours of stimulation with PMA (10 ng/ml) plus anti-CD3 mAb (200 ng/ml). Messenger RNA (mRNA) was extracted using an mRNA direct isolation kit (Qiagen Gmbh, Hilden, Germany). The concentration and quality of mRNA samples were estimated by determining absorbance at 260/280 nm and in all subsequent procedures all samples and all reactions were handled and performed under identical circumstances by the use of master mixes. T-cell clones with definite Th1, Th0 and Th2 cytokine profiles stimulated with PMA plus anti-CD3 antibody provided positive control mRNA. Using the same amount of mRNA (50 ng), cDNA was synthesized with M-MuLV-RT (New England Biolabs, Beverly, MA, USA) and oligo-dT primers according to the enzyme suppliers' protocol. cDNA-mix of all samples was amplified under equal conditions by a 30 cycle PCR using IL-2, IFN-γ, IL-4, and IL-5 Stratagene (La Jolla, CA, USA) primers according to the manufacturer's instructions. Post-PCR samples were analyzed on a 2% Metaphor agarose gel (FMC, Rockland, ME, USA), stained with ethidium bromide and photographed under ultraviolet light. β-actin was assayed in all specimens to verify the efficiency of cDNA synthesis from the extracted RNA.

**Table 2.** Phenotype of T-cell clones derived from kidney graft biopsies and efficiency of the cloning procedure

| Case number and diagnosis | Phenotype and number of T-cell clones obtained[a] CD4 | CD8 | Clonal efficiency |
|---|---|---|---|
| Acute interstitial rejection (grade I-II) | | | |
| 1. | 26 (38) | 42 (62) | 91% |
| 7. | 31 (48) | 33 (52) | 83% |
| 8. | 29 (55) | 24 (45) | 71% |
| 11. | 32 (76) | 10 (24) | 53% |
| 13. | 41 (77) | 12 (23) | 70% |
| 16. | 14 (64) | 8 (36) | 29% |
| 24. | 16 (64) | 9 (36) | 23% |
| 32. | 49 (73) | 18 (27) | 86% |
| 33. | 23 (46) | 27 (54) | 66% |
| 34. | 18 (46) | 21 (54) | 52% |
| Borderline histology | | | |
| 2. | 35 (67) | 17 (33) | 66% |
| 3. | 56 (73) | 21 (27) | 98% |
| 4. | 29 (62) | 18 (38) | 61% |
| 5. | 44 (66) | 23 (34) | 87% |
| 15. | 34 (62) | 21 (38) | 68% |
| 28. | 32 (67) | 16 (33) | 61% |
| CsA toxicity | | | |
| 20. | 31 (55) | 25 (45) | 66% |
| 28. | 15 (52) | 14 (48) | 30% |
| 30. | 29 (62) | 18 (38) | 63% |

[a] Percent values are in parenthesis

**Table 3.** Different patterns of proliferative response to staphylococcal enterotoxins by T-cell clones derived from a kidney graft biopsy

| Patient #24 (AIR) T-cell clones and cytokine patterns | Degree of responsiveness to: SEA | SEB | SED | SEE |
|---|---|---|---|---|
| CD4 Th1 | | | | |
| 24/3 | + | − | +++ | + |
| 24/4 | +++ | +++ | − | − |
| 24/5 | − | − | ++ | − |
| 24/9 | + | ++ | + | + |
| 24/10 | +++ | ++ | − | + |
| 24/11 | − | + | ++ | − |
| 24/14 | +++ | +++ | − | +++ |
| 24/15 | +++ | − | − | − |
| 24/16 | ++ | − | ++ | + |
| 24/19 | − | − | + | ++ |
| 24/21 | + | − | + | ++ |
| 24/23 | − | +++ | − | − |
| 24/25 | − | − | +++ | ++ |
| CD4 Th0 | | | | |
| 24/6 | +++ | − | − | +++ |
| 24/8 | − | +++ | +++ | − |
| 24/13 | + | + | − | +++ |
| CD8 Th1 | | | | |
| 24/1 | − | − | +++ | + |
| 24/2 | − | − | − | − |
| 24/7 | +++ | − | − | +++ |
| 24/12 | ++ | +++ | − | − |
| 24/17 | +++ | − | − | − |
| 24/18 | + | ++ | ++ | − |
| 24/20 | − | + | ++ | − |
| 24/24 | − | − | ++ | − |
| CD8 Th0 | | | | |
| 24/22 | ++ | − | − | ++ |

T-cell blasts from each clone ($5 \times 10^4$) were co-cultured for 60 hours with irradiated allogeneic PBMC ($5 \times 10^4$) in the absence or presence of staphylococcal enterotoxins A, B, D and E (5 ng/ml). A mitogenic index lower than 5 is indicated as −, from 5 to 20 as +, from 20 to 100 as ++, and higher than 100 as +++.

## Results

### *Phenotype and diversity of the clonal progeny of graft-infiltrating T-cells*

Core biopsy specimens of the kidney graft were obtained in 10 patients with grade I or II AIR and 6 patients with a histology of BLR (Table 1). Tissue fragments were cultured for nine days in IL-2-conditioned medium in order to preferentially expand T-cells activated *in vivo*. Biopsy specimens were then disrupted and T-cell blasts were cloned under limiting dilution in the presence of irradiated allogeneic feeder cells, PHA and IL-2, according to a highly efficient cloning technique that allows the clonal expansion of virtually every single T-cell [32]. A total of 829 T-cell clones were derived from these 16 kidney graft biopsies included in this study. As shown in Table 2, in the series of 483 T-cell clones obtained from the 10 patients with AIR, 279 were $CD3^+CD4^+CD8^-$ (mean % ± SD = 58 ± 14) and 204 were $CD3^+CD4^-CD8^+$ (42 ± 14). In the series of 346 T-cell clones obtained from the 6 patients with BLR, the CD4 and CD8 phenotype distribution was similar, 230 T-cell clones being $CD3^+CD4^+CD8^-$ (66 ± 4) and 116 $CD3^+CD4^-CD8^+$ (34 ± 4). CD4/CD8 ratios in acute and borderline rejection, therefore, showed no significant difference (1.72 ± 1.01 vs. 1.94 ± 0.39, respectively), nor did ratios in acute rejection differ substantially between grade I and grade II (1.49 ± 0.95 vs. 2.32 ± 1.25, respectively). On the 132 clones derived from the two CsA toxicity biopsies, 56 ± 9% were $CD4^+$ and 44 ± 5 $CD8^+$. No attempt was made to study cell yield before cloning, since the size of biopsy specimens was variable. Finally, no clones could be derived from a "normal" kidney graft biopsy (**Methods**).

In general, the frequency of proliferating clones (cloning efficiency), as determined according to the minimum $\chi^2$ method reported by Taswell [33], was sufficiently high (range 23 to 98%; Table 2), and no substantial difference in cloning efficiency was found between AIR, BLR cases and CsA toxicity (62 ± 23%, 73 ± 15% and 53 ± 20%, respectively).

A reasonable question to be answered was whether the large series of T-cell clones obtained were polyclonal or merely reflected the IL-2-induced selective expansion of a few graft-infiltrating T-cells. To test this possibility, T-cell clones were assessed for their proliferative response to superantigens, such as staphylococcal enterotoxins A, B, D and E, in the presence of allogeneic APCs. As shown in Table 3, in which the results for a representative series of clones (patient #24) are set out, the great majority of T-cell clones showed individual patterns of response to the four superantigens, indicating that they had expressed different T-cell receptor (TCR) Vβ rearrangements. On the basis of these data we concluded that the cohort of 961 T-cell clones obtained was adequately representative of the graft infiltrating T-cells present in each biopsy.

### *Cytokine profiles of the clonal progeny of graft-infiltrating T-cells*

To characterize their cytokine secretion profile, all clones were stimulated for 36 hours with PHA, and cytokine (IFN-γ, IL-4 and IL-5) secretion was assessed in the culture supernatant. As expected, no detectable cytokine production was found in any unstimulated T-cell clones, irrespective of their origin. In contrast,

**Table 4.** Percent distribution of cytokine secretion patterns in PHA-stimulated T-cell clones derived from kidney grafts

| Source of T-cell clones | N | Cytokine secretion profile[a] | | | |
|---|---|---|---|---|---|
| | | Th1 | Th0 | Th2 | Null[b] |
| Grade I/II acute rejection | | | | | |
| CD4+ clones | 279 | 70 ± 14 | 27 ± 13 | 2 ± 3 | 1 ± 3 |
| CD8+ clones | 204 | 90 ± 8 | 6 ± 6 | 0 | 4 ± 4 |
| All clones | 483 | 80 ± 15 | 17 ± 15 | 1 ± 2 | 3 ± 4 |
| Borderline rejection | | | | | |
| $CD4^+$ clones | 230 | 54 ± 7 | 27 ± 12 | 4 ± 7 | 15 ± 8 |
| $CD8^+$ clones | 116 | 55 ± 17 | 19 ± 9 | 0 | 26 ± 23 |
| All clones | 346 | 55 ± 13[c] | 23 ± 11[d] | 2 ± 5[d] | 20 ± 17[c] |
| CsA toxicity | | | | | |
| $CD4^+$ clones | 75 | 52 ± 8 | 32 ± 6 | 3 ± 2 | 13 ± 4 |
| $CD8^+$ clones | 57 | 63 ± 11 | 26 ± 4 | 0 | 11 ± 8 |
| All clones | 132 | 57 ± 10 | 30 ± 6 | 1 ± 1 | 12 ± 6 |

Data are mean ± SD.

[a] T-cell blasts from each clone ($10^6$/ml) were stimulated for 36 hours with PHA (1% vol/vol) and culture supernatants were assayed for their IFN-γ, IL-4 or IL-5 content by appropriate assays, as reported in the **Methods** section.

[b] No secretion of IFN-γ, IL-4 or IL-5.

[c] $P < 0.0001$ vs. grade I/II acute rejection, NS vs. CsA toxicity

[d] $P$ = NS vs. grade I/II acute rejection or vs. CsA toxicity

**Table 5.** Proliferative response and IFN-γ production induced by donor-derived EBV-B-cell lines in alloreactive T-cell clones generated from biopsies obtained from the kidney graft recipients

| Recipient graft-derived T-cell clones (phenotype) | Proliferation (MI) induced by | | IFN-γ production (U/ml) induced by | |
|---|---|---|---|---|
| | EBV.B16 | EBV.B3 | EBV.B16 | EBV.B3 |
| Recipient #16 (AIR) | | | | |
| 16/2 (CD4 Th1) | 337 | <5 | 61 | <3 |
| 16/7 (CD4 Th1) | 82 | <5 | 49 | <3 |
| 16/21 (CD4 Th1) | 504 | <5 | 68 | <3 |
| 16/11 (CD8 Th1) | 55 | <5 | 42 | <3 |
| 16/16 (CD8 Th1) | 128 | <5 | 51 | <3 |
| Recipient #3 (BLR) | | | | |
| 3/19 (CD4 Th1) | <5 | 406 | <3 | 23 |
| 3/56 (CD4 Th1) | <5 | 93 | <3 | 19 |
| 3/9 (CD8 Th1) | <5 | 753 | <3 | 21 |
| 3/15 (CD8 Th1) | <5 | 398 | <3 | 17 |
| 3/37 (CD8 Th1) | <5 | 29 | <3 | 16 |

Culture conditions are reported in the **Methods** section.

upon mitogen stimulation the majority of both $CD4^+$ and $CD8^+$ T-cell clones derived from AIR biopsies exhibited a cytokine secretion profile consistent with the Th1 pattern (Table 4). Indeed, 70 and 90% of $CD4^+$ and CD8+ AIR clones respectively produced IFN-γ, without detectable IL-4 or IL-5 secretion. In the same series of clones, only 2% of the $CD4^+$ and none of the $CD8^+$ T-cell clones expressed the Th2 profile (production of IL-4 and/or IL-5 without detectable IFN-γ secretion), whereas 27% of $CD4^+$ and 6% of $CD8^+$ clones showed combined production of Th1-type and Th2-type cytokines (Th0 pattern). In spite of their good viability, a small number of T-cell clones (1% of $CD4^+$ and 4% of $CD8^+$) were categorized as 'null' clones since no measurable IFN-γ, IL-4 or IL-5 production could be detected in their supernatants. Taken as a whole, of the 483 T-cell clones derived from grade I/II AIR, 80% were Th1, 17% were Th0 and only 1% Th2, suggesting that graft infiltration in AIR is dominated by Th1-type effector T-cells (Table 4). The Th1 pattern was also predominant in the series of 346 clones derived from cases of BLR. However, proportions of $CD4^+$ and $CD8^+$ T-cell clones expressing the Th1 profile were significantly lower (54 and 55%, respectively; $P < 0.0001$) than in the series of AIR clones. Interestingly, this difference was not due to a high proportion of Th2 (2%) or Th0 (23%) clones, but rather to high proportions of both $CD4^+$ (15%) and $CD8^+$ (26%) 'null' clones unable to secrete IFN-γ, IL-4 or IL-5. Despite the variability in the proportion of 'null' clones between different BLR patients (range 0 to 30%), 'null' clones overall accounted for 20% of clones, as compared to 3% in AIR ($P < 0.0001$). The cytokine secretion profile of clones ($N = 132$) derived from the three CsA toxicity biopsies was not significantly different from the BLR group (Th1 57%, Th0 30%, Th2 1%, Null 12%). The failure of 'null' clones to secrete detectable amounts of IFN-γ, IL-4 or IL-5 in response to PHA was confirmed by assessing the expression of cytokine mRNA in response to PMA plus anti-CD3 antibody in 36 randomly selected 'null' clones from BLR patients. Indeed, none of these 36 T-cell clones stimulated for 18 hours with PMA plus anti-CD3 expressed IL-2, IFN-γ, IL-4 or IL-5 mRNA, as assessed by RT-PCR amplification, whereas all of them showed a clear signal for β-actin (data not shown).

An attempt was made to assess the proportion of anti-donor alloreactive T-cell clones and their cytokine profile in response to alloantigen stimulation. This was possible in only one patient with AIR (#16) and one with BLR (#3) for whom EBV transformed donor-derived B-cell lines were available. In the series of T-cell clones from the patient with AIR, 2 out of 8 (25%) $CD8^+$ and 3 out of 14 (21%) $CD4^+$ clones proliferated in response to the appropriate donor-derived EBV-B cell line. In the series of clones from the patient with BLR, alloreactivity was shown by 3 out of 21 (14%) $CD8^+$ and 2 out of 56 (4%) $CD4^+$ clones (Table 5). On alloantigen stimulation, all the alloreactive $CD8^+$ and $CD4^+$ T-cell clones, whether from the patient with AIR or the patient with BLR, confirmed their Th1 profile, as assessed by PHA stimulation. Interestingly, however, at an equal number of T-cell blasts in culture, antigen-induced IFN-γ production was considerably higher in alloreactive clones from the patient with AIR than in those from the patient with BLR (Table 5).

The quantitative aspects of mitogen-induced cytokine production by all T-cell clones derived from both AIR and BLR were then carefully examined. In both the AIR and BLR series, $CD4^+$ clones usually produced larger quantities of IFN-γ (about twice as much) compared to $CD8^+$ clones (Table 6). More importantly, however, a large majority of both $CD4^+$ and $CD8^+$ clones from patients with AIR were able to produce significantly higher quantities of IFN-γ (about 3 times as much) compared to T-cell clones with same phenotype derived from patients with BLR (Table 6) or CsA toxicity (mean production by all clones 32 ± 8 U/$10^6$ cells), but no futher difference in levels of IFN-γ production was found between T-cell clones from grade I (105 ± 58 U/$10^6$ cells/ml) and grade II (89 ± 24 U/$10^6$ cells/ml) acute rejection. Interestingly, no significant difference was found in mean production by Th0 and the rare Th2 clones, whether from patients with AIR or BLR, of IL-4 (0.929 ± 0.558 vs. 0.848 ± 0.284 ng/$10^6$ cells/ml) or IL-5 (2.1 ± 0.7 vs. 2.8 ± 0.6 U/$10^6$ cells/ml).

### *Histological and clinical correlation*

Even though assessed in single clones, IFN-γ production *in vitro* correlated quite closely with the density of the cellular infiltrate

**Table 6.** Quantitative analysis of mitogen-induced IFN-γ and IL-4 production by T-cell clones derived from kidney grafts

| Source of T-cell clones | N | Cytokine production | |
|---|---|---|---|
| | | IFN-γ *U/ml* | IL-4 *ng/ml* |
| Grade I/II acute rejection | | | |
| $CD4^+$ clones | 279 | 122 ± 60 | ND |
| $CD8^+$ clones | 204 | 63 ± 31 | ND |
| All clones | 483 | 100 ± 50 | 0.9 ± 0.5 |
| Borderline rejection | | | |
| $CD4^+$ clones | 230 | 44 ± 10[a] | ND |
| $CD8^+$ clones | 116 | 18 ± 4[b] | ND |
| All clones | 346 | 36 ± 7[c] | 0.8 ± 0.3[d] |
| CsA toxicity | | | |
| $CD4^+$ clones | 75 | 39 ± 9 | ND |
| $CD8^+$ clones | 57 | 22 ± 6 | ND |
| All clones | 132 | 32 ± 8 | 0.8 ± 0.2 |

Data are mean ± SD. ND is not done.
[a] $P = 0.007$ vs. grade I/II acute rejection, NS vs. CsA toxicity
[b] $P = 0.004$ vs. grade I/II acute rejection, NS vs. CsA toxicity
[c] $P = 0.008$ vs. grade I/II acute rejection, NS vs. CsA toxicity
[d] $P = 0.75$ vs. grade I/II acute rejection or CsA toxicity

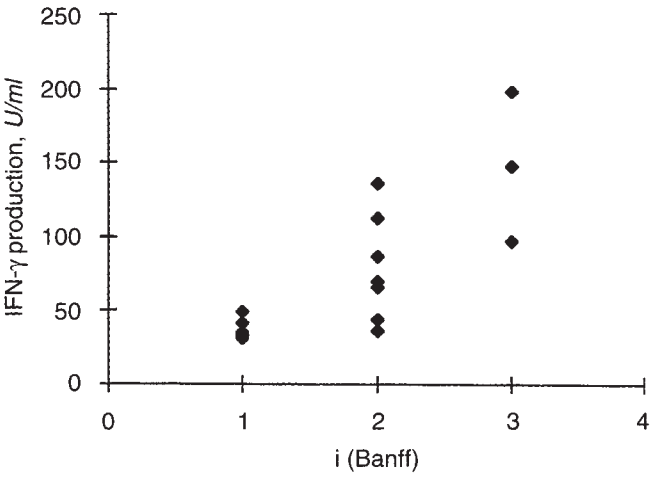


**Fig. 1.** *The level of IFN-γ production by T-cell clones derived from biopsies correlates with the intensity of graft mononuclear cell infiltration.* For each biospy, the mean IFN-γ production ($U/10^6$ cells) of all T-cell clones after *in vitro* stimulation with PHA was plotted against the "i" item of the Banff classification. *r* indicates the correlation coefficient as determined by linear regression analysis ($r = 0.83$).

observed histologically and quantified using the "i" item in the Banff classification applied to characterize biopsy samples from kidney grafts [29]. This is clearly shown in Figure 1, where the degree of mononuclear cell infiltration *in vivo* (that is, the degree of "i" in the Banff grading) was plotted against mean levels of IFN-γ production *in vitro* by the T-cell clones derived from the same biopsy. Regression analysis showed that the degree of cell infiltration and the level of IFN-γ production in the clones were significantly linked ($r = 0.83$, $P < 0.01$). This correlation between item "i" and levels of IFN-γ production *in vitro* was observed for both $CD4^+$ ($r = 0.78$, $P < 0.01$) and $CD8^+$ ($r = 0.81$, $P < 0.01$) T-cell clones. As expected, levels of IL-4 production in T-cell clones did not correlate with the degree of infiltration, nor with any other parameter in the Banff classification (data not shown). It is of note that item "i" in the Banff grading was the only clinical parameter that correlated with levels of IFN-γ production *in vitro*; other parameters related to clinical outcome, such as peak creatinine level or increment from nadir (L. Gaber et al, unpublished observations) showed no correlation.

## Discussion

This study provided evidence that a substantial majority of T-cell clones generated from kidney allografts undergoing grade I/II AIR exhibited a polarized Th1 pattern of cytokine secretion, regardless of their CD4 or CD8 phenotype. A minority of clones showed combined production of Th1 and Th2 cytokines (mixed Th0 pattern), while those with a polarized Th2 profile were extremely rare. When T-cell clones were derived from kidney allografts undergoing BLR, the major qualitative difference observed was a less homogeneous and polarized distribution of Th patterns, with lower but still predominant proportions of Th1 clones and higher proportions of clones that remained unclassified ("null clones"), due to their consistent failure to express at least one of the four cytokines (IFN-γ, IL-2, IL-4 or IL-5) commonly used to categorize human Th cells [28].

The high efficiency of the cloning procedure makes it reasonable to suppose that the distribution of Th1/Th2 patterns among clones is an adequate representation of the actual situation in graft infiltrates during episodes of grade I/II or borderline rejection. Indeed, the clonal efficiency achieved in this study was substantially higher than that previously obtained in T-cells cloning from rejected graft tissue using EBV-transformed donor-cell lines as feeder cells in the absence of exogeneous IL-2 [34, 35]. Our cloning approach has already been successfully used to characterize the functional profile of T-cells infiltrating the thyroid in Hashimoto's thyroiditis [36], the retroorbital tissue in Graves' ophtalmopathy [37], the bronchial mucosa in allergic bronchial asthma [30] and the gastric antrum in *Helicobacter pylori* infection [38]. One reasonable objection could be that the high number of T-cell clones merely reflects IL-2-induced selective expansion of a few T-cells. That, however, is unlikely. Although direct analysis of Vβ repertoire using monoclonal antibodies or molecular biology was not feasible because of contaminating feeder cells, the different TCR Vβ rearrangements expressed by the majority of graft-derived T-cell clones were demonstrated by their almost individual response patterns to the four different staphylococcal superantigens [39].

In previous studies, all the clones that have been derived from a graft undergoing definitive rejection by cloning in the presence of donor-derived feeder cells recognized epitopes presented by donor MHC molecules [6]. Of the present series of graft-derived T-cell clones, however, as indicated by data from two patients (#3 with BLR and #16 with AIR), proliferation and cytokine production in response to donor-derived EBV B-cell lines occurred in only a fraction (4 to 21% of $CD4^+$ and 14 to 25% of $CD8^+$). The conclusion can therefore be drawn that the strategy of "*in situ* culture" with IL-2 and highly efficient cloning using polyclonal mitogen yielded a quite reliable picture of graft infiltrates, which was an important prerequisite if the interpretation of Th1/Th2 patterns in the clones was to have any clinical relevance.

There is no doubt that the initial specific immune response is essential to focus the effector mechanisms on the relevant antigen(s) and to trigger the series of events that subsequently lead to

massive nonspecific recruitment of immune cells within the affected tissue. Thus, non-antigen-specific cells generally represent the dominant population in inflammatory infiltrates [28, 30, 38, 40], and the main source of cytokines that can in turn cause T-cell activation by antigen-independent mechanisms [41]. The initial preferential development of antigen-specific cells into the Th1 or Th2 phenotype is believed to depend on the cytokines predominant at the site of initial antigen presentation, the type of APCs, the nature of the costimulating molecules involved, and the dose of stimulating antigen(s) [42]. This initial commitment may be critical for the subsequent differentiation of cells recruited nonspecifically at the site of inflammation.

The presence of a cellular infiltrate with a predominantly Th1 pattern is the hallmark of acute allograft rejection in rodents, while in some models of allograft tolerance infiltration is associated with dramatically inhibited Th1-cytokine transcription [10, 20]. In humans, an increase in the expression of IFN-γ in kidney allografts has been shown to correlate with episodes of rejection [13], and earlier observations made by Benvenuto et al [43] reported the production of IFN-γ, but not IL-4, in a series of clones derived from two end-stage rejection kidneys. In that study, however, the clones that were the major source of IFN-γ were $CD8^+$ rather than $CD4^+$ [43]. By assessing the production of IFN-γ, IL-4 and IL-5 concomitantly in these large series of T-cell clones derived from a number of patients undergoing episodes of grade I/II AIR or BLR, our study provides further evidence that Th1 is the dominant cytokine pattern in kidney graft rejection, with virtually no clone producing IL-4 or IL-5 in the absence of IFN-γ (Th2 pattern). Although the majority of studies performed at the mRNA levels were unsuccessful in detecting significant IL4 mRNA accumulation during rejection experimentally or in clinical situations [3, 15, 19, 20, 24], a single group reported elevated transcription [14], this discrepancy could be due to the nonquantitative PCR method used.

It has been suggested that Th2 cells play a role, mediated principally by IL-4, in the induction or maintenance of allograft tolerance in adult rodents [reviewed in 44], but this possibility has not yet been verified in humans. Indeed, kidney allograft biopsies from "tolerant" recipients (that is, with a graft that remains unaffected) are difficult to obtain and are usually free of any leukocyte infiltrate, as in the case included in this study (**Methods**). The very low proportion of Th2 clones obtained in this study raises the question of whether the pattern of clones derived *in vitro*, rather than reflecting the *in situ* pattern and the actual behavior of graft-infiltrating Th cells *in vivo*, resulted from *in vitro* artifacts due to the cloning procedure. However, by using the same cloning protocol, allergen-specific Th0 and Th2 clones were easily generated from the bronchial mucosa of grass pollen-sensitive asthmatic patients challenged with the specific allergen *in vivo* [30].

More interestingly, a significant correlation was found between the semiquantitative estimate of cellular infiltration into the graft (the Banff grading) with the quantitation of IFN-γ production at clonal level. IFN-γ is believed to recruit macrophages into the graft, cause macrophage activation, enhance CTL activation, and amplify the ongoing immune response by up-regulating the expression of both MHC and costimulating molecules (such as B7) on graft parenchymal cells and APCs [2, 25]. In addition, IFN-γ promotes differentiation to Th1 cells, both *in vitro* [45] and *in vivo* [46] and provides T-cells with a priming signal for high IFN-γ production and cytolytic activity [47]. Interestingly, some authors have reported that IFN-γ mRNA accumulation could occur before the clinically symptomatic rejection crisis [10, 13]. However, our results show that the clones derived from ongoing AIR are clearly distinguishable from those obtained from BLR (or nonrejected kidneys) in terms of IFN-γ production. Furthermore, the IFN-γ production from the case #28 (BLR stage that ultimately resulted in a severe steroid-resistant rejection) was not associated with high IFN-γ production. Rather, our data suggest that the clones remained committed toward Th1 differentiation during acute rejection.

The link between the degree of IFN-γ production and the intensity of interstitial infiltration in the graft suggests that T-cells expanded *ex vivo* still retain some properties, such as a capacity for growth [4, 35], which can be correlated with a clinical parameter and contribute to a better understanding of rejection mechanisms. Moreover, while the CD4/CD8 ratios in patients with AIR and BLR were similar, both the patterns of cytokine secretion (that is, Th1/Th0 ratios) and the levels of IFN-γ produced differed significantly between episodes of grade I/II AIR and episodes of BLR, the higher values being associated with more severe interstitial infiltration. Interestingly, in cases of grade I/II AIR or BLR, the mean magnitude of IFN-γ production could not be correlated with lower levels of cyclosporine A (data not shown).

Taken together, these data reasonably conclude that acute kidney graft rejection elicits a powerful inflammatory response that recruits and activates both allospecific and nonspecific Th1 effector cells, which are primed to high IFN-γ production. However, a number of observations argue against the possibility that the magnitude of graft infiltration and the severity of its destructive activity depend only on *in situ* IFN-γ produced. First, no significant T-cell growth activity has been reported for IFN-γ during allogeneic responses [48, 49]. In addition, a graft infiltration occurs in the experimental donor blood transfusion-induced tolerance state despite abolished IFN-γ transcription [20, 26]. However, the effect of anti-IFN-γ mAb on graft survival is still controversial. If neither we [50] nor others [51] were able to inhibit the rejection process with anti-IFN-γ in rats, others have reported a prolongation of skin allograft survival [52]. Therefore, it is difficult to conclude if, in the human situation, there is a causal relationship between the local IFN-γ production and the magnitude of cellular response. We favor the possibility that the intensity of both graft infiltration in biopsies and IFN-γ production in the clones results from the process of allorecognition through TCR-stimulation of committed cells, as also suggested by the strong production of IFN-γ induced in alloreactive clones by stimulation with donor EBV B-cells. However, as other important cytokines found to be elevated (mRNA accumulation) by others during ongoing rejections, such as IL-10 [15], or IL-6 [53], were not studied, the correlation between IFN-γ production and the "i" item of the Banff grading does not necessarily imply an exclusive role of this cytokine in rejection.

Our results also suggest that the clinically problematic "borderline" type of rejection, sometimes defined by histological analysis, does not represent a single homogeneous situation, but corresponds rather to a low-grade rejection episode (average IFN-γ production one third less than in grade I/II) with some heterogeneity in the Th1/Th0/Th2 phenotype pattern, a feature which is also in keeping with the variety of clinical outcomes from these episodes.

## Acknowledgments

The first two authors contributed equally to this work. This work was in part funded by Biotech Contract (BIO2-CT92-0300) and by the Italian Ministery of Health (Istituto Superiore di Sanita SF.OA.TO).

*Reprint requests to Prof. Jean-Paul Soulillou, INSERM U437, ITERT, 30, boulevard Jean Monnet, 44093 Nantes Cedex 1, France.*
*E-mail: jps@sante.univ-nantes.fr*

## Appendix

Abbrevations are: AIR, acute interstitial rejection; APC, antigen-presenting cell; BLR, borderline rejection; CTL, cytotoxic T-lymphocyte; DGF, delay graft function; EBV, Epstein Barr virus; IFN-γ, interferon-gamma; IL, interleukin; MHC, major histocompatibility complex; PCR, polymerase chain reaction; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; SE, staphylococcal enterotoxin; TCR, T-cell receptor; Th, T-helper.

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