Kidney International, Vol. 50 (1996), pp. 1019–1025

Detection of donor-specific hyporesponsiveness following late failure of human renal allografts

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Detection of donor-specific hyporesponsiveness following late failure of human renal allografts. Limiting dilution assays to measure the frequency of interleukin-2-secreting peripheral blood T cells were carried out in patients, whose renal allografts had failed due to acute rejection (9 patients) and in patients whose grafts failed more than two years after transplantation without any recent evidence of acute rejection. Using a modified form of the assay we demonstrate that nearly half of 18 patients whose renal transplants had failed after more than two years have low or undetectable HTLp frequencies against donor, but not third-party DR antigens. No such difference was observed in any of the nine patients studied whose transplants were lost from early acute rejection. These results provide the first indication that, as in rodent models of transplantation, T cell unresponsiveness towards donor MHC antigens can occur following prolonged residence of an allograft in humans. Furthermore, the results suggest that chronic rejection may be driven by mechanisms other than direct allorecognition. The assay may be a valuable tool to study the evolution of donor-specific direct T cell alloresponsiveness in patients with well-functioning grafts.

Over the past twenty years, with the advent of improved immunosuppressive protocols, there has been a marked improvement in the one-year graft survival of cadaver renal transplants. Unfortunately, there has been virtually no change in the subsequent rate of graft loss over the same period, and the half-life of cadaveric renal transplants surviving 12 months averages seven to eight years. After the first year graft loss is most often attributed to chronic rejection, the underlying mechanisms of which are unclear, but may be immunologically mediated. In rodent models of transplantation acute rejection resulting in graft loss may be prevented by a short course of a variety of immunosuppressive agents, following which long-term graft acceptance generally occurs and chronic rejection is not a prominent feature. Furthermore, in experimental models prolonged graft residence may be associated with donor-specific helper T cell unresponsiveness or tolerance. This has not been demonstrated before in humans.

It is well established that $CD4^+$ T cells are central to acute graft rejection [1], and data from experimental animal models of transplantation suggest that the subset of T cells secreting interleukin-2 (IL-2) are particularly important. This is consistent with evidence that T cell-mediated cytotoxicity and/or delayed type hypersensitivity are the major effector mechanisms of graft rejection. The precursor frequencies of alloreactive IL-2-secreting $CD4^+$ T cells might therefore be related to the strength of the alloresponse following transplantation. Limiting dilution analysis provides a method of precisely quantitating the alloreactive T cell repertoire. A sensitive limiting dilution assay to measure precursor frequencies of IL-2-producing T cells, in which an indicator cell line is added directly to the microcultures, has been described [2, 3]. A modification of the assay, based on differential sensitivity of IL-2 production to γ-irradiation, distinguishes unstimulated or naive from primed or conditioned T helper cells. T cells that have previously seen antigen and been stimulated are subsequently capable of IL-2 production even after γ-irradiation and have been described as primed or conditioned. These assays have been used previously to study a murine model of transplantation using either subcutaneously implanted synthetic sponges injected with allogeneic spleen cells or skin grafts [3]. Conditioned helper T cell precursor frequencies were high in blood, while conditioned cytotoxic T cell precursor frequencies were undetectable, although they were high within the grafts and the draining lymph nodes. Furthermore, conditioned helper T cell precursor frequencies remained detectable for at least 60 days following rejection of skin grafts [4]. The relative resistance of primed as compared to naive T cells to cyclosporine is a further reason why primed frequencies might be expected to be of greater predictive significance than total or naive T cell frequencies in clinical transplantation.

Preliminary work from this laboratory showed that in two renal transplant recipients whose allograft failed more than two years after transplantation, the primed helper T lymphocyte precursor (HTLp) frequencies in the peripheral blood were low against HLA-DR alloantigens expressed by the failed grafts, compared to frequencies measured against other HLA-DR alloantigens [5, 6]. We now report that nearly half of 18 patients whose renal transplants had failed after more than two years have low or undetectable HTLp frequencies against donor, but not third-party DR antigens. In contrast, in the nine patients studied whose transplants were lost from early acute rejection, HTLp frequencies against donor and third-party DR antigens were of the same order of magnitude. These data suggest that helper T cell precursor frequency measurements will be valuable in predicting the strength of the *in vivo* alloresponse following transplantation. This prediction is supported by our recent findings in bone marrow transplant recipients in which anti-recipient alloreactive T

Received for publication November 6, 1995
and in revised form April 10, 1996
Accepted for publication April 11, 1996

Table 1. Patient details

Patient	Patient DR type	Donor DR type	Time txp functioned[a]	Txp *in situ*[b]	IS[c]	Fail-test time *years*[d]
1	1, 13	6, 7	59 days	no	steroids	14
2	7, 8	1, 15	166 days	no	triple	3
3	13, 13	3	7 days	no	triple	2
4	1, 2	1, 4	8 days	no	triple	10
5	1, 13	(i) 2	120 days	no	pred/aza	6
		(ii) 4, 7	127 days	yes	triple	5
6	7, x	(i) 2, 7	39 days	no	steroids	8
		(ii) 3, 11	0 days	no	triple	7
7	5, 6	3, 7	30 days	no	pred/aza	9
8	2, 9	2, 7	24 days	no	triple	6
9	1, x	4, 13	356 days	no	CsA only	8
10	2, 6	2, 5	3.5 years	no	pred/aza	5
11	5, 6	2, 4	2.25 years	yes	triple	5
12	5, 6 or 8	1, 4	4.0 years	no	triple	4
13	7, x	4, 12	5.5 years	yes	triple	1
14	3, 5	1, 7	2.1	yes	triple	3
15	6, x	4, x	6.8 years	yes	triple	1
16	3, 6	6, 7	9.5 years	yes	pred/aza	1
17	3, 15	4, 7	2.1 years	no	triple	1
18	7, 13	2, 4	4.9 years	yes	triple	2
19	10, 11	3, 11	2 years	yes	triple	4
20	8, 10	4, 7	3.0 years	no	triple	1
21	3, 7	4, 5	3.25 years	yes	triple	2
22	13, x	2, 6	5.6 years	yes	CsA only	3
23	4, x	15, 4	6.6 years	yes	triple	3
24	2, ?6	?	5.6 years	yes	pred/aza	3
25	3, x	3, 4	2.1 years	yes	triple	1
26	5, 6	9, x	6.9 years	yes	triple	1
27	2, x	3, x	2.9 years	no	triple	4

[a] Time the renal transplant functioned
[b] Whether or not the failed graft was still *in situ* at the time of study
[c] Immunosuppressive treatment given during the period the transplant functioned (triple-prednisolone, azathioprine and cyclosporine)
[d] Time between graft failure and study

cell precursor frequencies in donor blood correlated with the incidence of graft-versus-host disease [7].

Methods

Patients

Twenty-seven renal transplant recipients whose grafts had failed were studied. The chronic loss group consisted of 18 patients whose kidney grafts failed due to chronic rejection more than two years after transplantation. In the acute loss group of 9 patients, transplants had failed within the first year of transplantation (7 within 2 months), following aggressive acute rejection. All patients had been back on dialysis and off all immunosuppressive drugs for at least one year. In all cases the previous transplants had expressed at least one mismatched HLA-DR antigen. Patient details are shown in Table 1.

CTLL-2 cells

This murine cell line responds to murine IL-2 and interleukin-4 (IL-4) but only to human IL-2. The cells are maintained in culture in RPMI-1640 supplemented with glutamine (2 mmol/liter), penicillin/streptomycin (50 U/ml), 5% fetal calf serum, 1 mmol/liter pyruvate and 5×10^{-5} M 2-mercaptoethanol (all from Flow Laboratories) and human recombinant IL-2 (rIL-2, 10 U/ml; Boehringer Mannheim, Germany). They were sub-cultured every two to three days. Prior to use they were washed three times and cultured in medium without IL-2 for 18 hours.

Table 2. DR types of the EBV lines used as stimulator cells

Workshop no.	DR type
9001	DR1
9017	DRw15
9019	DR3 DRw17
9029	DR4 Dw4
9030	DR4 Dw13
9038	DR5(12) B6
9043	DRw11 Dw5
9065	DRw13 Dw18
9053	DRw13 Dw19
9050	DR7 Dw17
9067	DR8 Dw8.1

The numbers are those assigned by the Xth Histocompatibility Workshop [8].

Responder cells

Peripheral blood mononuclear cells (PBMC) were separated from patients' blood by density gradient centrifugation over Lymphoprep (Nygaard). PBMC were depleted of $CD8^+$ cells by incubation with anti-CD8 monoclonal antibody (OKT8, ATCC) followed by magnetic beads (Dynal) separation. Efficacy of depletion was measured by flow cytometry and the final population was always $\leq 2\%$ $CD8^+$. The depleted cells were stored in liquid nitrogen until assayed.

Stimulator cells

A range of B-lymphoblastoid cell lines (B-LCL) of defined homozygous DR type from the Xth International Histocompatibility Workshop [8] were used as stimulator cells (Table 2). They were maintained in culture in supplemented RPMI-1640 tissue culture medium with 5% fetal calf serum.

T cell clones

To establish the sensitivity of the assay, a number of human $CD4^+$ anti-DR1 T cell clones [9] as responder cells were assayed in limiting dilution against DR1- and control DRw17-expressing B-LCL under identical conditions to those used for patient $CD4^+$ T cells.

Limiting dilution assay

The assay is sensitive and is based on a bioassay for IL-2 production using the CTLL indicator cell line. The assay has been modified from that previously described [2, 3].

Total limiting dilution assay. The assay was performed in supplemented RPMI with 5% human AB serum. Each responder was tested against B-LCL expressing donor DR antigens and against at least four other lines expressing third party DR alloantigens. The number of third-party cell lines tested depended upon the number of responder cells available. Replicates of 24 wells at 7 doubling dilutions of responder cells (from 1 to 2×10^4 per well) in 50 μl of medium were aliquoted into wells, and medium alone was added to 24 control wells. Irradiated (200 Gy) B-LCL cells (2×10^4 in 50 μl) were added to all wells. After 20 hours the plates were irradiated (20 Gy), followed by the addition of 2×10^3 CTLL cells in 50 μl to each well. After a further eight hours the

Table 3. Measured precursor frequencies of anti-DR1 clones

Clone	Total 1/frequency	95% Confidence interval	χ^2	*P* value	Primed 1/frequency	95% Confidence interval	χ^2	*P* value
G3	1.5	1.1–2.0	4.6	> 0.1	3.3	2.5–4.4	4.6	> 0.1
G3	1.8	1.4–2.4	2.9	> 0.1	4.2	3.1–7.1	5.2	> 0.1
G8	2.4	1.8–3.1	1.4	> 0.1	3.7	2.9–5.0	9.5	> 0.1
G11	4.2	3.2–7.1	5.6	> 0.1	71.5	45.2–113.1	14.6	= 0.05
G12	2.4	1.9–3.2	5.9	> 0.1	8.4	6.4–10.9	13.2	> 0.05
PG4	4.3	3.2–5.8	10.8	> 0.1	v low			
PG12	2.1	1.6–2.8	3.6	> 0.1	3.0	2.3–3.9	1.5	> 0.1

A range of anti-DR1 specific human T cell clones were used as responder cells in limiting dilution assays to measure total and primed frequencies (see **Methods**). The frequencies against no stimulator cells and DRw17 expressing cells were undetectable.

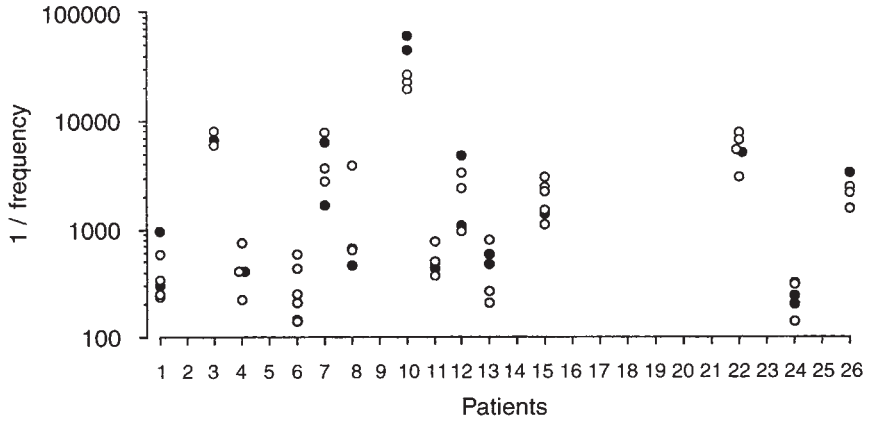

Fig. 1. *Scatter plot of total HTLp frequencies against different DR alloantigens measured in CD4⁺ T cells from patients with failed renal transplants.* Transplants failed within one year in patients 1 to 9, and after two years in patients 10 to 27. Symbols are: (●) HTLp frequencies against stimulator cell lines expressing donor DR-antigens; (○) HTLp frequencies against stimulator cell lines expressing third-party DR-antigens.

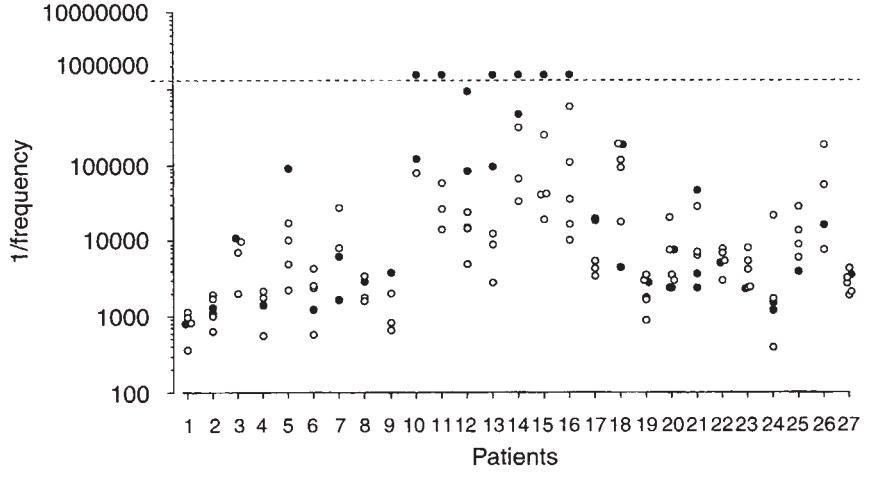

Fig. 2. *Scatter plot of primed HTLp frequencies against different DR alloantigens measured in CD4⁺ T cells from patients with failed renal transplants.* Transplants failed within one year in patients 1 to 9, and after two years in patients 10 to 27. Symbols are: (●) HTLp frequencies against stimulator cell lines expressing donor DR-antigens; (○) HTLp frequencies against stimulator cell lines expressing third-party DR-antigens. The interrupted line indicates the level of sensitivity of the assay.

wells were pulsed with 0.2 μCi of ^{3}H-thymidine (25 mCi/mmol; Amersham International plc, Amersham, UK) in 25 μl, and the plates were harvested 16 hours later and counted by liquid scintillation spectrometry (LKB, Turu, Finland). In all experiments a dose-dependent proliferation of CTLL-2 cells was confirmed by incubation with a range of rIL-2 concentrations.

Primed limiting dilution assay. Responder cells were irradiated (20 Gy) prior to culture with B-LCL stimulator cells prepared as for the total assay. Replicates of 24 wells of 7 doubling dilutions of responder cells (from 2 to 4 × 10^4 per well) were aliquoted into wells in 50 μl of medium together with 2 × 10^4 irradiated (200 Gy) B-LCL. CTLL cells (2 × 10^3) were added one hour later, and the remainder of the assay was then identical to the total assay.

Calculation of precursor frequencies. Wells were scored positive if the counts were greater than the mean plus 3 SDs of control wells (with stimulator, CTLL but no responder cells). The frequency, confidence interval and χ^2 value for each assay were calculated by the maximum likelihood method [10] using GLIM software (NAG Ltd., Oxford, UK). For all data sets presented for which a measurable frequency was obtained, the *P* value was > 0.05, confirming that the experiments conformed to single-hit kinetics. Assays for which the *P* value was < 0.05 were discarded.

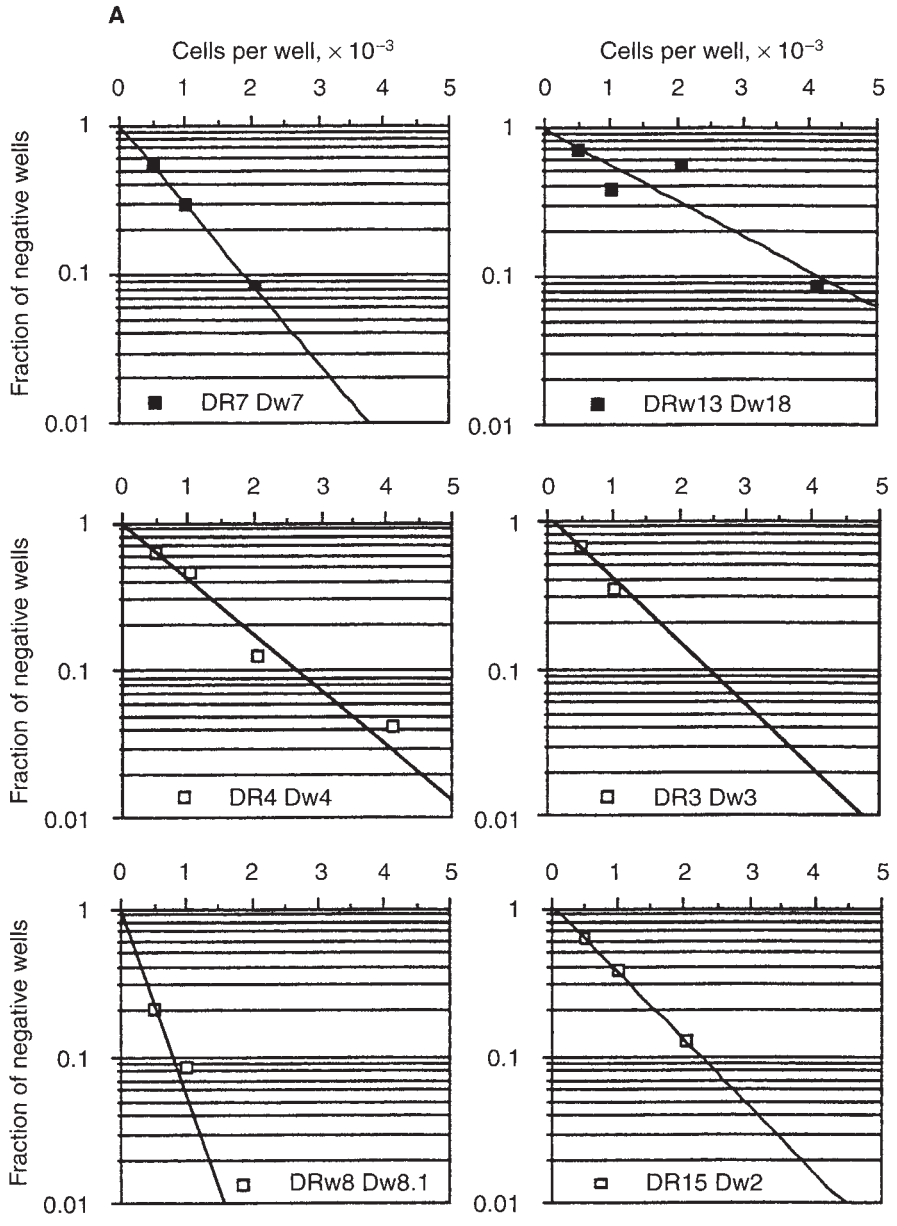

Fig. 3. *Limiting dilution plots of primed HTLp frequency assays for 2 patients.* (**A**) patient 1 whose transplant failed early (56 days) and (**B**) patient 13 whose transplant failed late (5.6 years). The lines represent those calculated to fit the data best (**Methods**) and from which the frequency was derived. Symbols are: (■) stimulator cell lines expressing donor DR-antigens; (□) stimulator cell lines expressing third-party DR-antigens.

Reproducibility of the assays. During development of the assay reproducibility was established using blood from healthy volunteers and patients on dialysis. Assays were performed on different occasions using lymphocytes prepared on the same day as well as on different days over a six months period. In general the repeat frequencies were similar with overlapping 95% confidence intervals, and with similar relative frequencies when several stimulator cells were used.

Results

The HTLp assay is capable of detecting IL-2 secretion from a single cell

To assess the sensitivity of the assay, limiting dilution cultures were performed using anti-DR1 alloreactive T cell clones as responder cells and a DR1-positive B cell line as stimulator cell. The results of assays using five different clones are shown in Table 3. Since it is unlikely that every single T cell in a clonal population will be in a position to respond to antigen, we conclude that the total assay is capable of detecting a single IL-2-secreting T cell. All T cell clones would be expected to be primed, although the primed assay consistently measured a lower frequency than the total assay (Table 3). Presumably γ-irradiation impairs the ability of the T cells to secrete IL-2, although the various clones exhibited differences in radiation susceptibility. For three clones (G3, G8 and PG12), the measured primed frequency was only slightly lower than the total frequency (1 to 2 times lower), while for others (G11 and PG4) the primed frequency was much lower. It is likely that optimal stimulation of these latter clones produce less

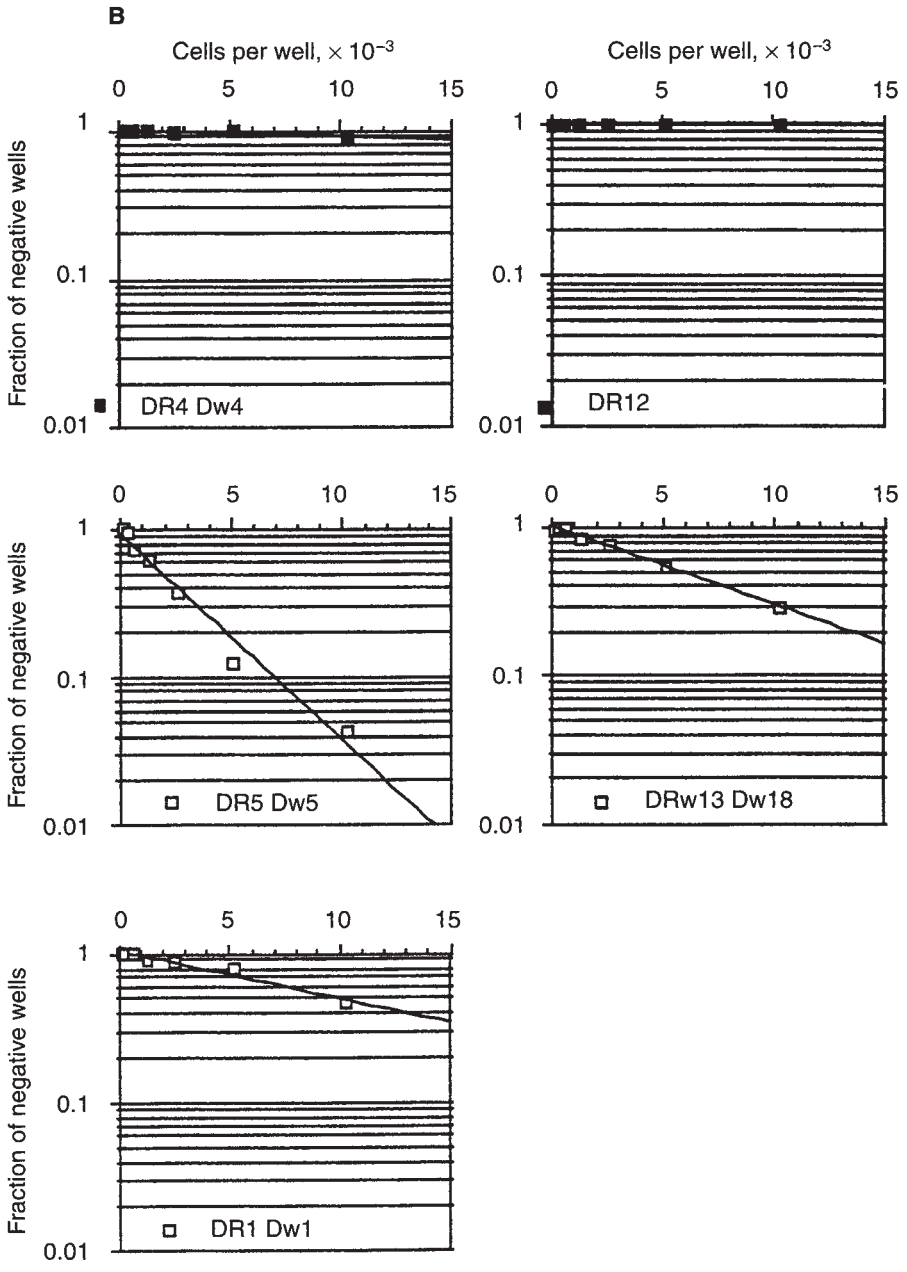

Fig. 3. *Continued.*

IL-2 per cell since the measured total frequencies were approximately 1 in 4 rather than being nearer to 1 in 1, as was the case for the other clones tested.

No differences in total HTLp frequencies were detected between the two patient groups

Seventy total HTLp assays were performed in 14 patients. Five assays were excluded either because $P < 0.05$ or for other technical reasons. There were no differences between the total HTLp frequencies measured against B-LCL cells expressing donor and third-party DR antigens in any of the first fifteen patients studied (Fig. 1). Consequently, only the primed HTLp frequencies were measured in the remaining 13 patients.

The primed HTLp frequencies against donor DR alloantigens were undetectable in 7 out of 17 chronic loss patients

One hundred and fifty primed HTLp frequencies were measured in 27 patients with the rejection of 9 assays either because $P < 0.05$ or for other technical reasons. These HTLp frequencies were lower than the total HTLp frequencies in all patients. The results are displayed in Figure 2.

For patients whose grafts failed early, there were no differences between HTLp frequencies measured against the B-LCL cells expressing donor and third-party DR alloantigens, except for patient 5. Within the group whose transplants functioned for more than two years, 10 patients similarly showed no difference in

frequencies towards donor and third-party DR alloantigens. However, strikingly in eight patients, the frequencies against one or more of the donor DR-antigens were an order of magnitude lower than any of the frequencies against third-party DR-antigens, and were very low or undetectable in seven patients. Figure 3 shows representative limiting dilution plots from two patients (numbers 1 and 13 from Table 1 and Fig. 2) with different patterns of HTLp frequencies against donor and third-party DR alloantigens.

There were no obvious differences between these two groups of patients in terms of the immunosuppression they had received (Table 1). None of the nine patients whose grafts failed early had the transplant *in situ* at the time of study (patient 5 had the DR4,7 but not the DR2 graft *in situ*, and frequency data were only available against DR2 stimulator cells). In the group of patients whose grafts failed after two years, the majority retained the transplant *in situ* although the proportion was similar in those with (5 of 8) and without (8 of 10) donor hyporesponsiveness.

Discussion

Helper T cell responses towards donor HLA antigens in patients who have returned to dialysis following failure of a renal transplant due to acute or chronic rejection have not previously been addressed. When a transplant fails due to acute rejection, it is reasonable to assume that sensitization occurs, since experimental models of transplantation suggest that such rejection results from direct recognition of allogeneic, especially class II, MHC molecules. In contrast, when graft failure results from chronic rejection, the mechanisms of which are obscure, there is little basis for making any assumption regarding the expansion of donor-specific T cells. The findings reported here suggest that in a significant proportion of patients whose grafts failed due to chronic rejection, rather than sensitization low or unresponsiveness occurred in T cells with a direct allospecificity for donor DR alloantigens. This finding, as discussed below, is immunologically important but suggests that direct allorecognition is not responsible for chronic rejection. There have been a large number of factors implicated as mechanisms underlying chronic rejection [11]. Although immunological mechanisms have been implicated, especially in experimental models (see below), many non-immunological factors including damage sustained during reperfusion, low numbers of functioning nephrons, hypertension, hyperlipidaemia and drug toxicity have also been implicated. Since this study addressed the question of the T cell responsiveness towards donor and other alloantigens after rejection of renal transplants, non-immunological parameters were not analyzed.

In experimental models of transplantation, the prolonged residence of a vascularised allograft induces donor-specific T cell tolerance. Although the mechanisms of this tolerance are not fully understood, it is likely that it is, in part, the result of the encounter by recipient T cells of donor alloantigens on the surface of parenchymal cells of the allograft. Such parenchymal cells lack specialised accessory cell function, and do not provide the key co-stimulatory signals required for full activation of IL-2-secreting T cells. It has been extensively documented *in vitro* that ligand recognition on the surface of antigen-presenting or allostimulator cells that lack co-stimulatory potential can inactivate the IL-2-secreting pathway in the responder cell [12, 13]. In a rat system, Braun et al have shown that γ-interferon-induced, MHC class II-expressing, renal tubular epithelial cells induce a state of proliferative non-responsiveness in alloreactive T cells *in vitro* [14]. Furthermore, the injection into an animal carrying a renal allograft of *in vitro*-primed alloreactive T cells specific for donor MHC alloantigens failed to induce graft rejection. In contrast, if the same T cells were injected into a sub-lethally irradiated animal at the time of transplantation, graft rejection ensued [14]. This presumably reflects the ability of bone marrow-derived donor antigen-presenting cells, transplanted with the graft, to stimulate the injected T cells *in vivo*. However, in animals bearing an established graft, from which donor bone marrow-derived cells had been lost, the renal parenchymal cells were unable to activate the alloreactive cells [14, 15]. The observations made in the chronic loss patients studied here may parallel these experimental findings. The low or undetectable donor-specific HTLp frequencies were not found in all of the chronic loss patients. This is perhaps not surprising, particularly when one considers all the relevant factors which might falsely obscure such a result (see below). However, we believe the observation that donor-specific low or unresponsiveness occurs at all is highly informative.

These experiments do not reveal the mechanisms responsible for the low or undetectable donor-specific HTLp frequencies in the chronic patients. However, the LDA data conformed to single hit kinetics suggesting that the presence of a population of suppressor cells is unlikely to be the explanation. It is more likely that hyporesponsive patients, exhibiting tolerance in their repertoire of T cells with direct anti-donor specificity, have a reduced number of donor DR alloantigen-specific T helper cells. This could be due either to clonal deletion or to the induction of anergy.

Since all the patients' transplants in this study eventually failed, an important issue that arises from these data is the mechanism of chronic rejection. Both immunological and non-immunological factors have been implicated, and it is possible that more than one mechanism is responsible [12]. The results obtained in these patients suggest that this process does not reflect the activity of T cells with a direct specificity for donor HLA class II alloantigens. This does not exclude the possibility that an immunological mechanism may be involved. It has long been appreciated that two pathways of MHC allorecognition exist [15, 16]. The direct pathway is responsible for the uniquely high precursor frequency of alloreactive T cells, and involves interaction between the alloreactive T cell's receptor and the intact MHC alloantigen on the surface of an allogeneic cell. The indirect pathway, on the other hand results from the display of processed antigens as peptides bound to self MHC restriction elements on the surface of cells expressing a self MHC restriction element. This has been demonstrated to occur [17], indeed, in a rat model of transplantation antibodies to recipient MHC class II antigens blocked rejection of rat kidneys expressing allogeneic MHC [18], and it has been argued that indirect pathway T cells may contribute to chronic rejection [19]. T cells with indirect anti-donor specificity would not have been detected in the assays used here since the stimulator cells expressed only donor, and not self, DR molecules. Assays to measure the precursor frequencies of T cells with indirect allospecificity are currently being developed.

The modification of the assay that distinguished anti-donor from anti-third party HTLp frequencies, at least in a proportion of the patients, involved γ-irradiation of the responder cells. As shown with the alloreactive T cell clones, irradiation reduces the sensitivity of the assay, presumably due to impairment of IL-2 secretion by responder cells, although not all clones had the same radiation sensitivity. These findings indicate that this form of the assay is not detecting primed, rather than naive, cells but may

detect alloreactive T cells with higher avidity or with the capacity to secrete relatively large amounts of IL-2. Addition of cyclosporin to the cultures may have a similar differential effect on T cells, as demonstrated recently for cytotoxic T cells [20]. Whatever the difference between the information provided by the two forms of the assay, it may well be that the alloreactive cells detected by the primed assay have the most aggressive properties *in vivo*, such that this assay has predictive power in the transplant context. This possibility is currently being explored.

Two additional questions arise from these data. First, why was T cell low or unresponsiveness towards donor DR antigens only detected in a proportion of patients with late graft failure? This may reflect the inaccuracy of donor HLA typing. Clearly it would have been preferable to have used only patients and donors whose DR antigens had been accurately typed using molecular biological methods. However, because we wished to study patients at least one year after the loss of grafts which had functioned for many we often had to rely on the original serological typing of the donor. Thus incorrect B-LCL may have been used in assessing donor-specific alloreactivity. Furthermore, sharing of DQ and/or DP types and/or DRB3/DRB4 gene products by the recipient and the stimulator B-LCL may have created the possibility that EBV-specific helper T cells contributed to the measured frequencies. These confounding factors would all result in the measurement of falsely high frequencies, so that the observation of T cell low or unresponsiveness towards donor DR antigens in nearly half of the chronic loss patients is even more striking.

Second, why was hypo- or unresponsiveness detected so long after removal of the allograft in some patients? Experimental data exists to suggest that tolerance has a relatively short memory. In rodents, donor-specific tolerance appears to depend on the persistence of alloantigen [21] and persists for approximately 100 days after removal of an antigen [22]. There is no data to indicate the longevity of T cell tolerance in the absence of antigen in humans. It is conceivable that donor alloantigen persisted after transplant removal, in the form of long-lived bone marrow-derived cells that have been reported to seed the periphery of renal transplant recipients [23].

These results provide the first indication that T cell low or unresponsiveness towards donor HLA-DR antigens occurs in humans. They also suggest that a mechanism other than direct allorecognition by recipient T cells may be responsible for driving chronic rejection. The application of this assay may be a valuable tool to study the evolution of donor-specific direct T cell alloresponsiveness in patients with well-functioning grafts, and these studies are the focus of continuing studies.

Acknowledgments

This work was supported by the Wellcome Trust and The National Kidney Research Fund. We also thank Professor Peter Morris (Oxford, UK) and Dr. Chisholm Ogg (London, UK) for permission to study some of their patients.

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