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Loss of Direct and Maintenance of Indirect Alloresponses in Renal Allograft Recipients: Implications for the Pathogenesis of Chronic Allograft Nephropathy

Richard J. Baker, Maria P. Hernandez-Fuentes, Paul A. Brookes, Afzal N. Chaudhry, H. Terry Cook, and Robert I. Lechler

Chronic allograft nephropathy (CAN) is the principal cause of late renal allograft failure. This complex process is multifactorial in origin, and there is good evidence for immune-mediated effects. The immune contribution to this process is directed by CD4+ T cells, which can be activated by either direct or indirect pathways of allorecognition. For the first time, these pathways have been simultaneously compared in a cohort of 22 longstanding renal allograft recipients (13 with good function and nine with CAN). CD4+ T cells from all patients reveal donor-specific hyporesponsiveness by the direct pathway according to proliferation or the secretion of the cytokines IL-2, IL-5, and IFN-γ. Donor-specific cytotoxic T cell responses were also attenuated. In contrast, the frequencies of indirectly alloreactive cells were maintained, patients with CAN having significantly higher frequencies of CD4+ T cells indirectly activated by allogeneic peptides when compared with controls with good allograft function. An extensive search for alloantibodies has revealed significant titers in only a minority of patients, both with and without CAN. In summary, this study demonstrates widespread donor-specific hyporesponsiveness in directly activated CD4+ T cells derived from longstanding recipients of renal allografts, whether they have CAN or not. However, patients with CAN have significantly higher frequencies of CD4+ T cells activated by donor Ags in an indirect manner, a phenomenon resembling split tolerance. These findings provide an insight into the pathogenesis of CAN and also have implications for the development of a clinical tolerance assay.


C hronic allograft nephropathy (CAN) is the principal cause of late renal allograft failure and represents a major obstacle to successful long-term engraftment. CAN is a complex process, involving both immune and nonimmune mechanisms. Several lines of evidence implicate immunological factors in the pathogenesis of CAN. First, the t1/2 of allografts are inversely related to the number of HLA mismatches, whether they be from live or cadaveric donors (1). Second, several studies have shown a strong association between the development of acute rejection and the subsequent development of CAN, suggesting that the two processes may be related (2, 3). Third, the presence of activated lymphocytes in protocol biopsies of renal allografts is directly activated CD4+ T cells indirectly activated by allogeneic peptides when compared with controls with good allograft function. An extensive search for alloantibodies has revealed significant titers in only a minority of patients, both with and without CAN. In summary, this study demonstrates widespread donor-specific hyporesponsiveness in directly activated CD4+ T cells derived from longstanding recipients of renal allografts, whether they have CAN or not. However, patients with CAN have significantly higher frequencies of CD4+ T cells activated by donor Ags in an indirect manner, a phenomenon resembling split tolerance. These findings provide an insight into the pathogenesis of CAN and also have implications for the development of a clinical tolerance assay.


Abbreviations used in this paper: CAN, chronic allograft nephropathy; LDA, limiting dilution assay; LRD, living related donor; MFI, mean fluorescence intensity; RFM, ratio of fluorescence means.
in a cohort of longstanding renal transplants (n = 22), which di-
vide into a subgroup with good graft function (n = 13) and another
group with CAN (n = 9). The results of these studies are presented
below.

Materials and Methods

Patients

Twenty-two recipients of live-related allografts were selected by virtue of
being longstanding recipients of allografts (median duration, 157 mo;
rage, 96–333), and more importantly because it was possible to isolate
fresh cells from the original donor to set up cellular assays. The median age
of the patients at transplantation was 32 years (range, 19–47 years). This
cohort of patients was divided into two categories, those with CAN and
those without. CAN was defined as biopsy-proven changes of CAN, as
assessed by a single pathologist (H. T. Cook) according to the 1997 Banff scheme (23) (eight of nine patients), or at least two of the
following clinical criteria: serum creatinine >170 μmol/L; proteinuria >
++ on dipstick; or hypertension requiring medication. Patient characteris-
tics are summarized in Table I. Third party cells were derived from vol-
unteers around the laboratory who had all been previously tissue typed.

HLA typing

At the time of transplant, class I HLA-A, B, and class II HLA-DR were
typed by microlymphocytotoxicity test based on the assay developed by
Terasaki et al. (24). On the day of the functional assays described in this
work, samples of 10 ml of blood in EDTA were taken from the donors and
recipients for retrospective typing. Samples were then typed by sequence-
specific amplification using the PCR with a unified PCR-sequence-specific
amplification system allowing the detection of HLA-A, B, C, DRB1,
DRB2, DRB4, DRB5, and DQB1 (25, 26).

LDAs

PBMC were obtained by density gradient centrifugation over Lymphoprep
(Nycomed, Oslo, Norway). CD4+ T cells from transplant recipients were
obtained by incubation with different mAbs: CD14, CD33 and CD19,
CD16, and CD56 (BD Biosciences, San Jose, CA); CD8 (Serotec, Kidling-
ton, U.K.), followed by negative selection on magnetic beads (Dynal Bio-
tech, Oslo, Norway). Efficacy of depletion was measured by flow cytom-
etry, and in all cases CD4+ fractions were >95% pure.

All assays were performed in RPMI 1640 (Life Technologies, Paisley,
U.K.) supplemented with penicillin/streptomycin (Life Technologies), 2
mM l-glutamine (Life Technologies), and 10% human AB serum (Harlan,
Loughborough, U.K.). Each responder was tested against donor and third
party allogeneic cells (PBMC). Assays against donor and third party were
always performed at the same time. As donor and recipient shared one
HLA haplotype, third party cells were chosen on the basis of sharing the
matched DR type between donor and recipient.

Replicates of 24 wells at seven double dilutions of responder cells
(from 1 x 105 per well) in 100 μl of medium were aliquoted, and medium
alone was added to the 24 control wells. Irradiated (30 Gy) stimulator
PBMC (5 x 105 in 100 μl) were added to all wells. Two plates were
prepared in duplicate. From the first set, IL-2, IFN-γ, and proliferation
were measured; from the second set, the remaining cytokines were
measured.

Proliferation assays

After 6 days, 50 μl 1 μCi/ml [3H]thymidine was added. Plates were har-
vested after a further 12 h. Radioactivity was measured in a scintillation
counter (Wallac, Turku, Finland). Wells were scored positive when counts
were higher than the mean ± 3 SDs of the control wells (irradiated stimu-
lator cells only).

IL-2 estimation

After 72 h, 50 μl of supernatant was harvested into another plate, and 5 x
104 CTL-2 in 75 μl of medium was added to each well. The frequency of
IL-2-producing cells was obtained by bioassay with the IL-2-dependent
CTL-2 line, as described previously (27, 28).

IFN-γ, IL-5, IL-10, IL-13, and TGF-β ELISAs

After 6 days of culture, supernatants were harvested from each well and
frozen (−20°C) until further use. Paired Abs for each cytokine were used
(IL-2 and IFN-γ from Immunokontact, Frankfurt, Germany; TGF-β from
R&D Systems, Abingdon, U.K.; IL-13 from BD Biosciences; IL-10-coat-
ning Ab (clone 9D7); and biotinylated Ab from Biosource, Nivelles, Bel-
gium), and the ELISAs were performed using a standard protocol. Wells
were scored positive when greater than the limit of detection of the assay

Table I. Patient characteristics

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a Tx, Transplantation.

b P, Parent; S, sibling.

c MM, Number of HLA mismatches, A, B, DR.

d Early AR, Acute rejection episode within the first 6 mo.

e Initial maintenance immunosuppression including cyclosporin.

f CRT, Serum creatinine (mmol/L) at time of assay; ESRF, end stage renal failure.

g HT, Hypertensive.

h Biopsy-proven chronic rejection.
as determined by the standard curve. The limit of detection varied between 1 and 5 pg/ml for IFN-γ and IL-10 and between 5 and 20 pg/ml for IL-5, IL-13, and TGF-β.

Calculation of precursor frequencies

For all the assays, the frequencies with confidence intervals were calculated with the modified score function, which is based on a maximum likelihood method (27). A complete set of FCS files with appropriate confidence intervals. Donor-specific hyporesponsiveness was characterized by an antidonor frequency that was lower than the anti-third party frequency, with nonoverlapping confidence intervals. As judged by proliferation (Fig. 1A), donor-specific hyporesponsiveness was present in 6 of 9 patients with CAN, as opposed to 11 of 13 patients with good graft function. This difference was not statistically significant. These results were in accordance with descriptions of donor-specific hyporesponsiveness measured by simple mixed lymphocyte reactions, although previous reports have concentrated on patients with good graft function alone (9–11, 13).

Fig. 1B, showing IL-2-secreting frequencies of CD4+ T cells, demonstrates donor-specific hyporesponsiveness in all 22 patients studied. The most striking aspect of these findings was the similarity between those patients with CAN and those without. This result implies that the direct pathway is not involved in the process of CAN. We have previously described a similar phenomenon in some patients suffering from late renal allograft failure (12) and also in a group of recipients of cardiac allografts who had developed transplant vasculopathy (16).

The frequencies of alloreactive T cells, as measured by IFN-γ and IL-5 secretion, are shown in Fig. 1. C and D, respectively. These cytokines were chosen as surrogate markers for Th1 and Th2 polarization, as originally described by Mosmann (33). In particular, IL-5 was chosen in preference to IL-4 because previous work (27) from our laboratory has shown that levels are higher and more consistent than IL-4. In addition, levels of IL-5 follow similar kinetics to IFN-γ, enabling the measurement of both cytokines at the same time point (62). Evidence for this form of polarization of CD4+ responses has been demonstrated in autoimmune, infectious, and allergic-type diseases, but the relevance of this phenomenon to transplantation remains at best controversial. While it is true that a number of animal models of tolerance display some features of Th2-type responses and acute rejection is associated with a number of facets of Th1-type responses, attempts to produce tolerance by immune deviation have been uniformly unsuccessful across fully allogeneic MHC barriers (34, 35). The relevance of cell polarization to the outcome of clinical transplantation is not
clear, although IL-10, traditionally a Th2-type cytokine in murine systems, is one of the best markers of acute rejection (36). Our results show that longstanding recipients of renal allografts show significant donor-specific hyporesponsiveness, according to the secretion of both cytokines, whether they have CAN or not. In Fig. 2, the donor-specific frequencies for both cytokines are superimposed, revealing that IFN-γ secretion frequencies were significantly higher than IL-5 frequencies in three of nine patients with CAN, as opposed to 3 of 12 patients without, a result that is not significantly different. Interestingly, a similar analysis of responses to third party controls shows the opposite trend, namely, 75% of patients exhibited predominantly Th1-type responses in both groups of patients (Fig. 2B). This suggests that there may be an overall decline in donor-specific Th1 responses with preservation of Th2 cells in transplant patients. The role of Th2 polarization in the indirect pathway is discussed below.

Turning to direct responses to class I HLA molecules, frequencies of donor-responsive cytotoxic cells were significantly reduced in five of seven patients with CAN and also in 12 of 12 patients with good graft function (Fig. 3). The reduced CTL frequencies in the CAN patients highlight one of the fundamental distinctions between the acute and chronic rejection processes. During acute allograft rejection, the finding of tubulitis and the prominence of CD8+ T cells in inflammatory infiltrates suggest an important pathogenic role for these cells. This contention is further supported by the increase in donor-specific CTLs demonstrated during episodes of acute rejection (37). However, it should be noted that the third party cells expressed a larger number of HLA class I mismatches than the living related donors, and this may have contributed to the lower antidonor frequencies (data not shown).

In recent years, there has been a great deal of interest in the role of the indirect pathway of CD4+ T cell activation in chronic allograft rejection. The contribution of the indirect pathway was first proposed on the basis of rodent renal transplant experiments. Having established that donor dendritic cells made a crucial contribution to the immunogenicity of an allograft, the rejection of donor dendritic cell-depleted kidney grafts was attributed to the presentation of donor alloantigens by recipient dendritic cells. This was referred to as the indirect pathway (38). Additionally, there has been increasing evidence that the direct pathway is down-regulated in a donor-specific fashion, as alluded to above. Finally, there have been clear demonstrations of sensitization through the indirect

**FIGURE 1.** Frequencies of directly activated CD4+ T cells. In longstanding recipients of renal allografts, donor-specific hyporesponsiveness is a general feature of the alloimmune responses that occur through the direct pathway. CD4+ T cells from 22 living related donor (LRD) patients were cultured with irradiated donor cells (●), or equally mismatched third party cells (□), in a LDA. For all the figures, the nine patients on the left had CAN. Frequencies of alloreactive cells were then calculated using either cytokine secretion or proliferation as a readout. Reciprocal frequencies are plotted on a logarithmic scale. Ninety-five percent confidence intervals for the calculated frequencies are shown. A, Proliferation was measured after 6 days of culture by 12 h of [3H]thymidine uptake and radioactivity measurement in a beta counter. B, IL-2 production was measured with a CTLL bioassay after 72 h of culture. IFN-γ (C) and IL-5 (D) production was measured by ELISA in the supernatants harvested after 6 days of culture. Donor-specific hyporesponsiveness is defined as a lower antidonor than anti-third party frequency with nonoverlapping confidence intervals. The fraction of patients in which this condition is met is shown in each figure for both groups of patients.
pathway in patients suffering from chronic rejection of renal, cardiac, and lung transplants (17–20). In Fig. 4, we have used a modification of a freeze-thawing technique described by Yamada (30) to prepare a mixture of donor membrane proteins, which were then used to pulse autologous APCs. Because no whole cells were present, only CD4^+ T cells previously stimulated through the indirect pathway in vivo would respond in measurable numbers to such antigenic stimulation. It can be seen that frequencies of indirectly activated T cells were generally higher in patients with CAN, and the median frequency was significantly higher than in the group with good allograft function (p = 0.021; Mann-Whitney U test). In this experiment, we did not measure anti-third party frequencies because previous experiments from our laboratory had shown them to be below the limit of detection for the system used (18).

Additional experiments were conducted to investigate the role of alloantibodies in the development of CAN. For each patient, a sample of serum was collected at the same time as the cellular assays were set up. In each case, a retrospective flow cytometric cross-match was performed against fresh donor T (CD3^+) and B cells (CD19^+), to detect anti-class I HLA and anti-class II HLA Abs, respectively. Fig. 5A shows the results of both flow cytometric T cell cross-matches and anti-class I HLA Abs measured by ELISA in all of the patients studied. In two of nine patients with CAN, anti-class I HLA Abs were detected by ELISA compared with none of the patients with good graft function. In contrast, only one of nine patients with CAN had a positive T cell cross-match, compared with 4 of 13 patients with good function. It can readily be seen that there is no concordance between the results of these two assays, as the positives were all in entirely different patients.
Furthermore, there were no significant differences between patients with or without CAN. In Fig. 5B, similar results are shown for B cell cross-matches and anti-HLA class II Abs detected by ELISA. In this case, one of nine patients with CAN had both a positive B cell cross-match and anti-class II Abs detected by ELISA. In the group of patients with good graft function, 1 of 13 patients had anti-class II Abs by ELISA, whereas 2 of 13 (different) patients had mildly positive B cell cross-matches. Although results are inconclusive, there is little to suggest that circulating alloantibodies are currently contributing to CAN in the majority of these patients.

**Discussion**

This study represents the first simultaneous comparison of direct and indirect antidonor T cell immunity in a cohort of renal transplant recipients. The most striking finding was the universal hyporesponsiveness in donor-specific direct pathway CD4⁺ T cells, irrespective of the development of CAN. Such a degree of hyporesponsiveness has not previously been described, and it may relate to the unusually long time interval that had elapsed between the transplant operation and the performance of these assays (median 13 years). These data accord with recent clinical studies in renal allograft recipients that have revealed evidence of donor-specific hyporesponsiveness, although in a smaller proportion of patients (12, 37). Similar findings have also been reported in recipients of both liver and cardiac allografts (14–16). There have been previous assertions that the development of donor-specific hyporesponsiveness might allow the prediction of patients with a good clinical outcome, and even that such assays might form the basis of predicting successful withdrawal of immunosuppression (39, 40). The results described in this work raise a note of caution that hyporesponsiveness appears to be uniform among patients whether they have CAN or not. Thus, it is doubtful whether such assays, measuring only direct alloresponses, can ever form the basis of a tolerance assay. Whether this result exonerates the direct pathway completely from contributing to the development of CAN is unclear since residual antidonor responses remained detectable in 60% of patients. However, the maintenance of the indirect response warrants further scrutiny as a contributor to the development of CAN.

The mechanism whereby directly activated CD4⁺ T cell donor-specific hyporesponsiveness is brought about remains a subject of speculation. We have performed experiments specifically designed to investigate the possibility of immune deviation as a contributory mechanism, as there is some evidence that allograft acceptance is associated with a Th2-type CD4⁺ T cell response in animal models (34). There is surprisingly little data available in human transplantation, although evidence from protocol renal biopsy series suggests that up-regulation of cytokines such as IL-10 and IL-4 is not associated with a good histological appearance (41). We cannot conclude that direct antidonor-polarized Th2 responses avoid the appearance of chronic rejection, since in our data there was no difference between the two groups of patients. A general antidonor Th2 bias might be a feature of longstanding renal transplants and additional experiments after a shorter interval since transplantation may help to clarify this point. Evidence was sought in these assays for the secretion of other immunosuppressive cytokines that might be maintaining hyporesponsiveness. However, analysis of the same LDA plates by ELISAs revealed no evidence of IL-10, IL-13, or TGF-β secretion in the patients that had reduced frequencies of IL-2-secreting T cells (data not shown).

Two alternative mechanisms for the observed hyporesponsiveness are the deletion, or the induction of anergy, in donor-specific T cells. Based on the rodent experiments referred to above, we would argue that alloantigen presentation by the parenchymal cells of the graft plays a key role, since rat kidney allografts that were depleted of donor bone marrow-derived cells were spontaneously accepted, without immunosuppression, in some strain combinations (38). This led to the proposal that the graft parenchymal cells were antigenic, but not immunogenic. More importantly, in vitro studies with primary cultures of IFN-γ-treated thyroid and renal epithelial cells demonstrated the capacity of these cells to induce allo-specific unresponsiveness, due to T cell anergy, in CD45RO⁺ CD4⁺ T cells (42).

Immune responsiveness can be recovered in anergic CD4⁺ T cells by the addition of exogenous IL-2 (43), and data from our...
laboratory have shown that in some patients donor-specific CD4+ T cell frequencies can be restored upon incubation with IL-2, suggest- ing that a form of anergy may be operating (44). In keeping with the proposition that anergy is induced by encounter with renal epithelial cells, we have shown recently in patients receiving renal allografts that donor-specific hyporesponsiveness in the direct pathway emerges preferentially in the subset of CD4+ T cells capable of trafficking through the allograft, thereby interacting with parenchymal cells (CD4+CD45RO+) (45). One recent study in- vestigated a live-related recipient who had stopped all immuno- suppression. T cells were analyzed by the extremely sensitive method of RT-PCR, looking for clonotypic alloreactive T cells (46). This patient had previously been shown to exhibit donor- specific hyporesponsiveness in functional assays (47). Despite this fact, a high level of donor HLA-specific T cell clonotypic mRNA was detected in the patient’s circulation, implying that the T cells were still circulating, but had either become anergic or were in some way being suppressed.

The recovery of donor-specific alloresponsiveness in recipient CD4+ T cells suggests that deletion of alloreactive T cells has not taken place; however, it is possible that the magnitude of the anti- donor response is diminished in some way by clonal deletion, and indeed recent evidence from murine models suggests that tolerance induction is dependent upon significant shrinkage in the allore- active repertoire due to activation-induced cell death (48, 49). There- fore, it may be desirable to reduce the repertoire size by creating circumstances that favor a wave of deletion and then allow encoun- ter with graft cells to promote anergy over ensuing months. Previous work from animal models has suggested that deletion may be required to reduce the alloreactive T cell repertoire down to levels that are then controllable by regulatory mechanisms (35). This complex subject clearly requires further study.

With such prominent donor-specific hyporesponsiveness in the direct pathway, suspicion falls upon the indirect pathway to pro- vide the ongoing CD4+ T cell stimulation that may contribute to the development of CAN. Significantly increased frequencies of indirectly activated donor-specific CD4+ T cells were recorded in our patients with CAN. This is in marked contrast to the pretrans- plant situation in which the direct response is extremely vigorous in 1-haplotype-mismatched pairs, while the indirect response is usually unrecordable. Our findings are consistent with other groups that have described sensitization to donor-derived allelogeneic HLA peptides in patients with CAN, when compared with controls with good graft function (17). In addition, studies in patients with chronic rejection of other solid organs such as hearts and lungs have also shown evidence of activation of indirect pathway T cells (18–20). An intriguing recent finding has been the suggestion that the indirect responses in CAN are polarized toward a Th1 type of response, and this warrants further investigation (50).

Elegant rodent experiments, performed with class II knockout mice, have suggested that the generation of IgG donor-specific alloantibodies requires processing of donor MHC Ag through the indirect pathway to bring about isotype switching (51). This find- ing has also been confirmed by other groups (52, 53). If this mech- anism is also operating in human transplantation, then conversely, the presence of donor-specific IgG alloantibodies represents cir- cumstantial evidence of ongoing T cell activation through the in- direct pathway. In addition, some recent studies have suggested that alloantibodies are significantly more prevalent in patients with CAN, particularly those directed against allelogeneic class II mole- cules (54). In our patients, we were unable to consistently detect circulating alloantibodies. However, a possible role of anti-HLA alloantibodies in the pathogenesis of CAN cannot be dismissed without a more extensive search for Ab-mediated disease. Novel techniques, such as the staining of peritubular capillaries for the C4d component of complement or ultrastructural analysis of the peritubular capillaries for multilayering of the basement mem- brane, might prove more sensitive (55–58).

There has recently been a great deal of interest in the idea of developing a clinical tolerance assay. Ideally, such an assay would form the experimental basis for measured drug withdrawal. This has become increasingly important with the evolution of strategies for the induction of donor-specific tolerance. From this work, we think it unlikely that assays of direct alloreactivity will prove use- ful in this respect. Likewise, assays for circulating alloantibodies would not appear to be discriminatory. We would suggest that such an assay would require monitoring of the indirect pathway of al- lorecognition. Our own assays have limitations. They are time con- suming and require large numbers of both donor and recipient cells. They are somewhat insensitive at low responder frequencies (1/500,000), despite the fact that such responses may still be bio- logically relevant. However, they give a very accurate comparison between two different stimuli. Given all these circumstances, it is also possible that the final tolerance assay will involve a panel of different tests used in combination with other promising candidates such as rapid ELISPOT assays (22, 59) and trans vivo analysis in nude mice (60, 61).

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References


