Chronic Rejection: Insights from a Novel Immunosuppressive-Free Model of Kidney Transplantation

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Abstract. The use of immunosuppressive drugs in models of chronic rejection may limit their usefulness for mechanistic studies. We have developed a new minor histocompatibility-mismatched rat kidney transplant model without the need for immunosuppression. Kidneys from LEW (RT1) donors were transplanted to congenic WF.1L (RT1l) recipients and compared with the reversed strain combination and isogenic controls. Urinary protein excretion was measured serially in all recipients; kidneys were harvested 90, 120, and 180 d after transplantation for morphologic analysis and cytokine gene expression. In vitro lymphocytic reactivity and cytokine analysis of mixed lymphocyte reaction (MLR) culture supernatants by ELISA was also carried out. LEW into WF.1L kidney grafts developed proteinuria starting 120 d after transplantation and were associated with morphologic changes of focal segmental glomerulosclerosis together with interstitial cell infiltrates, upregulated gene expression of IL-1β, IL-2, and TNF-α/β, as well as IL-2, IFN-γ, and TNF-α production by lymphocytes in MLR culture supernatants. WF.1L kidneys transplanted into LEW recipients did not develop chronic rejection and had upregulation of Th2 cytokines, both within the allograft and in MLR supernatant of recipient lymphocytes cultured with WF.1L cells. Furthermore, these lymphocytes produced both Th1 and Th2 cytokines when cultured with WF cells, unlike lymphocytes from the LEW isografts, which produced Th1 cytokines when challenged with WF cells. These studies show that indirect allorecognition can cause strain-dependent chronic rejection associated with Th1-like cytokine production, whereas production of Th2 cytokines is associated with protection from the development of chronic rejection.

The term chronic rejection as applied to kidney transplants was first used by Harlan et al. to describe histologic changes in kidney allografts that occurred later and were different from those seen in acute rejection (1). An immunological basis was assumed to be the cause of the lesions seen; hence, the term chronic rejection was used. It is now well recognized that nonimmunological factors can exacerbate the development of this chronic alloimmune response. A newer term, chronic allograft nephropathy, is now commonly used to emphasize the multifactorial nature of the damage seen in the transplanted kidneys. The term chronic rejection is now usually reserved for purely immunologically mediated injury (2). There is still no doubt that an alloimmune response lies at the heart of most, if not all, chronic allograft nephropathy. The nature of this chronic alloimmune response is also believed to be different from that of acute rejection; therefore, it is important to develop good models of chronic rejection to understand this form of injury and how best to treat it (3).

Although all solid organs develop some form of chronic alloimmune-mediated injury, the exact nature of this injury varies from organ to organ and the degree of injury even varies in different organs transplanted across the same strain combination (4–6). Hence, insights gained from cardiac or other solid organ transplant models may not be wholly relevant to the development of chronic rejection in kidney allografts.

The most commonly used chronic rejection model for kidney transplantation is the rat F334-to-LEW transplant model (5,7,8). Although this has provided many insights into the development of chronic rejection, it suffers from one major drawback: it requires the use of a short course of cyclosporine to modify the acute rejection and prevent a high attrition rate among the transplanted animals. Cyclosporine itself has been shown to contribute to the development of chronic allograft nephropathy; therefore, its use in a model complicates mechanistic studies into the development of chronic rejection, not only because of its immunosuppressive effects but also because of its nephrotoxic effects (9). In addition, calcineurin inhibitors are thought to interfere with the induction of transplantation tolerance (10,11).

To address these concerns, we decided to develop a new model of chronic kidney rejection in the rat that does not require the use of immunosuppressive drugs. Guttmann et al.
have shown that LEW recipients of MHC-matched congenic WF.1L (RT1\(^{b}\)) cardiac tissue develop chronic cardiac rejection beginning 60 d after transplantation and becoming full blown by 120 d (12–14). In this study, we examined whether this minor histocompatibility-mismatched model may be suitable for the analysis of chronic kidney rejection in an otherwise untreated animal. WF.1L and LEW kidneys were transplanted into LEW and WF.1L recipients, respectively, and morphologic and functional changes were compared with syngeneic controls.

**Materials and Methods**

**Animals and Operative Technique**

Inbred male rats, 8 to 10 wk of age and weighing 200 to 250 g, were used throughout the experiments. Pathogen-free LEW (RT1\(^{b}\)) rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). The congenic WF.1L (LEW)/GUT (RT1\(^{l}\)) rats were a generous gift from Dr. Ronald Guttmann (McGill University, Montreal, Canada). The WF.1L rats were originally derived from backcrossing LEW to WF (RT1\(^{b}\)), and the haplotype was fixed by selecting I/L progeny at F6. Animals were bred by brother/sister mating for more than 20 generations before being shipped to our laboratory (14). All animal experimentation described was conducted in accord with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Our laboratory has been breeding WF.1L animals for the past 3 yr.

The kidneys were flushed with 3 ml of cold lactated Ringer’s solution before removal and stored transiently in the same cold solution (4°C) before engraftment. They were transplanted heterotopically with a short aortic segment anastomosed end-to-side to the abdominal aorta of the recipient, and the renal vein was joined in a similar manner to the inferior vena cava. The ureter was divided, and the proximal third was anastomosed end-to-end to the recipient ureter. All anastomoses were performed with 10-0 Prolene (Ethicon, Somerville, NJ). The left kidney was then removed. The time between the release of the vascular clamps and the return of obvious uniform cortical blood flow was approximately 35 to 45 s. The contralateral right native kidney was removed 10 d later, at which time the graft had recovered from any dysfunction resulting from transient ischemia and the integrity of the ureteral anastomosis in the transplant could be examined.

**Experimental Groups**

Kidneys were transplanted from LEW donors into WF.1L recipients (LEW into WF.1L) as well as in the reversed strain combination (WF.1L into LEW). LEW into LEW and WF.1L into WF.1L isografts served as controls for both experimental groups, and no immunosuppressive drugs were given to any of the groups.

**Renal Function**

All animals had monthly 24-h urine collections between day 30 and day 180 (n = 5/group). Protein excretion was determined by measuring turbidity after precipitation of urinary protein and interaction with 3% sulfosalicylic acid (Fisher Scientific, Fair Lawn, NJ). Turbidity was assessed by absorbance at a wavelength of 595 nm using a UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan) (7).

**Histology**

Graft morphology was examined at 90, 120, and 180 d. Representative portions of all allografts and isografts were fixed in 10% buffered formalin at the time of removal for histomorphological examination. Paraffin sections were stained with hematoxylin and eosin, periodic acid–Schiff, Mason’s trichrome for collagen, and Verhoeff’s elastin for elastic fibers and evaluated by light microscopy.

Two examiners blinded to the group from which the sections came examined matched trichrome-stained sections independently. A semi-quantitative scoring system for fibrosis was used, with specimens graded from 0 to 3, where 0, 1, 2, and 3 indicated minimal, mild, moderate, and severe fibrosis, respectively. A similar scale was used to score the degree of interstitial mononuclear cell infiltration.

Portions of the kidney grafts harvested at 180 d after transplantation were also snap frozen and stored at −80°C for the determination of cytokines and growth factors using an RNase protection assay.

**Mixed Lymphocyte Reaction**

Donor-strain lymph nodes or splenocytes were harvested, and mononuclear cells were separated by passing the tissue through a 60-gauge sterile stainless steel sieve. The stimulating cells (3 × 10^5 per well) were then irradiated (3000 rads) and cocultured with responder T cells (3 × 10^5 per well) in 96-well flat-bottom plates (Costar, Cambridge, MA) containing RPMI 1640 medium (BioWhitaker, Walkersville, MD), 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 × 10^{-5} mol/L 2-mercaptoethanol, and 5 mmol/L Hepes. The plates were incubated at 37°C with 5% CO\(_2\) for 96 h. Proliferation was assayed by measuring the DNA incorporation of [\(^{3}\)H]thymidine (New England Nuclear-Dupont, Boston, MA), added for the final 6 h of culture, using a β-scintillation counter (Beta Plate; Wallac, Gaithersburg, MD). All experiments were done in quadruplicate, and the results are expressed as Δcpm (experimental minus background) ± SEM. Background cultures were set up with responder cells alone.

**ELISA for Rat TNF-α, IFN-γ, IL-2, IL-4, and IL-10**

From the mixed lymphocyte reaction (MLR) culture, supernatants were harvested after 48 h of incubation at 37°C with 5% CO\(_2\) to determine TNF-α, IFN-γ, IL-2, IL-4, and IL-10 production. Culture supernatants were assayed by ELISA using BioSource Cytoscreen Rat ELISA Kits (BioSource, Camarillo, CA).

**RNase Protection Assay**

RNase protection was performed using the Riboquant Multi-Probe RNase Protection assay system (PharMingen, San Diego, CA). RNA was isolated from kidney grafts using Trizol. [\(^{32}\)P]-labeled probes were synthesized from the rCK-1 Multi-Probe Template Set (PharMingen) and were hybridized overnight with RNA samples in hybridization buffer according to the manufacturer’s instructions. Samples were digested with RNase and T1 mix in RNase buffer, and protected probes were purified and run on a 5% acrylamide gel in 0.5% TBE buffer. Kidney graft control RNA and a dilution of the probe set (serving as size markers) was run in parallel. The gel was absorbed onto filter paper, dried, and exposed on Kodak photographic paper at −70°C for 24 h. The RNA was analyzed by a phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA), allowing accurate quantification of mRNA.

**Statistical Analyses**

The results are expressed as arithmetic means ± SEM. The MLR data are presented as differences between the proliferative responses of the tested cells minus the proliferative responses of the responder and stimulator cells tested alone (Δcpm). Statistical significance among groups was ascertained using the t test and the Mann-Whitney
test. Urine protein excretion was compared among all groups using the Kruskal-Wallis test for several independent samples and the Mann-Whitney test for two independent samples. The differences were considered to be significant at \( P < 0.05 \).

**Results**

**Survival and Renal Function**

All recipients in the four groups survived the 180-d follow-up period. Proteinuria was determined sequentially for all recipients in all groups (Figure 1). LEW kidneys transplanted into LEW recipients showed no evidence of increased urinary protein excretion by day 180 after transplantation (9.6 ± 5 mg). WF.1L kidneys transplanted into LEW recipients developed mild proteinuria (16.7 ± 6.0 mg), but not significantly different from WF.1L isografts (15.26 ± 4.9 mg). By contrast, WF.1L recipients of LEW kidneys developed progressive proteinuria evident by day 60 and becoming significantly different from the other groups by day 120 (28.3 ± 6.1 mg at day 180; \( P < 0.0001 \)).

**Histology**

Graft morphology from each of the groups harvested at day 180 after transplantation mirrored the proteinuria data. Transplanted kidneys from the two isogenic control groups were essentially normal when examined 180 d after transplantation (Table 1). Allografts from the LEW-into-WF.1L group showed histologic changes of chronic rejection, with persistent focal cellular interstitial infiltration and focal glomerulosclerosis (Figure 2 and Table 1). Kidneys from the reverse strain combination, WF.1L kidneys transplanted into LEW recipients, showed very minor morphologic changes, with only mild glomerulosclerosis and occasional cellular infiltrates (Figure 2). Arteriosclerotic alterations of cortical small vessels or interstitial fibrosis were not significantly different when compared with both isograft groups.

**Mixed Lymphocyte Reaction**

To investigate whether the differences seen in the development of chronic rejection in the LEW-into-WF.1L and WF.1L-into-LEW transplants was attributable to recipient immunoresponsiveness, we tested the proliferative response of lymphocytes harvested from each group to irradiated antigen-presenting cell (APC) from naive LEW, WF.1L, and WF animals.

As expected, the primary MLR response of splenic and lymph node (LN) lymphocytes from naive WF.1L and LEW animals to irradiated minor histocompatibility-mismatched LEW or WF.1L APC was minimal (splenic cells, 1237 ± 186 \( \text{versus} \) 1523 ± 201, respectively; Figure 3C). Recipient splenic and LN lymphocytes from the LEW animals primed with a WF.1L kidney (which developed chronic rejection) showed a significant response to naive irradiated APC of donor origin (Figure 3A). Interestingly, a comparable response was observed in recipient lymphocytes to naive irradiated APC of donor origin from the reverse strain combination, which did not develop chronic rejection.

The proliferative response of recipient lymphocytes from both allografted groups was higher when cultured with naive irradiated APC from WF compared with WF.1L (splenic cells group 1, 8045 ± 1456 \( \Delta \text{cpm} \); group 2, 5998 ± 721 \( \Delta \text{cpm} \); Figure 3, A and B). However, the proliferation of lymphocytes from LEW allografted animals to WF APC was significantly lower than the response of lymphocytes taken from LEW isografted animals (splenic cells, 15,937 ± 2284 [not shown] \( \text{versus} \) 5998 ± 721; Figure 3), indicating that prior transplantation of LEW animals with a WF.1L organ blunts the immunoresponsiveness of LEW lymphocytes to WF APC.

**Enzyme-Linked Immunosorbent Assay**

Culture supernatants obtained from the MLR assays were examined for cytokine production by ELISA. Lymphocytes from animals that developed chronic rejection (LEW into WF.1L) had a Th1 phenotype, with production of TNF-\( \alpha \), IFN-\( \gamma \), and IL-2 but not IL-4 and IL-10, when stimulated with cells mismatched for minor or major histocompatibility antigens (Figure 4A). Even though LEW recipients of WF.1L kidneys mounted a significant alloimmune response in the MLR-to-WF.1L APC, the supernatant indicated a Th2 response, with production of IL-4 and IL-10 rather than Th1 cytokines. LEW animals previously transplanted with LEW isografts produced a strong Th1 response in terms of IFN-\( \gamma \) and IL-2 but small amounts of IL-4 and IL-10 when stimulated with WF cells. Priming LEW animals with a WF.1L allograft, before stimulating with WF APC, changed the cytokine response to a mixed Th1/Th2 response, with increased production of TNF-\( \alpha \), IFN-\( \gamma \), IL-2, IL-4, and IL-10. Thus, it is possible that the Th2 response (presumably against the minor histocompatibility antigens) in these animals regulates the more aggressive Th1 response to WF MHC.

**Molecular Changes**

To confirm that the cytokine expression was not merely an \textit{in vitro} phenomenon, we examined cytokine gene expression in allografts harvested at regular intervals by RNase protection assay for mRNA levels of IL-1\( \alpha \) and -\( \beta \), IL-2, -3, -4, -5, -6, and -10, TNF-\( \alpha \) and -\( \beta \), and IFN-\( \gamma \) and compared the results with those from naive organs. These data mirrored the cytokine expression.
expression found in the culture supernatants. At 90 and 180 d after kidney transplantation, IL-1β, IL-2, and TNF-α gene expression were upregulated in grafts from the LEW-into-WF.1L group compared with those from the WF.1L-into-LEW and LEW-into-LEW groups (Figure 5). By contrast, IL-4 was upregulated in the WF.1L kidneys transplanted into LEW recipients.

Discussion

For more than two decades, the F344-to-LEW model of kidney transplantation has been the most popular model of chronic renal allograft rejection (5). The F344 (RT1^fr) rat has a variant l haplotype that differs from LEW (RT1^l) in the class I E/C region but is identical to LEW in the immunodominant RT1.A and RT1.B regions (15). In this model, cyclosporine is usually given for 10 d to overcome acute allograft rejection. Renal allografts in this strain combination survive for a prolonged period and develop marked functional impairment and histologic changes compatible with chronic rejection/chronic allograft nephropathy. As cyclosporine is also known to be nephrotoxic, the use of cyclosporine makes this model less than ideal. Several congenic models have been used to analyze the role of the major and minor histocompatibility antigen differences in the rejection process, mainly for differentiation between isolated MHC loci (e.g., the RT.1A and RT.1B/D complex), responsible for the induction of the immune response (16–19). It is now clear that the experimental process that initially defined the “major” histocompatibility complex similarly defined the “minor” loci by the slower rejection caused by their antigens. The combination of several minor histocompatibility discrepancies may bring about rejection as rapidly as a whole MHC difference (20,21). Recently, a heterotopic cardiac allograft model in the WF.1L (LEW)-to-LEW inbred rat strain combination was identified as an experimental model for systematic studies of chronic vascular rejection (12,14). RT1^A serotyping and analysis of the LEW-type
TNF-α polymorphism by PCR methods have shown that this congenic line has the LEW immunodominant class I MHC as well as the MHC class II loci RT.1B and RT.1D of the haplotype. In this MHC-identical combination, indefinite survival (>100 d) of the cardiac allografts occurred in the absence of recipient immunosuppression. The grafts revealed morphologic features of progressive chronic vascular rejection that were highly reproducible as a function of time. Extensive infiltration by T cells and macrophages within the vessel wall were variably associated with minimal nonocclusive intimal accumulation of actin and myointimal cells or with changes of occlusive or subocclusive intimal thickening associated with minimal vascular wall infiltration by macrophages. Advanced vasculopathy was already present 60 d after transplantation.

To study chronic renal allograft rejection based on this cardiac transplant model, we developed a new MHC-matched, but minor histocompatibility-mismatched, rat kidney model, the LEW-into-WF.1L model. This new model develops the functional and histologic features of chronic renal allograft rejection without the need of calcineurin inhibitor-based immunosuppression. Although a variable degree of focal interstitial mononuclear cell infiltration was seen in the kidney allografts compared with the cardiac allograft model mentioned above, significant vascular changes, even after 180 d, were not detectable in either the LEW-into-WF.1L or the WF.1L-into-LEW kidney transplant model. Differences in the way that kidney and cardiac transplants respond to immunosuppressive/immunoregulatory regimens in the same model are well documented (6). Vasculopathy is often not a dominant finding in kidney allografts undergoing chronic rejection compared with cardiac allografts. When it does occur, it appears to be associated with sensitization and late acute rejection (22), neither of which occurs in this model. The lack of vascular changes in the kidney model may be attributable to differences in the relative importance of the direct/indirect pathway of antigen recognition, the type of antigen-processing cell, the route of antigen processing, and so forth.

WF.1L recipients of LEW kidneys developed increased urinary protein excretion and grade 1 to 2 lesions of chronic rejection according to the Banff classification 180 d after transplantation (23). By contrast, LEW recipients of WF.1L kidneys developed little chronic rejection. Strain-dependent development of chronic rejection has been documented in the F334-to-LEW combination (24). In this model, F344 kidneys transplanted into LEW recipients develop chronic rejection, and reversing the strain combination does not result in the development of chronic rejection. The reasons for this are not totally apparent but are likely attributable to the combination of antigen processing and the repertoire of T cells available to recognize foreign antigens. Minor alloantigens require APC processing before activating a T cell response and hence are dependent on the indirect pathway of allosensitization (25,26). Most described studies of allograft rejection based on minor histocompatibility-mismatched strains have used heart transplant models either in the rat or in the mouse (27–29). Kidney grafts from the LEW-to-WF.1L congenic strain combination (group 1) revealed a slow but significant process of chronic rejection, with typical signs of glomerulosclerosis and representative cytokines, i.e., TNF-α, IFN-γ, IL-1β, and IL-2, associated with chronic rejection after 90 d, before the develop-
ment of significant proteinuria. A similar pattern of cytokine expression was seen in grafts from this group harvested 180 d after transplantation, implying an association between this cytokine profile and the development of chronic rejection. Cells obtained from these animals demonstrated alloreactivity in vitro not only against major but also against minor mismatch antigens that may reflect the in vivo ongoing slow rejection process. Although the overall proliferative responses were reduced compared with those of cells obtained from naïve controls, this observation could be explained by the fact that the alloreactive T cell clone size is relatively small in a minor mismatched combination and by the effect of age on the vigor of the T cell response (28). In the supernatants of these cultures, TNF-α, IFN-γ, and IL-2 were detectable in this strain combination (LEW into WF.1L). Interestingly, in the reversed strain combination, in which no significant signs of chronic rejection were observed, IL-4 and IL-10 were detectable not only in the culture supernatants but also in the kidney grafts of those animals, reflecting type 2 cytokine expression, previously associated with transplantation tolerance (30). It has been speculated that Th2 responses may be responsible for the development of chronic rejection (31); in contrast, we found that chronic rejection was associated with a Th1 response, in keeping with human studies that have shown that alloreactive T cells cloned from chronically rejecting patients were universally of the Th1 phenotype (32).

Figure 4. Cytokine analysis of the culture supernatants obtained from mixed lymphocyte reaction assays. Group 1 animals showed TNF-α, IFN-γ, and IL-2 but barely detectable IL-4 and IL-10 production (A) when tested against minor and major mismatch antigens. In contrast, the pattern was reversed in group 2 animals (B), with greater production of IL-2, IL-4, and IL-10 observed in culture supernatants of the group 2 splenic lymphocytes. (C and D) Cytokine production in mixed lymphocyte reaction supernatants from groups 3 and 4, respectively. Data are presented as means ± SEM (n = 4; P < 0.0001).

Figure 5. Cytokine gene expression in kidney allografts of groups 1 to 4. (A) By 90 d after kidney transplantation, IL-1β, IL-2, and TNF-α and -β gene expression was significantly upregulated in grafts from LEW into WF.1L (group 1) compared with those from WF.1L into LEW (group 2) and the syngeneic controls LEW into LEW (group 3) and WF.1L into WF.1L (group 4). In contrast, IL-4 gene expression was only upregulated in group 2. IL-5 and IL-6 were detectable but not significantly different from those in the control grafts. Data are presented as cytokine/GAPDH ratio (n = 4/group). (B) Differences in cytokine gene expression were still present at 180 d after kidney transplantation.
matched WF kidneys into LEW recipients leads to an aggressive form of acute rejection, and lymphocytes from the LEW isografts produced a predominantly Th1 response to WF cells. WF.1L kidneys transplanted into LEW recipients did not develop chronic rejection (33). LEW lymphocytes from these animals produced predominantly Th2 cytokines when challenged with WF.1L cells and a mixed Th1/Th2 response when challenged with WF cells.

These studies reiterate the complexity of the alloimmune response in the development of chronic rejection and highlight the importance of donor-recipient pairing in the development of this form of injury.

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References
analysis using a retransplantation model. Transplantation 60: 1073–1079, 1995


31. Shirwan H: Chronic allograft rejection: Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? Transplantation 68: 715–726, 1999
