Ultrastructural analysis of the Fisher to Lewis rat model of chronic allograft nephropathy

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Introduction

Chronic allograft nephropathy (CAN) is the leading cause of renal transplant failure. CAN has been hypothesized to be caused by cell-mediated permanently ongoing rejection of the present alloantigen, recurrent intermittent acute rejection episodes, or alloantigen-independent processes that activate graft endothelial cells and induce an unspecific inflammatory response. The relative contribution of alloantigen-dependent and -independent events in CAN is unclear.

One important factor leading or contributing to CAN is chronic alloimmune injury. Observations on human CAN suggest that it may be possible to differentiate changes typical of chronic alloimmune injury from changes caused by alloantigen-independent processes of CAN or other forms of renal allograft diseases. Three lesions, either alone or in combination, indicate the presence of alloimmune injury:

- Transplant capillaropathy (circumferential multiplication of the peritubular capillary basement membrane) and transplant glomerulopathy (reduplication of the glomerular basement membrane) have recently been validated clinicopathologically as ultrastructural indicators of chronic alloimmune injury.
- Transplant arteriopathy (indicative of ongoing cellular rejection) is also absent.

To investigate the presence of these markers, F344-to-LEW kidneys were examined by electron and light microscopy 32, 40 and 52 weeks after implantation. F344 rats with or without 30-min ischemia of the left kidney following right nephrectomy served as controls. All transplanted rats displayed marked proteinuria. On electron microscopy, transplant capillaropathy, transplant glomerulopathy, and T-cell cytotoxicity (indicator of ongoing cellular rejection) were absent. On light microscopy, the arteries were devoid of intimal fibrosis. Focal-segmental glomerulopathy resembling hyperfiltration injury was encountered, with mild interstitial infiltration, fibrosis, and tubular atrophy. The proteinuria and kidney pathology were more severe in transplanted than in ischemic or uninephrectomized rats. Because chronic-active rejection could not be detected between weeks 32 and 52, we propose that the alloantigen-dependent initial graft injury subsides, but induces the late events: glomerular hyperfiltration, proteinuria, and glomerulosclerosis. Accordingly, the model – in the late phase – is suitable to investigate alloantigen-independent factors of CAN and lacks markers of alloantigen-dependent processes.

Summary

Chronic allograft nephropathy (CAN) is the leading cause of graft loss following kidney transplantation. One factor contributing to CAN is chronic alloimmune injury. However, the involvement of alloantigen-dependent and -independent factors in CAN is unclear. The pathomechanism of CAN has been extensively studied by utilizing the Fischer-to-Lewis (F344-to-LEW) rat model. Transplant capillaropathy (circumferential multiplication of the peritubular capillary basement membrane) and transplant glomerulopathy (reduplication of the glomerular basement membrane) have recently been validated clinicopathologically as ultrastructural indicators of chronic alloimmune injury.

Keywords
chronic allograft nephropathy, Fischer-to-Lewis rat model, T-cell cytotoxicity, transplant arteriopathy, transplant capillaropathy, transplant glomerulopathy.
transplant capillaropathy [1], transplant glomerulopathy [2], and transplant arteriopathy (for definitions, see Table 1; for illustrations, see Fig. 1a–d) [3]. Transplant capillaropathy and transplant glomerulopathy can be best identified by electron microscopy (EM). Ultrastructural evaluation of renal allograft tissue offers the advantage of the recognition of contact-dependent T-cell cytotoxicity of the endothelial cells and/or tubular epithelial cells (Fig. 1e). One of the main targets of an ongoing or repeated alloimmune injury is the graft vasculature. The detection of transplant capillaropathy, transplant glomerulopathy and transplant arteriopathy, if possible, should enable a differentiation between alloantigen-dependent and other, alloantigen-independent events, and hence a prediction, to some degree, of the efficacy of immunosuppressive strategies.

One of the most frequently used animal models of CAN is rat kidney transplantation from Fischer (F344, RT1\(^{su}\)) donors to Lewis (LEW, RT1\(^{u}\)) recipients (F344-to-LEW). This model was introduced by White and Hildemann in 1968 [4,5], and modified by Tilney and his co-workers in 1992 [6]. From the literature it is known that in this model CAN develops on the basis of initial alloimmune injuries [7]. However, after about week 16, CAN proceeds with similar speed even if the graft is transplanted back to the original donor strain, and accordingly the alloantigen plays a major role no longer [8]. Our aim, therefore, was to clarify the involvement of alloimmune injury in long-term lesions. Experiments on the F344-to-LEW model are generally terminated between 16 and 32 weeks after grafting. We carried out a systematic ultrastructural search for signs of transplant capillaropathy, transplant glomerulopathy, and T-cell-mediated cytotoxicity in animals killed between 32 and 52 weeks after engraftment. F344 rats either with or without 30-min ischemia of the left kidney following right nephrectomy served as controls.

### Materials and methods

**Experimental animals**

Naïve male inbred LEW (RT\(^{u}\)) and F344 (RT\(^{su}\)) rats weighing 220–260 g were used throughout the experiment. All animals were obtained from Charles River, Isaszeg, Hungary, housed under standard conditions, and fed rat chow and water ad libitum. All experimental procedures were in accordance with the guidelines set by the Institutional Animal Care and Use Committee of Semmelweis University, and the Hungarian law on animal care and protection [1998/XVIII, 243/1998(XII.31)]. NIH ‘Principles of Laboratory Animal Care’ (NIH Publication No. 86-23, Revised 1985) were followed.

**CAN model**

The F344 rats served as donors and LEW rats as recipients. Fischer rats (n = 48) were randomized into five groups (32, 40 and 52 weeks allograft and uninephrectomy and ischemia groups) before surgery. Transplantation was performed under general anesthesia, as described previously [9]. Briefly, the left donor kidney was perfused with 4°C Ringer lactate, removed, and positioned orthotopically into the recipient, whose renal vessels had been isolated and clamped, and whose left native kidney had been removed. End-to-end anastomosis of the renal artery, vein and ureter was performed, using 10–0 prolene sutures. The duration of total graft ischemia was set to 30 min. The transplanted animals (Tx group) were treated with cyclosporine A (CSA; 1.5 mg/kg/day subcutaneously) during the first 10 days after surgery. On day 10, the contralateral native kidney was removed.

### Assessment of alloantigen-independent injury

Two groups of F344 rats (four in each) were applied. In one group, right kidney nephrectomy was performed (RNx) under general anesthesia. In the other group, following right kidney removal, the left kidney was decapsulated, and the renal artery and vein were isolated and occluded with a clamp for 30 min (RNx + left ischemia).

### Termination of the experiment: end-points

About 32, 40 or 52 weeks after surgery, rats were anesthetized, the intra-aortic blood pressure was measured...
Figure 1 Lesions of chronic renal allograft rejection in humans. (a) Transplant arteriopathy: the lumen (L) of the arcuate artery is markedly narrowed by intimal fibrosis (asterisks; trichrome). (b) Transplant glomerulopathy: thickened, double-contoured capillary loops, increase in mesangial matrix, and proliferation of mesangial cells. Note the overall involvement (periodic acid-Schiff, PAS). (c) Transplant glomerulopathy: the glomerular basement membrane (GBM) is widened; a new subendothelial basal lamina (arrowheads) is formed (L, lumen; P, podocyte). (d) Transplant capillaropathy: the peritubular capillary endothelium (E) is thickened and nonfenestrated, and displays a serrated contour along the interstitial aspect of the cell body. The basement membrane (arrowheads) has more than 7 layers (L, lumen; IS, peritubular interstitium). (e) Apoptotic (APO) tubular cell in the vicinity of a tubular wall-localized lymphocyte (Ly; TBM, tubular basement membrane).

Figure 2 Morphology of the F344-to-Lew model 52 weeks after grafting. (a) The artery is devoid of intimal fibrosis (periodic acid-Schiff, PAS; L, lumen). (b) Nonspecific focal-segmental glomerulosclerosis; the arrowhead points to subendothelial hyaline deposit (PAS). (c) The glomerular basement membrane (asterisks) appears normal; the foot processes of the podocytes (P) are effaced (L, lumen; MES, mesangium). (d) Normal peritubular capillary (L, lumen; BM, capillary basement membrane).
directly in the aorta by using a Hemosys system (Experimetria Ltd, Budapest, Hungary), and the serum creatinine and blood urea nitrogen (BUN) levels were determined. The rats were bled thereafter, and the left kidneys were fixed and processed for light microscopy and EM according to standard techniques. About 32 Tx animals were killed at week 32 (Tx/32 weeks), four Tx animals at week 40 (Tx/40 weeks), and four Tx animals at week 52 (Tx/52 weeks). At week 32, ultrastructural examination was carried out only in the four animals which displayed the highest level of proteinuria and in which the most severe kidney pathology was observed by light microscopy (Tx/32 weeks-severe). These animals had functional and morphologic parameters of a preterminal stage of the disease. This positive selection ensured the examination of significant allograft disease.

Morphologic investigations

Light microscopy
Consecutive sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), elastin stain, Crossmon’s trichrome, and the silver impregnation of Jones. The reading of lesions started from one arbitrarily chosen glomerulus. From this, the stage was moved in only one direction, and 30 consecutive glomerular profiles were evaluated. The following alterations were read.

1. Hyalinosis: Luminal hyaline thrombi and/or subendothelial hyaline deposits in the capillaries and/or hyaline droplets in the podocytes.
2. Mesangial matrix (MES) expansion: Present if the extracellular matrix exceeded the matrix area of the glomeruli by at least 25%.
3. Focal-segmental glomerulosclerosis (FSGS): Capillary collapse and attachment to Bowman’s capsule with or without collagen deposition (trichrome: blue).

The number of glomeruli with lesions was expressed as a percentage of the total number of glomeruli counted.

The extent of cortical tubulointerstitial (TI) changes was the sum of the following changes (each lesion was scored with 1-point if present).

1. Interstitial inflammation: Mononuclear cell infiltrates in more than 10% of the peritubular interstitium.
2. Interstitial fibrosis: In more than 6% of the cortical area.
3. Tubulitis: Lymphocytes in nonatrophic tubular walls.
4. Tubular atrophy: Profiles with thick and/or wrinkled tubular basement membrane and loss of the segmental features of the nephron.

Electron microscopy
Transplant glomerulopathy, transplant capillaropathy (Table 1), and T-cell-mediated cytotoxicity were searched for. The latter phenomenon is manifested as the apoptosis or lysis of endothelial cells or tubular epithelial cells in the vicinity of lymphocyte(s) adhering to the target cell.

Functional measurements
For protein and creatinine analysis, 24-h urine samples were collected in metabolic cages (Techniplast, Buguggiate, Italy) every 4 weeks. Urinary protein concentrations were determined by the biuret method with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Munich, Germany). Absorbance was determined at 595 nm with a Philips PU8700 spectrophotometer (Philips, Southampton, UK). The serum and urine creatinine concentrations and the BUN levels were determined photometrically with a Reflotron IV automat (Boehringer, Mannheim, Germany). The creatinine clearance was calculated.

Statistical analysis
The functional, histologic and ultrastructural data on the transplanted animals were compared with those on the controls. Data are presented as means ± SEM. Parametric data were compared by one-way ANOVA and nonparametric data were tested by using the Kruskal–Wallis one-way analysis of ranks. Probability values were calculated by means of the paired Student’s t-test for parametric data, and the Mann–Whitney test for nonparametric data. A P-value of <0.05 was considered significant.

Results

Light microscopy
Tx animals
Transplant arteriopathy or transplant glomerulopathy were not detected (Fig. 2a,b). FSGS (Fig. 2b) was observed, with the appearance of subendothelial or luminal hyaline deposits and hyaline droplets in the podocytes. The MES displayed a mild-to-moderate expansion, which was primarily localized in areas of focal adhesions of glomerular capillaries to Bowman’s capsule. Focal-segmental mild mesangial hypercellularity was noted. A large number of hyaline droplets was encountered in the proximal tubular epithelial cells. There were focal, mainly periarterial interstitial mononuclear infiltrates, involving up to 25% of the cortical interstitium, a few tubular cross-sections with tubulitis of one to five lymphocytes in the tubular wall, focal interstitial fibrosis not exceeding 25% of the cortical area, and focal tubular atrophy involving <25% of the area of the cortical tubular profiles (Table 2).
**Animals with alloimmune-independent injury**

The morphologic alterations were milder in both of these groups when compared with the Tx animals (Table 2).

**Electron microscopy**

**Tx animals**

At weeks 32, 40 and 52, the number of glomerular profiles per animal studied were 13, 13 and 6 (median; range: 4–13), respectively, and the number of peritubular capillary profiles per animal examined were 21 (median; range: 20–22), 17 (median; range: 11–21) and 37 (median; range: 25–50), respectively.

**Glomeruli**: Several profiles displayed focal-segmental changes characterized by MES expansion, an increase in the number of cytoplasmic processes of the mesangial cells (Fig. 2c), the occasional solidification of capillary loops, with adhesion to Bowman’s capsule, multilamellated basement membrane of Bowman’s capsule, and effacement of the foot processes above the collapsed segment. Vacuoles filled with proteinaceous material were noted in the cytoplasm of the podocytes. In some areas, the insudation of large subendothelial, homogenous, moderately dense deposits were encountered, and the cells in the vicinity of the deposits contained large vacuoles of lipid and protein droplets. Newly formed subendothelial basal lamina involving the entire circumference of the capillary loop similar to that seen in transplant glomerulopathy was not noted.

**Peritubular capillaries**: Most of the profiles were without noteworthy changes (Fig. 2d). Very rarely, the capillary basement membrane was segmentally duplicated along the wide interstitial aspect of the capillary profile, a pattern of peritubular capillaries that is normal in humans. Transplant capillaropathy was not verified. Signs of ongoing inflammatory response, i.e. activation of the peritubular capillary endothelial cells [10], seen as cellular hypertrophy, the disappearance of fenestration, the increased adherence of lymphocytes and mononuclear cells to the endothelial layer, and the transcapillary migration of inflammatory cells, were not detected.

**Interstitium, tubules, arterioles, and arteries**: There was a mild and focal interstitial inflammation, involving lymphocytes and mononuclears. Lymphocytes localized within the tubular wall were encountered infrequently (one cell/profile). The neighboring epithelial cells were devoid of signs of T-cell-mediated cytotoxicity. Protein droplets were present in the proximal tubular epithelial cells. Nonspecific focal tubular atrophy and a focal increase in interstitial collagen bundles completed the alterations. The profiles of small arteries and arterioles appeared normal.

**Animals with alloimmune-independent injury**

At weeks 40 and 52, at least 3 glomeruli and 15 peritubular capillary profiles were analyzed per animal. The changes in the glomeruli and TI corresponded to those observed on light microscopy. Glomerular subendothelial deposits were absent, and the focal segmental changes were significantly less common when compared with those for the Tx animals. The peritubular capillaries, arterioles and small arteries appeared normal.

**Functional studies**

Throughout the follow up period, the weight differences between the groups did not reach the level of statistical significance (Table 3). The kidney function (as determined via the serum creatinine and the creatinine clearance at the end of the study) was also similar. These values were within the upper-normal range. The BUN level was the only parameter which exhibited pathologic values; it was significantly higher in the Tx animals than in either of the alloantigen-independent injury groups. In all the animals, the proteinuria progressed over time (Fig. 3). In the Tx animals, however, a proteinuria level of over 50 mg/24 h had developed by week 12, and for the remainder of the study period it was significantly higher than that in the controls. At the end of the study, the proteinuria peaked at above 250 mg/24 h in the Tx...
animals, whereas it remained below 100 mg/24 h in both control groups \((P < 0.01)\). The blood pressure remained within the upper-normal range at week 52. The mean arterial blood pressure was highest in the Tx animals, but the differences between the groups were not significant (Table 3).

**Table 3.** Functional data 32, 40 and 52 weeks after operation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Tx/32 weeks</th>
<th>Tx/32 weeks-preterminal</th>
<th>Tx/40 weeks</th>
<th>Tx/52 weeks</th>
<th>RNx + left ischemia</th>
<th>RNx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td>418 ± 33</td>
<td>408 ± 25</td>
<td>432 ± 12</td>
<td>455 ± 24</td>
<td>447 ± 6</td>
<td>474 ± 31</td>
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<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td>107 ± 11.2</td>
<td>120 ± 21</td>
<td>110 ± 7.9</td>
<td>114 ± 13.3</td>
<td>108 ± 13.9</td>
<td>106 ± 14.9</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td></td>
<td>1.4 ± 0.08</td>
<td>2.1 ± 0.09*</td>
<td>1.4 ± 0.01</td>
<td>1.6 ± 0.02</td>
<td>1.2 ± 0.05</td>
<td>1.2 ± 0.07</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td></td>
<td>0.8 ± 0.08</td>
<td>0.5 ± 0.06*</td>
<td>0.9 ± 0.04</td>
<td>0.7 ± 0.02</td>
<td>1.0 ± 0.05</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dl)</td>
<td></td>
<td>22 ± 3.3</td>
<td>32 ± 4.6*</td>
<td>20 ± 1.8</td>
<td>24.1 ± 2.3*</td>
<td>18 ± 2.1</td>
<td>15.4 ± 1.2</td>
</tr>
</tbody>
</table>

Table 3. Functional data 32, 40 and 52 weeks after operation.

*P < 0.05.

Tx: transplanted animals 32, 40 and 52 weeks after engraftment; Tx/32 weeks-preterminal: animals with preterminal disease from Tx/32; RNx + left ischemia: rats with removal of the right kidney and 30-min ischemia in the left kidney; RNx: rats with right nephrectomy.

level, a normal serum creatinine level, normotension, and focal-segmental glomerulopathy. CAN in humans develops in continuously immunosuppressed recipients with a relatively poor human leukocyte antigen (HLA) match. The allograft biopsy can display transplant capillaropathy, transplant glomerulopathy, and transplant arteriopathy, either alone or in combination. These lesions have been validated clinicopathologically as indicators of an insidiously ongoing alloimmune injury [11]. Studies performed on the F344-to-Lew model early after engraftment have demonstrated that alloantigen-dependent processes contribute to graft destruction. Accordingly, graft infiltrating activated lymphocytes are present in the first 12 weeks after engraftment [7]. Alloantigen-specific antibodies have recently been described [12], and the elimination of the initial immunosuppression accelerates graft loss [13], while early back-transplantation to the donor strain prevents chronic rejection [8]. However, back-transplantation after 16 weeks does not alter the clinical course of CAN [8]. Thus, it can be hypothesized that the alloantigen-dependent processes are important in the initiation of CAN, but may not contribute to the process in the late phase.

As peritubular and glomerular capillaries in the F344-to-Lew model have not been previously examined for the presence or absence of the above-mentioned microvascular marker lesions, the present study was conducted to establish conclusive evidence concerning the involvement of alloimmune injury in the late phase of CAN in the F344-to-Lew model.

Pathologic findings indicating rejection have a focal distribution; accordingly special emphasis was given throughout the study to avoid sampling errors. Transplant capillaropathy was carefully searched for in semi-thin sections, and a large number of peritubular capillaries were examined ultrastructurally. Transplant capillaropathy or peritubular capillary profiles with three or four circumferential basement membrane layers

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In the present study, the observed features of the model included marked proteinuria, a slightly elevated BUN level, a normal serum creatinine level, normotension, and focal-segmental glomerulopathy. CAN in humans develops in continuously immunosuppressed recipients with a relatively poor human leukocyte antigen (HLA) match. The allograft biopsy can display transplant capillaropathy, transplant glomerulopathy, and transplant arteriopathy, either alone or in combination. These lesions have been validated clinicopathologically as indicators of an insidiously ongoing alloimmune injury [11]. Studies performed on the F344-to-Lew model early after engraftment have demonstrated that alloantigen-dependent processes contribute to graft destruction. Accordingly, graft infiltrating activated lymphocytes are present in the first 12 weeks after engraftment [7]. Alloantigen-specific antibodies have recently been described [12], and the elimination of the initial immunosuppression accelerates graft loss [13], while early back-transplantation to the donor strain prevents chronic rejection [8]. However, back-transplantation after 16 weeks does not alter the clinical course of CAN [8]. Thus, it can be hypothesized that the alloantigen-dependent processes are important in the initiation of CAN, but may not contribute to the process in the late phase.

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suggestive of ongoing peritubular capillary damage were not noted. Additional efforts were made to analyze a sufficient number of glomeruli so as not to miss duplication or multiplication of the glomerular basement membrane (GBM), a key feature of transplant glomerulopathy. The results were negative. Furthermore, the entire kidneys were examined light microscopically for the presence of transplant arteriopathy, but the result was negative as well.

Mild interstitial infiltrates and, on occasion, tubulitis were present in the tissue sections. To determine whether these changes were part of an ongoing cellular alloimmune response, we searched for signs of contact-dependent T-cell cytotoxicity, but again with negative results. The data indicate that a chronically active alloimmune injury were not present between weeks 32 and 52. The alterations mostly resembled those observed in glomerular hyperfiltration injury [14]. However, functional and morphologic deterioration was more severe in the Tx animals than in the RNx animals or the postischemic kidneys of the F344 rats, indicating that the initial injury to the allografts was more severe than ischemia-reperfusion alone.

Tilney and his co-workers reported 100% death because of acute rejection in animals not receiving CSA vs. 80% survival if the animals received initial CSA treatment [13]. On flow cytometry, the immunoglobulin (Ig)M and IgG alloantibodies peaked at 2–4 weeks, with a gradual decline to baseline thereafter [7]. Similarly, the group of Joosten et al. [12] recently reported that, in rats without immunosuppression, grafting was followed by the appearance of antidonor antibodies, which disappeared at week 8 [12]. Initial immunosuppression with CSA decreased the intensity of the cellular alloimmune injury, and completely prevented antibody formation [12]. Thus, possibly, the initial immunosuppressive therapy in this model prevents the alloimmune injury at the late phase. Furthermore, Tullius and his co-workers [7] also demonstrated an early reversible and a late self-perpetuating phase of allograft damage in the F344-to-LEW model in experiments where the graft was transplanted back to the donor strain at different time-points [8].

We observed previously that the blockade of alloantigen-dependent processes by interleukin (IL)-2 inhibition significantly slowed the progression of the allograft disease in F344-to-LEW animals up to week 16, but was largely ineffective thereafter [15]. These data and the observations from the present study suggest that the F344-to-LEW model has two, partially overlapping phases: an early phase in which rejection and alloantigen-independent factors damage the graft in parallel, and a late phase in which mainly glomerular hyperfiltration operates.

Literature data in support of the lesser importance of alloimmune injury in the late phase have recently accumulated. Alloantigen-independent pathogenetic factors in this model include ischemia [11,16], infection [17], proteinuria [6], hypertension [13], brain death [18], and nonheart-beating donors [19]. Alterations similar to those associated with F344-to-LEW allografts also develop as a result of hyperfiltration in F344-to-F344 isografts [20], and native postischemic F344 kidneys [11,16], but not in Brown-Norway isografts [21], which is evidence in favor of strain-specific alloantigen-independent factors. Hyperfiltration injury can be reduced by inhibition of the rennin-angiotensin system [22]. Accordingly, it has been concluded by Tilney and his co-workers that the F344-to-LEW model is to a large extent mediated by angiotensin II-dependent mechanisms [22]. The pathologic changes that we have observed fully support this view.

In conclusion, although an alloimmune response may be present early after engraftment in the F344-to-LEW model of CAN, the typical morphologic signs of chronic alloimmune injury to the graft vasculature observed in human samples could not be detected, and T-cell-mediated cytotoxicity indicative of an ongoing/active alloimmune response was not verified 32–52 weeks after engraftment. In the F344-to-LEW allografts, lesions resembled glomerular hyperfiltration injury, and proteinuria dominated functionally. It is suggested, therefore, that alloantigen-dependent factors are not major players in the late phase in the F344-to-LEW model of CAN. Thus, the model, in its present form, is suitable to study alloantigen-independent processes of CAN. Regarding the involvement of an ongoing alloimmune injury, conclusions to the pathophysiology of human CAN should be drawn with caution.

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