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Four Stages and Lack of Stable Accommodation in Chronic Alloantibody-Mediated Renal Allograft Rejection in Cynomolgus Monkeys

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Abstract

The etiology of immunologically mediated chronic renal allograft failure is unclear. One cause is thought to be alloantibodies. Previously in Cynomolgus monkeys, we observed a relationship among donor-specific alloantibodies (DSA), C4d staining, allograft glomerulopathy, allograft arteriopathy and progressive renal failure. To define the natural history of chronic antibody-mediated rejection and its effect on renal allograft survival, we now extend this report to include 417 specimens from 143 Cynomolgus monkeys with renal allografts. A subset of animals with long-term renal allografts made DSA (48%), were C4d positive (29%), developed transplant glomerulopathy (TG) (22%) and chronic allograft arteriopathy (CAA) (19%). These four features were highly correlated and associated with statistically significant shortened allograft survival. Acute cellular rejection, either Banff type 1 or 2, did not correlate with alloantibodies, C4d deposition or TG. However, endarteritis (Banff type 2) correlated with later CAA. Sequential analysis identified four progressive stages of chronic antibody-mediated rejection: (1) DSA, (2) deposition of C4d, (3) TG and (4) rising creatinine/renal failure. These new findings provide strong evidence that chronic antibody-mediated rejection develops without enduring stable accommodation, progresses through four defined clinical pathological stages and shortens renal allograft survival.

Keywords

Allograft; antibody; chronic; kidney; monkey; rejection

Introduction

Cellular or antibody-mediated rejections and nonrejection-mediated renal injuries from drugs, recurrent or *de novo* disease, vascular or viral diseases all contribute to late renal allograft loss. Distinguishing between rejection or non-rejection is not easy either clinically or pathologically. One important putative etiology of late renal allograft failure is donor-specific alloantibodies (DSA), identified in tissue by peritubular capillary staining of C4d (1,[2]), which correlates well with the presence of donor-reactive major histocompatibility complex (MHC) serum alloantibodies (3–[15]).

A substantial fraction of patients with chronic rejection have circulating antibodies and deposition of C4d (6,[12],[16]–[20]), which is associated with the later development of transplant glomerulopathy (TG) (12,[16],[20]). However, C4d is occasionally found in human renal allografts

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with normal function (21–[24]), and in one series preceded TG (20), which led us to postulate that C4d may predict later chronic antibody-mediated rejection (25,[26]).

To test this hypothesis, we have evaluated long-term renal allografts in Cynomolgus monkeys (Macaca fascicularis) with mixed chimerism protocols (27–[33]). Many of these kidney allografts survive long term without rejection, but some later develop chronic rejection with alloantibodies, TG and transplant vasculopathy (29), thereby providing a unique opportunity to study the clinical–pathological parameters involved in the development of chronic allograft rejection without the effects of exogenous immunosuppression. Previously in 17 animals we studied the association of alloantibodies, C4d deposition, TG and arteriopathy (33), but too few animals were available to test statistically the effect of alloantibodies or graft pathology on actual renal allograft survival or to test the hypothesis of four stages in the natural history of chronic alloantibody-mediated rejection. With new findings in 143 animals, we define the natural history of chronic alloantibody-mediated rejection in four stages (alloantibody, C4d deposition, TG, rising creatinine/renal failure), which markedly shortens allograft survival, and occurs without apparent enduring accommodation. In addition, ancillary findings include an association of the development of arteriopathy with prior TG, endarteritis, C4d/alloantibodies and glomerulitis and the development of interstitial fibrosis with prior TG, glomerulitis and C4d/alloantibodies.

Methods

Animals

The purpose of this study was to identify the relationship among alloantibodies, C4d, allograft pathology and late graft failure, rather than the frequency of these events in specific treatment protocols (27–[33]). Therefore, we evaluated all animals treated with a variety of mixed chimerism protocols from 1993 to 2007, not on chronic immunosuppression, and with renal allograft surviving more than 50 days (n = 143). The endpoint was death from any cause, including infection, spontaneous death, renal failure or euthanasia to terminate the experiment in animals with normal renal allograft function. Recipient and donor Cynomolgus monkey pairs (3–8 kg Charles River Primates, Wilmington, MA) were selected for ABO compatibility but mismatched for Cynomolgus leukocyte (CyLA) MHC antigens (27,[28]). All surgical procedures and postoperative care of animals were carried out in accordance with National Institute of Health guidelines and were approved by the Massachusetts General Hospital Subcommittee on Animal Research.

Regimens

The standard preparative regimen included nonlethal total body irradiation (TBI) (1.5 Gy) on day −6 and −5 relative to transplantation, local thymic irradiation (TI) (7 Gy) on day −1, i.v. ATG (ATGAM, Pharmacia and Upjohn Co., Kalamazoo, MI.) (50 mg/kg/day) on days −2, −1 and 0, and i.v. donor bone marrow transplantation (DBMT) on day 0, infused at 0.4 to 4 $\times 10^8$/kg. Monkeys underwent heterotopic renal transplantation and splenectomy on day 0 and bilateral native nephrectomies under ketamine hydrochloride/diazepam anesthesia, supplemented by halothane (27). Cyclosporin (CyA, Novartis, Basel, Switzerland) was given i.m. beginning on day 1, tapered from an initial dose of 15 mg/kg/day to maintain therapeutic serum levels (>300 ng/mL), and discontinued on day 28 posttransplant, after which the serum CyA levels become undetectable by days 60 to 70. In the anti-CD40L protocol, transplantation was followed by a short course of anti-CD154 monoclonal antibody (5c8, Immerge Biotherapeutics, 20 mg/kg × 2), usually without splenectomy (34). Controls included selective elimination of major components of the protocols. Modifications of the standard protocol included substitution of peripheral blood stem cells for bone marrow cells, addition of other antibodies (e.g. antibodies to CD28, CD30, Rituxan, CTLA4Ig) or other drugs (rapamycin,

fludarabine). Data from many of these animals have been published previously (27–[33]). The frequency of alloantibodies and positive C4d staining was indistinguishable among groups, in part because some groups contained fewer than five animals, so that data from all 143 animals were pooled for pathological review and survival analysis. Nevertheless, differences may have been missed due to sample size.

Detection of alloantibodies

Antidonor-specific alloantibodies were detected by flow cytometric analysis on sera simultaneously sampled for creatinines (29,[32],[33]). The positive threshold was two standard deviations above pretransplant sera. Overall, 175 serum samples from 54 animals were available for correlation with pathology specimens. Sera from nine Cynomolgus animals were previous tested to verify cross-reactivity to purified human class I and class II alloantigens (32).

Pathology studies

Four hundred seventeen samples studied included 269 biopsies for elevated creatinines (incident biopsies), or stable function (protocol biopsies), 5 nephrectomies and 143 autopsies. After euthanasia, complete autopsies were performed, and sections were stained with hematoxylin and eosin and periodic acid Schiff (PAS) stains and scored using Banff criteria (35,[36]). TG was defined as >25% glomerular capillaries with glomerular basement membrane (GBM) duplication in the most involved glomerulus in PAS stains (Banff cg2). Chronic allograft arteriopathy (CAA) was scored only on autopsy and nephrectomy specimens due a paucity of arteries on biopsy. Electron microscopy and C4d staining was performed as previously described (33). C4d staining was considered positive if most of the capillaries in 6 or more 10 high-power fields showed circumferential C4d staining of the peritubular capillaries.

Statistical analysis

Data were analyzed using ANOVA and post-hoc corrections, Fisher exact test, Kaplan–Meier and Life Table statistics (SPSS Software, version 10, Chicago, IL).

Results

Development of chronic rejection

The purpose of this study was to analyze the natural history of chronic rejection in all animals with renal allografts surviving 50 days and not to determine relative outcomes among protocols so that data are pooled for analysis. TG was found in 31 of 143 animals (22%, and 17% of all samples). First detected at 82–818 days with the median first appearance of 161 days for all samples, GBM duplication was usually extensive, affecting on average 89 ± 14% of the glomerular capillaries (60–100%). Most of the samples with TG also had mesangial hypercellularity (> 1 mm) (74%) and/or mononuclear cell accumulation (>1 g) (68%) in greater than 50% of glomeruli (transplant glomerulitis). In contrast, of those with less than 50% GBM duplication, 6% had mesangial hypercellularity and 17% had glomerulitis. Thus, GBM duplication was highly correlated with diffuse mesangial expansion and glomerulitis (both $p < 0.001$). An example of the histological progression of TG and C4d in animal 4498 with chronic antibody-mediated rejection is illustrated in Figure 1. This animal had a normal-looking renal biopsy on day 106 (Figure 1A), lacked C4d staining (Figure 1F) and had a creatinine of 1.2. Alloantibodies were first detected on day 162 and remained positive. The biopsy on day 182 showed a mild glomerulitis without TG (Figure 1B), without C4d staining (Figure 1G), and with a creatinine of 1.4. On day 225, TG was present (Figure 1C) with positive C4d staining (Figure 1H), and a slightly elevated creatinine of 1.8. TG worsened on a follow-

up biopsy on day 352 (Figure 1D), and autopsy on day 371 (Figure 1E), with persistent C4d staining (Figures 1I and J) and rising creatines of 2.4 and 4.8, respectively. Previously electron microscopy confirmed prominent basement membrane duplication in five cases and identified no immune complexes (33). Seven additional autopsied kidneys from four animals with alloantibodies and C4d, and three without alloantibodies and C4d were analyzed ultrastructurally. In the three cases without alloantibodies or C4d, basement membrane duplication and excess peritubular laminations were not identified, data not shown. In the four cases with alloantibodies and C4d, the glomerular basement membrane showed frequent and marked duplication with isolated glomerular basement membrane laminations (Figure 2A, and B), plus peritubular capillary laminations, up to seven layers (Figure 2C). These findings are similar to those found in the ultrastructural analysis of human TG (37).

C4d deposition

Samples from 29% of the animals showed prominent C4d deposition in peritubular capillaries in at least one sample (41/143), and 31% of all samples were C4d positive (79/251) (33) (Figure 1). The first time of appearance of PTC C4d deposition was 65–770 days after transplant (median of 117 days). Twenty-two animals with C4d-positive biopsies remained positive on repeat biopsies (1–5 subsequent biopsies); one was negative at autopsy 10 days later and 18 did not have a subsequent biopsy. Of the 11 animals with focal C4d (less than 50% of fields) four became positive on subsequent biopsies (22, 65, 91 and 111 days later), two became negative (4 and 42 days later), one remained focal and four had no follow-up sample.

Negative controls including 9 native kidney controls, 6 biopsies of allografts with polyomavirus interstitial nephritis and 7 allograft biopsies with posttransplant acute tubular injury (ATN) all showed no peritubular capillary C4d staining (data not shown).

Experimental protocols and antibody to donor T cells and B cells

Overall, alloantibodies were detected in 48% of the animals tested (26/54). The antibodies were first detected 53–839 days after transplantation (median of 106 days) (29,[31–33]).

Correlation between C4d and alloantibodies

For individual animals, alloantibodies correlated with C4d deposition in the allografts of 54 animals tested. Nineteen of 26 animals with alloantibody (73%) had positive C4d, whereas only 3 of 28 without alloantibody (10%) had positive C4d. This relationship is statistically significant, $p < 0.001$. Similarly among concurrent samples, alloantibodies and C4d strongly associated ($p < 0.001$, $n = 177$) (Table 1).

Alloantibodies and C4d are risk factors for TG

Alloantibodies are a risk factor for the development of TG (Table 1). Fourteen of 26 animals with alloantibody developed TG (54%), whereas only 1 of 28 animals (4%) without antibodies developed TG ($p < 0.001$). C4d is also a risk factor for the development of TG. Among the 54 animals with known alloantibody status, 14 of 26 animals positive for C4d (54%) developed TG, whereas only 1 of 28 (4%) without C4d developed TG ($p < 0.001$). Using all animals, including 89 with an unknown antibody status, 9 of 15 (60%) with C4d developed TG, whereas only 7 of 74 (9%) without C4d developed TG ($p < 0.001$). In 93 animals with adequate numbers of biopsies to determine if C4d proceeded the development of TG, in 19 animals C4d preceded TG, whereas in only 3 animals TG was identified before C4d. Both C4d and TG were occurring concurrently in 71 animals. Among concurrent samples alloantibodies, C4d and TG were strongly associated ($p < 0.001$, $n = 264$). The presence of either alloantibodies or C4d staining correlated with the later development of TG if not present concurrently ($p < 0.001$).

Correlation of alloantibodies, C4d and CAA

CAA was found in 27 of 143 (19%) of the autopsy and nephrectomy samples (Table 1). Using only autopsies to assure adequate sampling of arteries, alloantibodies before autopsy were a risk factor for CAA ($p < 0.001$). Overall, 13 of 26 animals with alloantibodies had CAA at autopsy (50%), whereas only one animal of 24 without alloantibodies had CAA at autopsy (4%). Positive C4d staining before autopsy predicted CAA. Using 110 animals surviving 62 days or longer because the earliest detection of CAA was 62 days, of the 39 with C4d peritubular capillary staining, 20 had CAA (51%), but of 71 without C4d, only 8 had CAA (11%, $p = 0.001$). TG before autopsy and arteriopathy at autopsy were strongly associated. Using 96 animals surviving 82 days or longer because the earliest detection of TG was 82 days, of the 30 animals with TG, 18 also had CAA (60%), whereas of 66 without TG, only 7 had CAA (11%; $p < 0.001$) (Table 1).

Additional pathological correlations

Fibrosis of greater than 50% of the cortex was correlated with peritubular C4d deposition, TG and alloantibodies on concurrent specimens, ($p < 0.001$) but not with acute cellular rejection (Banff types 1, 2 or suspicious) (Table 1). Concurrent or prior episodes of acute cellular rejection were not associated with the presence of alloantibodies, C4d or TG, but TG on biopsy was associated with later development of CAA, and fibrosis. Endarteritis (Banff acute cellular rejection, type 2) was a risk factor for CAA ($p < 0.001$) but not for C4d or TG (all $p > 0.5$) because 12 of 15 animals (80%) with a type 2 rejection had CAA, whereas only 8 of 60 without a type 2 rejection had CAA (13%). C4d staining of peritubular or glomerular capillaries on biopsy predicted the later development of CAA, TG and fibrosis ($p < .001$) (Table 1).

Sequential stages of chronic alloantibody-mediated rejection

If alloantibodies, C4d deposition, TG and renal failure are causally related, a temporal sequence should exist (Figure 3). To test this hypothesis in 18 autopsied animals with allografts surviving 200 days or longer and with adequate interval sampling, the geometric mean of the first day of appearance of alloantibodies, C4d, TG and rising creatinine was 158, 210, 252 and 367 days with respective mean creatinines of 1.4, 1.9, 2.2 and 6.0 (Figure 3, top) with diagrammatic representation of the stages of chronic alloantibody-mediated rejection (Figure 3, bottom). First to appear was circulating alloantibodies (42% of cases), C4d (18% of cases) or a combination of C4d and alloantibodies (37% of cases). Only rarely (3%) was the first manifestation TG without C4d or alloantibodies.

Over time alloantibodies, C4d, TG generally became congruent (Figure 4) supporting our hypothesis that these are three closely related events. In 18 animals with alloantibodies, 9 (50%) were C4d positive at the time alloantibodies was detected or within 34 days. Eight more became C4d positive 35–225 days later so that 94% of antibody-positive animals were or became C4d positive (Figure 4). In 18 animals with alloantibodies, 5 were TG positive (28%) at the time alloantibodies was detected or within 28 days. Ten more became TG positive 37–543 days later so that 89% of alloantibodies positive animals were or became TG positive.

Survival

To test the relationship between survival times and presence of alloantibodies, survival times were analyzed in two ways. In the first method animals were separated into three groups, (Figure 5): (1) acute cellular rejections, (2) other (nonrejections, mostly obstruction) and (3) long-term survivors, which were partitioned to test if the presence or absence of alloantibodies, positive C4d staining, TG or CAA were risk factors for survival of long-term animals. In this analysis, survival time was defined as death from any cause or the survival interval at the time of a normal-looking biopsy if the animal was alive. Of these long-term survivors, the presence

of alloantibodies, C4d, TG or CAA reduced the respective mean and median survival time by at least 58% (Table 2 and Figure 5). In the second method Kaplan–Meirer analysis was performed censoring animals that died from nonrejection causes (mostly obstruction). This analysis showed that some animals without alloantibody (or C4d, TG or CAA) might survive indefinitely when nonrejection causes are censored from the data (Table 2 and Figure 5) and that the presence of alloantibody (or C4d TG, or CAA) markedly shortened the survival times (Table 2, $p < 0.01$).

Discussion

This study confirms that nonhuman primates develop chronic alloantibody-mediated renal allograft rejection, very similar to humans (12,[16],[20]), without confounding nonrejection etiologies of late renal allograft failure often present in humans. Major new findings document markedly shortened allograft survival caused by alloantibodies, consistent with the excess renal allograft loss in humans associated with alloantibodies (38–[40]). The clinical pathological progression strongly supports the four proposed stages of chronic alloantibody-mediated rejection (25,[26]). Additionally, the overall progressive nature of antibody-mediated chronic rejection and renal dysfunction observed in this study fails to support the hypothesis that persistent MHC alloantibody and C4d deposition merely identify a state of quiescent accommodation without ongoing or eventual pathological injury and allograft dysfunction (41). In addition, ancillary findings include an association of the development of arteriopathy with prior TG, endarteritis, C4d/alloantibodies and glomerulitis, and the development of interstitial fibrosis with prior TG, glomerulitis and C4d/alloantibodies.

The absence of maintenance immunosuppression in this model makes extrapolation to human data difficult because most humans generally receive continuous treatment. In this study, the average interval between alloantibodies and renal failure was 0.6 years compared in humans of 2.7 years for class I and 3.9 years for class II alloantibodies (40). This variation between monkeys and humans may reflect treatment in humans or biological variation in the disease itself. Using a variety of tolerance protocols in Cynomolgus monkeys, we have pooled data for statistical and survival analysis. The tightness of the observed correlations, the temporal sequence and the ultimate congruence of alloantibodies with these pathological findings provide strong evidence that they are closely associated events, if not causally related, and support the etiological relevance of alloantibodies over minor variations in treatments and make this pattern highly relevant to human renal allografted patients, who all receive variations in therapy (38–[40]). Other investigators using different models also emphasize the value of nonhuman primate models of chronic rejection to describe similar pathologic features, TG and CAA, although without C4d (42,[43]).

Although C4d and alloantibodies were strongly correlated, a fraction of discordant cases exist with C4d deposition but without detectable alloantibodies or the reverse on any given biopsy similar to occasional discordance observed in humans (3,[8],[44]–[46]). Positive C4d staining without concurrently detectable serum alloantibodies (which occurs in about 10% of human biopsies) might be due to non-HLA alloantibodies (47–[49]) or adsorption of antibodies by the graft. This study supports the later possibility because most of the alloantibody-negative, C4d-positive animals became alloantibody positive on subsequent testing or lost the C4d staining. Other investigators have shown that eluates of renal allograft nephrectomies removed for chronic rejection contain alloantibodies in 71% of cases but only 31% of these patients had detectable alloantibodies in the circulation (50). Why C4d staining occasionally lags the presence of serum alloantibodies (Figures 2–4) is unclear but may involve complement regulatory proteins or accommodation (51–[53]), lack of complement fixing antibodies (54) or just insufficient antibody to activate complement. Our serial studies also showed that most examples of discordance between C4d and alloantibodies subsequently became congruent on

follow-up, that is, increasing frequency of C4d or TG over time (Figure 4). However, congruence was not absolute, and a few animals with alloantibodies never acquired C4d or TG. Some human studies have reported that a minority of TG cases were associated with alloantibodies and positive C4d (55–[57]), whereas Sis et al. identified that in 54 allograft kidneys with TG, 70% had alloantibodies and 36% had positive C4d (58). Nevertheless, 27% of cases could not be associated with either alloantibodies or positive C4d (58). Some of these human cases might be attributable to calcineurin inhibitor-induced thrombotic microangiopathy. Similarly, in our study of 31 animals with TG, 8 lacked C4d or alloantibody (26%). With increased sampling (Figure 4) 11% of animals with TG had no antecedent alloantibody or C4d, suggesting that sampling error and selection bias may increase the frequency of alloantibody-associated TG. Other human studies provide support that the strongest predictor of TG is C4d deposition (12,[18],[20],[59]). Also supporting the relationship between alloantibodies and C4d is the inability in this study to correlate acute cellular rejection and TG. The slightly lower frequency of human alloantibody-associated TG compared to monkeys is unclear but could be related to continuous immunosuppression especially calcineurin inhibitors, which are known to cause thrombotic microangiopathy (TMA)-associated TG. Twelve animals in this study were identified without both positive C4d positive peritubular capillary (PTC) staining, glomerulitis (Table 1) (60) or concurrent TG. Seven of these 12 animals later developed TG also suggesting that all cases of TG cannot be attributed solely to alloantibody/C4d or calcineurin inhibitor TMA. Variations in antibody titer, complement regulation, endothelial gene expression, accommodation or antibody/complement-independent mechanisms may all contribute to the heterogeneity of TG development.

The pathogenesis of CAA is unclear but is likely related to T cell, alloantibody or natural killer (NK) cell-mediated mechanisms (26). This study does not distinguish among these potential mechanisms but identified alloantibody/C4d and/or ACR2 (endarteritis) as risk factors for CAA. Of 26 animals with CAA, two lacked precursor alloantibodies/C4d or endothelialitis, 7 had only precursor endothelialitis, 10 only had precursor alloantibodies/C4d and 7 had both alloantibodies/C4d and endothelialitis. These data support the hypothesis that the pathological injury from either cellular or antibody-mediated rejection contributes to CAA, consistent with other observations (43,[61]). Alternatively, alloantibodies may not mediate CAA *per se* but are just a marker of sensitization for the cellular immunity that actually causes CAA (endothelialitis) but was missed due to sampling error because endothelialitis is more prominent in arcuate-sized renal arteries (62,[63]). Consistent with this hypothesis is that in autopsies CAA was observed more frequently with Banff type 2 rejections. Possibly, alloantibody and complement activation can recruit or activate T cells or dendritic cells (64) or B cells acting as antigen-presenting cells could promote acute cellular rejection (65,[66]). NK cells by ligation of Fc receptors and alloantibody could mediate allograft vasculopathy by interferon activation (67,[68]). CAA was predicted by positive C4d staining and alloantibodies. Overall, 68% of the monkeys that had CAA showed C4d-positive peritubular staining, comparable to 61% reported in humans (6).

How alloantibodies and complement activation promote glomerulopathy or possibly arteriopathy are unknown. Antibodies to class I MHC antigens can stimulate endothelial and smooth muscle proliferation and expression of fibroblast growth factor (FGF) receptors (69). Terminal complement components (C5b-9) trigger the production of FGF and platelet-derived growth factor (PDGF) by endothelial cells (70). Thus, antibodies and activated complement might cause chronic antibody-mediated rejection by inducing gene products that promote endothelial activation with subsequent basement membrane duplication and arterial smooth muscle proliferation (71,[72]).

To establish accommodation in some models requires acute complement depletion (73), especially the absence of activation of the terminal complement cascade (74,[75]), the up-

regulation of complement regulatory components (DAF and CD59) (51,[76]) or the induction of anti-apoptotic proteins (Bcl-2, Bcl-xl, HO-1) or other cytoprotective proteins (nitric oxide, indolamine 2,3 dioxygenase) (52,[77],[78]). Low alloantibody titer or sub-saturating quantities of alloantibody may preferentially induce anti-apoptotic gene expression and accommodation (79,[80]). More likely chronic antibody-mediated rejection may represent partially accommodated acute antibody-mediated rejection (26), in which accommodation only mitigates the mechanisms of acute injury but does not prevent gene expression of proteins that eventually causes capillary, interstitial or vascular remodeling, the pathological hallmarks of injury associated with clinical dysfunction. Our studies suggest that in the absence of immunosuppression anti-MHC alloantibodies may not be associated with stable accommodation in contrast to carbohydrate antigen/ABO incompatibility, which may lead to more stable accommodation (81–[84]).

Alloantibodies and chronic rejection are significant problems in tolerance protocols (42,[85],[86]). This study confirms the difficulty of inducing tolerance in some types of T helper cells and in B cells, stressing the importance of the indirect pathway in allograft rejection (87–[89]) and are especially relevant to those clinical protocols designed to induce tolerance, T regulatory cells or to reduce or eliminate immunosuppression because the activation requirements of Th1, Th2, T regulatory cells or B cells may be different (90). Because complement activation is a T-cell costimulatory pathway, persistent complement activation in an allograft may increase the risk for the development of acute cellular rejection (64,[65]). Our results also emphasize the importance of timely and serial serological assays for alloantibodies and C4d staining because among various assays presently available only these predicted the critically important but clinically silent progressive intragraft pathological changes that can ultimately lead to graft loss from chronic antibody-mediated rejection (14,[32],[91]).

In summary, the natural history of chronic antibody rejection in this model takes place in the absence of antigen-independent etiologies of chronic allograft nephropathy, occurs in four progressive stages, shortens renal allograft survival, is likely mediated by alloantibodies and develops without enduring stable accommodation.

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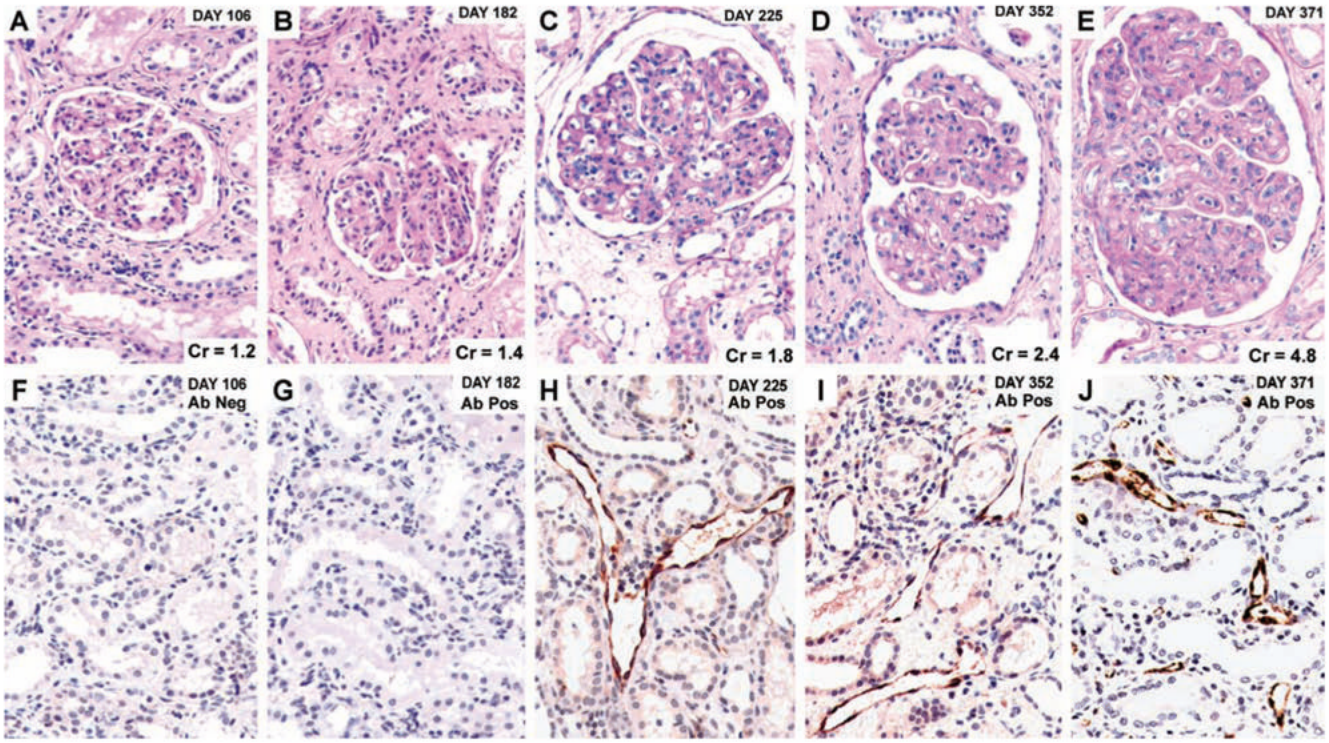

Figure 1. Natural history of chronic alloantibody-mediated rejection in animal no. 4498

Protocol biopsies are on days 106 (Cr = 1.2), 182 (Cr = 1.4), 225 (Cr = 1.8) and 352 (Cr. 2.4) and autopsy on day 371 (Cr = 4.8). Hematoxylin-eosin stained images (A–E) and C4d immunohistochemistry and hematoxylin counterstained images (F–J). Alloantibody was detected on day 162, and C4d staining is positive and TG is present on days 225, 352 and 371. Original magnification, 400×.

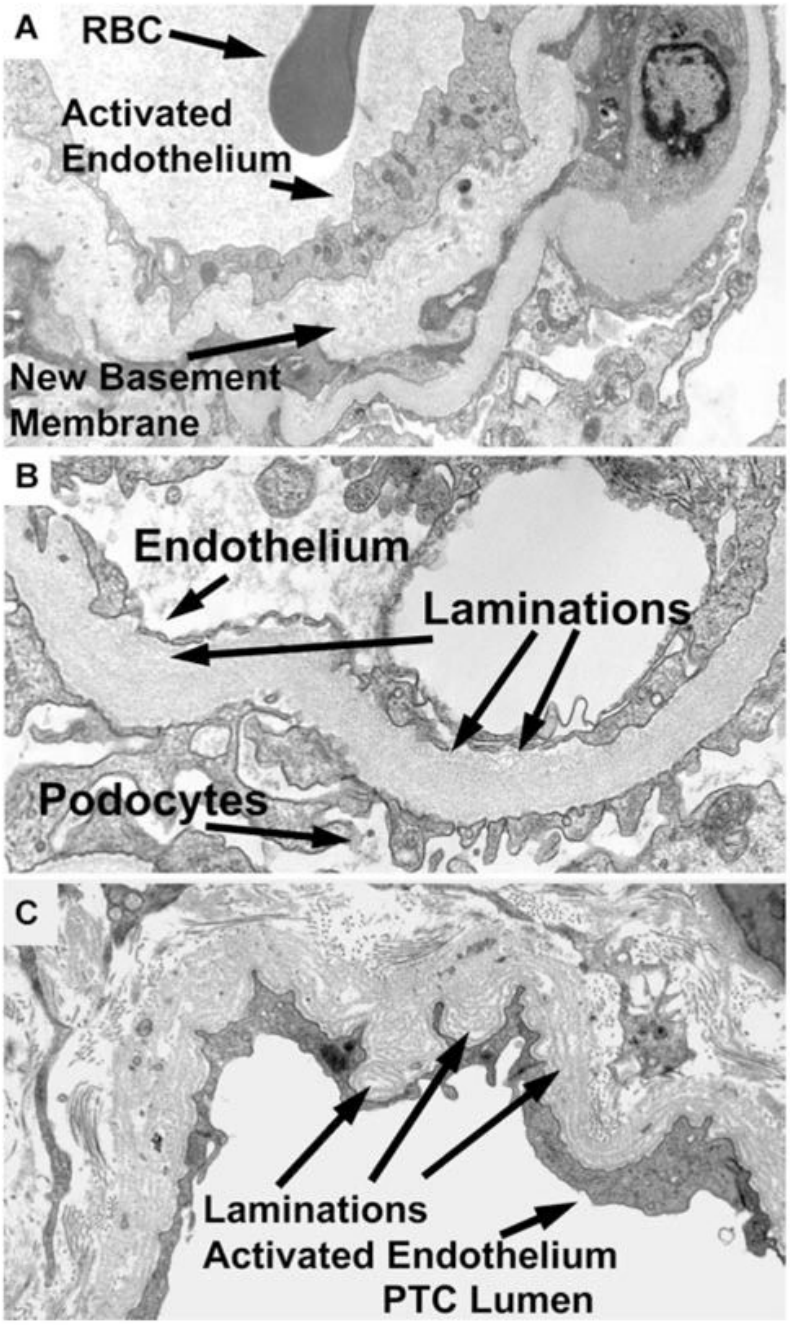

Figure 2. Digital images from the electron microscopy of TG and peritubular capillary laminations

(A) Glomerular loop with new basement membrane (arrow), that is duplication, activated endothelium (arrow) and lumen with a red blood cell (RBC, arrow). Original magnification 7100×. (B) Glomerular loop with thickened basement membrane laminations (arrow), and activated endothelium (arrow). Podocytes for reference (arrow). Original magnification, 15 000×. (C) Peritubular capillary with laminations (arrow). and activated endothelium (arrow). Peritubular capillary lumen for reference (PTC lumen). Original magnification, 9100×.

First Appearance

		Ab	C4d	CAG	Rising Cr/ GraftLoss
Days To	Mean	158	210	252	367
	SD	145	180	165	217
	Range	61-652	97-755	113-695	231-838
Cr at Days To	Mean	1.4	1.9	2.2	5.8
	SD	0.6	0.8	0.9	2.0
	Range	0.9-2.9	1-3.5	1-3.7	2.3-9

N = 18

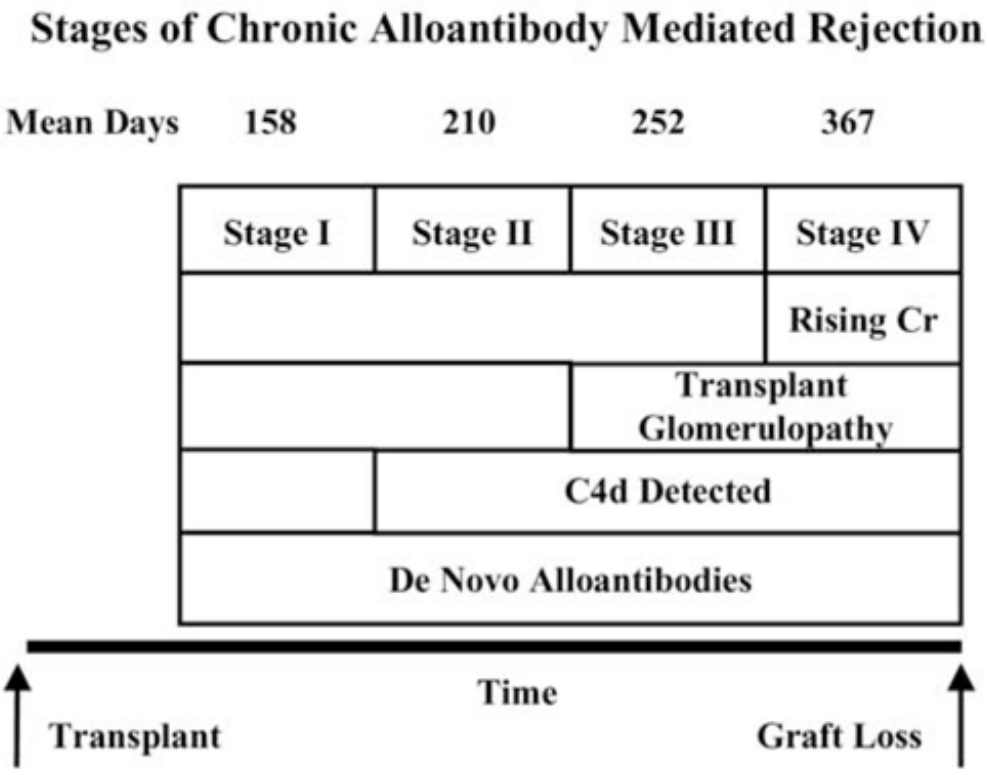

Figure 3. Sequential appearance of alloantibody, C4d deposition, TG and rising creatinine in 18 animals

Stages of chronic alloantibody-mediated rejection. (Top) The first appearance of alloantibody, C4d, transplant glomerulopathy and rising creatinines in 18 animals. (Bottom) Proposed four stages of chronic alloantibody-mediated rejection.

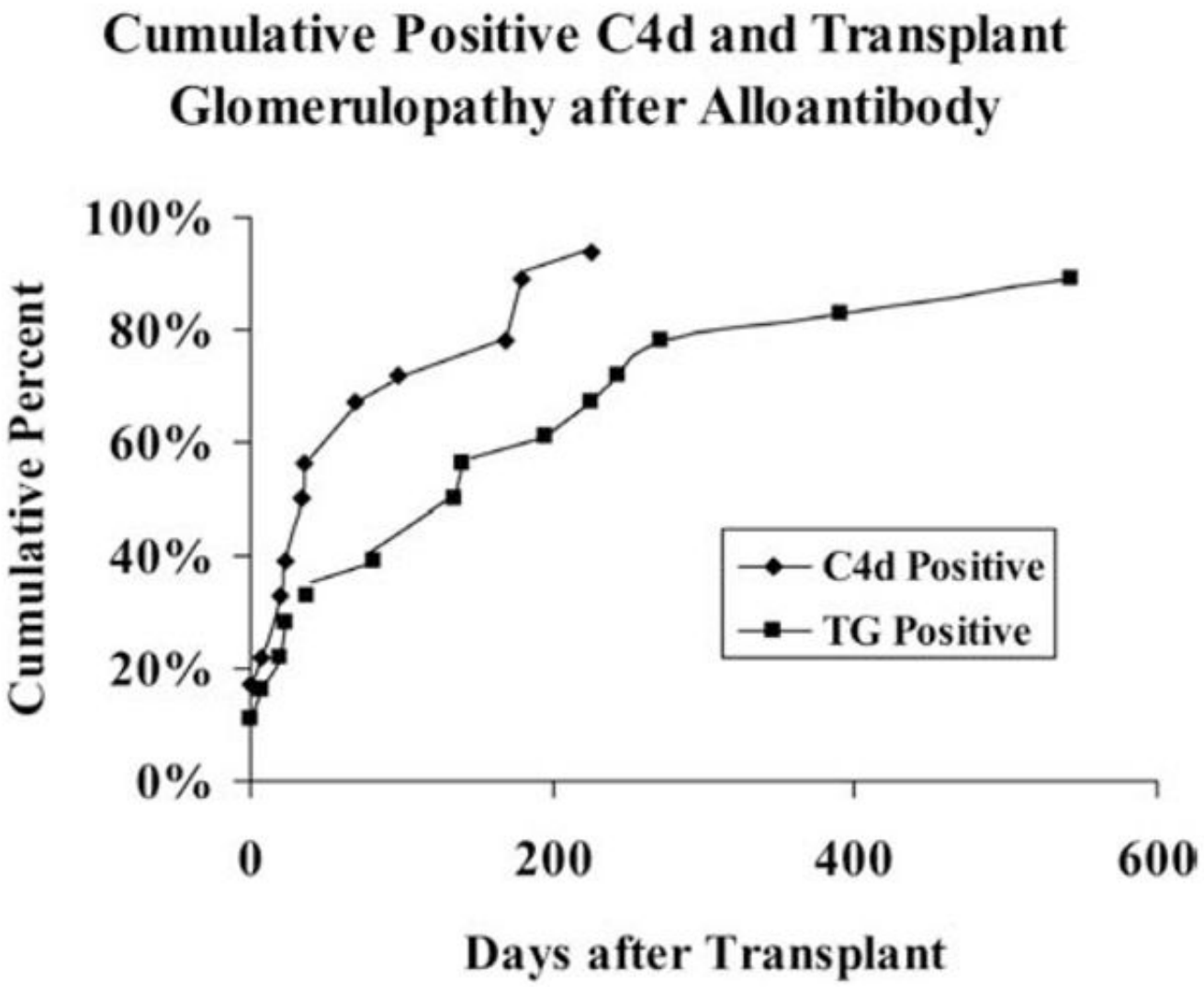

Figure 4.
Cumulative increasing frequencies of positive transplant glomerulopathy and C4d staining.

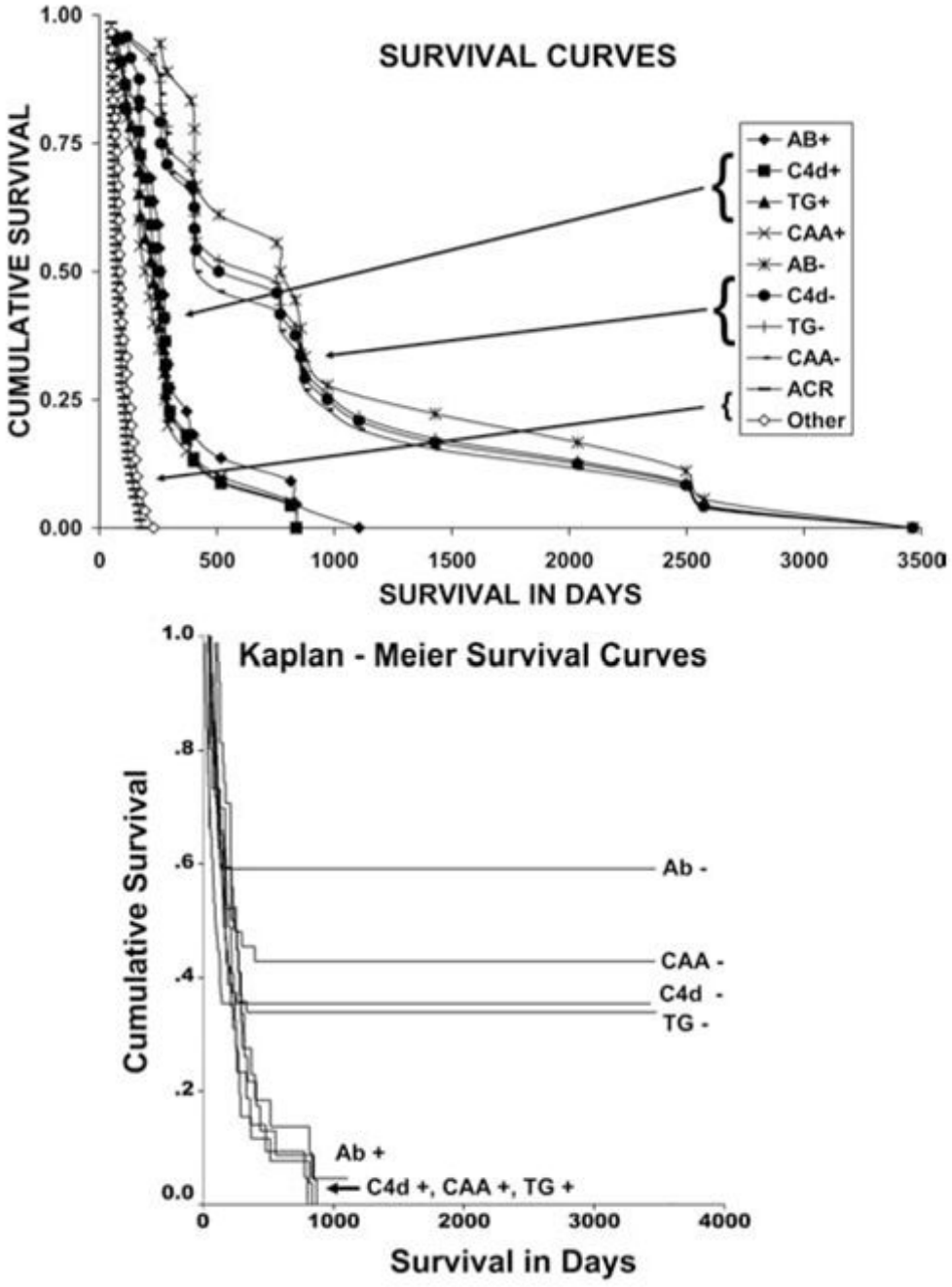

Figure 5. Survival curves of 143 animals

(Top) Survival interval until death from any cause including termination of the experiment. Number of animals: Alloantibody negative (18), alloantibody positive (22), C4d negative (24), C4d positive (22), TG positive and negative (23), chronic arteriopathy positive (26) and negative (20), ACR (64) and other (33). ACR = acute cellular rejection (mostly ACR2, mean and median survival, 87 or 76 days). Other is death from nonrejection: obstruction (most cases) or infection, BK nephritis (1), or posttransplant lymphoproliferative disease (1) (mean or median survival, 105 or 90 days). $P < 0.001$ for respective positive and negative groups. (Bottom) Kaplan–Meier survival curves of 143 animals with data censored for nonrejection. Number of animals: Alloantibody negative (15), C4d negative (52), TG negative (57), CAA

negative (37), alloantibody positive (25), C4d positive (25), TG positive (26) and CAA positive (26). $P < 0.01$ for respective positive and negative groups.

Table 1

	Concurrent pathology				
Pathology on biopsy	**Banff arterial inflammation >v0**	**Presence of alloantibodies**	**TG> cg1**	**Positive C4d PTC**	**Banff acute> suspicious**
TG> cg1	0.600	<0.001		<0.001	0.012
Fibrosis ≥50%	0.820	0.001	<0.001	<0.001	0.77
Banff acute > suspicious	<0.001	0.110	0.100	0.060	
Banff arterial inflammation >v0		0.970	0.910	0.990	<0.001
Positive C4d PTC	0.150	<0.001	<0.001		0.15
Presence of alloantibodies	0.970		<0.001	<0.001	0.22
Banff infiltrate >i1	0.230	0.280	0.380	0.670	<0.001
Banff glomerular inflammation >g1	0.001	0.001	<0.001	0.001	0.002
N > 150					

	Later pathology				
Pathology on biopsy	**Banff acute > suspicious**	**Positive C4d PTC**	**Chronic arteriopathy**	**TG > cg1**	**Fibrosis ≥50%**
TG > cg1	>0.05	<0.001	<0.001		<0.001
Fibrosis ≥50%	>0.05	0.050	0.150	0.040	
Banff acute > suspicious		0.110	0.630	0.120	0.67
Banff arterial inflammation >v0	<0.001	0.600	<0.001	0.730	0.74
Positive C4d PTC	>0.05		<0.001	<0.001	<0.001
Presence of alloantibodies	0.150	<0.001	<0.001	<0.001	<0.001
Banff infiltrate >i1	< 0.001	0.720	0.680	0.360	0.36
Banff glomerular inflammation >g1	>0.05	<0.001	<0.001	<0.001	0.001
N > 150; for CAA (autopsies) >47					

Table 2

	Survival times (days)					
	N	**Mean**	**SD**	**Median**	**SD**	**p-Value**
Antibody negative	18	1056	222	770	79	
Antibody positive	22	348	56	274	18	p <0 .001
C4d negative	24	950	185	755	282	
C4d positive	22	303	43	256	33	p <0 .001
TG negative	23	964	186	755	282	
TG positive	23	287	42	230	35	p <0.001
CAA negative	20	912	174	508	287	
CAA positive	26	286	49	215	38	p <0.001
	Kaplan–Meier survival times (days)					
	N	**Mean**	**SD**	**Median**	**SD**	**p-Value**
Antibody negative	15	2086	277			
Antibody positive	25	313	54	252	31	p <0.01
C4d negative	52	1288	187			
C4d positive	25	282	42	230	30	p <0 .01
TG negative	57	1252	184			
TG positive	26	267	41	195	36	p <0.01
CAA negative	37	1559	220			
CAA positive	26	237	40	174	6	p <0.01

p-value refers to either medians or means.

p-value refers to means.