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Hydrolysis of Coagulation Factors by Circulating IgG Is Associated with a Reduced Risk for Chronic Allograft Nephropathy in Renal Transplanted Patients

Bharath Wootla,* Antonino Nicoletti,* Natacha Patey, † Jordan D. Dimitrov,* Christophe Legendre, ‡ Olivier D. Christophe,§ Alain Friboulet,¶ Srinivas V. Kaveri,* Sébastien Lacroix-Desmazes,* and Olivier Thaunat†* 1

Chronic allograft nephropathy (CAN), a major cause of late allograft failure, is characterized by a progressive decline in graft function correlated with tissue destruction. Uncontrolled activation of the coagulation cascade by the stressed endothelium of the graft is thought to play an important role in the pathophysiology of CAN. In this study, we demonstrate that circulating IgG from renal-transplanted patients are endowed with hydrolytic properties toward coagulation factors VIII and IX, but fail to hydrolyze factor VII and prothrombin. The hydrolytic activity of IgG was reliably quantified by the measure of the hydrolysis of a fluorescent synthetic substrate for serine proteases: proline-phenylalanine-arginine-methylcoumarinamide (PFR-MCA). A retrospective case-control study indicated that an elevated hydrolysis rate of PFR-MCA by circulating IgG correlated with the absence of CAN lesions on protocol graft biopsy performed 2 years posttransplantation. We propose that circulating hydrolytic IgM may counterbalance the procoagulation state conferred by the activated endothelium by disrupting the amplification loop of thrombin generation which is dependent on factors VIII and IX. Interestingly, low rates of PFR-MCA hydrolysis, measured 3 mo posttransplantation, were predictive of CAN at 2 years down the lane. These data suggest that PFR-MCA hydrolysis may be used as a prognosis marker for CAN in renal-transplanted patients. The Journal of Immunology, 2008, 180: 8455–8460.

Advances during the last 20 years in immunosuppressive therapy and surgical techniques have resulted in a regular improvement of short-term (i.e., 1-year) rate of renal graft survival. Contrasting with this progress, the half-life of the transplants has remained the same due to chronic allograft nephropathy (CAN), 3 which represents a major cause for graft loss after the first year of transplantation (1). The pathophysiology of CAN remains elusive. Accumulating evidence suggest however that the uncontrolled activation of the coagulation cascade by the activated endothelium of chronically rejected graft is an important contributor to graft damages. Indeed, 1) extensive fibrin deposition has been evidenced within the vessels of chronically rejected grafts (1), 2) tissue factor expression is up-regulated in chronically rejected graft (2, 3), and 3) hirudin (a specific inhibitor of thrombin) attenuates experimental rat cardiac chronic rejection (4).

Our group has recently demonstrated the presence in plasma of IgG endowed with serine protease-like activity able to hydrolyze certain coagulation factors (5). In the present study, we hypothesized that hydrolyzing IgG may reduce the uncontrolled activation of the coagulation cascade within rejected allografts and prevent the development of CAN. To address this issue, we have investigated whether hydrolyzing IgG are present in the serum of renal graft recipients and whether the hydrolyzing activity of serum IgG is associated with a decreased risk for CAN.

Materials and Methods

Study population

The present study relies on the Biobank established by the Renal Transplantation Department of the Necker Hospital (Paris, France), where biopsies and serum samples are routinely collected at regular time points for all grafted patients. For the first part of the study, we randomly selected 20 sera at various time points after renal transplantation. For the case-control study, we retrospectively selected from the database 20 patients that 1) had received a first cadaveric kidney graft and 2) displayed normal graft histology on the 3 mo posttransplantation protocol biopsy. One group (n = 10) of patients displayed chronic allograft nephropathy defined as ≥grade 2 (Banff classification) at the 2-year histological examination (group CAN), while the second group (n = 10) displayed normal biopsies (group without CAN). Clinical characteristics of patients from the two groups are presented in Table I.
followed by detection in Western blot of the biotin-labeled material (data not shown). IgG was quantified by OD measurements at 280 nm.

**Biotinylation of Ags**

Recombinant human factor VIII (FVIII; Kogenate FS; BayerHealthcare) was reconstituted in distilled water to a final concentration of 600 µg/ml, desalted by dialyzing against borate buffer (100 mM borate [pH 7.0], 150 mM NaCl, and 5 mM CaCl2). Sulfo-NHS-LC-biotin (440 µl at 25 µg/ml) was allowed to react with 600 µg of FVIII with gentle agitation in the dark for 2 h at 4°C. Biotinylated FVIII was dialyzed against catalytic buffer containing 5 mM CaCl2, for 3 h at 4°C, aliquoted, and stored at −20°C until use. The protocol was essentially identical for the biotinylation of human proteins factor IX (FIX; BeneFIX; Baxter), activated factor VII (FVIIa; NovoSeven; Novo Nordisk) and prothrombin (Kordia).

**Hydrolysis of biotinted Ags**

Biotinylated FVIII, FIX, FVIIa, and prothrombin (185 nM) were incubated in 40 µl of catalytic buffer containing 5 mM CaCl2, with the purified patients’ IgG (10 µg/ml; 66.67 nM) in the dark for 24 h at 37°C. Samples were mixed with Laemmli’s buffer without 2-ME (1:1, v/v) and 25 µl of each sample was subjected to 10% SDS-PAGE. Protein fragments were then transferred onto nitrocellulose membranes (Schleicher & Schuell Microscience). Following overnight blocking in TBS containing 0.2% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled alkaline phosphatase (KPL) diluted 1:4000 in blocking buffer, for 60 min at room temperature. After washing in TBS containing 0.1% tween-20 and TBS, labeled protein was revealed using the BCIP/NBT kit (Kirkgaard & Perry Laboratories). Blots were scanned using a SnapScan 600 (Agfa) scanner and rates of hydrolysis were calculated by densitometric analysis.

**Hydrolysis of PFR-MCA**

IgG (66.67 nM) were mixed with 100 µM PFR-MCA (Peptide) in 40 µl of catalytic buffer containing 5 mM CaCl2 (pH 7.7) in white 96-well U-bottom plates and incubated in the dark for 24 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined by the fluorescence of the leaving group (aminomethylcoumarin; λ exc 465 nm, λ em 360 nm) using a spectrofluorometer (GENios; Tecan Trading). Fluorescence values were compared with a standard curve of free MCA and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured in wells containing the substrate alone, was subtracted from the value observed in the presence of the Abs. Data are expressed as the quantity of released free MCA computed at time 0 subtracted from the quantity of released MCA computed at a given time point per amount of time per amount of IgG. The significance of the increase in the hydrolytic activity of patient IgG as compared with that of IVIg was assessed by ANOVA and Fischer post hoc tests.

**Results**

Circulating IgG of renal-transplanted patients display hydrolytic activity against coagulation factors

We purified IgG from 20 randomly selected serum samples of renal-transplanted patients and tested their ability to hydrolyze human recombinant FVIII, FIX, FVIIa, and prothrombin. The choice for FVIII, FIX, and prothrombin as proteolytic targets for catalytic IgG was motivated by their key role in the amplification loop of thrombin generation in the coagulation cascade, while FVIIa is a key molecule of the extrinsic pathway of coagulation. Absence of contamination of the IgG samples by adventitious proteases was ensured by the use of a double-step purification procedure that involves a step of purification based on affinity and a step of purification based on protein size under denaturing conditions. IgG from renal-transplanted patients were endowed with proteolytic activity. The results demonstrate an heterogeneity in the hydrolytic activity of circulating IgG. Four catalytic profiles were identified. Representative examples of these four proteolytic behaviors are provided in Fig. 1, A and B. IgG from some patients hydrolyzed both FVIII and FIX (patients P1 and P2), while IgG from other patients hydrolyzed FIX (patient P3), or FVIII (patient P4) or presented with no proteolytic activity (patient P5). IgG from transplanted patients did not display hydrolytic activity toward FVIIa and prothrombin (data not shown).

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**Table I. Characteristics of the study population**

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Group CAN (n = 10)</th>
<th>Group wCAN (n = 10)</th>
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<td>1</td>
<td>3</td>
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<td>Caucasian</td>
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<td>Age (year)</td>
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<td>3</td>
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<tr>
<td>Group wCAN</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>p</td>
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<td>nd</td>
<td>0.59</td>
<td>0.59</td>
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<table>
<thead>
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<th>Cold ischemia time (h)</th>
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<td>B</td>
<td>Dr</td>
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<tr>
<td>A</td>
<td>1.33 ± 0.50</td>
<td>1.00 ± 0.71</td>
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<tr>
<td>0.82 ± 0.75</td>
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<tr>
<td>0.10</td>
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<tr>
<td>B</td>
<td>1.00 ± 0.71</td>
<td>1.00 ± 0.71</td>
<td>0.87</td>
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<tr>
<td>0.82 ± 0.87</td>
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<tr>
<td>% of panel-reactive Ab</td>
<td>6.67 ± 13.46</td>
<td>5.46 ± 18.09</td>
<td>0.87</td>
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<td>Immunosuppressive regimen</td>
<td>Cyclosporine</td>
<td>Azathioprine</td>
<td>Sirolimus</td>
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<td>Cyclosporine</td>
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<tr>
<td>10</td>
<td>10</td>
<td>10</td>
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</tr>
</tbody>
</table>

No. of biopsy-proven rejection episodes | 0.56 ± 0.53 | 0.55 ± 0.69 | 0.97 |

* nd. Not determined. Statistic significance was assessed using one-way ANOVA.
The heterogeneous specificity of catalytic IgG from different patients precluded a systematic analysis with a single Ag hydrolysis assay. We therefore relied on a generic substrate for serine protease-like activity (7): PFR-MCA.

Incubation of patients’ IgG with the peptide PFR-MCA resulted in hydrolysis of the peptide and release of the fluorescent MCA tag. Released fluorescence allowed for the calculation of rates of hydrolysis. Hydrolysis of PFR-MCA was dose- and time-dependent (data not shown). IVIg, used as a control, demonstrated a marginal PFR-MCA-hydrolyzing activity of 0.06 mmol/min per mol consistent with our previous observations (5) (Fig. 1C).

Patients with IgG hydrolyzing both FVIII and FIX demonstrated the highest rates of hydrolysis of PFR-MCA (patients P1 and P2). Rates of PFR-MCA hydrolysis were intermediate in the case of IgG hydrolyzing either FVIII (patient P4) or FIX (patient P3) and were low for IgG that were devoid of proteolytic activity (patient P5; Fig. 1C).

Low rates of IgG-mediated hydrolysis of PFR-MCA correlate with CAN

We postulated that a high hydrolytic activity of circulating IgG against PFR-MCA may be associated with a better control of the coagulation cascade that could translate into a reduced risk for CAN.

We undertook a pilot case-control study to determine whether the level of hydrolytic activity against PFR-MCA is predictive of the occurrence of CAN. The follow-up procedure in the Renal Transplantation Department of Necker Hospital includes serum collection and graft biopsies performed at regular time points. We selected 10 patients that received a first cadaveric kidney graft and who displayed potent (grade 2 of the Banff classification (8)) CAN lesions on the biopsy performed 2 years after the transplantation (group CAN; Fig. 2B). Importantly, the possibility that histological lesions were already present in the grafts before the...
transplantation was ruled out by checking that the biopsy performed 3 mo posttransplantation was normal. It is widely accepted that both the immune response of the recipient (responsible for chronic rejection) and various non-alloantigen-dependent factors (including the direct nephrotoxicity of the drugs used as immuno-suppressive therapy) are important contributors to CAN lesions. A careful analysis relying on the criteria detailed in the recent Banff 2005 Meeting Report (9) was therefore performed to determine the relative contributions of each mechanism in the development of the lesions (Table II). Forty percent (4 of 10) of the patients in the CAN group displayed features suggestive of chronic rejection that was either cellular mediated (n = 3, Fig. 2C) or Ab mediated as assessed by the presence of circulating antidonor alloantibodies and a positive C4d staining on the biopsy (n = 1, Fig. 2D). Forty percent (4 of 10) of the patients had developed CAN lesions due to the nephrotoxicity of the calcineurin inhibitors (Fig. 2, E and F). Pathological changes attributable to both processes were evidenced in the biopsy of the two remaining patients (20%), suggesting a mixed pathophysiology.

Ten control patients with similar clinical characteristics (Table I) but normal biopsy 2 years posttransplantation were then retrieved from the database (group wCAN, Fig. 2A).

Two years after transplantation, the hydrolytic rates of circulating IgG against PFR-MCA were higher in the group devoid of lesions than in the group with CAN (6.17 ± 3.29 vs 2.52 ± 2.56 mmol/min per mol, respectively; p = 0.006). Interestingly, this difference was also observed 3 mo after transplantation (Fig. 2B; 8.94 ± 6.12 vs 3.80 ± 2.15 mmol/min per mol, respectively; p = 0.008) at a time when all patients displayed normal biopsy. These data suggest that a high hydrolytic activity of circulating IgG may be protective against CAN and that the hydrolytic rate of circulating IgG may be used as a biological marker to predict the occurrence of CAN.

**Hydrolytic activity of circulating IgG is a potential predictive marker for CAN**

The receiver operating characteristic (ROC) curve is a classical tool to evaluate the accuracy of a diagnostic test. The percentage of false-positive cases was plotted against the percentage of true-positive cases for 10 possible cutoff values using the levels of IgG-mediated PFR-MCA hydrolysis measured at 3 mo posttransplantation (Fig. 2C). Cases were patients with CAN lesions on the renal biopsy performed 2 years posttransplantation. An area under the ROC curve of 1 represents a perfect test and an area of 0.5 represents a worthless test. With an area under the ROC curve close to 0.9, the rate of IgG-mediated PFR-MCA hydrolysis exhibits excellent accuracy in predicting CAN. C. The glomerular filtration rate was estimated using the MDRD formula (25) at 3 mo, 1 year, and 2 years posttransplantation. The accuracy of the test indicated by the area under the ROC curve (AUC). An area of 1 represents a perfect test and an area of 0.5 represents a worthless test. With an area under the ROC curve close to 0.9, the rate of IgG-mediated PFR-MCA hydrolysis exhibits excellent accuracy in predicting CAN.

**FIGURE 3.** Pilot retrospective case-control study of the clinical value of PFR-MCA hydrolysis by circulating IgG in renal transplantation. A, Retrospective longitudinal case-control comparison of the rate of PFR-MCA hydrolysis by IgG from renal-transplanted patients with or without CAN. Box plot representing the distribution of the rate of PFR-MCA hydrolysis by circulating IgG collected 3 mo (left panel) or 2 years (right panel) posttransplantation from renal-transplanted patients with (CAN) or without (wCAN) CAN at 2 years. **, p < 0.01 CAN vs wCAN (Mann-Whitney U nonparametric test). B, ROC curve for PFR-MCA hydrolysis by circulating IgG was obtained by plotting the percentage of false-positive cases against the percentage of true-positive cases for 10 possible cutoff values using the rate of PFR-MCA hydrolysis of circulating IgG obtained 3 mo posttransplantation. Cases were patients with CAN on renal biopsies 2 years posttransplantation. The accuracy of the test indicated by the area under the ROC curve (AUC). An area of 1 represents a perfect test and an area of 0.5 represents a worthless test. With an area under the ROC curve close to 0.9, the rate of IgG-mediated PFR-MCA hydrolysis exhibits excellent accuracy in predicting CAN. C. The glomerular filtration rate was estimated using the MDRD formula (25) at 3 mo, 1 year, and 2 years posttransplantation. *, p < 0.05 CAN vs wCAN (one-way ANOVA). D, The proteinuria was quantified in the 24-h urine collection at 3 mo, 1 year, and 2 years posttransplantation. Log scale. *, p < 0.05 CAN vs wCAN (one-way ANOVA).
ROC curve of 0.9 demonstrated an excellent predictive value of the level of hydrolytic activity of circulating IgG for CAN.

Several biological parameters have been shown to be predictive for late renal graft failure. Among the most widely used are the estimated glomerular filtration rate (10) and the proteinuria (11). Interestingly, we found that the level of hydrolytic activity of circulating IgG had a superior predictive value of CAN when compared with these two established markers. Indeed, estimated glomerular filtration rate decline was only detectable at 2 years in the CAN group when histological changes were already patent (Fig. 3C). Proteinuria was not different in the two groups at 3 mo after transplantation and increased in the CAN group after 1 year; however, the difference between the two groups (wCAN vs CAN) failed to reach statistical significance (Fig. 3D).

Discussion
In the present study, we demonstrate that 1) circulating IgG of renal-transplanted patients are endowed with hydrolytic activity against coagulation factors VIII and IX and that 2) a high hydrolytic rate of circulating IgG correlates with a lesser prevalence of CAN. Interestingly, the difference in hydrolytic activities between patients that will develop CAN and patients that will not 2 years posttransplantation can be evidenced as early as 3 mo posttransplantation.

The presence of circulating hydrolytic IgG has been reported in various pathological situations including inflammatory, autoimmune, and infectious diseases (12–15). Their deleterious role has been suspected on the basis of their association with disease and confirmed in the case of hemophilia (16), multiple sclerosis (17), and HIV-1-related immune thrombocytopenia (18). In contrast, we have recently demonstrated that a high hydrolytic activity of circulating IgG is associated with a higher survival rate in severe sepsis (5). The present data complements our previous observations by suggesting that hydrolytic IgG may carry a protective effect against CAN.

Interestingly, although sepsis and CAN display obviously different pathophysiology, the two situations share in common a defective control of the coagulation cascade. Indeed, both sepsis and CAN are characterized by an abnormal activation of the endothelium, which is systemic in sepsis (19) and restricted to the endothelium of the graft in transplantation (20). Activation of the endothelium tilts the coagulation balance toward a hypercoagulatory state. Our study documents that hydrolytic activity of circulating IgG of some renal-transplanted patients is directed toward coagulation factors VIII and/or IX, an observation reminiscent of our findings in severe sepsis (5). Interestingly, these two factors play a central role in the amplification loop for the generation of thrombin. We have previously demonstrated that IgG-mediated FVIII hydrolysis results in inactivation of the procoagulant activity of FVIII (21). It is thus tempting to speculate that hydrolytic circulating IgG exert their preventive action against CAN by disrupting the amplification loop of the coagulation cascade, therefore counterbalancing the abnormal activation of the endothelium.

Accumulating evidence suggests that Ab play a crucial role in the pathophysiology of CAN (22). Consequently, current therapeutic strategies to treat or prevent alloantibody-mediated rejection include B cell depletion therapy in combination with plasmapheresis (23). Our data suggest that some IgG subpopulations may be endowed with a beneficial effect, i.e., regulation of the coagulation cascade by hydrolytic IgG. In the future, more efficient therapies against CAN should thus rely on a targeted control of pathogenic IgG rather than on the global elimination of circulating Ab. Interestingly, patients are heterogeneous in their ability to generate hydrolytic IgG. Experimental studies aiming at the identification of the B cells responsible for the production of hydrolytic IgG and of the factors that influence their synthesis would pave the way to therapeutic immunointervention.

As yet, no efficient therapy is available to treat CAN. Recent encouraging evidence suggests however that graft failure might be delayed through adequate management if initiated early (24), outlining the need for a reliable predictive marker to identify the patients prone to develop CAN. Our present results suggest that the rate of IgG-mediated PFR-MCA hydrolysis at 3 mo posttransplantation (i.e., at a time when graft biopsies are still normal) were significantly lower in the case of patients with CAN on the 2-year biopsies. The IgG hydrolytic activity could therefore represent a very early marker, the decrease of which would be detectable before any tissue damage is apparent. The measurement of IgG-mediated PFR-MCA hydrolysis is an affordable, simple, noninvasive test that can be done at large scale and that displays a satisfactory predictive value. A large clinical prospective study shall allow both to validate the test and to provide additional information including the sensitivity and specificity, the subgroups of patients for whom this test may be more specifically beneficial, and the optimal cutoff value.

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Disclosures
The authors have no financial conflict of interest.

References


