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POTENTIAL FOR GLOMERULAR C4D AS AN INDICATOR OF THROMBOTIC MICROANGIOPATHY IN LUPUS NEPHRITIS

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ARTHRITIS AND RHEUMATISM 2008;58:2460–2469

Abstract

OBJECTIVE In patients with systemic lupus erythematosus (SLE) and lupus nephritis, the presence of antiphospholipid antibodies (aPL) is considered to be an indication of increased risk of thrombotic microangiopathy, a serious complication of SLE. Previous studies have demonstrated a critical role for activation of the classical pathway of complement that leads to thrombotic injury in the presence of aPL. This study was undertaken to investigate whether C4d deposition in lupus nephritis is related to circulating aPL and the presence of renal microthrombi.

METHODS Deposition patterns of C4d in 44 renal biopsy samples obtained from 38 patients with biopsyproven lupus nephritis were determined by staining with a polyclonal anti-C4d antibody. A phosphotungstic acid–hematoxylin stain was used to identify fibrin microthrombi. Clinical data (serum creatinine levels and presence or absence of aPL) were obtained and correlated with findings in the renal biopsy specimens. Patients were categorized as having aPL (n = 20) or not having aPL (n = 18).

RESULTS A strong relationship between the intensity of glomerular C4d staining and the presence of microthrombi was found ($P < 0.002$). Intense glomerular C4d deposition was present in 7 of 8 biopsy samples showing renal microthrombi. Neither C4d deposition nor the presence of microthrombi was correlated with aPL status.

CONCLUSION Our findings suggest that activation of the classical pathway of complement plays a pathogenic role in the development of renal tissue injury leading to thrombosis, irrespective of the type of circulating antibodies present. Immunodetection of glomerular C4d deposition in renal biopsy samples could be a convenient method of identifying patients at risk of thrombotic microangiopathy.

Introduction

Lupus nephritis develops in up to 60% of patients with systemic lupus erythematosus (SLE) during the course of the disease.[1] Standard clinical practice is to perform a renal biopsy if clinical parameters suggest renal involvement; the lupus nephritis class[2] can be determined from the biopsy and has an impact on both the choice of treatment and the patient's prognosis. In renal biopsy specimens obtained from SLE patients, evidence of thrombotic microangiopathy is occasionally detected.

Although the occurrence of thrombotic microangiopathy in patients with SLE is rare, about 3% according to various studies[3-6], clinicians are well aware of the serious complications. In its most fulminant form, thrombotic microangiopathy presents with multiple fibrin thrombi in glomeruli and/or arterioles and capillaries and is accompanied by a severe decline in renal function.[7] SLE patients with thrombotic microangiopathy also exhibit extrarenal complications, including thrombocytopenia, microangiopathic hemolytic anemia, neurologic manifestations, and even multiple organ failure.[8]

Thrombotic microangiopathy in SLE patients is typically associated with the presence of antiphospholipid antibodies (aPL), namely anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC).[8-11] However, aPL are detected in up to 40% of SLE patients[12-14], and in many patients with high aPL titers thrombotic microangiopathy never develops[4;10] Conversely, thrombotic microangiopathy has been reported in several SLE patients without aPL.[8] These observations are consistent with suggestions that other factors are necessary to trigger the endothelial damage leading to thrombotic events.

Recently, the role of complement in aPL-induced thrombosis has been thoroughly investigated. Pierangeli *et al* have shown that activation of the complement cascade is necessary for aPL-mediated thrombophilia.[15-16] In addition, Navratil *et al*[17] reported that platelet-bound C4d, a sensitive marker for the classical pathway of complement activation, is highly specific (though not sensitive) for the presence of aPL in SLE patients. Those investigators suggested that platelet-bound C4d may provide clues to the pathogenic mechanisms responsible for the thrombotic and vascular complications of SLE.[17]

C4d is a marker of humoral rejection in renal transplant biopsy samples, and its presence can be interpreted as a footprint of the classical pathway of complement activation and thus of alloantibody-induced damage.[18] In the transplanted kidney, humoral rejection is associated with glomerular and interstitial changes, in which thrombotic lesions may also occur.[19] A mechanism similar to humoral rejection possibly occurs in SLE, in which many different autoantibodies produce an analogous situation. Few reports have described C4d depositions in nontransplanted kidneys.

C4d positivity has been reported in membranous nephropathy and membranoproliferative glomerulonephritis and even in normal kidneys, suggesting that C4d positivity need not always be representative of activation of the classical pathway of complement.[20] C4d was detected by Kim and Jeong [21] in glomeruli in 21 cases of lupus nephritis, and the intensity of C4d staining correlated with the extent of immune complex depositions. In that study, the aPL status of the patients was unknown, and the presence of microthrombi was not investigated.

The recent finding of C4d expression on platelets from SLE patients[17], as well as the results of recent work on complement- and aPL-mediated thrombosis, support the notion of a possible role of C4d in the pathogenesis of thrombosis in SLE. We hypothesized that immunodetection of C4d in renal biopsy samples could facilitate detecting SLE patients at risk of the development of thrombotic complications. In the present study, we investigated the relationship between C4d deposition in renal biopsy samples, the presence of microthrombi in biopsy samples, and aPL status in patients with lupus nephritis.

Patients and methods

PATIENTS We retrospectively studied 38 patients with clinically evident SLE who visited the outpatient clinic at the Department of Nephrology and Internal Medicine of the Leiden University Medical Center between 1983 and 2006. All patients met at least 4 of the American College of Rheumatology criteria for SLE[22], and lupus nephritis was confirmed in all patients by ▸1 renal biopsy. The biopsy specimens were reevaluated and classified according to the most recent modification of the World Health Organization classification from the International Society of Nephrology/Renal Pathology Society (ISN/RPS).[2] Follow-up biopsy samples were available for 4 patients. Three of these patients had 2 biopsies, and 1 patient had 4 biopsies. The medical records of all patients were reviewed independently of the analysis of pathologic features. Most clinical data were available, although some were missing for the older cases. All patients were investigated for the presence of aPL as defined by the Sapporo criteria for antiphospholipid syndrome (APS).[23] In the present study, patients were categorized as having aPL (n = 20) or not having aPL (n = 18). An overview of the clinical data is given in Table 1.

BIOPSY SAMPLES Biopsy samples were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections (4 micrometer thick) were placed on positively charged slides and kept in a stove at 60°C for 1 hour. Sections were deparaffinized and rehydrated through a series of xylene and graded alcohols. Endogenous peroxidase was blocked in 3% H_2O_2 for 30 minutes. Antigen retrieval was performed by boiling the slides in a microwave at 1,000W for 10 minutes in 10 mM citrate buffer (pH 6.0).

The primary rabbit anti-human C4d polyclonal antibody (BI-RC4d; Biomedica Gruppe, Vienna, Austria) was applied at a dilution of 1:50 in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS), and slides were incubated overnight at room temperature. The slides were then incubated with anti-rabbit EnVision (K5007; DakoCytomation,Glostrup, Denmark) for 30 minutes, and staining was visualized with the Vector Nova Red Substrate Kit (SK-4800; Vector, Peterborough, UK). Sections were washed with PBS (pH 7.4) between each step (3 times for 5 minutes each time). Finally, sections were counterstained with Mayer's hematoxylin, air-dried, cleared in xylene, and coverslipped. A renal specimen from a humoral allograft rejection patient with C4d-positive staining confirmed by immunofluorescence was used as a positive control. Consecutive sections were stained for fibrin with Mallory's phosphotungstic acid–hematoxylin. Fresh frozen tissue sections were incubated with fluorescein isothiocyanate–conjugated polyclonal antibodies directed against human IgA, IgG, IgM, C3, and C1q (Dako).

QUANTIFICATION OF MORPHOLOGY, IMMUNOHISTOCHEMICAL STAINING, HISTOCHEMICAL STAINING, AND IMMUNOFLUORESCENCE The biopsy specimens were scored by a renal pathologist (IMB) who had no prior knowledge of the clinical and laboratory findings in the patients. Peritubular capillary C4d staining was scored as 0 or 1, where 0 represented the absence of C4d in peritubular capillaries, and 1 represented positive peritubular capillary C4d staining in any area of the biopsy sample. C4d positivity in arterioles was scored as 0 or 1, where 0 represented the absence of C4d, and 1 represented the presence of C4d.

Glomerular C4d staining was scored semiquantitatively using the following scoring system: 0 (no glomerular staining), 1 (mild to moderate glomerular staining), and 2 (intense glomerular staining). Typical examples of the different intensities of glomerular C4d staining are shown in Figures 1A–C. Consecutive sections were stained with phosphotungstic acid–hematoxylin and carefully examined for the presence of arterial and/or arteriolar thrombosis and glomerular microthrombi.

A glomerular microthrombus stained with phosphotungstic acid–hematoxylin is shown in Figure 1D. Positive identification of at least 1 microthrombus, either in glomeruli, interstitial capillaries, or small arterioles, was scored as 1. Absence of microthrombi was scored as 0. Intensity of glomerular staining for IgG, IgA, IgM, C3, and C1q was semiquantitatively scored as 0, 1, or 2.

LABORATORY EVALUATION Standard methods were used to determine the levels of antinuclear, anti-DNA, antiextractable nuclear antigen, and anti-C1q antibodies. C3, C4, and C1q levels were also determined by standard protocols. Levels of IgG and IgM aCL were determined using the Varelisa Cardiolipin Antibodies enzyme-linked immunosorbent assay kit (Phadia, Nieuwegein, The Netherlands). Laboratory diagnosis of LAC was performed in citrated plasma prepared by double centrifugation. Two different phospholipid-dependent coagulation assays were used. An activated partial thromboplastin time (APTT) was measured, and, if the APTT was prolonged, patient plasma was mixed with normal plasma (automated APTT reagent; BioMerieux, Marcy l'Etoile, France) at a 1:1 ratio to test for the presence of an inhibitor. Persistence of a prolonged APTT clotting time indicated the presence of an inhibitor. Furthermore, the presence of LAC was tested using a dilute Russell's viper venom time (dRVVT)–based assay (LA screen/LA Confirm; Life Diagnostics, Frenchs Forest, Australia). If the dRVVT obtained using the LA Screen reagent in the patient sample was prolonged ‣20% compared with normal plasma, studies with normal plasma were performed to exclude possible clotting factor deficiencies. If the dRVVT remained prolonged after mixing, then the phospholipid dependency of the possible inhibitor was tested with the LA Confirm reagent, which contains a high phospholipid concentration. Normalization of dRVVT with the LA Confirm reagent confirmed the presence of LAC.

CLINICAL FOLLOW-UP The glomerular filtration rate (GFR) was estimated for all patients at the time of biopsy, as well as during yearly followup visits, using the Modification of Diet in Renal Disease (MDRD) formula.[24] Followup data were used to investigate whether renal biopsy findings at presentation were related to renal outcome. Data regarding the end points of occurrence of end-stage renal failure requiring dialysis and the date and cause of death were obtained from medical records.

STATISTICAL ANALYSIS Categorical variables were compared using Fisher's exact test or the chi-square test. For the statistical analysis of immunofluorescence data, we used the nonparametric Spearman's rank correlation. For the analysis of creatinine level and GFR, we used the Kruskal-Wallis one-way analysis of variance (ANOVA). All analyses were performed using SPSS software, version 12.0.1 (SPSS, Chicago, IL). P values less than 0.05 were considered significant.

Results

Relationships between the presence of aPL, the presence of microthrombi, and C4d staining. Forty-four renal biopsy samples from 38 patients with lupus nephritis were examined. For patients with multiple biopsy samples, only the first biopsy sample in which lupus nephritis was diagnosed was analyzed. The mean (+/-SD) age of patients at the time of biopsy was 31.9 +/- 11.2 years (range 14–66 years). Twenty patients (52.6%) were positive for aPL, according to the Sapporo criteria. IgG aCL was present in 17 of 36 patients (47%), and LAC was present in 10 of 31 patients (32%). A significant relationship was found between the presence of microthrombi and the intensity of glomerular C4d staining. In 15 renal biopsy samples, intense glomerular C4d staining was observed. Of these biopsy samples, 7 had microthrombi, whereas microthrombi were absent in almost all other biopsy samples (P ‹0.002, by Fisher's exact test) (Table 2). Within the renal tissue specimens, microthrombi were found almost exclusively in glomeruli. In some cases, microthrombi were also found in the interstitial arterioles and capillaries. Thrombi were typically not found in areas of active inflammation. Among the 8 patients with biopsy specimens showing microthrombi, 5 were positive for aPL, compared with 15 of 30 patients without microthrombi. This difference was not statistically significant.

Neither the intensity of glomerular C4d staining nor the deposition of C4d in peritubular capillaries was related to aPL status (Table 3). Glomerular C4d staining and C4d in peritubular capillaries were

not related to the presence of aCL or LAC alone, or to the ISN/RPS classification for lupus nephritis. C4d positivity in arterioles occurred in only 3 patients, and none of these arterioles contained thrombi. Relationship between glomerular C4d staining and the presence of antibodies (IgG, IgA, IgM), C3, and C1q. Table 4 shows the relationship between immune complex deposits, glomerular C4d, and aPL. Consistent with results previously reported by Kim and Jeong[21], we found that increased intensity of C4d deposition was significantly correlated with the presence of capillary C3 (r = 0.348, P = 0.023). We also found a nearly significant correlation between the intensity of C4d staining and the presence of capillary IgG (r = 0.316, P = 0.064). There was also a nearly significant correlation between the intensity of C4d staining and the presence of aPL(r = 0.315, P = 0.054).

RESULTS OF CLINICAL FOLLOW-UP The serum creatinine level and the GFR were available for all patients over at least 3 years of followup and for some patients for up to 15 years of followup. Of the 38 patients, 4 died before the study started, and of those, 3 had developed endstage renal failure requiring dialysis. Of the 3 patients who died with end-stage renal failure, 2 had biopsies showing renal microthrombi and intense C4d staining.

Two other patients had developed end-stage renal failure by the time our study started. In these patients, renal biopsy did not show microthrombi, but in 1 patient the biopsy did show intense C4d staining. The mean (+/-SD) GFR after 3 years of followup was 73 +/- 38 ml/minute in the group with no C4d staining (n = 5), 68 +/- 22 ml/minute in the group with mild C4d staining (n = 18), and 62 +/- 23 ml/minute in the group with intense staining (n = 15). There were no statically significant differences between these 3 groups (P = 0.34 by Kruskal-Wallis one-way ANOVA).

FOLLOW-UP BIOPSY FINDINGS Multiple biopsy samples were available for 4 patients. Three patients each had 2 biopsies, and 1 patient had 4 biopsies. Follow-up biopsies showed consistently positive or negative staining in peritubular capillaries (3 of 4 patients had all positive results, while 1 patient had no peritubular capillary staining in either sample). Likewise, followup biopsies showed equally intense glomerular C4d staining at all time points. The patient with 4 biopsy samples showed both intense glomerular C4d staining and positive peritubular capillaries in all biopsy samples (Figure 2). In the diagnostic evaluation for this patient, microthrombi were first detected on the third biopsy. Additional stainings made for the purpose of the present study showed that microthrombi were also present in the first biopsy sample. During the course of the disease, results of tests for aCL and LAC were consistently negative. Because the clinical history of this patient was very illustrative of our results regarding the combination of intense glomerular C4d staining with the presence of microthrombi, we briefly present the case history here.

CASE HISTORY The patient, a 27-year-old woman, presented with butterfly exanthema, alopecia, and renal involvement with proteinuria and active urinary sediment, and SLE was diagnosed. Positive antinuclear factor and anti–double-stranded DNA antibodies were present, and no aPL were found. The first renal biopsy specimen obtained indicated lupus nephritis class V, and prednisone therapy and azathioprine were initiated. At age 28 years, a second renal biopsy specimen obtained, showing a diffuse proliferative glomerulonephritis compatible with lupus nephritis class IV (A/G). Additionally, renal vein thrombosis developed, but the patient was still negative for aPL. She was treated with vitamin K antagonists as anticoagulant therapy. Pulse cyclophosphamide (CYC) was administered, and the patient's condition stabilized. Because aPL did not seem to play a role, anticoagulant therapy was terminated after 2 years. Because the patient's renal function continued to decline during the following 2 years, and proteinuria continued to increase during pulse CYC and prednisone therapy, a third renal biopsy specimen was obtained. Intra- and extracapillary proliferative lupus nephritis class IV (A/C) and evidence of a thrombotic microangiopathy were found. Shortly thereafter,

the patient presented with upper body ataxia and aphasia. These neurologic complications were most likely associated with the thrombotic microangiopathy in combination with active SLE. The patient remained negative for aPL, but complement values were very low. She was treated with 10 plasmapheresis sessions and hemodialysis, and her GFR declined to 15 mmoles/liter. At age 39 years, prolonged hemodialysis was started. Two years later, acute neurologic deterioration and respiratory distress occurred, and the patient was admitted to the intensive care unit, where she died, likely due to diffuse alveolar hemorrhage in the lungs and bleeding in and around multiple organs during active lupus nephritis. At autopsy, the kidneys showed extensive chronic damage, classified as lupus nephritis class IV-C (G). In the brain, diffuse chronic and global ischemia was detected, accompanied by a few vessels in the white matter with intraluminal thrombi and extensive cerebral gliosis. For the present study, all of the kidney biopsy specimens obtained from the patient were stained for C4d, and all of them were positive for similar high-intensity glomerular C4d staining (Figure 2).

Discussion

We observed a striking relationship between the intensity of glomerular C4d deposition and the presence of renal microthrombi in lupus nephritis. This report describes the novel finding of the presence of C4d in lesions indicative of thrombotic microangiopathy in nontransplant biopsy specimens. It is likely that activation of the classical pathway of complement is a crucial pathogenic intermediate in the development of thrombosis in lupus nephritis. In the group of patients included in the present study, there was no definitive relationship between C4d deposition and the presence of aPL. The presence of aPL was not correlated with the presence of microthrombi. This suggests that additional factors, such as other autoantibodies that have not yet been identified and assessed, may trigger activation of the classical pathway of complement in these patients. Although its function is closely related to the classical pathway, C4d is also involved in the lectin pathway. Therefore, we cannot exclude the possibility that C4d deposition partly reflects activation of the lectin pathway due to, for example, IgM autoantibodies.[25]

The case report presented in this study illustrates that C4d deposition can be found in the kidney years prior to the time when actual thrombotic microangiopathy is revealed. Regarding the clinical outcome in the patients in the present study, there appeared to be a trend toward a less favorable renal outcome in the group of patients whose biopsy samples showed more intense C4d staining. However, this trend did not reach statistical significance. Of the 5 patients in whom end-stage renal failure developed, 2 had renal microthrombi and 3 had intense C4d staining, suggesting that the presence of intense C4d staining may be related to a less favorable clinical outcome. Based on the findings of our study, positive C4d staining in a renal biopsy sample from a patient with lupus nephritis should raise suspicion of thrombotic complications, even in the absence of aPL. However, further studies are needed to define the clinical implications of C4d deposition as a biopsy finding, because in this retrospective study, our results were obviously biased by several factors that were uncontrolled, such as ongoing disease activity at the time of measurement and co-medication. Our findings therefore indicate the need for future prospective and controlled studies in larger groups of patients, in order to investigate the potential of C4d staining in the renal biopsy specimen to predict the serious clinical complication of thrombotic microangiopathy in SLE and APS.

Our study was initiated by the results of the recent study by Navratil *et al*[17], which showed a specific relationship between platelet C4d and the presence of aPL in SLE. Platelet C4d was detected in 18% of 105 patients with SLE, and was 100% specific for a diagnosis of SLE compared with healthy controls, and 98% specific for SLE compared with patients with other diseases. The authors suggested that, apart from indicating that platelet-bound C4d may be a biomarker for SLE, their findings provided a clue to the pathogenic mechanisms responsible for the myriad thrombotic and vascular complications

of lupus associated with aPL. Thus far, the exact role of aPL in the development of lupus-related thrombotic microangiopathy has not been elucidated. The ability of aPL to activate endothelial cells in vitro and in vivo, induce platelet activation, and interact with elements of the coagulation cascade has been well established.[26] These findings indicate that aPL seem to play an important role in the initial phase of a cascade leading to thrombotic events. In addition to this, many studies have shown complement activation to be essential. The detrimental role of complement is underlined in experimental studies, such as that by Nangaku *et al*[27], demonstrating that thrombotic microangiopathy is prevented if C5b–9 (the membrane attack complex) is temporarily inhibited. Pierangeli *et al*[15] demonstrated that C3- and C5-deficient mice were resistant to aPL-induced thrombosis. How would complement activation result in a thrombotic event? A novel finding is the role of tissue factor activation as an inducer of thrombosis downstream of complement. It was recently demonstrated that aPL-induced complement activation and downstream signaling via C5a receptors in neutrophils lead to the induction of tissue factor, a key initiating component of the blood coagulation cascade[28]. Another study identified tissue factor as an important mediator of the C5a-induced oxidative burst in neutrophils in the setting of aPL-induced fetal injury[29]. These findings are interesting in light of our results, since they may explain why such a strong relationship was found between the presence of C4d and microthrombi in this study. In patients with SLE, APS may become clinically apparent only after a major thrombotic event. In many hospitals, patients with SLE are not routinely tested for aPL but are tested only 'on clinical indication.' In some instances, the detection of microthrombi in the renal biopsy specimen is the first clue that thrombotic microangiopathy is present. However, the detection of microthrombi in renal biopsy specimens is not very sensitive, which is likely due to sampling error.

We have illustrated the difficulty of identifying and managing thrombotic microangiopathy in an SLE patient in the case history presented in this study. In this patient, the presence of thrombotic microangiopathy became evident fairly late in the clinical course, namely when a microthrombus was detected on the third renal biopsy specimen, which was obtained 7 years after disease presentation. Earlier tests for aPL were negative, which contributed largely to the decision to stop oral anticoagulant therapy after renal vein thrombosis. The patient experienced severe renal and neurologic complications, which were confirmed at autopsy to have been caused by microthrombi. In this patient, C4d staining was notably intense in all biopsy samples, as shown in Figure 2, and could have indicated the risk of a thrombotic microangiopathy much earlier in the time course of the disease. Our results further suggest the potential for C4d staining in guiding the therapeutic strategy, such as the earlier and prolonged use of immunosuppressive therapy or anticoagulant therapy. Recently, immunoreactivity to C4d protein was reported to be significantly stronger in the placentas of patients with aPL than in the placentas of healthy controls.[30] These findings are consistent with the results of murine studies by Girardi *et al*[31; 32], indicating that low molecular weight heparin, even at doses that do not interfere with coagulation, protects pregnancies against aPL-induced damage mor thrombosis, because it blocks the activation of complement. Interestingly, anticoagulant treatment without any anticomplement effect was not protective, suggesting that anticoagulation in and of itself is not sufficient for patients with APS-related miscarriage. If complement is indeed a critical factor in the development of endothelial damage and microthrombi in kidneys of patients with SLE and/or APS, the possibility that treatment with heparin at subcoagulant doses could have beneficial effects in these patients should be investigated. Notably, the patient described in the case history presented in this study was treated with vitamin K antagonists as anticoagulant therapy after renal vein thrombosis and received several plasmapheresis sessions when thrombotic microangiopathy was discovered, but this therapeutic intervention was not successful. She never received heparin during the course of her disease.

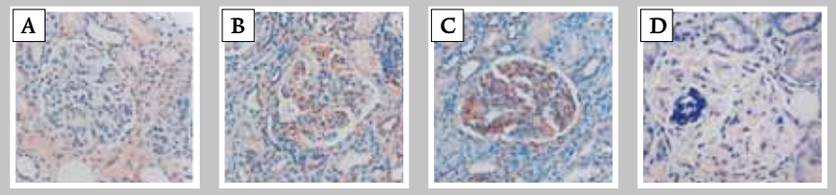
A shortcoming of the present study regards the detection of microthrombi in renal biopsy specimens. Given their relatively sparse

presence, it is likely that microthrombi are easily missed in the small tissue sample obtained at renal biopsy. Therefore, we cannot exclude the possibility that renal microthrombi were missed due to sampling error in the 8 patients whose biopsy samples showed intense glomerular C4d staining but no thrombotic lesions when stained with phosphotungstic acid–hematoxylin. It is also possible that some focal microthrombi were missed in patients whose biopsy samples showed only mild or no staining for C4d.

In this study, only 1 patient had renal microthrombi in the absence of C4d deposition. In future studies, more patients should be included, and thrombotic microangiopathy should be investigated more closely, by examining more biopsy slides or by taking into account other clinical parameters indicative of thrombotic microangiopathy.

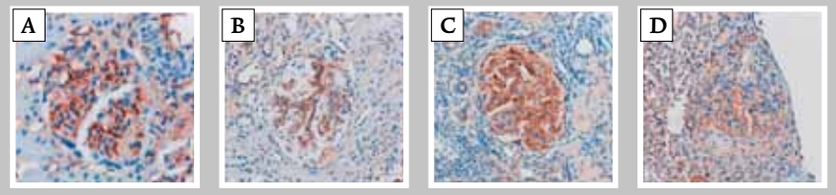
Importantly, not all patients with SLE and aPL develop thrombotic microangiopathy. According to Harris and Pierangeli[33], a 'second hit,' e.g., infection or trauma, is necessary to trigger thrombosis at the vessel site where aPL have deposited. Conversely, not all patients with SLE and thrombotic microangiopathy have aPL. This suggests that the deposition of antibodies other than aPL may lead to a thrombotic event, or that aPL are only transiently present in some SLE patients. Although the etiology of neither situation is completely understood, it seems wise to look at complement deposition, and deposition of C4d in particular, in all patients with lupus nephritis. Because activation of the classical pathway of complement is likely to be a marker of endothelial damage leading to thrombotic microangiopathy in lupus nephritis, C4d immunodetection may be a useful tool in determining whether patients are at risk of this complication. In our experience, the serologic detection of aPL is only marginally related to evidence of microthrombi in the kidney. Of 8 patients with microthrombi, aPL were found in 5, and of 30 patients without microthrombi, aPL were present in 15. This difference was not statistically significant. The results of the present study strongly support the notion that activation of the classical pathway of complement is a crucial factor in the development of thrombosis in lupus nephritis. Furthermore, we propose that C4d may be an important additional tool in the evaluation of renal biopsy specimens obtained from patients with SLE. Future prospective studies are needed to investigate this possibility. Our findings also have therapeutic implications, in that staining renal biopsy specimens for C4d could be an easy and elegant method of identifying patients with lupus nephritis who are at risk of thrombotic microangiopathy.

FIG 1 DIFFERENT INTENSITIES OF GLOMERULAR C4D STAINING IN LUPUS NEPHRITIS



Renal biopsy samples obtained from patients with lupus nephritis, showing different intensities of glomerular C4d staining and a glomerular microthrombus. A, No glomerular C4d staining. B, Mild to moderate glomerular C4d staining. C, Intense glomerular C4d staining. D, A glomerular microthrombus stained bright blue with a phosphotungstic acid–hematoxylin stain for fibrin. (Original magnification X400.)

FIG 2 FOUR BIOPSY SAMPLES OF ONE PATIENT



Four renal biopsy samples obtained from a patient with lupus nephritis complicated by a thrombotic microangiopathy during the disease course. The patient was negative for antiphospholipid antibodies. Glomerular C4d staining intensity was uniformly strong in all biopsy samples. (A) First biopsy sample in which lupus nephritis was diagnosed. Microthrombi were not detected. (B) Biopsy sample obtained after development of renal vein thrombosis and a decline in renal function. (C) Biopsy sample showing an intensely stained glomerulus. Thrombotic microangiopathy was diagnosed, and neurologic complications developed. (D) Biopsy sample obtained at autopsy, showing extensive chronic damage. C4d deposition was still detectable. (Original magnification X400.)

TABLE 1 CLINICAL CHARACTERISTICS AND LABORATORY FINDINGS IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

Age at biopsy, mean ± SD (range) years	31.9 ± 11.2 (14–66)
Sex, no. female	35
ISN/RPS class for lupus nephritis, no. of samples†	
I	0
II	1
III	9
IV	24
V	2
VI	0
Unclassified	2
Immunologic disorders, no. (%) positive‡	
Antiphospholipid antibody disorders§	20 (53)
Anticardiolipin antibodies, IgG	17 (47)
Anticardiolipin antibodies, IgM	11 (32)
Lupus anticoagulant	10 (32)
Antinuclear antibodies	34 (90)
Anti-EMA antibodies	22 (59)
Anti-dsDNA antibodies	28 (74)
Anti-C1q antibodies	8 (33)
Serum creatinine level, median (range) μmoles/liter	95.0 (56–485)
GFR, median (range) μmoles/liter	70.1 (9.6–119.4)
Serum C3 level, median (range) μmoles/liter	45.5 (1–115)
Serum C4 level, median (range) μmoles/liter	17.0 (2–46)
Serum C1q level, median (range) μmoles/liter	11.5 (1–21)

* ISN/RPS = International Society of Nephrology/Renal Pathology Society; anti-dsDNA = anti–double-stranded DNA; GFR = glomerular filtration rate.

† Subclasses are not shown.

‡ IgG anticardiolipin antibodies were tested in 36 patients; IgM anticardiolipin antibodies were tested in 34 patients; lupus anticoagulant was tested in 31 patients; anti–extractable nuclear antigen (anti-ENA) antibodies were tested in 37 patients; and anti-C1q antibodies were tested in 24 patients.

§ Defined by the Sapporo criteria, specifically by the presence of IgG anticardiolipin antibody or lupus anticoagulant

TABLE 2 RELATIONSHIP OF GLOMERULAR C4D STAINING TO THE PRESENCE OF MICROTHROMBI IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

NO DETECTABLE MICROTHROMBI (N = 30)	MICROTHROMBI PRESENT (N = 8)	TOTAL (N = 38)
4	1	5
18	0	18
8	7†	15

* Values are the number of patients. P ◂ 0.002 for the presence of microthrombi in samples with intense staining versus samples with no staining or mild to moderate staining.

TABLE 3 RELATIONSHIP OF C4D STAINING AND PRESENCE OF MICROTHROMBI TO APL STATUS IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

	PATIENTS WITH APL (N = 20)	PATIENTS WITHOUT APL (N = 18)
C4d peritubular capillary staining		
Positive	10	12
Negative	10	6
C4d glomerular staining		
No staining	1	4
Mild to moderate staining	9	9
Intense staining	10	5
Presence of microthrombi		
Microthrombi present	5	3
No detectable microthrombi	15	15

* Values are the number of patients. APL = antiphospholipid antibodies

TABLE 4 RELATIONSHIP BETWEEN IMMUNE COMPLEX DEPOSITS, GLOMERULAR C4D STAINING, AND THE PRESENCE OF ANTIPHOSPHOLIPID ANTIBODIES IN THE 38 PATIENTS WITH LUPUS NEPHRITIS*

PATIENT	GLOMERULAR C4D STAINING	APL	ARTERIOLAR C4D	MICRO-THROMBI	IGG	IGM	IGA	C3	C1Q
1	0	0	0	0					
2	0	0	0	0					
3	0	0	0	1	2	2	2	2	2
4	0	0	0	0	2	1	1	2	1
5	0	1	0	0	1	1	2	1	2
6	1	0	0	0	1	1	0	2	1
7	1	0	0	0	1	0	1	0	0
8	1	0	0	0	1	2	2	2	2
9	1	0	0	0	1	1	1	0	1
10	1	0	0	0	1	2	1	1	2
11	1	1	0	0	1	1	1	1	1
12	1	1	0	0	2	2	1	1	1
13	1	1	0	0	1	1	1	1	1
14	1	0	0	0	1	1	0	1	0
15	1	0	0	0	0	1	0	1	1
16	1	0	0	0	2	2	1	2	2
17	1	0	0	0	2	1	1	2	1
18	1	1	0	0	0	1	1	1	1
19	1	1	0	0	2	2	1	2	2
20	1	1	0	0	1	1	2	2	2
21	1	1	0	0					
22	1	1	1	0	1	1	1	1	1
23	1	1	0	0	1	1	1	1	1
24	2	0	0	1	2	2	1	2	0
25	2	0	0	0	1	1	1	1	1
26	2	0	0	0	2	2	2	2	2
27	2	0	0	1	2	2	1	2	2
28	2	0	1	0	2	1	0	2	1
29	2	1	0	1	2	2	2	2	2
30	2	1	0	0	0	1	1	1	1
31	2	1	0	0	1	0	2	2	1
32	2	1	0	1	2	2	2	2	2
33	2	1	0	0	2	2	1	2	2
34	2	1	0	0	2	2	1	2	2
35	2	1	0	1	2	2	2	2	2
36	2	1	1	1	2	0	1	2	2
37	2	1	0	1	2	2	1	2	2
38	2	1	0	0	1	2	2	1	2

* Values are the score. Glomerular C4d staining of each sample was scored on a scale of 0–2, where 0 = no glomerul ar staining, 1 = mild to moderate glomerular staining, and 2 = intense glomerular staining. Samples were given a score of 1 or 0 for the presence or absence, respectively, of antiphospholipid antibodies (aPL), arteriolar C4d, and microthrombi. The intensity of immunofluorescence for IgG, IgM, IgA, C3, and C1q was scored on a scale of 0–2. Data on IgG IgM, IgA, C3, and C1q were not available for patients 1, 2, and 21.

REFERENCES

1 Cameron JS. Lupus nephritis. J Am Soc Nephrol 1999;10:413–24.

2 Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited [published erratum appears in J Am Soc Nephrol 2004;15:835–6]. J Am Soc Nephrol 2004;15:241–50.

3 Descombes E, Droz D, Drouet L, Grunfeld JP, Lesavre P. Renal vascular lesions in lupus nephritis. Medicine (Baltimore) 1997;76:355–68.

4 Appel GB, Pirani CL, D'Agati V. Renal vascular complications of systemic lupus erythematosus. J Am Soc Nephrol 1994;4:1 499–515.

5 Grishman E, Venkataseshan VS. Vascular lesions in lupus nephritis. Mod Pathol 1988;1:235–41.

6 Magil AB, McFadden D, Rae A. Lupus glomerulonephritis with thrombotic microangiopathy. Hum Pathol 1986;17:192–4.

7 Bridoux F, Vrtovsnik F, Noel C, Saunier P, Mougenot B, Lemaitre V, *et al.* Renal thrombotic microangiopathy in systemic lupus erythematosus: clinical correlations and long-term renal survival [published erratum appears in Nephrol Dial Transplant 1998;13: 1328]. Nephrol Dial Transplant 1998;13:298–304.

8 Jain R, Chartash E, Susin M, Furie R. Systemic lupus erythematosus complicated by thrombotic microangiopathy. Semin Arthritis Rheum 1994;24:173–82.

9 Hughson MD, Nadasdy T, McCarty GA, Sholer C, Min KW, Silva F. Renal thrombotic microangiopathy in patients with systemic lupus erythematosus and the antiphospholipid syndrome. Am J Kidney Dis 1992;20:150–8.

10 Tektonidou MG, Sotsiou F, Nakopoulou L, Vlachoyiannopoulos PG, Moutsopoulos HM. Antiphospholipid syndrome nephropathy in patients with systemic lupus erythematosus and antiphospholipid antibodies: prevalence, clinical associations, and long-term outcome. Arthritis Rheum 2004;50:2569–79.

11 Nochy D, Daugas E, Huong DL, Piette JC, Hill G. Kidney involvement in the antiphospholipid syndrome. J Autoimmun 2000;15:127–32.

12 Cervera R, Piette JC, Font J, Khamashta MA, Shoenfeld Y, Camps MT, *et al*, for the Euro-Phospholipid Project Group. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000patients. Arthritis Rheum 2002;46:1019–27.

13 Hughes GR. The antiphospholipid syndrome: ten years on. Lancet 1993;342:341–4.

14 Alarcon-Segovia D, Deleze M, Oria CV, Sanchez-Guerrero J, Gomez-Pacheco L, Cabiedes J, *et al.* Antiphospholipid antibodies and the antiphospholipid syndrome in systemic lupus erythematosus: a prospective analysis of 500 consecutive patients. Medicine (Baltimore) 1989;68:353–65.

15 Pierangeli SS, Girardi G, Vega-Ostertag M, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody–mediated thrombophilia. Arthritis Rheum 2005;52:2120–4.

16 Pierangeli SS, Chen PP, Gonzalez EB. Antiphospholipid antibodies and the antiphospholipid syndrome: an update on treatment and pathogenic mechanisms. Curr Opin Hematol 2006;13:366–75.

17 Navratil JS, Manzi S, Kao AH, Krishnaswami S, Liu CC, Ruffing MJ, *et al.* Platelet C4d is highly specific for systemic lupus erythematosus. Arthritis Rheum 2006;54:670–4.

18 Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N, *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. J Am Soc Nephrol 1999;10:2208–14.

19 Crespo M, Pascual M, Tolkoff-Rubin N, Mauiyyedi S, Collins AB, Fitzpatrick D, *et al.* Acute humoral rejection in renal allograft recipients. I. Incidence, serology and clinical characteristics. Transplantation 2001;71:652–8.

20 Zwirner J, Felber E, Herzog V, Riethmuller G, Feucht HE. Classical pathway of complement activation in normal and diseased human glomeruli. Kidney Int 1989;36:1069–77.

21 Kim SH, Jeong HJ. Glomerular C4d deposition indicates in situ classic complement pathway activation, but is not a marker for lupus nephritis activity. Yonsei Med J 2003;44:75–80.

22 Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.

23 Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, *et al.* International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop [published erratum appears in Arthritis Rheum 1999;42:1997]. Arthritis Rheum 1999;42:1309–11.

24 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D, for the Modification of Diet in Renal Disease Study Group. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Ann Intern Med 1999;130: 461–70.

25 McMullen ME, Hart ML, Walsh MC, Buras J, Takahashi K, Stahl GL. Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. Immunobiology 2006; 211:759–66.

26 Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GL, Harris EN. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 1999;99:1997–2002.

27 Nangaku M, Alpers CE, Pippin J, Shankland SJ, Kurokawa K, Adler S, *et al.* CD59 protects glomerular endothelial cells from immune mediated thrombotic microangiopathy (TMA). J Am Soc Nephrol 1997;8:590–7.

28 Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, *et al.* A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J Immunol 2006; 177:4794–802.

29 Redecha P, Tilley R, Tencati M, Salmon JE, Kirchhofer D, Mackman N, *et al.* Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. Blood 2007;110:2423–31.

30 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. Am J Obstet Gynecol 2007;196:167–5.

31 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat Med 2004;10:1222–6.

32 Salmon JE, Girardi G. Antiphospholipid antibodies and pregnancy loss: a disorder of inflammation. J Reprod Immunol 2008;77:51–6.

33 Harris EN, Pierangeli SS. Primary, secondary, catastrophic antiphospholipid syndrome: is there a difference? Thromb Res 2004;114:357–61.