Platelet C4d Is Associated With Acute Ischemic Stroke and Stroke Severity

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Background and Purpose—Platelets bearing complement C4d were recently reported to be 99% specific for a diagnosis of systemic lupus erythematosus (SLE) and associated with neuropsychiatric lupus. We compared the prevalence of platelet C4d and investigated the clinical associations of platelet C4d in patients with acute ischemic stroke.

Methods—We recruited 80 patients hospitalized for acute ischemic stroke. Stroke severity was measured by the National Institutes of Health Stroke Scale (NIH-SS). Infarct volume was determined by MRI. Platelet C4d was measured by flow cytometry.

Results—Mean age was 57.9 years (range: 24.6 to 86.8 years), 58% were male, and 91% were white. Eight patients (10%) with acute ischemic stroke were platelet C4d-positive, which was significantly higher in prevalence compared to healthy controls (0%, P<0.0001) and non-SLE patients with immune/inflammatory disease (2%, P=0.004). The median NIH-SS score and infarct volume for acute stroke patients were 6 (interquartile range [IQR]: 2 to 13) and 3.4 cc (IQR: 1.1 to 16.6), respectively. Platelet C4d-positive patients were more likely to have a severe stroke compared to those with negative platelet C4d (NIH-SS median: 17.5 versus 5, P=0.003). Positive platelet C4d was independently associated with stroke severity (P=0.03) after controlling for age, anticardiolipin antibody (aCL) status, and total anterior circulation of stroke involvement, and also with infarct volume (P=0.005) after controlling for age, aCL status, and old stroke by MRI.

Conclusions—Platelet C4d is associated with severe acute ischemic stroke. Platelet C4d may be a biomarker as well as pathogenic clue that links cerebrovascular inflammation and thrombosis. (Stroke. 2008;39:3236-3241.)

Key Words: complement activation; platelet; ischemic stroke; stroke severity; infarct volume; NIH stroke scale

We have recently identified complement activation product C4d bound to the surface of platelets (platelet C4d; PC4d) in 18% of patients with systemic lupus erythematosus (SLE),1 a systemic autoimmune disease in which autoantibody production and complement activation are key participants in the pathogenesis of organ injury. Platelet C4d was highly specific (99%) for SLE in comparison to more than 30 other autoimmune and inflammatory disorders. Platelet C4d positivity was also significantly associated with the presence of antiphospholipid antibodies in these SLE patients. In our unpublished observation, platelet C4d was significantly associated with a history of neuropsychiatric event (seizures and/or psychosis) in SLE patients. These studies suggested that platelets bearing C4d may be present in patients with other primary and secondary cerebrovascular disorders, which were not included in previous studies.

We elected to investigate platelet C4d in acute ischemic stroke for two primary reasons. First, a growing body of literature has documented complement activation as a critical mechanism of tissue injury in cerebral ischemia and ischemia-reperfusion.2–9 Second, abundant evidence suggests that therapeutic strategies focused on platelet or complement inhibition hold great promise.3,4,7,10–15 Together, these considerations suggest that platelets (as the cellular participant in thrombosis) bearing the complement activation product C4d (as a molecular mediator of cerebrovascular inflammation) may be an informative biomarker in patients with acute ischemic stroke.

In this study, we compared the prevalence of platelet C4d in patients with and without acute ischemic stroke and investigated the association between platelet C4d and acute stroke severity.
Materials and Methods

Patients hospitalized for acute ischemic stroke (n = 80) were recruited from the Stroke Unit at the University of Pittsburgh Medical Center (UPMC) from June 2005 to November 2006. This sample represents consecutive patients in whom informed consent could be obtained. The diagnosis of ischemic stroke was determined by a neurologist based on neurological examination and evidence of acute infarct on MRI or computed tomography (CT). The National Institutes of Health Stroke Scale (NIH-SS) was assessed for each patient at the time of admission. A board-certified vascular neurologist classified consecutive patients in whom informed consent could be obtained. The diagnosis of ischemic stroke was determined by a neurologist based on neurological examination and evidence of acute infarct on MRI or computed tomography (CT). The National Institutes of Health Stroke Scale (NIH-SS) was assessed for each patient at the time of admission. A board-certified vascular neurologist classified strokes into etiologic and localization subtypes. The etiologic subtypes were defined by the Trial of Org 10172 in Acute Stroke (TOAST) criteria,16 which categorizes ischemic strokes into the following groups: cardioembolic, large artery atherosclerotic, small vessel occlusion, stroke of other determined etiology, and stroke of undetermined etiology. Localization subtypes were determined by Bamford classification: large anterior circulation infarcts with both cortical and subcortical involvement (total anterior circulation infarcts), more restricted and predominantly cortical infarcts with both cortical and subcortical involvement (partial anterior circulation infarcts), vertebrobasilar arterial territory (posterior circulation infarcts), and infarcts in the territory of the deep perforating arteries (lacunar infarcts).17 Demographic and clinical data including vascular risk factors, prior medical history, medication use, and neuroimaging were recorded for each patient.

Blood sample collections were initiated within 72 hours after hospital admission and when conditions (ie, hospital stay) permitted. Analyses included anticardiolipin (aCL) IgG and IgM antibodies, platelet C4d, and plasma C3 and C4 levels. The isotype specific measurements of aCL IgM and IgG antibodies were determined using the EL-aCL ELISA kit (TheraTest Labs Inc). Plasma C3 and C4 levels were measured by nephelometry (Beckman Coulter). Platelet C4d was detected as previously described.1 Briefly, whole blood was incubated with mouse monoclonal antihuman C4d antibody (Quidel Corporation) or a mouse IgG1k isotype control (Becton Dickinson) that was labeled with Alexa Fluor 488 using a mouse IgG1 Zenon labeling kit (Molecular Probes). The blood was then diluted and analyzed immediately by flow cytometry. Platelets were identified with a phycoerythrin (PE)-conjugated anti-CD42b body (Pharmingen). Platelet C4d-specific fluorescence intensity values of greater than or equal to 2.15 were considered to be positive.1

All patients underwent either MRI or CT of the brain within 48 hours of hospital admission. Two radiologists measured infarct volume by consensus: a third-year radiology resident (SF) and a board-certified neuroradiologist (B.F.B.). Infarct volume was measured on fluid attenuation inversion recovery (FLAIR) sequences, using diffusion weighted sequences as a guide when acuity of infarct was indeterminate on FLAIR images alone. On each axial image, areas of infarction were outlined with a freehand region of interest, and a three-dimensional volume was computed by summing the areas of infarction on each slice and multiplying by the slice interval (GE Advantage Workstation, Version 4.0). After an overall measurement of infarct volume, the pre- and postcentral sulci, thalamus, and basal ganglia were individually evaluated to determine whether 50% or more of each of these specific territories had infarcted. Finally, the brain stem was analyzed and any areas of infarction within the brain stem were noted. The radiologists were blinded to all clinical and laboratory data.

To explore whether platelet C4d was associated with acute ischemic stroke, we compared the prevalence in acute ischemic stroke patients to those with other diseases and healthy controls, who were recruited as a part of our original study in SLE. These comparison groups were comprised of the following:

Patients With Other Diseases

Patients (n = 246) with other rheumatologic, inflammatory, or hematologic diseases were recruited from subspecialty clinics affiliated...
with the University of Pittsburgh. They included systemic sclerosis, inflammatory myopathy, chronic hepatitis C infection, rheumatoid arthritis, primary Sjögren syndrome, vasculitis, sickle cell disease, cutaneous lupus, primary antiphospholipid syndrome, and cardiovascular disease.

Healthy Controls
Healthy controls (n=307) were recruited through advertisements posted on the University of Pittsburgh campus. To confirm their healthy status, participants completed a brief questionnaire regarding medical conditions such as pregnancy, reactive airway disease, hematologic disease, cardiovascular disease, malignancy, and other chronic medical conditions such as renal and autoimmune diseases.

The Institutional Review Board of the University of Pittsburgh approved this study, and all participants or next-of-kin (if the stroke patient was unable to provide informed consent) provided their written informed consent.

Data were presented with mean (standard deviation) or median (interquartile range/IQR: 25th to 75th percentile) based on the distribution of the continuous variables. Categorical variables were analyzed using Fisher’s Exact or Chi-square test. Spearman rank correlation was used to determine the correlation between two variables. The two stroke outcome variables of interest were stroke severity (as measured by NIH-SS) and infarct volume. NIH-SS scores and stroke volumes were transformed to normality by square root and log, respectively. Variables which had univariate association with stroke outcome variable at \( P \leq 0.15 \) entered the stepwise forward selection. Multivariable linear regression was utilized to assess for independent association of platelet C4d with each stroke outcome variable. All tests used 2-tailed tests with significance level of 0.05. Analyses were performed using the STATA/SE version 9.0 for Windows (Stata Corporation).

Results
Characteristics of Patients
The demographics and characteristics of the 80 acute ischemic stroke patients are shown in Table 1. The overall mean age of the ischemic stroke patients was 57.9 years (range: 24.6 to 86.8 years) with 30% aged less than 50 years. More than half (58%) of the patients were male. Median NIH-SS score was 6 (IQR: 2 to 13), and median infarct volume was 3.4 cc (IQR: 1.1 to 16.6). Of these unselected patients, 3 patients had one of these rheumatic diseases: systemic lupus erythematosus/SLE, rheumatoid arthritis/RA, and Takayasu arteritis. There was no significant difference in the demographics, cardiovascular risk factors, and medications at home (ie, antiplatelet and anticoagulation therapy) between patients with positive platelet C4d and those with negative platelet C4d (data not shown). However, patients with positive platelet C4d were more likely to have received thrombolytics (\( P=0.02 \)) and platelet glycoprotein IIb/IIIa inhibitor (Eptifibatide; \( P=0.02 \)) than were platelet C4d-negative patients.
Eight patients (10%) were positive for platelet C4d in patients with acute ischemic stroke compared to none of the 246 healthy controls ($P<0.0001$) and 7 of the 307 patients with immune and inflammatory diseases other than SLE ($P=0.004$).

**Association Between Platelet C4d and Stroke Severity**

Patients in this study who were PC4d-positive had significantly more severe stroke (NIH-SS median: 17.5 versus 5, $P=0.003$) and greater infarct volume (median: 17.4 cc versus 2.9 cc, $P=0.06$) than those who were PC4d-negative (Table 2). PC4d-positive patients were also more likely to have stroke in the total anterior circulation compared to those with negative PC4d (50% versus 7%, $P=0.004$). There were no significant differences in timing of blood collection from symptom onset or other pertinent laboratory parameters in PC4d-positive patients.

Nearly a quarter of the stroke patients (22.5%) were positive for aCL antibodies; however, only one of these patients was also positive for platelet C4d. Three of the aCL-positive patients had rheumatic disease. Both the SLE and RA patients had moderate-to-severe stroke (NIH-SS ≥5), while the patient with Takayasu’s arteritis had mild stroke (NIH-SS <5). Only the SLE patient was positive for platelet C4d (2.52).

Using Spearman rank correlations, platelet C4d correlated with stroke severity by NIH-SS ($r_s=0.34$, $P=0.002$) and infarct volume ($r_s=0.24$, $P=0.06$) as shown in Table 3. NIH-SS was moderately correlated with infarct volume ($r_s=0.56$, $P<0.0001$). Platelet C4d did not correlate with either plasma C3 ($r_s=-0.13$, $P=0.27$) or C4 ($r_s=-0.05$, $P=0.69$). Age, hemoglobin, platelet count, plasma C3, cardioembolic subtype of ischemic stroke, and location of stroke (total anterior circulation) were correlated with stroke severity by NIH-SS. aCL antibodies, large-vessel and small-vessel ischemic stroke subtypes, and stroke locations (total anterior and lacunar infarct) were significantly correlated with infarct volume.

Using multivariable linear regression after forward stepwise selection (Table 4), platelet C4d (beta coefficient = 1.07, $P=0.03$) was independently associated with stroke severity by NIH-SS after adjusting for age, presence of aCL antibodies, and location of stroke (total anterior circulation). Platelet C4d (beta coefficient = 2.63, $P=0.004$) was also associated with infarct volume after adjusting for age, presence of aCL antibodies, and evidence of prior stroke by MRI. Adjustment for sex, race, and treatments such as thrombolitics and antiplatelet/anticoagulation therapy did not influence the regression models. Platelet C4d (beta coefficient = 2.17, $P=0.01$) continued to be significantly associated with infarct volume after additional adjustment for the large-vessel subtype of ischemic stroke and lacunar infarcts. There was no interaction between platelet C4d and the presence of aCL antibodies or prior stroke by MRI. After excluding the 1 SLE patient with positive platelet C4d, the association of platelet C4d with stroke severity by NIH-SS (beta coefficient = 1.06, $P=0.04$) and infarct volume (beta coefficient = 2.25, $P=0.02$) remained statistically significant. Presence of platelet C4d but not aCL antibodies continued to be significantly associated with NIH-SS and infarct volume after excluding the 3 patients with rheumatologic diseases.

**Discussion**

In this study, we demonstrated that platelet C4d is significantly associated with acute ischemic stroke and with stroke

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**Table 3. Clinical Correlates of Stroke Severity Using Spearman Rank Correlation**

<table>
<thead>
<tr>
<th></th>
<th>NIH-SS (n=80)</th>
<th>Infarct Volume (n=66)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$</td>
<td>$P$ Value</td>
</tr>
<tr>
<td>Age</td>
<td>0.28</td>
<td>0.01</td>
</tr>
<tr>
<td>Old stroke by MRI</td>
<td>−0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>WBC</td>
<td>0.04</td>
<td>0.74</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>−0.28</td>
<td>0.01</td>
</tr>
<tr>
<td>Platelet count</td>
<td>−0.26</td>
<td>0.03</td>
</tr>
<tr>
<td>Platelet C4d positive</td>
<td>0.34</td>
<td>0.002</td>
</tr>
<tr>
<td>Plasma C3</td>
<td>−0.33</td>
<td>0.003</td>
</tr>
<tr>
<td>Plasma C4</td>
<td>−0.22</td>
<td>0.05</td>
</tr>
<tr>
<td>aCL antibody positive</td>
<td>0.18</td>
<td>0.12</td>
</tr>
<tr>
<td>Ischemic stroke subtypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>Large-vessel</td>
<td>0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>Small-vessel</td>
<td>−0.18</td>
<td>0.10</td>
</tr>
<tr>
<td>Location of stroke</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total anterior</td>
<td>0.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lacunar</td>
<td>−0.14</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Table 4. Age-Adjusted Association of Platelet C4d With Stroke Severity by NIH Stroke Scale and Infarct Volume Using Multivariable Linear Regression**

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>NIH Stroke Scale ($R^2=0.27$)</th>
<th>Infarct Volume ($R^2=0.26$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ Coefficient</td>
<td>SE</td>
</tr>
<tr>
<td>P-C4d positivity</td>
<td>1.07</td>
<td>0.49</td>
</tr>
<tr>
<td>aCL positivity</td>
<td>0.75</td>
<td>0.32</td>
</tr>
<tr>
<td>Total anterior circulation</td>
<td>1.53</td>
<td>0.47</td>
</tr>
<tr>
<td>Old stroke by MRI</td>
<td>−1.91</td>
<td>0.66</td>
</tr>
</tbody>
</table>

*Known risk factors for stroke such as hypertension, dyslipidemia, smoking history, and diabetes did not contribute to the final multivariable regression models.
severity by NIH-SS and infarct volume in these patients. The potential impact of these observations can be considered from 5 different perspectives. The first perspective links 2 seemingly unrelated conditions, ischemic stroke and SLE. We had previously shown that platelet C4d was 99% specific for a diagnosis of SLE and significantly associated with neuropsychiatric manifestations of the disease (our unpublished data, 2007). However, we had not included patients with other cerebrovascular disorders in our previous studies, and it was the association with neuropsychiatric lupus that led to the current investigation. The results of the current investigation, together with our previous studies, suggest that platelet C4d may participate in mechanisms of both primary and secondary cerebrovascular injury.

The second perspective links the cellular element of thrombosis with an activation product of the complement system, an inflammatory cascade that is already known to intersect with the coagulation cascade at multiple points. Our current findings corroborate previous work that demonstrated activation of complement in acute ischemic stroke2–8 and also suggest a novel interaction between complement activation and platelets in cerebral ischemic injury.

The third perspective suggests that inhibition of platelets and inhibition of complement activation are 2 promising therapeutic approaches to cerebral ischemia that should be considered in synergy rather than as targeting independent pathogenic events. Based on our data, we speculate that deposition of C4d on platelet surfaces may influence platelet aggregation or platelet interactions between circulating leukocytes and endothelial cells. Previous studies further support this hypothesis. For example, during the ischemia and reperfusion injury observed in acute stroke, endothelial activation occurs through the upregulation of adhesion molecules such as E-selectin and P-selectin,10,18 which can be upregulated by activated complement, thereby promoting platelet plugging. Furthermore, strong expression of P-selectin has been observed after ischemic stroke in wild-type mice but not in complement deficient (C3)-mice.3 Conversely, activated platelets are capable of activating the complement system, in part through a P-selectin–dependent process. Finally, complement C3b can bind to the surface of activated platelets; and this binding can be partially inhibited by P-selectin antibodies.10

With regard to therapy, it should also be noted that patients who were PC4d-positive were more likely to have received thrombolytics and platelet glycoprotein IIb/IIIa inhibitor compared to those who were PC4d-negative. We do not know the effect of these therapies on complement activation on platelets. However, patients with more severe stroke may have been more likely to have received such intervention. This might therefore explain the association of treatment with PC4d in the stroke cohort.

The fourth perspective suggests that PC4d should be considered as a risk factor for ischemic stroke and for more severe stroke. Although it might be argued that PC4d is generated as a result of the ischemic stroke and is more likely to be detectable with a more severe event, this seems to be unlikely for several reasons. First, PC4d is detected in patients with SLE who have not suffered from any clinical cerebrovascular event. Second, in patients who are PC4d-positive, the entire platelet population, rather than a subset of the cells, is positive for C4d (flow cytometric and confocal microscopic data not shown). If the ischemic event led to C4d deposition on the platelets it is unlikely that all of the cells would be homogeneously positive. Third, if PC4d were a relatively nonspecific result of ischemic injury, it would not be limited to 10% of the stroke patients. The PC4d is more likely a preceding risk factor for severe stroke.

Finally, we consider the potential links among platelets, complement, and aCL, which are known risk factors for vascular occlusive disorders and recurrent fetal loss. In a previous study, we demonstrated that platelet C4d was independently associated with aCL in SLE patients.1 Several studies have also shown the presence of aCL antibodies as a stroke risk factor in young adults (maximum age cutoffs ranged from 40 to 51 years), primarily in patients without SLE.19–23 Similar to findings previously reported in the literature, we found the presence of aCL antibodies in 22.5% of our acute ischemic stroke patients, and we observed that aCL was independently associated with higher infarct volume. More importantly, we demonstrated an independent positive association of platelet C4d with stroke severity by NIH-SS and infarct volume even after adjusting for covariates and/or potential confounders which included aCL, stroke subtype and location. These observations suggest that although there may be a mechanistic link between platelet C4d and aCL, they are independent risk factors for acute ischemic stroke.

In summary, our findings indicate that platelet C4d is associated with severe acute ischemic stroke. These observations have important implications for understanding the cellular and molecular mechanisms of cerebrovascular injury and for therapeutic strategies. These initial observations in our relatively small cohort can now be explored in larger multi-center studies.

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Disclosures
S.M. and J.M.A. are consultants for Cellatope Corporation. J.M.A. has equity in Cellatope Corporation. The remaining authors report no conflicts.

References


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