Erythrocyte C4d and Complement Receptor 1 in Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Complement activation and ineffective clearance of complement-bearing immune complexes via erythrocytes contribute to the pathogenesis of systemic lupus erythematosus (SLE). Abnormally high levels of erythrocyte C4d and low levels of complement receptor 1 (CD35) have been reported in SLE and might have diagnostic utility. We investigated whether erythrocyte C4d and complement receptor 1 were specific for SLE and whether there was any association with disease activity.

Methods. Expression of complement receptor 1 (CD35) and complement protein C4d on erythrocytes was measured by indirect immunofluorescence and flow cytometry on the same day as the blood draw, in patients with SLE, patients with rheumatic disease, and in normal controls.

Results. Within the SLE population, there was no association with disease activity measured by the physician's global assessment or SELENA SLE Disease Activity Index, nor with past or current lupus nephritis. Assays were not specific for SLE, with higher levels also seen in antiphospholipid syndrome.

Conclusion. Overlap of erythrocyte C4d and CD35 between SLE and other rheumatic diseases limits their utility as diagnostic tests. However, longitudinal investigation of these assays is warranted, especially given the higher levels in some patients with primary antiphospholipid syndrome. (First Release Aug 15 2008; J Rheumatol 2008;35:1989–93)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS COMPLEMENT

The classification of systemic lupus erythematosus (SLE) is based on the American College of Rheumatology (ACR) criteria published in 1982[1] and later revised[2]. Although these criteria were developed for the classification of SLE patients for inclusion in clinical or laboratory research studies, they are often used for diagnostic purposes as well[3]. Serological tests with acceptable sensitivity and specificity would improve both diagnostic and classification capability. Serum anti-dsDNA has been the most specific laboratory test for diagnosis of SLE, but has a sensitivity of only 40%[4].

Low serum complement levels are not included in the classification criteria; although commonly found in SLE, they can occur as well in other rheumatic diseases. Low levels of complement components can reflect complement activation, but also occur with genetic deficiencies of complement, such as C4[5].

Measurement of C4d bound to erythrocytes (C4d-E) has been proposed as a sensitive and quantitative assay of the degree of complement activation and disease activity in patients with SLE[6]. A recent study found that the determination of C4d (produced by proteolytic cleavage of C4) and complement receptor 1 (CR1) levels on erythrocytes were specific for the diagnosis of SLE[7].

Reticulocytes are the youngest erythrocytes observed in circulation and are usually present from days 0 to 2 of the erythrocyte cycle. CR1 is present on both reticulocytes and erythrocytes. Low CR1 expression on reticulocytes could be attributed to either a downregulation of the erythrocyte lineage in the bone marrow or an acquired process. Reduced expression of CR1 on mature erythrocytes likely arises from an acquired process[8].

Our study investigated whether C4d and CR1 on the surface of erythrocytes and reticulocytes are specific for SLE and whether there was any correlation with clinical disease activity.

MATERIALS AND METHODS

Study participants. The Johns Hopkins University Institutional Review Board approved this study. Informed consent was obtained from all participants. Consecutive patients with SLE, as diagnosed by one physician (MP), were recruited for this study during their routine visits to the Johns Hopkins University Lupus Center. As part of their routine care, one physician (MP) performed history and physical examination on each patient. This physician was blinded to the results of the tests for erythrocyte-bound complement. Disease activity was measured by use of physician's estimate of activity and the SELENA Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[9]. Patients with other rheumatic or autoimmune

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diseases were also recruited, including antiphospholipid syndrome (APS), chronic cutaneous lupus, rheumatoid arthritis (RA), and undifferentiated connective tissue disease (UCTD). Controls with no history of SLE or other autoimmune diseases were recruited during their phlebotomy visits.

Ninety percent of SLE patients were female, mean age 45.2 years, and 63% were Caucasian and 37% African American. The cumulative ACR criteria included 96% positive antinuclear antibodies, 52% malar rash, 23% discoid rash, 55% photosensitivity, 54% oral ulcers, 40% renal SLE, 75% hematologic SLE, 34% pleurisy, 21% pericarditis, 4% seizures, 2% psychosis, 72% arthritis, 47% anti-dsDNA, 17% anti-Sm, 31% false-positive test for syphilis, 48% anticardiolipin-positive.

Characteristics of the study subjects. The study population for the erythrocyte component consisted of 111 patients with SLE (of those, 4 had secondary APS), 10 primary APS, 1 chronic cutaneous lupus, 4 RA, 7 UCTD, and 45 non-SLE controls. The study population for the reticulocyte component consisted of 17 patients with SLE, 5 primary APS, 1 chronic cutaneous lupus, 2 UCTD, and 20 non-SLE controls.

Methods for complement red blood cell assays. Expression of complement receptor 1 (CD35) and complement protein C4d on the surface of erythrocytes was measured essentially as described by Manzi, *et al*[7]. Blood was collected in citrate/CTAD tubes and transported to the laboratory at room temperature. All remaining steps were performed at 0–4°C. The assays were performed on the day of blood collection. A 20 µl aliquot was washed in complement FACS buffer (CFB; phosphate buffered saline/1% heat-inactivated fetal calf serum/0.02% NaN_3). The cells were counted and resuspended in CFB at 10 million per ml. One million cells (100 µl) were incubated with 10 µg/ml anti-human CD35 (Becton-Dickinson), anti-human C4d (Quidel), or IgG1 isotype control for 30 min on ice, and washed once in CFB. Each antibody reaction was performed in duplicate. Cell pellets were resuspended in 100 µl of 1:100 goat anti-mouse IgG-FITC (Jackson Immunoresearch, West Grove, PA, USA), incubated for 30 min on ice in the dark, washed once in CFB, and resuspended in 0.5 ml of CFB. The samples were analyzed on a FacsCalibur flow cytometer, with instrument settings held constant from day to day. In addition, a preserved control blood sample (CD Chex Plus, Streck Laboratories, Omaha, NE, USA) was assayed each day in parallel with the samples, to confirm consistent readings. Values were reported as specific mean fluorescence intensity (sMFI), defined as the MFI of the antibody reaction minus the MFI of the isotype control reaction.

Reticulocyte C4d levels were assayed in whole blood in a similar fashion, in parallel with the erythrocyte measurements. After incubation with the FITC-coupled secondary antibody, cells were washed with CFB and resuspended in anti-CD71-PE (Becton-Dickinson) for 30 min on ice in the dark, then washed, resuspended in CFB, and analyzed by flow cytometry. sMFI values were determined as above on the CD71-positive pool (mean frequency = 0.40% of total; range 0.06%–1.40%). Activated lymphocytes were also CD71+. However, preliminary experiments demonstrated that the CD3+ pool did not overlap with the pool representing reticulocytes (data not shown).

Statistical methods. Means were compared by Student's t-test; percentages were compared by Fisher's exact test. A p value of 0.05 was taken as statistically significant.

RESULTS

The levels of C4d (Table 1) and CD35 (Table 2) on erythrocytes were compared to the disease activity of the patients who had SLE. C4d-E was positively and CD35 was negatively associated with the SELENA SLEDAI, in a statistically significant fashion. However, the magnitude of the correlation was small, suggesting that other factors were more important in explaining the variance in disease activity. Similarly, although statistically significant, the magnitude of the association of C4d-E with ESR, C3, and anti-dsDNA was low. The R^2 for anticardiolipin was quite small at 0.12 ($p < 0.001$). All associations with CD35 were of low magnitude, although many were statistically significant (Table 2).

The levels of the C4d-E and CD35-E were also analyzed for any significant association with the clinical phenotypes exhibited by the SLE subjects (Table 3). The strongest association was of high levels of C4d-E, and to a lesser extent, low levels of CD35-E, with antiphospholipid antibody. Platelet counts ranged from 43 to 700 among SLE participants. Higher C4d-E was found with the direct Coombs test and with a history of hemolytic anemia. Low CD35-E was found with malar rash. Several neurologic manifestations clustered with high levels of CD35-E.

The C4d and CD35 levels in SLE were also compared to the other disease states that were included in this study. Figure 1 depicts the levels of C4d on erythrocytes within the subsets of patients. As shown in Figure 1, there was significant overlap between primary APS and SLE. While the SLE group and the normal controls had considerable overlap, a subset of the SLE patients had levels higher than that seen in controls. Figure 2 depicts the levels of CD35 on reticulocytes. All disease states overlapped with controls. Figure 3 shows a similar depiction for C4d levels on reticulocytes. Once again, there was overlap between primary APS and SLE.

Table 1. Correlation of levels of C4d on erythrocytes (C4d-E) with measures of disease activity, serologic tests, and anticardiolipin antibody (aCL).

	Level of C4d-E (sMFI)		
	R^2	p	Correlation
SELENA SLEDAI	0.078	0.0031	Positive
Physician's global assessment of disease activity	0.036	0.049	Positive
Erythrocyte sedimentation rate	0.1129	0.001	Positive
Serum C3, mg/dl	0.0634	0.009	Negative
Serum C4, mg/dl	0.1959	< 0.0001	Negative
Anti-dsDNA (*Crithidia*)	0.0480	0.025	Positive
IgG aCL	0.1213	0.0004	Positive
IgM aCL	0.1255	0.0002	Positive

sMFI: specific mean fluorescence intensity.

Table 2. Correlation of levels of CD35 on erythrocytes (CD35-E) with measures of disease activity, serologic tests, and anticardiolipin antibody (aCL).

	Level of CD35-E (sMFI)		
	R^2	p	Correlation
SELENA SLEDAI	0.057	0.013	Negative
Physician's global assessment of disease activity	0.015	0.210	Negative
Erythrocyte sedimentation rate	0.0691	0.007	Negative
Serum C3, mg/dl	0.029	0.078	Positive
Serum C4, mg/dl	0.045	0.028	Positive
Anti-dsDNA (*Crithidia*)	0.043	0.033	Negative
IgG aCL	0.053	0.021	Negative
IgM aCL	0.03	0.085	Negative

sMFI: specific mean fluorescence intensity.

Table 3. Univariate analysis of levels of C4d-E and CD35-E with SLE manifestations. Values are specific mean fluorescence intensity (sMFI).

	Level of C4d-E (sMFI)			Level of CD35-E (sMFI)		
	Negative	Positive	p	Negative	Positive	p
Malar rash	26.1 ± 21.7	27.9 ± 19.6	0.680	35.9 ± 17.1	25.4 ± 13.0	0.0016
Seizure	27.3 ± 20.9	22.4 ± 8.1	0.643	31.0 ± 16.1	19.1 ± 7.6	0.1459
Lupus meningitis	26.3 ± 9.4	60.5 ± 48	0.019	29.9 ± 15	56.9 ± 39.7	0.017
Organic brain syndrome	27.3 ± 21.0	22.3 ± 6.8	0.643	29.6 ± 15.3	44.4 ± 9.8	0.044
Peripheral neuropathy	28.0 ± 21.2	18.1 ± 7.6	0.225	28.8 ± 14.1	48.4 ± 25.7	0.0015
Livedo reticularis	24.9 ± 17.6	34.9 ± 28.0	0.059	32.4 ± 16.5	23.2 ± 11.6	0.025
Anticardiolipin	23.1 ± 12.7	32.6 ± 25.1	0.018	32.8 ± 15.7	26.9 ± 16.1	0.0922
Lupus anticoagulant (dRVVT)	21.6 ± 12.3	41.3 ± 30.1	< 0.0001	33.5 ± 16.7	22.7 ± 11.4	0.0047
FP-RPR	23.3 ± 14.3	48.7 ± 35.0	< 0.0001	31.9 ± 15.6	20.4 ± 10.9	0.0219
Hemolytic anemia	25.4 ± 18.2	38.4 ± 31.4	0.0483	30.1 ± 15.3	32.7 ± 20.9	0.623
Direct Coombs test	23.7 ± 16.9	46.8 ± 29.5	0.0005	31.5 ± 15.8	19.8 ± 8.5	0.025
Low serum C4	19.6 ± 10.6	34.3 ± 25	0.0005	24.9 ± 16.3	26.1 ± 14.6	0.009

dRVVT: dilute Russell viper venom time. FP-RPR: false-positive rapid plasma reagin.

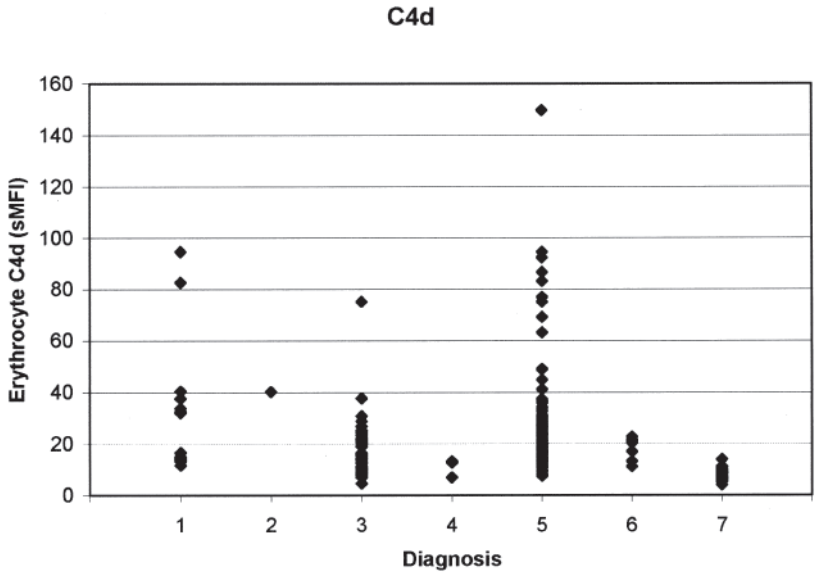

Figure 1. Level of C4d on erythrocytes. 1: primary APS; 2: cutaneous lupus; 3: nonrheumatologic controls; 4: RA; 5: SLE; 6: undifferentiated connective tissue disease; 7: assay control.

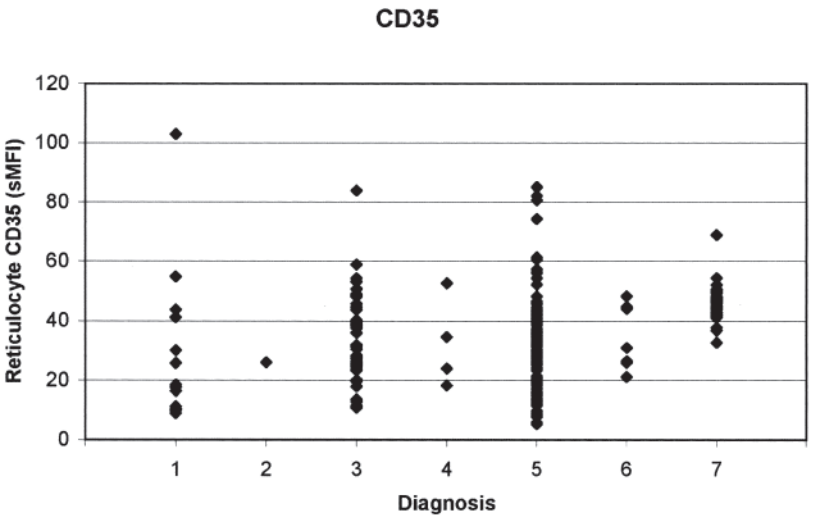

Figure 2. Level of C35 on reticulocytes. 1: primary APS; 2: cutaneous lupus; 3: nonrheumatologic controls; 4: RA; 5: SLE; 6: undifferentiated connective tissue disease; 7: assay control.

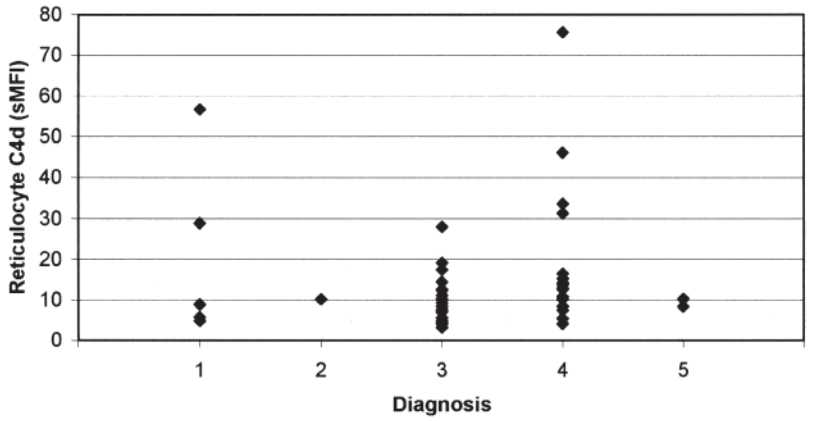

Figure 3. Level of C4d on reticulocytes. 1: primary APS; 2: cutaneous lupus; 3: nonrheumatologic controls; 4: SLE; 5: undifferentiated connective tissue disease.

DISCUSSION

The comparison of the levels of C4d and CD35 on erythrocytes and C4d on reticulocytes showed that there was overlap between SLE and other rheumatic diseases (Figures 1, 2, and 3). The overlap was greatest between SLE, APS, and cutaneous lupus. Previous studies have proposed that these assays could be useful as an additional test with a high diagnostic sensitivity and specificity for SLE[6,7]. Our study did not reproduce those findings. Thus, although these assays differentiate the autoimmune phenotype from normal, they are not specific for SLE. Indeed, these results point to potential complement activation in patients with clinically stable primary APS.

Our study separated rheumatic controls by their diagnosis, allowing us to identify overlap between SLE, chronic cutaneous LE, and APS in the assays. Our study also visually presented the data, because analysis of the group means could have missed this overlap. Our study also differs in that our "normal" control group consisted of nonrheumatic outpatients, not healthy volunteers.

Although complement activation is part of the pathogenesis of SLE, the magnitude of the association of C4d-E and CD35-E with the SELENA SLEDAI was small. This suggests that it is unlikely to be clinically useful, even though there was statistical significance. The correlation with the SELENA SLEDAI can be explained by the association of these assays with C3, C4, and anti-dsDNA, which are components of the SLEDAI.

The phenotype most associated with C4d-E and CD35-E was that of antiphospholipid antibodies. In murine models, complement activation has been recognized to be important in the pathogenesis of both pregnancy loss[10] and thrombosis[11]. In rats, thrombus formation due to anti-ß$_2$-glycoprotein I is complement-dependent[12]. In humans, complement

activation was found in 9 of 13 with cerebral ischemia due to antiphospholipid antibodies[13]. Levels of C4d-E in our study were substantially higher in those with either anticardiolipin or the lupus anticoagulant. It has been reported that some patients with primary APS may go on to develop SLE[14,15]. Thus, it would be of interest to longitudinally follow the patients with primary APS with the most abnormal values, to determine whether SLE develops later and whether the subset with complement activation is more likely to have later thrombosis or pregnancy loss.

In summary, C4d and CD35 levels on erythrocytes or reticulocytes neither separate patients with SLE from other rheumatic diseases nor associate in an important way with disease activity. High levels in some patients with APS might suggest an "inflammatory" component to this disease, normally considered to be primarily thrombotic[16], or could reflect antibodies to platelet membrane proteins that activate the complement cascade. New findings include the strong association of high C4d-E with antiphospholipid antibodies and with hemolytic anemia, suggesting the pathogenesis of these disease manifestations includes complement activation, and of high CD35-E with organic brain syndrome, lupus meningitis, and peripheral neuropathy.

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