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Reticulocytes Bearing C4d as Biomarkers of Disease Activity for Systemic Lupus Erythematosus

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Objective. **There is an urgent need for biomarkers with which to monitor disease activity in patients with systemic lupus erythematosus (SLE). We recently showed that abnormal levels of C4d, an activation-derived fragment of complement component C4, are deposited on the surface of erythrocytes from patients with SLE. This study focused on reticulocytes, the youngest and shortest-lived erythrocytes (lifespan 24–48 hours), with the objective of testing our hypothesis that when reticulocytes emerge from the bone marrow, they are immediately exposed to and acquire C4d at levels proportionate to the extent of complement activation at that time, thereby reflecting disease activity in SLE.**

Methods. **We conducted a cross-sectional study of 156 patients with SLE, 140 patients with other diseases, and 159 healthy controls. Levels of C4d on the surface of reticulocytes were examined using a 2-color flow cytometric assay. The results were analyzed for correlations with SLE disease activity.**

Results. **A wide range of increased levels of reticulocyte C4d was specifically detected in SLE patients. These levels fluctuated in SLE patients and correlated with clinical disease activity, as determined by the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and the Systemic Lupus Activity Measure (SLAM). Specifically, in cross-sectional analyses, patients with reticulocyte C4d levels in the highest quartile compared with those in the lowest quartile had significantly higher SELENA–SLEDAI ($P = 0.00002$) and SLAM ($P = 0.02$) scores. Longitudinal observation demonstrated that the reticulocyte C4d levels changed in relation to the clinical course in individual patients.**

Conclusion. **These findings support our hypothesis that C4d-bearing reticulocytes may serve as biomarkers of disease activity in patients with SLE.**

Systemic lupus erythematosus (SLE) is the most clinically and serologically diverse autoimmune disease. The spectrum of disease among patients with SLE is broad and ranges from subtle symptoms to life-threatening multiple organ failure. Because of its heterogeneous presentation and unpredictable course, clinical management of SLE remains one of the greatest challenges to physicians. The lack of reliable, specific biomarkers of SLE not only hampers the precise assessment of disease activity and the prompt identification of patients at risk of disease flares and organ damage, but it also prohibits the accurate evaluation of responses to treatment.

A large body of evidence accumulated over several decades has demonstrated that abnormalities in complement activation and clearance of immune complexes by erythrocytes are fundamental to the pathogenesis of SLE (1,2). Although serum levels of C3, C4, and their activation-derived products are frequently used to monitor disease activity in SLE, their value as biomark-

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ers is a subject of controversy (3–13). These observations have led us to consider measurement of complement activation products bound to circulating blood cells (14,15).

It has been known for decades that proteolytic fragments of complement component C4, particularly C4d, are present on the surfaces of normal erythrocytes (16,17). Erythrocytes, the most abundant blood cells, have immediate access to complement activation products generated systemically or locally. We therefore postulated that complement-bearing erythrocytes may serve as biologic beacons of inflammation in vivo, reflecting disease activity in patients with SLE (14,15).

Erythrocytes, which have a lifespan of ~120 days, may bind and retain activation-derived C4d throughout their lifetime. Accordingly, the level of erythrocyte-bound C4d may reflect the cumulative result of complement activation and disease activity during the preceding 120 days. Alternatively, reticulocytes maintain distinct phenotypic features, e.g., residual cellular RNA and high surface levels of the transferrin receptor, for 1–2 days before fully maturing into erythrocytes (18). Reticulocytes, if released into the peripheral circulation during an active disease state, might immediately be exposed to and bind C4d generated from activation of the complement system. Due to their transient lifespan, reticulocytes bearing C4d are likely to reflect the current state of complement activation, rather than past events. Therefore, we hypothesized that levels of reticulocyte-bound C4d, rather than levels of C4d on the entire erythrocyte population, may reflect more accurately the current level of disease activity in a given patient with SLE.

In the present study, we tested this hypothesis by determining the levels of reticulocyte-bound C4d in patients with SLE, patients with other inflammatory and immune-mediated diseases, and healthy control subjects with the use of a flow cytometric assay. We also investigated potential correlations between reticulocyte C4d levels and disease activity in SLE.

PATIENTS AND METHODS

Study participants. All study participants were 18 years of age or older and provided written informed consent. No one was excluded based on sex or ethnicity. The University of Pittsburgh Institutional Review Board approved this study.

SLE patients. From April 2002 through October 2003, 156 consecutive patients with SLE who met the American College of Rheumatology (ACR) 1982 (19) or 1997 (20) revised classification criteria were recruited for this study during routine visits to the University of Pittsburgh Lupus Diagnostic and Treatment Center. Patients who were pregnant were excluded. As part of their routine care, all patients underwent a history and physical examination by one physician (SM or AHK), who was blinded to the reticulocyte/erythrocyte-bound complement results. Disease activity was assessed at the time of the visit using the Systemic Lupus Activity Measure (SLAM) (21) and the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (22).

Patients with other diseases. From April 2002 through March 2004, 140 randomly selected patients with 11 other rheumatic, inflammatory/autoimmune, or hematologic diseases, including scleroderma (n = 43), myositis (n = 30), Sjögren's syndrome (n = 16), rheumatoid arthritis (n = 32), Wegener's granulomatosis (n = 5), hepatitis C (n = 3), vasculitis (n = 2), primary Raynaud's phenomenon (n = 5), hemophilia (n = 1), psoriatic arthritis (n = 2), and antiphospholipid syndrome (n = 1), were recruited. The diagnoses were confirmed by their treating subspecialist physicians from various outpatient facilities at the University of Pittsburgh Medical Center.

Healthy control subjects. A total of 159 healthy control subjects were recruited through local advertisements posted around the University of Pittsburgh campus. To confirm their healthy status, participants completed a brief questionnaire querying for obvious medical conditions.

Flow cytometric characterization of reticulocytes and erythrocytes. At the time of each participant's study visit, a 3-ml sample of blood was collected into Vacutainer tubes containing EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Blood samples were used for experiments on the same day they were collected. Whole blood cells were washed with phosphate buffered saline (PBS), diluted in PBS, and aliquotted for indirect immunofluorescence staining. Mouse monoclonal antibody specific for human C4d (reactive with C4d-containing fragments of C4; Quidel, San Diego, CA) or for the isotype-matched control MOPC-21 was added to cell suspensions at a concentration of 10 μg/ml. Phycoerythrin-conjugated goat anti-mouse IgG $F(ab')_2$ (Cappel, West Chester, PA) was used at a concentration of 10 μg/ml.

Following antibody staining, cell suspensions were incubated with thiazole orange (ReticCount reagent; Becton Dickinson) to identify reticulocytes or with PBS as the vehicle control. Stained cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA). Erythrocytes were electronically gated based on their forward and side-scatter properties. Reticulocytes were electronically gated based on their forward-scatter property and positive staining with ReticCount. Levels of C4d on the surface of reticulocytes or erythrocytes were expressed as specific median fluorescence intensity (SMFI), which was calculated as the C4d-specific median fluorescence intensity minus the isotype control median fluorescence intensity.

To ensure the day-to-day reliability of reticulocyte C4d measurements, the FACSCalibur flow cytometer was calibrated daily using CaliBrite 3 beads and FACSComp software (Becton Dickinson Immunocytometry Systems). The instrument settings were also calibrated daily using blood samples stained with an isotype control IgG to ensure that the back-

ground fluorescence intensity was ≤3.8. To determine the reliability and reproducibility of the reticulocyte C4d measurement, randomly selected samples were independently tested by different investigators and/or were repeatedly tested by the same investigator. To explore the utility and reliability of reticulocyte C4d measurements using stored blood samples, selected blood samples were stored at 4°C and tested daily up to 3 days after collection.

Statistical analysis. Descriptive statistics, including means, medians, standard deviations, and ranges, were computed for continuous data, and frequency distributions were determined for categorical variables. Differences in levels of reticulocyte C4d and erythrocyte C4d among the 3 study groups were compared by Kruskal-Wallis test, followed by Wilcoxon's rank sum test, to determine the statistical significance of the differences between each of the paired study groups. Spearman's rank correlations were used to determine the association between levels of reticulocyte C4d and erythrocyte C4d and disease activity, as measured by the SLAM and SELENA–SLEDAI. Wilcoxon's rank sum test was used to analyze the significance of differences in SLAM or SELENA–SLEDAI scores between the first quartile group and the other 3 quartile groups of SLE patients with different levels of reticulocyte C4d and erythrocyte C4d. Chi-square test for trend was used to evaluate associations between quartiles of reticulocyte C4d levels and the presence of specific clinical and serologic manifestations of SLE.

The statistical significance of the various tests was examined by 2-sided hypothesis testing using Stata/SE 8.2 software for Macintosh (Stata, College Station, TX). *P* values less than 0.05 were considered significant. To determine the reliability of the reticulocyte C4d flow cytometric assay, intraclass correlation coefficients were measured from the results obtained by different raters using the same set of samples from 10 patients and the repeated measures of the assays obtained by the same rater.

RESULTS

Characteristics of the study participants. The study population consisted of 156 patients with SLE, 159 healthy controls, and 140 patients with other immune-mediated, inflammatory, or hematologic diseases. The SLE patient group had a mean ± SD age of 43.78 ± 12.18 years, were 82.7% Caucasian, and 95.5% female. Additional demographic and clinical features of the SLE patients are shown in Table 1. The cohort included patients with new-onset as well as longstanding disease, represented a broad range of disease activity, as reflected in the SLAM and SELENA–SLEDAI scores, and had a wide spectrum of organ involvement. The healthy control group had a mean age of 43.55 ± 13.45 years, were 85% Caucasian, and 91% female. The group of patients with other diseases had a mean age of 52.20 ± 13.81 years, were 93% Caucasian, and 79.6% female.

Table 1. Clinical characteristics of the 156 study patients with SLE*

Characteristic	Result
Age, mean ± SD (range) years	43.78 ± 12.18 (18–80)
Race, % Caucasian	82.7
Sex, % female	95.5
Disease duration, mean ± SD (range) years	12.21 ± 9.49 (0.01–47.06)
Clinical manifestations, % positive since SLE diagnosis†	
Malar rash	53.2
Discoid rash	14.1
Photosensitivity	52.6
Oral ulcers	55.8
Arthritis	89.1
Serositis	49.4
Renal disease	25.8
Neurologic disease	7.7
Hematologic manifestations, % positive since SLE diagnosis†	57.7
Anemia	14.1
Leukopenia	42.3
Thrombocytopenia	20.5
Immunologic tests, % positive since SLE diagnosis†	80.6
Anti-Sm (n = 154)	13.6
Antiphospholipid antibodies (n = 154)	44.8
Antinuclear antibodies	96.2
Anti-SSA, anti-SSB, rheumatoid factor, etc. (n = 141)	36.6
Anti-dsDNA, (% positive at study visit) (n = 155)	69.0 (39.5)
Raynaud's phenomenon, % positive since SLE diagnosis†	43.2
SLAM score at study visit, mean ± SD (range)	5.79 ± 3.75 (0–20)
SELENA–SLEDAI at study visit, mean ± SD (range)	2.82 ± 2.91 (0–20)
Serum C3, % below normal at study visit (n = 155)	39.4
Serum C4, % below normal at study visit (n = 155)	51.0
ESR, mean ± SD (range) mm/hour (n = 151)	21.10 ± 19.80 (0–117)

* SLE = systemic lupus erythematosus; SLAM = Systemic Lupus Activity Measure; SELENA–SLEDAI = Safety of Estrogens in Lupus Erythematosus: National Assessment version of the Systemic Lupus Erythematosus Disease Activity Index; ESR = erythrocyte sedimentation rate.
† Manifestation was recorded as positive in individual patients if it was ever present since the diagnosis of SLE.

Comparison of the levels of C4d on reticulocytes from the 3 study groups. Previous studies conducted by us (15) and by other investigators (16,17) have shown the presence of C4d, a complement activation product, on the surface of erythrocytes. To evaluate the potential of reticulocyte C4d levels as a biomarker of disease activity, we conducted a cross-sectional study to examine and compare the presence of C4d on reticulocytes from healthy subjects, patients with SLE, and patients with

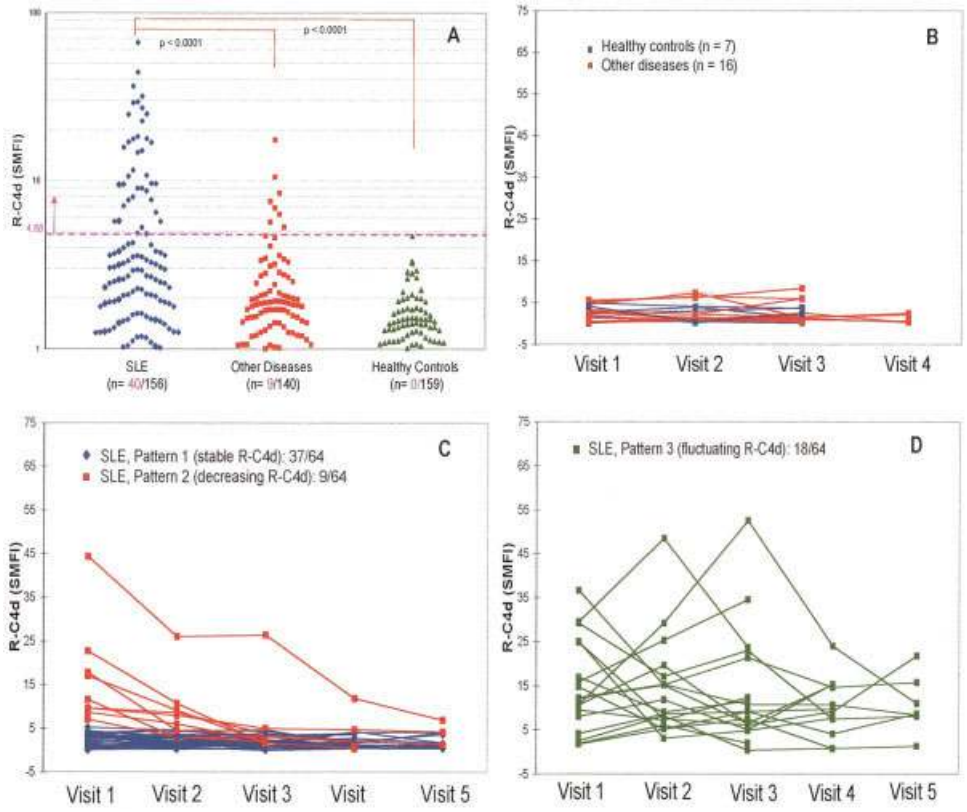

Figure 1. Reticulocyte C4d levels in 156 systemic lupus erythematosus (SLE) patients, 140 patients with other diseases, and 159 healthy control subjects over time, demonstrating significant elevations in SLE patients and fluctuations over time. **A,** Significantly higher levels of C4d were seen on reticulocytes from patients with SLE compared with those from patients with other diseases as well as those from healthy controls, based on the reticulocyte-bound C4d (R-C4d)–specific median fluorescence intensity (SMFI). Pink horizontal line represents an empirically determined "cutoff" point. The numbers of subjects in each group who had SMFI values higher than this point are shown in pink in at the bottom of the figure (see text for details). **B,** Reticulocyte C4d levels remained stable over time in healthy controls and patients with other diseases, as shown by the SMFI values in 7 healthy controls and 16 patients with non-SLE autoimmune diseases (1 with scleroderma, 7 with inflammatory myopathies, 1 with Sjögren's syndrome, 6 with rheumatoid arthritis, and 1 with antiphospholipid antibody syndrome) who were examined at 3 or 4 different study visits over a period of 22 months. **C** and **D,** Reticulocyte C4d levels fluctuated over time in a significant proportion of SLE patients, as shown by the SMFI values in 64 patients with SLE examined at 3–5 different study visits over a period ranging from 7 months to 20 months. In 37 patients, reticulocyte C4d levels remained stably low, and in 9 patients, levels were elevated at the first visit, but were decreased at subsequent visits (**C**). Remarkable fluctuations in reticulocyte C4d levels were observed in 18 of the SLE patients (**D**). Four representative patients with different reticulocyte C4d patterns were selected for the case studies described in the text and illustrated in Figure 2.

other diseases. Initial studies showed that the reticulocyte C4d flow cytometric assay was highly reliable and, thus, indicated excellent reproducibility. The interrater reliability coefficient was 0.96 (95% confidence interval 0.88–0.91) among the 3 different raters, and the intraclass correlation coefficient was 0.98 (95% confidence interval 0.93–0.99) within the same rater. Furthermore, repeated measurements of the same blood sample stored at 4°C showed minimal variation in the reticulocyte C4d levels and the percentages of reticulocytes over a 3-day period (data not shown), suggesting stability and reliability of reticulocyte C4d on stored blood samples. These latter results also excluded the possibility that significant amounts of C4d may be generated as a result of in vitro complement activation after blood collection.

The experimental results obtained in the 3 study

Table 2. Comparison of levels of reticulocyte-bound and erythrocyte-bound C4d in patients with SLE, patients with other diseases, and healthy controls*

Group	Reticulocyte-bound C4d	Erythrocyte-bound C4d
SLE patients (n = 156)		
Mean ± SD	5.50 ± 9.01	18.36 ± 27.92
Median	2.30	9.58
Range	0–66.81	0.5–22.7
Patients with other diseases (n = 140)		
Mean ± SD	1.79 ± 2.12	72.1 ± 7.50
Median	1.26	4.93
Range	0–17.6	0.4–47
Healthy control subjects (n = 159)		
Mean ± SD	1.4 ± 0.72	4.30 ± 3.51
Median	0.85	3.42
Range	0–4.68	0–27

* Values are the C4d-specific median fluorescence intensity. Patients with other diseases had other rheumatic, inflammatory/autoimmune, or hematologic diseases. All differences in the levels of reticulocyte-bound C4d as well as erythrocyte-bound C4d between the systemic lupus erythematosus (SLE) patients and the patients with other diseases as well as the healthy controls were significant at $P < 0.0001$, by Wilcoxon's rank sum test.

cohorts showed that variable, yet generally low, levels of C4d could be detected on reticulocytes from all healthy controls and most patients with other diseases (Figure 1A and Table 2). In contrast, significantly elevated levels of C4d were detected on reticulocytes from many of the patients with SLE (Figure 1A). When the reticulocyte C4d SMFI values were compiled for the entire study population, the mean ± SD level of reticulocyte C4d in SLE patients (5.50 ± 9.01 [range: 0–66.81]) was significantly higher than that in healthy controls ($P < 0.0001$) as well as that in patients with other diseases ($P < 0.0001$) (Table 2). As shown in Figure 1A, none of the healthy controls and only 9 of the 140 patients with other diseases (6.4%) had reticulocyte C4d SMFI levels above 4.68, whereas 40 of 159 patients with SLE (25.2%) had levels above 4.68. The reticulocyte C4d level was not affected by the percentage of reticulocytes detected in the peripheral blood of each of the individual patients (data not shown).

The reticulocyte C4d levels remained relatively constant in the healthy control subjects and in the patients with other diseases, as shown by longitudinal observations. Figure 1B summarizes the results obtained in representative healthy controls and in patients with other diseases. These subjects had remarkably similar reticulocyte C4d levels over days, months, and years. However, the reticulocyte C4d levels in a significant proportion of SLE patients varied considerably over time (Figures 1C and D).

Correlation between reticulocyte C4d levels and SLE disease activity. Our cohort of SLE patients representing a wide range of disease activity enabled us to determine the capacity of reticulocyte C4d levels to reflect disease activity at a single clinic visit. Both disease activity indices were significantly associated with the reticulocyte C4d level, although the correlation with the SELENA–SLEDAI score ($r = 0.45$, $P < 0.00001$) was better than with the SLAM score ($r = 0.23$, $P = 0.003$). For an additional analysis, we ranked and sorted the SLE study participants into 4 increment groups according to their reticulocyte C4d levels, with patients in the bottom quartile having the lowest reticulocyte C4d levels. The disease activity of each patient at the study visit was determined using the SELENA–SLEDAI and the SLAM disease activity indices (Table 3). Other laboratory parameters that may reflect lupus disease activity (e.g., anti–double-stranded DNA [anti-dsDNA], complete blood cell counts, serum complement levels) were also examined in relationship to the quartiles of reticulocyte C4d values.

When the disease activity scores of all SLE patients were compiled, the mean SELENA–SLEDAI scores were significantly different among the 4 groups (39 patients per group) by pairwise comparison ($P = 0.022$, $P = 0.0001$, and $P = 0.00002$ for the second, third, and fourth quartiles, respectively, versus the first quartile) (Table 3). The mean SLAM scores also differed among the 4 groups, although the differences were statistically significant only between the SLE patients in the fourth quartile (highest reticulocyte C4d levels) and those in the first quartile (lowest reticulocyte C4d levels) ($P = 0.02$). The reticulocyte C4d level also correlated significantly with specific disease variables, including anti-dsDNA ($P = 0.007$), anemia ($P = 0.025$), and thrombocytopenia ($P = 0.016$) (Table 3).

The numbers of patients with low serum C3 and C4 levels increased as the reticulocyte C4d levels rose in each quartile group (10, 9, 15, and 25 patients with low serum C3 levels among the 39 patients in each of the first to the fourth quartiles, and 13, 17, 19, and 28 patients with low serum C4, respectively). The mean standardized serum C3 and C4 levels were significantly different among the 4 groups ($P < 0.0001$ for serum C3 and for serum C4, by Kruskal-Wallis test), with the lowest mean serum C3 and C4 levels detected in the fourth quartile group. Moreover, an inverse correlation was observed between serum C3 and reticulocyte C4d levels (Spearman's correlation coefficient $r = -0.42$, $P < 0.0001$) as

Table 3. Correlation between levels of reticulocyte-bound C4d, levels of erythrocyte-bound C4d, and disease activity in the 156 SLE study patients*

Group	SELENA–SLEDAI score		SLAM score		Laboratory finding		
	Mean ± SD (median) [range]	*P* (versus first quartile)†	Mean ± SD (median) [range]	*P* (versus first quartile)†	% positive for anti-dsDNA antibodies	% with anemia (hematocrit <35%)	% with thrombocytopenia (platelets <150,000/μl)
Reticulocyte-bound C4d							
First quartile (0–1.12)	1.36 ± 1.35 (2) [0–4]	–	4.64 ± 2.41 (5) [0–12]	–	24	20	0
Second quartile (1.12–2.29)	2.64 ± 3.11 (2) [0–12]	0.022	5.26 ± 3.48 (5) [0–14]	NS	30	26	10
Third quartile (2.29–4.84)	2.85 ± 1.87 (2) [0–7]	0.0001	6.20 ± 4.28 (6) [1–20]	NS	54	33	15
Fourth quartile (4.84–66.8)	4.34 ± 3.84 (4) [0–20]	0.00002	7.08 ± 4.18 (7) [1–20]	0.02	56	50	18
P	<0.0001‡	–	<0.0001‡	–	0.007§	0.025§	0.016§
Erythrocyte-bound C4d							
First quartile (0–5.1)	1.64 ± 2.37 (2) [0–12]	–	4.97 ± 2.92 (5) [0–12]	–	29	21	0
Second quartile (5.1–9.6)	2.46 ± 2.27 (2) [0–12]	NS	5.48 ± 3.11 (5) [0–14]	NS	28	31	15
Third quartile (9.6–17.9)	3.23 ± 2.92 (2) [0–14]	0.002	6.26 ± 4.23 (5) [0–20]	NS	54	23	10
Fourth quartile (17.9–22.7)	3.95 ± 4.44 (4) [0–20]	<0.001	6.46 ± 4.44 (6) [0–20]	NS	53	41	18
P	<0.001‡	–	NS‡	–	0.02§	0.20§	0.059§

* Each quartile group consisted of 39 patients. SLE = systemic lupus erythematosus; SELENA–SLEDAI = Safety of Estrogens in Lupus Erythematosus: National Assessment version of the Systemic Lupus Erythematosus Disease Activity Index; SLAM = Systemic Lupus Activity Measure; anti-dsDNA = anti–double-stranded DNA; NS = not significant.
† By Wilcoxon's rank sum test.
‡ By Kruskal-Wallis test.
§ By chi-square test for trend (toward an increase).

well as between serum C4 and reticulocyte C4d levels ($r = -0.36$, $P < 0.0001$).

In comparison, the erythrocyte C4d levels were significantly associated with the SELENA–SLEDAI score ($r = 0.37$, $P < 0.00001$), although not as strongly as were the reticulocyte C4d levels ($r = 0.46$, $P < 0.00001$). The erythrocyte C4d level was not significantly associated with the SLAM score ($r = 0.14$, $P = 0.07$). Table 3 also shows the disease activity scores in SLE patients ranked according to quartiles of erythrocyte C4d levels.

Responses of reticulocyte C4d levels to changes in SLE disease activity, as shown in case studies. Reticulocyte C4d levels in healthy controls and in patients with other diseases were low and stable over time (Figure 1B). In contrast, we observed 3 different patterns of reticulocyte C4d levels in patients with SLE. The first group of patients had stable, low levels of reticulocyte C4d (Figure 1C). The second group of patients had a significantly elevated reticulocyte C4d level at the first visit, which decreased at subsequent visits (Figure 1C). The third group of patients had reticulocyte C4d levels that fluctuated over time (9–20 months). Case studies of 4 of the patients with fluctuating levels of reticulocyte C4d are presented below.

Case reports. We present the following 4 case reports to demonstrate the capacity of reticulocyte C4d levels to fluctuate with the clinical course of SLE. In these illustrative examples, reticulocyte C4d measurement is compared with 2 "gold standard" laboratory tests used in the clinical care of patients with SLE (the serum C4 level and the dsDNA antibody titer), the 2 disease activity indices (SLAM and SELENA–SLEDAI), and a nonspecific measure of systemic inflammation (the erythrocyte sedimentation rate [ESR]). In addition, we compare the utility of erythrocyte C4d with that of reticulocyte C4d.

Patient JN. The patient, a 19-year-old Caucasian woman, was diagnosed as having SLE in April 2002. Her SLE was manifested by inflammatory arthritis, rash, alopecia, oral ulcers, fatigue, fevers, antinuclear antibody at a titer of 1:320 (homogeneous pattern), anti-dsDNA antibody at a titer of 50 (normal <2), anticardiolipin antibody of IgG type (29 IgG phospholipid units/ml), and slightly elevated ESR at 28 mm/hour (normal 0–20). She was noncompliant with her medications (hydroxychloroquine and methotrexate) and was taking only prednisone, 5 mg/day, at the time of her first study visit on November 18, 2002 (Figure 2A). At this visit, she reported fatigue and had evidence of active arthritis and oral ulcers. Her laboratory tests showed an undetectable serum C4 level (<10 mg/dl), slightly elevated ESR (25 mm/hour), anti-dsDNA antibodies (titer 50), and elevated levels of reticulocyte C4d (11 SMFI) and erythrocyte C4d (33 SMFI). She was restarted on hydroxychloroquine and methotrexate therapy.

In December 2002, she presented to the emergency room with a high fever and headache. At that time, her serum C4 level remained undetectable, and her ESR had decreased to 20 mm/hour. In contrast, her reticulocyte (29 SMFI) and erythrocyte (47 SMFI) C4d levels had both increased significantly, suggesting an increase in disease activity. The anti-dsDNA antibody titer was not determined. She was admitted to the hospital, and a thorough evaluation for infection yielded negative results. She was discharged with no change in therapy.

On January 10, 2003, she was hospitalized because of the new development of proteinuria, renal insufficiency, and worsening of her constitutional symptoms. A renal biopsy was performed, and a diagnosis of mesangial glomerulonephritis was made. At this time, her serum C4 was increased to a detectable level (11 mg/dl) and her anti-dsDNA titer was decreased to 10. Both of these findings suggested possible improvement or no change in her lupus activity. In contrast, her erythrocyte (79 SMFI) and reticulocyte (52 SMFI) C4d levels had increased markedly by this time, suggesting a disease flare, which was consistent with both the clinical impression and the findings of the renal biopsy. After intensive treatment with a 3-day pulse of 1,000 mg of methylprednisolone succinate sodium (Solu-Medrol; Pharmacia & Upjohn, Kalamazoo, MI) followed by oral prednisone, hydroxychloroquine, and mycophenolate mofetil (CellCept; Roche, Indianapolis, IN), her condition improved, with a decrease in her serum creatinine level (from 1.2 mg/dl to 0.7 mg/dl) and resolution of her fever and arthralgia.

By January 29, 2003, her reticulocyte C4d level had decreased from 52 SMFI to 24 SMFI, whereas her erythrocyte C4d did not change significantly (from 79 SMFI to 77 SMFI). In contrast, her serum C4 level remained low and not significantly changed (12 mg/dl). The clinical impression at this time was improvement and response to intervention, which, despite her elevated erythrocyte C4d (77 SMFI) and ESR (42 mm/hour) values and the abnormal serum C4 level (12 mg/dl), was consistent with the marked decrease in her reticulocyte C4d level. Her prednisone dosage was lowered.

The clinical impression was confirmed on March 31, 2003, when the patient returned for followup evaluation. At this time, she had no symptoms, and her

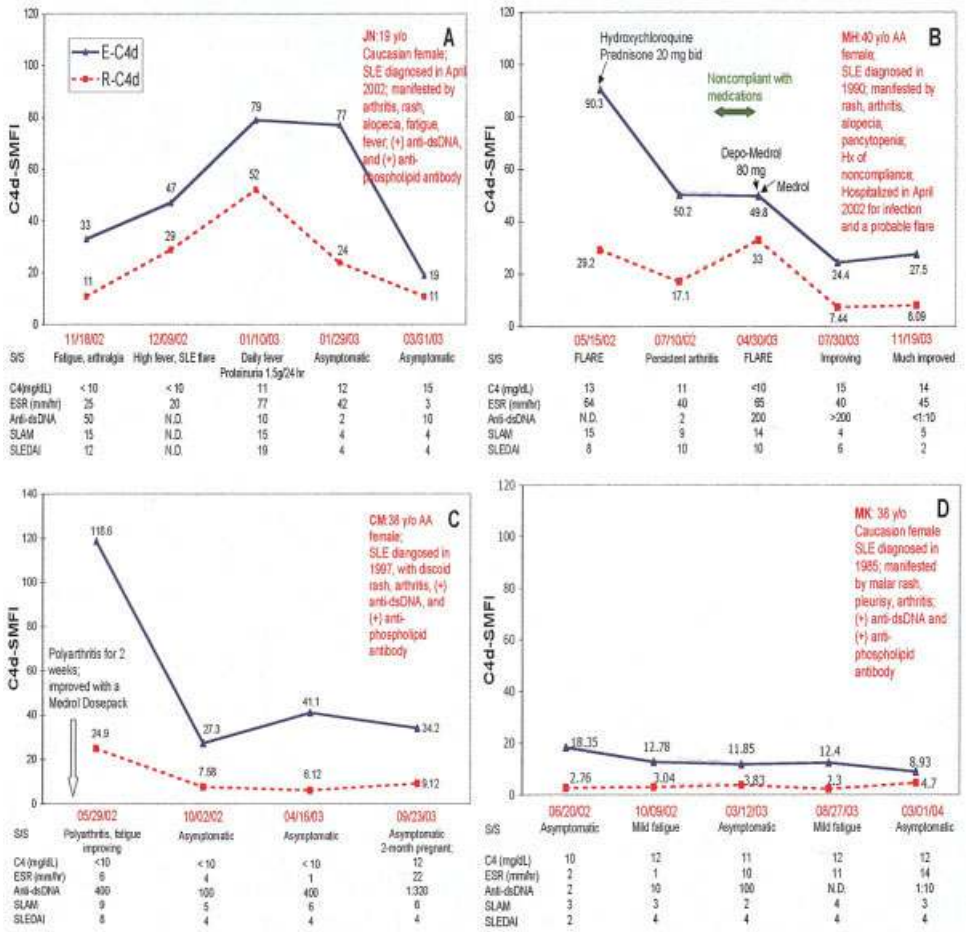

Figure 2. Fluctuations in reticulocyte C4d levels in 4 patients with systemic lupus erythematosus (SLE) and correlations with the clinical course of SLE. **A–D,** Serial measurements of reticulocyte-bound C4d (R-C4d) and erythrocyte-bound C4d (E-C4d) were obtained in 4 representative patients with SLE. Reticulocyte-bound C4d–specific median fluorescence intensity (SMFI) values are shown for each sampling point along the respective graphs; dates of sampling are shown in red underneath the graphs. See text for additional clinical history and results of other laboratory tests. Normal values are as follows: serum C4 level 20–59 mg/dl; erythrocyte sedimentation rate (ESR) 0–20 mm/hour; and anti–double-stranded DNA (anti-dsDNA) titer <2 or <1:10, depending on the type of assay used. Depo-Medrol, Medrol, and Medrol Dosepak are proprietary names of 3 different formulations of methylprednisolone produced by Pharmacia & Upjohn. Each patient's initials are shown at the top. y/o = years old; AA = African American; Hx = history; S/S = signs and symptoms; N.D. = not done; SLAM = Systemic Lupus Activity Measure; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index (the Safety of Estrogens in Lupus Erythematosus: National Assessment [SELENA] version).

reticulocyte (11 SMFI) and erythrocyte (19 SMFI) C4d levels had decreased. However, her serum C4 level remained low at 15 mg/dl, and her anti-dsDNA antibody titer was increased at 10, which was the same value observed at the peak of the flare on January 10, 2003.

Patient MH. The patient, a 40-year-old African American woman, was diagnosed as having SLE in 1990, and she had a history of noncompliance with her medications. She was hospitalized because of an ear infection and worsening arthritis a few weeks prior to her first study visit in May 2002. By the time of the first study visit, her symptoms had improved during therapy with prednisone 40 mg/day. The detection of a markedly elevated erythrocyte C4d (90.3 SMFI) and a moderately high reticulocyte C4d (29.2 SMFI) were consistent with a recent SLE flare. Response to therapy was confirmed by a significant reduction in her erythrocyte and reticulocyte C4d levels at her next visit in July 2002.

At the third study visit in April 2003, she was experiencing worsening arthritis and a skin rash. She reported noncompliance with all medications during the preceding 2 months. At that time, her reticulocyte C4d level was significantly elevated as compared with the previous visit, consistent with her reported flare. Her SLE flare was successfully treated with an injection of methylprednisolone acetate (Depo-Medrol) followed by oral methylprednisolone (Medrol) (both from Pharmacia & Upjohn). She reported marked resolution of all symptoms during the subsequent several months, which was consistent with the normal range of her reticulocyte C4d levels (Figure 2B).

Patient CM. The patient, a 38-year-old African American woman, was diagnosed as having SLE in 1997. Her SLE was manifested by discoid lesions, arthritis, and antiphospholipid antibody syndrome. In April 2002, she reported a 2-week history of flare, which was manifested as polyarthritis. This was treated with oral methylprednisolone (Medrol Dosepak).

By the time of her first study visit in May 2002, she reported marked improvement, and she subsequently remained asymptomatic (Figure 2C). Her reticulocyte C4d levels decreased with response to therapy, and remained low during the 16-month asymptomatic interval, despite persistently positive anti-dsDNA titers and abnormally low serum C4 levels.

Patient MK. The patient, a 38-year-old Caucasian woman, was diagnosed as having SLE in 1985. Her SLE was manifested by malar rash, photosensitivity, arthritis, and pleurisy. During 5 study visits spanning 20 months, she remained asymptomatic. Her reticulocyte C4d levels remained normal, despite abnormal levels of serum C4 and fluctuating anti-dsDNA titers (Figure 2D).

DISCUSSION

There is an urgent need for lupus biomarkers (23,24). Despite decades of advances in our understanding of the pathogenesis of SLE, few lupus biomarkers have been validated and widely accepted (25,26). Perhaps the most important consequence of this deficiency is the reluctance of the pharmaceutical industry, with promising therapeutics in hand, to embark on lengthy and costly clinical trials without reliable and accepted measures of clinical improvement (27).

It was established more than 25 years ago that proteolytic fragments of complement C4 are deposited at low levels on the surfaces of all normal erythrocytes (16,28), although the biologic significance of this phenomenon is unknown. In humans, C4 exists in 2 isoforms, C4A and C4B, which are encoded by 2 highly homologous (>99%) genes (29–31). Both isoforms exhibit further polymorphism at the protein and DNA levels, with >40 alleles (including null alleles) identified to date (29–32). Despite their striking sequence identity, C4A and C4B differ significantly in their functional features with regard to the expression of antigenic determinants, reactivity of the thioester site, hemolytic activity, and ability to bind/solubilize immune complexes (31–35). The activated thioester in C4A reacts predominantly with amino group–containing substrate and binds efficiently to immune complexes, whereas the activated thioester in C4B reacts preferentially with hydroxyl groups on carbohydrate substrates and binds effectively to erythrocytes (31–35). However, it should be noted that 2 erythrocyte blood group antigens, Rodgers and Chido, have been shown to correspond to the C4d region of C4A and C4B, respectively (16,28), suggesting that both C4A and C4B can bind to and persist on the surface membranes of normal erythrocytes. C4-null alleles or partial deficiency of C4A and/or C4B has been associated with human SLE (36–39).

In the present study, we identified reticulocyte C4d as a promising biomarker that should be rigorously evaluated for its potential in monitoring disease activity and making therapeutic decisions in patients with SLE. The rationale for our decision to focus on reticulocyte C4d was compelling. First, complement activation is known to participate in the pathogenesis of tissue damage in SLE (1,2). Second, serum C3 and serum C4 have been widely used in attempts to monitor disease activity in SLE. Although the utility of these measurements is a subject of controversy, it is generally held that in some patients, particularly those with lupus nephritis, these are useful tests (3–5,10,13,14). Surprisingly, these tests have not been replaced or improved upon despite enormous advances in our understanding of complement biology during the last 20 years.

We recently demonstrated that erythrocytes in patients with SLE are characterized specifically by abnormally high levels of erythrocyte C4d and abnormally low levels of erythrocyte CR1 (15). The capacity of C4-derived complement activation products to attach covalently to cell surfaces via thioester bonds suggests that erythrocytes, once tagged with C4d, may carry the molecule during the remainder of their lifespan (40,41).

This rationale led to the current studies of reticulocytes. The reticulocyte is a relatively neglected subject of investigation, viewed primarily as a transient stage of erythrocyte development. However, novel characteristics of the reticulocyte led to its consideration as an ideal

candidate for a lupus biomarker. It is short-lived, emerging from the bone marrow and circulating for only 1–2 days before developing into a mature erythrocyte. This suggests that phenotypic surface alterations of the reticulocyte most likely occur during its transient circulation. The reticulocyte is also readily and rapidly distinguished by flow cytometry from the more abundant mature erythrocytes. In addition, reticulocytes are immediately exposed to products of complement activation that are generated both locally and systemically and, therefore, serve as perfect targets for C4d deposition and circulating sentinels of the inflammatory response.

We initially studied C4d because it is present on normal erythrocytes, whereas C3d is not (16). Earlier work, however, showed that moderate levels of C3d could be detected on the erythrocytes of patients with autoimmune hemolytic anemia and cold agglutinin disease (42,43). Once we identified abnormally elevated erythrocyte and reticulocyte C4d levels, we considered the possibility that erythrocyte and reticulocyte C3d may be even more sensitive biomarkers, since C3 activation is more distal in the enzymatic amplification cascade. We explored this possibility and discovered that both erythrocyte and reticulocyte C3d levels are specifically abnormal in SLE (data not shown). However, in contrast to what might be expected, elevated levels of C4d were detected more frequently than were elevated levels of C3d (data not shown).

There are at least 3 possible explanations for this observation. First, the mechanism responsible for generating reticulocyte C4d following activation of the classical pathway of complement may not routinely lead to the stable assembly of adequate levels of C3 convertases and deposition of C3 activation products on the reticulocyte surface. Second, a significant proportion of C4d detected on erythrocytes and reticulocytes from SLE patients may be independently derived through yet-to-be-elucidated mechanisms that do not lead to subsequent activation and proteolytic degradation of C3. This possibility is supported by our preliminary study, in which we detected considerable levels of C4d on reticulocytes and erythrocytes even in a C3-deficient environment (Liu C-C, et al: unpublished observations).

The third possible explanation is that reticulocytes bearing C3-derived ligands may be cleared more rapidly than those bearing only C4-derived ligands. Schreiber and Frank (44,45) originally demonstrated that erythrocytes coated with cold agglutinin IgG/IgM and complement C3b are cleared/sequestrated rapidly by phagocytes in the spleen and liver. Other investigators have subsequently shown that erythrocytes bound by immune complexes opsonized with complement C3b are quickly stripped off by phagocytic cells, leaving behind C3dg fragments that are covalently bound on cells (43,46,47). Nevertheless, it is possible, and perhaps likely, that some erythrocytes coated with C3-derived ligands may be cleared from the circulation during these processes before completing a 120-day life cycle. However, similar studies have not been performed to determine the fate of erythrocytes bearing C4-derived ligands. The observation that C4d is present on healthy erythrocytes that survive for 120 days suggests that the biology and pathobiology of C4d-bearing erythrocytes is likely to be distinct from that of C3d-bearing erythrocytes. Because reticulocyte C4d was detected with greater sensitivity than was reticulocyte C3d, we focused on investigating the role of reticulocyte C4d as a biomarker of SLE disease activity.

Biomarker validation is a complex process that ultimately requires multicenter trials of diverse patient cohorts. A particular lupus biomarker may be most informative at a specific time point in the disease process, such as at the time of diagnosis, during the course of the inflammatory phase of the disease, or during an assessment of end-organ damage. In addition, a particular biomarker may indicate systemic or specific organ inflammation. Due to the heterogeneous and complex nature of SLE, it is unlikely that a single biomarker will demonstrate universal utility. However, the present study was performed to sample the diverse spectrum of lupus in an effort to generate compelling evidence in support of reticulocyte C4d as a promising lupus biomarker and with the hope of igniting and accelerating the validation process. As shown in Table 1, the demographic, clinical, and laboratory manifestations of the patients enrolled in this study indicate that this cohort is representative of the broad spectrum of the disease. These broad ranges of disease manifestations and activities were reflected by a broad range of reticulocyte C4d levels (Figure 1A and Tables 2 and 3). In addition, as we observed with erythrocyte C4d (15), the levels of reticulocyte C4d in a normal subject remain remarkably stable over days, weeks, and even years (Figure 1B). This suggested that even minor fluctuations may be biologically relevant.

It should be noted that a small fraction (6.4%) of patients with other diseases (n = 140) had elevated reticulocyte C4d levels that were comparable to those in some of the SLE patients (SMFI >4.68). Clinical diagnoses in these patients included rheumatoid arthritis in 1, idiopathic inflammatory myopathy in 2, Sjögren's syndrome in 2, scleroderma in 2, vasculitis in 1, and

hemophilia in 1. These data suggest that the reticulocyte C4d level may have utility in monitoring diseases other than SLE.

Initial examination of the capacity of the reticulocyte C4d level to reflect disease activity using the entire cohort of SLE patients showed that the value was moderately, yet significantly, correlated with the SELENA–SLEDAI scores ($r = 0.45$, $P < 0.0001$). Correlation between reticulocyte C4d levels and SLAM scores was significant, but rather weak ($r = 0.23$, $P = 0.003$). Upon further analysis using quartile groups of patients divided according to reticulocyte C4d levels (Table 3), we found that not only did the reticulocyte C4d levels correlate with both the SELENA–SLEDAI and SLAM scores, but the stronger correlation with the SELENA–SLEDAI was also supported by our hypothesis and rationale. The reticulocyte C4d level as an "instant messenger" of disease activity might be expected to more closely reflect the SELENA–SLEDAI score, which was designed to measure disease activity over the preceding 10 days, as compared with the preceding 30 days for the SLAM. We have begun a prospective study of patients with SLE to determine whether fluctuations in reticulocyte C4d levels can identify and predict meaningful clinical end points.

Preliminary indications that a biomarker will be a valid measure of disease activity and response to therapy will be most apparent in patients with a clinically obvious disease course and response to therapy, the interpretation of which does not require complex measures such as the SLAM and SELENA–SLEDAI that are themselves imperfect. For example, the diagnosis of SLE in patient JN was certain, as determined by the ACR criteria and the presence of anti-dsDNA antibodies in particular. The occurrence of a disease flare was also unmistakable, both subjectively, by physician's assessment, and objectively, by the presence of proteinuria and the findings on renal biopsy. As described in the case report, her reticulocyte C4d level steadily rose during the flare and abruptly decreased following therapeutic intervention (Figure 2A). In contrast, other laboratory tests were less reliable. Serum C4 was initially undetectable (<10 mg/dl) and was therefore not useful in capturing a subsequent flare. In fact, the serum C4 level was found to be increased (11 mg/dl) during the peak of the flare, although a decreased serum C4 level is typically thought to indicate a flare. Similarly, the anti-dsDNA titer remained unchanged at 10 from the time of the flare to the time of remission more than 10 weeks later. Finally, it is important to note differences in fluctuations of the erythrocyte C4d levels and the reticulocyte C4d levels. Whereas both values paralleled the disease course, less than 3 weeks after therapeutic intervention, the reticulocyte C4d level was significantly decreased, yet the erythrocyte C4d level was not significantly changed. By the time of the next clinic visit 2 months later, the erythrocyte C4d had also decreased to near-normal levels, thus supporting our focus on reticulocyte C4d as a novel biomarker of disease activity.

The potential of reticulocyte C4d as a lupus biomarker is further supported by longitudinal observation of 3 additional patients with varying disease courses (Figures 2B–D). These illustrative cases demonstrate that the reticulocyte C4d level remains low (normal) during prolonged asymptomatic intervals, whereas the serum C4 level remains low (abnormal) and at times undetectable (<10 mg/dl) and the anti-dsDNA titer remains positive at the same study visits. It is also apparent that the reticulocyte C4d level rises and falls in parallel with the clinical course.

From a clinical practice perspective, performance of the reticulocyte C4d assay is simple and practical both for physicians and for laboratory investigators. We have observed that the percentage of reticulocytes and the level of reticulocyte C4d remain stable for 3 days after sample collection and storage at 4°C (data not shown). These preliminary results suggest that reticulocytes do not mature in vitro as rapidly as they do in vivo, which provides a convenient window of time in which to review the "snapshot" of complement activation and disease activity present at the time of blood collection. This may permit physicians and investigators to transfer blood samples to commercial laboratories or other research institutions where the test will be performed. Because flow cytometry technology has increasingly become standard in most medical centers and research institutes, the reticulocyte C4d assay, which shows excellent intraassay/intrainvestigator and interassay/interinvestigator reliability and reproducibility, can easily be adapted for laboratory monitoring of SLE.

In summary, there is an urgent need for lupus biomarkers with which to monitor disease activity and to reliably identify and predict important clinical end points. This study identifies reticulocyte C4d as a promising candidate, based upon a biologically plausible mechanism, cross-sectional analyses, and longitudinal study of individual patients. Prospective multicenter validation of reticulocyte C4d may enhance clinical care and, specifically, facilitate clinical trials of new therapies for patients with SLE.

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