Usefulness of complement activation products in Chinese patients with systemic lupus erythematosus

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Abstract

Objective

To evaluate the roles of complement activation products C3d and C4d binding to lymphocytes in the diagnosis of systemic lupus erythematosus (SLE) in a cohort of Chinese patients.

Methods

96 patients with SLE, 44 patients with other autoimmune disease and 40 healthy control individuals were enrolled in this study. The levels of C3d and C4d binding to peripheral CD4+ T and CD19+ B lymphocyts (designated as T-C3d, T-C4d, B-C3d, B-C4d) was assessed by flow cytometry. The diagnostic values of these biomarkers were determined by receiver-operator characteristic analysis.

Results

The levels of T-C3d, T-C4d, B-C3d, B-C4d were significantly higher in SLE patients than patients with other disease and healthy controls ($p<0.01$). As diagnostic tools, T-C4d and B-C4d were 61.1% sensitive/94.3% specific and 63.9% sensitive/94.3% specific in differentiating SLE patients from patients with other disease and healthy controls, respectively. T-C4d and B-C4d were significantly associated with SLE disease activity as measured by the SLE disease activity index (SLEDAI) ($p<0.001$), low serum C3 ($p<0.001$), low serum C4 ($p=0.006$), anti-dsDNA (IIF) ($p=0.001$), and anti-dsDNA (ELISA) ($p=0.001$).

Conclusion

Complement activation products C3d and C4d binding to lymphocytes can reflect the disease activity of SLE and can be used as biomarkers for SLE.

Key words

systemic lupus erythematosus, complement, C3d, C4d, lymphocyte, biomarker

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Introduction

Systemic lupus erythematosus (SLE) is one of the most clinically and serologically diverse autoimmune diseases. For monitoring patients with SLE in clinical practice and observational studies, the European league against rheumatism have given some useful recommendations (1). Traditionally anti-double stranded DNA (anti-dsDNA) titers, and serum complement levels C3 and C4 have been used to diagnose and monitor patients with SLE (2, 3). High C3 could also be considered as a hallmark of inflammation (4). However some researchers found them less useful (5-8).

Complement system activation has linked more intimately to autoimmune diseases (9) and been playing a great role in the pathogenesis of SLE (10, 11). Complement activation products (CAP) may be also correlated with SLE. As early as 1988, Senaldi *et al*. found that serum levels of complement split product C4d was elevated in SLE patients. Serum C4d and C3d concentration was correlated with SLE disease activity (12). Levels of serum C3d were comparable in patients with active renal and extrarenal SLE (13). For the past several decades, the data from several studies have demonstrated that complement activation products binding to circulation cells, such as erythrocytes (14, 15), reticulocytes (16), platelets (17) and lymphocytes (18), can serve as diagnostic biomarkers and/or mornitoring disease activity. In this study we evaluated the levels of lymphocyte bound complement activation product C3d/C4d (LB-CAP) in patients with SLE, with other autoimmune disease, and healthy control subjects by flow cytometric assay. We also investigated the potential correlations between LB-CAP levels and disease activity as well as clinical manifestations in our SLE patients.

Patients and methods

Study participants and blood specimens

All study participants were 18 years of age or older and provided written informed consent. This study was approved by the Peking University First Hospital Ethics Committee.

Consecutive 96 SLE patients and 44 patients with other autoimmune diseases were recruited from July 2011 to February 2012. They were either in-patients or out-patients of the Department of Rheumatology and Clinical Immunology in Peking University First Hospital. All SLE patients met the 1982 (19) or 1997 (20) American College of Rheumatology revised criteria for the classification of definite SLE. SLE disease activity was measured using the SELENA-SLEDAI (21) and modified-SELENA-SLEDAI (low complement and positive anti-dsDNA were not included in SELENA-SLEDAI). The demographic and clinical characteristics of the 96 SLE patients are listed in Table I.

The other autoimmune diseases included rheumatoid arthritis (n=21), primary Sjögren's syndrome (n=11), systemic vasculitis (n=6), systemic sclerosis (n=2), psoriatic arthritis (n=4).

Healthy control subjects: 40 healthy control individuals were recruited through the Center of Healthy Examination of Peking University First Hospital. To confirm their healthy status, these participants completed a brief questionnaire regarding their obvious medical conditions.

Flow cytometry characterisation

At the time of enrolment, a 9 ml peripheral blood sample from each study participant was collected in a blood-collection tube containing EDTA as an anticoagulant (Greiner, German). The mononuclear cells were isolated by Ficoll density gradient centrifugation method within 24 hours and then stored in liquid nitrogen.

Mouse monoclonal antibodies that recognise human C3d (Abcam, USA) or C4d (Quidel, San Diego, CA) were conjugated with fluorescein isothiocyanate (FITC) using the Easylink FITC Conjugation kit (Abcam, USA). Anti-human CD4 or CD19 monoclonal antibodies conjugated with phycoerythrin (PE) and allophycocyanin (APC) were purchased from BD company in the United States. All antibodies were used at a concentration of 5 ug/ml. After standard staining procedures, cells were acquired by BD Influx™ flow

Table I. Clinical characteristics of the 96 patients with SLE.

Characteristics	SLE patients
Age, (mean ± SD)	34.9±11.6
Disease duration, median (IQR) years from diagnosis to study entry	3, (1.6)
Malar rash (n=52)	54.2%
Discoid rash (n=1)	1.4%
Photosensitivity (n=16)	16.7%
Oral ulcer (n=30)	31.3%
Arthritis (n=53)	55.2%
Serositis (n=9)	9.4%
Renal disease (n=59)	61.5%
Neurologic disease (n=15)	15.6%
Raynaud's phenomenon (n=6)	6.3%
Haematologic manifestations	
Leukopenia (n=64)	66.7%
Anaemia (n=54)	56.3%
Thrombocytopenia (n=35)	36.5%
Immunologic test result ever positive	
Antinuclear antibodies (n=99)	100%
Anti-dsDNA (IIF or ELISA) (n=77)	80.2%
Anti-Sm (n=22)	22.9%
Antiphospholipid antibody (n=20)	20.8%
Anti-SSA (n=54)	56.3%
Anti-SSB (n=16)	16.7%
Anti-nRNP (n=36)	37.5%
Anti-rRNP (n=20)	20.8%
Immunologic test result positive at study visit	
Anti-dsDNA (IIF) (n=12)	12.5%
Anti-dsDNA (ELISA) (n=33)	34.4%
Reduced serum C3 level (n=35)	36.5%
Reduced serum C4 level (n=33)	34.4%
SELENA-SLEDAI score, median (range)	4, (0-20)
Modified SELENA-SLEDAI score, median (range)	4, (0-18)

IQR: interquartile range; IIF: indirect immunofluorescence; ELISA: enzyme-linked immunosorbent assay.

Table II. C3d and C4d on $CD4^+$ T cells and $CD19^+$ B cells.

Level	SLE	OAD	HC	*p*-value (SLE *vs.* OAD)	*p*-value (SLE *vs.* HC)	*p*-value (OD *vs.* HC)
T-C3d	5.9 ± 3.9	3.7 ± 1.5	1.0 ± 0.4	0.008	<0.001	0.02
T-C4d	8.7 ± 5.8	3.7 ± 1.2	1.4 ± 0.5	<0.001	<0.001	0.18
B-C3d	6.3 ± 3.8	3.8 ± 1.3	1.5 ± 0.4	0.002	<0.001	0.043
B-C4d	9.9 ± 6.3	4.1 ± 1.6	3.5 ± 1.1	<0.001	<0.001	0.744

Data shown are: mean ± SD of the specific median fluorescence intensity (SMFI).
OAD: other autoimmune diseases; HC: healthy controls.

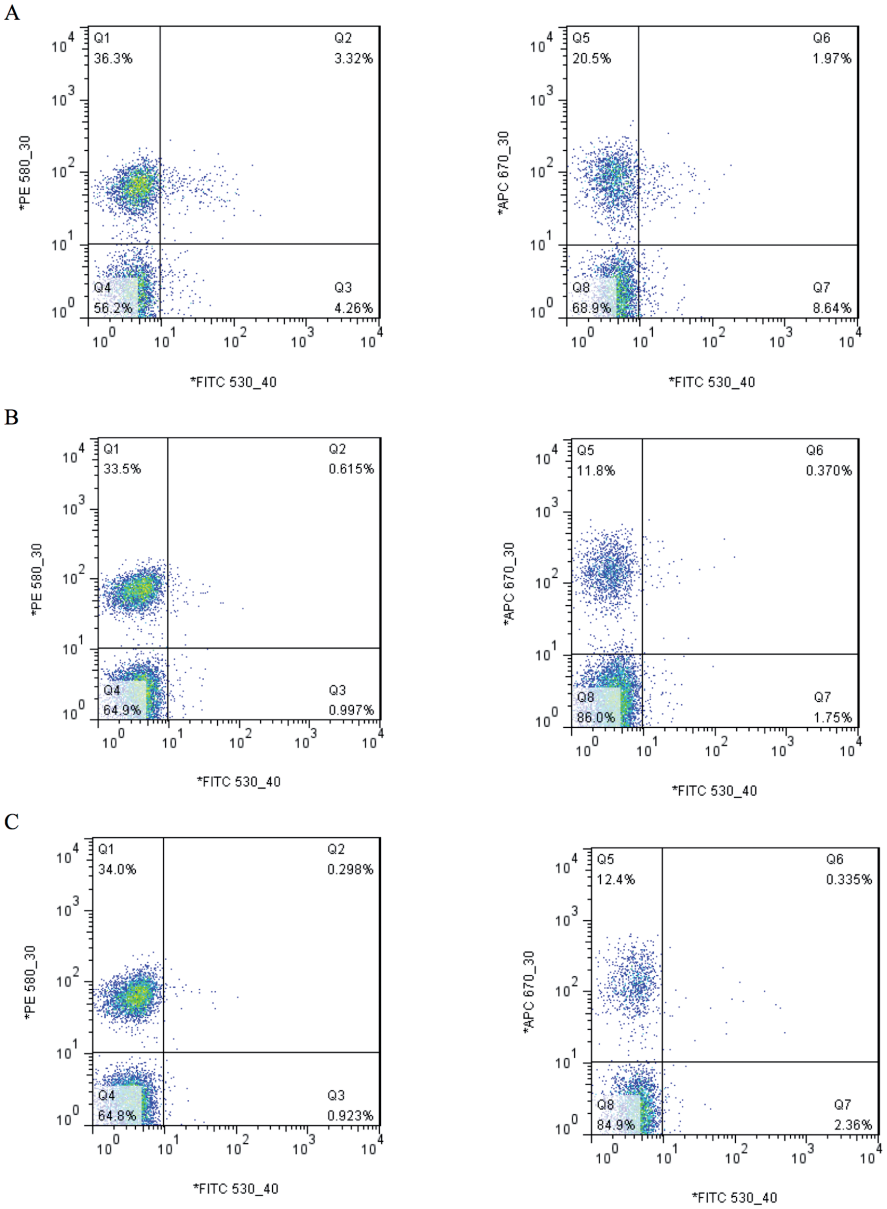

Fig. 1. Pseudocolour graph of flow cytometric analysis of C4d on the $CD4^+$ T cells (PE labelled) and $CD19^+$ B cells (APC labelled). Q2 quadrant represents the level of C4d on $CD4^+$ T cells. Q6 quadrant represents the level of C4d on $CD19^+$ B cells. **A**, **B**, and **C** represent a patient with SLE, a patient with other disease, and a healthy control, respectively.

cytometer and analysed by Spigot software (BD Immunocytometry System). The flow cytometer was calibrated by using Rainbow Fluorescent Particles (BD, USA). Levels of C3d or C4d on the surface of lymphocytes were expressed as specific median fluorescence intensity (SMFI) minus the isotype control SMFI.

Statistical analysis

Descriptive statistics was computed for continuous data including mean ± SD, medians, and interquartile range (IQR). Analysis of variance or Wilcoxon's rank sum tests for continuous variables and chi-square tests for categorical variables were used in analysing the demographic characteristics and experimental tests between the SLE group and the comparative groups (patients with other

Table III. Correlations between levels of T-C3d, T-C4d, B-C3d, and B-C4d.

Correlation coefficient / *p*	T-C3d	T-C4d	B-C3d	B-C4d
T-C3d		0.920/<0.001	0.747/<0.001	0.627/<0.001
T-C4d	0.920/<0.001		0.758/<0.001	0.686/<0.001
B-C3d	0.747/<0.001	0.758/<0.001		0.846/<0.001
B-C4d	0.627/<0.001	0.686/<0.001	0.846/<0.001	

Table IV. Correlations between biomarkers and SLEDAI and clinical manifestations.

	T-C4d		B-C4d		C3↓[¥]		C4↓[§]	
	r_s**	*p*	r_s	*p*	r_s	*p*	r_s	*p*
SELENA-SLEDAI	0.826	<0.001	0.827	<0.001	0.484	<0.001	0.399	<0.001
mSELENA-SLEDAI	0.750	<0.001	0.772	<0.001	0.412	<0.001	0.350	0.003
C3↓	0.410	<0.001	0.382	0.001	–	–	–	–
C4↓	0.318	0.006	0.254	0.031	–	–	–	–
Anti-dsDNA (IIF)	0.405	0.001	0.407	0.001	–	–	–	–
Anti-dsDNA (ELISA)	0.391	0.001	0.372	0.001	–	–	–	–
Photosensitivity	0.270	0.022	0.362	0.002	–	–	–	–

*: correlation coefficient; ¥: low serum C3; §: low serum C4.

Table V. Multivariate logistic regression analysis of LB-CAP.

	Odds ratio (95% CI)	*p*-value
T-C3d	1.64 (1.26–2.13)	<0.001
T-C4d	1.73 (1.30–2.30)	<0.001
B-C3d	1.72 (1.32–2.25)	<0.001
B-C4d	1.61 (1.24–2.09)	<0.001

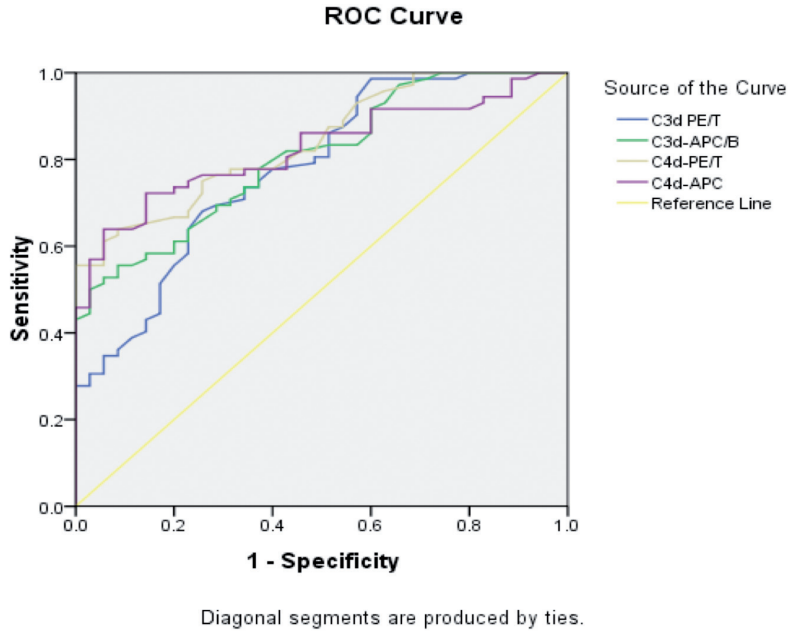

Fig. 2. ROC curve for indicators: T-C3d, T-C4d, B-C3d, and B-C4d.

diseases (OD) and healthy control subjects). Correlations between LB-CAP and SELENA-SLEDAI, modified-SELENA-SLEDAI were determined using the Spearman's rank correlation technique. Multivariate logistic regression analysis was used to determine an index value. Utility of LB-CAP in diagnosing SLE was assessed by the receiver-operating characteristic (ROC) analysis.

Results

Characteristics of study participants

The study population consisted of 96 SLE patients, 44 patients with other autoimmune diseases, and 40 healthy controls. The mean age of SLE patients was 34.9±11.6 years (range 18–75 years); all of them were Han nationality, and 83.3% were female. The mean ± SD age of the patients with other disease was 36.8±13.8 years (range 21–70 years); all of these patients were Han aslo, and 86.4% were female. The mean age of the healthy control subjects was 40.5±15.3 years (range 21–73 years); all of them were Han, and 84.6% were female. The age and gender of the three study groups were matched. Demographic and clinical characteristics of SLE patients are summarised in Table I.

Levels of C3d/C4d on lymphocyte from the three study groups

The mean ± SD levels of the entire study population of SLE, other diseases and healthy control subjects were T-C3d (5.9±3.9; 3.7±1.5; 1.0±0.4), T-C4d (8.7±5.8; 3.7±1.2; 1.4±0.5), B-C3d (6.3±3.8; 3.8±1.3; 1.5±0.4), B-C4d (9.9±6.3; 4.1±1.6; 3.5±1.1), respectively (Table II).

Levels of C3d and C4d were significantly elevated on both $CD4^+$ T lymphocyte and CD19+ B lymphocytes in SLE patients compared to patients with other autoimmune diseases and healthy controls ($p<0.05$) (Fig. 1). Levels of C4d on $CD19^+$ B cells and $CD4^+$ T cells were more elevated in patients with other autoimmune diseases than those of the healthy controls, but the difference was not significant ($p>0.05$). Levels of T-C3d, T-C4d, B-C3d, and B-C4d within a given patient were correlated (Spearman's correlations, $p<0.001$) (Table III).

Relationship between the biomarkers and SLE disease activity, as well as clinical manifestations

Unparametric correlation analysis showed that T-C4d and B-C4d were significantly associated with SLE disease activity index (SELENA-SLEDAI and modified-SELENA-SLEDAI) ($p<0.001$), low serum C3 ($p<0.001$), low serum C4 ($p=0.006$), anti-dsDNA

(IIF) (p=0.001), and anti-dsDNA (ELISA) (p=0.001). Lower serum C3 and C4 were also correlated with SELENA-SLEDAI and modified-SELENA-SLEDAI (p<0.001), but the correlation coefficients were less than 0.5 (Table IV). Further analysis showed that T-C4d and B-C4d were associated with photosensitivity (p=0.006, 0.031, respectively), but the correlation coefficients were lower than 0.5.

Utility of T-C3d, T-C4d, B-C3d, and B-C4d measures for SLE diagnosis

Multivariate logistic regression analysis showed that these LB-CAP indexes were risk factors of SLE. The OR and 95% CI were 1.64 (1.26–2.13), 1.73 (1.30–2.30), 1.72 (1.32–2.25), 1.61 (1.24–2.09) for T-C3d, T-C4d, B-C3d, and B-C4d respectively (Table V).

Receiver-operating characteristic (ROC) analysis was used to examine the diagnostic utility of T-C3d, T-C4d, B-C3d, and B-C4d in distinguishing patients with SLE from patients with other diseases and healthy controls. The differentiating power of the four biomarkers was estimated using the area under the ROC curve (AUC). As shown in Figure 2, the AUCs for the T-C3d, T-C4d, B-C3d, and B-C4d assay were 0.780; 0.841; 0.807 and 0.824 respectively. Based on these data, according to Youden's index, it was estimated that T-C4d (SMFI >5.25) and B-C4d (SMFI >5.65) were 61.1% sensitive/94.3% specific and 63.9% sensitive/94.3% specific in differentiating patients with SLE from patients with other autoimmune diseases and healthy controls, respectively.

Discussion

Complement plays an important role in the complex pathogenesis of SLE (10). The complement system was thought to be activated during flares of SLE. Measurements of serum complements have been considered as the "gold standard" in monitoring disease activity of SLE patients (11), whereas other researchers have found them to be minimally useful (7).

In the recent decades, researchers found that complement activation products C4d covalently bound on cellular components are likely to live longer than their soluble counterparts and hence detected more reliably (22, 23). In the late nineties, C4d staining was introduced in daily clinical practice, playing a role in antibody-mediated mechanisms in allograft rejection (24). C4d is now increasingly recognised as a potential biomarker for other autoimmune diseases and tumours. Complement fragments C3d and C4d were found to present on circulation cells, such as erythrocytes, reticulocytes, platelets and lymphocytes. C3d and C4d, especially C4d bounding to circulation cells were significantly elevated in SLE patients and thus they were thought to be the potential biomarkers of SLE (25). In this study, we examined the complement activation products C3d and C4d present on lymphocytes in SLE patients. Since no significant differences were observed between $CD4^+$ T-CAPs and $CD8^+$ T-CAPs in previous studies. We believe that $CD4^+$ T cells play a more important role than $CD8^+$ T cells in autoimmune diseases. Therefore we only evaluated $CD4^+$ T cells in our study (18).

As what we expected, the levels of T-C3d, T-C4d, B-C3d and B-C4d were significantly higher in patients with SLE than patients with other autoimmune diseases and healthy control subjects. The levels of C4d presenting on T and B cells were significantly elevated in SLE patients than those of C3d. However C4d presenting on T and B cells were not significantly different in patients with other autoimmune diseases from healthy controls. These were not consistent with the results from Chau-Ching Liu (18).

Multivariate analysis showed that LB-CAPs were risk factors for SLE. However concerning the level of C4d on T and B cells were not significantly different between patients with other autoimmune diseases and healthy controls, the diagnostic performance of C3d and C4d presenting on lymphocytes was assessed in distinguishing SLE patients from patients with other autoimmune diseases and healthy controls. Based on the two higher AUC, T-C4d and B-C4d were chosen to be the potential biomarkers of SLE. The specificity of the two biomarkers was up to 94.3%. The specificity was better in our Chinese SLE patients than that of Caucasian lupus patients (18).

In this article, both low serum C3, C4 and T-C4d, B-C4d were correlated with SLEDAI score. The correlation coefficients of T-C4d, B-C4d were dramatically higher than that of low serum C3 and C4. That is to say complement activation product C4d on lymphocytes may be a better biomarker in reflecting the disease severity.

Due to the heterogeneous and complex nature of SLE, it is unlikely that a single biomarker will demonstrate universal utility. Some researchers found that an assay panel combining anti-dsDNA, ANA, anti-MCV, E-C4d and B-C4d is sensitive and specific for the diagnosis of SLE (26). We also believe that a correct diagnosis include not only laboratory parameters but also clinical symptoms and signs.

In summary, this is the first report of lymphocyte bearing complement activation products in a Chinese Han patients with SLE, with other autoimmune diseases and healthy people. Our data suggested that complement activation product C4d presenting on lymphocytes has high sensitivity and specificity not only in the diagnosis of SLE, but also in mornitoring the disease activity.

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