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Masayuki Yasuda · Yasuo Suenaga · Hiroaki Nishimukai
Masashi Nobunaga

Serum C4 levels in patients with systemic lupus erythematosus in remission

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Abstract Twenty-six patients with systemic lupus erythematosus (SLE) showing systemic lupus activity measure (SLAM) and SLE disease activity index (SLEDAI) scores ≤2, as well as a lower C4 concentration than the mean C4 levels of healthy controls, were selected to evaluate the C4 levels of SLE patients in remission. Serum complement (CH50), complement components (C4, C3, and B), complement split products (C4d, iC3b, and Bb), phenotypic expression of C4 allotype, C4 production by peripheral blood monocytes, peripheral blood lymphocyte subpopulation, and interferon-gamma (IFN-γ) production were examined. In patients with SLE in remission, the C4 consumption (C4d/C4) was found to increase, and this was considered to be the most important factor for determining the serum concentration of C4. However, the relevance of the C4 allotypic expression was minimal. The IFN-γ-stimulated production of C4 by peripheral blood monocytes in SLE patients in remission was also less than that of the healthy controls. The IFN-γ-stimulated production of C4 in SLE patients in remission correlated with the peripheral blood CD4-positive cells. Less IFN-γ was produced by lymphocytes of SLE in remission than by those of healthy adults. We conclude that the serum C4 levels in SLE patients in remission reflect the degree of C4 consumption as well as the disease state, rather than genetic influences such as a C4A defect.

Key words C4 · C4A defect · C4d · C4 production · Systemic lupus erythematosus (SLE)

M. Yasuda[1] (✉) · Y. Suenaga · M. Nobunaga
Department of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumibaru, Beppu 874-0838, Japan

H. Nishimukai
Department of Legal Medicine, Ehime University School of Medicine, Ehime, Japan

Present address:
[1]Center for Rheumatology and Rehabilitation, Beppu National Hospital, 1473 Uchikamado, Beppu 874-0011, Japan
Tel. +81-977-67-1111; Fax +81-977-67-5766
e-mail: yasudamk@beppu.hosp.go.jp

Introduction

It has been widely recognized that patients with systemic lupus erythematosus (SLE) frequently show abnormally reduced serum complement levels. Since the serum complement is one of the effector molecules of inflammation in rheumatic diseases, biologically active fragments generated from the early components of the complement may thus contribute to both local and systemic inflammatory reactions.[1] Since the decreased complement levels as well as the increased circulating immune complex levels are associated with disease activity and tissue injury, a correction in the abnormal complement profiles has been shown to be followed by a better prognosis.[2]

We have experience of SLE patients in remission who do not show either clinical symptoms or abnormal laboratory findings, but they still show decreases in their C4 level. If a low C4 is due to the activation and consumption of the classical pathway of the complement system, we should treat these patients to improve their low C4 level. On the other hand, if the low C4 is not due to an increased consumption, but instead to decreased C4 synthesis, then no complement-mediated tissue injury is present and thus there is no urgent need for extensive therapy.

In order to elucidate the reasons for and clinical significance of the presence of an isolated C4 decrease in SLE patients in remission, we studied the consumption and synthesis of C4. The former was examined by detecting complement split products and a C4A defect, since the rate of C4A partial defects in SLE patients has been reported to be higher than that in healthy adults.[3–10] The latter was examined based on the in vitro C4 production by peripheral blood monocytes without any specific stimulators, as well as when stimulated with interferon-gamma (IFN-γ). The monocytes in vitro have been shown to synthesize all of the complement components, and IFN-γ is known to be one of the specific stimulators for C4 production.[11–13] In addition, we studied the peripheral blood lymphocyte surface markers and IFN-γ production, since the synthesis

of complement protein is reported to be the result of cell-to-cell interactions, as well as due to humoral factors.

Materials and methods

Patients and normal controls

Patients with SLE in remission who had regularly attended the outpatient clinic were chosen. They all fulfilled the diagnostic criteria for SLE.[14] Their C4 concentrations were all less than the lower limit of normal C4 levels (45.0 ± 18.0 mg/dl) when their disease was diagnosed, and when their blood was collected their C4 concentrations had never been higher than 45 mg/dl. Blood samples from 11 healthy adults were used as normal controls.

In vitro complement production and complement assay

Mononuclear cell collection and characterization

The preparation and characterization of human monocytes were performed following Tsukamoto et al.[15] Heparinized peripheral blood was drawn, and mononuclear cells were separated by Ficoll Conray centrifugation at 2000 *g* for 10 min. Mononuclear cells were placed in fetal calf serum (FCS)-coated petri dishes in 5% CO_2 at 37°C for 1 h. Nonadherent cells were collected as lymphocytes, and adherent cells were collected as monocytes. More than 90% of the adherent cells were monocytes. Mononuclear cell surface markers were examined by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using monoclonal antibodies. The monoclonal antibodies against CD4 and CD8 were from Becton Dickinson Immunocytometry Systems.

Monocyte production of C4 and lymphocyte production of IFN-γ

Monocytes at 1×10^6/ml in RPMI1640 supplemented with 10% FCS were incubated for 7 days in 10% CO_2 at 37°C with or without 1000 u/ml IFN-γ (Otsuka Pharmaceutical, Japan) to obtain spontaneous production of C4. The C4 concentration in the culture supernatant was measured by the solid-phase enzyme linked immunosorbent assay (ELISA) method using diluted (×1000) polyspecific goat antibody to C4 (Boehringer Mannheim, Mannheim, Germany), and diluted (×2000) antigoat IgG bound with alkaline phosphatase (Boehringer Mannheim). The C4 concentration is expressed in milliunit.

Lymphocytes at 10^6/ml were incubated for 24 h in 10% CO_2 at 37°C with or without 100 u/ml interleukin-2 (IL-2) (Seikagaku, Tokyo, Japan) for spontaneous production of IFN-γ. IFN-γ concentration was measured using a solid-phase ELISA kit, and the concentration is expressed in picograms per milliliter.

Determination of the serum complement and plasma complement split product

Serum C4, C3, and B were measured by single radial immunodiffusion, and their values were expressed in milligrams per deciliter. Bb, iC3b, and C4d were measured using solid-phase ELISA kits (Quidel, San Diego, CA, USA) and expressed in micrograms per milliliter.[3] The C4d ratio (C4d/C4), Bb ratio (Bb/B), and iC3b ratio (iC3b/C3) were calculated by simply dividing their figures without adjusting the units.[3]

Determining the phenotypic expression of C4 allotype

The C4 phenotype was determined as previously described.[16,17] In brief, ethylenediaminetetraacetic acid (EDTA) plasma samples were treated with carboxypeptidase B and neuraminidase. Following electrophoresis in 0.7% agarose gel, the C4A and C4B bands were detected by immunofixation with anti-C4 antiserum.

Statistical analysis of the data

Statistical analysis of the data was performed using the JMP 2 computer software (SAS Institute, Cary, NC, USA). A one-way analysis of variance and Student's *t*-test were used to compare the mean values of the different groups. The rate of defects in C4A and C4B were compared by Fisher's exact test. The correlations between the individual parameters were evaluated by a linear regression. In order to select a subset of effects for a regression model of complement, a forward step-wise regression was performed by limiting the regressor effect probability to 0.250. All *P* values were determined based on two-tailed tests, and *P* values of less than 0.05 were considered to be significant.

Results

Patients' profile

A total of 26 patients with SLE were investigated. The serum C4 concentrations of 11 patients had constantly been lower than 27 mg/dl, while that of the other 15 patients was between 27 and 45 mg/dl at the time of blood collection. All patients were women and were aged 39.4 ± 11.8 years (mean ± SD), with a disease duration of 9.8 ± 6.8 years. All fulfilled the 1982 American Rheumatism Association diagnostic criteria for SLE.[14] At the time of blood collection, all patients were receiving prednisolone at an average of 7.2 mg/day, ranging from 3 to 20 mg/day, and 16 of them also took immunosuppressants. Cyclophosphamide was administered to 7 patients at 50 mg every day or every other day, azathioprine to 2 patients at 50 or 100 mg/day, and mizoribine to 7 patients at 50 or 100 mg/day.

The disease activity of the patients was evaluated using systemic lupus activity measure (SLAM) and SLE disease activity index (SLEDAI).[18,19] Regarding clinical symptoms, only Raynaud's phenomenon was observed in the fingers of

two patients during the winter. Urine protein was positive in two patients without heme-granular or red blood cell casts. Antibody to double-stranded DNA was negative in all patients. No leukocyte count of less than 3000/μl or thrombocytopenia of less than 10^5/μl was observed. Consequently, the SLAM score was ⩽2 depending on Raynaud's phenomenon and/or erythrocyte sedimentation rate (ESR) (mild), and the SLEDAI score was 0 or 2 depending on the presence of the low complement.

Serum complement levels and plasma complement split products

Patients with SLE showed a lower mean serum complement component (C4, C3) and higher mean complement split products (C4d, iC3b, Bb), as well as their mean ratio (C4d/C4, iC3b/C3, Bb/B), than the healthy controls (Table 1). However, only C4, C4d, and C4d/C4 were significantly different from those of the healthy controls.

C4 production by monocytes

The spontaneous production of C4 by monocytes was slight in both the SLE patient groups and healthy adults (Table 2).

Table 1. Serum or plasma concentration of complement components and split product

	SLE (n = 26)	Healthy (n = 11)	P[a]	t[a]
C4	31.1 ± 9.3	45.6 ± 7.3	<0.01	4.045
C3	99.6 ± 23.4	113.1 ± 17.0	ns	1.509
B	26.4 ± 5.8	25.6 ± 4.9	ns	0.380
C4d	4.67 ± 2.06	2.62 ± 1.54	<0.05	2.597
iC3b	21.1 ± 7.0	19.7 ± 4.0	ns	0.505
Bb	1.38 ± 0.46	1.18 ± 0.30	ns	1.174
C4d/C4	0.161 ± 0.073	0.057 ± 0.034	<0.01	3.877
iC3b/C3	0.219 ± 0.078	0.174 ± 0.038	ns	1.460
Bb/B	0.052 ± 0.014	0.046 ± 0.008	ns	1.114

ns, not significant
[a] Comparison between patients with systemic lupus erythematosus (SLE) and healthy adults

Table 2. Monocyte production of C4

	SLE	Healthy	P[a]	t[a]
C4 (no)[b]	0.032 ± 0.094 (26)	0.083 ± 0.128 (10)	ns	0.196
C4 (IFN)	0.792 ± 0.616 (19)	1.421 ± 0.805 (9)	<0.05	2.287

The number of patients is shown in parentheses
[a] Comparison between patients with SLE and healthy adults
[b] Stimulators: no, no stimulation; IFN, interferon-gamma

In the presence of IFN-γ, the production of C4 in both groups significantly increased, but the amount in the SLE patients was lower than that in the healthy adults.

Determination of the phenotypic expression of C4 allotype

No complete deficiency of C4A (C4AQ0,Q0) was observed in any sample, but a C4A defect was found in as many as 18 SLE patients, including two cases of C4AQ0–BQ0 (Table 3). The frequencies of the C4AQ0 and C4BQ0 allotypes in the SLE patients and healthy adults were not significantly different from each other (Fisher's exact test; P (C4A defect) = 0.0911, P (C4B defect) = 0.2880).

The mean serum C4 levels of the SLE patients with or without a C4A and/or a C4B defect were not significantly different from each other (data not shown).

Next, the effects of C4A (or C4B) defects on the C4 production of SLE patients and healthy adults were examined (Table 4). The mean C4 production of healthy adults with a C4A defect was statistically lower than that of adults without a C4A defect in the case of both spontaneous C4 production and IFN-γ-stimulated C4 production. Among the SLE patients, on the other hand, no difference was observed in the C4 production between the C4AQ0-positive group and the C4AQ0-negative group.

Characterization of lymphocytes in SLE patients

Lymphocyte surface markers

In patients with SLE, the percentage of CD4-positive cells was lower than that of healthy adults (Table 5). An increase

Table 3. Distribution of C4AQ0 and C4BQ0 allotypes in the SLE and healthy groups

	SLE	Healthy
C4AQ0,Q0	0	0
C4AQ0,3	10	3
C4AQ0,4	6	0
C4BQ0,Q0	1	1
C4BQ0,1	1	2
C4BQ0,2	1	0
C4AQ0-BQ0	2	0
No defect	4	2
Not available	1	0
No. of cases	26	8

Table 4. Effects of a C4A partial defect on C4 production by monocytes

	SLE		Healthy	
	No defect (n = 7)	Partial defect (n = 18)	No defect (n = 5)	Partial defect (n = 3)
C4 (no)[a]	0.066 ± 0.174	0.021 ± 0.040	0.158 ± 0.150	0.013 ± 0.023
C4 (IFN)	0.696 ± 0.477	0.772 ± 0.673	1.578 ± 0.664	0.530 ± 0.240

[a] Stimulators: no, no stimulation; IFN, interferon-gamma

Table 5. Lymphocyte surface markers and lymphocyte production of IFN-γ

	SLE ($n = 26$)	Healthy ($n = 11$)	P[a]	t[a]
%CD4	32.45 ± 13.71	41.55 ± 3.64	<0.05	2.152
%CD8	36.53 ± 11.24	30.00 ± 5.40	ns	1.825
IFN-γ (no)[b]	34.0 ± 96.3	949.2 ± 1951.4	<0.05	2.432
IFN-γ (IL-2)	3619.8 ± 5131.3	9439.6 ± 6036.8	<0.01	2.299

[a] Comparison between patients with SLE and healthy adults
[b] Stimulators: no, no stimulation; IL-2, interleukin 2

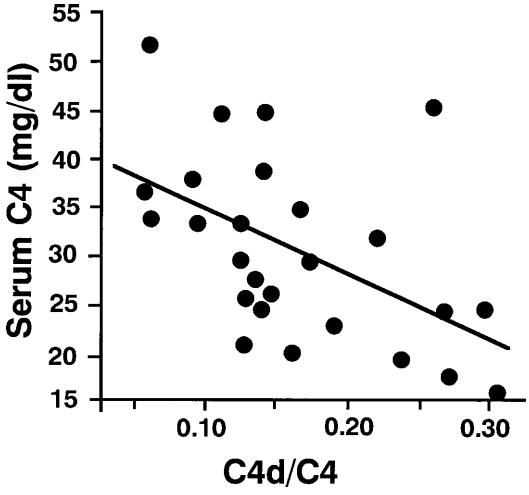

Fig. 1. Correlations between serum C4 concentration and C4d ratio in systemic lupus erythematosus (SLE) patients. The serum C4 concentration was not correlated with C4d, but showed a significant inverse correlation with the extent of C4 consumption expressed as the C4d ratio

in the percentage of CD8-positive cells was observed, but the difference was not statistically significant.

IFN-γ production by lymphocytes

In the absence of stimulators, the lymphocytes of SLE patients produced a significantly smaller amount of IFN-γ than those of healthy adults.

Factors correlating with the serum C4 concentration and C4 production

The serum C4 concentration and C4 production were analyzed to assess the correlations among the complement components, split products, split-product ratio, lymphocyte surface markers, C4A defects, and spontaneous as well as IL-2-stimulated production of IFN-γ as variables.

The serum C4 concentration of SLE patients showed a significant inverse correlation with the C4d ratio ($r = 0.516$, $n = 26$, $t = 2.95$, $P < 0.01$; Fig. 1), but was not correlated with any other complement components or their split products. Serum C4 concentration, regressed by a forward stepwise regression (mg/dl), $= 45.57 - 82.05 \times$ (C4d ratio) $+ 41.80 \times$ (spontaneous C4 production) $- 3.92 \times$ (C4A defect). $R^2 = 0.5018$, $n = 21$. C4d ratio: F ratio = 14.5896, $P = 0.0010$. Spontaneous C4 production: F ratio = 6.0513, $p = 0.0227$. C4A type: F ratio = 1.3852, $P = 0.2524$ (positive C4A defect = 1, no C4A defect = 0).

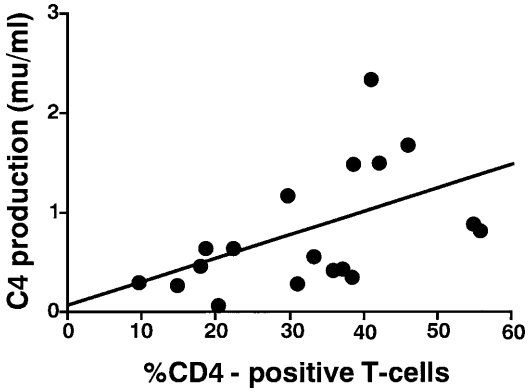

Fig. 2. Correlations between IFN-γ-stimulated C4 production and the percentage of CD4-positive T-cells. In the presence of IFN-γ, SLE patients produced a larger amount of C4 than without IFN-γ-stimulation. IFN-γ-stimulated C4 production was correlated with the peripheral blood percentage of CD4-positive T-cells

The IFN-γ-stimulated production of C4 in the SLE patients correlated with the percentage of CD4-positive cells ($r = 0.519$, $P < 0.05$, $n = 18$; Fig. 2). On the other hand, spontaneous C4 production in both SLE patients in remission and healthy adults were not significantly correlated with any variables.

Discussion

Since the serum complement concentration has been recognized to reflect the sum of the complement consumption and production, we examined both effects on serum C4 concentration. The patients selected were all in clinical remission without any constitutional signs of active disease.[18,19] As laboratory indices for inflammation and immunological abnormalities, a low C4 level or a tendency to show a low C4 level was a common finding.

We first examined C4 consumption. The C4d ratio in SLE patients in remission was significantly higher than that in healthy adults. In addition, a step-wise regression analysis of the serum C4 concentration indicated the importance of C4 consumption (C4d ratio).

The Bb ratio and the iC3b ratio in the SLE patients were not significantly higher than those in healthy adults. This finding conflicts with previous reports which showed an elevation in C3d, the C3d/C3 ratio, and the Bb/B ratio in SLE-active patients.[3–5,20] As we have indicated, the SLE patients in remission did not show either constitutional signs or ongoing organ damage. Therefore, C4 activation was not considered to be followed by C3 activation as well as tissue damage. The reasons for these absences remain speculative; C4-activating substances such as immune complexes might not be capable of C3 activation, and/or inhibitors for a classical pathway as well as an alternative pathway might sufficiently suppress the C3 activation.[1–3]

We then examined C4 production by peripheral blood monocytes as an experimental C4 production system in vitro.[13] No influence of a C4A defect on the monocyte production of C4 was observed, and such an influence was only slightly observed on serum C4 concentration. Previous authors have reported the serum C4 concentration in SLE patients with a C4A defect to be somewhat higher than in those without any C4A defect, thus suggesting that a C4A defect is not one of the major determinants of serum C4 concentration.[8] We also conclude that the effects of a C4A defect on the serum C4 concentration as well as C4 production are minimal in SLE patients in remission.

Regarding the role of lymphocytes in the monocyte production of C4, we showed a significant correlation between the percentage of CD4-positive T cells and the IFN-γ-stimulated production of C4. The IFN-γ-stimulated production of C4 by monocytes has been reported previously.[11–13,15] The decrease in the number of CD4-positive T cells in SLE patients in remission might be followed by a reduction in IFN-γ production. However, the significant correlations observed between the IFN-γ-stimulated production of C4 and the percentage of CD4-positive T cells does not indicate a direct influence of IFN-γ produced by the contaminating CD4-positive T cells in monocyte preparation, because the amount of IFN-γ used in our experiment was able to maximally stimulate the monocyte production of C4. The participation of some kind of cytokine or cell-to-cell interactions in vitro, which mimicked the production of C4, may thus be more likely.[15] On the other hand, the monocytes might have already been primed by some humoral factors or cells, including CD4-positive T cells for a stronger reaction to IFN-γ in vivo before cell separation.

The lymphocytes of the SLE patients in remission did not produce ordinary amounts of IFN-γ. This finding correlates with those found in previous studies on active SLE patients.[21–23] The PHA-stimulated IL-2 production as well as the response to IL-2 in SLE patients in remission is reported to be as low as that in SLE-active patients.[22] Several authors have suggested the presence of genetically controlled immunological defects in SLE lymphocytes.[24] On the other hand, Murakawa and Sakane[25] reported that a decreased phytohemagglutinin (PHA)-stimulated IL-2 production can be reversed by pharbol myristate acetate (PMA), which enhances the protein kinase C (PKC) activity, thus indicating that abnormalities are not genetically controlled, but instead are based upon intracellular signal transduction.[25]

Taken together, the SLE patients in remission are thus also suggested to possess similar immunological abnormalities to those in SLE-active patients. However, we cannot conclude whether the functional abnormalities in SLE patients in remission are based on cell responsiveness to cytokines, or the effects of drugs such as corticosteroids and immunosuppressants.[26]

In addition, we should mention some of the limitations of our present experiment. Since the major C4 production site in the living body is the liver, it is difficult to extend our results from in vitro studies using monocytes to circulating C4.[13] The next limitation is that our monocytes and lymphocytes were a mixture of both cells. The production of C4 by cloned cells allows us to obtain further insight into the mechanisms of C4 production and the evaluation of serum C4 determination as one of the disease activity measures for SLE.

We conclude that the serum C4 levels in SLE patients in remission are mainly a reflection of C4 consumption, and partly of the decreased C4 production, as well as the disease state, rather than genetic influences such as a C4A defect. We must also recognize that C4 levels in SLE patients in remission reflect not only abnormalities in the complement system, but also in cell-to-cell interactions and humoral factors.

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