Mod Rheumatol (2001) 11:127–131

ORIGINAL ARTICLE

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Autoantibodies against C-reactive protein (CRP) in sera of patients with systemic rheumatic diseases

Received: July 1, 2000 / Accepted: January 9, 2001

Abstract An assessment of the frequency of serum autoantibodies against modified C-reactive protein (mCRP) in systemic rheumatic diseases and the association of these autoantibodies with clinical and laboratory findings in patients with systemic lupus erythematosus (SLE). Serum levels of autoantibodies against mCRP were measured by an enzyme-linked immunosorbent assay in 125 patients with SLE and in 213 patients with other systemic rheumatic diseases. The frequency of patients with high antimodified CRP antibody levels was 32% in SLE, 22% in systemic sclerosis (SSc), 19% in polymyositis/dermatomyositis (PM/DM), 43% in primary Sjögren's syndrome (pSS), 29% in rheumatoid arthritis (RA), 33% in mixed connective tissue disease (MCTD), and 43% in overlap syndrome. Serum levels of anti-mCRP antibody were significantly lower in SLE patients with persistent proteinuria ($P < 0.001$), cellular casts ($P < 0.01$), and hypoalbuminemia ($P < 0.05$). Serum anti-mCRP antibody levels in SLE showed a direct correlation with serum IgG levels ($P < 0.001$), serum anti-SS-A antibody levels ($P < 0.01$), serum anti-SS-B antibody levels ($P < 0.01$), and serum anti-U1-RNP antibody levels ($P < 0.05$). Inhibition experiments revealed that nonnative epitopes on the CRP molecule, termed mCRP, were the main target of the anti-mCRP antibodies detected. Autoantibodies against mCRP were frequently found in sera from patients with systemic rheumatic diseases, and may have a role in the immunopathogenesis of systemic rheumatic diseases, which are characterized by persistent inflammation.

Key words Anti-CRP antibodies · C-reactive protein · Modified CRP · Systemic lupus erythematosus · Systemic rheumatic disease

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Introduction

Systemic rheumatic diseases are characterized by aberrations such as the production of various autoantibodies, B-cell hyperactivity, and T-cell abnormalities. Thus far, autoantibodies to C-reactive protein (CRP) have been reported in one case of systemic lupus erythematosus (SLE).[1] Furthermore, autoantibodies to CRP and other acute-phase proteins have been reported previously in a group of patients suffering from toxic oil syndrome (TOS). Antibodies against CRP in patients with TOS were shown to be mostly of the IgG class and directed against a modified form of CRP (mCRP).[2] In a subsequent study, the incidence of anti-mCRP was shown to be high in SLE but low in other autoimmune diseases.[3]

CRP is an acute-phase protein in humans and other mammalian species. It is synthesized primarily by the liver, and increases up to 1000-fold in serum as a response to various stimuli such as bacterial infection, inflammation, or tissue injury.[4,5] Two distinct conformational forms of CRP have been described, native CRP and a denatured form, termed mCRP.[6] Factors such as binding to a solid phase such as plastic, urea chelation, heat, or acid cause a nonproteolytic conformational change of the native CRP molecule, leading to the formation of mCRP and expression of new antigenic epitopes termed neo-CRP.[7]

Various biological functions have been demonstrated for mCRP, such as its ability to stimulate platelets, leukocytes, monocytes,[8] and megakaryocytes.[9] mCRP antigenicity has been shown to be expressed on the surface of B cells and natural killer cells,[10] and in inflamed rabbit liver and muscle.[11] It was reported that mCRP binds specifically to immune complexes in vivo, and thus might potentiate effector reactions that participate in immune complex removal.[6] Recently, mCRP was shown to be expressed on the surface of rat liver Kupffer cells, and to exhibit a selectin-like activity participating in adhesion.[12]

In the present study, we measured serum anti-mCRP antibody levels in patients with systemic rheumatic diseases such as SLE, systemic scleroderma (SSc), polymyositis/

dermatomyositis (PM/DM), primary Sjögren's syndrome (pSS), rheumatoid arthritis (RA), mixed connective tissue disease (MCTD), and overlap syndrome, and investigated the possible correlation between clinical and laboratory parameters and anti-mCRP antibody levels in patients with SLE.

Patients and methods

Patients

We performed a retrospective study of 356 consecutive patients with systemic rheumatic diseases who were actively followed at the Collagen Disease Clinic of the International Medical Center of Japan. There were 125 patients with SLE, 24 with SSc, 16 with PM/DM, 77 with pSS, 102 with RA, and 12 with MCTD. Serum samples from 49 healthy volunteers were tested as controls.

SLE, RA, and SSc were diagnosed according to the American College of Rheumatology criteria.[13–15] Primary SS was diagnosed according to the Copenhagen criteria,[16] PM/DM was diagnosed according to Bohan's criteria,[17] and MCTD was diagnosed according to Kasukawa's criteria.[18]

For the 125 SLE patients, we used the patients' notes, which charted the entire clinical history of the disease, to investigate the presence of the following clinical and laboratory indicators: malar or discoid rash, photosensitivity, oral ulcers, alopecia, arthritis, arthralgia, pericarditis, pleuritis, convulsions, psychosis, thrombosis, spontaneous abortions, sicca syndrome, persistent proteinuria ($\geq$0.5 g/day or $\geq$3+ if quantification was not performed), cellular casts, decreased creatinine clearance (<60 ml/min), hemolytic anemia, leukopenia (<4000/mm^3), lymphopenia (<1500/mm^3), thrombocytopenia (<10^5/mm^3), the presence of fluorescent antinuclear antibodies (FANA), LE cells and a positive LE test, anticardiolipin antibodies, circulating immune complexes, low C3 (<60 mg/dl) and low C4 (<10 mg/dl) levels, biological false positivity for syphilis, lupus anticoagulants, hypoalbuminemia (<3.5 g/dl), and an elevated erythrocyte sedimentation rate (>30 mm/h).

Anti-double-stranded (ds) DNA antibody, anti-single-stranded (ss) DNA antibody, anti-U1-RNP antibody, anti-Sm antibody, anti-SS-A antibody, and anti-SS-B antibody were assayed using enzyme-linked immunosorbent assay (ELISA) kits (Medical Biological Laboratory, Nagoya, Japan). Serum levels of immunoglobulin (Ig) G, IgA, and IgM were measured by laser nephelometric assay.

ELISA for antibody binding to CRP

Antibody activity to mCRP was determined by an ELISA as described previously. CRP was obtained from Chemicon International Inc. (CA, USA). Polystyrene microtitre plates were coated with 100 µl CRP (2.5 µg/ml in 0.15 M Tris-HCl buffer, pH7.6) by overnight incubation at 4°C. After blocking with 200 µl 1% BSA in Tris-HCl buffer for 30 min, sera were diluted 1:200 and incubated overnight at 4°C in duplicate CRP-coated wells and duplicate bovine serum albumin (BSA)-coated wells. Bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat antihuman IgG antibody (1:2000). The peroxidase substrate OPD and H_2O_2 was used as the chromagen. Absorbancy at 490 nm (A490) was measured using an automated spectrophotometer.

Inhibition assay

CRP was modified in 8 M urea containing 10 mM EDTA according to the method described prevoiusly.[7] The preparation was then dialyzed against phosphate buffered saline (PBS) and insoluble material was removed by centrifugation. The capacity of urea/EDTA-modified CRP and native CRP to block antibody binding in sera to solid-phase CRP was measured by adding increasing amounts of native or modified CRP to sera with elevated anti-mCRP activity. The final serum concentration was 1:200, and the incubation time at room temperature was 1.5 h. The residual IgG antibody binding capacity to solid-bound CRP was determined by ELISA as described above.

Statistical analysis

Data are given as the mean and standard deviation (SD) or the median and interquartile range. Data analyses were performed using a statistical analysis software package (StatFlex, ViewFlex, Tokyo, Japan) to evaluate the Wilcoxon *T*-test, Mann–Whitney *U*-test and Spearman's rank correlation test. Differences with P values less than 0.05 were considered to be statistically significant.

Results

IgG anti-mCRP antibodies in connective tissue disease

The frequency of patients with high anti-mCRP antibody levels ($\geq$0.170; mean + 2SD of antibody levels in 49 normal subjects) was 40 of 125 (32%) for SLE, 9 of 24 (22%) for SSc, 3 of 16 (19%) for PM/DM, 33 of 77 (43%) for pSS, 30 of 102 (29%) for RA, 4 of 12 (33%) for MCTD, and 3 of 7 (43%) for overlap syndrome. The median levels of anti-mCRP antibody in patients with SLE ($P < 0.001$), SSc ($P < 0.01$), SS ($P < 0.001$), RA ($P < 0.001$), and overlap syndrome ($P < 0.01$) were significantly higher than those in normal volunteers (Fig. 1).

Relationship of anti-mCRP antibody with clinical and laboratory findings in SLE

SLE patients were stratified according to the presence or absence of 34 clinical and laboratory parameters during past or present illness. As shown in Table 1, the levels of anti-mCRP antibody were significantly lower in patients who had had the following clinical and laboratory abnormalities:

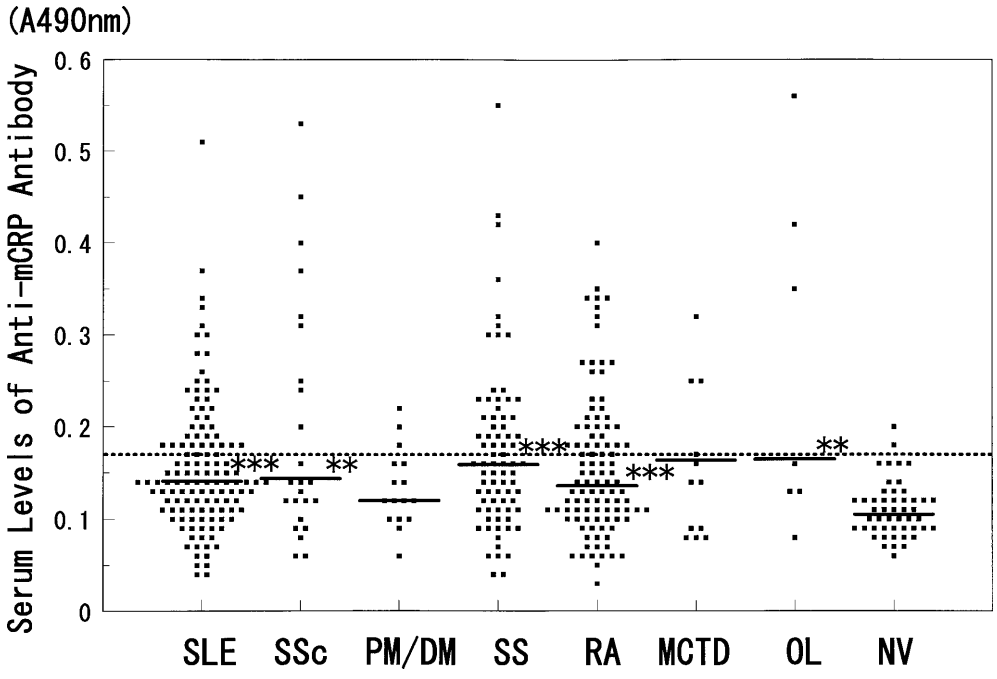

Fig. 1. Serum levels of anti-mCRP antibody in patients with systemic rheumatic diseases. *Horizontal bars* indicate the median values. *NV*, normal volunteers. The *dotted line* indicates the upper limit of the normal range (mean + 2SD value of NV). ***$P < 0.001$, **$P < 0.01$, significantly different compared with NV. *CRP*, C-reactive protein; *SLE*, systemic lupus erythematosus; *SSc*, systemic scleroderma; *PM/DM*, polymyositis/dermatomyositis; *SS*, Sjögren's syndrome; *RA*, rheumatoid arthritis; *MCTD*, mixed connective tissue disease; *NV*, normal volunteers

Table 1. Comparison of serum levels of anti-CRP antibody levels in lupus patients with and without clinical or laboratory findings during the whole course of the disease

			Anti-CRP antibody levels	
		Patients (*n*)	Median	(Q1–Q3)
Persistent proteinuria	Present	50	0.133	(0.099–0.156)*
	Absent	65	0.154	(0.125–0.205)
Cellular casts	Present	34	0.119	(0.092–0.153)**
	Absent	81	0.145	(0.125–0.204)
Hypoalbuminemia	Present	54	0.127	(0.102–0.168)***
	Absent	61	0.146	(0.126–0.213)

Among 34 clinical and laboratory findings, only those showing a statistically significant difference are indicated
CRP, C-reactive protein
* $P < 0.001$; ** $P < 0.01$; *** $P < 0.05$

persistent proteinuria ($P < 0.001$), cellular casts ($P < 0.01$), and hypoalbuminemia (<3.5 g/dl, $P < 0.05$). These tendencies were unchanged after serum IgG levels were taken into account.

Serum levels of anti-mCRP antibody in lupus patients showed a positive correlation with the following laboratory findings: serum IgG levels ($P < 0.001$), serum anti-SS-A antibody levels ($P < 0.01$), serum anti-SS-B levels ($P < 0.01$), and serum anti-U1-RNP antibody levels ($P < 0.05$) (Table 2).

Table 2. Spearman's correlation coefficients (*r*) between anti-mCRP antibody and laboratory findings

	r	*P*
IgG	0.472	<0.001
Anti-SS-A	0.249	<0.01
Anti-SS-B	0.258	<0.01
Anti-U1-RNP	0.197	<0.05

Inhibition of anti-mCRP antibodies

Binding of CRP to polystyrene causes conformational changes exposing nonnative regions of the pentameric CRP molecule, a form termed mCRP. To test whether anti-mCRPantibodies in autoimmune sera were directed against epitopes expressed on both native and modified CRP or on modified CRP alone, we compared the capacities of urea/EDTA-modified CRP and native CRP to block antibody binding to plate-bound CRP in 12 representative sera with high anti-mCRP antibody levels from two patients with SLE, three with SSc, three with SS, two with RA, and two with overlap syndrome. As shown in Fig. 2, a negligible capacity to inhibit antibody binding was seen with native CRP added at up to 40μg/ml, whereas mCRP caused a dose-dependent decrease in antibody binding, with signifi-

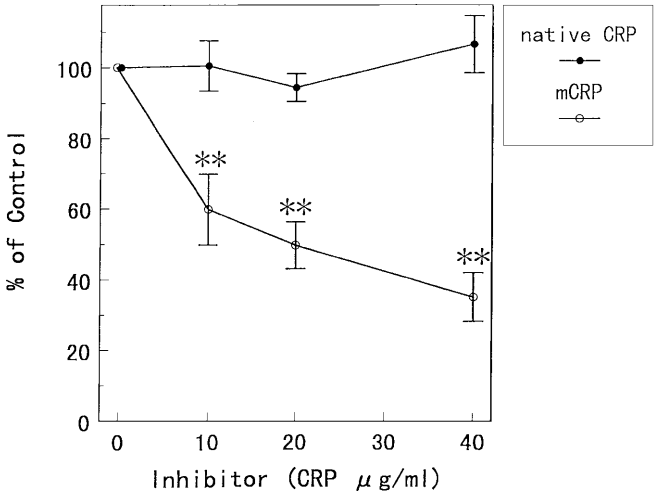

Fig. 2. Inhibition of anti-mCRP antibody binding to solid-phase CRP by native CRP and urea/EDTA-modified CRP. These are composite results (mean ± SEM) from 12 patients with high anti-mCRP antibody. $^{**}P < 0.01$, significantly different from data without inhibitor

cant inhibition of 40%, 50%, and 65% at concentrations of 10, 20, and 40 μg/ml, respectively ($P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively, Wilcoxon T-test).

Discussion

Antibodies against mCRP were first described in a patient with SLE[1] and later in patients suffering from TOS.[2] Bell et al.[3] showed that the incidence of anti-mCRP is high in SLE (39 of 50, 78%) but low in other autoimmune diseases: 3 of 40 (8%) in discoid lupus erythematosus, and 3 of 42 (7%) in SSc. Our study demonstrated that not only patients with SLE, but also those with other autoimmune diseases (SSc, SS, RA, and overlap syndrome) have a high incidence of antibodies against mCRP. This is the first report demonstrating that patients with autoimmune diseases other than SLE have a high incidence of anti-mCRP antibodies.

As to the relationship of anti-mCRP antibody levels with clinical and laboratory findings in SLE patients, anti-mCRP antibody levels were found to be significantly lower in patients who had had persistent proteinuria, cellular casts, or hypoalbuminemia. SLE is considered to be the prototype of human diseases that are mediated by immune complexes.[19] The glomerulonephritis of this disease is believed to result primarily from the deposition of immune complexes on the glomerular basement membrane. Recently, CRP was shown to bind specifically to model immune complexes (heat-aggregated IgG) and to potentiate effector reactions for immune complex removal.[6] It might be possible that circulating anti-mCRP antibody levels are reduced in patients with lupus nephritis because of adsorption to mCRP bound to immune complexes deposited in glomerular basement membranes.

Low CRP responses have frequently been observed in SLE despite higher levels of circulating IL-6.[20,21] It is possible that the presence of anti-mCRP antibody might be related to the low CRP response in SLE by interfering with the determination of CRP or by accelerating the clearance of CRP.[1] Alternatively, the decreased levels of circulating CRP in SLE may be explained by the adsorption of CRP on deposited immune complexes[6] or by impairment in the hepatocyte response to IL-6.[20]

Positive correlations were found in SLE between serum levels of anti-mCRP antibody and those of total IgG. One possible hypothesis that could explain this relationship is that various inflammatory cytokines, including IL-6, are induced by the chronic inflammation that accompanies the autoimmune phenomena in SLE. These cytokines are able to induce acute-phase proteins such as CRP in the liver. Since CRP has been shown to be capable of binding B-cells or natural killer cells through their Fcγ receptor,[10] and of exhibiting new epitopes in the form of mCRP,[6] this may lead to an immune response to mCRP by elevating thelevels of cryptic epitopes to the threshold required for T cell activation. In terms of total IgG, inflammatory cytokines are capable of activating B cells polyclonally and of elevating serum total IgG levels.

CRP has been found to bind to several nuclear constituents such as small nuclear ribonucleoproteins (snRNP), chromatin, and histones,[1,22,23] and it may participate in the recognition and clearance of nuclear material from damaged cells.[1] Recently, apoptotic cells were shown to display nucleosome ribonucleoprotein (RNP) blebs on their surface.[24] This further supports the possibility of enhanced binding of CRP and subsequent clearance. The binding of CRP to target proteins (SS-A, SS-B, and U1-RNP) in autoimmune diseases may also contribute to its role as an autoantigen in the context of epitope spreading,[25,26] and explain the correlation of anti-mCRP antibody levels with anti-SS-A, anti-SS-B, and anti-U1-RNP antibody levels. However, there is at least a possibility that nuclear antigens present in sera could bind CRP, and that antibodies against nuclear antigens would be found which would produce anti-mCRP antibody activity.

As a result of modification of native CRP molecules, e.g., by simple binding to a solid-phase surface, determinants termed mCRP are exposed.[6,7] Our inhibition studies using urea/EDTA-modified CRP demonstrated that the targets of mCRP antibodies in SLE, as well as in other systemic rheumatic diseases, are probably epitopes expressed on the mCRP molecule.

It is not clear whether differences in the epitopes recognized by anti-mCRP antibodies are present in each disease. In future studies, it will be necessary to consider the relationship of serum anti-mCRP antibody levels to CRP levels, and to conduct a longitudinal follow-up of anti-mCRP antibodies.

In conclusion, it seems likely that chronic inflammation in systemic rheumatic disease leads to increased presentation of cryptic epitopes on CRP molecules, which may elicit an immune response by elevating thelevels of cryptic epitopes to the threshold required for T cell activation. In the context of the potential effect of CRP and mCRP on host defense, the roles of anti-mCRP antibodies in the

immunopathogenesis of the multifarious lesions of systemic rheumatic disease remain to be clarified.

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