CONCISE REPORT

Antibodies to C reactive protein

B J Rosenau, P H Schur

Ann Rheum Dis 2006;65:674–676. doi: 10.1136/ard.2005.037895

Background: C reactive protein (CRP) is a known indicator of inflammation. Serum CRP is often raised in patients with inflammatory conditions.

Objective: To determine whether individuals make antibodies to CRP and whether this might affect serum CRP concentrations. **Methods:** An enzyme linked immunosorbent assay was developed for the detection of antibodies to CRP. Specificity of the reaction was determined by inhibition of the reaction.

Results: Sera from 413 patients were tested and 25 were found to be positive, particularly in patients with rheumatic diseases. Levels of anti-CRP did not correlate with serum CRP levels.

Conclusions: The presence of low CRP levels may not reflect the presence of antibodies to CRP.

Serum concentrations of C reactive protein (CRP) are a well known marker of inflammation. Levels of CRP are increased in many patients with rheumatoid arthritis, polymyalgia rheumatica, giant cell arteritis, infections, and malignancies, as well as in atherosclerosis.¹ In patients with systemic lupus erythematosus (SLE), CRP is less likely to be raised unless there is a co-existing infection.² The erythrocyte sedimentation rate (ESR)—which is also a non-specific indicator of inflammation—more accurately reflects SLE disease activity in patients without associated infection. Sjowall *et al* detected antibodies to CRP in 20 of 56 patients with SLE (36%) and anti-dsDNA antibodies, but found no significant correlation between serum CRP levels and anti-CRP antibodies.³

On the basis of these observations—and our recent findings of autoantibodies to serum amyloid A (SAA), another acute phase reactant⁴—we initiated the current study to determine the presence of antibodies to CRP in a variety of connective tissue conditions and explore its relation to serum CRP and the ESR.

METHODS

We examined 190 consecutive sera from patients with connective tissue diseases submitted to the clinical immunology laboratory for CRP measurement. Subsequently, based on the initial observations, we studied an additional 145 consecutive sera from patients with SLE and from 78 patients with rheumatoid arthritis. The medical records of all the patients were reviewed for their diagnoses.

An enzyme linked immunosorbent assay (ELISA) protocol was developed as follows. Irradiated plates (Costar 3590, Cambridge, Massachusetts, USA) were coated with CRP from human plasma (Sigma catalogue No C-4063; St Louis, Missouri, USA) at a concentration of 10 µg/ml in TRIS buffer pH 7.4, 50 µl/well. The plates were incubated overnight at 4°C. They were then washed with 0.1% Tween/TRIS (wash solution) twice, blocked with 1% bovine serum albumin (BSA), 0.1% Tween/TRIS (block solution) 200 µl/well, and

incubated for one hour at room temperature. After washing five times with wash solution, 50 µl of serum diluted 1:50 in block solution were added to a well containing antigen plus block, and a well containing block only to control for reactivity to block. Wells containing a highly reactive serum, a negative donor pool, or buffer served as controls. All were incubated overnight at 4°C. The plates were then washed five times with wash solution. Conjugate (Sigma A-3150 goat anti-human IgG alkaline phosphatase) was diluted 1:1000 in block solution, and 50 µl/well were added to each well.

The plates were incubated for one hour at room temperature and then washed five times with wash solution. Fifty microlitres of substrate (p-nitrophenyl phosphate; Sigma N2765 20 mg in 10 ml glycine buffer) were added to each well and incubated at room temperature for one hour, 20 minutes. This long incubation period was necessary to reach the peak of the reaction. The optical densities (OD) were read on a plate reader at 405 nm. The difference between the OD in the well with antigen plus block and the OD in the well with block only was recorded as the result.

Fifty blood donors were tested by this method to determine the mean OD $\pm$ 2 SD for healthy individuals. Levels of anti-CRP above 2 SD, that is 0.9369 OD, were considered to be positive. The medical records of the 253 patients were reviewed for diagnoses.

CRP was tested for by nephelometry.⁵ ESR was determined by standard methods.⁶

Specificity of detecting anti-CRP antibodies

Specificity of the anti-CRP method was determined by solid phase inhibition. A highly reactive serum was diluted fourfold from 1:25 to 1:6400 in TRIS buffer. Each dilution was added to a well coated with antigen and one uncoated well. After overnight incubation at 4°C, the antibody fluids from plate 1 were transferred to a freshly coated plate (plate 2), which was incubated overnight at 4°C. The test was completed on plate 1 in order to obtain a baseline test result (see table 2). Following overnight incubation the test on plate 2 was completed. Fluid phase inhibition was not successful.

RESULTS

We tested 413 patients, of whom 83 were positive (20%). The frequency of positivity in different conditions was as follows: 413 had a diagnosis of some form of arthritis, 190 had SLE, 103 had rheumatoid arthritis, 27 had osteoarthritis, 27 had some form of arthralgia or myalgia, and 66 had some other form of inflammatory arthritis.

Using phosphate buffered saline (PBS) or a carbonate-bicarbonate buffer for coating instead of TRIS buffer gave virtually identical results (not shown). An experiment comparing 1 µg/ml of antigen with 10 µg/ml gave virtually identical results (not shown).

Table 2 summarises the testing for specificity. Serum was first incubated with antigen on a plate overnight (plate 1), and then the supernatant was assayed on a second plate

Abbreviations: CRP, C reactive protein; SAA, serum amyloid A

Table 1 Frequency of anti-CRP in different diseases

Disease	Frequency*
Rheumatoid arthritis	23/103
SLE	43/190
Other connective tissue diseases	14/120

*Frequency positive/frequency patients. Some patients had multiple diagnoses.

CRP, C reactive protein; SLE, systemic lupus erythematosus.

(plate 2). Both plates were then assayed as described above. The difference between plate 1 and 2 represents inhibition of the antibody. As can be seen from table 2, as the serum was diluted out, the degree of inhibition disappeared as the antibody disappeared.

There was no association between anti-CRP levels and either CRP or ESR. Anti-serum amyloid A (SAA), as previously described,⁴ and anti-CRP levels tended to parallel each other, but the number of samples was small. Further testing is planned.

DISCUSSION

We have shown that some patients have antibodies to CRP, although the frequency of these antibodies is similar to that reported by others. In our studies 20% of 413 sera had detectable antibodies to CRP, as compared with 13% of 118 sera tested by Bell *et al.*,⁷ and 25% of 241 sera tested by Sjowall *et al.*³ However, we observed a lower frequency of anti-CRP antibodies in patients with SLE (23%) compared with Bell *et al.* (78% of 50 patients)⁷ and Sjowall *et al.* (48% of 27 patients).³ These differences between our studies and others may reflect a difference in methodology for detection of these antibodies, or a difference in the populations studied. We also observed anti-CRP in 23% of the patients with rheumatoid arthritis.

Two major variables in methodology were addressed during development of our ELISA method. Using the carbonate-bicarbonate buffer, as described by Bell *et al.*,⁷ and phosphate buffered saline, as described by Sjowall *et al.*,³ gave virtually identical results to those we obtained using the Tris buffer system described above. In addition, using 1 µg/ml, as used Sjowall *et al.*,³ and 10 µg/ml gave virtually identical results. We selected the TRIS buffer system because it has worked best in other ELISA systems we have used. We selected an antigen concentration of 10 µg/ml because it worked as well as 1 µg/ml, and ensured we were getting enough antigen on the plate. Another difference between the present methodology and that used by Bell *et al.*⁷ and Sjowall *et al.*³ was that in our ELISA system we blocked our plates with bovine serum albumin after incubating them with antigen. In our experience^{3,8,9} and that of others¹⁰ it is important to block ELISA plates to prevent non-specific reactions.

With regard to the populations tested, we studied 413 consecutive sera from patients whose blood had been sent to the hospital's clinical immunology laboratory for CRP testing. The other studies included patients with selected defined diagnoses. Our study represents a larger population than a

study cohort. Although the percentage positive for anti-CRP in some of our patients with different diseases may be less than in selected cohorts, we feel that our results are more likely to represent what is present in the general clinical community. In fact, although the frequencies of SLE patients with anti-CRP antibodies were higher in the cohort studies of Sjowall *et al.*³ and Bell *et al.*,⁷ the frequency of positivity in all patients was quite similar in those studies and our present one.

The significance of anti-CRP antibodies in specific rheumatic diseases is not clear. We know that immune complexes may be harmful, as has been shown in SLE. On the other hand, Sjowall *et al.* suggested that antibodies to CRP might result in lower serum CRP values, perhaps through an immune complex formation clearance mechanism.³ However, in the present study we found no association between CRP and anti-CRP levels.

It is also important to note that these anti-CRP and anti-SAA antibodies⁴ are probably directed not to the native form of the protein but to neoepitopes or misfolded proteins. This hypothesis is based on observations with both the SAA/anti-SAA and the CRP/anti-CRP systems, where we could demonstrate inhibition only with plate bound antigen and not fluid phase antigen.

CRP is produced mainly in the liver in response to proinflammatory cytokines, especially interleukin 6.¹¹ CRP exists in the serum as a non-glycosylated protein comprised of five identical non-covalently bound subunits.¹¹ Under certain conditions, such as changes in acidity, alkalinity, and high and low calcium concentrations, the CRP molecule dissociates irreversibly into monomers (mCRP).¹² This dissociation occurs when CRP binds to cells.¹³ Some of this binding occurs on the surface of cells undergoing apoptosis.¹⁴ As we found anti-CRP in patients in various inflammatory disorders such as SLE and rheumatoid arthritis, it is possible that CRP is binding to inflamed apoptotic cells, and this contributes to their antigenicity.

The current observations regarding the antigenicity extend the observations of autoantibodies in humans. It appears that many human proteins can become autoantigenic. These include ox-LDL,¹⁵ β2-glycoprotein 1,¹⁶ prothrombin,¹⁷ cardiolipin,¹⁸ IgG,¹⁹ idiotype,²⁰ α1-antitrypsin,²¹ fibrinogen,²¹ ceruloplasmin,²¹ and SAA.⁴ Why autoantibodies develop to these proteins but not to other plasma proteins will require further investigation. Studies are also needed to determine why CRP becomes immunogenic, what immune/pathological mechanisms are involved, and what are the biological implications of this antibody.

In conclusion, we have observed antibodies to CRP in various connective tissue diseases, including SLE. The lack of association between anti-CRP and serum CRP suggests that the low serum levels of CRP seen in some patients do not reflect the presence of these antibodies. Further studies in this area are under way.

Authors' affiliations

B J Rosenau, P H Schur, Division of Rheumatology/Immunology/Allergy, Department of Medicine, and the Department of Clinical Laboratories, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

Table 2 Inhibition assay

Serum dilution	1:25	1:100	1:400	1:1600	1:6400
Plate 1 (baseline)	2.290	1.650	0.710	0.203	0.113
Plate 2 (after transfer)	0.741	0.602	0.172	0.192	0.186

The values represent the optical densities of the wells coated with antigen minus the optical densities in uncoated wells.

Correspondence to: Professor Peter H Schur, Department of Clinical Laboratories, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA; pschur@partners.org

Accepted 14 September 2005

Published Online First 21 September 2005

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