

High Diagnostic Value of Anticalpastatin Autoantibodies in Rheumatoid Arthritis Detected by ELISA Using Human Erythrocyte Calpastatin as Antigen

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ABSTRACT. *Objective.* To develop a quantitative method of measuring autoantibodies against human calpastatin in rheumatoid arthritis (RA) and to determine their diagnostic value compared with other autoimmune and articular diseases.

Methods. We performed a highly sensitive ELISA for IgG and IgM anticalpastatin autoantibodies in human sera using human erythrocyte calpastatin as an antigen. Samples were diluted 1:2000 for the measurement of IgG and 1:400 for IgM.

Results. IgG anticalpastatin antibodies were found in the sera of 48 of 58 patients (82.8%) with RA. In contrast, IgG anticalpastatin antibodies were found in the sera of only 2 of 11 (8.3%) patients with osteoarthritis (OA). Compared to sera from patients with other autoimmune diseases, anticalpastatin antibody sensitivity for RA was better than that of systemic lupus erythematosus (5.6%), systemic sclerosis (0%), mixed connective tissue disease (0%), and Sjögren's syndrome (20%). IgG anticalpastatin antibodies also showed high specificity (96.1%) for RA. Almost 90% of patients with RA were positive for IgG or IgM anticalpastatin antibodies.

Conclusion. We have developed a simple, sensitive, specific, and quantitative ELISA for anticalpastatin antibodies that may have a high diagnostic value for RA. (J Rheumatol 2004;31:17–22)

Key Indexing Terms:
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CALPASTATIN
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Rheumatoid arthritis (RA) is an autoimmune disease characterized by extracellular matrix degradation, destruction of joint cartilage, and loss of function¹. The proteolytic pathways associated with cellular interactions seem to play an important role in joint cartilage destruction. In this process, 3 major enzyme families have been implicated: matrix metalloproteinases (MMP)², serine proteinases³, and

cysteine proteinases^{4,5}. Among them, calpains, calcium-dependent cysteine proteinases (EC 3.4.22.17), and calpastatin, their natural endogenous protein inhibitor, seem to be important. Levels of calpains are increased in quantity in the synovial fluid of patients with RA^{6–8}. Cysteine proteinases are secreted *in vitro* by tumor necrosis factor- α or interleukin 1-stimulated synovial fibroblast-like cells⁵. Further, calpains can degrade cartilage proteoglycan and calpastatin can inhibit this degradation⁹.

Anticalpastatin autoantibodies have recently been identified independently in sera of RA patients by 2 groups^{10,11}. Both groups identified anticalpastatin antibodies using screening of HeLa cells or the human placenta λ gt11 cDNA expression library using RA sera, followed by Western immunoblotting against recombinant calpastatin fusion proteins as an antigen. By this method, anticalpastatin antibodies were positive in 46–57% of sera from patients with RA. This sensitivity is lower than that of rheumatoid factor (RF), another well known autoantibody in RA. However, Mimori, *et al* found that the IgG fraction of RA sera that contains anticalpastatin antibodies is able to block calpastatin-mediated inhibition of calpain protease activity¹⁰, leading to the important role of anticalpastatin antibodies in the pathology of RA¹².

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We developed a highly sensitive ELISA for the detection of anticalpastatin antibodies in human sera using human erythrocyte calpastatin as the antigen. We also compared our results using sera from patients with RA and with other autoimmune diseases and other articular disease to determine the diagnostic value of the method.

MATERIALS AND METHODS

Sera. All patients with RA (n = 58), osteoarthritis (OA, n = 24), systemic lupus erythematosus (SLE, n = 18), systemic sclerosis (SSc, n = 19), and Sjögren's syndrome (SS, n = 5) fulfilled the criteria of the American College of Rheumatology^{13,14}. Patients with mixed connective tissue disease (MCTD, n = 9) fulfilled the criteria of the Ministry of Health, Labour and Welfare in Japan. No patient was treated with steroids. Sera from healthy donors (n = 30) were provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan). Samples were stored at -80°C until use.

The clinical and laboratory data reported here were obtained at the time the serum samples were drawn. These data included the measurement of serum C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).

ELISA for anticalpastatin antibodies. A 96-well flat bottom plate (Iwaki, Chiba, Japan) was coated with human erythrocyte calpastatin (2 µg/ml; Calbiochem-Novabiochem Corp., La Jolla, CA, USA) in 0.1 M borate buffer (pH 8.4) at 4°C overnight. After washing 3 times with Tris buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl), the wells were blocked with 2% bovine serum albumin (BSA)/1% gelatin/TBS at 37°C for 1 h. The wells were washed 3 times with TBS. In case of measuring IgG or IgM anticalpastatin antibodies, samples were diluted 1:2000 or 1:400 with 1% BSA/TBS, respectively. Diluted samples (100 µl) were added to wells at room temperature for 90 min. After washing 3 times with TBS containing 0.05% Tween 20 (TTBS), the bound antibodies were detected with peroxidase-conjugated rabbit IgG anti-human IgG or IgM antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA) using orthophenylenediamine as substrate. The plates were read at 492 nm. Absorbance values greater than the mean value plus 2 standard deviations (SD) calculated for the controls were considered positive. Using serial dilutions of the antisera, the linearity of the absorbance of IgG anticalpastatin autoantibody assay was conserved in the range from 1:1000 to 1:4000 dilution in this ELISA method.

Western blotting. Human erythrocyte calpastatin was run on sodium dodecyl sulfate-polyacrylamide gels (7.5% gel) and electroblotted by standard Western blotting techniques onto polyvinylidene difluoride membranes (Pro Blott; Applied Biosystems Inc., Foster City, CA, USA). After the electroblotting, membranes were cut into strips and incubated overnight in 1:50 diluted sera of RA patients who were IgG anticalpastatin antibody positive or negative. Mouse anti-human calpastatin monoclonal antibody (clone CSF3-3; Takara Shuzo Co., Tokyo, Japan) was used as a control. Then the membranes were developed using alkaline phosphatase-conjugated anti-human or mouse IgG antibodies (Jackson Immuno Research) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate.

Assay of MMP-3 and MMP-9. The concentrations of MMP-3 and MMP-9 in sera were determined by a one-step sandwich ELISA system using a human MMP-3 kit (Panaclear MMP-3; Daiichi Pure Chemicals Co., Tokyo, Japan) and an MMP-9 ELISA kit (MMP-9, human ELISA Biotrak System; Amersham Pharmacia Biotech, Buckinghamshire, England), respectively¹⁵.

Assay of IgM RF. IgM RF was measured using the rheumatoid factor IgM ELISA kit (Orgentec Diagnostika GmbH, Mainz, Germany). An IgM RF titer ≥ 20 IU/ml was considered positive.

Statistical analysis. Statistical analyses, including p values, were carried out using Super ANOVA (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant if p was less than 0.05 by Fisher's exact test.

RESULTS

Detection of anticalpastatin antibodies in sera of patients with RA and OA by ELISA. Using human erythrocyte calpastatin as an antigen, we performed ELISA for the detection of anticalpastatin antibodies in human sera. Concentrations of anticalpastatin antibodies in sera from patients with RA, OA, and controls are illustrated in Figure 1 and summarized in Table 1. IgG anticalpastatin antibody levels in patients with RA were significantly higher compared with OA patients and controls. IgG anticalpastatin antibodies were positive in 48 of 58 (82.8%) patients with RA. Although the levels of IgM anticalpastatin antibodies in RA were higher than OA and controls, the frequency of IgM anticalpastatin antibody positivity (20/58, 34.5%) was lower than that of IgG anticalpastatin antibody positivity. Further, when measuring IgG anticalpastatin antibodies, samples were diluted 1:2000 compared to 1:400 dilution for IgM. From these results it is suggested that the IgG isotype may be more frequently found in RA sera than IgM. RA patients produced anticalpastatin antibodies of either isotype in a high frequency (52/58, 89.7%), but only 16/58 (27.6%) had both. There was almost no difference in the frequency or levels of IgG or IgM anticalpastatin antibodies comparing OA and controls.

When the sera were analyzed by ELISA using the 27 C-terminal amino acids of calpastatin as an antigen for the presence of IgG anticalpastatin antibodies (Anti-calpastatin IgG ELISA kit; Progen Biotechnik, Heidelberg, Germany)¹⁶, only 8.7% of RA sera were above the cutoff of the assay (data not shown). This result was quite similar to Lackner and colleagues' (8.9%)¹⁷.

Western blotting with RA sera. To confirm the ELISA results, we performed Western blotting using IgG anticalpastatin positive or negative sera from RA patients. Figure 2 shows a typical pattern of Western blotting. Purified human erythrocyte calpastatin was run on SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting. An IgG anticalpastatin positive RA serum sample showed the same 60 kDa calpastatin band as a mouse monoclonal antibody that reacts with domain IV of calpastatin (clone CSF3-3) (Figure 2, lanes 1 and 3). However, an IgG anticalpastatin negative serum sample showed no band (lane 2). In this experiment, we used RA sera diluted 1:50; however, we could not see any band using sera diluted 1:2000 that we used in the ELISA (data not shown).

Sensitivity and specificity of IgG anticalpastatin antibody for RA. Figure 3 illustrates the levels of anticalpastatin antibodies and Table 2 summarizes the frequency of IgG anticalpastatin antibodies in sera from patients with systemic rheumatic diseases. IgG anticalpastatin antibodies were both sensitive (82.8%) and specific (96.1%) for RA. In contrast, sera from patients with other systemic rheumatic diseases, like SLE (1/18, 5.6%), SSc (0/19, 0%), MCTD (0/9, 0%), and SS (1/5, 20%), showed lower sensitivities. The likeli-

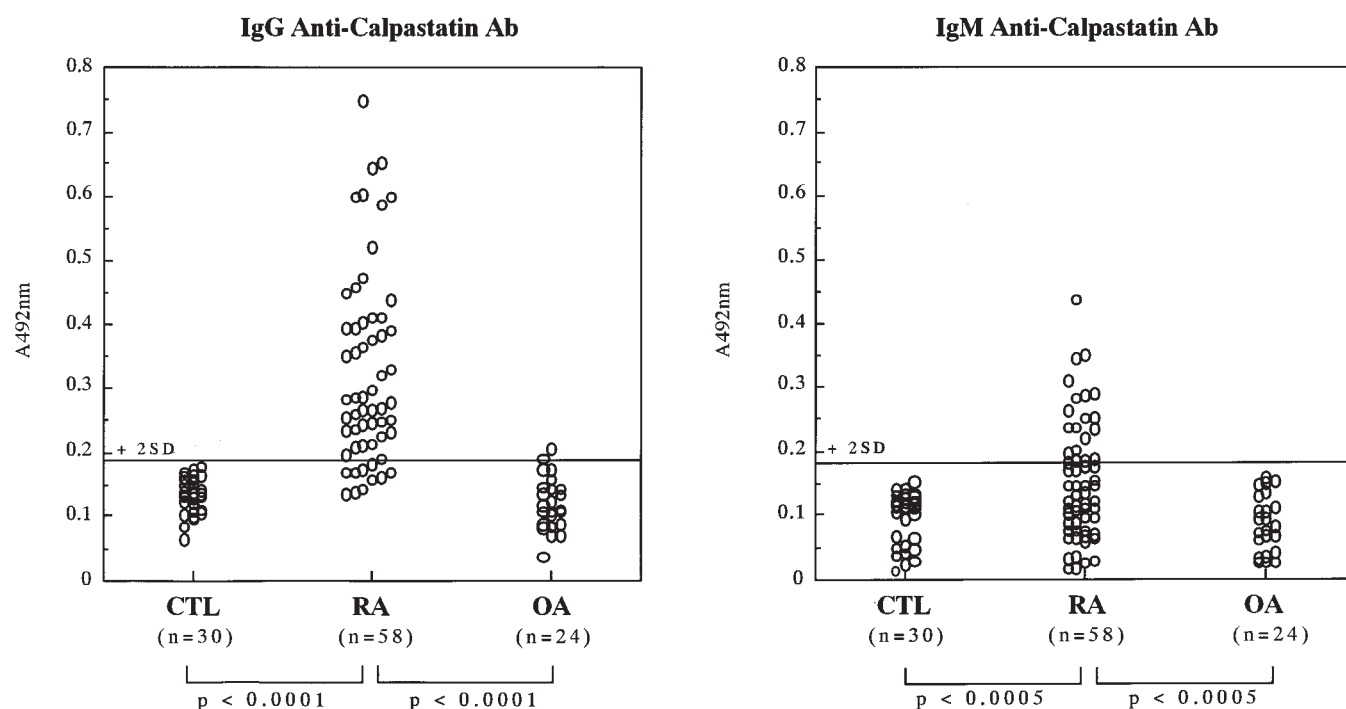


Figure 1. Levels of anticalpastatin antibodies (Ab) in sera from patients with RA and OA and controls (CTL). Horizontal lines show mean plus 2 standard deviations (2 SD) of absorbance of CTL.

Table 1. Frequency of anticalpastatin antibodies in sera from patients with RA and OA. Values are the number (%) positive. Anticalpastatin antibodies were detected by ELISA.

Sera, n	Anticalpastatin Antibody			
	IgG	IgM	IgG or IgM	IgG and IgM
RA, n = 58	48 (82.8)	20 (34.5)	52 (89.7)	16 (27.6)
OA, n = 24	2 (8.3)	0 (0)	2 (8.3)	0 (0)

hood ratio positive for RA was 21.2, which was a much higher value compared to the other diseases. However, IgM anticalpastatin antibodies were not so sensitive compared with IgG anticalpastatin antibodies: sensitivities were 34.5% for RA, 11.1% for SLE, 10.5% for SSc, 0% for MCTD, and 0% for SS. Even the highest likelihood ratio was 4.42 for RA. IgG anticalpastatin may be more useful than IgM anticalpastatin for the diagnosis.

Diagnostic value of individual autoantibodies for RA. To evaluate the diagnostic value of IgG anticalpastatin for RA, we compared the sensitivity and specificity of IgG anticalpastatin antibody with those of other autoantibodies for RA (Table 3). Although IgM RF, an autoantibody directed against Fc fragment of IgG, is one of the best known autoantibodies in RA, its sensitivity and specificity were lower than those of IgG anticalpastatin (66% and 87%, respectively)¹⁸.

Correlations between IgG anticalpastatin antibodies and

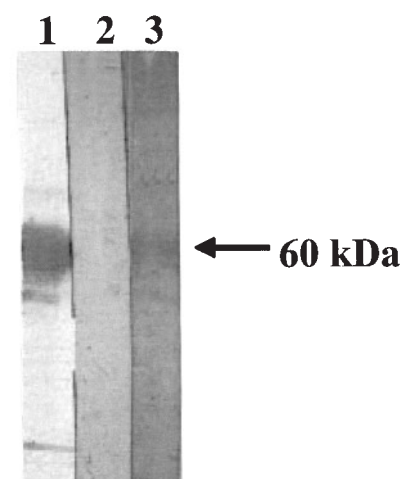


Figure 2. Western blotting of a calpastatin-specific monoclonal antibody and RA sera using purified human erythrocyte calpastatin as antigen. Purified human erythrocyte calpastatin was subjected to SDS-PAGE (7.5% gel), followed by Western blotting. Lane 1: mouse monoclonal antibody that reacts with domain IV of calpastatin (clone CSF3-3); lane 2: anticalpastatin antibody ELISA-negative RA serum; lane 3: anticalpastatin antibody ELISA-positive RA serum.

clinical and biological characteristics of RA. We investigated the clinical correlation of IgG anticalpastatin antibodies for 20 RA patients for whom detailed clinical and biological characteristics were available (Table 4). Seropositivity for IgG anticalpastatin antibodies was deter-

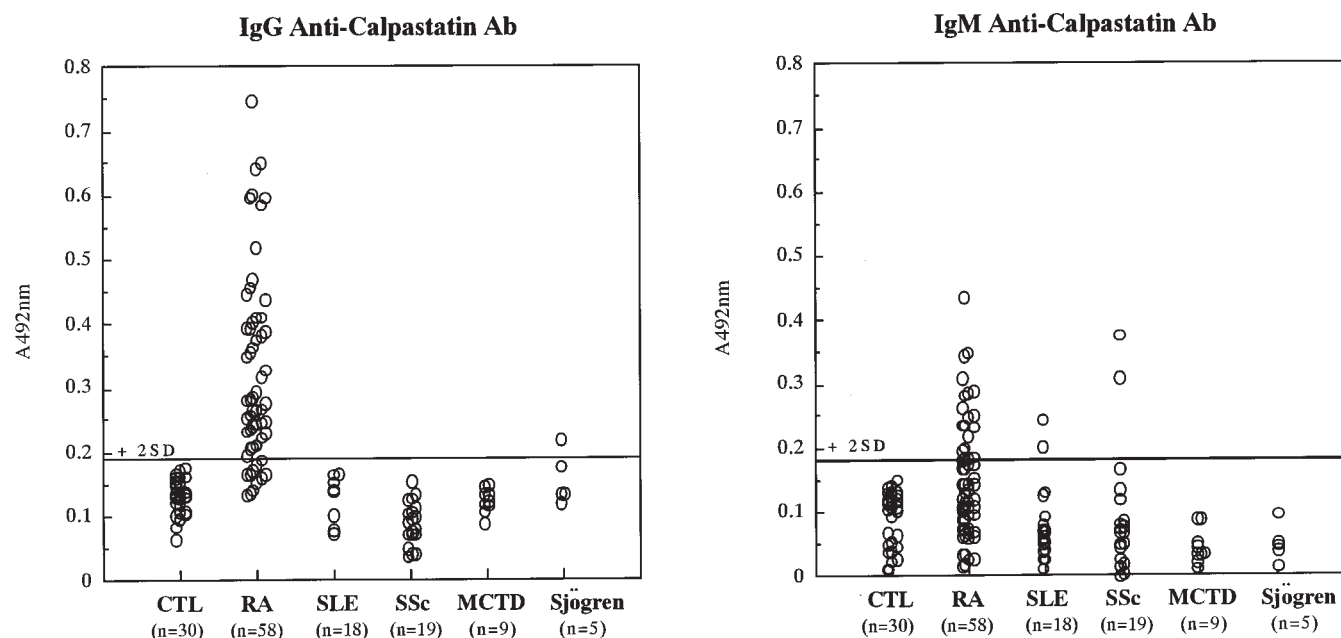


Figure 3. Levels of anticalpastatin antibodies (Ab) in sera from patients with various systemic rheumatic diseases. Horizontal lines show mean plus 2 standard deviations (2 SD) of absorbance of sera from controls (CTL) and patients with systemic lupus erythematosus (SLE), systemic sclerosis (SSc), mixed connective tissue disease (MCTD), and Sjögren's syndrome.

Table 2. Frequency of IgG anticalpastatin antibodies in systemic rheumatic diseases.

	Sensitivity, %	Specificity, %	Likelihood Ratio*	
			LR ⁺	LR ⁻
RA (n = 58)	82.8	96.1	21.2	0.18
SLE (n = 18)	5.6	45.7	0.11	2.04
SSc (n = 19)	0	44.0	0	2.25
MCTD (n = 9)	0	49.5	0	2.0
Sjögren's (n = 5)	20	52.4	0.42	1.51

* LR⁺: sensitivity ÷ (100 - specificity); LR⁻: (100 - sensitivity) ÷ specificity. RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; MCTD: mixed connective tissue disease.

Table 3. Diagnostic value of individual autoantibodies for RA.

	Sensitivity, %	Specificity, %
IgG anticalpastatin	82.8	96.1
IgM RF ¹⁸	66.0	87.0
Anti-Sa ¹⁸	22.0	98.0
Antikeratin (AKA) ²⁰	51.6	96.7
Antifilaggrin (AFA) ²⁰	57.0	93.4

mined on the basis of an ELISA cutoff level as described. No statistically significant difference was found between RA patients who were either positive or negative for IgG anticalpastatin autoantibodies. We also measured the levels of MMP-3 and -9, which are considered to play a crucial role in cartilage destruction of joints, frequently observed in RA². There was a mild correlation between MMP-9 and IgM RF ($r = 0.419$, $p < 0.01$) and no correlation between MMP-3 and IgM RF (data not shown). However, there were

no significant correlations between MMP-3, MMP-9, and IgG anticalpastatin antibodies.

DISCUSSION

Many autoantibodies are helpful as serological markers in the diagnosis of various diseases. In RA, IgM RF is detected in nearly 70% of patients. However, it can also be found not only in other systemic rheumatic diseases but also in healthy controls. Anti-Sa antibody, directed towards an unknown antigen that is abundant in rheumatoid synovium and placental tissue¹⁹, is known to have high specificity for RA (98%); however, its sensitivity is only 22%¹⁸. Recently autoantibodies like antikeratin (AKA) and antifilaggrin (AFA), which preferentially recognize citrullinated antigens, have been proposed as good diagnostic tools for RA because of their high specificity, more than 90%. However, their sensitivities are less than 60%²⁰. Anti-Sa, AKA, and

Table 4. Clinical and biological characteristics of 20 patients with RA. Except where indicated, values represent either number of patients or mean \pm 2 standard deviations. A significant difference was not identified in biological characteristics.

Characteristic	IgG Anticalpastatin Antibody	
	Positive, n = 16	Negative, n = 4
Men/Women	1/15	0/4
Age, yrs	56.3 \pm 8.6	57.3 \pm 12.8
RA duration, yrs	6.7 \pm 5.4	5.3 \pm 5.3
Stage		
I	1	0
II	1	1
III	1	0
IV	13	3
Class		
1	2	0
2	6	2
3	8	2
CRP, mg/dl	2.14 \pm 2.76	3.18 \pm 3.12
ESR, mm/h	60.3 \pm 19.4	54.5 \pm 28.7
MMP-3, ng/ml	384 \pm 360	481 \pm 314
MMP-9, ng/ml	152 \pm 147	157 \pm 230
IgM RF, IU/ml	80.8 \pm 142	31.1 \pm 44.6

AFA are more specific to RA, but their sensitivities are not as high as IgM RF^{18,20}.

Recently, several groups found that anticalpastatin antibodies were associated with RA^{10,11}, venous thrombosis²¹, and autoimmune infertility^{22,23}. Mimori, *et al* detected anticalpastatin antibodies in 57% of RA patients, and in lower percentages of patients with SLE, polymyositis/dermatomyositis, and SSc and healthy controls^{10,24}. Després, *et al* detected anticalpastatin antibodies in 45.5% of RA patients. However, they did not detect anticalpastatin antibodies in SLE¹¹. Since both groups used the same Western blotting method, they explained this discrepancy might reflect racial differences.

Lackner, *et al* and Vittecoq, *et al* reported an ELISA using a synthetic peptide corresponding to the C-terminal 27 amino acids of calpastatin as the antigen^{17,25}. The sensitivities of Lackner's and Vittecoq's groups were 8.9% and 19.5%, respectively. These were rather lower than those observed by Western blotting. The peptide-based ELISA seems to be less sensitive than the immunoblotting method. Human organ calpastatin, like muscle, consists of an N-terminal domain (domain L) and 4 repetitive domains (domain I, II, III, and IV)²⁶. In contrast, human erythrocyte calpastatin consists of domains II, III, and IV. The domain L is not homologous with the other 4 and has no inhibitory activity. Each of the 4 C-terminal domains binds to and inhibits calpain. Each domain has a central conserved sequence (TIPPPXYR), which is the binding site to calpain²⁷. Further, erythrocyte calpastatin exists as tetrameric protein and its molecular weight is about 250–280 kDa^{28,29}. Taking other groups' results into consid-

eration, we considered that the use of a large protein as the antigen would be an efficient tool for detection of anticalpastatin antibodies. We utilized human erythrocyte calpastatin as the antigen in our ELISA because it is a commercially available native human calpastatin and is better suited for the standardization of a diagnostic test. In this method, we used only a small sample (diluted to 1:2000) and were able to increase the sensitivity of the assay to almost 83%, with a specificity of 96% (Table 2). Moreover, the sensitivity of IgG or IgM anticalpastatin-positive RA was almost 90%. Lackner, *et al* found that the use of a large antigen in Western blotting did not lead to a substantially higher prevalence of anticalpastatin autoantibodies in RA¹⁷. The discrepancy between our data and others' may be explained by the hypothesis that there are conformational epitopes constructed from regions of several domains.

Correlations between anticalpastatin antibodies and clinical and biological characteristics were investigated. No statistically significant difference was found between anticalpastatin antibody positive and negative patients with RA. And also no correlation was found between anticalpastatin antibodies and clinical/biological variables. We investigated the correlations between MMP and clinical/biological characteristics, because MMP also seem to be implicated in joint cartilage destruction. MMP-9 and RF have a mild correlation, but MMP-3 and RF do not. This result is in agreement with that of Yamanaka, *et al*³⁰. Recently, they reported that the serum concentration of MMP-3 is a useful marker for predicting bone damage in early RA.

Among various autoantibodies, anticalpastatin antibodies are very specific to RA and can be an independent marker of RA. Moreover, it seems to be most efficient to combine anticalpastatin antibody with other autoantibodies, such as RF. Longitudinal measurements of anticalpastatin antibody levels in RA sera will be important to clarify their correlation with disease activity.

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