Antibodies to Viral Citrullinated Peptide in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To analyze the frequency of anti-viral citrullinated peptide (anti-VCP) antibodies in sera from patients with rheumatoid arthritis (RA) by an Epstein-Barr virus (EBV)-derived peptide in which arginine is replaced with citrulline.

Methods. Anti-VCP antibodies were determined in 627 serum samples, 300 from patients with RA and 327 from controls, including connective tissue diseases, chronic arthritides, and healthy donors. Among patients with RA, a possible correlation with systemic involvement, disease severity, and disease activity was investigated; in 94 RA patients antibodies to cyclic citrullinated protein (anti-CCP) were also measured.

Results. Anti-VCP antibodies were found in 45% of RA sera versus less than 5% of controls; anti-VCP levels correlated with anti-CCP levels ($p < 0.0001$), rheumatoid factor ($p = 0.02$), and erythrocyte sedimentation rate ($p = 0.0058$). No correlation was found with extraarticular manifestations of the disease or with disease severity.

Conclusion. Anti-VCP antibodies are helpful in discriminating RA from other chronic arthritides or connective tissue disorders. The level of positivity is positively correlated with the anti-CCP level, suggesting that VCP can be considered a novel substrate to detect anti-citrullinated peptide/protein antibodies (ACPA). The reactivity of RA-specific antibodies with a viral citrullinated antigen raises questions on the role of EBV in the induction of ACPA. (First Release Mar 1, 2006; J Rheumatol 2006;33:647–51)

Key Indexing Terms:
ANTI-CITRULLINATED PEPTIDE/PROTEIN ANTIBODIES RHEUMATOID ARTHRITIS
CITRULLINATED PEPTIDES EPSTEIN-BARR VIRUS

The serological diagnosis of rheumatoid arthritis (RA) has always been difficult because the most frequent and best characterized autoantibody, rheumatoid factor (RF), is not disease-specific and is not found in the early stage of the disease. RF occurs frequently in many inflammatory and infectious diseases and in healthy elderly individuals. Very often it is not present in the first years of the disease, when it is not easy to differentiate the diagnosis from other chronic arthritides on clinical grounds alone.

Anti-perinuclear factor (APF) and anti-keratin antibodies (AKA) were described long ago as autoantibodies frequently found in the sera of patients with RA, and are highly specific for the disease. However, technical difficulties in their measurement never allowed their routine use. The discovery of deiminated filaggrin as the target of APF and AKA[1,2] represented a major breakthrough in RA research and allowed new methods for serological diagnosis of RA[3]. A further step represented the identification of the sequences of filaggrin that are deiminated (i.e., arginine is substituted by citrulline) and are thus recognized by a high percentage of RA sera[4,5]. ELISA based on such sequences have been developed and used to screen patient sera. A more sensitive assay was obtained in which the peptide structure is modified to optimally expose the citrulline moiety (cyclic citrullinated peptide, CCP) allowing the detection of antibodies in up to 70% of RA patients[6,7].

Anti-filaggrin antibodies react with several proteins expressed in synovial tissue and mainly with deiminated fibrinogen[8]. On the other hand, the Sa antigen, specifically recognized by antibodies present in 50% of patients with RA[9], has been identified as deiminated vimentin[10]. Thus, RA-specific antibodies that recognize different deiminated proteins represent a family of antibodies of overlapping specificities that can be collectively named anti-citrullinated peptide/protein antibodies (ACPA)[11].

A comparative evaluation of sequences recognized by ACPA shows that the critical feature is the presence of citrulline flanked by neutral aminoacids such as glycine, serine, or threonine. Similar amino acid repeats are commonly found in viral proteins[4].

One of the nuclear proteins encoded by Epstein-Barr virus (EBV), EBNA I, contains in its N-terminal region a sequence (35-58) characterized by 6 gly-arg repeats.

We synthesized this sequence substituting arginine with citrulline (viral citrullinated peptide, VCP) to test sera from patients with connective tissue disorders: anti-VCP antibod-

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Accepted for publication December 7, 2005.

ies were detected in 45% of RA sera and in less than 5% of normals or disease controls[12]. Moreover, anti-VCP antibodies bound *in vitro* deiminated recombinant EBNA I and immunoprecipitated *in vivo* deiminated EBNA I from the lysate of EBV-infected cell lines[13].

Our aim was to analyze the frequency and clinical correlations of anti-VCP antibodies in a large group of patients with RA and in patients with chronic arthritides and connective tissue disorders.

MATERIALS AND METHODS

Patients. Sera were obtained from 300 RA patients (223 women and 77 men, mean age 61.4 ± 14.3, range 18-90 years; mean disease duration 11 years, range 6 mo-45 yrs) and from disease control subjects, including 51 with systemic sclerosis (SSc), 33 with mixed cryoglobulinemia (MC), 36 with systemic lupus erythematosus (SLE), 40 with psoriatic arthritis (PsA), 32 with ankylosing spondylitis (AS), 24 with polymyalgia reumatica (PMR), 15 with palindromic rheumatism (PaR), and 6 with hepatitis C (HCV)-related arthritis. Sera from 18 patients with infectious mononucleosis (IM) were also tested. Seventy-two healthy subjects (NHS) (blood donors and healthy laboratory personnel, age- and sex-matched with the patients) served as controls. All subjects provided their informed consent.

The diagnosis of RA[14], SLE[15], and SSc[16] was based on American College of Rheumatology criteria; MC was diagnosed in the presence of Meltzer's triad (purpura, weakness, and arthritis/arthralgia) and cryoglobulins in the sera; AS was diagnosed according to the revised New York criteria[17], and PsA according to the Vasey and Espinoza criteria[18].

Patients with RA were evaluated for systemic involvement (presence of xerostomia, xerophthalmia, peripheral vasculitis, rheumatoid nodules), disease activity [erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), active synovitis, morning stiffness lasting more than 30 minutes], and disease severity (presence of erosions in hands and/or feet).

Methods. Anti-VCP antibodies were detected by ELISA as described[12]. Briefly, ELISA plates (Nunc Maxi-Sorp F96, Denmark) were coated with a synthetic linear multiple antigen peptide (corresponding to the amino acid sequence 35–58 of the EBNA1 protein: GPA GPR GGG RGR GRG RGR GGH NDGG) in which all the arginine residues are substituted with citrulline, diluted at 5 μg/ml in phosphate buffered saline (PBS). After blocking with PBS 3% bovine serum albumin, sera diluted 1/200 in PBS, 1% BSA, and 0.05% Tween were incubated for 3 h at room temperature (RT). After washing, anti-human IgG $F(ab')_2$ fragment labeled with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:3000 in PBS, 1% bovine serum albumin, and 0.05% Tween was added to the wells and incubated for 3 h at room temperature. After washing, the bound enzyme activity was measured using para-nitrophenyl-phosphate as substrate (Sigma Chemical Co.).

Results are expressed as the percentage of an internal positive sample; positivity threshold of the test is 27% (higher than the upper limit of normal, which was established as the 95th percentile of a group of healthy controls).

Anti-CCP antibodies were detected by a commercial kit (QUANTA LITE™ CCP, Inova Diagnostics, San Diego, CA, USA) following manufacturer's instructions; the positivity threshold was 25 U. RF was measured by nephelometry. Upper limits for CRP and ESR were 5 mg/l and 30 mm/h, respectively.

Statistical analysis. Chi square, Fisher's exact, Spearman's rank correlation coefficient, and Mann-Whitney tests were used when appropriate.

RESULTS

We measured anti-VCP antibodies in 300 serum samples from patients with RA and in 327 serum samples from controls (120 connective tissue disease, 117 non-RA chronic arthritides, 18 IM, and 72 healthy control samples) (Table 1).

Anti-VCP antibodies were detected in 45% of patients with RA, and in a significantly lower percentage of controls: 2% SSc ($p < 0.0001$), 3% MC ($p < 0.0001$), 0% SLE ($p < 0.0001$), 3% PsA ($p < 0.0001$), 3% AS ($p < 0.0001$), 17% PMR ($p < 0.01$), 13% PaR ($p < 0.02$), 17% HCV (NS), 6% IM ($p < 0.001$), and 6% NHS ($p < 0.0001$) (Figure 1, Table 1).

Anti-VCP antibodies were rarely found in sera from patients with other connective tissue disorders, such as SSc, SLE, and MC. Notably, MC sera contained high levels of RF, but no anti-VCP antibodies, thus excluding any interference of RF on their detection. Anti-VCP antibodies were infrequently detected in PsA and AS. Only 1 out of 18 IM sera was positive for anti-VCP antibodies, confirming the striking association of these antibodies with RA. Four out of 24 PMR and 2 out of 15 PaR sera were positive for anti-VCP antibodies. The test identified RA patients with a sensitivity of 45% and a specificity of 95%, comparable to the first version of the CCP assay[19].

Anti-VCP antibodies were more frequently detected in RA patients positive for RF, (94/165, 57%) compared to patients negative for RF (21/81, 26%) ($p < 0.0001$). In a subgroup of 65 patients, we analyzed by the Spearman rank test the correlation between the levels of anti-VCP antibodies and ESR (mm/h), CRP (mg/l), and RF (UI/ml). A statistically significant correlation was seen with anti-VCP compared to RF ($p < 0.05$) and ESR ($p < 0.01$), but only weak correlation was found with CRP ($p < 0.05$).

We also analyzed some clinical features of RA such as systemic involvement, disease severity, and disease activity. The levels of anti-VCP antibodies were not significantly different in patients with or without erosive arthritis, active arthritis, xerostomia, xerophthalmia, or peripheral vasculitis.

Ninety-four RA sera were also tested by the commercially available anti-CCP assay: 74 (79%) sera were positive in the anti-CCP assay and 45 (48%) in our test; the 2 assays were highly correlated ($p < 0.0001$) (Figure 2).

DISCUSSION

Our data show that an EBNA I-derived citrullinated peptide can be used to detect RA-associated antibodies. The ELISA based on this peptide is able to discriminate RA from other connective tissue disorders, including other chronic arthritides.

The "wild type" EBNA I 35-58 sequence is recognized by antibodies present in normal sera as well as in sera from patients with connective tissue disorders, acute EBV infection, and EBV-associated neoplasms[20]. In contrast, the modified peptide we obtained by substituting arginine with citrulline detected antibodies almost exclusively in RA patients.

The highest number of false positives was found in the PMR (4/24, 17%) and in the PaR (2/15, 13%) groups. However, RA can present with clinical features resembling PMR, especially in older patients. In 2 PMR patients positive for anti-VCP antibodies, the diagnosis was subsequently changed to RA, after the patients developed chronic ero-

Table 1. Clinical and demographic features of patients and controls.

	Female/Male	Age, yrs Mean ± SD (range)	Anti-VCP, > 27% (%)	RF, titer 1:40 (%)	CRP, > 5 mg/l (%)
RA	223/77	61.5 ± 14.2 (18–90)	136/300 (45)	201/300 (67)	210/300 (70)
SSc	45/6	59.1 ± 13.4 (31–79)	1/51 (2)	5/51 (9.8)	11/51 (22)
MC	23/10	65.6 ± 11.7 (43–84)	1/33 (3)	27/33 (81)	NA
SLE	31/5	40.4 ± 8.9 (24–55)	0/36 (0)	2/36 (5.5)	NA
PsA	18/22	59.1 ± 12.8 (31–82)	1/40 (3)	1/40 (2.5)	24/40 (60)
AS	9/23	47.6 ± 11.3 (29–72)	1/32 (3)	2/32 (6.25)	13/32 (40)
PmR	18/6	72 ± 7.2 (56–82)	4/24 (17)	5/24 (20.8)	19/24 (80)
PaR	7/8	62.1 ± 11.6 (40–80)	2/15 (13)	5/15 (33)	9/15 (60)
HCV	4/2	61 ± 13 (39–73)	1/6 (17)	5/6 (83)	2/6 (33)
IM	NA	NA	1/18 (5.5)	NA	NA
NHS	40/32	38.2 ± 9.8 (25–60)	4/72 (5.5)	NA	NA

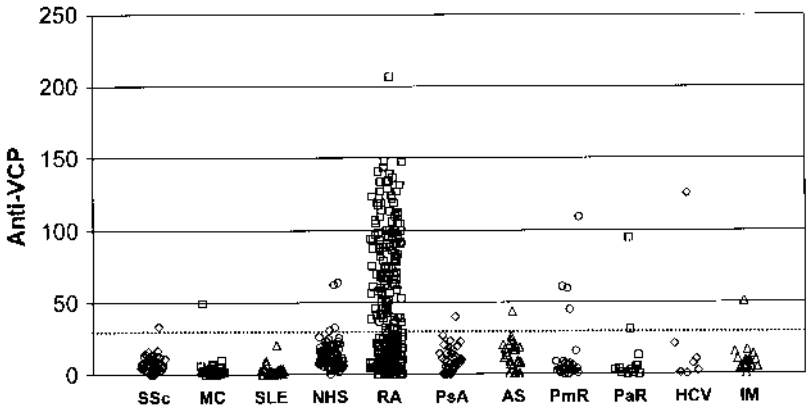

Figure 1. Anti-VCP antibodies in patients with RA and other chronic arthritides. Sera diluted 1:200 were incubated on VCP-coated plates, and bound antibodies were detected by alkaline phosphatase-labeled anti-IgG antibodies. Results are expressed as percentage of a reference serum. The upper limit of normal, set at 95th (97.5) percentile of normal sera (NHS), was 27% (dotted line). Anti-VCP were detected in: 136/300 RA, 1/51 SSc, 1/33 MC, 0/36 SLE, 1/40 PsA, 1/32 AS, 4/24 PMR, 2/15 PaR, 1/6 HCV arthritis, and 1/18 IM.

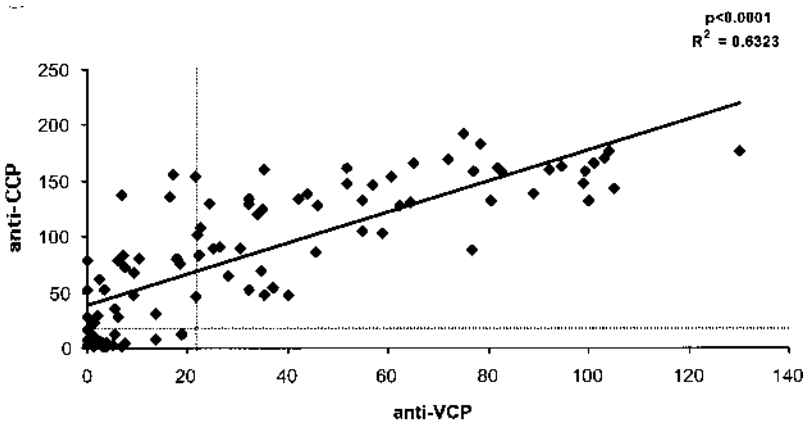

Figure 2. Anti-VCP and anti-CCP antibodies in RA sera. Anti-VCP and anti-CCP antibodies were tested in 94 RA sera. The upper limit of normal was 25 U for the CCP assay and 27% for the VCP assay. Seventy-four out of 94 sera (79%) were positive in the CCP assay and 45/94 (48%) in the VCP assay. The levels of antibodies measured in the 2 assays were positively correlated ($p < 0.0001$).

sive arthritis. It is of interest that 50% of patients diagnosed with PaR will develop RA[21]. One of the positive PaR patients has recent onset oligoarthritis (which may evolve into RA suggesting an atypical RA onset). Thus, in disorders like PMR and PaR the presence of anti-VCP antibodies may identify patients whose disease may evolve into RA.

It was recently reported that anti-CCP antibodies are extremely helpful in discriminating RA from chronic HCV-associated arthritis: in fact, anti-CCP antibodies were not detected in 8 patients with HCV-related arthritis[22]. We examined only 6 patients with HCV-related arthritis, and one had high levels of anti-VCP antibodies; he shows a chronic polyarticular involvement without erosions, and is treated with low doses of steroids and methotrexate.

Anti-VCP antibodies are more frequently detected in RF-positive patients and their titer is correlated with RF titer, suggesting that both antibodies are expressed in the same subgroup of RA patients.

Similarly, anti-CCP antibodies have been more frequently detected in RF-positive RA patients[19], but not in other conditions characterized by the presence of RF[23]. A correlation between the titers of anti-CCP and RF has been observed in RA[24], and particularly in elderly onset RA[25,26].

Anti-CCP antibodies have been shown to correlate also with disease severity, evaluated on the basis of radiological damage and functional impairment[27,28]. A similar correlation with the presence of bone erosions has not been found in anti-VCP positive patients. However, our RA population was heterogeneous in terms of disease duration. The presence of several cases of recent onset RA may have weakened the correlation of anti-VCP antibodies with signs of radiological damage. Prospective studies including patients with recent onset RA are in progress to correctly evaluate the predictive role of anti-VCP antibodies on disease severity.

Anti-VCP antibodies were not related to extraarticular manifestations of the disease, as previously reported for other ACPA[29].

The relationship of anti-VCP antibodies with disease activity is presently unclear: antibody titer was correlated with ESR and weakly with CRP, which is a better indicator of disease activity. Followup studies have been planned, to establish whether or not anti-VCP antibodies are modified by therapy and fluctuate with spontaneous or therapy-induced remissions. Similarly, conflicting results have been obtained with anti-CCP antibodies and it is not yet clear whether their levels are associated with disease flares[25,30].

Anti-VCP antibodies displayed the strong association with RA that is typical of anti-filaggrin and AKA and their titer was correlated with the titer of anti-CCP antibodies. Moreover, we have shown[13] that affinity purified anti-VCP antibodies bind CCP and deiminated fibrinogen.

Taken together, these observations indicate that anti-VCP antibodies belong to the ACPA family and that VCP may be considered a substrate for the detection of ACPA.

It has been shown that several self proteins are deiminated *in vivo* and some of them (namely filaggrin, fibrin, vimentin) are recognized by ACPA. The EBNA I protein, expressed in the nuclei of EBV-infected cells during latent infection[31], is an example of exogenous antigen that can be deiminated *in vivo* and thus become a target of RA-specific antibodies.

Deimination is a post-translational modification mediated by peptidylarginine deiminase (PAD) and deeply influenced by tissue expression and activity of these enzymes. Recently, the genetic factors regulating PAD activity have been analyzed in patients with RA. In a Japanese population, it was shown that a PADI4 haplotype is associated with higher mRNA stability and with susceptibility to RA[32]. Patients with RA would be characterized by a higher activity of PAD and more extensive protein deimination. Although an association of PADI4 alleles with RA has been excluded in Caucasians[33,34], genetic factors affecting the expression and/or activity of PAD may play an important role in RA.

The immune response to deiminated antigens is strongly influenced by HLA status. ACPA production is associated with DR haplotypes carrying the shared epitope and conferring susceptibility to RA[35,36], while the ability of these DR haplotypes to bind citrullinated peptides has been shown both *in vivo* and *in vitro*[37].

EBV infection is extremely frequent, and more than 95% of adults have been infected by the virus. An altered immune response to EBV that causes persistence of a higher number of infected cells and a higher viral protein load has been proposed as a contributing factor to the pathogenesis of RA[38-41].

Our data suggest a new interaction of EBV with the immune system in RA: in individuals with the appropriate genetic background, *in vivo* deiminated viral proteins encoded by EBV might contribute to the production of ACPA.

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