IgG, IgA, IgM antibodies to a viral citrullinated peptide in patients affected by rheumatoid arthritis, chronic arthritides and connective tissue disorders

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Objectives. Anti-citrullinated protein/peptide antibodies (ACPA), a family of antibodies with overlapping specificities, represent a specific marker of rheumatoid arthritis (RA). The aim of the present study is to investigate the prevalence and clinical significance of IgG, IgA and IgM ACPA by a newly described assay employing a viral citrullinated peptide (VCP).

Methods. IgG, IgA and IgM anti-VCP antibodies have been measured in sera from 146 patients affected by RA and 404 controls, including 204 chronic arthritides, 111 connective tissue disorders and 89 healthy subjects. The affinity of the different isotypes for VCP was analysed by liquid phase inhibition assays.

Results. Among RA patients, 40 were single positive for IgG anti-VCP, five for IgA and 11 for IgM. Ten patients were double positive for IgG and IgA, four for IgG and IgM, six for IgA and IgM. In 15 RA patients IgG, IgA and IgM anti-VCP antibodies were detected. No correlation could be found between the isotype and the clinical manifestations or duration of the disease. IgA anti-VCP were strongly associated with RA, whereas IgM anti-VCP were detected also in a low percentage of systemic lupus erythematosus, psoriatic arthritis and mixed cryoglobulinaemia (MC) patients. IgG anti-VCP displayed a higher affinity for the antigen than IgA or IgM.

Conclusions. These data show that anti-VCP of IgG and IgA isotype discriminate RA from other chronic arthritides and disease controls and suggest an independent production of each isotype.

Key words: ACPA, Isotype, Rheumatoid arthritis, Chronic arthritides, Connective tissue disorders.

Introduction

Anti-perinuclear factor (APF) and anti-keratine antibodies (AKA) are specific markers of RA. The target antigens of APF and AKA are human epidermal filaggrin and other pro-filaggrin-related proteins of various epithelial tissues. It has been shown that anti-filaggrin antibodies (AFA) bind to antigenic determinants which bear the amino-acid citrulline, generated by the post-translational modification of arginine by peptidyl arginine deiminase. Sequences derived from filagrin in which arginine has been substituted with citrulline have been used as antigens on the solid phase to measure Rheumatoid arthritis (RA)-associated antibodies [1]. Cyclic peptides corresponding to these sequences optimally expose citrulline residues and are thus employed in a very sensitive assay cyclic citrullinated peptide (CCP) widely used for AFA detection [2, 3]. However, AFA react also with other citrullinated proteins/peptides: fibrin [4], vimentin [5], collagen [6] and recently a viral peptide [7] have been described as targets of RA-specific antibodies. Thus, these antibodies are presently referred to as ‘ACPA’ [8].

ACPA are produced early in the disease course [9] and have also been detected in healthy subjects that later developed RA [10], but are persistently produced in patients. The frequency, clinical associations and predictive value of ACPA have so far been established detecting IgG ACPA.

Recently, the heterogeneity of ACPA has been outlined by Veerport et al. [11], that measured anti-CCP of IgA and IgM isotype in CCP-IgG positive sera from patients affected by established RA or undifferentiated arthritis. A high proportion of RA sera contain also IgA anti-CCP (62%) and IgM anti-CCP (61%); in undifferentiated arthritis, the contemporary presence of anti-CCP antibodies of different isotypes is strongly predictive of an evolution towards RA. However, no data are available on the presence of ACPA of IgA and IgM isotype in other systemic auto-immune disorders; on the other hand, it is unknown whether ACPA IgA or IgM are associated with specific disease manifestations of RA.

The aim of the present study was:

• to evaluate the prevalence of IgG, IgA and IgM ACPA in patients affected by RA, chronic arthritides and connective tissue disorders by a recently described assay based on a viral citrullinated peptide (VCP) [12];
• to analyse the association of IgA and IgM anti-VCP with clinical features and specific disease manifestations;
• to establish whether or not the detection of IgA and IgM anti-VCP antibodies increases the sensitivity of the assay based on the viral peptide.

Patients and methods

Patients

RA patients. One hundred forty-six RA patients, diagnosed according to the ACR classification criteria [13], were enrolled. RA patients were evaluated for systemic involvement (presence of xerostomia, xerophthalmia, peripheral vasculitis and rheumatoid nodules), disease activity, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), active synovitis, morning stiffness lasting >30 min and disease severity (presence of erosions in hands and/or feet). Written consent according to the Declaration of Helsinki was obtained from the patients included in the study. The study was approved by the local ethical committee.

Controls

Other arthritides. Fifty-two ankylosing spondylitis (AS) patients, 128 psoriatic arthritis (PsA) patients and 19 undifferentiated arthritis (UA) patients were enrolled. AS was diagnosed according to the revised New York criteria [14]; PsA was diagnosed according to the criteria of Vasey and Espinoza [15]. Sixty-six PsA patients (51%) had symmetrical polyarthritis,
60 (47%) asymmetrical oligoarthritis and two (1.5%) spondylitis. Psoriasis was always confirmed by a dermatologist.

Undifferentiated arthritis patients displayed oligo- or poly-arthritis that did not fulfill the criteria for the diagnosis of RA or spondyloarthritides after at least 1 year of follow-up. Demographic and clinical features of chronic arthritides patients are given in Table 1.

Other disease controls. One hundred and eleven disease control subjects were enrolled, including 20 systemic sclerosis (SSc), 19 mixed cryoglobulinaemia (MC), 24 systemic lupus erythematosus (SLE), 35 polymyalgia rheumatica (PmR), 13 Sjögren’s Syndrome (SS). The diagnosis of SLE [16] and SSc [17] was based on the ACR criteria; MC was diagnosed in the presence of Meltzer’s triad (purpura, weakness and arthritis/arthralgia) and of cryoglobulins in the sera; SS was diagnosed according to the criteria of the American European Consensus Group [18].

Healthy donors. Eighty-nine blood donors from the Hospital of Pisa were included as healthy controls.

After informed consent was obtained, serum samples were stored at −20°C until tested.

Methods

ELISA for anti-VCP antibodies detection. Anti-VCP antibodies were detected by ELISA as previously described [12]. ELISA plates were coated with a synthetic multiple antigen peptide (MAP) corresponding to the amino-acid sequence 35-58 of the EBNA1 protein, in which all the arginine residues are substituted with citrulline. Results are expressed as the percentage of an internal positive sample; positivity threshold of the test (higher than the 95th percentile of healthy controls) is 27% of an internal positive sample; positivity threshold of the test substituted with citrulline. Results are expressed as the percentage of the EBNA1 protein, in which all the arginine residues are substituted with citrulline.

Inhibition assays. For the inhibition assays, 11 sera from RA patients single positive for IgG or IgA or IgM anti-VCP antibodies were tested on VCP at different dilutions. The dilution corresponding to 50% of the maximum binding of each serum was pre-incubated with VCP or with a control peptide (MAP-Scl 70 corresponding to the amino acids 487-508 of the human topoisomerase I) for 30 min at 37°C before being transferred to the VCP-coated plates. Thereafter, ELISAs were carried out as for direct binding assay.

Statistical analysis. ANOVA (analysis of variance), Bonferroni post-hoc, χ²-test, Fisher exact, Spearman rank correlation coefficient and Mann–Whitney tests were used.

Results

The frequency of IgG, IgA and IgM anti-VCP antibodies in chronic arthritides patients is given in Table 1.

Among RA patients, 40 were single positive for IgG anti-VCP, five for IgA and 11 for IgM. Ten patients were double positive for IgG and IgA, four for IgG and IgM, and six for IgA and IgM. In 15 RA patients, IgG, IgA and IgM anti-VCP antibodies were detected (Fig. 1).

Two of the PsA patients were positive for IgG anti-VCP and six for IgM anti-VCP; none of these patients showed the contemporary presence of IgG and IgM anti-VCP.

Among AS patients, three were positive for IgA anti-VCP and two for IgM anti-VCP. None of the UA showed reactivity for VCP.

Among disease controls, one SLE patient was positive for IgG anti-VCP and two for IgM anti-VCP; one PMR patient was positive for IgG anti-VCP; two MC patients were positive for IgM anti-VCP; no reactivity was detected in SS and SSc patients (Fig. 2A, B and C). Analysing these data by ANOVA, anti-VCP of any isotype differentiated RA from disease controls (P < 0.0001). Bonferroni post-hoc test showed that IgG and IgA anti-VCP antibodies were able to differentiate RA from each of the disease controls (P < 0.001); the levels of IgM anti-VCP antibodies distinguished RA from AS, PmR, PsA, SSc, and UA (P < 0.001), but not from MC (P = 0.1817) and SLE (P = 0.3458). Anti-VCP levels, on the contrary, did not differentiate any other disease from the remaining controls.

Combining IgG and IgA anti-VCP antibodies, the sensitivity of the assay for RA increased to 55% with a specificity of 95%.

Taking into account the clinical features of RA patients (systemic involvement, disease severity and disease activity), the presence or the levels of anti-VCP antibodies of any isotype were not significantly different in patients with or without erosive arthritis, active arthritis, xero stomia, xero ptalmia or peripheral vasculitis.

The two PsA patients positive for IgG anti-VCP and the six positive for IgM anti-VCP antibodies showed polyarthritis that was active in one patient only.

Table 1. Demographic and serological characteristics of chronic arthritides patients

<table>
<thead>
<tr>
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<th>RA</th>
<th>PsA</th>
<th>AS</th>
<th>UA</th>
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<tbody>
<tr>
<td>Number of patients (F:M)</td>
<td>146 (101:45)</td>
<td>134 (58:76)</td>
<td>51 (14:37)</td>
<td>19 (15:4)</td>
</tr>
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<td>Age in years (mean ± s.d., range)</td>
<td>56 ± 14 (16–80)</td>
<td>52 ± 12 (22–82)</td>
<td>39 ± 12.1 (22–62)</td>
<td>50 ± 19 (13–82)</td>
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<td>Disease duration (mean ± s.d., range)</td>
<td>8.3 ± 7.6 (0–34)</td>
<td>6.6 ± (1–43)</td>
<td>17 ± 13 (1–55)</td>
<td>3.8 ± 6.1 (1–25)</td>
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<td>RF positivity (≥40 UI) (%)</td>
<td>105/146 (72%)</td>
<td>0/134 (0%)</td>
<td>0/134 (0%)</td>
<td>3/19 (16%)</td>
</tr>
<tr>
<td>C reactive protein positivity (≥5 g/l) (%)</td>
<td>100/146 (69%)</td>
<td>87/134 (65%)</td>
<td>29/51 (57%)</td>
<td>12/19 (62%)</td>
</tr>
<tr>
<td>IgG anti-VCP (%)</td>
<td>69/146 (47%)</td>
<td>2/134 (1.5%)***</td>
<td>0/51 (0%)***</td>
<td>0/19 (0%)***</td>
</tr>
<tr>
<td>IgA anti-VCP (%)</td>
<td>96/146 (65%)</td>
<td>0/134 (0%)***</td>
<td>3/51 (5%)*</td>
<td>0/19 (0%)*</td>
</tr>
<tr>
<td>IgM anti-VCP (%)</td>
<td>36/146 (25%)</td>
<td>6/134 (4.4%)***</td>
<td>2/51 (4%)**</td>
<td>0/19 (0%)**</td>
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*p-values compared with RA patients by Fisher’s exact test.

***P < 0.0001; **P < 0.001; *P < 0.01.

FIG. 1. Anti-VCP isotypes distribution in RA patients.
On the contrary, the presence of IgM anti-VCP in the patients affected by SLE and MC was not associated with chronic arthritis.

Liquid phase inhibition assays were performed with sera from RA patients, single positive for IgG or IgA or IgM anti-VCP. In all of the 11 sera analysed, pre-incubation with the liquid phase antigen inhibited the binding of IgG but not of IgA or IgM antibodies, indicating a higher affinity of IgG for VCP. A representative example is given in Fig. 3.

**Discussion**

The data presented in this article show that antibodies specific for a deimmunized viral peptide (VCP) belonging to IgA or IgM isotype can be found in RA and in a few cases are the only anti-VCP present in sera.

According to liquid phase inhibition assays, IgG anti-VCP are endowed with high affinity, while IgA and IgM anti-VCP, even when present in high titre, display a low affinity for the antigen. IgA anti-VCP shows the same specificity for RA typical of IgG anti-VCP. On the contrary, IgM anti-VCP, although strongly associated with RA, can be detected also in chronic arthritides and connective tissue disorders. As previously shown for anti-VCP [12] and anti-CCP antibodies [19, 20] of IgG class, IgA anti-VCP are not detected in PMR or in HCV-associated disorders like MC. Thus, the detection of ACPA may allow the correct diagnosis when elderly RA patients present with signs and symptoms of PMR.

Anti-VCP IgA, either alone or co-expressed with IgG or IgM, do not identify patients with exocrine gland involvement. Other IgA auto-antibodies (anti-SSA and anti-SSB) are instead very frequent in RA patients with secondary Sjögren's syndrome [21].

Anti-VCP IgA, either alone or co-expressed with IgG or IgM, are not associated with erosive or with active disease.

Anti-CCP antibodies, on the contrary, have been shown to correlate with disease severity, evaluated on the basis of radiological damage and functional impairment [22–25]. A similar correlation with the presence of bone erosions has not been found in anti-VCP (IgG and/or IgA) positive patients. However, the RA population analysed in the present study is heterogeneous, in terms of disease duration and only prospective studies on recent onset RA may allow to correctly evaluate the predictive role of anti-VCP antibodies on disease severity.

It has been reported that RA sera contain mutated high affinity IgM rheumatoid factors, strongly associated with vasculitis. In SLE, IgM anti-DNA may play a role in cutaneous vasculitis [26] and it has been shown that anti-phospholipid [27] IgM antibodies have pathogenic potential. IgM anti-VCP, on the contrary, do not display any association with clinical or serological features of RA, or with disease duration.

As observed by Verpoort et al. [11], for IgM anti-CCP antibodies IgM anti-VCP are present in patients with long-standing RA, suggesting that B cells producing IgM ACPA are persistently expanded in RA. Follow-up studies will be helpful to establish whether or not their production is regulated independently of the IgG.

The presence of IgM or IgA anti-VCP in sera negative for anti-VCP antibodies suggests an independent production of
each isotype. At variance with this result, anti-CCP antibodies of IgA and IgM isotype are only detected in CCP-IgG-positive sera. Thus, in the case of anti-VCP, the detection of IgG and IgA antibodies improves the sensitivity of the assay for RA, allowing better discrimination of RA from other chronic arthritides, including PsA.

This inflammatory joint disease, associated with psoriasis and classified as a form of spondyloarthropathy, may show a wide spectrum of articular involvement and the differential diagnosis between PsA and RA may be not easy on solely clinical grounds, particularly in the early phase of the disease. Thus, auto-antibody detection acquires an important role. The presence of rheumatoid factor, previously considered as exclusion criteria for the diagnosis of PsA, is now accepted if the typical clinical and radiological features are present. ACPA detection, on the contrary, may allow correct discrimination of RA and PsA. A variable prevalence of ACPA in PsA, ranging from 5.6% to 15.7%, has been reported in previous studies depending on the technique used to detect ACPA and the classification criteria for PsA [28-31]. Using highly specific criteria for patient inclusion, anti-VCP antibodies of IgG and IgA isotype are detected in a very low percentage of PsA patients (1.5%), thus confirming that ACPA in this disease are present very rarely and exclusively in patients with polyarticular involvement.

In conclusion, anti-VCP of IgG, IgA or IgM isotype are strongly associated with RA and the detection of IgA antibodies improves the sensitivity of this assay. ACPA of IgG and IgA isotype can be considered highly specific markers of RA that allow the differential diagnosis with other chronic disorders affecting the joints such as spondyloarthritides. The measurement of IgA and IgM specific for other citrullinated substrates will establish whether this observation can be extended to the assays for ACPA detection based on fibrinogen or vimentin, leading to a further evolution in the serological diagnosis of RA.

Rheumatology key messages

- Anti-VCP of IgA and IgM isotype can be found in RA patients negative for IgG.
- IgA and IgM are low affinity anti-VCP.
- IgM anti-VCP are also present in long-standing RA.

The authors have declared no conflicts of interest.

References