Prevalence and clinical significance of anti-cyclic citrullinated peptide antibodies in juvenile idiopathic arthritis

T Avčin, R Cimaz, F Falcini, F Zulian, G Martini, G Simonini, V Porenta-Bešić, G Cecchini, M O Borghi, P L Meroni

Background: Antibodies against cyclic citrullinated peptide (anti-CCP) are considered to be specific for rheumatoid arthritis (RA).

Objective: To assess the clinical significance of anti-CCP in a cohort of patients with juvenile idiopathic arthritis (JIA).

Methods: Anti-CCP were tested by an enzyme linked immunosorbent assay (ELISA) in serum samples from 109 patients with JIA (30 boys, 79 girls), with a mean age of 8.7 years (range 0.6–20.3) and mean disease duration of 3.6 years (range 3 months to 15.6 years). As control groups, anti-CCP were also tested in sera of 30 healthy children, 25 patients with juvenile onset systemic lupus erythematosus (SLE), and 50 adult patients (30 with RA, 20 with SLE).

Results: Positive anti-CCP values were found in sera of two patients with JIA (2%), one with polyarthritis, and one with oligoarthritis. Statistical analysis showed that anti-CCP were not associated with the presence of antinuclear antibodies, raised erythrocyte sedimentation rate, or erosions. In the control groups, none of the patients with juvenile onset SLE and only one of 20 adults with SLE were positive for anti-CCP, but 19/30 (63%) adults with RA showed anti-CCP positivity.

Conclusions: Anti-CCP can be detected in children with JIA, but are less frequently present than in adults with RA.

A number of autoantibodies, including antiperinuclear factor (APF), antikeratin antibodies (AKA), and anticyclic citrullinated peptide antibodies (anti-CCP), are now known to be specifically associated with rheumatoid arthritis (RA). There is an evolving understanding of the antigens to which these RA associated antibodies are directed, and it appears that they are closely related. In particular, it has been shown that APF and AKA identify a common antigen, the (pro)filaggrin protein present in the keratohyalin granules in the cytoplasm of differentiating epidermal cells as well as in the stratum corneum tissue of rat oesophagus. Recently, Schelleken et al convincingly demonstrated in patients with RA that APF and AKA specifically bind to substrates containing the modified amino acid citrulline, which is present also in the amino acid sequence of filaggrin. In addition, a peptide based enzyme linked immunosorbent assay (ELISA) was developed using citrullinated cyclic peptide substrates for the detection of anti-CCP and it was suggested that this assay might be used as a functional replacement of the immunofluorescence tests used for the detection of APF and AKA.

Juvenile idiopathic arthritis (JIA) is a systemic autoimmune disease of unknown cause, which is characterised by chronic inflammation of the joints similar to RA. It is the most common chronic rheumatic disease in children and one of the most common chronic illnesses of childhood. The diagnosis of JIA depends primarily on clinical manifestations of the disease, with only limited serological support. The only serological tests routinely used are the presence of rheumatoid factor (RF), which is found in a small percentage of patients with polyarticular disease, and antinuclear antibodies (ANA), which are a marker for early onset oligoarticular disease with uveitis. A number of clinical studies have shown that sera from patients with JIA may contain many other antibodies, including anticardiolipin, APF, AKA, and anti-RA33 antibodies. However, none of them appears to be useful for diagnosis and assessment of the disease course.

PATIENTS AND METHODS

Patients

A cohort of 109 patients with JIA (30 boys, 79 girls) recruited from four paediatric rheumatology centres (University of Padova, University of Florence, University of Milan, Italy, and University of Ljubljana, Slovenia) participated in the study. Their mean age was 8.7 years (range 0.6–20.3) and mean disease duration was 3.6 years (range 3 months to 15.6 years). Serum samples were obtained from 109 patients with JIA: 52 with polyarticular, 51 with oligoarticular, and six with systemic disease. In the group of patients with polyarthritis, 25 had a polyarticular, 13 an oligoarticular, and 14 a systemic onset type. Twenty patients with JIA were further investigated by serial determinations at a median interval of seven months (range 1–45) from the first to the last sample. Moreover, paired synovial fluid and serum samples were obtained from eight patients.

Abbreviations: AKA, antikeratin antibodies; ANA, antinuclear antibodies; anti-CCP, anti-cyclic citrullinated peptide antibodies; APF, antiperinuclear factor; AU, arbitrary units; ELISA, enzyme linked immunosorbent assay; ESR, erythrocyte sedimentation rate; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; SF, synovial fluid; SLE, systemic lupus erythematosus.
patients with JIA (two with polyarthritis, six with oligoarthritis). At the time of the study, 47 patients were receiving treatment with methotrexate, 39 were receiving oral glucocorticosteroids, and one patient was receiving treatment with etanercept.

The control group comprised 30 apparently healthy children at their regular routine visits in the community based health centres. In addition, 25 patients with juvenile onset systemic lupus erythematosus (SLE) acted as the paediatric rheumatic disease control group and 50 adult patients (30 with RA, 20 with SLE) acted as the adult disease control group.

Anti-cyclic citrullinated peptide antibodies
Blood (2–3 ml) was collected during routine venepuncture performed for periodic assessment of laboratory tests. Samples were centrifuged, and sera were divided into aliquots and stored at –70°C until assayed. Samples were tested without knowing the clinical details of the patients. The presence of anti-CCP was determined by a commercial ELISA test (Immunoscan RA, Euro-Diagnostica, Arnhem, The Netherlands). The manufacturer’s instructions for the kit were followed, without modifications. Briefly, 100 µl/well of calibrator and sera samples diluted 1:50 were applied in duplicate on microtitre plates coated with synthetic peptides containing citrulline, and incubated for 60 minutes at 37°C in a humid incubation chamber. The plates were then washed three times, and 100 µl/well of conjugate solution was added. After 60 minutes of incubation at 37°C, the plates were washed again three times and 100 µl/well of substrate solution was added. The colour reaction was stopped after 30 minutes and the absorbency values were read immediately at 450 nm. A control serum was used to monitor plate to plate variation, with the results expressed in arbitrary units (AU). The cut off point was calculated as the mean plus three standard deviations of the values in a group of healthy children (30 subjects matched for age and sex). Samples with >70 AU were considered positive.

Paired synovial fluid (SF) and serum samples were obtained from eight patients. The SF samples were taken by needle puncture, centrifuged at 800 g, and the supernatant stored at –70°C until assayed. For anti-CCP testing in these samples, sera and SF were diluted to obtain comparable total IgG protein concentrations.

Rheumatoid factor and antinuclear antibodies
RF was determined by a nephelometric commercial test (RFIL, Tina-quant, Roche Diagnostic, Mannheim, Germany). ANA were determined by a standard indirect immunofluorescence technique on HEP-2 cells. ANA serum titres at >1/40 were considered positive.

Immunoglobulin G (IgG) concentration by sandwich ELISA
IgG concentration in both serum and SF samples was evaluated by a sandwich ELISA, as previously described.

Ethics
The study was approved by the ethics committees of the different institutions. Informed consent for drawing extra blood at the time of routine venepuncture was obtained from parents or guardians of all participating children.

Statistical analysis
Patient groups were compared using the χ 2 test for proportions. For tables with cells with small frequencies we used Fisher’s exact test. Differences were considered significant whenever p<0.05. Statistical analysis was performed using StatXact-4 for Windows statistical software (Cytel Software Corporation, Cambridge, MA, USA).

RESULTS
Figure 1 shows the cumulative results of the anti-CCP assays in all the patients studied. Positive anti-CCP values were found in sera of 1.8% (2/109) patients with JIA. Both positive patients with JIA had relatively low anti-CCP values (below 100 AU). In the paediatric rheumatic disease control group, none of the patients with juvenile onset SLE was positive for anti-CCP. In contrast, anti-CCP were found in 19/30 (63%) adults with RA, which was significantly higher than in patients with JIA (p<0.0001). Positive anti-CCP values were also found in 1/20 (5%) adult patients with SLE.

Figure 2 presents the anti-CCP values for each subtype of JIA. Positivity was 1/52 (1.9%) in the group with polyarthritis and 1/51 (2.0%) in the group with oligoarthritis (p=NS). None of the six patients with systemic disease had positive anti-CCP. Analysis of anti-CCP according to the JIA onset type showed that 1/25 (4%) patients with polyarticular onset, 1/64 (1.6%) with oligoarticular onset, and none of the 20 patients with systemic onset type were anti-CCP positive. The differences in the incidence of anti-CCP between various JIA onset types were not significant (p=NS). In our cohort there was only one girl who had RF positive polyarticular disease, who was negative for anti-CCP. Table 1 shows that no statistically significant association was found between anti-CCP positivity and the presence of ANA, raised erythrocyte sedimentation rate (ESR), or erosions.

Serial determinations of anti-CCP in serum were carried out in 20 patients with JIA, of whom two had low positive anti-CCP at the first determination. In both those patients anti-CCP antibodies were detected only transiently, and no patient displayed persistent positivity for anti-CCP.

We also determined the total IgG concentrations and the anti-CCP IgG antibody activity in paired SF and serum samples from eight patients with JIA (table 2). Total IgG were significantly higher in sera than in SF (p<0.001, Student’s t
DISCUSSION

Anti-CCP antibodies are now considered as an important serological marker for the diagnosis of RA and as a possible prognostic marker for the development of erosive disease. In our cohort of patients with JIA, anti-CCP were found in only 2/109 (1.8%) patients, a figure that is significantly lower than in RA. This finding supports previous observations that anti-CCP are virtually confined to patients with RA and this is not surprising because JIA is a heterogeneous group of disorders, only a small proportion of which can be considered the paediatric counterpart of RA (that is, seropositive polyarticular disease).

Published studies have reported varying prevalences of APF and AKA in patients with JIA ranging from 0% to 53%.

In a more recent study performed by Serra et al., APF were observed in 49% and AKA in 2% of patients with JIA.

The discrepancy between the reported results of APF and AKA in JIA may have several causes. It was suggested that major causes of variability were probably methodological differences of serum dilution and the criteria used to assess positivity in APF and AKA assays.

The discordance in APF and AKA positivity found in patients with JIA might also be explained by the possibility that the association of these two antibodies in JIA is not as close as that reported for RA, and that they are directed against various target antigen(s), which cannot be identified by immuno-fluorescence. Finally, heterogeneity in patient selection may be partially responsible for the discrepancies among the different studies.

Our study presents data based on an anti-CCP ELISA, which allows straightforward identification of antibodies reacting with peptides containing citrulline. Our results show that anti-CCP are rare in JIA. Furthermore, the presence of anti-CCP was not associated with any of the disease subtypes or with the presence of ANA, raised ESR, or erosions. These results are at variance with those found in adult RA serum samples, suggesting that anti-CCP have limited diagnostic value in JIA. A limitation of our original cohort is the small percentage of anti-CCP positivity might be higher. It should be pointed out that our patients were recruited in a large multicentre study, without any inclusion criteria other than the clinical diagnosis. However, in eight subsequently recruited RF positive patients there was only one anti-CCP positive patient, suggesting that anti-CCP antibodies display a low prevalence also in this subtype of JIA.

Sequential determination of anti-CCP performed in 20 patients with JIA showed no tendency for anti-CCP to increase over time. On the contrary, during the follow up period two patients initially positive became negative, which indicates that anti-CCP positivity in JIA may be a transient phenomenon and should be confirmed.

Interestingly, when tested at equal IgG protein concentrations SF samples displayed a higher anti-CCP activity than paired sera. Although the sample series is too small to draw any definite conclusions, these findings apparently favour local anti-CCP antibody production. In adult patients with RA, Reparón-Schuji et al showed that the SF compartment contains a population of B cells that spontaneously produce IgM anti-CCP. In addition, a recent paper reported convincing evidence that deaminated forms of fibrin may be the antigenic target for anti-filaggrin autoantibodies, raising the possibility that deaminated fibrin may represent the initial autoantigenic stimulation in rheumatoid synovium.

In conclusion, our data show that anti-CCP can be detected also in patients with JIA, but are generally present at low levels and less common than in adults with RA. These antibodies were reported to have a prevalence of up to 76% in patients with RA, with a very high specificity ranging between 95% and 100%.

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