Determination of Anti-Cyclic Citrullinated Peptide Antibodies in the Sera of Patients with Juvenile Idiopathic Arthritis

JASON M. LOW, ANIL K. CHAUHAN, DANIEL A. KIETZ, UMAR DAUD, PERI H. PEPMUELLER, and TERRY L. MOORE

ABSTRACT. ***Objective.*** Anti-cyclic citrullinated peptide (anti-CCP) antibodies have been found in sera of 76% of patients with rheumatoid arthritis (RA), mainly in rheumatoid factor (RF) positive patients, with a specificity of 96%. We evaluated the presence of anti-CCP antibodies in patients with juvenile idiopathic arthritis (JIA) and assessed the possibility of synthetic citrullinated peptides as antigenic determinants in JIA.

Methods. The presence of anti-CCP antibodies was determined using 3 synthetic citrullinated peptide variants and 2 commercial kits (Inova Diagnostics and Axis-Shield Diagnostics) optimized for detecting JIA-specific antibodies in serum by an ELISA based assay. We evaluated 66 patients with JIA (16 RF positive polyarthritis, 18 RF negative polyarthritis, 19 oligoarthritis, and 13 systemic arthritis). We also tested 9 adult RA patients, 34 patients with systemic lupus erythematosus (SLE), and 25 healthy persons as controls.

Results. Significant concentrations of anti-CCP antibodies were detected in the majority of RF positive JIA patients with polyarthritis. Using the 2 synthetic linear peptides, 12/16 (75%) were positive; 9/12 (75%) were positive with the Inova kit and 9/10 (90%) were positive with the Axis-Shield kit. However, utilizing the synthetic linear peptides, significant concentrations of anti-CCP antibodies were detected in 51/66 (77%) JIA patients, including 15/18 (83%) RF negative polyarthritis, 16/19 (84%) oligoarthritis, and 8/13 (62%) systemic arthritis patients. No healthy control showed elevated antibody levels. In contrast, 4/9 (44%) patients with adult RA and 2/6 (33%) with SLE had elevated anti-CCP levels. The synthetic cyclic variant cfc-1-cyc yielded significant anti-CCP levels for 13/14 (93%) patients with RF negative polyarthritis, 6/10 (60%) with oligoarthritis, and 3/7 (43%) with systemic arthritis, and 8/9 (88%) RF positive patients. No healthy control had increased anti-CCP levels. However, 4/9 (44%) adult RA and 9/34 (26%) SLE patients were found to have elevated anti-CCP levels. Using the Inova and Axis-Shield kits, much smaller percentages were found in the RF negative patients, with only 4/16 (25%) in the oligoarthritis and RF negative polyarthritis patients with the Inova kits and 0/25 (0%) by the Axis-Shield kits. The Inova kit revealed elevated anti-CCP antibodies in 5/9 (56%) adult RA patients and in 8/34 (24%) SLE patients. No healthy control had elevated anti-CCP antibodies. However, the Axis-Shield kits did not detect anti-CCP antibodies in adult RA (0/9) or SLE (0/34) patients. Moreover, 0/25 (0%) healthy individuals exhibited anti-CCP levels. The presence of anti-CCP antibodies correlated more frequently with the presence of RF.

Conclusion. This study confirms the presence of anti-CCP antibodies in patients with JIA, especially those with RF positive polyarthritis, by all ELISA based methods. Use of synthetic peptides also revealed anti-CCP antibodies in a percentage of RF negative patients with polyarthritis, oligoarthritis, and systemic arthritis; there was a loss in specificity, but an increase in sensitivity. These results suggest that antibodies to these antigenic peptides may be markers for JIA, and indicate a possible role of citrulline-containing epitopes in the pathogenesis of JIA. (J Rheumatol 2004;31:1829–33)

Key Indexing Terms:
ANTI-CYCLIC CITRULLINATED ANTIBODIES ANTIPERINUCLEAR FACTOR
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From the Division of Pediatric Rheumatology, Departments of Internal Medicine and Pediatrics, Saint Louis University Health Sciences Center, St. Louis, Missouri, USA.

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J.M. Low, MS (Research), Graduate Student, Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine; A. Chauhan, PhD, Adjunct Assistant Research Professor of Internal Medicine, Division of Rheumatology, Saint Louis University Health Sciences Center, ProGen Biologics, Inc., Ballwin, MO; D.A. Kietz, MD, Assistant Professor of Pediatrics, Division of Pediatric Rheumatology, University of Pittsburgh Health Sciences Center, Pittsburgh, PA; U. Daud, MD, Subspecialty Resident in Pediatric Rheumatology, Saint Louis University Health Sciences Center; P.H. Pepmueller, MD, Associate Professor of Pediatrics and Internal Medicine, Division of Rheumatology, Saint Louis University Health Sciences Center;

T.L. Moore, MD, Director, Division of Adult and Pediatric Rheumatology, Professor of Internal Medicine, Pediatrics, and Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center.

Address reprint requests to Dr. T.L. Moore, Division of Rheumatology, Saint Louis University, Room 211A Doisy Hall, 1402 S. Grand Blvd., St. Louis, MO 63104. E-mail: mooretl@slu.edu

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Juvenile idiopathic arthritis (JIA) is characterized by chronic inflammation of one or more joints in both children and adolescents[1]. Patients with JIA are divided into 7 subgroups, which include oligoarthritis, rheumatoid factor (RF) positive polyarthritis, RF negative polyarthritis, extended oligoarthritis, systemic arthritis, psoriatic arthritis, and enthesitis-related arthritis[2].

Serological diagnosis of JIA is difficult and is primarily restricted to females with late-onset polyarthritis, with the presence of IgM-RF in 7–10% of patients detected by nephelometry and slightly higher percentages by enzyme-linked immunoabsorbent assays (ELISA)[3]. However, about 60–70% of patients are positive for hidden 19S IgM-RF[4]. This test, however, requires serum separation and is not available in most laboratories. Anti-perinuclear factor (APF) is an antibody (or antibodies) that is found in 83% of patients with seropositive JIA polyarthritis and in 37% with seronegative JIA polyarthritis[5,6]. Anti-keratin antibodies (AKA), APF, and a monoclonal antibody for human profilaggrin produce an identical staining pattern in immunofluorescence and have been identified as a 40 kDa protein neutral/acidic isoform of filaggrin[7-9]. Filaggrin is released by proteolytic cleavage, and during this stage, the protein is dephosphorylated and roughly 20% of the arginine residues are converted into citrulline by the enzyme peptidylarginine deiminase[10]. Using several citrulline-containing peptide variants in an ELISA, Schellekens, *et al*[7] found anti-cyclic citrullinated peptide (anti-CCP) antibodies could be detected in 76% of rheumatoid arthritis (RA) sera with a specificity of 96%. The specific structure of these antibodies and the presence of these antibodies early in the disease, even before other disease manifestations occur, further indicated a possible role of citrulline-containing epitopes in the pathogenesis of RA and some utility for diagnosis and therapeutic evaluation[7,11].

We evaluated the presence of anti-CCP antibodies in JIA using 3 citrullinated peptide variants and 2 commercial kits optimized for detecting JIA-specific antibodies in a serum-binding ELISA based test. The resulting data indicate that anti-CCP antibodies are also seen in JIA. Our further studies also indicate a possible role for citrulline-containing epitopes in the pathogenesis of JIA.

MATERIALS AND METHODS

Serum samples. Sera were collected from the outpatient clinics of the Saint Louis University Health Sciences Center and Cardinal Glennon Children's Hospital. A total of 66 sera were collected from patients diagnosed with JIA, including 16 with RF positive polyarthritis, 18 RF negative polyarthritis, 19 oligoarthritis, and 13 systemic arthritis. Patients' mean age was 11.6 years and the mean disease duration was 12.4 years (range 1 to 24 yrs; median 13 yrs). Also tested were 9 adult patients with RA, 34 adult and child patients with systemic lupus erythematosus (SLE), and 25 healthy controls. The study was approved by the Institutional Review Board of the Saint Louis University Health Sciences Center.

Peptide synthesis and ELISA. Both linear and cyclic peptides were selected for synthesis from amino acid sequences deduced from known cDNA sequences of human profilaggrin as originally described by Gan, *et al*[10] and subsequently modified by Schellekens, *et al*[7,11] (Table 1). The peptides were synthesized by Research Genetics (Invitrogen Corporation, Huntsville, Alabama, USA) for our assays. The peptides were at least 98% pure as deduced from their elution pattern on reverse-phase high performance liquid chromatography (RP-HPLC) at the relative absorption of 214 nm. Key linear peptides used in this study were cfc1, cfc1-319, cfc1-318, cfc1-317, and cfc1-316, with the substitution of citrulline for arginine at peptide sequence 312 as described[7]. A cyclic peptide (cfc1-cyc) formed by substituting serine residues by cysteine in cfc1 was also synthesized[11].

Peptides were coupled covalently to 96-well assay plates (TPP Corp., Callino, Switzerland) at 1 µg per well. Coupling was performed at 4°C for 16 h at pH 9.0. Nonspecific binding was evaluated when wells were coupled with 2 µg of bovine serum albumin (BSA) per well instead of peptide. All wells were blocked with 2% BSA for 1 h and subsequently washed 6 times with phosphate buffered saline/0.05% (vol/vol) Tween-20 (PBS-T). Sera were diluted 200-fold in 0.05% PBS-T and incubated 1.5 h at room temperature. After washing 6 times with 0.05% PBS-T, 100 µl of anti-human IgG conjugated to horseradish peroxidase (HRP; Sigma Chemical Co., St. Louis, MO, USA) diluted 1:1000 in PBS-T was added to the wells. After incubation (1 h at room temperature) plates were washed 6 times with 0.05% PBS-T, and bound antibodies were detected with 3, 3′-5, 5′-tetramethyl-benzidine (TMB; BioRad Laboratories, Hercules, CA, USA) as substrate. The reaction over 10 min was stopped by adding 100 µl of 2 M sulfuric acid/well. In addition to the 3 synthetic peptide ELISA, IgG anti-CCP antibodies were measured using both the Inova QuantaLite ELISA kit (Inova Diagnostics Inc., San Diego, CA, USA) and the Axis-Shield Diastat ELISA kit (Axis-Shield Diagnostics Ltd., Dundee, Scotland). All commercial assays were used according to the manufacturer's directions. After subtraction of the blank control and highest healthy value, the resulting OD_{450} ×1000 was defined as the optimal cutoff value. All sera were tested in duplicate to ensure consistency and the results averaged. A control serum was included on all plates to monitor plate-to-plate variation. Variation was never significant and therefore values were not corrected.

RESULTS

The 6 peptides synthesized and described by Schellekens, *et al*[7,11] were cfc1, cfc1-319, cfc1-318, cfc1-317, cfc1-316, and cfc1-cyc. It was found that results were reproducible from utilizing linear variants cfc1-318 and cfc1-319 and the cyclic variant, cfc1-cyc, in the ELISA (Table 1). Subsequently, cfc1, cfc1-317, and cfc1-316 were removed

Table 1. Sequences of synthetic peptides.

Peptide	Sequence*
cfc1	SHQESTXGRSRGRSGRSGS
cfc1-316	SHQESTXGRSR
cfc1-317	SHQESTXGRSRG
cfc1-318	SHQESTXGRSRGR
cfc1-319	SHQESTXGRSRGRS
cfc1-cyc	HQXHQESTXGRSRGRCGRSGS

* X: citrulline; C: bonding carbon.

from the study. Peptides purified by RP-HPLC eluted as a single peak were at least 98% pure, as deduced from their elution pattern. Identity of the 3 peptides was confirmed by mass spectrometry. To identify which peptide was the best antigenic substrate for the anti-CCP antibodies, the performance of both the synthetic citrullinated peptide variants and the 2 anti-CCP kits was determined and compared. The most reproducible results were ascertained from applying both the Inova and Axis-Shield kits. ELISA data using both linear variant peptides yielded significant concentrations of anti-CCP antibodies in 51 out of 66 (77%) patients with JIA, including 15/18 (83%) RF negative polyarthritis, 16/19 (84%) oligoarthritis, 8/13 (62%) systemic arthritis, and 12/16 (75%) RF positive polyarthritis patients (Figure 1A). No healthy controls showed any elevated antibody levels. Four of 9 patients with adult RA and 2 of 6 with SLE had elevated antibody levels. In JIA patients, the presence of anti-CCP antibodies did not correlate statistically with presence of RF, except in patients with RF positive polyarthritis, who exhibited greater sensitivity with higher optical density (OD) values than the other 3 subgroups. In contrast, the cyclic variant peptide cfc1-cyc generated data with both greater specificity and sensitivity compared to the linear variants. Utilizing cfc1-cyc, significant anti-CCP antibody levels were observed for 13/14 (93%) RF negative polyarthritis, 6/10 (60%) oligoarthritis, 3/7 (43%) systemic arthritis, and 8/9 (88%) RF positive polyarthritis patients (Figure 1B). Again, patients with RF positive polyarthritis produced greater OD values with the corresponding subgroups, indicating an increased sensitivity. None of 17

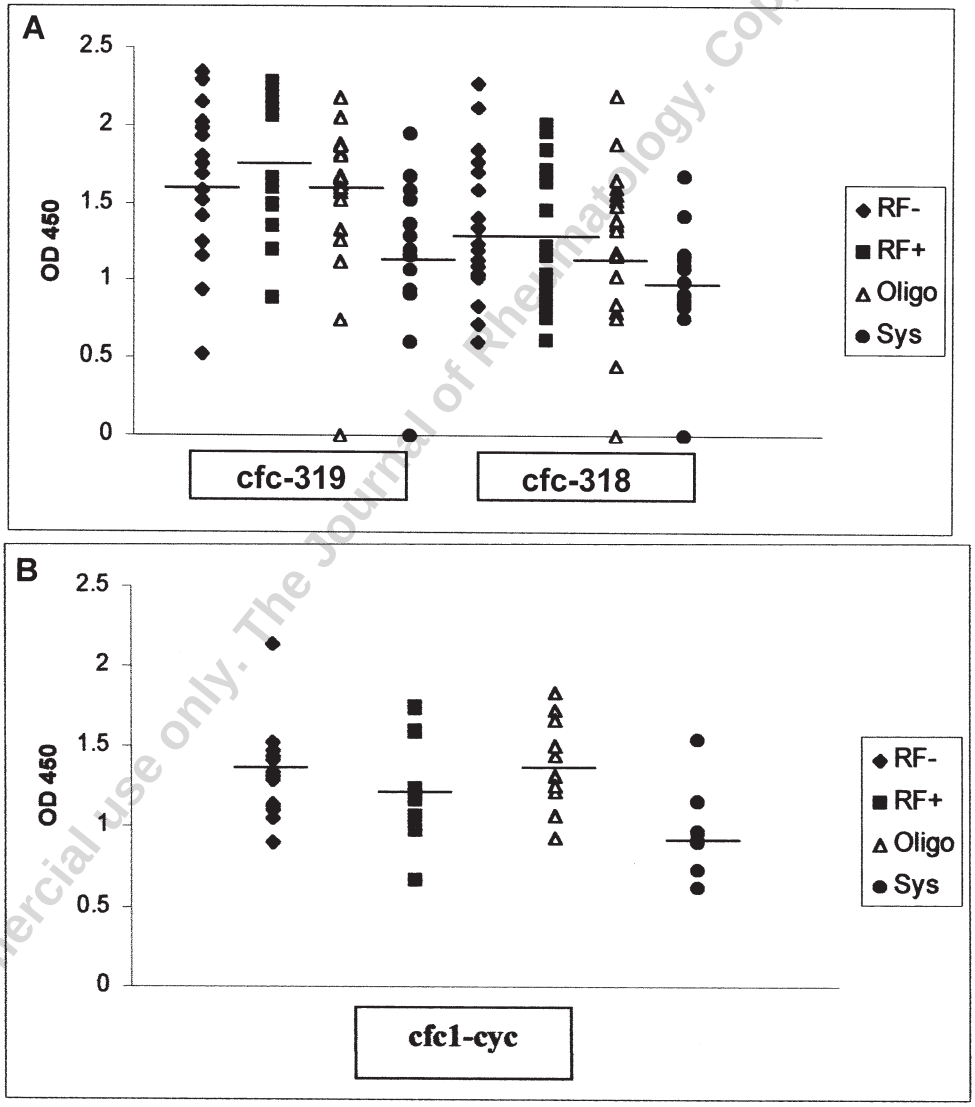

Figure 1. Percentage optical density (OD) at 450 nm of anti-citrullinated antibody levels utilizing (A) linear variant peptides cfc-319 and cfc-318, and (B) the cyclic variant peptide cfc1-cyc for patients with different subtypes of JIA (RF negative polyarticular, RF positive polyarticular, oligoarticular, and systemic). Horizontal lines indicate means of pooled data for each JIA subtype.

healthy controls exhibited increased antibody levels. In contrast, 4/9 (44%) adult RA and 9/34 (26%) SLE patients were found to have elevated anti-CCP antibodies. To further assess anti-CCP antibody levels in the patient subsets, ELISA data were collected using kits from both Inova Diagnostics and Axis-Shield Diagnostics. For both kits, optimal cutoff values were obtained from regression analysis and the subsequent construction of an internal standard curve, as well as comparison with both high and low positive values. Both kits gave results indicating greater specificity for patients with RF positive JIA. The increase in specificity was seen predominantly in RF positive sera. Inova and Axis-Shield kits yielded positive values in 9/12 (75%) and 9/10 (90%) patients with RF positive polyarthritis, respectively (Figure 2A). Inova kits also produced positive values in 2/11 (18%) RF negative polyarthritis and 2/5 (40%) oligoarthritis patients, but none in patients with systemic arthritis (Figure 2B). Similarly to the cfc1-cyc variant, the Inova kit exhibited elevated anti-CCP antibody levels in 5/9 (56%) adult RA and 8/34 (24%) SLE patients, and 0/25 (0%) healthy individuals. These data are consistent with findings from Inova Diagnostics (Piette AL, *et al*, unpublished data). In contrast, the Axis-Shield kit did not yield significant anti-CCP levels for patients other than the RF positive polyarthritis subset, with 0/15 (0%) for patients with RF negative polyarthritis, 0/10 (0%) oligoarthritis, 0/7 (0%) systemic arthritis, 0/9 (0%) adult RA, and 0/34 (0%) with SLE. In addition, 0/25 (0%) healthy individuals exhibited elevated anti-CCP levels.

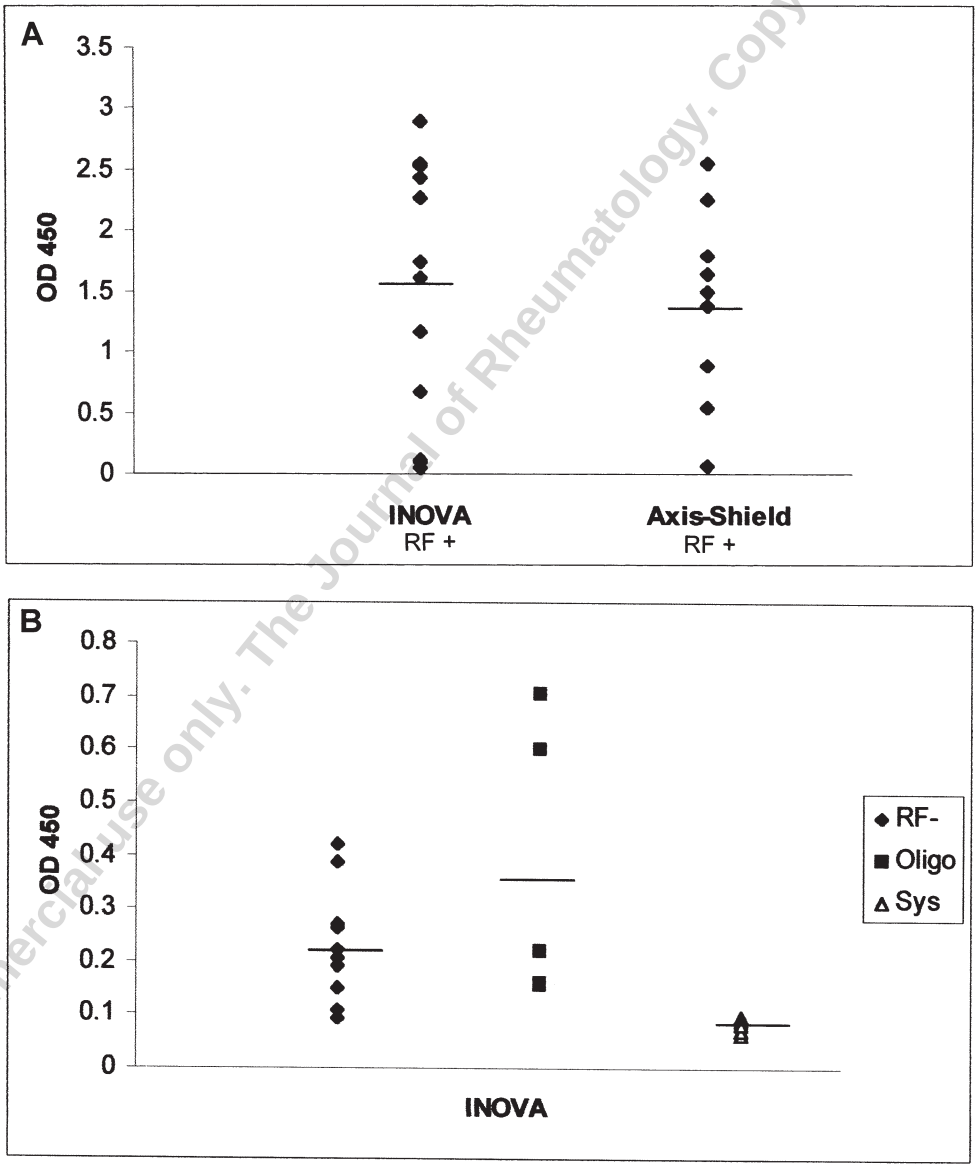

Figure 2. Percentage optical density (OD) at 450 nm of anti-citrullinated antibody levels utilizing (A) Inova and Axis-Shield kits for RF positive patients and (B) the Inova kit for the remaining disease subtypes (RF negative polyarticular, oligoarticular, and systemic). Horizontal lines indicate means of pooled data for each JIA subtype.

DISCUSSION

We observed that anti-CCP antibodies can be detected in patients with JIA, especially those patients with RF positive polyarthritis. In addition, we found anti-CCP antibodies in patients with SLE, albeit with lower specificity. Subsequent studies have shown a patient with psoriatic arthritis and one with mixed connective tissue disease that were positive for anti-CCP antibodies. These non-RA disease groups did not have RA-like arthritis, supporting the observation that anti-CCP are not RA-specific, but rather are highly sensitive in RA and JIA. Further, this study demonstrates that citrulline can be considered an antigenic determinant recognized by antibodies in JIA sera, among other diseases.

We also demonstrate that an ELISA based test for the presence of anti-CCP antibodies in JIA can be adapted utilizing both synthetic linear and cyclic peptides as the antigen. With predefined sequences, large quantities of synthetic peptides can be produced with a high degree of purity. However, an innate problem exists with the use of relatively short (linear) peptides. Because they generally do not adopt a stabilized conformation in solution, only a fraction of the peptides adopt a form comparable to that of the original antigenic site on the complete protein antigen. This results in a direct negative effect on the affinity of the antibody for the peptide, despite the peptide sequence comprising the whole antigenic site, and the increased possibility for nonspecific binding[11]. The findings with the linear variants, cfc-318 and cfc-319, exemplify this principle, as the corresponding rates of positive values were elevated for the patients with RF negative polyarthritis, oligoarthritis, and systemic arthritis JIA and the SLE patients when compared to values utilizing both the cyclic variant and the kits. It is important to note that both the Inova and Axis-Shield kits use identical antigenic peptides provided by the same source. Previous Inova kits with different antigenic peptides gave data similar to those found with the 3 synthetic peptides.

The etiology of the antigenicity of citrulline has been debated. In most cases arginine is modified to citrulline, and this modified antigen could possibly evoke a polyclonal antibody response against citrulline. The antibodies to citrulline in JIA are similar to the percentages in adult patients with RA, the majority being RF positive[7,11]. Since previous observations indicate that anti-CCP antibodies are most commonly found in patients with RA, it is not surprising to find them in certain subsets of patients with JIA, especially patients with RF positive polyarthritis. However the presence of anti-CCP antibodies did not correlate directly with presence of RF as in adults[7,11]. Further, Avcin, *et al*[12] detected anti-CCP antibodies in 2% of patients with JIA, which is much less common than in adults with RA. Two limitations are noted that would account for the discrepancy between the results of Avcin, *et al*[12] and our study. First, their study included a small number of seropositive polyarticular patients. We hypothesize that a larger seropositive polyarticular cohort would reveal a significantly higher correlation with anti-CCP antibodies. Further, the patients in their group were from a large multicenter study with no inclusion criteria other than their clinical diagnosis[12]. In addition, anti-CCP antibody positivity in JIA is transient over the course of the disease. Therefore, the time (in relation to disease activity) at which the sera were obtained would influence the anti-CCP antibody concentration. Previously in the separation of immune complexes from the sera of JIA patients we had noted a 40 kDa band[13]. Isolation of immune complexes was by affinity chromatography, sodium dodecyl sulfate gradient, and Western blot analysis. Four bands were noted, a 70 kDa band of IgM heavy chain, a 50 kDa band of IgG heavy chain, a 60 kDa band, possibly a heat-shock protein, and a 40 kDa band, which may be a cyclic citrullinated protein. Further studies may give more information on the antigenic determinants responsible for the production of anti-CCP antibodies.

Our data indicate anti-CCP antibodies can be detected in sera from patients with JIA and may indicate a possible role of citrulline-containing epitopes in the pathogenesis of JIA.

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