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Diagnostic Accuracy of the Anti-Citrulline Antibody Assay for Rheumatoid Arthritis

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Background: **Rheumatoid arthritis (RA) is the most common autoimmune rheumatic disease, but specific and practicable tests for its diagnosis are lacking. We evaluated the diagnostic accuracy of a new commercial ELISA in detecting anti-cyclic citrullinated peptide (CCP) antibodies for the diagnosis of RA.**

Methods: **Anti-CCP antibodies were determined in 330 serum samples: 98 from RA patients and 232 from controls, including patients with connective tissue diseases, other rheumatic diseases, viral infections, Lyme disease, autoimmune thyroiditis, cancer, and monoclonal gammopathy, and sex- and age-matched healthy subjects. Intra- and interassay CVs were 5–13% and 9–17%, respectively. Rheumatoid factor (RF) was also assayed in every sample, and results were compared to anti-CCP for sensitivity and specificity.**

Results: **At a cutoff value of 50 units, sensitivity was 41% (confidence interval, 31–50%) and specificity was 97.8% (95–100%). Anti-CCP-positive RA patients had a mean antibody concentration of 1100 units (range, 57–3419 units), and anti-CCP-negative RA patients and controls had mean values of 7.6 and 6.8 units, respectively (range, 1–39 units). The area under the ROC curve was 0.71 (95% confidence interval, 0.63–0.78). RF had a higher sensitivity (62%) and a lower specificity (84%) than anti-CCP. When the two antibodies were used together, specificity was 99.6%.**

Conclusion: **Anti-CCP antibody testing may be useful if performed concomitantly with RF assay to diagnose patients with suspected early RA.**

Rheumatoid arthritis (RA)[6] is the most frequent autoimmune rheumatic disease, affecting ~1–2% of the population in Western countries *(1)*. RA is diagnosed primarily according to clinical manifestations, and serologic support is restricted to the determination of IgM rheumatoid factor (RF), which has a low specificity because it may be found in healthy elderly individuals, healthy immunized subjects, and patients with other autoimmune diseases or chronic infections.

Two other antibodies, anti-perinuclear factor (APF) *(2)* and anti-keratin antibodies (AKAs) *(3)*, are considered possible diagnostic markers for RA; both recognize the antigenic protein filaggrin *(4, 5)*. Although quite specific, these antibodies have never gained widespread popularity because of technical difficulties in substrate standardization and the subjective nature of the interpretation of the immunofluorescence tests.

Recently, a new serological test, the anti-cyclic citrullinated peptide (anti-CCP) ELISA was developed *(6)* and, based on preliminary data, it has an excellent specificity for the diagnosis of RA, especially in patients with early disease *(7, 8)*.

Citrulline is an unusual amino acid resulting from an enzymatically posttranslationally modified arginine residue. Citrulline is present on a few human proteins, including filaggrin. Profilaggrin, which is present in the keratohyalin granules of human buccal mucosa cells, is proteolytically cleaved into several filaggrin subunits during cell differentiation. During this stage, the protein is dephosphorylated, and some arginine residues are converted into citrulline by the enzyme peptidylarginine deiminase *(6)*.

In this study we evaluated the diagnostic and analyti-

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[6] Nonstandard abbreviations: RA, rheumatoid arthritis; RF, rheumatoid factor; APF, anti-perinuclear factor; AKA, anti-keratin antibody; CCP, cyclic citrullinated peptide; HCV, hepatitis C virus; AITD, autoimmune thyroid disease; MGUS, monoclonal gammopathy of undetermined significance; and CI, confidence interval.

cal performances of a new commercial ELISA for the detection of anti-CCP antibodies.

Materials and Methods

We studied 330 serum samples: 98 from RA patients (88 women and 10 men; mean age, 65 years; range, 43–89 years) diagnosed according to the American College of Rheumatologists criteria *(9)* and consecutively recruited from the Rheumatology outpatient clinic. Thirty-six (36.7%) of these patients were classified as having early RA because the diagnosis was made <1 year before this study and radiological examinations revealed no lytic lesions at the wrists, hands, and feet. To provide data on assay specificity, 232 controls selected on the basis of their clinical diagnoses were also studied and consisted of 43 patients with connective tissue diseases (24 Sjögren syndrome, 14 systemic lupus erythematosus, 3 systemic sclerosis, 1 mixed connective tissue disease, and 1 dermatopolymyositis); 24 patients with other rheumatic diseases (15 polymyalgia rheumatica and 9 psoriatic arthritis); 3 patients with juvenile RA; 31 patients with various viral infections [16 hepatitis C virus (HCV) infection, 8 hepatitis B virus, 4 parvovirus B19, and 3 Epstein-Barr virus]; 20 with Lyme disease; 29 with autoimmune thyroid diseases (AITDs); 14 with different kinds of cancer, and 10 with monoclonal gammopathy of undetermined significance (MGUS), as well as 58 sex- and age-matched healthy subjects.

Among the RA patients, the variables recorded were age, gender, time from diagnosis, and laboratory data (antinuclear antibodies, IgM-, IgA-, and IgG-RF).

Anti-CCP antibodies were tested by ELISA (Immunoscan RA; EuroDiagnostica); RF was measured by laser nephelometry for the IgM isotype and by ELISA for the IgA and IgG isotypes; antinuclear antibodies were assayed by indirect immunofluorescence on HEp-2 cells. Each of these tests was performed and evaluated by operators who were blinded to other serological results and unaware of the patients' clinical data.

To evaluate assay reproducibility, we also determine intra- and interassay imprecision; to this end, three samples containing low (100 units), intermediate (600 units), and high (2300 units) antibody concentrations were assayed six times in five independent analytical runs on different days.

Statistical analysis was performed using the SPSS 6.0 for Windows statistical package (SPSS). Means, SD, and confidence intervals (CIs) were used where appropriate. Nonparametric analyses were used to compare the two different groups of patients, and multiple regression models were used to assess the importance of the different variables relative to anti-CCP status. Two-sided P values <0.05 were considered significant throughout. ROC curves were used to calculate cutoff values for optimal sensitivity and specificity *(10)*. Moreover, in preparing this report, the guidelines proposed by Bruns et al. *(11)* were followed.

Results

SENSITIVITY AND SPECIFICITY OF ANTI-CCP

Forty of 98 RA patients (40.8%) were positive for anti-CCP at very high concentration, whereas only 7 of 232 control sera (3.0%) showed a positive reaction, in particular sera from 1 HCV-seropositive patient, 3 patients with Lyme disease, 2 with AITD, and 1 with MGUS. None of the patients with connective tissue diseases, juvenile rheumatoid arthritis, other inflammatory rheumatic diseases, or cancer was positive, as was none of the 58 healthy controls (Fig. 1). Among these seven presumptive false positives, the patient with MGUS, one of the two patients with AITD, and the one patient with HCV infection had very high antibody concentrations of 1029, 1654, and 1395 units, respectively; the other four had lower concentrations (66–154 units). These seven patients were all re-

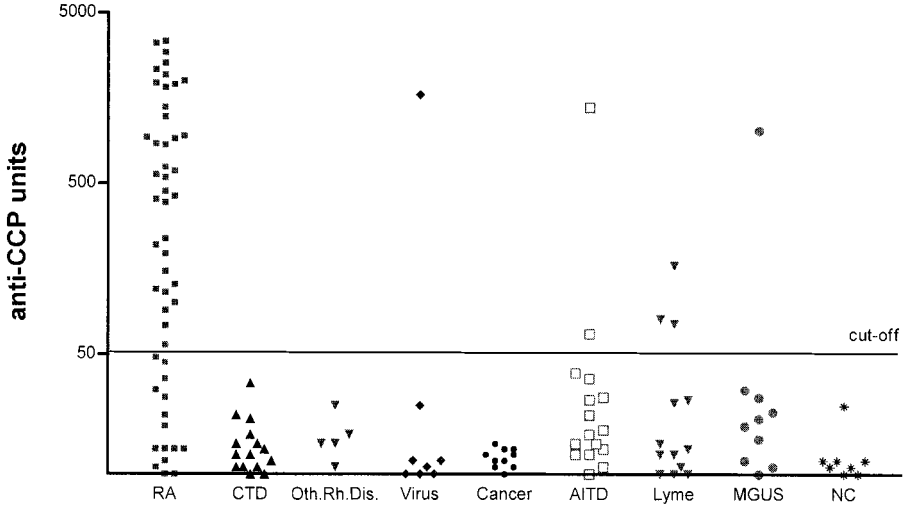

Fig. 1. Distribution on a log scale of the test results according to anti-CCP units for the different groups of patients.

A cutoff value set at 50 units guarantees a good specificity because all but seven of the non-RA patients have an antibody concentration below the threshold. *CTD*, connective tissue disease; *Oth. Rh. Dis.*, other rheumatoid diseases; *NC*, healthy controls.

called, and a very careful anamnesis and clinical work-up were done. After this inquiry, it became evident that at least two of the patients with high antibody concentrations, the one with MGUS and one with AITD, also had RA. The HCV-positive patient died of colon cancer, but according to her general practitioner, she had no symptoms or signs of RA. The three patients with Lyme disease had no signs of RA, and the other AITD patient was lost to follow-up. To avoid introducing a possible bias, the other 225 patients in the control group were recalled to verify the presence or absence of RA symptoms. None manifested clinical signs of RA. However, in 13 subjects follow-up was not possible; 5 patients with cancer and 2 with MGUS had died, and 6 subjects with AITD could not be located. After this correction, the optimal cutoff value was determined by means of the ROC curve (Fig. 2). At a cutoff value of 50 units, sensitivity was 41% (CI, 31–50%) and specificity was 97.8% (CI, 95–100%; Table 1). The area under the ROC curve was 0.71 (CI, 0.63–0.78).

ANTIBODY CONCENTRATION

The assay reagent set included four calibrators from 50 to 3200 units; therefore, we could obtain data on antibody concentration as well. Sample diluent was also included as the blank in each assay to calculate the zero unit value. RA CCP-positive patients had a mean antibody concentration of 1100 ± 764 units (range, 57–3419 units). RA CCP-negative patients had a mean value of 7.6 ± 6.7 units, and controls had a mean of 6.8 ± 5.1 units, with a range of 1–39. The intraassay CV was 4.8–13%, and the interassay CV was 9–17%.

ANTI-CCP AND RF

We also looked at RF prevalence: IgM-RF was positive in 62.2% (61 of 98) of the RA cases and in 16% (36 of 232) of controls. Therefore, the sensitivity was 62% (CI, 57–67%) and specificity was 84% (CI, 82–87%).

IgA-RF, IgG-RF, and antinuclear antibodies, tested only in RA patients, were positive in 45%, 33%, and 8% of the cases, respectively. In the RA group, a good association was found between anti-CCP and IgM-RF (χ^2, 13.8; $P = 0.0002$), IgA-RF (χ^2, 18.8; $P = 0.00001$), and IgG-RF (χ^2, 7.0; $P = 0.008$). Anti-CCP antibodies alone were present in 5 cases, and IgM-RF was the only antibody in 26. In the control group, there were 4 false-positive cases for anti-CCP and 34 for IgM-RF; only 1 case was falsely positive for both anti-CCP and IgM-RF antibodies (Table 2).

We also compared the results of anti-CCP and IgM-RF for sensitivity and specificity. IgM-RF showed a higher sensitivity (62% vs 41%) and a lower specificity (84% vs 98%) than anti-CCP.

We also analyzed how these parameters would be affected if anti-CCP and IgM-RF were combined in pa-

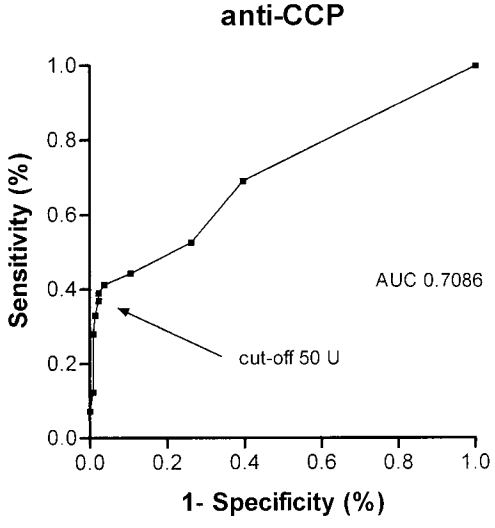

Fig. 2. ROC curve showing that a cutoff value set at 50 units has the highest sensitivity, while maintaining a high specificity.

Below 40 units the assay rapidly loses specificity. *AUC*, area under the curve.

Table 1. Anti-CCP findings in RA patients and control groups.[a]

		Anti-CCP findings	
	Total, n	Positive, n	Negative, n
RA patients	98	40	58
Patients with non-RA pathologies	174	5	169
JRA[b]	3	0	3
CTDs	43	0	43
Other rheumatoid diseases	24	0	24
Viral infections	31	1	30
Lyme	20	3	17
AITD	29	1	28
Cancer	14	0	14
MGUS	10	0	10
Healthy controls	58	0	58
Total	330	45	285

[a] At optimal cutoff values as determined by ROC curves, sensitivity was 41% and specificity was 97.8%.

[b] JRA, juvenile rheumatoid arthritis; CTDs, connective tissue diseases.

Table 2. Test results for anti-CCP in relation to RF presence or absence.[a]

	RA patients (n = 98)		Controls (n = 232)	
	n	%	n	%
Anti-CCP positive				
RF positive	35	36	1	0.4
RF negative	5	5	4	1.7
Anti-CCP negative				
RF positive	26	26	35	15.1
RF negative	32	33	192	82.8

[a] Although the two antibodies were significantly correlated (χ^2, 13.8; $P = 0.0002$), in the RA group anti-CCP was present alone in 5 cases and RF was the only antibody in 26, showing that they are different markers for RA. In the control group, 5 cases were false-positive for anti-CCP and 36 for RF; in only 1 case were they both positive.

tients with only one of the two markers (anti-CCP or IgM-RF) positive as well as in patients with both markers (anti-CCP and IgM-RF) positive. When assayed together, the specificity would increase from 98.7% for anti-CCP alone to 99.6% (Table 3).

A significant correlation was also found between anti-CCP reactivity and early arthritis (χ^2, 8.2; $P = 0.004$). We found no correlation between the presence or absence of anti-CCP antibody and age or gender.

Discussion

It has long been known that the keratohyalin bodies present in human buccal mucosa cells contain filaggrin, a protein that is recognized by APF and AKAs, specific antibodies present in RA subjects. These antibodies are detectable by indirect immunofluorescence techniques, but they have never become part of the diagnostic repertoire of clinical laboratories because of difficulties in the availability and storage of the antigenic substrates, as well as objective difficulties in interpreting the fluoroscopic patterns *(12, 13)*.

The recent development of synthetic peptides containing citrulline *(6)*, an amino acid present in the filaggrin molecule and produced after its deimination, has enabled the development of an ELISA test; from preliminary data obtained during experimental trials, this test appears to have the same high specificity as APF and AKAs and to be able to eliminate the standardization problems related to immunofluorescence procedures. In this study, we evaluated the diagnostic accuracy of this new ELISA test, which is now commercially available. In the 98 RA subjects studied, the diagnostic sensitivity of the test was 41% and the specificity was 98%; these values confirm those obtained initially by Schellekens et al. *(8)*.

To explain the low sensitivity, it must be considered that anti-CCP antibodies are a heterogeneous group of antibodies directed against different epitopes on the citrulline molecule, that each patient's serum contains different subsets of antibodies, and that the synthetic peptide used in this assay represents a relatively small set of antigenic determinants that do not entirely encompasses the antigenic determinants present on the as yet unknown antigenic molecule in the joint *(14)*.

The specificity is instead the most valuable aspect of this assay, so much so that it may be proposed as the most important examination in the diagnosis of RA. The net and surprising difference in antibody concentration between anti-CCP-positive and -negative samples is noteworthy. Indeed, positive samples showed high antibody concentrations, with a mean value of 1100 units, whereas negative samples were never higher than 39 units, with a mean value of 7–8 units. This is the first study to report quantitative data on anti-CCP antibody concentrations. Although our results require confirmation in larger studies, they show that a high antibody concentration is almost exclusively associated with RA.

It was also interesting to evaluate anti-CCP and RF behavior in RA patients in relation to the duration of disease. In patients with early arthritis (diagnosis made <1 year before this study), the correlation with anti-CCP was highly significant, thus indicating that this assay may be used even in the early phases of disease. This aspect is important because an early diagnosis of RA may modify the therapeutic strategy substantially, suggesting the use of more aggressive pharmacological treatments that can delay the progression of joint damage and thus substantially change the natural history of the disease *(15–17)*.

Preliminary studies have demonstrated that the presence of anti-CCP antibodies also has a prognostic significance because it was shown that anti-CCP-positive patients develop significantly more severe radiologic damage than anti-CCP-negative patients *(7, 14, 18)*. Therefore, serial assay of these antibodies could be useful in monitoring the clinical course.

In conclusion, we believe that the anti-CCP antibody assay is a very valuable tool for the diagnosis of RA. This ELISA test avoids many of the problems of the APF and AKA tests regarding quantification of the results and standardization of the assay. Its low sensitivity does not allow its use as a screening test, but because of its high specificity, especially when high antibody concentrations are present, it may become one of the most useful serologic tests for the diagnosis of RA. Moreover, when associated with RF determination, it provides nearly 100% specificity and thus could be helpful in the differential diagnosis of RA and other rheumatic diseases. In addition, this test may be very influential for the choice of the best therapeutic strategy in patients with recent-onset arthritis.

Table 3. Sensitivity and specificity of anti-CCP and IgM-RF, alone and combined, in patients with one of the two antibodies present, and in patients with both antibodies.

	Sensitivity, %	Specificity, %
Anti-CCP	41	98
IgM-RF	62	84
Anti-CCP or IgM-RF	70	83
Anti-CCP and IgM-RF	33	99.6

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