Diagnostic tests for rheumatoid arthritis: comparison of anti-cyclic citrullinated peptide antibodies, anti-keratin antibodies and IgM rheumatoid factors

S. Bas, T. V. Perneger[1], M. Seitz[2], J.-M. Tiercy[3],
P. Roux-Lombard[4] and P. A. Guerne

Division of Rheumatology, Department of Internal Medicine, University Hospital, Geneva, [1]Quality of Care Unit and Institute of Social and Preventive Medicine, University Hospital, Geneva, [2]Department of Rheumatology and Clinical Immunology/Allergology, Inselspital, Bern, [3]Transplantation Immunology Unit, Department of Internal Medicine, University Hospital, Geneva and [4]Division of Immunology and Allergology, Department of Internal Medicine, University Hospital, Geneva, Switzerland

Abstract

Objectives. To examine the value of anti-cyclic citrullinated peptide (anti-CCP) antibodies, anti-keratin antibodies (AKA) and immunoglobulin M rheumatoid factors (IgM RF) in discriminating between rheumatoid arthritis (RA) and other rheumatic diseases, and to determine whether the clinical manifestations or severity of erosions in RA are associated with anti-CCP positivity.

Methods. In a cross-sectional study, we determined the concentrations or titres of these three markers in 179 RA patients and 50 controls. Erosions were quantified using the Larsen score in 129 patients.

Results. Sensitivity was highest for IgM RF (75%), followed by anti-CCP antibodies (68%) and AKA (46%). Specificity was highest for anti-CCP antibodies (96%), followed by AKA (94%) and IgM RF (74%). A correlation with clinical manifestations and severity of erosions was observed mainly for IgM RF positivity.

Conclusions. With their excellent specificity, anti-CCP antibodies can be useful in establishing the diagnosis of RA, but IgM RF is a better predictor of disease severity.

KEY WORDS: Rheumatoid arthritis, Anti-cyclic citrullinated peptide antibodies, Anti-keratin antibodies, IgM rheumatoid factor.

Rheumatoid arthritis (RA) has been associated with several autoantibodies, including rheumatoid factors (RF), anti-perinuclear factor (APF), anti-keratin antibodies (AKA) and anti-filaggrin antibodies (AFA) [1]. The epitopes targeted by APF, AKA and AFA have been characterized recently [2, 3]. These autoantibodies bind antigenic determinants that contain the unusual amino acid citrulline [2], formed by a post-transcriptional modification of arginine residues by peptidyl-arginine deiminase [4]. To detect these autoantibodies, Schellekens *et al.* [2] developed an enzyme-linked immunosorbent assay (ELISA), using as antigen a cyclic variant of a citrullinated peptide (CCP) derived from the sequence of human filaggrin. The diagnostic and prognostic value of this test has been evaluated in patient populations with recent-onset RA (< 1 yr) and a follow-up of 1 yr [1, 5], 3 yr [6] and 6 yr [7]. The evaluations were compared with those obtained for RF [1, 5–7], APF [6], AKA and AFA as well as antibodies to Sa and RA-33 [1]. The anti-CCP antibody test has subsequently become available commercially (Euro-Diagnostica, Arnhem, The Netherlands) and its diagnostic accuracy has been compared with that of the RF test [8].

However, the performance of this test in the diagnosis of RA is still unclear, as sensitivities ranging from 41 to 68% have been reported [1, 5–8]. We therefore compared

the sensitivity and specificity of the anti-CCP antibody test with those of tests for the autoantibodies most commonly used in the diagnosis of RA: AKA and immunoglobulin (Ig) M RF.

Some uncertainty also remains about the value of these autoantibodies as markers of disease severity. Some studies have reported an association of AKA with disease expression (activity, severity or outcome) [9–14] but others have not [15–17]. Schellekens *et al.* [5] have found anti-CCP antibodies and IgM RF equally associated with erosive disease, while Kroot *et al.* [7] found IgM RF more associated with radiological damage than anti-CCP antibodies. We therefore also investigated the correlation of anti-CCP antibodies with clinical severity and joint damage progression in comparison with AKA and IgM RF.

Patients and methods

Patients

Two groups were compared. The RA patients comprised 179 consecutive, unselected patients who had been diagnosed with RA according to the revised criteria formulated by the American College of Rheumatology (ACR) [18]. The clinical characteristics of these patients have been described previously [15]. Briefly, nodules and serositis were defined as the presence of either of these manifestations at any time during evolution. Sicca syndrome was taken into account in the presence of anamnestic xerophthalmia, xerostomia, a positive Schirmer's test result or a positive biopsy result. As data on joint destruction and erosions proved difficult to interpret, we subsequently requested recent hand X-rays (obtained less than 2 yr previously) for all patients, and obtained them for 129 patients. Joint damage progression was assessed with a standardized Larsen score as a function of disease duration at the time the X-ray was taken. X-rays were analysed blindly with regard to clinical and laboratory data, by the same reader (a rheumatologist). The Larsen score was established using standard reference films [19]. The wrists, meta-carpophalangeal joints 2–5 and proximal interphalangeal joints 2–5 were scored on the following scale: 0 = no abnormalities, 1 = slight abnormalities (joint space narrowing or band-like osteoporosis); 2 = small but definite erosions; 3 = erosions of medium severity; 4 = severe destructive abnormalities; 5 = mutilating abnormalities. The score for the wrist was then multiplied by 2, so that the total score ranged from 0 to 100.

The non-RA control patients were 50 patients recruited in the out-patient clinic of the University Hospital of Geneva [crystal-induced arthritis ($n = 21$), connective tissue diseases ($n = 9$), seronegative spondyloarthropathies ($n = 7$), other inflammatory diseases, including Crohn's disease, polymyalgia rheumatica and sarcoidosis ($n = 7$), osteoarthritis ($n = 6$)].

A serum sample was drawn from each patient, aliquoted and stored at $-80°C$ until use.

Anti-CCP antibody determination by enzyme immunoassay

The ELISA kits used to detect IgG anti-CCP antibodies were purchased from Euro-Diagnostica. The assay was performed according to the manufacturer's protocol. Different tests were carried out to evaluate the performance of the kit. The average recovery was 94% (anti-CCP antibodies spiked to three levels in three serum samples), the intra-assay coefficient of variation (CV) was 4–10% and the inter-assay CV 6–20%.

AKA determination by indirect immunofluorescence

The test was performed as described previously [15]. Positive sera were titrated and the greatest serum dilution exhibiting evidence of fluorescence was considered the titration end-point.

IgM RF determination

Flat-well microtitre plates (Immunoplate I, catalogue no. 439454; Nunc Life Technologies, Basel, Switzerland] were used as the solid phase and were coated with human IgG Fc fragment (5 µg/ml) (Organon Teknika Cappel, Durham, NC, USA]. Non-specific binding sites were blocked with 1% foetal calf serum (Gibco, Life Technologies, Basel, Switzerland). Appropriate dilutions of serum (usually 1/25, 1/50 and 1/100 for patient serum and 13 serial two-fold dilutions from 1/20 for standard serum) were incubated for 90 min at 37°C. The reactivity of each serum dilution was also tested in a non-coated well. Bound IgM RF was detected with alkaline phosphatase-labelled F[ab′]$_2$ fragment of polyclonal goat IgG anti-human IgM (μ-chain-specific) from Organon Teknika Cappel for 1 h 30 min at 37°C. Substrate [1 mg/ml paranitrophenyl phosphate (Fluka, Buchs, Switzerland) in 10% diethanolamine] was added for 45 min at 37°C. Colour development was stopped by the addition of 3 M NaOH. The optical density (OD) at 410 nm was determined with a Dynatech MR 5000 Microplate reader (Dynatech, Alexandria, VA, USA) linked to a Macintosh computer running the Biocalc program (Dynatech). The net OD values [OD value for the Fc-coated well minus non-specific OD (OD for the non-coated well)] were transformed automatically after curve-fitting with the four-parameter log-logistic model transformation [20] into international units by the use of the standard dilution curve (curve derived from 13 serial two-fold dilutions of a pool of sera calibrated with the WHO RF reference serum [21]).

Immunogenetic analysis

HLA DR generic typing and DRB1*01 and DRB1*04 subtyping were performed as described elsewhere [22].

Statistical analysis

We determined the cut-off value for positivity of the anti-CCP antibody test from the receiver operating characteristic (ROC) curve, and computed the area under the ROC curve. The area under the curve indicates, for a randomly drawn RA patient and

control, the probability that the RA patient will have a higher test value than the control; a value of 0.5 indicates no discrimination and 1 perfect discrimination [23].

The sensitivity (among RA patients) and specificity (among other patients) were computed for each of the three tests, along with the 95% confidence intervals (CI); differences were tested with McNemar's test. We also computed a measure of agreement (the kappa statistic) to examine whether the tests tended to identify the same patients as positive or negative. The kappa statistic measures agreement beyond chance; a value of 0 implies no agreement beyond chance and 1 implies perfect agreement. Values less than 0.4 were interpreted as representing poor agreement, 0.4–0.75 fair agreement and >0.75 excellent agreement [24]. Because of relatively small sample sizes, all these tests and statistics were computed using exact algorithms (StatXact 3; Cytel Corporation, Cambridge, MA, USA).

In the RA patients we examined whether patient characteristics (sex, age, duration of disease, number of ACR RA criteria other than RF positivity, clinical manifestations of RA, and the HLA-DRB1 shared epitope) were associated with positive test results.

Associations between serology test results and the progression of radiological lesions (Larsen score) over time among 129 patients who had X-rays available were assessed by non-parametric regression (Lowess regression). Differences between slopes (in Larsen points per year) were tested in linear regression models. Because the exploratory non-parametric regression models suggested a change in slope at about 12 yr of disease duration in the subgroup of seronegative patients, we used linear regression models that allow for a change in slope, and tested whether this change was statistically significant.

Results

Descriptive data

We analysed data from 179 RA patients and 50 control patients who had rheumatological diseases other than RA. The two groups were similar in their proportion of women (75% and 68% respectively) and median age (62 and 67 yr respectively).

Positivity for anti-CCP antibodies

For this test, the ROC curve achieved excellent discrimination between RA patients and controls, with an area under the ROC curve of 0.91 (95% CI 0.88–0.95). We then compared two plausible cut-off values to define a positive test: 1500 U/ml (sensitivity 63%, specificity 98%) and 1000 U/ml (sensitivity 68%, specificity 96%). On the basis of these analyses, we selected the threshold value of 1000 U/ml to define a positive test for all further analyses.

Sensitivity and specificity of determinations of anti-CCP antibodies, AKA and IgM RF

Sensitivity for RA was highest for the IgM RF test, followed by the anti-CCP antibody and AKA tests (Table 1). The AKA test was significantly less sensitive than the other two tests. The agreement between these three tests in their ability to detect RA patients as positive was fairly low ($\kappa < 0.4$).

Both the anti-CCP antibody and the AKA tests had specificity exceeding 90%. The difference between the two was not significant, and both were significantly better than the IgM RF test.

As the control group included many patients with crystal-induced arthritis or osteoarthritis, and thus may be inadequate for this type of study, a second evaluation of the specificity of the anti-CCP antibody test was performed with a small subgroup of patients with only RA-like presentation: connective tissue diseases ($n = 11$), seronegative spondyloarthropathies ($n = 9$), other inflammatory diseases, including Crohn's disease, polymyalgia rheumatica or sarcoidosis ($n = 8$). The same specificity (96%) was obtained.

There was no agreement whatsoever beyond what could be expected by chance between the three tests in their tendency to generate false-positive results among the control patients.

Sensitivity of the three tests for the detection of RA, according to patient characteristics

Differences in test sensitivity between men and women and across age-groups were not statistically significant. There were no differences in test sensitivity according to disease duration or number of ACR RA criteria (other than RF positivity) fulfilled.

TABLE 1. Comparison of three tests in the detection of RA: sensitivity and specificity, statistical significance of differences in sensitivity and specificity, and agreement in identifying RA patients as positive and controls as negative

	Sensitivity (95% CI)	Specificity (95% CI)
Anti-CCP antibodies	0.68 (0.60 to 0.74)	0.96 (0.86 to 1.00)
AKA	0.46 (0.38 to 0.53)	0.94 (0.83 to 0.99)
IgM RF	0.75 (0.68 to 0.81)	0.74 (0.60 to 0.85)
	Intertest difference in sensitivity (P^a)	Intertest difference in specificity (P^a)
Anti-CCP vs AKA	<0.0001	1.00
Anti-CCP vs IgM RF	0.08	0.003
AKA vs IgM RF	<0.0001	0.013
	Agreement among RA patients[b]	Agreement among control patients[b]
Anti-CCP vs AKA	0.36 (0.24 to 0.48)	−0.05 (−0.1 to 0.00)
Anti-CCP vs IgM RF	0.36 (0.22 to 0.51)	0.07 (−0.14 to 0.28)
AKA vs IgM RF	0.27 (0.16 to 0.39)	0.03 (−0.19 to 0.25)

[a]McNemar's test.
[b]Kappa (95% CI).

IgM RF, but not anti-CCP antibodies or AKA, was significantly more frequent among patients who had subcutaneous nodules (86.7 *vs* 70.9%, $P < 0.05$), sicca syndrome (91.4 *vs* 70.8%, $P < 0.05$) or marked joint deformities (86.8 *vs* 65.7%, $P < 0.01$).

Among RA patients, carriers of the HLA-DRB1 shared epitope were significantly more likely to be positive for AKA: 72 (74.2%) of 97 AKA-negative patients were positive for the HLA-DRB1 shared epitope *vs* 72 (87.8%) of 82 AKA-positive patients (Fisher's exact test, $P = 0.024$). The association was weaker for anti-CCP antibodies: 43 (74.1%) of 58 anti-CCP antibody-negative patients were positive for the HLA-DRB1 shared epitope *vs* 101 (83.5%) of 121 anti-CCP antibody-positive patients (Fisher's exact test, $P = 0.16$).

No significant association was observed between the HLA-DRB1 shared epitope and parameters of disease severity (number of ACR RA criteria and extra-articular manifestations) (data not shown).

Associations with disease progression

X-rays were available for 129 patients. The average Larsen score was 36.5 (s.d. 21.4, range 0–100). The Larsen score was associated with disease duration: for disease duration less than 3 yr, the average Larsen score was 22.1, for disease duration 3–12 yr, the score was 36.0 and for disease duration exceeding 12 yr it was 50.4 (linear trend test, <0.0001); each year of disease duration was associated with a 1.2-point increase in the Larsen score (95% CI 0.9–1.5).

The exploratory non-parametric analysis suggested that, for all three tests, the association between disease duration and Larsen score was steep and nearly linear among seropositive patients, but much weaker, especially for disease duration greater than 12 yr, among seronegative patients (Fig. 1). To test whether the change in slope at 12 yr in seronegative patients was statistically significant, we applied a linear regression model which included a term for the interaction of serostatus with change in slope at 12 yr. This parameter was significantly different from 0 for anti-CCP antibodies ($P < 0.001$), AKA ($P = 0.050$) and IgM RF ($P < 0.001$).

Discussion

This study establishes the value of anti-CCP antibodies in the diagnosis of RA and the merit of this test in comparison with the AKA and IgM RF tests.

The specificity obtained for the anti-CCP test (96%) was similar to that found by other groups: 91–98% [1, 5, 8]. However, although there is consensus for the different studies about specificity, there is considerable variation in diagnostic sensitivity, ranging from 41–68% [1, 5–8]. This variation can be attributed to the different serum dilutions tested or, more probably, to the different cut-off values. Indeed, the cut-off level for positivity was either determined from OD_{450} ($\geqslant 0.11$ [6]
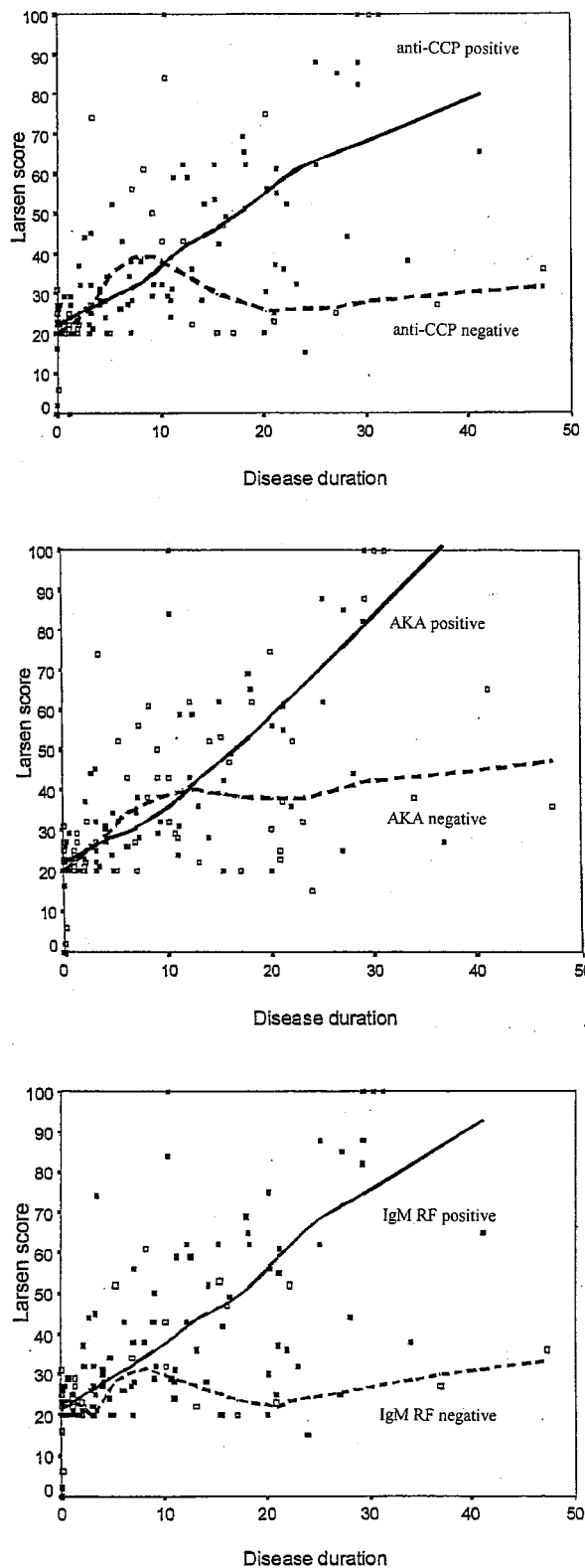

FIG. 1. Scatterplots of Larsen score against disease duration for 129 patients with RA, by serostatus for anti-CCP antibodies (upper panel), AKA (middle panel) and IgM RF (lower panel). Non-parametric regression lines are superimposed. Seropositive (filled dots, solid lines) and seronegative (open dots, dotted lines) patients are shown separately.

or 0.3 [1]) or expressed in units (50 [8] or determined from a ROC curve [5, 7]). In the present study, the sensitivity of anti-CCP antibody determination was 68% and was also established from an ROC curve, according to the group who developed this ELISA [2, 5]. Using a threshold of 1000 U/ml, the sensitivity of anti-CCP antibody determination (68%) did not significantly differ from that obtained for IgM RF (75%) but was significantly higher than that obtained for AKA (45%). The specificity of the anti-CCP antibody test (96%) was significantly higher than that obtained for the IgM RF test (74%).

This study also indicates that the three tests examined do not provide equivalent information. The anti-CCP antibody test has moderate sensitivity and excellent specificity, the AKA test has poor sensitivity but excellent specificity, and the IgM RF test has moderate sensitivity and specificity. Thus, while none of the tests, if negative, rules out the diagnosis of RA, a positive test for anti-CCP antibodies or AKA practically establishes this diagnosis. Hence these tests, although not uniformly better than the IgM RF test, may prove more useful in selected cases in clinical practice. In this regard, the anti-CCP antibody test appears more promising, being more sensitive than the AKA test, even when another cut-off was chosen for anti-CCP positivity [1]. Overall, the discriminative ability of the anti-CCP test was impressive: if a patient with RA and a control were to be selected at random, the odds are 10:1 that the RA patient would have the higher anti-CCP antibody concentration.

Nevertheless, the substantial disagreement between these tests, both regarding true positives among RA patients and false positives among controls, suggests that there is considerable room for improvement in the serological diagnosis of RA.

The three markers were also associated with more severe radiological lesions, particularly in patients with disease duration of more than 12 yr. In a previous study, we observed that severity of erosions was correlated with RF detected by agglutination of latex but not with AKA [15]. In the present study the best correlation with severity of erosions was obtained for IgM RF; anti-CCP antibodies were also found to be significantly correlated, but AKA appeared to be at the limit of significance. Whether these tests are able to predict the occurrence of clinical or radiological manifestations of RA remains unclear, as our study was cross-sectional and therefore subject to possible selection bias. Prospective studies would probably have produced more information than a cross-sectional study of patients with definite RA. However, in prospective studies, it is necessary to assume that treatments have not biased the results. They are therefore subject to ethical problems or to the possible loss of sensitivity as new and more effective treatments, adjustable to disease severity, become available. Here we analysed a cross-sectional sample of RA patients with the non-parametric Lowess regression, considering progression of joint damage as a function of time. This approach takes into account the fact that the Larsen score is broadly dependent on disease duration and probably represents a good alternative to a prospective study.

Two groups have, however, studied prospectively the association of anti-CCP antibodies and IgM RF with radiological damage. Schellekens et al. [5] observed that the ability of the two tests, performed at the first visit, had similar ability to predict erosive disease at 2 yr of follow-up. In multiple regression analysis, Kroot et al. [7] observed that radiological damage after 6 yr of follow-up was significantly predicted by IgM RF and anti-CCP status at entry. The strongest predictor was IgM RF and the additional predictive value of anti-CCP positivity was moderate [7]. Our results are compatible with these last observations.

As in a previous study [15], a significant association between the HLA-DRB1 shared epitope and AKA positivity was observed. The association with anti-CCP antibodies was weaker.

In conclusion, our results suggest that anti-CCP antibodies, AKA and IgM RF reflect clinically relevant disease processes in RA patients. Anti-CCP antibodies and AKA are weaker than IgM RF as markers of disease severity. However, in clinical practice, both IgM RF and anti-CCP antibodies may be useful, IgM RF for their good sensitivity and as a marker of disease severity and anti-CCP antibodies for their high specificity and their presence in some RA-seronegative patients.

Acknowledgements

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