

EXTENDED REPORT

Evaluation of anti-citrullinated filaggrin antibodies as hallmarks for the diagnosis of rheumatic diseases

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Background: Anti-filaggrin antibodies (AFA) are among the most specific antibodies for rheumatoid arthritis, so procedures for their detection should be included in early biological diagnoses. AFA can be detected by indirect immunofluorescence (anti-keratin antibodies, AKA) or by new enzyme immunoassays (EIA). Their comparative performance needs to be established.

Objective: To compare these technical procedures to optimise the serological diagnosis of rheumatoid arthritis.

Methods: Results obtained using AKA and EIA were compared in 271 sera from 140 patients with rheumatoid arthritis at various stages, 98 patients with other autoimmune diseases, and 33 healthy subjects. EIA were successively undertaken with citrullinated linear filaggrin peptide (home made EIA) or cyclic citrullinated peptide (CCP2, commercial kits). Rheumatoid factor (RF) was assessed by EIA in all patients.

Results: Anti-CCP2 kits showed the best sensitivity and specificity (65% and 96%, respectively). Among the 140 patients with rheumatoid arthritis, those with very recent disease (less than six months' duration, n=21) were studied as a separate group. In this group, the sensitivity of anti-CCP2 kits decreased to ~50%. Nevertheless this assay remained the most accurate when compared with AKA or home made EIA using linear filaggrin peptides. The combination of anti-CCP2 and RF only slightly increased the sensitivity of the diagnosis of very early rheumatoid arthritis.

Conclusions: Kits using citrullinated cyclic peptides (CCP2) were more suitable than either AKA or EIA using linear filaggrin peptides for the diagnosis of early rheumatoid disease.

Recent papers have highlighted the early treatment of rheumatoid arthritis with disease modifying antirheumatic drugs (DMARD) in. Several independent studies have shown that even a brief delay in starting DMARD treatment can have a significant impact on disease activity years later.^{1,2} However, these findings have shifted the medical problem from "how to treat" to "how to make an early diagnosis of rheumatoid arthritis".

The diagnosis of rheumatoid arthritis is currently based on American College of Rheumatology (ACR) criteria.³ However, these criteria were established in a population of patients selected on the basis of their disease status, with a view to classifying different forms of the disease rather than different ways of diagnosing it. New criteria are emerging for the detection of very early rheumatoid arthritis (less than six months in duration). A recent study has demonstrated the value of biological markers by using diagnostic models of erosive arthritis.² Markers such as rheumatoid factor (RF) and anti-cyclic citrullinated (filaggrin) peptide antibodies (anti-CCP antibodies) were selected from among five other criteria. Persistence of disease associated with erosive arthritis was particularly associated with the presence of anti-CCP antibodies.²

Anti-filaggrin antibodies are a subset of a large panel of antibodies, previously called anti-perinuclear factor (APF) when detected in buccal mucosal cells, or anti-keratin antibodies (AKA) when detected in epithelial cells of the stratum corneum of rat oesophagus. AKA and APF have been shown to recognise both a 37–40 kDa protein and three profilaggrin related proteins extracted from human epidermis or rat oesophagus.⁴ In humans, this protein has been identified as a neutral/acidic isoform of filaggrin that may

be involved in the aggregation of cytokeratin intermediate filaments. Recent data have shown that citrullination of epitopes was essential to detect antibodies related to rheumatoid arthritis.^{5–7} Moreover, antibodies to citrullinated peptides appear to be highly specific hallmarks of the disease.^{5–8} In addition, they are associated with the more active and severe forms of rheumatoid arthritis.⁹ The immune response to citrullinated epitopes is believed to be involved in the pathophysiology of rheumatoid arthritis. Very recent data have shown that the citrullination of peptides could enhance the stability of the HLA-DRB1*0401 MHC class II peptide complex, and therefore could enhance the presentation of citrullinated epitopes to the immune system.^{10,11}

For the detection of these specific antibodies, various technical procedures have been used. Immunofluorescence methods,⁹ western blotting,¹² and enzyme immunoassays^{5,7} have been used with varying degrees of sensitivity and specificity. A new generation of commercial enzyme immunoassay (EIA) kits (CCP2) using a cyclic peptide that is not derived from filaggrin is now available for rheumatoid arthritis diagnosis.

Our aim in this study was to establish the relative performance of several anti-citrullinated peptide antibody detection methods in order to optimise the diagnosis of rheumatoid arthritis. We evaluated 271 sera to compare both the sensitivity and specificity of AKA, anti-linear citrullinated linear filaggrin peptide antibodies,⁵ and anti-CCP2 antibodies.

Abbreviations: AFA, anti-filaggrin antibodies; AKA, anti-keratin antibodies; CCP, cyclic citrullinated peptide; EIA, enzyme immunoassay; RF, rheumatoid factor

We also compared the performance of each of three different commercial anti-CCP2 antibodies kits.

METHODS

Patients

Sera ($n = 271$) were obtained from four clinical departments of either rheumatology or internal medicine. One hundred and forty patients were diagnosed as having definite rheumatoid arthritis, according to the revised ACR criteria.³ Seventy five patients had rheumatoid arthritis of at least two years' duration, while 65 and 21 patients, respectively, had had the disease for no more than two years and no more than six months. Though our study was not prospective, the diagnosis of rheumatoid arthritis was reviewed 6–18 months after the samples were collected to ensure that the clinical data were complete in all the patients.

Controls consisted of a group of healthy individuals ($n = 33$) and patients with other autoimmune diseases ($n = 98$), which included Sjögren's syndrome, according to the new revised criteria¹³ ($n = 47$) and systemic lupus erythematosus (SLE), according to the ACR criteria ($n = 51$). Sera were stored, as several aliquots, at -80°C until used.

Procedures

Enzyme immunoassay anti-linear filaggrin citrullinated peptides antibodies

We employed a home made procedure similar to previously published methods.⁵ The most reactive linear filaggrin peptide (SHQESTCitGitSRGRSGRSGS), referred to as cfc6 in Schellekens' seminal paper,⁵ was used as antigen. In this peptide, arginine residues 312 and 314 of the control peptide referred to as cf0 (SHQESTRGRSRGRSGRSGS) was replaced by two citrulline residues. Peptides were synthesised and purified (to $>95\%$) with high performance liquid chromatography (HPLC; Neosystem, Strasbourg, France). All sera were tested in duplicate. Control wells were coated with bovine serum albumin (BSA) and all the data were corrected by subtracting their mean background values. In a second part of the study, IgG antibody reactivity relating specifically to the citrullinated form of cfc6 was expressed as: [mean optical density (OD)_{cfc6} - OD in blank_{cfc6} well] - [mean OD_{cf0} - OD in blank_{cf0} well].

Anti-CCP2 ELISA, AKA evaluation, and RF procedures

An anti-CCP2 antibody assay was undertaken using the Dia-Stat commercial kit (Axis-Shield, Dundee, UK) according to the manufacturer's instructions. The presence of AKA was evaluated by indirect immunofluorescence on rat oesophagus stratum corneum (Biorad, Marnes la Coquette, France) after a 1:5 serum dilution.

IgM RF was measured using an enzyme linked immunosorbent assay (ELISA; Biomedical Diagnostic, Marne la Vallée, France) according to the manufacturer's instructions. A level of >20 IU/ml was considered positive.

In a second part of the study, the performance of two additional anti-CCP2 antibodies kits (Euro-Diagnostica, Arnhem, Netherlands, and Inova, San Diego, California, USA) was evaluated using sera from rheumatoid arthritis patients ($n = 46$) and a control group (patients with connective tissue diseases, $n = 22$). These sera were collected from the first cohort of sera.

Statistical analysis

Sensitivity of the different technical procedures was defined as positive values in the rheumatoid arthritis population, while specificity was evaluated from false positive values in the control population (healthy subjects plus the SLE and

Sjögren's syndrome patients). Statistical analysis was done using a non-parametric Mann-Whitney U test with StatView statistical analysis software. Probability (p) values of <0.05 were considered statistically significant.

RESULTS

IgG reactivity to the linear citrullinated filaggrin peptide (home made EIA)

The cut off value of the home made anti-filaggrin antibody assay (called LCP, for "linear citrullinated peptide") was defined at OD 140 from the IgG response to cfc6 in the healthy subjects group (mean value $+3$ SD), as previously reported.⁵ As shown in fig 1A, AFA were found in 94 of 140 rheumatoid arthritis patients (67.1%), but also in 32 of the 98 patients with either SS or SLE (32.6%), and in one of the

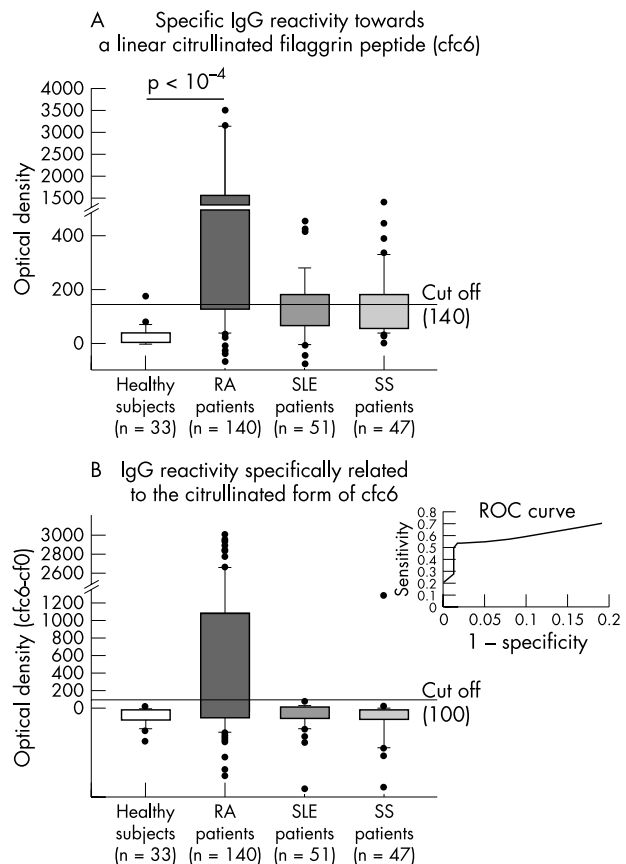


Figure 1 (A) Analysis of the serum IgG response to the citrullinated filaggrin peptide (cfc6) in different populations of subjects. Patients with rheumatoid arthritis had a significantly higher serum IgG response to cfc6 than those with other connective diseases or the healthy controls. However, a cut off value established at OD 140 (mean optical density value of the healthy subjects $+3$ SD) did not accurately discriminate the patients with rheumatoid arthritis from those with the other connective tissue diseases. IgG anti-filaggrin peptide was detected by this procedure in more than 30% of the cases of Sjogren's syndrome and SLE. Results are expressed as mean optical densities in the reactive well subtracted from the OD in blank well. (B) Evaluation of the serum IgG response related to the citrullinated form of cfc6 in different populations. Subtraction of the optical density of the IgG response to cf0 from that of the IgG reactivity to cfc6 discriminated the IgG response of the rheumatoid arthritis group to filaggrin peptide. A receiver operating characteristic (ROC) curve (insert) established a cut off value at 100 arbitrary units that discriminated rheumatoid arthritis patients with a sensitivity of 50% and a specificity of 98%. The boundaries of the boxes indicate the 25th and 75th centiles; whiskers above and below the boxes indicate the 90th and 10th centiles. RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjogren's syndrome.

healthy subjects. The specificity of this method using linear citrullinated filaggrin peptide was 68.1%.

In a second phase, we measured the IgG reactivity relating specifically to the citrullinated form of cfc6. In this, the IgG response to cfo was subtracted from the reactivity to cfc6 in each patient, as previously described.⁶ We found that only the rheumatoid arthritis group had a specific IgG antibody response related to the citrullinated condition of cfc6 ($p < 10^{-4}$). An ROC curve established a threshold value at 100 arbitrary units (AU) (fig 1B), resulting in a sensitivity value of 50% and a specificity reaching 98% (fig 1B)

Comparison of diagnostic performance using different AFA and RF detection methods

As shown in table 1, the sensitivity of the anti-CCP2 antibodies assay was around 65%, which was better than the AFA methods (~48% for both home made EIA, using a linear filaggrin peptide, and AKA) and equivalent to the RF assay (60%). The sensitivity of all assays fell when the evaluation was restricted to rheumatoid arthritis with onset less than two years previously. Sensitivity was maintained at ~45% with anti-CCP2 antibodies in the rheumatoid arthritis subgroup with very recent onset of disease (≤ 6 months; $n = 21$). On the other hand, sensitivity fell to 20% and 10%, respectively, for home made EIA and AKA in this early disease subgroup. In this group of patients, only one had anti-CCP2 positive and RF negative serum, while one had anti-CCP2 negative and RF positive serum. While the combination of RF and anti-CCP2 showed a sensitivity of ~80% for rheumatoid arthritis of more than two years' duration, the sensitivity of this combination fell for very early disease (to around 60%). Percentages of false positives were low with most EIA methods (4% and 2% for the anti-CCP2 antibody kit and the home made EIA, respectively). They were higher with the immunofluorescence AKA method (10%). Specificity of RF was evaluated at ~70% in this population.

As shown in table 2, the performance of the three commercial anti-CCP2 kits was quite comparable in terms of sensitivity and specificity.

DISCUSSION

In this study, we evaluated anti-citrullinated peptide antibodies as biological hallmarks for the diagnosis of early rheumatoid arthritis. Our data showed that the anti-CCP2 antibody assay was more effective than methods involving linear citrullinated filaggrin epitopes (home made EIA) or indirect immunofluorescence (AKA). These results could be useful in the management of rheumatoid arthritis. There is growing evidence that early therapeutic intervention leads to earlier disease control and less joint damage.¹⁴⁻¹⁹

Table 2 Comparative sensitivity and specificity of three commercial anti-CCP2 antibody kits and RF

	CCP2			
	Diastat®	Inova®	Euro-Diagnostica®	RF (BMD®)
RA (n=45)	37 (82%)	38 (85%)	38 (85%)	42 (94%)
Control (n=22)	2 (9.1%)	1 (4.5%)	2 (9.1%)	12 (53%)

CCP, cyclic citrullinated peptide; RA, rheumatoid arthritis; RF, rheumatoid factor.

New generations of RF assays (ELISA, nephelometry) have increased the sensitivity of this biological test²⁰; nevertheless, it performed poorly in the differential diagnosis of connective diseases associated with joint inflammation, such as Sjögren's syndrome.²¹ Moreover, the sensitivity of RF dramatically decreases in rheumatoid disease of short duration, as noted in this study. Using EIA, RF sensitivity fell from ~70% (duration >2 years) to ~55% (duration <6 months), as previously reported.²² In other studies, the sensitivity of RF was ~80% for disease duration of more than two years²³ and ~65% for duration less than two years.²⁴ Our performance figures could reflect the use of a commercial EIA kit or patient selection, or both.

Recently, new criteria for the early diagnosis of erosive arthritis have been proposed.² Useful indices include anti-CCP antibody assays, the detection of which might be related to persistent erosive arthritis.^{2 25-27}

Large subsets of anti-filaggrin antibodies have been described, identified using various methods of which indirect immunofluorescence is the most common. However, performance in terms of sensitivity varies from 35% to 70% in well established disease,^{26 28-30} and from 10% to 50% in early disease (less than two years from onset).^{24 31} These differences in sensitivity could be related to the use of non-standardised substrates leading to interassay and interlaboratory variations. Using an enzyme immunoassay, Schellekens *et al* have shown that around 50% of serum samples from patients with rheumatoid arthritis have IgG recognising citrullinated pep₃₀₆₋₃₂₄ (arg₃₁₂₊₃₁₄→cit) filaggrin epitopes, whereas patients with other rheumatoid diseases and healthy controls do not.³ Furthermore, these investigators have shown definitively that the reactivity of rheumatoid arthritis sera was related to the specific recognition of the arginine→citrulline substitution, in the context of some (pro-) filaggrin epitopes.^{3 7}

Reports of a first generation EIA using a cyclic citrullinated filaggrin peptide (CCP1) have shown variable sensitivity: 68% by Schellekens and co-workers,^{7 25} but only 41% (95% confidence interval, 31% to 50%) by Bizarro *et al*.²⁶ Previous

Table 1 Evaluation of anti-citrullinated peptide antibody using different procedures, and rheumatoid factor by enzyme immunoassay in patients with rheumatoid arthritis and a control population

	CCP2, commercial kit	(Cfc6-cfo) Home made EIA	AKA (IFI)	RF (EIA)	CCP2 negative RF positive	CCP2 positive RF negative	CCP2 or RF positive
RA (n=140)	90 (64.3)	67 (47.9)	68 (48.6)	84 (60.0)	15 (10.7)	12 (8.6)	105 (75.0)
RA >2 y (n=75)	58 (77.3)	45 (60.0)	46 (61.3)	50 (66.7)	4 (5.3)	7 (9.3)	62 (82.7)
<2 y (n=65)	32 (49.2)	22 (33.8)	22 (33.8)	34 (52.3)	10 (15.4)	5 (7.7)	35 (53.8)
<6 months (n=21)	10 (47.6)	4 (19.0)	2 (9.5)	12 (57.1)	1 (4.8)	1 (4.8)	13 (61.9)
SS (n=47)	2 (4)	1 (2)	5 (10)	22 (47)	-	-	-
SLE (n=51)	0 (-)	2 (4)	3 (6)	16 (31)	-	-	-
Healthy subjects (n=33)	0 (-)	0 (-)	0 (-)	3 (10)	-	-	-
Specificity (%)	96.4	97.1	93	69	-	-	-

Values are n (%) unless specified.

AKA, anti-keratin antibodies; CCP, cyclic citrullinated peptide; EIA, enzyme immunoassay; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SS, Sjogren's syndrome; y, year.

conflicting data could have resulted from the population studied or the kit used, or both.

Recently a new generation of anti-CCP (anti-CCP2) has been commercially distributed. These data led us to compare different procedures with anti-filaggrin antibody detection kits in order to optimise the biological methods for diagnosing rheumatoid arthritis. First, we developed a home made EIA using a linear citrullinated filaggrin peptide, as previously described.⁵ We observed false positive results with sera from patients with connective tissue diseases such as Sjögren's syndrome and systemic lupus erythematosus (though specificity was at least 70% in our first studies). These false positive results could have resulted from the presence of various epitopes on the filaggrin peptides. These epitopes (found on both citrullinated and non-citrullinated filaggrin peptides) may react with antibodies present in sera from patients with Sjögren's syndrome or systemic lupus erythematosus.

Such a hypothesis would raise doubts about methods such as immunoblotting¹² or line immunoassays³² which use epitopes of citrullinated filaggrin as targets. In contrast, the lack of specificity of EIA may be corrected by using a parallel evaluation of the immunoglobulin reactivity against a citrullinated filaggrin peptide and its non-citrullinated counterpart. The subtraction of the reactivity directed against the non-citrullinated peptide allows evaluation of the immune reactivity specifically linked to the citrullinated form of the filaggrin epitope, as previously reported.⁶ This restricting approach enhanced specificity (to more than 97% in our assay) but sensitivity was equivalent to AKA (50%). The combination of both anti-linear citrullinated filaggrin peptide (LCP) antibodies and AKA (with at least one assay positive) appears useful, with a gain in sensitivity to 65% (data not shown).

In the present study, we found that both the home made EIA (LCP) and the AKA procedures may be usefully substituted by a single assay using a cyclic citrullinated peptide. Indeed, the highest levels of specificity (more than 96%) and sensitivity (around 65%) were observed when we used this procedure with various sera from patients with rheumatoid arthritis, Sjögren's syndrome, or systemic lupus erythematosus. The good level of specificity shown by anti-CCP2 kits could be related to the spatial structure of the cyclic epitope coated on the solid phase, as claimed by the manufacturer. Using a non-filaggrin citrullinated peptide, CCP2 kits have evidently enhanced the specificity of the method, as recently demonstrated in an aged population.³³ Moreover, the cyclic epitope of the citrullinated peptide could better mimic the structural conformation of various citrullinated targets for antibodies in patients with rheumatoid arthritis. This hypothesis would explain the increase in sensitivity of CCP2 kits in relation to EIA using linear citrullinated peptides or indirect immunofluorescence.

The performance of anti-CCP seems to be cohort dependent. In some rheumatoid arthritis cohorts, the sensitivity is only 41%,²⁶ in others it is over 80%.²⁵ Our results are similar to those previously published.^{25 27 34} However, in our study, sensitivity decreased to less than 50% when we focused our evaluation on the very early stages of the disease (duration less than six months). These results could call into question initial data reporting precocity of anti-CCP antibodies in terms of performance for early rheumatoid arthritis diagnosis.³⁵ Nevertheless, we observed that anti-CCP2 antibodies remained the most accurate assay when compared with EIA using either LCP or AKA, as the sensitivity of these two last assays decreased to 20% for LCP antibodies and 10% for AKA.

From our data, the combination of anti-CCP and RF might be worth considering in view of the relatively poor sensitivity of either assay for very early rheumatoid arthritis (duration

less than six months). Independently, the two procedures had a sensitivity of around 45–60% in this subgroup. The combination of the two tests (with at least one test positive) led to an improved sensitivity of around 60%. By contrast, in rheumatoid arthritis of more than two years' duration, the combination of RF and anti-CCP had a sensitivity of 75%. Though the sensitivity is relatively low in early disease, we believe that the combination of an RF test and an anti-CCP test should still be used to increase the likelihood of identifying patients with rheumatoid arthritis who do not have RF but who have other autoantibody markers.

Finally, we showed in our study that the performance of the three different anti-CCP antibodies kits was quite comparable. This could reflect the fact that there is only one industrial source of cyclic peptide (Euro-Diagnostica).

Rheumatologists and biologists need to be informed that the various anti-citrullinated peptide (from filaggrin or not) antibody detection procedures are not equivalent in term of efficacy, sensitivity, and specificity. Currently, the best levels of sensitivity and specificity are obtained with commercial kits using cyclic citrullinated peptides (CCP2). However, the sensitivity of anti-CCP2 appears to be decreased in very early rheumatoid arthritis.

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