Performance of two ELISAs for antifilaggrin autoantibodies, using either affinity purified or deaminated recombinant human filaggrin, in the diagnosis of rheumatoid arthritis

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Abstract

Objective—To develop a standardisable enzyme linked immunosorbent assay (ELISA), using human filaggrin, for detection of antifilaggrin autoantibodies in rheumatoid arthritis (RA). To compare the diagnostic performance of the ELISA with those of reference tests: “antikeratin antibodies” (“AKA”), and antibodies to human epidermis filaggrin detected by immunoblotting (AhFA-IB).

Methods—Two ELISAs were developed using either affinity purified neutral-acidic human epidermis filaggrin (AhFA-ELISA-pur) or a recombinant human filaggrin deaminated in vitro (AhFA-ELISA-rec) as immunosorbent. Antifilaggrin autoantibodies were assayed in 714 serum samples from patients with well-characterised rheumatic diseases, including 241 RA and 473 other rheumatic diseases, using the two ELISAs. “AKA” and AhFA-IB tests were carried out in the same series of patients. The diagnostic performance of the four tests was compared and their relationships analysed.

Results—The titres of “AKA”, AhFA-IB, and the AhFA-ELISAs correlated strongly with each other. The diagnostic sensitivity of the AhFA-ELISA-rec, which was better than that of AhFA-ELISA-pur, was 0.52 for a specificity of 0.95. This performance was similar to those of “AKA” or AhFA-IB. However, combining AhFA-ELISA-rec with AhFA-IB led to a diagnostic sensitivity of 0.55 for a specificity of 0.99.

Conclusion—A simple and standardisable ELISA for detection of antifilaggrin autoantibodies was developed and validated on a large series of patients using a citrullinated recombinant human filaggrin. The diagnostic performance of the test was similar to that of the “AKA” and AhFA-IB. Nevertheless, combining the AhFA-ELISA-rec with one of the other tests clearly enhanced the performance.

Various autoantibodies have been described in the serum of patients with rheumatoid arthritis (RA). Among them, the antiperinuclear factor (APF) that recognises perinuclear granules in human buccal keratinocytes as seen by indirect immunofluorescence (IIF), and the so-called “antikeratin antibodies” (“AKA”) that are labelled by IIF in the cornified layer of the rat oesophagus epithelium, were found to be the most specific serological criteria for the diagnosis of RA (reviewed by Hoet and van Venrooij). Moreover, they appear at very early stages of the disease, when specific treatment might delay joint destruction, and their presence and titre have been shown to be related to RA activity and severity. Nevertheless, although these antibodies are increasingly considered as useful diagnostic and, even, prognostic markers, their detection remains restricted to specialised laboratories, essentially because of technical difficulties. Therefore, the advent of new detection methods, easy to standardise and to perform, is desirable.

On a rat oesophagus epithelium extract separated under non-denaturing conditions, “AKA” were shown, by immunoblotting (IB), to recognise three late differentiation proteins immunologically related to (pro)filaggrin. Assessed on a small series of patients, the diagnostic sensitivity of such an IB assay was 30% higher than that of the classical IIF method, but the complex labelling pattern of the antigens prevented the use of this IB as a routine test.

We showed that “AKA” recognise a neutral-acidic isof orm of filaggrin in human epidermis, and that APF recognises a protein closely related to epithelial (pro)filaggrin. APF and “AKA” were therefore shown to belong to the same autoantibody family directed to (pro)filaggrin, and we suggested calling them as a whole “antifilaggrin autoantibodies”. Recently, we and others showed that the epitopes recognised by antifilaggrin autoantibodies on the various proteins contained citrulline residues generated through deamination of arginines by a peptidyl-arginine deiminase (PAD). These results subsequently allowed the development of several immunoassays for the detection of antifilaggrin autoantibodies.

We first developed a diagnostic test by IB on filaggrin enriched extracts from human epidermis. This test (AhFA-IB), validated on a large series of patients, had a diagnostic performance similar to that of the APF and “AKA” tests. Although it has been shown to be easier to standardise than the classical IIF methods, and yet, its use has remained restricted to a few specialised laboratories.

More recently, two enzyme linked immunosorbent assays (ELISAs), using either human
Table 1  Detail of patients

<table>
<thead>
<tr>
<th>Diagnosis of rheumatoid arthritis</th>
<th>Number</th>
<th>Age Median (range)</th>
<th>No (%) men</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>241</td>
<td>59 (18–93)</td>
<td>49 (20)</td>
</tr>
<tr>
<td>Controls</td>
<td>473</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory diseases</td>
<td>158</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic lupus erythematous</td>
<td>21</td>
<td>47 (20–73)</td>
<td>4 (19)</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>10</td>
<td>56 (39–71)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Other connective tissue diseases*</td>
<td>21</td>
<td>59 (24–82)</td>
<td>4 (19)</td>
</tr>
<tr>
<td>Psoriatic arthritis</td>
<td>43</td>
<td>47 (25–75)</td>
<td>33 (77)</td>
</tr>
<tr>
<td>Ankylosing spondylitis</td>
<td>40</td>
<td>45 (17–79)</td>
<td>26 (65)</td>
</tr>
<tr>
<td>Other inflammatory diseases†</td>
<td>23</td>
<td>63 (21–86)</td>
<td>14 (61)</td>
</tr>
<tr>
<td>Non-inflammatory diseases</td>
<td>315</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoarthrosis</td>
<td>104</td>
<td>55 (16–83)</td>
<td>49 (47)</td>
</tr>
<tr>
<td>Compressive neuropathy</td>
<td>26</td>
<td>55 (31–77)</td>
<td>13 (50)</td>
</tr>
<tr>
<td>Paget’s disease of bone</td>
<td>68</td>
<td>68 (37–85)</td>
<td>41 (60)</td>
</tr>
<tr>
<td>Reflex sympathetic dystrophy</td>
<td>29</td>
<td>51 (27–68)</td>
<td>14 (48)</td>
</tr>
<tr>
<td>Malignant bone metastases</td>
<td>49</td>
<td>66 (39–85)</td>
<td>32 (65)</td>
</tr>
<tr>
<td>Other non-inflammatory diseases‡</td>
<td>39</td>
<td>69 (42–85)</td>
<td>10 (26)</td>
</tr>
</tbody>
</table>

°Vasculitis (9), mixed connective tissue diseases (12).
†Microcrystalline arthropathies (14), infectious arthritides (9).
‡Multiple myeloma (33), bone metastases (13), Hodgkin’s disease (3).
§Osteoporosis (32), benign gammopathies (7).

epidemis filaggrin purified by high performance liquid chromatography (HPLC) or a citrulline-substituted synthetic peptide derived from filaggrin, as immunosorients, were proposed as an alternative to “AKA” and APF.

In this work, we developed a method for screening for filaggrin antibodies in RA and other rheumatic diseases, using ELISAs against two different epitopes of human filaggrin: a recombinant human filaggrin and an anti-human filaggrin detected by IIF and AhFA detection by IB.

Patients, material, and methods

SERUM SAMPLES

Serum samples were obtained from 241 patients diagnosed with RA according to the criteria of the American College of Rheumatology and from 473 patients with non-RA rheumatic diseases (control samples). All the patients were from the departments of rheumatology of the Purpan and Rangueil hospitals, Toulouse, France (table 1). The serum samples were split into aliquots and stored at −80°C until assayed.

“AKA” AND AHFA-IB DETECTION AND TITRATION

The detection of “AKA” was performed by IIF on cryosections of rat oesophagus as described elsewhere. Filaggrin were detected by immunoblotting (AhFA-IB) on filaggrin enriched extracts of human epidermis containing essentially the neutral-acidic variants of filaggrin, according to a previously described method. “AKA” and AhFA-IB titre-like values (referred to as titres in the paper) were estimated on a semiquantitative scale (from 0 to 8, 0.25 steps).

FILAGGRIN EXTRACTION AND PARTIAL PURIFICATION OF THE NEUTRAL-ACIDIC VARIANTS OF FILAGGRIN

The extraction and partial purification of the neutral-acidic variants of filaggrin has been described elsewhere. Briefly, human breast epidermis was cleaved from dermis by heat treatment and homogenised in 0.2 ml/cm² of 40 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.1% sodium azide, and 0.5 mM phenylmethylsulphonyl fluoride. The soluble proteins were precipitated with absolute ethanol, recovered by centrefugation, and resuspended in water after 20 minutes drying at 80°C. After another centrefugation, the supernatant contained the partially purified (≥80%) neutral-acidic variants of filaggrin and was designated as a filaggrin enriched extract.

FILAGGRIN PURIFICATION

The neutral-acidic isoforms of filaggrin were purified from the filaggrin enriched extract by affinity chromatography on mouse monoclonal antibodies (mAbs) directed to different epitopes of human (pro)filaggrin and belonging to the anti-human filaggrin (AHF) series of mAbs produced and characterised in our laboratory. The protein was purified on a 2 ml affinity column (CarbolinkTM, Pierce Chemical Co, Rockford, IL, USA) coupled, as recommended by the manufacturer, with 2.8 mg of periodate-oxidised AHF mAbs, in the following proportions: AHF1 (5%), AHF2 (20%), AHF3 (35%), AHF5 (30%), AHF7 (10%). The filaggrin enriched extract was lyophilised, dissolved in phosphate buffered saline (PBS), and loaded onto the column. After incubation for three hours at room temperature or overnight at 4°C, the column was washed with 1 M NaCl, 10 mM phosphate buffer pH 7.4, and bound filaggrin was eluted with 0.2 M glycine-HCl pH 2.5, and immediately neutralised by addition of 0.05 volume of 2 M Tris base. The purified filaggrin, subsequently used to coat ELISA plates, was obtained by pooling the eluted fractions.

PRODUCTION AND PURIFICATION OF HUMAN RECOMBINANT FILAGGRIN

The production and purification of human recombinant filaggrin as a gluthathione-S-transferase (GST)-filaggrin fusion protein has been described elsewhere.

IN VITRO DEIMINATION OF HUMAN RECOMBINANT FILAGGRIN

PAD purified from rabbit skeletal muscle was purchased from Sigma (St Louis, MO, USA). Recombinant GST-filaggrin was incubated for two hours at 37°C, with or without PAD (3.5 IU/mg of protein), in 0.1 M Tris-HCl pH 7.4, 10 mM CaCl₂, 5 mM dithiothreitol. When deiminated recombinant filaggrins prepared under several conditions were compared by immunoblotting with a series of AFA positive RA sera, the above conditions allowed maximum immunoreactivity to be reached each time. Deimination was stopped by addition of 20 mM EDTA, and buffer exchange was accomplished versus 20 mM Tris pH 7.4. The recombinant GST-filaggrin, deiminated or not, was then lyophilised and stored at −80°C until used.
Table 2 Diagnostic sensitivities (0.05 confidence intervals) of antifilaggrin autoantibodies detected by indirect immunofluorescence on rat oesophagus epithelium (“AKA”), by immunoblotting on human epidermis filaggrin (AhFA-IB) and by ELISA (AhFA-ELISA-pur or AhFA-ELISA-rec)

<table>
<thead>
<tr>
<th></th>
<th>Specificity &gt; 95%</th>
<th>Specificity &gt; 99%</th>
</tr>
</thead>
<tbody>
<tr>
<td>“AKA”</td>
<td>0.51 (0.45 to 0.58)</td>
<td>0.40 (0.34 to 0.46)</td>
</tr>
<tr>
<td>AhFA-IB</td>
<td>0.59 (0.53 to 0.66)</td>
<td>0.37 (0.30 to 0.43)</td>
</tr>
<tr>
<td>AhFA-ELISA-pur</td>
<td>0.48 (0.41 to 0.54)</td>
<td>0.22 (0.17 to 0.27)</td>
</tr>
<tr>
<td>AhFA-ELISA-rec</td>
<td>0.52 (0.46 to 0.59)</td>
<td>0.31 (0.25 to 0.37)</td>
</tr>
</tbody>
</table>

ELISA DETECTION OF ANTIFILAGGRIN AUTOANTIBODIES

Antibodies to either purified filaggrin or recombinant filaggrin deaminated in vitro were detected by ELISA using a protocol resulting from optimisation of a previously described method. Assay plates, 96 well (MaxiSorp, Nunc, Denmark), were coated with 100 µl (2.5 µg/ml) of each antigen diluted in PBS pH 7.4, incubated for one hour at 37°C, then overnight at 4°C. After three washes with PBS, 0.05% Tween 20, 0.5% gelatine (Prolabo, Manchester, UK), the plates were blocked with PBS, 2.5% (w/v) gelatine, for one hour at 37°C, washed again, and serum samples diluted 1:100 in 100 µl PBS, 0.5% gelatine, 0.05% (v/v) Tween 20 (diluting buffer) were incubated for one hour at 37°C. Serum-free control wells (blank) received 100 µl of diluting buffer. After washing above, 100 µl of peroxidase-conjugated goat antibody to human γ heavy chains of immunoglobulins (Southern Biotech Inc, Birmingham, AL, USA), were coated with 100 µl (2.5 µg/ml) of each antigen diluted in PBS pH 7.4, incubated for one hour at 37°C. The bound antibodies were detected with o-phenylenediamine dihydrochloride substrate (Sigma) dissolved in 0.035 M citric acid, 0.1 M sodium phosphate pH 5, supplemented with 10 µl/10ml of a 30% H2O2 solution. The reaction was stopped by 50 µl of 3 M sulphuric acid. The absorbance was determined at 492 nm by an automated plate reader (Multiskan, Labsystem). All the serum samples were tested four times using two different plates and two independent dilutions, and the four results were averaged. For the AhFA-ELISA-pur, the optical density (OD) values obtained on filaggrin were directly referred to as titres. For the AhFA-ELISA-rec, the titre was considered to be the difference between the OD obtained on the deaminated recombinant filaggrin and that obtained on the non-deaminated recombinant filaggrin. A pool of highly positive RA serum samples was tested with each plate and used as a reference to correct interassay variations. The means of intra-assay and interassay variations (standard deviation/mean) were less than 8% for both ELISAs.

STATISTICAL ANALYSES

Data analyses were performed using STATISTICA for Windows (StatSoft, Tulsa, OK, USA)

Median differences were tested with the Mann-Whitney U test, correlations were sought by computing Spearman rank correlation coefficients, and the χ² test was used to compare percentages. A test was considered significant for p≤0.01.

Results

TITRE DISTRIBUTION AND DIAGNOSTIC INDEXES

For “AKA”, AhFA-IB, and the AhFA-ELISAs whatever the immunosorbent, the titres were significantly higher in RA than in control sera (p<0.05).

For all the tests (“AKA”, AhFA-IB, and AhFA-ELISAs) the diagnostic sensitivities were determined at titre thresholds allowing diagnostic specificities of either 0.95 or 0.99 to be reached (table 2). For “AKA” and AhFA-IB, the diagnostic sensitivities were found to be similar to those we previously reported, indicating that the sample of patients was representative. The sensitivities of the two AhFA-ELISAs did not significantly differ from each other whatever the diagnostic specificity. Moreover, at 0.95 specificity, the diagnostic sensitivities of the two ELISAs were comparable with those of “AKA” and AhFA-IB. However, at 0.99 specificity, the diagnostic sensitivities of “AKA” and AhFA-IB were significantly higher (p<0.05) than that of AhFA-ELISA-pur. At the same specificity, the diagnostic sensitivity of AhFA-ELISA-rec was also lower than that of “AKA” or AhFA-IB, but this difference was not statistically significant.

CORRELATIONS AND CONCORDANCES

Antibody titres of “AKA”, AhFA-IB, and the AhFA-ELISAs were found to be independent of the age of the patients. On the other hand, as reported previously, the proportion of men positive for “AKA” and/or AhFA-IB and/or the AhFA-ELISAs was significantly higher than the proportion of women (roughly 70% v 50%).

The titres of “AKA”, AhFA-IB, and the AhFA-ELISAs were found to be significantly correlated with each other (p<0.05). However, correlation coefficients were higher between AhFA-IB and AhFA-ELISA-pur (r=0.86) than between AhFA-IB and AhFA-ELISA-rec (r=0.59). The correlation coefficient between AhFA-ELISA-rec and “AKA” was the lowest (r=0.50).

For further analyses of the two ELISAs only ELISA-rec was considered because it had a better diagnostic performance. The results (positive or negative) obtained with “AKA”, AhFA-IB, and AhFA-ELISA-rec at the 0.95 specificity threshold were found to be largely concordant (table 3). Indeed, among the 241 RA sera, 131 (54%) were concordant for the three tests, and 412 (87%) in the control sera. When only the positive serum samples are considered, 129/241 (54%) patients with RA were positive with at least two tests compared with only 5/473 (1%) of the controls, showing that the false positives of each test were mainly single positives. Consequently, if a serum is considered positive when two out of the three tests are positive, the diagnostic sensitivity is 0.54 and the specificity 0.99.

The performance of a combination of AhFA-ELISA-rec with one of the other tests was analysed; the most efficient combination was AhFA-ELISA-rec (titre >0.05) and AhFA-IB (titre >0.0). This combination allowed 132 RA samples to be diagnosed (diagnostic...
Table 3  Distribution of sera according to the presence of antifilaggrin autoantibodies detected by the “AKA”, AhFA-IB, and AhFA-ELISA-rec tests (the thresholds used are those which correspond to a diagnostic specificity of 0.95 for each test)

<table>
<thead>
<tr>
<th>“AKA”</th>
<th>AhFA-IB</th>
<th>AhFA-ELISA-rec</th>
<th>RA No (%)</th>
<th>Controls No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>54 (22)</td>
<td>411 (87)</td>
<td>18 (7)</td>
<td>17 (4)</td>
</tr>
<tr>
<td>+</td>
<td>20 (8)</td>
<td>20 (4)</td>
<td>20 (8)</td>
<td>20 (4)</td>
</tr>
<tr>
<td>+</td>
<td>23 (10)</td>
<td>1 (0.2)</td>
<td>3 (0.2)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>+</td>
<td>6 (2)</td>
<td>1 (0.2)</td>
<td>23 (10)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>+</td>
<td>77 (32)</td>
<td>1 (0.2)</td>
<td>20 (8)</td>
<td>20 (4)</td>
</tr>
</tbody>
</table>

| 241 (100) | 473 (100) |

+ = positive; blank = negative.

Discussion
Numerous groups have demonstrated the diagnostic value of “AKA” and APF, but detection of these autoantibodies has remained largely restricted to specialised laboratories, essentially because of technical difficulties. We thus undertook the development and validation, on a large series of patients, of an easily standardisable diagnostic test, based on ELISA detection of autoantibodies to human filaggrin. We evaluated the diagnostic performance of a first ELISA using the neutral-acidic isoforms of filaggrin purified from human epidermis as immunosorbent, and of a second ELISA, using a recombinant human filaggrin deiminated in vitro. Although the diagnostic sensitivities of the ELISAs were not significantly different from each other, only the AhFA-ELISA-rec showed diagnostic performance that was not significantly different from those of “AKA” and AhFA-IB. The difference between the two ELISAs may be partly explained by the fact that the purified filaggrin contains partially deiminated neutral isoforms of filaggrin, which are less antigenic, and that the recombinant filaggrin may have a higher percentage of deiminated arginine residues. Moreover, in the AhFA-ELISA-rec, we used the non-deiminated recombinant filaggrin as a negative control, which allowed subtraction of a non-specific reactivity to the molecule, targeting epitopes devoid of citrulline. Indeed, when analysing only OD obtained on the deiminated protein instead of the OD difference between deiminated and non-deiminated recombinant filaggrin, the diagnostic sensitivity was notably lower (data not shown).

The titres of “AKA”, AhFA-IB, and the AhFA-ELISAs were significantly correlated with each other. The strongest correlations were obtained, as expected, between AhFA-ELISAs and AhFA-IB, the three tests using human filaggrin. Conversely, the weakest (though significant) correlation coefficient was found between “AKA” and AhFA-ELISA-rec, showing that the overlapping between the sets of antibodies detected by these two methods is more restricted. In accordance with our finding, Aho et al found a better correlation between the tests using a human antigen (APF and AhFA-ELISA) rather than between those performed on antigens from different species (“AKA” and AhFA-ELISA). In our study the correlation coefficient between AhFA-IB and AhFA-ELISA was higher when purified rather than when deiminated recombinant filaggrin was used as immunosorbent. This is easily explained by the fact that AhFA-IB and AhFA-ELISA-pur use the same neutral-acid variants of human epidermis filaggrin as target antigen, whereas AhFA-ELISA-rec uses a recombinant protein. Indeed, the water soluble variants of human epidermis filaggrin correspond to several (10–12) filaggrin polypeptides which are polymorphic in sequence and charge, whereas the recombinant filaggrin corresponds to only one filaggrin polypeptide. Moreover, the arginine residues modified by the rabbit PAD in vitro may be partly different from the residues physiologically deiminated during epidermis differentiation. Therefore, it appears that purified and deiminated recombinant filaggrins present sets of epitopes which are partly different.

Palosuo et al developed and validated an ELISA for the detection of antifilaggrin autoantibodies, using as immunosorbent, filaggrin extracted from human epidermis and subsequently purified by sequential HPLC. The diagnostic sensitivity proposed for the test was higher than those of the ELISA-rec we developed (0.71 for a specificity of 0.95 and 0.47 for a specificity of 0.99). However, the number of patients with RA studied was considerably smaller (55 v 241) and, in addition, the positivity thresholds were set using 100 normal blood donors and not patients with non-RA rheumatic diseases, who have to constitute the control group when validating such a diagnostic test, as was the case in our study (473 patients). Furthermore, in the series of patients with RA analysed by these authors, the sensitivity of “AKA” was high (0.51 for a specificity of 0.99), whereas at the same specificity, it was only 0.40 in our sample of patients with RA, the latter performance being more representative for this test.’ This underlines the importance of the composition of the samples of patients used when validating diagnostic tests.

Although the ELISAs using purified filaggrin may appear to be efficient diagnostic tools, human epidermis filaggrin remains a difficult antigen to obtain, and its extraction and purification difficult to standardise. We think that use of a recombinant human filaggrin deiminated in vitro is better suited to standardise a diagnostic test.

Schellekens et al recently proposed another ELISA for detection of antifilaggrin autoantibodies using a cyclic variant of a citrullinated peptide derived from the sequence of human filaggrin (CCP-ELISA). In a series of 134 RA and 815 control sera, this test proved to have a diagnostic sensitivity of 0.68 for a specificity of 0.98. This good diagnostic performance was confirmed on several cohorts of patients from
different groups. However, the CCP-ELISA had a diagnostic sensitivity ranging from 0.45 to 0.80 with a specificity ranging from 0.96 to 1.00. Unfortunately, the performance of the CCP-ELISA was not compared with the reference tests “AKA”, APF, or AhFA-IB, determined on the same series of patients. A comparison has been made for a smaller group of patients (134 RA and 154 controls) for APF, the diagnostic sensitivity of which is 0.74 for a specificity of 0.83.18 In this group the sensitivity of the CCP-ELISA was also 0.68. Within a subgroup of our patients (206 RA, 153 controls) where the sensitivity of AhFA-ELISA-rec was 0.51 for a specificity of 0.93, APF showed a sensitivity of 0.65 for a specificity of 0.94.20 These partial results may indicate a higher sensitivity for the CCP-ELISA compared with our AhFA-ELISA-rec. This has to be confirmed on larger series of patients.

In a series of patients with recent onset RA, van Jaarsveld et al detected 52% positive RA serum samples with the CCP-ELISA v 66% with APF.19 In a similar series of patients, Aho et al detected 49% positive RA serum samples with their AhFA-ELISA v 47% with APF,20 whereas Goldbach-Mansky et al found 41% positive RA serum samples with the CCP-ELISA v 33% with the same AhFA-ELISA.21 Lastly, in the same type of recent onset RA, Kroot et al identified 66% of patients with RA positive for the CCP-ELISA.22 The data from these studies, considered as a whole, clearly confirm the diagnostic importance of antifilaggrin autoantibodies in patients with recent onset arthritis. However, the discrepancies between the performance of the tests, which may result from the composition of the series of patients and from subtle interlaboratory technical differences in performing the same test, underline the difficulties in comparing the results with each other objectively in the absence of a common standardized reference test.

An analysis of rheumatoid factor (RF) titres, available from a previous study on a subset of sera (198 patients with and 150 patients without RA),23 showed that the specificity and sensitivity of the AhFA-ELISA-rec (0.93 and 0.49, respectively) and RF (0.90 and 0.55, respectively) were not significantly different. However, consistent with our previous findings when the “AKA”, AhFA-IB, and APF tests were used at the 0.95 threshold, 26 (29%) of the 90 RF negative patients with RA were positive for the AhFA-ELISA-rec. In this subset of 348 patients, combining AhFA-ELISA-rec (titre >0.076) with RF, did not lead to a better diagnostic performance (specificity 0.98, sensitivity 0.47) than that obtained when combining AhFA-ELISA-rec with AhFA-IB (specificity 0.98, sensitivity 0.51).

In conclusion, among the AhFA-ELISAs we developed and validated in this study, AhFA-ELISA-rec, which had a diagnostic performance similar to that of “AKA” or AhFA-IB, is proposed for diagnostic use, and could replace “AKA” or AhFA-IB. Nevertheless, because each of the three tests allowed the detection of antifilaggrin autoantibodies in subgroups of patients with RA which did not entirely overlap, it seems to be most efficient to combine AhFA-ELISA-rec with one of the reference tests, particularly with AhFA-IB. Indeed, this combination clearly enhances the detection of antifilaggrin autoantibodies for the diagnosis of RA.

We recently showed that deaminated α and β chains of fibrin are the major target antigens of antifilaggrin autoantibodies in the rheumatoid synovium.24 The development of new immunoassays using these antigens may permit detection of all of the subsets of antifilaggrin autoantibodies of different specificities found in the various subgroups of patients with RA. This constitutes the next challenge in the development of immunoassays to detect these RA-specific autoantibodies.

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