Improvement in Diagnosis of Rheumatoid Arthritis Using Dual Indirect Immunofluorescence and Immunoblotting Assays for Antifilaggrin Autoantibodies: A Retrospective 3 Year Study

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ABSTRACT. ***Objective.*** To determine the clinical usefulness of measuring antistratum corneum (ASC) and antifilaggrin autoantibodies (AFA) to discriminate between rheumatoid arthritis (RA) and other rheumatic or autoimmune diseases, using an indirect immunofluorescence (IIF) assay, along with a complementary immunoblotting technique (IB) when IIF detection of ASC was negative.

Methods. Sera from 346 patients were studied: 189 sera from patients with RA seen in the same clinic, 92 from patients with non-RA rheumatic diseases, 24 from nonrheumatic autoimmune diseases, and 41 from healthy blood donors. ASC and AFA were detected using IIF and IB, respectively.

Results. ASC detection using IIF showed a specificity of 97.5% for RA with 44.4% sensitivity. When both IIF and IB techniques were used, sensitivity for RA increased significantly (up to 53.4%; $p < 0.01$) with no decrease in specificity ($p < 0.01$).

Conclusion. These data confirm the usefulness of 2 different techniques performed simultaneously for detecting ASC/AFA, and the usefulness of these biological markers for discriminating between RA and other rheumatic diseases in clinical practice. (J Rheumatol 2002;29:276–81)

Key Indexing Terms:
ANTIFILAGGRIN AUTOANTIBODY RHEUMATOID ARTHRITIS IMMUNOBLOTTING

Rheumatoid arthritis (RA) is the most frequent human systemic autoimmune disease. Diagnosis is still difficult, especially at the onset of clinical symptoms. Like other non-organ-specific systemic autoimmune diseases, RA shows a wide variety of circulating autoantibodies (Ab) such as rheumatoid factor (RF), anticollagen, anti-Sa, anticalpastatin, antiperinuclear (APF), antistratum corneum (ASC), and antifilaggrin (AFA) Ab[1-11]. RF is the only Ab chosen to be a biological criterion in the diagnosis of RA following the criteria of the American Rheumatism Association (ARA)[12]. However, this marker shows poor diagnostic specificity, as it is found in various other autoimmune diseases and in normal healthy persons[13]. Further, its detection is not helpful at the onset of disease, since positivity usually appears after the first year of active arthritis[14]. Among the wide variety of circulating Ab in RA, APF and ASC/AFA are increasingly recognized as major Ab with diagnostic significance because of their higher specificity and sensitivity compared to the other Ab. Indeed specificity of APF reaches 74 to 99%[15-17], with a mean sensitivity of 59.7%. Specificity of ASC/AFA varies between 72 and 100%, with a mean sensitivity of 45%[7,18-25]. Besides this high specificity this marker does not appear to be affected by the duration of the disease compared to RF[2,26]. APF recognize antigens in perinuclear granules located in superficial cells in human buccal mucosa squamous epithelium. Classically APF is detected by indirect immunofluorescence (IIF) on fresh human buccal mucosa cells. Inadequate standardization of this substrate, i.e., selection of "good donors," results in high interlaboratory variations for assay sensitivity[27]. Consequently APF are not considered a suitable marker for RA diagnosis. ASC/AFA, mainly of the IgG isotype, were described by Young, *et al*[28] and were initially called antikeratin or antistratum corneum antibody. Indeed, ASC are essentially detected by IIF using cryosections of rat esophagus giving rise to a laminar labelling within the stratum corneum of the epithelium. Later filaggrin was identified as the target antigen of the ASC in the stratum corneum[8,9]. Filaggrin of an apparent molecular weight of 37 kDa derives from the

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profilaggrin, with intermediate forms existing from this protein to the final molecule, i.e., filaggrin. This molecule is associated with the intermediate filament and is involved in the aggregation of cytokeratin filaments during the cornification of the epidermis[29].

To date IIF determination of ASC/AFA presents the disadvantage of subjectivity of interpretation, difficulties of standardization, and semiquantitative results. These drawbacks largely explain the wide variations in sensitivity found with the first determinations: 10 to 81% depending on the study[2,25,26,30-36]. Efforts to standardize the IIF technique using cryosections of rat esophagus give rise to an expected sensitivity around 40%. To enhance the sensitivity of ASC/AFA, an immunoblotting assay (IB)[10,11] and an enzyme linked immunosorbent assay[36,37] have been developed. With these new techniques, sensitivity reached 49 to 52%[10,37]. In our study, filaggrin extracted from human epidermis was used as a source of antigen for IB. This technique was performed on IIF negative sera in an attempt to improve the threshold of sensitivity without decreasing specificity.

We tested the usefulness of ASC/AFA measurements to try to discriminate RA from other rheumatic or autoimmune diseases in routine clinical practice. With a dual detection system (IIF and IB), ASC/AFA determinations were found to be a useful biological tool in RA diagnosis, showing good sensitivity and high specificity.

MATERIALS AND METHODS

Serum samples. Two hundred eighty-one patients seen in the same clinic with primary diagnosis of inflammatory or noninflammatory rheumatic disease gave blood samples consecutively. Sera were collected and frozen at –20°C until assayed. One hundred eighty-nine sera from RA patients who fulfilled the criteria of the ARA[12] were tested. Seventy sera from patients with various other definite rheumatic inflammatory diseases served as negative inflammatory controls. Twenty-two sera from patients presenting symptoms mimicking rheumatic disease but whose diagnoses were determined as noninflammatory were also tested as negative "noninflammatory controls." Twenty-four sera from patients with autoimmune thyroiditis were considered as "nonrheumatic autoimmune disease controls." Forty-one sera from healthy blood donors were used to calibrate both techniques. The mean ages were 57 years (range 17–84), 45 (12–76), 54 (32–79), 47 (21–87), and 42 (range 21–73) years for the 5 groups, respectively.

Indirect immunofluorescence. For each serum sample diluted at 1/5, the presence of ASC was determined using a described IIF method[18,20]. In those reports, this dilution was accepted as having the best sensitivity with no loss of specificity. Ig were detected with fluorescein isothiocyanate conjugated goat Ab to human IgG, IgA, and IgM (H+L) (Biorad, Marnes la vallée, France) diluted 1/140. The labelling of the stratum corneum of rat esophagus epithelium (Biorad) was evaluated by 2 readers unaware of the clinical context. Sera were considered ASC positive when the characteristic linear laminated pattern of labelling was restricted to the stratum corneum of rat esophagus epithelium. The different pattern of stratum corneum fluorescence, irregular and heterogenous due to IgM and IgA, was considered as negative. If the first 2 readers disagreed, a third reader's determination was taken into account.

Filaggrin extraction and purification. Filaggrin was extracted from human epidermis as described by Vincent, *et al*[7,10]. Briefly, epidermis was cleaved from dermis by heat treatment in phosphate buffered saline (PBS), 8.5 mM KH_2/K_2HPO_4, 150 mM NaCl, 5 mM EDTA, and 0.5 mM PMSF for 5 min at 56°C and then in PBS, 8.5 mM KH_2/K_2HPO_4, 150 mM NaCl, and 0.5 mM PMSF for 5 min at 4°C and homogenized in 0.2 ml/cm^2 of ice cold buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% nonidet P-40, and 0.1 mM PMSF). The suspension was centrifuged at 15,000 g for 15 min at 4°C and the supernatant was collected. Proteins in this extract were precipitated with 1/10 (v/v) absolute ethanol, recovered by centrifugation at 15,000 g for 15 min at 4°C, dried at 80°C, and resuspended in water. With this method an extract enriched in the neutral/acidic isoforms of epidermal filaggrin is obtained[7,10].

Electrophoresis and immunoblotting of filaggrin. The partially purified neutral/acidic filaggrin was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes (0.45 μm) (Schleisser & Schull, Dassel, Germany). They were probed with human RA sera or control sera diluted 1/100. Human Ig were detected with peroxidase conjugated goat Ab to human IgG, IgA, IgM (Biorad) diluted 1/140. The immunoreactive band between 37 and 40 kDa, which was relatively well defined with low titer sera, showed a more diffuse pattern with high titer sera (Figure 1). This pattern was previously found to be typically produced by Ab from RA sera on human epidermal filaggrin[7]. A mouse monoclonal Ab (AKH1, Biomedical Technologies Inc., Boston, MA, USA) was used as a positive control.

Statistical analysis. The chi-squared test was used to analyze qualitative variable difference, i.e., sensitivity and specificity of ASC/AFA, with or without the IB technique. A p value < 0.01 was considered significant.

RESULTS

IIF detection of ASC. Eighty-eight sera (25.4%), including 84 RA sera and 4 non-RA sera out of a total of 346, labelled the stratum corneum of rat esophagus epithelium (Table 1). The sensitivity for RA was 44.4%. The diagnostic speci-

Table 1. Detection of AFA using IIF in the 5 categories of subjects.

	No.	IIF Positive (%)
Rheumatoid arthritis	189	84 (44.4)
Inflammatory rheumatic diseases	70	3 (4.3)
Systemic lupus erythematosus	16	0
Sharp's disease	4	0
Seronegative spondyloarthritis	35	
Arthropathic psoriasis	6	0
Ankylosing spondylitis	15	2
Reactive arthritis	14	0
Myositis	3	0
Still's disease	2	0
Vasculitis	3	0
Other inflammatory diseases*	7	1†
Noninflammatory rheumatic diseases	22	1 (4.5)
Osteoarthritis	7	1
Fibromyalgia	11	0
Other noninflammatory diseases**	4	0
Noninflammatory nonrheumatic disease:		
thyroiditis	24	0 (0.0)
Healthy controls	41	0 (0.0)
Total	346	

*Behçet's syndrome (1), chondrocalcinosis (1), Lyme disease (1), calcified periarthritis (1), polychondritis (1), leukosis (1), sarcoidosis (1). ** Retractive capsulitis (1), intervertebra disk hernia (1), sciatica (1), sympathetic algodystrophy (1). † Sarcoidosis (1).

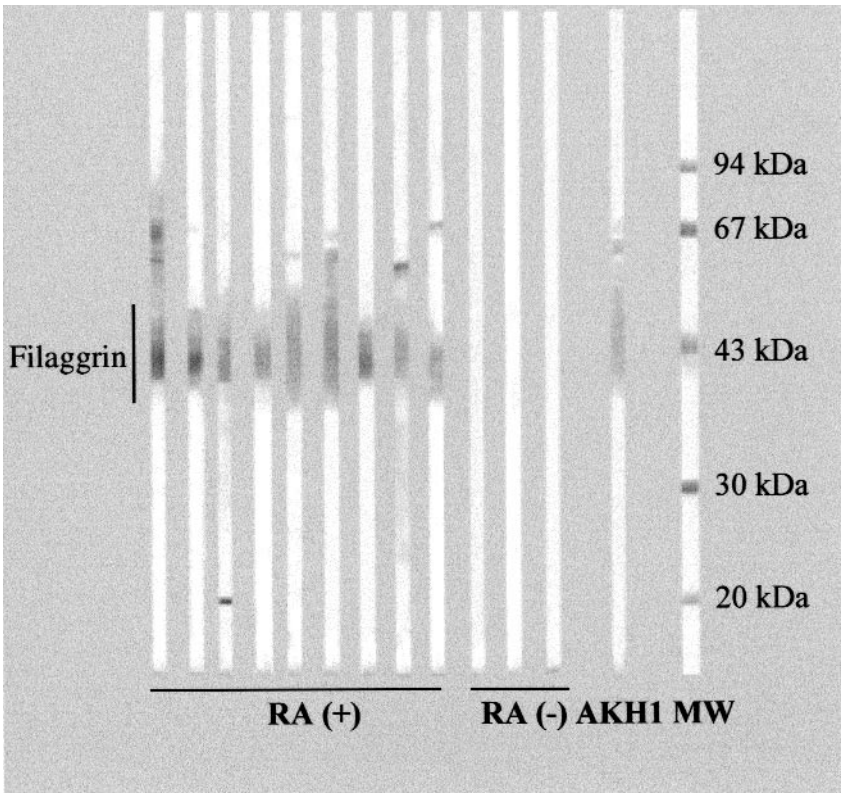

Figure 1. Detection of ASC/AFA by IB with extracts of human epidermis enriched in filaggrin. The positive RA sera showed a large band about 37 to 40 kDa consistent with filaggrin (lanes RA+). The negative RA sera showed no reactivity (lanes RA–). AKH1, a mouse Mab specific for human filaggrin, clearly identified a large band about 40 kDa (lane AKH1). MW: molecular weight markers.

ficity for RA compared to non-RA inflammatory rheumatic disease was 95.7%.

Immunoblotting detection of ASC/AFA. To assess whether IB assays increased the sensitivity of ASC/AFA detection without decreasing the specificity, the IB AFA detection assay was performed on 105 sera from patients with RA found to be IIF negative, on 116 sera from patients with other diseases, and on 41 sera from healthy blood donors found to be IIF negative or positive. The results of 17 sera (16.2%) studied from IIF negative RA patients showed a band labelled with an apparent molecular weight of 37–40 kDa; this single band smeared with high titer sera and reactivity with a higher molecular weight that indicated that it might correspond to intermediate forms of filaggrin (Figure 1, Table 2). The association of IIF and IB detection assays significantly increased the sensitivity for ASC/AFA in RA to 53.4% ($p < 0.01$).

In the group of patients with rheumatic diseases, 3 sera among 4 IIF positive sera were IB positive. In the healthy controls, group 1 serum, which was IIF negative, was IB positive (Table 2). Using both IIF and IB detection assays, the specificity for RA was the same as the specificity previously found using the IIF assay.

Diagnosis of non-RA patients with ASC/AFA positive sera. The 4 positive non-RA rheumatic patients were carefully reexamined. The 3 patients belonging to the inflammatory non-RA group also presented high levels of RF, and 2 were diagnosed as having ankylosing spondylitis, fulfilling the New York criteria[38]. One was positive only by IIF. He had been treated for epicondylitis that occurred 10 months after surgical treatment for lung cancer. The other one was positive by both IIF and IB, and fulfilled the American College of Rheumatology (ACR) criteria for RA with evidence of erosion. The third patient had left ankle arthritis and was positive by both IIF and IB. The diagnosis of sarcoidosis was established after exclusion of a valid diagnosis of RA because of a history of proven pulmonary involvement 10 years ago.

In the patients with noninflammatory rheumatic disease, one patient was both IIF and IB positive and also presented a high level of RF. This was a 78-year-old woman who fulfilled the ACR criterion for hand osteoarthritis[39].

A negative IIF serum from one healthy control was IB positive. He did not present any symptoms of rheumatic or autoimmune disease; however, he is being followed for any clinical evidence of rheumatism.

Table 2. Detection of ACS/AFA using combination of IIF and IB techniques.

	IB+	IIF+ and/or IB+
Rheumatoid arthritis	17/105*	101/189 (53.4%)
Inflammatory rheumatic disease	2/70	3/70 (4.3%)
Noninflammatory rheumatic disease	1/22	1/22 (4.5%)
Noninflammatory nonrheumatic disease: thyroiditis	0/24	0/24 (0%)
Healthy controls	1/41	1/41 (2.4%)

* IB were only performed on the 105 sera found to be negative by IIF. The positive IIF RA sera were not tested by IB.

DISCUSSION

Studies of ASC/AFA have reported that the specificity of these antibodies for RA was high, ranging from 72 to 100%[2,15,25,26,30-37]. Our study confirms this result for the IIF assay with a value of 95.7% compared to the non-RA inflammatory rheumatic diseases. The 3 positive sera in the non-RA rheumatic group were also positive for RF. The diagnosis of sarcoidosis in one case was by elimination, and remains unconfirmed because no sign of sarcoidosis relapse could be detected at the time of diagnosing arthritis. One patient diagnosed with AS perfectly fulfilled the ACR criteria for RA, and presented digital joint erosion closer to that found in RA than in AS. This observation suggests that the patient could have both diseases or that the diagnostic criteria were not strict enough[40]. The third positive serum in this group came from a patient with AS with a recent history of lung carcinoma and could be considered as a paramalignant arthropathy, similar to those found in another study[41]. In the noninflammatory rheumatic group, one positive serum was from a 78-year-old patient with evidence of osteoarthritis. It is notable that this patient also had a high serum level of RF with no sign of inflammation. The prevalence of Ab increases with age, especially for RF[41] and anticardiolipins[42] and this could also apply to ASC/AFA. However, in a recent report this hypothesis was challenged[43].

Despite the high specificity of ASC/AFA a major disadvantage of this marker is its relatively low sensitivity in routine clinical practice using IIF detection[44]. To minimize this disadvantage a complementary IB detection strategy was adopted. The combination of these methods has shown an increased sensitivity (63.7%) over the IIF technique alone (44%)[10]. In agreement with these results, our study showed an IIF assay sensitivity of 44.4%, which increased significantly to 53.4% using the IIF and IB assays. Of note, this higher sensitivity using both techniques did not decrease the specificity of ASC/AFA detection for RA compared to the non-RA inflammatory rheumatic diseases. Consequently, the combination of IIF and IB detection for ASC/AFA, especially when IIF is negative, provides better sensitivity, taking advantage of the difference between both techniques.

Several explanations can account for the discrepancy in the results between the IIF negative/IB positive sera. First, background fluorescence of the stratum corneum could interfere with ASC/AFA detection, especially if anticytokeratin Ab are present in the sera. In this condition the interpretation of the IB assay is more precise, since cytokeratins with a molecular weight around 37 to 40 kDa are absent from the protein extract used for IB. Second, IB essentially detects sequential epitopes, some of which are probably inaccessible or absent in the IIF assay; this may also be explained by the results of a preliminary study where only 85% of the IIF positive RA sera were IB positive (data not shown). Consequently, IB and IIF detection of ASC/AFA would be complementary, as confirmed by our results, and one should not in any case replace the other. The comparison of our test with other results using IgG-specific secondary Ab[10] showed that the diagnosis performance is quite similar using secondary Ab to IgG, IgM, and IgA.

Along with IIF and IB, ELISA techniques have been developed to detect AFA using purified filaggrin from human skin[36], different citrullinated peptide variants of the carboxy-terminal part of filaggrin, and recently a single cyclic citrullinated peptide (cfc1-cyc)[45-49]. The ELISA using the citrullinated peptides in combination with IIF showed a higher sensitivity of 76% with a specificity of 96%[45]. The sensitivity found in other studies was 66% for IIF, 52% for ELISA, and 67% using both techniques[48]. Using a single cyclic citrullinated peptide, cfc1-cyc, Schellekens, *et al* recently reported a sensitivity of 68% with a high specificity of 96% using the average of optical densities of 154 control sera. However, this ELISA exhibited a sensitivity that varied from 45 to 80% depending on the cohort of patients studied[45]. The major disadvantage of ELISA using artificially citrullinated peptides is the need to cover the whole antigenic pattern of the filaggrin protein, providing a panel of representative peptides. Thus ELISA results depend largely on the nature of the peptides used. This characteristic may explain the great variation reported with this technique when peptides or positivity threshold are different. The major targets of ASC/AFA in the rheumatoid synovium were recently identified as deiminated forms of the α and ß chains of fibrin(ogen)[49]. To date a comparative analysis of the reactivity of large series of RA sera to filaggrin and to deiminated fibrin(ogen) is not available; as well, as a large overlap exists between ASC/AFA and Ab to the deiminated fibrin(ogen)[49], it is still convenient to use the entire filaggrin molecule present in human epidermis to detect AFA in clinical practice.

Our results provide evidence for an improved sensitivity of detection of ASC/AFA by techniques that are easy to perform and that give highly specific results in routine diagnosis.

There is a clear need for a specific biological marker for RA: ASC/AFA could be one such marker[50]. Efforts are needed to standardize IIF and especially IB techniques to allow this marker to be used as a biological criterion.

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