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Anti-α-fodrin antibodies do not add much to the diagnosis of Sjögren's syndrome

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Abstract

The presence of anti-α-fodrin autoantibodies has been reported to be a highly specific and sensitive test for the diagnosis of Sjögren's syndrome (SjS). We looked (in Nijmegen) for anti-α-fodrin, anti-Ro60, and anti-La autoantibodies in a cohort of 51 patients with rheumatic diseases (primary SjS [21], secondary SjS [6], rheumatoid arthritis [RA] [12], systemic lupus erythematosus [SLE] [6], and scleroderma [6]) and in 28 healthy subjects, using ELISA, immunoblotting, and immunoprecipitation. The same samples were analyzed with an alternative anti-α-fodrin ELISA in Hanover. The Nijmegen ELISA of the sera from primary SjS showed sensitivities of 43% and 48% for IgA- and IgG-type anti-α-fodrin antibodies, respectively. The Hanover ELISA showed sensitivities of 38% and 10% for IgA- and IgG-type anti-α-fodrin antibodies, respectively. The ELISAs for α-fodrin showed six (Nijmegen) and four (Hanover) anti-α-fodrin-positive RA sera. IgA and IgG anti-fodrin antibodies were also present in four patients with secondary SjS. The sensitivities of Ro60 and La-antibodies in the Nijmegen ELISA were 67% and 62%, respectively. Unlike anti-α-fodrin antibodies, all anti-Ro60 and anti-La positive sera could be confirmed by immunoblotting or RNA immunoprecipitation. Thus, anti-Ro and anti-La autoantibodies were more sensitive than anti-α-fodrin autoantibodies in ELISA and were more frequently confirmed by other techniques. Anti-La antibodies appear to be more disease-specific than anti-α-fodrin antibodies, which are also found in RA sera. Therefore, the measurement of anti-α-fodrin autoantibodies does not add much to the diagnosis of Sjögren's syndrome.

Keywords: alpha-fodrin, antibody, ELISA, sensitivity, Sjögren

Introduction

Sjögren's syndrome (SjS) is a chronic autoimmune exocrinopathy of unknown origin. Therefore the diagnosis of SjS, in the absence of a gold standard, is based on criteria containing a number of subjective and objective signs and symptoms. In the past three decades, several sets of criteria have been introduced [1–4], in which there has been a shift from emphasis on subjective symptoms, such as complaints of dry eyes or dry mouth, towards objective findings. Recently, a widely supported consensus was established to merge the most frequently used European (European Study Group [ESG]) and US (San Diego, San Francisco) classification criteria sets into one US/European set [5]. The authors of all three major classification criteria sets previously used took part in this consensus group. In the US/European classification criteria, more weight is put on the presence of anti-Ro and anti-La antibodies in the serum, and on the lymphocytic focus score (LFS) of the sublabial glands, both being objective signs. The cutoff point of a positive LFS was set at ≥1.0, which ended a long-lasting debate about whether an LFS of ≥1.0 (ESG criteria), >1.0 (San Francisco criteria), or ≥2.0 (San Diego criteria) was most applicable for the diagnosis of SjS. This agreement ultimately will produce uniform intercontinental disease prevalence data. However, the disease specificity of particularly anti-La anti-

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ELISA = enzyme-linked immunosorbent assay; ESG = European Study Group; FCS = fetal calf serum; LFS = lymphocytic focus score; NHS = normal healthy subjects; pGEX-4T2 = α fodrin cDNA in a GST expression vector; RA = rheumatoid arthritis; SjS = Sjögren's syndrome; SLE = systemic lupus erythematosus; SS-A = anti-Ro60 (antibodies); SS-B = Anti-La (antibodies); SSc = systemic sclerosis.

bodies is limited (besides being found in SjS, they are also found in systemic lupus erythematosus [SLE]), and the sensitivities of anti-Ro and anti-La antibodies range only from 60–75% and 30–50%, respectively [6–9]. Therefore, the search for more sensitive and specific diagnostic markers needs to be continued.

Haneji and co-workers [10] suggested a 120-kDa cleavage product of α-fodrin (a cytoskeletal protein) as a candidate autoantigen in SjS. They reported that the presence of anti-α-fodrin antibodies was very specific for the diagnosis of SjS and claimed a very high sensitivity (96%). In another report of the same group, however, these antibodies were also found in some sera of patients with SLE [11]. Their results suggested that anti-α-fodrin antibodies might replace anti-Ro and anti-La antibodies, as a more objective serological marker to improve the diagnostic value of classification criteria. This suggestion was supported by Witte and co-workers, who developed an ELISA for the detection of anti-α-fodrin antibodies and showed that IgA antibodies against α-fodrin provided an even higher sensitivity than IgG antibodies [12].

The objective of this study was to measure the presence of anti-α-fodrin antibodies in the sera of a cohort of patients with well-defined SjS at the Department of Rheumatology of the University Medical Center St Radboud, Nijmegen, The Netherlands. A second objective was to evaluate whether positive anti-fodrin ELISA results could be confirmed by at least one alternative biochemical technique such as immunoblotting or protein immunoprecipitation.

Materials and methods
Patients and measurement techniques
The sera of 21 patients (18 women and 3 men, aged 27–76 years, median 55 years) with well-defined primary SjS according to the US/European criteria [5] were tested along with the sera of 6 patients with secondary SjS (all women, aged 41–55 years), 28 normal healthy subjects (NHS) (19 women and 9 men, aged 24–61 years, median 43 years), 12 patients with rheumatoid arthritis (RA) and without signs of secondary SjS (8 women and 4 men, aged 32–72 years, median 47 years), 6 with SLE (all women, aged 33–56 years), and 6 with systemic sclerosis (SSc) (3 women and 3 men, aged 36–49 years).

All the patients tested were white. To fulfill the US/European classification criteria, all SjS patients had to have a biopsy of the sublabial salivary glands showing an LFS ≥1.0. Furthermore, immunohistochemical examination had to show a percentage IgA-containing plasma cells of less than 70, a feature that is also strongly associated with SjS and slightly more disease specific than the LFS [13,14]. The comorbidity of the six patients with secondary SjS is shown in Table 1. Secondary SjS was accompanied by SLE in four patients, by systemic sclerosis in another

patient, and by dermatomyositis in yet another. None of the SjS patients was receiving an immunosuppressant.

The presence of anti-α-fodrin antibodies in the sera was measured with three different biochemical techniques: ELISA, immunoblotting with recombinant fodrin, and immunoprecipitation of radiolabeled fodrin. Furthermore, a blinded set of our serum samples was analyzed by Witte and co-workers using the anti-α-fodrin ELISA developed in Hanover, Germany (referred to as Hanover ELISA) [12].

Expression of the 120-kDa α-fodrin fragment
For the expression of the antigenic fodrin fragment, we used the α-fodrin cDNA in a GST expression vector (pGEX-4T2), which was kindly supplied by Dr Y Hayashi (Tokushima University School of Dentistry, Tokushima, Japan). The cDNA was expressed in BL21 (DE3) cells, and the protein was affinity-purified using glutathione Sepharose beads (Amersham Pharmacia Biotech).

ELISA
The presence of IgA, IgG, and IgM anti-α-fodrin antibodies in sera in 100-fold dilution was assessed by ELISA. Plates were coated with purified α-fodrin–GST as antigen, and bound antibody was detected essentially as described by Schellekens and colleagues [15], using rabbit peroxidase-conjugated anti-human immunoglobulins (anti-IgG, anti-IgA, or anti-IgM, DAKO, Glostrup, Denmark).

Sera were considered positive when the optical density at λ 450 nm values after correction for background value exceeded the mean +2 SD of that of a pool of sera from NHS. All ELISAs were performed in duplicate. To check for possible false-positive results because of the presence of the GST moiety in the α-fodrin–GST product that was used as the antigen, the ELISA was also performed in the presence of a 10-fold excess of purified carrier GST.

Anti-La (SS-B) and anti-Ro60 (SS-A) autoantibodies were measured by ELISA, using recombinant La and Ro60 proteins. All sera were also analyzed by immunoblotting and RNA immunoprecipitation to confirm the presence of anti-Ro and anti-La antibodies, as previously described [16].

Immunoblotting
To confirm the ELISA results, reactivity of sera against recombinant fodrin–GST was evaluated by western blotting essentially as described elsewhere [17]. The human sera were diluted 5000-fold with blocking buffer. A second antibody directed against total human immunoglobulin was used (DAKO, Glostrup, Denmark). In a similar type of experiment, immunoblots containing extracts from apoptotic Jurkat cells, prepared according to the method of Zampieri and colleagues [18] and believed to contain the native and possibly modified apoptotic α-fodrin fragment of 120 kDa described by Haneji and

Table 1

Autoantibodies in Nijmegen sera from a cohort of patients with Sjögren's syndrome

Pt no.	IgA[a] Nijmegen	IgA[a] Hanover[c]	IgG[b] Nijmegen	IgG[b] Hanover[c]	IB[d]	IP[e]	Ro60[f]	La[g]	LFS[h]	Comorbidity
Primary Sjögren's syndrome										
1	+	+	+	−	−	−	+	−	+	
2	−	−	−	−	−	−	−	+	+	
3	+	−	−	−	−	−	−	−	+	
4	+	+	+	−	−	−	+	−	+	
5	+	+	+	−	+	+	+	+	+	
6	+	−	−	−	−	+	+	+	+	
7	+	−	−	−	−	−	−	−	+	
8	−	+	−	−	−	−	−	−	+	
9	−	+	+	−	−	−	+	−	+	
10	+	+	+	−	−	+	+	+	+	
11	−	−	−	−	−	−	+	+	+	
12	−	−	−	−	+	−	+	−	+	
13	−	−	+	−	−	−	+	+	+	
14	−	−	−	−	+	+	+	+	+	
15	−	−	+	−	−	−	+	+	+	
16	−	−	−	−	+	−	−	−	+	
17	−	−	+	−	−	−	+	+	+	
18	+	+	−	−	−	−	−	+	+	
19	−	−	−	−	+	−	−	−	+	
20	+	+	+	+	−	−	+	+	+	
21	−	−	+	+	−	−	+	+	+	
Secondary Sjögren's syndrome										
22	+	+	+	−	+	+	+	+	+	SLE
23	+	+	+	+	+	+	+	+	+	SLE
24	−	−	−	−	−	−	+	−	+	SLE
25	+	+	+	+	+	+	−	−	+	SLE
26	+	+	+	−	+	−	+	+	+	SSc
27	−	−	+	−	−	−	+	−	+	DM

[a]IgA, ELISA measuring presence of IgA antibodies directed against α-fodrin; [b]IgG, ELISA measuring presence of IgG antibodies directed against α-fodrin; [c]findings in Hanover for the sera originally tested in Nijmegen; [d]IB, immunoblotting results (α-fodrin); [e]IP, protein immunoprecipitation results (α-fodrin); [f]Ro60, ELISA measuring presence of antibodies directed against Ro60-antigen; [g]La, ELISA measuring presence of antibodies directed against La-antigen; [h]LFS, lymphocytic focus score ≥ 1.0 in sublabial minor salivary glands biopsy. DM, dermatomyositis; Pt, patient; SSc, systemic sclerosis; SLE, systemic lupus erythematosus.

coworkers, were used. Visualization was performed by chemiluminescence.

Immunoprecipitation of radiolabeled α-fodrin

For labeling of cellular proteins, HeLa cells were incubated in medium without methionine and supplied with 2% dialyzed fetal calf serum (FCS). After 1 hour, 10 μCi [^{35}S]methionine per ml was added and the concentration of dialyzed FCS was raised to 5%. After an additional 4 hours, 1 volume of complete medium containing 10% FCS (undialyzed) was added and incubation was continued for 16 hours at 37°C. The cells were collected by

centrifugation, washed, and homogenized in lysis buffer (50 mM Tris/HCl, pH 7.5, 0.5% NP40, 100 mM KCl, 1 mM dithioerythritol, 1 mM EDTA) containing a mixture of protease inhibitors. IgG antibodies from sera to be analyzed (SjS and controls) were coupled to protein A-agarose beads (Biozym, Landgraaf, The Netherlands) and immunoprecipitations were carried out as described by Raijmakers and colleagues [17].

Hanover ELISA confirmation
Another way to confirm our ELISA results was to compare our data with those obtained with the Hanover ELISA developed by Witte and colleagues [12]. A blinded set of serum samples was therefore analyzed in Hanover.

Results
Analysis for anti-α-fodrin, anti-La, and anti-Ro60 by ELISA
Using the purified α-fodrin–GST protein encoded by the cDNA construct obtained from Dr Hayashi, we developed an ELISA (hereafter referred to as the Nijmegen ELISA) that was used for the analysis of SjS and control sera. The Nijmegen IgA ELISA test appeared to be reasonably specific. Only one serum positive for anti-α-fodrin was found among the 12 RA sera. In the 28 NHS sera and 12 sera from SLE and SSc patients, no IgA anti-α-fodrin antibodies were detected. In the IgG ELISA, however, six RA sera were positive; the other control sera (NHS, SLE, and SSc) were negative.

Of the 21 sera from primary SjS, 10 were found positive in the IgG ELISA, indicating a disease sensitivity of IgG antibodies against α-fodrin of 48%. Nine (43%) of the 21 primary SjS sera were found to contain IgA antibodies against α-fodrin (Table 1) and 3 (14%) of the 21 contained IgM antibodies against α-fodrin (not shown). Five of the nine primary SjS sera with IgA antibodies to fodrin also contained IgG antibodies directed against this antigen.

To be sure that the antibodies measured were directed against the fodrin part of the fodrin–GST fusion protein, the ELISAs were also carried out in the presence of a 10-fold excess of purified GST protein. Essentially the same results were obtained.

The same sera (blinded) were also analyzed in Hanover by Witte and coworkers using their α-fodrin ELISA [12]. The Hanover results showed a disease sensitivity of 38% for IgA and of 10% for IgG antibodies against α-fodrin. Six of 8 sera from primary SjS that were positive in the Hanover IgA ELISA were also positive in the Nijmegen ELISA, so that 6 of 21 sera from primary SjS (29%) conclusively seemed to contain IgA antibodies directed against α-fodrin. While the data for IgA-positive sera from both ELISAs were quite congruent, data for IgG-positive sera showed clear discrepancies (Table 1). We do not know why.

We also looked for the classic anti-La and anti-Ro60 autoantibodies in the 21 sera from primary SjS. Fourteen sera contained anti-Ro60 (SS-A) antibodies (sensitivity 67%) and 13 sera contained anti-La (SS-B) antibodies (sensitivity 62%). These activities were confirmed by at least one other technique (immunoblotting and/or RNA precipitation). Anti-La and anti-Ro activities were absent in all control sera that were included in this study (data not shown). There was also considerable overlap between the presence of anti-α-fodrin and anti-Ro60 or anti-La. Of the 9 IgA-positive sera from primary SjS, 6 contained anti-Ro60 and 5 contained anti-La, while of the 10 IgG-positive sera, all contained anti-Ro60 also and 7 contained anti-La antibodies (Table 1).

Four of six sera from patients with secondary SjS contained IgA and IgG antibodies against α-fodrin. Three of these sera also contained anti-Ro and anti-La antibodies (Table 1). From these results we conclude that anti-α-fodrin antibodies are present in SjS sera and that the majority of these antibodies are of the IgG and IgA class, but that their frequency in SjS sera is not higher than that of the classic autoantibodies directed against the Ro60 and La antigens.

Analysis for anti-α-fodrin antibodies by western blotting and immunoprecipitation
We also analyzed the sera for anti-α-fodrin antibodies by two other techniques (immunoblotting and protein immunoprecipitation) to confirm the ELISA results. In general, these two techniques appeared to be less suited for this purpose. The size of the antigen (250 kDa) and its limited presence in cultured cells precluded both efficient blotting and efficient labeling by [^{35}S]methionine. There were also some background problems that in some cases made it difficult to distinguish between positive and negative sera. We therefore decided to count only those sera that were clearly positive.

Of the sera from primary SjS, five appeared to be positive in immunoblotting and four precipitated a protein with the expected molecular weight of fodrin. Two of those that immunoprecipitated fodrin were also positive on immunoblot, and three also contained IgA and/or IgG antibodies to α-fodrin as measured by ELISA (Table 1).

The presence of anti-fodrin antibodies in four sera of patients with secondary SjS was confirmed by immunoblotting (four of four) and by immunoprecipitation (three of four) (Table 1).

In our hands, the use of apoptotic extracts (to increase the amount of antigenic fodrin cleavage product) did not improve the suitability of these techniques for the detection of anti-α-fodrin antibodies (data not shown).

Discussion

The presence of anti-Ro and anti-La autoantibodies has been part of the classification criteria for Sjögren's syndrome (SjS), including the recently established US/European consensus group criteria, in which they play a more significant role than before [5]. According to these classification criteria, the presence of either anti-Ro or anti-La autoantibodies or a positive salivary gland biopsy (LFS ≥1.0) is mandatory for the classification of SjS. The disease sensitivities of anti-Ro and anti-La autoantibodies have been reported to be 60–75% and 30–50%, respectively [6–9], while the specificity of particularly anti-La autoantibodies is generally considered to be reasonably high. Anti-La autoantibodies are mostly found in SjS and SLE patients, and rarely in other diseases or normal healthy subjects. Nevertheless, there is certainly a need for an SjS-specific autoantibody showing a better disease sensitivity and specificity profile.

Haneji and colleagues suggested the presence of anti-α-fodrin autoantibodies as a highly specific diagnostic marker for SjS. In their initial paper, they reported that 96% of sera from primary SjS reacted with α-fodrin [10]. In follow-up studies, however, they also noticed the presence of these autoantibodies in SLE patients [11]. Witte and collaborators showed much lower disease sensitivities, of 64% and 47% in primary and secondary Sjögren's syndrome, respectively, when focusing on IgA antibodies against α-fodrin rather than IgG antibodies [12]. Their data suggest a sensitivity similar to that of anti-Ro antibodies. However, Witte and co-workers also noticed two positive sera in RA patients without symptoms of SjS, and one positive serum in the SLE group. These results indicate that the disease specificity of anti-α-fodrin antibodies might also be lower than reported previously.

In our cohort of patients with primary SjS, the ELISA tests of 21 sera showed sensitivities of 43% and 48% for IgA antibodies and IgG antibodies against α-fodrin, respectively; these are comparable to the percentages reported by Witte and co-workers. In the blinded set of control sera analyzed in Nijmegen and Hanover, no positive tests were found in the NHS, SLE, or SSc sera, suggesting that the ELISA tests in both laboratories are specific. However, of the 12 RA sera tested in Hanover, four gave positive results in the IgA ELISA, including the one that was also positive in Nijmegen. Of the six RA sera that were positive in the IgG ELISA in Nijmegen, three were also positive in the IgG ELISA of Hanover. These data, together with the previously reported positive RA sera by Witte and co-workers, indicate that the presence of anti-fodrin antibodies in a subset of RA patients cannot be ruled out. Although in this study disease specificity was not evaluated against a large variety of control sera from other diseases, these results suggest that the disease specificity of anti-fodrin antibodies is unlikely to exceed that of anti-La

antibodies, which are almost exclusively found in either SjS or SLE sera. Elucidating the precise disease specificity of anti-fodrin antibodies, which was not the aim of this study, can be done when larger cohorts of patients are available.

It should also be noted that anti-Ro- and anti-La- antibodies themselves are part of the US/European classification criteria, which may bias comparison between the classical versus anti-fodrin antibodies by means of these criteria. To overcome this potential problem, the presence of classical versus anti-fodrin antibodies in SjS patients can also be related to findings of the single next most important objective tool: the salivary gland biopsy. Since in our study all patients that were defined as having SjS at least had to have a positive LFS, the conclusions remain the same.

This study also showed that there are discrepancies between the two anti-fodrin ELISA systems. An explanation of this imperfect reproducibility between the two laboratories might be that the titers of anti-α-fodrin antibodies in patient sera were generally low in both ELISA systems. Consequently, small changes in the protocol would become important for the outcome. These observations underline once more the importance of an easy-to-perform alternative biochemical technique to confirm ELISA data [19].

Conclusion

Based on the difficulties encountered in this study to confirm the presence of anti-fodrin antibodies via alternative techniques, it is questionable whether anti-fodrin antibodies should replace the classic anti-Ro and anti-La antibodies in the classification criteria of SjS.

All anti-Ro and anti-La activities in our sera detected by ELISA could be confirmed using alternative biochemical techniques. Besides that, the observed sensitivity of these classic autoantibodies is higher than that of anti-α-fodrin antibodies, regardless of the anti-fodrin ELISA system (Hanover versus Nijmegen ELISA) that was used. In addition, a considerable overlap between the presence of anti-Ro60/anti-La antibodies and anti-α fodrin antibodies was observed. A potential contributing role for the measurement of anti-fodrin antibodies to detect SjS patients who are negative for anti-Ro60 and anti-La autoantibodies therefore appears unlikely. Anti-fodrin antibodies may still have some diagnostic value assuming that their incidence in SLE sera is low, which has yet to be confirmed in additional studies.

Based on the lower frequency, as compared to anti-Ro and anti-La, and the questionable specificity, we conclude that testing for anti-α-fodrin antibodies does not have much additional value for the diagnosis of Sjögren's syndrome.

Competing interests

None declared.

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