

# Antinuclear antibodies in routine analysis: the relevance of putative clinical associations

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## Abstract

**Defined antinuclear antibodies (ANA), such as antibodies to Ro/SS-A, La/SS-B, Sm, and nRNP, are often present in serum samples from patients with systemic lupus erythematosus (SLE) or other connective tissue diseases (CTD). Most data on associations between the presence of these antibodies and defined disease features have been obtained with the use of predefined groups of patients. In this work the issue of disease associations was approached from a different angle: patients suspected of having CTD were selected on the presence of these ANA in their serum samples and clinical data were subsequently scored according to a defined protocol. It was then tried to relate measured ANA and clinical symptoms.**

**No correlation was observed between the presence of antibodies to Ro/SS-A and specific clinical symptoms. The presence of antibodies to La/SS-B was associated with the diagnosis of Sjögren's syndrome combined with leucocytopenia. In patients positive for antibodies to Sm a significantly increased incidence of skin lesions, such as butterfly rashes and discoid lesions, was seen, together with signs of myocarditis. Myocarditis was also found to be associated with the presence of antibodies to nRNP.**

**The data presented in this study show that previously reported associations of these ANA with clinical symptoms are not confirmed when unselected patients are used.**

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For a long time systemic lupus erythematosus (SLE) has been considered to be an autoimmune disease, based on the presence of different kinds of antinuclear antibodies (ANA) in serum samples from patients. Antibodies to double stranded DNA (dsDNA) and Sm are fairly specific for SLE, but other specificities of ANA also occur in other connective tissue diseases (CTD).<sup>1</sup> More or less convincing associations have been described between the presence of antibodies to La/SS-B and Sjögren's syndrome, and antibodies to Ro/SS-A with neonatal lupus and subacute cutaneous lupus. Data on prevalence and prognostic significance are often controversial, however, probably based on the differences in methodology<sup>2</sup> used to measure the ANA and differences in the ethnic composition of the groups of patients studied. Whether ANA actually mediate disease phenomena in CTD is not yet clear, though current data

support a role of antibodies to DNA in lupus nephritis and of antibodies to Ro/SS-A in neonatal lupus, congenital heart block, or Sjögren's syndrome.<sup>3-6</sup>

Possible correlations between the presence of different ANA and disease symptoms can be studied from several premises. Most studies investigated the presence of ANA in groups of patients with defined disorders. A second approach is to study the relation between one particular disease manifestation and one specific antinuclear antibody in a defined group of patients. A third would be an epidemiological survey to see whether specific disease symptoms can be observed in groups of patients selected on the basis of one particular antibody present in the serum sample. The latter approach was adopted in this work.

## Patients and methods

### PATIENTS

From the serum samples sent to our department for routine determination of ANA during 1988 and 1989 we selected those that (a) were sent in for the first time and (b) were positive with an immunofluorescent screening test on HEp2 cells. In this way we hoped to avoid a bias in patient selection and to select patients with a short disease duration. Although we thought we would obtain, for the greater part, patients with an undefined disease, it was observed that in nearly 50% of the patients a clinical diagnosis had already been made before the serum sample was sent to us for determination of ANA. Patients were obtained from all major hospitals of north and south Holland, with the exclusion of academic hospitals, which perform their own serology of ANA. As our institute is well known for its research in SLE, there is no negative selection of patients because of nearby reference laboratories in this part of the country.

The doctors of the 339 patients selected this way were asked to complete a questionnaire containing questions related to a total of 34 defined clinical symptoms. Special attention was paid to the following clinical signs related to SLE, rheumatoid arthritis (RA), progressive systemic sclerosis, and Sjögren's syndrome. Systemic lupus erythematosus: butterfly rash, discoid lesions, photosensitivity, alopecia, mouth ulcerations, Raynaud's phenomenon, pleuritis, pericarditis, myocarditis, central nervous system disease, neuritis, psychosis, anaemia (haemoglobin 112.7 g/l), type of anaemia, lymphocytopenia ( $<1.5 \times 10^9/l$ ), leucocytopenia ( $<4 \times 10^9/l$ ), thrombocytopenia ( $<100 \times 10^9/l$ ), persistent proteinuria ( $>3.5$  g/l),

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casts, and decrease of more than 20% in the glomerular filtration rate.<sup>7</sup> Rheumatoid arthritis: morning stiffness (>15 minutes, specific joint pain caused by motion or palpation, synovitis (mono-, oligo-, or polyarticular; asymmetrical or symmetrical), subcutaneous noduli, erosions.<sup>8</sup> Progressive systemic sclerosis: sclerosis, myositis, oesophageal motility disturbances.<sup>9</sup> Sjögren's syndrome: xerostomia, keratoconjunctivitis.

Symptoms present at the time of investigation and symptoms present in the past were scored. Data obtained through these questionnaires were double checked by a careful analysis of the clinical charts by one of us (AJGS). In this way, reliable data could be obtained on 266 patients.

Every item included in the questionnaire was defined according to the dictionary of the rheumatic diseases prepared by the glossary committee of the American College of Rheumatology. Otherwise, definitions were based on the most commonly used textbooks of internal medicine or rheumatology (e.g. Harrison *et al*, Cecil *et al*, Copeman *et al*).

SCREENING OF ANTINUCLEAR ANTIBODIES

The presence of ANA in serum was determined using a standard immunofluorescence test. Briefly, spots of HEp2 cells, cultured in monolayers on slides, were incubated for 30 minutes at room temperature with serum samples diluted 1:40 in phosphate buffered saline (PBS: 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4). After being washed (three times for five minutes each) with PBS, the slides were incubated for

30 minutes with a mixture of FITC conjugated mouse monoclonal antihuman IgG (known to recognise all IgG subclasses) and polyclonal rabbit antihuman IgM (MH-16-F and KH-15-F, prepared in this institute), both diluted 1:250 in PBS (known to recognise all IgG subclasses). The slides were washed with PBS again (three times for five minutes each), counterstained with 0.01% Evans blue, and read using a fluorescence microscope. Serum samples giving a positive fluorescence in a 1:10 dilution are considered to contain ANA.

ANTIBODIES AGAINST RO/SS-A, LA/SS-B, nRNP, AND SM

Precipitating antibodies against Ro/SS-A, La/SS-B, nRNP, and Sm were detected by counter immunoelectrophoresis as described by Kurata and Tan,<sup>10</sup> using an extract of rabbit thymus powder (Pel Freez, Arkansas, USA) or human spleen as a source of antigen.

Briefly, 1.5 g of rabbit thymus powder was mixed for four hours with 25 ml PBS at 4°C. The mixture was centrifuged once, aliquoted, and frozen at -70°C. Such preparations contain about 10 mg/ml of protein.

Human spleen extract was made by homogenisation of spleen tissue obtained as necropsy. The homogenate was centrifuged and the supernatant applied to a DEAE-cellulose column (DE preswollen; Whatman Inc, Clifton, NY, USA). A 60-80% ammonium sulphate fraction made from the DEAE eluate was dissolved in distilled water and dialysed against PBS. For the specific determination of antibodies to Ro/SS-A, the purified human spleen extract was treated with trypsin to destroy La/SS-B activity.

Glass plates were covered with 1% agarose gel; holes, 4 mm in diameter, were punched 5 mm apart and filled with antigen and serum respectively. Electrophoresis was carried out at 120 V, 20 mA per plate for about 45 minutes. After extensive washing, first with PBS and then with distilled water, the plates were dried under pressure at 80°C. Precipitation lines were stained with a solution of 1% Coomassie brilliant blue in F solution (H<sub>2</sub>O:ethanol:acetic acid 9:9:2). Plates were read and an observed reactivity was classified as either antibodies to Ro/SS-A, La/SS-B, nRNP, or Sm by comparison

Table 1 Correlation between the presence of antibodies to Ro/SS-A in serum samples and defined clinical symptoms

| Clinical symptoms      | No of patients with this symptom          |                                            | Ratio (%A/%B)* |
|------------------------|-------------------------------------------|--------------------------------------------|----------------|
|                        | Positive for antibodies to Ro/SS-A (n=51) | Negative for antibodies to Ro/SS-A (n=215) |                |
| Photosensitivity       | 16                                        | 53                                         | 1.24           |
| Alopecia               | 12                                        | 33                                         | 1.6            |
| Sjögren's syndrome     | 5                                         | 16                                         | 1.43           |
| Mouth ulcers           | 8                                         | 17                                         | 1.96           |
| Myositis               | 15                                        | 85                                         | 0.74           |
| Pericarditis           | 6                                         | 16                                         | 1.71           |
| Polyneuropathy         | 5                                         | 13                                         | 1.67           |
| Oesophagus dysfunction | 2                                         | 12                                         | 0.67           |

\*%A=percentage of patients positive for antibodies to Ro/SS-A; %B=percentage of patients negative for antibodies to Ro/SS-A.

Table 2 Number of patients with a defined clinical symptom in relation to the presence of specific antinuclear antibodies (ANA) in their serum samples

| Clinical symptom                    | No (%)* of the 266 patients positive for ANA |                    |                    |                  |                    |
|-------------------------------------|----------------------------------------------|--------------------|--------------------|------------------|--------------------|
|                                     | Total antibodies                             | Antibodies to SS-A | Antibodies to SS-B | Antibodies to Sm | Antibodies to nRNP |
| Butterfly rash                      | 46 (17)                                      | 10 (22)            | 4 (9)              | 6 (13)           | 6 (11)             |
| Discoid lupus                       | 26 (10)                                      | 5 (19)             | 2 (8)              | 4 (15)           | 2 (8)              |
| Photosensitivity                    | 69 (26)                                      | 16 (23)            | 7 (10)             | 7 (10)           | 7 (10)             |
| Alopecia                            | 45 (17)                                      | 12 (27)            | 2 (4)              | 2 (4)            | 3 (7)              |
| Mouth ulcers                        | 25 (9)                                       | 8 (32)             | 2 (8)              | 2 (8)            | 3 (12)             |
| Sjögren's syndrome                  | 21 (8)                                       | 5 (24)             | 5 (24)             | 1 (5)            | 1 (5)              |
| Symmetrical polyarthritis           | 73 (27)                                      | 17 (23)            | 8 (11)             | 5 (7)            | 7 (10)             |
| Pleuritis                           | 45 (17)                                      | 13 (29)            | 3 (7)              | 4 (9)            | 8 (18)             |
| Pericarditis                        | 22 (8)                                       | 6 (27)             | 1 (5)              | 1 (5)            | 3 (14)             |
| Myocarditis                         | 19 (7)                                       | 5 (26)             | 1 (5)              | 3 (16)           | 6 (32)             |
| Persistent proteinuria (>0.5 g/day) | 37 (14)                                      | 8 (22)             | 3 (8)              | 4 (11)           | 2 (5)              |
| Cellular casts                      | 15 (6)                                       | 5 (33)             | 1 (7)              | 1 (7)            | 0 (0)              |
| Decrease in creatinine clearance    | 24 (9)                                       | 4 (17)             | 0 (0)              | 1 (4)            | 1 (4)              |

\*Percentages relate to the clinical symptoms—for example, of the 46 patients with a butterfly rash, 10 (22%) had antibodies to Ro/SS-A in their serum sample.

with reference serum samples (Arthritis Foundation, CDC, Atlanta, GA, USA).<sup>10 11</sup>

#### STATISTICS

Statistical analysis of the data was performed using Student's *t* test for mean values and with  $\chi^2$  analysis (with Yates's correction) for absolute numbers;  $p < 0.05$  was considered significant.

Table 3 Number of patients fulfilling more than three American Rheumatism Association (ARA) criteria for SLE

| Patients with antibodies to | No (%) of patients fulfilling more than three ARA criteria |
|-----------------------------|------------------------------------------------------------|
| Ro/SS-A (n=51)              | 27 (52)                                                    |
| La/SS-B (n=19)              | 6 (31)                                                     |
| Sm (n=16)                   | 9 (56)                                                     |
| nRNP (n=29)                 | 14 (48)                                                    |

Table 4 Correlation between the presence of antibodies to La/SS-B in serum samples and defined clinical symptoms

| Clinical symptoms                       | No of patients with this symptom   |                                    | Ratio (%A/%B)* |
|-----------------------------------------|------------------------------------|------------------------------------|----------------|
|                                         | Positive for antibodies to La/SS-B | Negative for antibodies to La/SS-B |                |
| Photosensitivity                        | 7                                  | 62                                 | 1.48           |
| Alopecia                                | 2                                  | 43                                 | 0.65           |
| Sjögren's syndrome                      | 5                                  | 16                                 | 4.33†          |
| Myositis                                | 10                                 | 90                                 | 1.47           |
| Pericarditis                            | 1                                  | 21                                 | 0.56           |
| Leucocytopenia ( $<4.0 \times 10^9/l$ ) | 6                                  | 31                                 | 2.46‡          |

\*%A=percentage of patients positive for antibodies to La/SS-B; %B=percentage of patients negative for antibodies to La/SS-B.

† $p < 0.008$ .

‡ $p < 0.03$ .

Table 5 Correlation between the presence of antibodies to Sm in serum samples and defined clinical symptoms

| Clinical symptoms                      | No of patients with this symptom |                               | Ratio (%A/%B)* |
|----------------------------------------|----------------------------------|-------------------------------|----------------|
|                                        | Positive for antibodies to Sm    | Negative for antibodies to Sm |                |
| Butterfly rash                         | 6                                | 40                            | 2.38†          |
| Discoid lupus                          | 4                                | 22                            | 2.80‡          |
| Alopecia                               | 2                                | 43                            | 0.77           |
| Raynaud's phenomenon                   | 7                                | 64                            | 1.69           |
| Symmetrical polyarthritis              | 5                                | 68                            | 1.19           |
| Sjögren's syndrome                     | 1                                | 20                            | 0.88           |
| Leucopenia ( $<4.0 \times 10^9/l$ )    | 4                                | 34                            | 1.79           |
| Persistent proteinuria ( $>0.5$ g/day) | 4                                | 33                            | 1.92           |
| Pleuritis                              | 4                                | 41                            | 1.56           |
| Myocarditis                            | 3                                | 16                            | 3.17§          |

\*%A=percentage of patients positive for antibodies to Sm; %B=percentage of patients negative for antibodies to Sm.

† $p < 0.05$ .

‡ $p < 0.01$ .

§ $p < 0.005$ .

Table 6 Correlation between the presence of antibodies to nRNP in serum samples and defined clinical symptoms

| Clinical symptoms                      | No of patients with this symptom |                                 | Ratio (%A/%B)* |
|----------------------------------------|----------------------------------|---------------------------------|----------------|
|                                        | Positive for antibodies to nRNP  | Negative for antibodies to nRNP |                |
| Myositis                               | 14                               | 86                              | 1.38           |
| Pleuritis                              | 8                                | 37                              | 1.81           |
| Pericarditis                           | 3                                | 19                              | 1.25           |
| Myocarditis                            | 6                                | 13                              | 4.00†          |
| Raynaud's phenomenon                   | 9                                | 62                              | 1.19           |
| Symmetrical polyarthritis              | 7                                | 66                              | 0.83           |
| Leucopenia ( $<4.0 \times 10^9/l$ )    | 6                                | 32                              | 1.43           |
| Persistent proteinuria ( $>0.5$ g/day) | 2                                | 35                              | 0.43           |
| Sjögren's syndrome                     | 1                                | 20                              | 0.37           |

\*%A=percentage of patients positive for antibodies to nRNP; %B=percentage of patients negative for antibodies to nRNP.

† $p < 0.05$ .

## Results

### CLINICAL CORRELATIONS OF ANTIBODIES

#### TO Ro/SS-A

Antibodies to Ro/SS-A were detected in 51 of the 266 patients positive for ANA. We found no significant correlation between the presence of antibodies to Ro/SS-A and particular defined clinical symptoms in these patients (table 1). There was only a slight increase in the occurrence of mouth or nasopharyngeal ulcerations. In contrast with this, we observed that patients positive for antibodies to Ro/SS-A in general showed, together with the patients positive for antibodies to Sm, the highest number of SLE related clinical symptoms per patient (table 2). In total, patients fulfilled a mean of 2.5 American Rheumatism Association criteria; looking at the subgroups positive for antibodies to Ro/SS-A, La/SS-B, Sm, and nRNP, the number of criteria were 2.9, 2.6, 3.1, and 2.3, respectively. There was hardly any difference in the frequency with which the diagnosis of SLE was made in the different subgroups, however (table 3).

### CLINICAL CORRELATIONS OF ANTIBODIES

#### TO La/SS-B

Antibodies to La/SS-B were only detected in 19 of the 266 patients positive for ANA. Looking for possible clinical correlations in these patients we calculated a strong association with the presence of Sjögren's syndrome. Leucocytopenia was also seen more often (table 4).

### CLINICAL CORRELATIONS OF ANTIBODIES TO Sm

Only in 16 of the 266 patients positive for ANA were antibodies to Sm found. Butterfly rash, discoid lupus skin lesions, and myocarditis were significantly more common in these patients (table 5) and a slight increase in the incidence of proteinuria was seen. No (further) data sustaining a correlation of antibodies to Sm with renal or central nervous system disease were obtained.

### CLINICAL CORRELATIONS OF ANTIBODIES

#### TO nRNP

Antibodies to nRNP were detected in 29 of the 266 patients positive for ANA. The most striking correlation found within this group of patients was with myocarditis (table 6). There was no increased frequency of myositis, pleuritis, or pericarditis in patients positive for antibodies to nRNP.

## Discussion

Studies of the clinical significance of antibodies to extractable nuclear antigens (antibodies to Ro/SS-A, La/SS-B, nRNP, and Sm) have usually been performed using serum samples from patients with well defined disease patterns. Such studies have shown some interesting correlations—for example, between the occurrence of antibodies to Sm and SLE, antibodies to La/SS-B and Sjögren's syndrome, and antibodies to Ro/SS-A and neonatal lupus and subacute cutaneous lupus (reviewed by



Tan<sup>11</sup>). Discrepancies have also been obtained and these are generally attributed to the use of different assay systems for the detection of the various ANA, or to the selection of the patients studied.

In the current study, we tried to use detection of the four main specificities of ANA in serum samples sent for the first time to our institute for routine screening of ANA. In this way, no selection regarding clinical syndrome or disease entity is made, yet the detection of autoantibodies is used in the way that it is generally used in a routine setting—that is, without previous knowledge of the disease status of the patient. The method we used for the detection of the specificities of ANA was counterimmunoelectrophoresis, which is still one of the most reliable assays for this purpose, as has been shown by the European Consensus Finding Study Group for detection of ANA (unpublished data). Using this approach and technology we could not confirm many of the relationships published previously.

Antibodies to Ro/SS-A have been described in association with primary Sjögren's syndrome, subacute lupus erythematosus, neonatal lupus erythematosus, lupus erythematosus negative for ANA, and photosensitive rashes.<sup>3,4</sup> The putative relation between the presence of antibodies to Ro/SS-A and subacute lupus erythematosus has further been substantiated by case reports of antibodies to Ro/SS-A positive patients who had had Sjögren's syndrome for a prolonged period of time and then suddenly developed the cutaneous lesions of subacute lupus erythematosus.<sup>4</sup> In our study, however, in which we started with undefined serum samples, we observed no clear correlation with one or more defined clinical symptoms. We paid special attention to all kinds of skin lesions described in CTD, such as butterfly rash, photosensitive rashes, discoid lesions of the scarring or non-scarring type, alopecia, and scleroderma. Overall, these results sustain the observation that in well defined SLE, no correlations with defined clinical symptoms could be observed.<sup>12,13</sup>

We did not study the relevance of differences in titre, which may have been of influence in our results. It has been reported that in SLE there are in fact two subgroups positive for antibodies to Ro/SS-A which differ quantitatively, clinically, and genetically.<sup>14</sup> From our data, it may be concluded that doubts can be raised about the use of detection of antibodies to Ro/SS-A as a diagnostic criterion for Sjögren's syndrome.<sup>15</sup> In our study, we found a relatively low incidence of antibodies to La/SS-B among the serum samples positive for ANA. The correlation of antibodies to La/SS-B with Sjögren's syndrome was confirmed by this study, however.

Antibodies to Sm are generally considered to be specific for SLE, yet they occur in only about 20% of patients. Discrepancies obtained in the various studies can easily be attributed to (a) racial differences between the groups of patients studied, (b) the selection of patients, and (c) the method used for the detection of antibodies to Sm. Within a well defined population of patients

with SLE a positive relation with the occurrence of nephritis<sup>16</sup> and pleuropericarditis<sup>17</sup> next to a negative correlation with the presence of central nervous system disease<sup>18</sup> has been described. These correlations could not be confirmed by others, however.<sup>19,20</sup> In a previous study, we found that antibodies to Sm did not correlate significantly with any particular manifestation of SLE.<sup>2</sup> In the present study, we observed an increased incidence of skin lesions such as butterfly rashes and discoid lesions associated with antibodies to Sm. This seems to agree with data presented in an abstract by Powers *et al.*,<sup>21</sup> who described 16 patients with antibodies to Sm as the only autoantibody characterised by these forms of skin lesions.

Antibodies to Sm are often seen in association with antibodies to nRNP. In the 19 serum samples positive for antibodies to Sm, antibodies to nRNP could be detected in 13 patients. These are, however, 16 of the serum samples positive for antibodies to nRNP that do not contain antibodies to Sm.

With respect to antibodies to nRNP, an increased incidence of Raynaud's phenomenon has been reported in patients with SLE containing this specificity for ANA in their serum samples.<sup>22-24</sup> Conflicting data have been published about the incidence of other clinical symptoms such as renal disease, central nervous system disease, and myositis in association with antibodies to nRNP. It has been reported that most patients with antibodies to nRNP in their serum samples will satisfy the diagnosis of SLE,<sup>25</sup> but no prognostic differences were found between patients positive and patients negative for antibodies to nRNP.<sup>22</sup> In this study, the most striking correlation between the presence of antibodies to nRNP and a clinical feature was found for myocarditis.

In conclusion, the clinical relevance of a particular antinuclear antibody can be studied from different starting premises. In most studies, one of the following approaches is applied: (a) patients are selected on the basis of a clinical diagnosis and the incidence of different symptoms is compared with the autoantibodies measured in the serum samples of these patients; or (b) starting from a particular defined clinical symptom, frequencies of autoantibodies in the various patient groups are studied. Using these approaches, several correlations have been obtained between autoantibodies and disease spectra. In our study, we used a third approach, by selecting the patients randomly only on the basis of the presence in serum samples of one of four (antibodies to Ro/SS-A, La/SS-B, Sm, and nRNP) antinuclear antibody specificities. Afterwards, we studied the occurrence of clinical symptoms in the four groups of patients thus obtained. In this way, many of the formerly obtained correlations could not be confirmed. It should be noted that the results can be influenced by the disease duration of the various patients. In patients with SLE the amount of disease symptoms present depends on the disease duration. In this study, however, the start point was having one particular antinuclear antibody and the diagnostic significance was evaluated afterwards. All results must be regarded from the

laboratory point of view, when at routine examination a serum sample is positive for one specific antinuclear antibody in this way. Correlations were found between the presence in serum samples of antibodies to Sm and the occurrence of butterfly rashes, discoid lesions, and myocarditis, between antibodies to nRNP and myocarditis, and antibodies to La/SS-B and Sjögren's syndrome.

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