

## Autoantibodies in SLE but not in scleroderma react with protein-stripped nucleosomes

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### Abstract

Autoantibodies against nucleosomes (ANuA) are known to be sensitive markers for systemic lupus erythematosus (SLE), but their clinical relevance seemed to be limited because sera from patients with progressive systemic sclerosis (PSS) also showed positive reactions with conventional ANuA ELISA test systems (anti-Nu1 ELISA). It was generally assumed that ANuA were associated with both diseases. Using discontinuous sucrose gradient centrifugation to generate pure nucleosomes, we discovered by chance that at the 30–50% sucrose interface an antigen (Nu2) banded which was demonstrably free of non-histone components and histone H1. The two different nucleosome preparations, Nu1 and Nu2, were used in parallel as antigenic substrates in standardised ELISA tests to analyse sera from SLE (295 patients), PSS (119) and patients with other rheumatic diseases (101). With Nu1, 62% of the SLE and 52% of the PSS sera showed positive reactions. Two sera from patients suffering from Sjögren's syndrome (SS) and one from polymyositis were also positive. Using the Nu2 preparation, 58% of the SLE but none of the PSS sera showed a positive reaction. One serum from a patient with SS was also positive. It could be shown that it was the PSS-specific autoantigen Scl-70 in the nucleosome preparation (Nu1) which contributed to the positive reactions of the PSS sera in conventional ANuA test systems, whereas in the Nu2 preparation no remaining Scl-70 was detectable. The present study definitely proved that ANuA are highly and specifically associated with SLE but not with PSS.

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### 1. Introduction

Systemic lupus erythematosus (SLE) is characterised by the presence of a multitude of different autoantibodies in the patients' sera directed against components of the cell nucleus, cytoplasm, cell membranes and other autoantigens. Antibodies against nucleosomes (ANuA) are among antinuclear antibodies (ANA) found in SLE patients, along with specific antibodies against double-stranded DNA (dsDNA), Sm and others.

The term "nucleosome" defines a unit of chromatin which consists of 146 base pairs of DNA wrapped around a protein core. The protein core is an octamer consisting of two molecules of each of the histones H2A,

H2B, H3 and H4 [1]. Neighbouring nucleosomes are joined together by linker DNA, which is associated with histone H1 located outside the nucleosome core.

Nucleosomes were suspected to play a major role in the induction of anti-dsDNA autoantibodies in SLE [2,3]. Anti-dsDNA and anti-histone antibodies belong to the nucleosome family just as anti-nucleosome-specific antibodies do, because nucleosomes share several common epitopes with dsDNA and histones [4]. Nucleosome-specific antibodies do not react with the individual components of nucleosomes, i.e. dsDNA and histones, as demonstrated using monoclonal antibodies from lupus mice and patients [4]. They recognise conformational epitopes resulting from interactions between DNA and histones, rather than linear epitopes [5]. Nucleosome-specific antibodies are directed predominantly against epitopes in the (H2A–H2B)–DNA

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complex and to a lesser extent against epitopes in the (H3–H4)<sub>2</sub>–DNA complex.

Although nucleosomes are among the most important autoantigens in SLE and the prevalence of ANuA in sera from SLE patients is high [6], the diagnostic use of this parameter has until now been greatly limited, since with conventional ANuA test systems many patients with progressive systemic sclerosis (PSS) also demonstrated significant positive reactions. It was even misleadingly assumed that ANuA were associated with both diseases.

PSS patients, like SLE patients, exhibit a number of anti-nuclear antibodies, of which antibodies against various DNA-associated proteins, for example centromere proteins and Scl-70, are characteristic for PSS [7]. Anti-centromere antibodies are pathognomonic markers for the limited form (CREST syndrome) of PSS while antibodies against Scl-70 occur in up to 75% of cases of PSS and are associated with the diffuse, proximal form of PSS [8,9].

Several procedures for the preparation of nucleosomes have been described, in which all types of eukaryotic cells have served as starting material [10]. Most commonly used was calf thymus because it yielded large quantities of mononucleosomes [11]. Frequently, the nuclei were digested by nuclease S7, which caused internucleosomal cleavage. The nuclease digestion was the key step for successful mononucleosome preparation. A less effective digestion often produced trace amounts of dinucleosomes, and over-digestion often yielded submononucleosome particles. Our first aim was to optimise the nuclease concentration and cleavage conditions to obtain higher yields of mononucleosomes with no remaining oligonucleosomes. This process was monitored by sucrose gradient centrifugation, which separated the mononucleosomes from the oligo- and polynucleosomes. One of the best-established methods to separate mononucleosomes from oligomers is sucrose gradient centrifugation with a linear gradient from 5 to 28% sucrose [12,13].

We characterised the single fractions of the nuclease digestion biochemically. For purification of the resulting products we used sucrose gradient centrifugation, but expanded the gradient from the conventionally used 28% up to 50% sucrose.

Furthermore, the resulting fractions were used as antigenic substrates in ELISA techniques and tested with sera from 515 patients with different rheumatic diseases, such as SLE, PSS, Sjögren's syndrome (SS) and polymyositis (PM), as well as sera from 204 healthy blood donors. We discovered that one of these fractions reacted only with SLE sera but not with scleroderma sera. In this study, this fraction was characterised and its value for the specific diagnostics of SLE was evaluated.

## 2. Patients and methods

### 2.1. Patients and sera

Sera used in this study were obtained from patients with clinically characterised diagnoses of systemic lupus erythematosus, progressive systemic sclerosis (the severe proximal form of sclerosis), Sjögren's syndrome and polymyositis and were obtained from the following centres: Rheumatology Clinic, Bad Bramstedt, Germany; University of Ankara, Turkey; German SLE study group, Erlangen, Germany; Rheumatology Clinic, Aachen, Germany; Warsaw School of Medicine, Warsaw, Poland; Institute of Molecular Genetics, Academy of Science of the Czech Republic, Prague, Czech Republic.

### 2.2. Preparation of 1st generation nucleosomes (Nu1)

The nucleosomes were prepared from calf thymus according to the procedure described previously [11]: 150 ml of 0.9% (w/v) NaCl solution were added to 20 g calf thymus. The material was mixed, subsequently homogenised and filtrated. The filtrate was then centrifuged (15 min, 1100 × g, 4 °C) and the nuclei containing pellet was washed three times with 0.9% (w/v) NaCl solution. The nuclei were purified according to the method of Subirana [14]. The purified nuclei were resuspended in digestion buffer (15 mM Tris/HCl, pH 7.5, 15 mM NaCl, 60 mM KNO<sub>3</sub>, 0.25 M Sucrose, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM DTT), and 750 units micrococcal nuclease (S7, Roche) were added. After 30 min incubation at 37 °C the suspension was centrifuged (15 min, 3500 × g, 4 °C) and the pellet resuspended in 10 ml 2 mM EDTA-solution and incubated for 45 min at room temperature on a rocking shaker. The suspension was centrifuged (15 min, 3500 × g, 20 °C) and the resultant supernatant used as Nu1.

### 2.3. Preparation of 2nd generation nucleosomes (Nu2)

The supernatant Nu1 was dialysed overnight against digestion buffer. The dialysate was chilled to 0 °C and adjusted to 0.55 M NaCl. To this solution 150 units of nuclease S7 per ml dialysate were added and incubated for 30 min at 37 °C. Subsequently the solution was again chilled to 0 °C and the resulting mononucleosomes were purified by discontinuous sucrose gradient centrifugation with 10, 30 and 50% (w/v) sucrose in TE buffer (10 mM Tris/HCl pH 7.4, 1 mM EDTA, 0.55 M NaCl, 22 h, 142,000 × g, 4 °C, fixed angle rotor 45 Ti, Beckman). Fractions were collected from the top in seven equal aliquots. The nucleosomes were found at the 30–50% (w/v) sucrose interface and termed 2nd generation nucleosomes (Nu2).

#### 2.4. SDS-PAGE and immunoblot analysis

The purity of the nucleosome preparations Nu1 and Nu2 was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 4–12% Bis-Tris (BT) NuPAGE with MOPS buffer as running buffer, 10% Bis-Tris NuPAGE gel with MES buffer, Invitrogen) followed by silver staining [15]. Purified Histones H2A, H2B, H3 and H4 (Roche) served as controls. The presence of H1 and Scl-70 was verified by immunoblotting using anti-H1 and anti-Scl-70-positive human sera. Purified H1 (Roche) and Scl-70 antigen (Fermentas) served as controls.

#### 2.5. Agarose gel electrophoresis

Nucleosomal DNA was extracted from the nucleosome preparations using the Wizard DNA Cleanup Kit (Promega, USA) and analysed together with native purified nucleosomes on a 2% agarose gel (Tris-acetate-EDTA, containing 1 µg/ml ethidium bromide for visualisation in UV light).

#### 2.6. ELISA

Microtiter plates (Nunc, Denmark) were coated with the two different nucleosome preparations Nu1 and Nu2 (2.5 µg/ml in PBS, pH 7.5) overnight at 4 °C, washed with PBS–0.05% (w/v) Tween-20 and blocked for 2 h with PBS–0.1% (w/v) casein. After washing, sera diluted 1:200 in PBS–0.1% (w/v) casein were added and allowed to react for 30 min at room temperature. Bound antibodies were detected using anti-human IgG peroxidase conjugate (EUROIMMUN, Germany) and stained with tetramethylbenzidine (EUROIMMUN, Germany). The OD was read at 450 nm using an automated spectrophotometer (Spectra Mini, Tecan, Germany). Antibodies against dsDNA, histones and Scl-70 were determined using commercial ELISAs (EUROIMMUN, Germany).

#### 2.7. Inhibition experiments

Inhibition experiments were performed to investigate the binding of PSS sera to Nu1 and Nu2 and to test the cross-reactivity of antibodies to Scl-70 and to Nu1. 4 SLE and 4 PSS sera were preincubated at different concentrations for 0.5 h at room temperature with Nu2 and subsequently the mixture incubated in the anti-Nu1 ELISA. Also, 4 PSS sera which were positive for Nu1 were preincubated in the same way with Nu1 and Nu2. Incubation of the anti-Scl-70 ELISA (EUROIMMUN) was performed according to the manufacturer's instructions.

#### 2.8. Statistical analysis

The data were analysed and the ROC curves established using the software program Analyse-it (Analyse-it

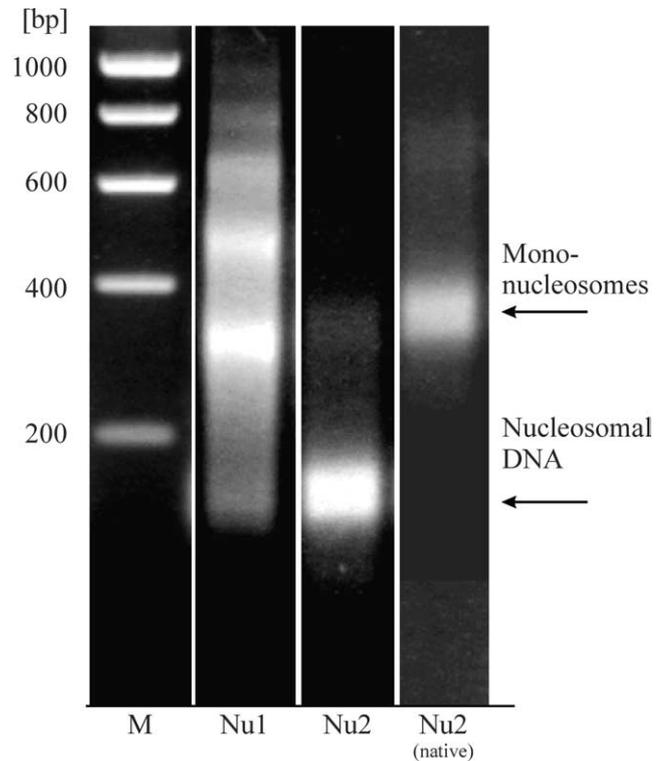


Fig. 1. Migration behaviour of nucleosomal DNA in 2% agarose gel electrophoresis. Nu1: DNA extracted from 1st generation nucleosomes migrated in a range between 150 and 800 bp which corresponds to DNA of mono- and oligonucleosomes. Nu2: DNA extracted from 2nd generation nucleosomes migrated as a single DNA band around 150 bp which corresponds to mononucleosomal DNA. Nu2 (native): native 2nd generation nucleosomes (DNA and histones) migrated as one single band with a mass equivalent to 350 bp. The quantitative mobility shift indicated that the DNA in Nu2 was completely bound in mononucleosomes. M represents the DNA markers.

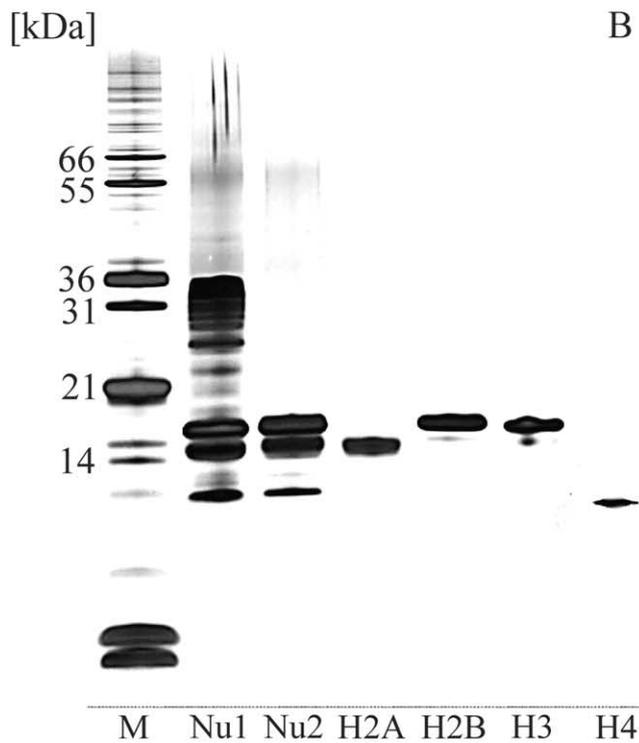
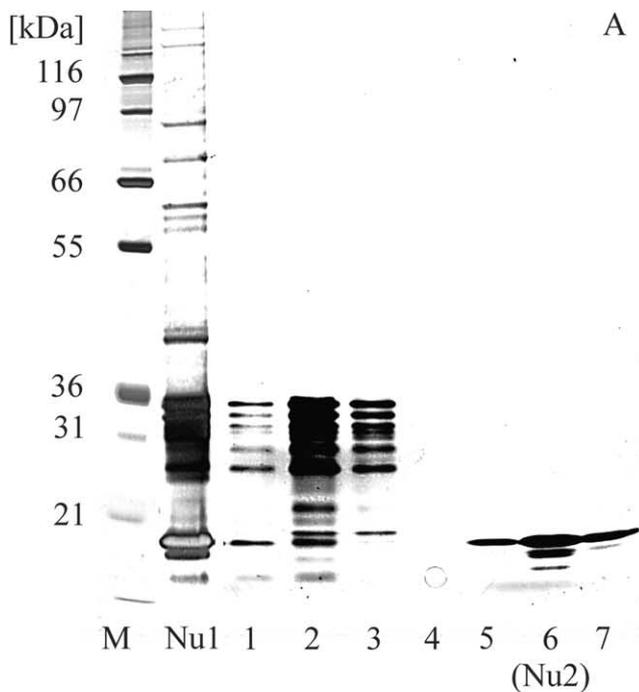
Software, Ltd). Sensitivity, specificity and positive predictive value were calculated with 295 clinically characterised SLE sera (positive), 220 patients with other rheumatic diseases (negative) and 204 blood donors (negative) using the following cut off values: Nu1 17 RU/ml, Nu2 19 RU/ml.

### 3. Results

#### 3.1. Comparative characterisation of 1st (Nu1) and 2nd (Nu2) generation nucleosomes

A conventional preparation of nucleosomes (Nu1) was produced as described in “Patients and Methods”. An aliquot of this Nu1 was subjected to dialysis, nuclease S7 digestion, high ionic strength conditions and sucrose gradient centrifugation. The fraction obtained from the 30–50% sucrose interface (fraction 6) contained the nucleosomes and was referred to as Nu2.

DNA from Nu1 and Nu2 was isolated and characterised by DNA-agarose gel electrophoresis. In the case of



Nu1, large bands, which were assigned to DNA of oligonucleosomal fragments, were observed blurred over a range from 150 to 800 bp (Fig. 1). Regarding the Nu2 preparation, only one strong band at about 150 bp was detectable: this position was assigned to nucleosomal DNA which is known to have a length of 146 bp [1].

Additionally, Nu2 was subjected to agarose gel electrophoresis without prior separation of the proteins from the DNA. A band with a mass equivalent to 350 bp was observed, which corresponded to native, histone-containing mononucleosomes [16].

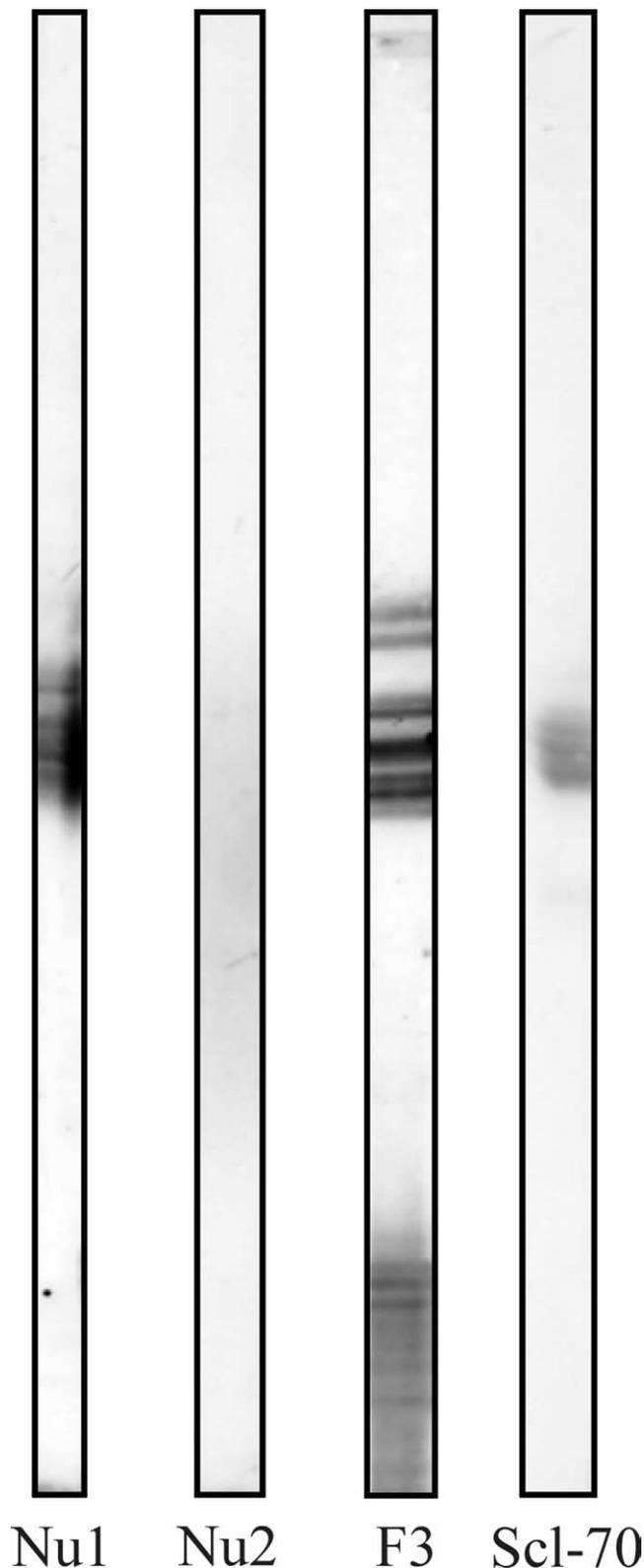
Nu1 and the fractions of the sucrose gradient centrifugation were further characterised by SDS-PAGE (Fig. 2A and B). Nu1 clearly showed a contamination with non-nucleosomal proteins (Fig. 2A). Fraction 6 (Nu2) containing the mononucleosomes, and fractions 5 and 7 exhibited exclusively bands in the region around 14 kDa, which could be attributed to the bands of the nucleosome core histones H2A, H2B, H3 and H4 as verified using commercial histone preparations analysed in parallel (Fig. 2A and B). The impurities of non-nucleosomal components were detectable in fractions 1 to 3.

Histone H1, which is located outside of the nucleosome core, was present in the Nu1 preparation, but not in Nu2, which could be proven by western blot analysis (data not shown).

### 3.2. Western blot analysis: presence of Scl-70 in the nucleosome preparation Nu1

Serum antibodies against Scl-70 are specific markers for the diagnosis of progressive systemic sclerosis (PSS). Nu1, Nu2 and fraction 3 from the sucrose gradient centrifugation as well as commercial Scl-70 antigen were subjected to SDS-PAGE with subsequent blotting of the proteins on to a nitrocellulose membrane. As analysed with an Scl-70 antibody positive PSS serum, the immunoblot of the Scl-70 preparation exhibited bands from 60 to 100 kDa (this protein is known to be quickly

Fig. 2. (A) 4–12% BT-SDS-PAGE (silver stained) of nucleosomes before (Nu1) and after sucrose gradient centrifugation (fractions 1 to 7). Nu1: displayed numerous bands higher than 21 kDa which derived from non-histone proteins. Fraction 6, which contained the mononucleosomes, displayed only bands around 15 kDa, and these were attributed to the histones of the nucleosome core. Fraction 6 contained the relevant antigen (Nu2, protein-stripped mononucleosomes), which served as a basis for the anti-nucleosome ELISA of the 2nd generation. Fractions 1 to 3 showed bands of proteins which were separated from the mononucleosomes by sucrose gradient centrifugation and which were not detectable in fraction 6. M represents the molecular weight markers. (B) 10% BT-SDS-PAGE (silver stained) of 1st (Nu1) and 2nd (Nu2) generation nucleosomes. Nu1 and Nu2 exhibited bands in the region of 15 kDa which can be assigned to the four nucleosome core histones. Purified histones (H2A, H2B, H3, H4) were used as controls. M represents the molecular weight markers.



degraded into fragments with molecular weights between 60 and 100 kDa [9,17]).

Western blot analysis of Nu1 and fraction 3 but not of Nu2 revealed also the presence of Scl-70 (Fig. 3). In neutralisation tests, in which the anti-Scl-70-positive serum was preadsorbed with purified Scl-70 prior to the immunoblotting, the bands were not stained, confirming that the reactions of Nu1, fraction 3 and Scl-70 immunoblots were specific to Scl-70 (data not shown).

In SDS PAGE analysis of fraction 3, no bands of Scl-70 were observable (Fig. 2A), since the protein silver staining exhibited a considerably lower sensitivity than western blot analysis (Fig. 3).

### 3.3. Anti-nucleosome reactivity in SLE and other rheumatic diseases

Microtiter plates were coated with Nu1 preparation and the corresponding antibodies tested by ELISA. Sera from 295 SLE patients and 204 healthy blood donors were analysed and the results were evaluated by means of Receiver Operating Characteristic (ROC) curves. If the cut off was fixed at 17 RU/ml, a specificity of 100% and a sensitivity of 62% were achieved, provided that the reactivity of PSS sera was not taken into consideration.

If sera from patients with Sjögren's syndrome (SS,  $n=48$ ) and polymyositis (PM,  $n=53$ ) were analysed in parallel, the specificity for SLE of the anti-Nu1 ELISA with the same cut off was slightly reduced to 99.0%. However, if sera from patients with PSS ( $n=119$ ) were included, the specificity of the anti-Nu1 ELISA decreased to 84.7%, since 52% of the PSS patients showed a positive reaction.

Following ROC analysis, the same tests containing Nu2 as target antigen resulted in an optimal cut off at 19 RE/ml with a specificity of 100% and a sensitivity of 58%. The inclusion of the sera from SS and PM patients in the evaluation resulted in a negligible change of the specificity for SLE to 99.7%. Remarkably, sera from PSS patients gave no positive reaction in the anti-Nu2 ELISA. In conclusion, the specificity of the anti-Nu2 ELISA for SLE with respect to serum panels including PSS was 99.8%.

A comparison of the areas under the ROC curves (AUC) showed that the AUC of the anti-Nu2 ELISA ( $0.847 \pm 0.035$ ) was greater than the AUC of the anti-Nu1 ELISA ( $0.793 \pm 0.038$ ), but overlapped within the confidence interval. The positive predictive value of the

Fig. 3. Scl-70 reactivities in different nucleosome preparations (Nu1 and Nu2) and fraction 3 of the sucrose gradient centrifugation (F3). The samples were analysed by SDS-PAGE followed by immunoblotting with an anti-Scl-70 positive serum. Nu1 and F3 exhibited bands in the region between 60 and 100 kDa which are attributed to Scl-70 and its degradation products. Nu2 exhibited no reactivity. Purified Scl-70 served as a control.

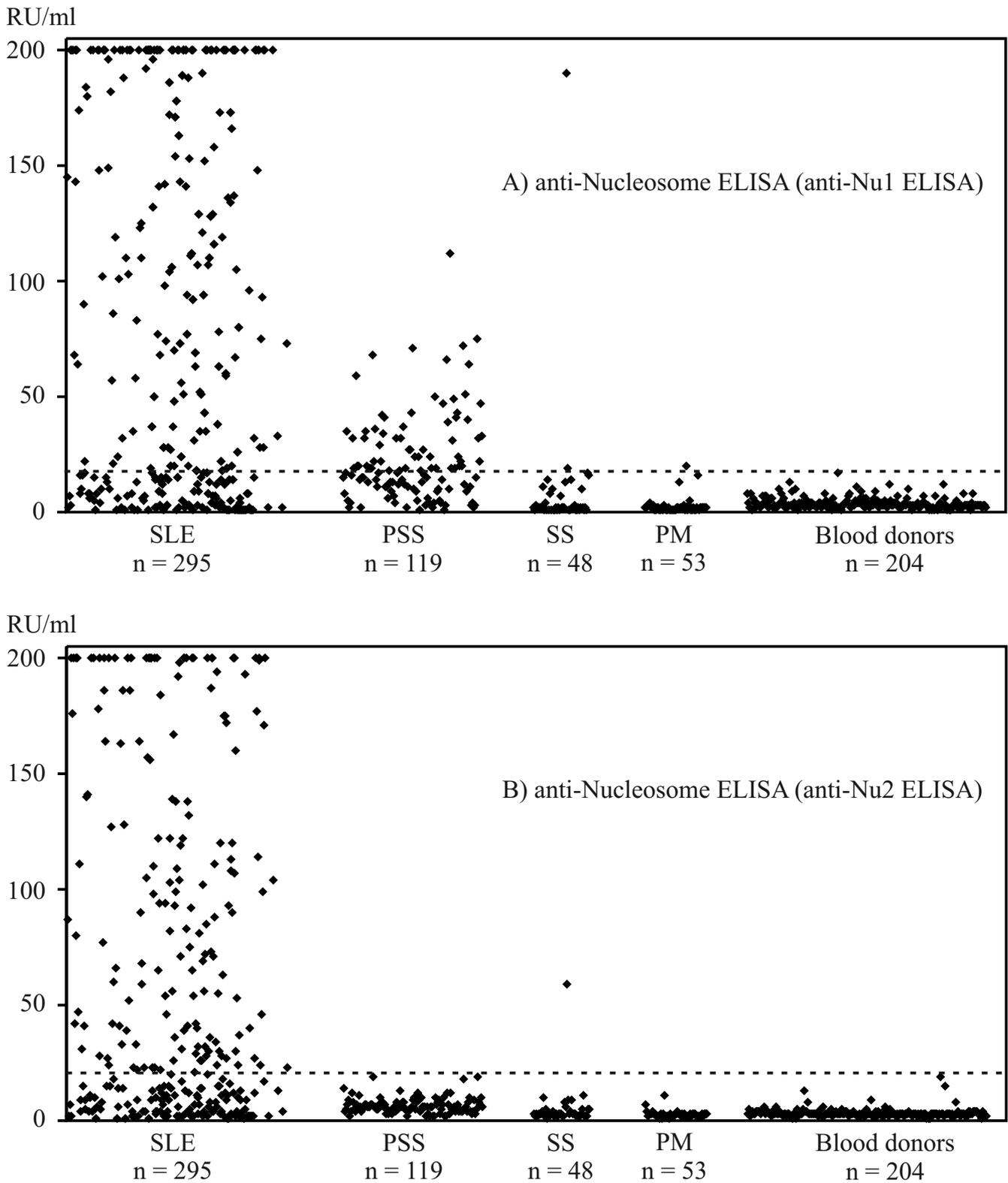


Fig. 4. Reactivities of sera from SLE, PSS, SS and PM patients and from blood donors in ELISA systems coated with Nu1 (A) and Nu2 (B). The dotted line represents the cut off. Anti-Nu1 ELISA: 62% of the SLE sera, 52% of the PSS sera, 4% of the SS sera and 2% of the PM sera were considered to be positive. Anti-Nu2 ELISA: 58% of the SLE sera and 1 SS serum were considered to be positive.

Table 1  
Frequency of positive reactions in both ELISA of the 1st (Nu1) and 2nd (Nu2) generation nucleosomes in sera from patients with various rheumatic diseases and from healthy blood donors

Patient group	<i>n</i>	Anti-Nu1 ELISA	Anti-Nu2 ELISA
Systemic lupus erythematosus	295	183 (62%)	171 (58%)
Progressive systemic sclerosis	119	62 (52%)	0
Sjögren's syndrome	48	2 (4%)	1 (2%)
Polymyositis	53	1 (2%)	0
Blood donors	204	0	0

anti-Nu2 ELISA was considerably higher (99.4%) than that of the anti-Nu1 ELISA (73.8%).

The reactivities of the different serum cohorts in both the anti-Nu1 and the anti-Nu2 ELISA systems are shown in Fig. 4 A and B. With the anti-Nu1 ELISA, sera from 183 SLE patients (62%) and 62 PSS patients (52%) gave reactions higher than 17 U/ml. With the anti-Nu2 ELISA, only the sera from SLE patients gave reactions higher than the cut off, with the exception of one SS serum. No PSS patient showed a positive reaction in the anti-Nu2 ELISA. The frequencies of positive results in both ANuA ELISA test systems for the different patient groups are shown in Table 1.

#### 3.4. Correlation between anti-Nu1 ELISA reactivity and anti-Scl-70 antibodies in PSS

In order to investigate whether contamination of the nucleosome preparation with Scl-70 was responsible for the false positive results, 119 sera from PSS patients were tested in the anti-Nu1 ELISA and anti-Scl-70 ELISA: 62 PSS sera were positive for anti-Nu1 and 103 positive for anti-Scl-70 antibodies. Of the 62 sera which reacted positively in the anti-Nu1 ELISA, 61 (98%) were also positive for anti-Scl-70 antibodies (Fig. 5).

#### 3.5. The Nu1 preparation is contaminated with Scl-70

The results presented indicate that the Nu1 preparation contained Scl-70 antigen as an impurity, whereas the Nu2 preparation consisted of pure nucleosomes. Inhibition experiments were performed to confirm this issue. Nu2 was added to sera of 4 SLE and 4 PSS patients at different concentrations and the mixture was subsequently used as a sample in the anti-Nu1 ELISA. Fig. 6A demonstrates that Nu2 was able to neutralise the reactivity of SLE sera, whereas in PSS no significant inhibition was detectable: the Nu2 preparation obviously did not contain the antigen causing the positive reaction of PSS sera in the anti-Nu1 ELISA, whereas the anti-nucleosome antibodies of SLE sera were neutralised.

Furthermore, Nu1 and Nu2 preparations were added to 4 PSS sera at different concentrations and the

mixtures incubated in an anti-Scl-70 ELISA. Nu1 exhibited a notable inhibition of anti-Scl-70 reactivity but Nu2 was not able to significantly neutralise anti-Scl-70 reactivity (Fig. 6B): the Nu1 preparation contained epitopes corresponding to Scl-70, which caused the neutralisation of anti-Scl-70 antibodies, whereas such epitopes were not present in Nu2 and thus no significant inhibition of anti-Scl-70 antibodies could be observed.

#### 3.6. Reactivities of SLE in anti-Nu2, anti-dsDNA and anti-histone ELISA

Antibodies against nucleosomes (determined using anti-Nu2 ELISA), dsDNA and histones were determined in parallel in sera from 89 SLE patients (Fig. 7). Fifty (56%) sera were positive for anti-Nu2 antibodies, 30 (34%) for anti-dsDNA and 20 (23%) for anti-histone antibodies. Making use of the two parameters anti-dsDNA and anti-histone, 42% of the SLE patients were serologically identified. However, the determination of anti-histone antibodies is not recommended from a diagnostic point of view, since these antibodies are not SLE-specific: they can also be found in sera from patients with rheumatoid arthritis and drug-induced lupus erythematosus.

By analysing the two SLE specific antibodies (anti-dsDNA and anti-Nu2) in parallel, the diagnostic hit rate increased significantly, identifying up to 59% of the SLE patients. Eighteen per cent of sera were positive for anti-Nu2 antibodies, but negative for anti-dsDNA and anti-histone antibodies. This implies that the anti-Nu2 antibodies represent a special entity in the spectrum of target antigens of SLE.

## 4. Discussion

It has been assumed up until now that, as well as SLE, progressive systemic sclerosis (PSS) is also associated with anti-nucleosome autoantibodies (ANuA). In the present study, it was possible to show that in PSS a correlation existed between positive results in a 1st generation anti-nucleosome ELISA (anti-Nu1 ELISA) and the presence of anti-Scl-70 antibodies. Furthermore, we demonstrated by immunoblot analyses and inhibition tests that Nu1 was contaminated with the PSS-related autoantigen Scl-70. Based on this knowledge, a nucleosome preparation from calf thymus was generated for the first time which exclusively reacted with sera of systemic lupus erythematosus (SLE) patients. We were able to clearly demonstrate that the earlier observed reactivities in PSS were false positives due to a contamination of conventional nucleosome preparations Scl-70 (DNA topoisomerase I).

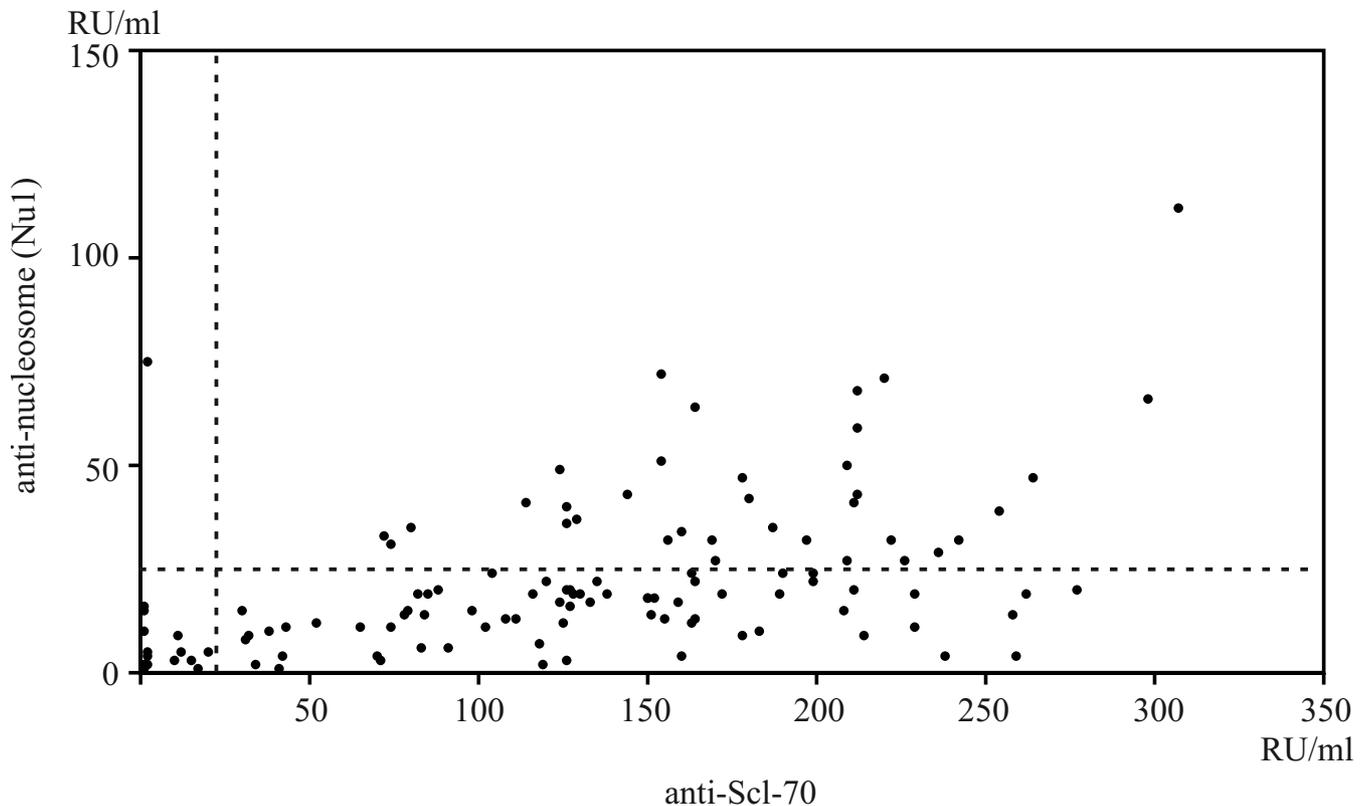


Fig. 5. Reactivities in PSS sera were determined using an anti-Scl-70 and an anti-Nu1 ELISA. The dotted lines represent the cut offs. Sixty-one (98%) of the 62 PSS sera considered to be positive in the anti-Nu1 ELISA were also positive for anti-Scl-70 antibodies.

#### 4.1. ANuA in PSS?

The detection rate of ANuA in PSS in the anti-Nu1 ELISA as observed by our group was in the same range as reported by Dick et al., who tested 50 PSS patients using four different commercial ANuA ELISA and determined between 20 and 68% to be positive [18], which was comparable to the results (46%) of Amoura et al. [19]. Bruns et al. used gel filtration-purified nucleosomes and found only 1 of 18 PSS sera to be ANuA positive [16]. However, they did not accurately characterise the patient cohort and possibly analysed sera mainly of the limited form of PSS. Such patients are known to exhibit antibodies against Scl-70 only in rare cases [20]. Under the purification conditions they described, it is not possible to remove Scl-70 completely, since high ionic strength conditions were not applied to the preparation before the gel filtration process, which would be necessary to strip off proteins. It could therefore be expected that their nucleosome preparation contained Scl-70 antigen and was reactive with sera of PSS patients. Furthermore, Bruns et al. did not identify Scl-70 as the main impurity, the removal of which from the nucleosome preparation is essential for obtaining an ELISA system specific for SLE.

#### 4.2. Removal of Scl-70 from a conventional nucleosome preparation

As early as 1975, Finch et al. described the purification of nucleosomes by sucrose gradient centrifugation following nuclear digestion using a gradient between 5 and 28% sucrose [12]. Up to the present, this method was considered the established process for purification of nucleosomes [13].

In this study a new technique was introduced to prepare nucleosomes which were demonstrably free from Scl-70 and other non-histone contaminants. A complete removal of Scl-70 was only possible by extending the sucrose gradient from 28% to 50% sucrose. Furthermore, displacement of Scl-70 from the nucleosomes required high ionic strength conditions to strip non-histone proteins from the DNA. However, the NaCl concentration should not exceed 0.75 M, since at this concentration the nucleosomes dissociated, releasing free DNA and histone complexes [21].

Numerous groups who have investigated ANuA in rheumatic diseases have used nucleosomes prepared according to Finch et al. as antigen. For ANuA studies on patients with SLE, Massa et al. used 5–30% sucrose gradients [22], while Chabre et al. applied a 5–20%

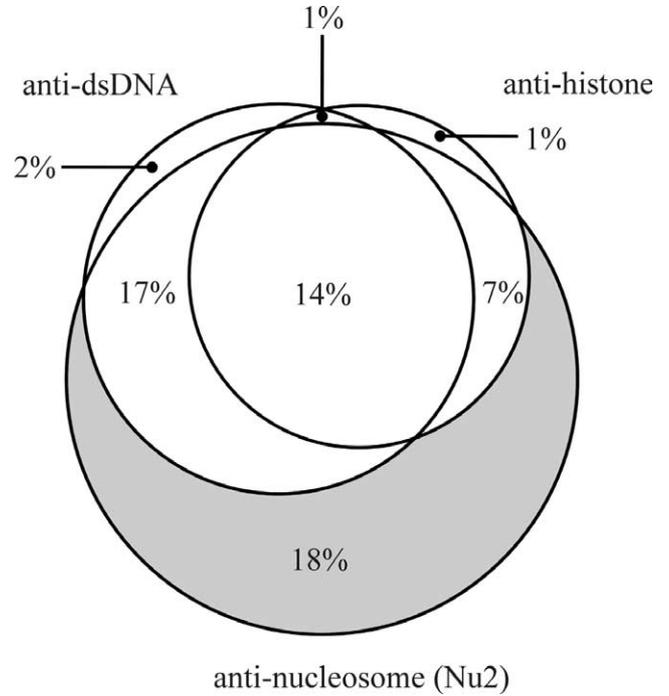
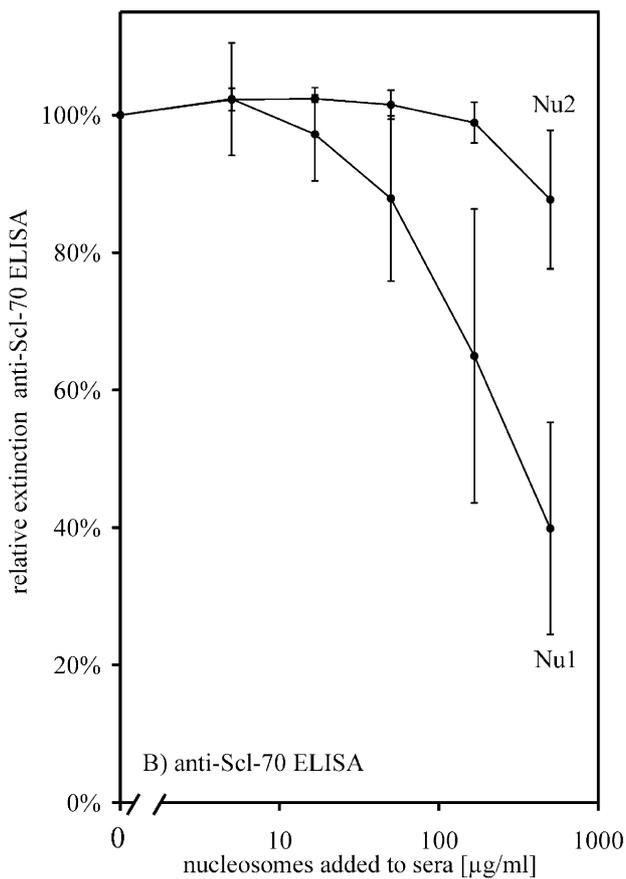
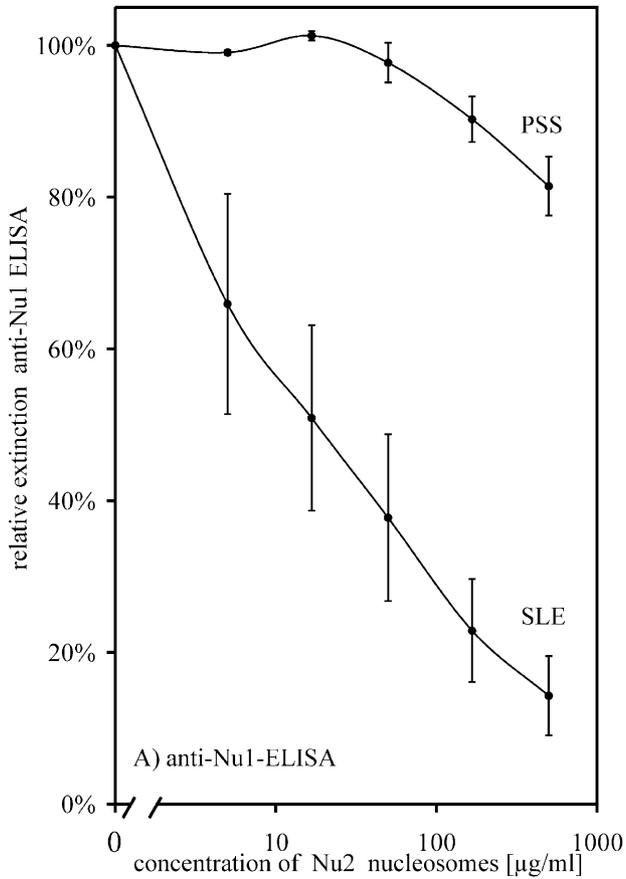


Fig. 7. Percentage of positive reactivities of 89 SLE patients in anti-nucleosome (Nu2), anti-dsDNA and anti-histone ELISA systems. Fifty (56%) sera were positive in the anti-Nu2 ELISA, 34 (38%) of these also contain anti-dsDNA and/or anti-histone antibodies. Thirty-six (40%) sera were considered to be negative.

sucrose gradient for their studies [23]. Since Scl-70 was contained in the 30% sucrose-containing fraction, as we were able to demonstrate, we assume that the nucleosomes of these authors were contaminated with Scl-70.

**4.3. The clinical relevance of ANuA and anti-dsDNA antibodies**

The clinical relevance of ANuA as a diagnostic marker for SLE, as regards testing using Nu2 as the target antigen, is due to its disease specificity of almost 100% and particularly to its high sensitivity of 56%, which is comparable with results reported elsewhere [16,19,24]. In the cohort used for this study, only 34% of the SLE patients could be diagnosed serologically by testing for anti-dsDNA antibodies, whereas the additional determination of ANuA increased the serological hit rate to 59%.

Fig. 6. Inhibition tests: (A) 4 SLE and 4 PSS sera were preincubated with varying concentrations of the Nu2 and tested in the anti-Nu1 ELISA. 86% of the reactivity was inhibited in SLE sera and 19% in PSS sera at a concentration of 500 μg/ml of Nu2 in the neutralisation solution. (B) 4 PSS sera were preincubated with varying concentrations of Nu1 and Nu2 and tested in an anti-Scl-70 ELISA. At the same concentration (500 μg/ml), 60% of the reactivity was inhibited with Nu1 and 12% with Nu2.

Nowadays, antibodies against dsDNA are regarded as the most prominent serological diagnostic marker for SLE. However, only anti-dsDNA results obtained from indirect immunofluorescence using *Crithidia luciliae* as substrate or from Farr assay are reliable, since anti-dsDNA ELISA systems exhibit a specificity of much less than 100% [25].

Conventional ANuA ELISA systems have also not been able to exhibit an optimal SLE specificity, as in the nucleosome preparations used up until now, the relevant target antigen contained a multitude of contaminants (e.g. see Fig. 2, lane Nu1). Apart from Scl-70, the new preparation technique also removes histone H1 and other non-histone components, promising an increased specificity of the ANuA ELISA. Initial encouraging results suggest that the ELISA based on the Nu2 as target antigen exceeds the specificity as well as the sensitivity of anti-dsDNA ELISA systems available to date. An international multi-centre study is currently investigating this issue with larger patient cohorts. Moreover, this multi-centre study should throw further light on the association of ANuA with disease activity and the various organ manifestations of SLE using Nu2 as the target antigen.

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