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PAPER

Autoantibodies to double-stranded DNA–Intermethod comparison between four commercial immunoassays and a research biosensor-based device

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Patients with systemic lupus erythematosus (SLE) often develop a wide variety of serological manifestations including the presence of antibodies to double-stranded DNA (anti-dsDNA). Positivity for anti-dsDNA constitutes one of the laboratory criteria for the diagnosis of SLE and is therefore clinically relevant. We analyzed the diagnostic accuracies of four commercial anti-dsDNA immunoassays and compared the results with a recently established surface plasmon resonance (SPR) biosensor chip with covalently chip-immobilized dsDNA. The anti-dsDNA measurements were performed retrospectively in 50 patients with clinically proven SLE, 39 patients with other autoimmunopathies and 20 healthy controls. Data were evaluated by Receiver-Operator Characteristic (ROC) analysis, with special regard to SLE patients suffering from lupus nephritis. The ROC analyses for the four immunoassays and the SPR biosensor resulted in the following area-under-the-curve (AUC) and diagnostic efficiency (DE) values in descending order: Bindazyme AUC, 0.89; DE, 0.88; ELiA AUC, 0.89; DE, 0.86; SPR biosensor AUC, 0.82; DE, 0.80; Farrzyme AUC, 0.77; DE, 0.77; Farr AUC, 0.77; DE, 0.70. When considering the 22 nephritis SLE patients the following AUC were observed: Bindazyme 0.98; EliA 0.95; SPR biosensor 0.93; Farr 0.89; Farrzyme 0.88. Although various methodologies for the determination of anti-dsDNA were compared, the overall diagnostic accuracy was found satisfactory in all immunoassays. Best data were found for the Bindazyme assay. We referenced the measurements to our in-house SPR biosensor device which showed good AUC and DE values. When optimized, this technique, allowing to monitor antigen/antibody interactions in real-time, may add a new analytical quality to the existing methods, potentially beneficial in diagnosis and clinical monitoring of SLE. *Lupus* (2010) **19,** 957–964.

Key words: autoantibodies; double-stranded DNA; ELISA; Farr assay; optical biosensor; surface plasmon resonance; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder, causing damage to virtually any organ of the human body. In the case of major organs being affected, e.g., when developing lupus nephritis, the disease can be lethal.[1,2] Clinical symptoms vary considerably, making correct and early diagnosis difficult. As therapeutical interventions are most effective at an early stage, there is a strong demand for early diagnosis. Therefore, the classification criteria of the American College of Rheumatology (ACR) contain, amongst clinical features, serological tests such as the detection of antinuclear antibodies and findings of 'antibodies to native DNA in abnormal titer'.[3,4] The latter are often present years before the onset of clinical symptoms[5] and are thus of high diagnostic and therapeutic significance.[6]

Double-stranded DNA autoantibodies (anti-dsDNA) are, due to their high specificity for SLE, especially helpful in terms of guiding the diagnosis in the right direction.[7] They are also feasible for follow-up, since rising titres of high-avidity IgG

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usually predict disease flares, particularly the onset or worsening of glomerulonephritis.[8,9]

Today three clinical chemistry methods are commonly used for the determination and quantification of anti-dsDNA: *Crithidia luciliae* indirect immunofluorescence (CLIF), ELISAs and radioimmunoassays (RIAs), namely the Farr assay. We have recently introduced a biosensor device for the detection of anti–dsDNA antibodies on the basis of surface plasmon resonance (SPR) technology, which has superior discrimination power between sera from SLE patients and controls.[10] The change of mass concentration at the interface caused by specific binding of the autoantibodies to surface-immobilized dsDNA is detected as changes in the refractive index via the SPR effect.[11,12]

The aim of our retrospective study was first to evaluate whether the newest generation ELISA methods EliA, Farrzyme and Bindazyme have similar diagnostic performance characteristics compared with the classical Farr RIA. The latter is often regarded as gold-standard for anti-dsDNA measurement since it is so far one of the most specific assays for SLE.[13] Secondly, these immunoassays were compared with our in-house SPR biosensor method. The system allows the kinetic monitoring of dsDNA and anti-dsDNA interactions in real time and under homogeneous conditions even providing information on binding kinetics and affinities under certain circumstances. As statistical criterion we applied the overall accuracy of each test method.

Materials and methods

Patients

We enrolled 50 SLE patients from the Dietrich Bonnhoeffer Clinical Centre in Neubrandenburg in the study. All fulfilled at least four of the ACR criteria and were tested positive for antinuclear antibodies (ANA).[3,4] Thereby, serum positivity for anti-dsDNA was consciously not considered as one of the ACR criteria. Twenty-two of the 50 SLE patients suffered from lupus nephritis. The diagnosis was proven by biopsy and/or clinical chemistry parameters (significant proteinuria, elevated serum creatinine). None of the nephritis patients, however, were under dialysis therapy. The control groups consisted of 39 patients with other autoimmune diseases and 20 apparently healthy people (recruited from the laboratory staff). The latter were assumed to be free of any acute or chronic disease on the basis of a medical and clinical chemistry examination. The 39 non-SLE patients were recruited both from the university hospital Klinikum rechts der Isar and the Klinikum München. Diagnoses have been provided by expert clinicians.

This study was in compliance with the Helsinki Declaration and had institutional review board approval from the ethics committee of the Medical faculty of the Technische Universität München. The demographic data are summarized in Table 1.

All patient serum samples were leftover human specimens that were not individually identifiable according to the FDA document 'Guidance for sponsors institutional review boards clinical investigators and FDA staff' (OMB control no. 0910–0582, issued 09/25/2006). The samples were accompanied by clinical information not making the source identifiable to the investigator.

All blood samples were collected without anticoagulant. After clot formation they were centrifuged at 1500 *g* for 20 min. Samples were then stored at −70°C in aliquots until analysis.

Assays

All commercial assays were calibrated against the former WHO standard Wo80. In a recent article of our group problems with this standard were discussed and possible solutions shown by applying the anti-dsDNA SPR biosensor.[14]

We used the anti-dsDNA Farr RIA from Trinity Biotech (Bray, Ireland). The assay uses radiolabeled dsDNA from the bacteriophage PM2 (6×10^6 Da) as the antigen to detect the specific antibodies in serum. dsDNA reacts with anti-dsDNA and is precipitated using a 50% $(NH_4)_2SO_4$ solution. The immunoprecipitate is then quantified by measuring the I-125 radioactivity. The Bindazyme anti-dsDNA

Table 1 Characteristics of patients and controls

Investigated group	*Subgroups*	n *(female/male)*	*Mean age (years)*
SLE	–	50 (47/3)	41.0 ± 14.3
	Lupus nephritis	22 (20/2)	36.9 ± 13.1
Controls	–	59 (39/20)	53.4 ± 12.9
	Wegener's granulomatosis	10 (5/5)	62.0 ± 14.4
	Rheumatoid arthritis	16 (11/5)	63.8 ± 13.4
	Myasthenia gravis	3 (2/1)	74.0 ± 5.2
	Primary biliary cirrhosis/ auto-immune hepatitis	7 (6/1)	54.7 ± 19.9
	Mixed connective tissue disease	2 (2/0)	85
	Coeliac disease	1 (0/1)	55
	Healthy subjects	20 (13/7)	36.2 ± 14.0

enzyme immunoassay (The Binding Site Ltd, Birmingham, UK) measures specific anti-dsDNA of IgG class in serum. Microwells are pre-coated with native calf thymus dsDNA as the antigen. The Farrzyme (The Binding Site) is a selective high avidity anti dsDNA (IgG class) enzyme immunoassay, employing the same antigen as the Bindazyme assay. The dsDNA source is also native calf thymus DNA. The EliA dsDNA (Phadia GmbH, Freiburg, Germany) is a selective IgG anti-dsDNA fluoroenzyme immunoassay using double-stranded plasmid DNA as the antigen and has to be performed on the ImmunoCAP 250 analyzer.

All commercial assays were performed according to the manufacturer's prescriptions.

Biosensor measurements

The SPR biosensor measurements were accomplished using a BIAcoreX (GE Healthcare, Freiburg, Germany) device. All measurements were performed at 25°C on two separate sensor chips streptavidin (SA) (also from GE Healthcare), consisting of a carboxymethyldextran-coating on the gold surface, pre-immobilized with streptavidin (FF1 and FF2). Preparation of dsDNA antigen and immobilization procedure were performed as described previously.[10] The assay calibration was established by use of the human anti-dsDNA monoclonal antibody dsDNA-mAb32, purchased from DIARECT AG (Freiburg, Germany).[14] The manufacturer specified the anti-dsDNA activity with 1.4×10^5 IU/ml at a protein concentration of 0.43 g/l.

We applied 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.1% albumin from bovine serum, 0.005% octyl β-D-glucopyranoside as running buffer for the measurements of the dsDNA anti-dsDNA interactions. A continuous flow rate of 30 µl/min was maintained. All buffers for the biosensor measurements were sterile-filtered using 0.2 µm FP CA-S filters (Whatman, Dassel, Germany) and exhaustively helium-degassed before use.

For the determination of anti-dsDNA in human samples human sera were diluted 1 : 100 with the respective running buffer prior to the injection step, and centrifuged at 10,000 *g* for 5 min. 90 µl of the prediluted sera were conducted over the surface to allow association, followed by a 300 s period of buffer injection to monitor the dissociation of immune complexes. Thereafter, the biosensor surface was regenerated with 60 s pulses of 50 mM NaOH/1 M NaCl until the signal returned to the baseline. Total measuring time for on serum sample was approx. 15 min. Signals were monitored as a function of time (SPR sensorgrams), expressed in arbitrary resonance units (RU) per 0.90 µl. As measuring value for the antibody interaction, we chose the RU values corresponding to maximal binding during the association phase *a*. All other attempts to use a combined variable which implements also the dissociation behaviour of anti-dsDNA (RU value after 300 s of dissociation *b*), e.g. $(a-b)/b$, did not improve results. By applying the above mentioned assay calibration, the RU values were converted into the respective IU/ml concentration specification.

Both FF1 and FF2 chips (with comparable surface antigen density) were tested for viability by repetitive measurements with the dsDNA-mAb32 calibrator and displayed an attenuation of the signal of less than 8% over approx. 60 measuring cycles. In Figure 1 the measurements with the FF1 chip are portrayed (mean 305 RU, standard deviation (SD) ± 24). Thus, the in-house biosensor surface met the requirements for multiple measurements of serum samples under routine conditions.[10]

Statistical evaluation

To investigate each assay with respect to its accuracy to detect patients with SLE, sensitivity and specificity data were calculated from 2×2 contingency tables for each possible cut-off value and plotted as Receiver-Operator Characteristic (ROC) curves. The area under the ROC curve (AUC), and its standard error were additionally calculated to interpret the probability that when one positive and one negative example are randomly picked, the classifier will assign a higher score to the positive example than to the negative.[15,16]

The significance of the AUC for each ROC curve and the statistical intermethod comparison of two independent AUCs were analyzed with the standard critical ratio test.[16,17] For defining the optimum cut-off we applied the Youden Index (YI). YI being equal to 1 − [false positive rate + false negative rate]. The respective cut-off was ascertained at the highest YI (highest theoretical value being 1).

As statistical criterion we applied also the overall accuracy/diagnostic efficiency (DE) of each test method. This represents the measure of 'true' findings (true-positive + true-negative results) divided by all test results within the total investigated collectives.

An intermethod comparison using the Bablok-Passing linear regression procedure could not be applied due to the fact that equality of

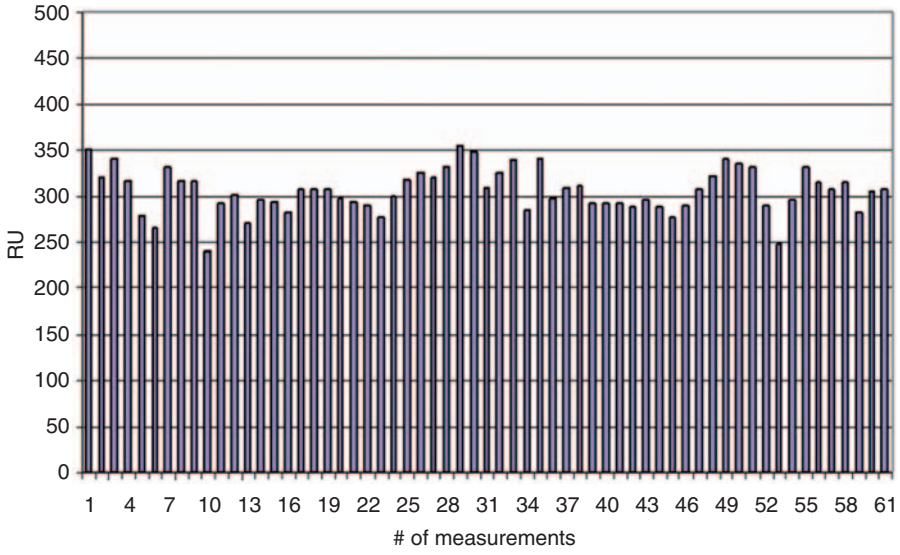

Figure 1 Repetitive measurements with the dsDNA-mAb32 calibrator for the FF1 chip as test for viability of the in-house SPR biosensor device with regenerative fluidics.

measurements from the different anti-dsDNA methods could not be assumed. Therefore we only implemented the Spearman correlation analysis for the four ligand assays and the biosensor method.

Statistical significance was accepted for $p < 0.05$. Patient data and measurements were collected in a Microsoft windows access data base and statistically analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA).

Results

Comparative descriptive data analysis for the four methods

The ligand assay methods had different individual measuring ranges and detected the SLE sera diversely, although they are all standardized to the same WHO standard Wo80. The SPR methodology is different and the quantifiable RU results cannot be simply compared to the four ligand assay data. SPR results were referred to the dsDNA-mAb32 calibrator. Table 2 depicts the various data sets and also shows the predefined diagnostic cut-off values for all applied methods, except for the SPR method.

With all methods no differences in the value ranges between the healthy controls ($n = 20$) and the control patients with autoimmunopathies (except for SLE) ($n = 39$) could be observed. Therefore both subgroups were pooled into one control group ($n = 59$).

Table 2 Data sets of all applied methods

Method (unit)	*Cut-off**	*Results for SLE patients (n = 50; mean ± SD)*	*Results for controls (n = 59; mean ± SD)*
SPR (IU/ml)	–	147.1 ± 107.4	63.7 ± 23.0
Farr (IU/ml)	7.0	14.6 ± 24.4	2.4 ± 1.7
Bindazyme (IU/ml)	30.0	146.0 ± 184.1	11.6 ± 4.7
Farrzyme (IU/ml)	30.0	49.9 ± 98.1	6.7 ± 3.4
EliA (IU/ml)	10.0	66.6 ± 127.3	3.8 ± 11.4

*According to the manufacturer's description.

Spearman rank-order correlation coefficients (r_s) between the four methods

Results are shown in Table 3. All r_s values are significant at the 0.01 level (two-tailed t-test $p < 0.01$).

ROC analyses for the four immunoassays compared to the SPR biosensor results, diagnostic efficiencies for all five methods

In the primary ROC analysis the complete data set of 50 SLE patients and 59 controls was included. To ascertain the accuracy for the diagnosis of SLE the DE value of each test method was calculated. All assays showed DE values significantly higher than 0.5. The highest values were found for the Bindazyme and the EliA assays, the SPR method being third (see Table 4).

All ROC curves (A, Bindazyme; B, EliA; C, SPR biosensor; D, Farr; E, Farrzyme) are exemplified illustrated in Figure 2. The AUC value of the

Table 3 Spearman rank-order correlation coefficients (r_s) between the five methods

	Bindazyme	*Farrzyme*	*Farr*	*ELiA*	*SPR*
Bindazyme	–	0.51	0.61	0.71	0.67
Farrzyme		–	0.48	0.47	0.38
Farr			–	0.56	0.59
ELiA				–	0.55
SPR					–

Table 4 AUC and DE values of each test method and AUC values in the subgroup of the 22 nephritis patients

ROC curves	*Bindazyme*	*ELiA*	*SPR*	*Farrzyme*	*Farr*
AUC (all SLE patients)	0.885	0.886	0.820	0.645	0.770
AUC (nephritis patients)	0.979	0.945	0.928	0.879	0.886
Youden (max) cut-off (IU/ml)	18.6	6.4	110.5	11.1	2.4
DE (all SLE patients)	0.88	0.86	0.80	0.77	0.70

Farr RIA is better than the one for the Farrzyme ELISA, even when the DE values of these two assays behave inversely.

As could be expected, comparing the subgroup of the 22 SLE patients with renal involvement to the control collective group, the ROC characteristics improved for all assays.

Discussion

With new methodologies emerging, the variation of anti-dsDNA assays is still increasing. Also, our SPR biosensor marks a new approach with promising possibilities.[10,14] The Farr assay is still considered as the gold standard for clinicians. Advantageous is the high specificity for diagnosing SLE and the discrimination from other autoimmune diseases. However, low affinity anti-dsDNA is often not detectable with this assay. On the other hand, ELISAs are by far the most widely used methods in routine laboratory practice. These are sensitive assays, in principle able to detect high and low affinity anti-dsDNA as well (in most cases IgG isotype). Their diagnostic efficiency for SLE, however, varies considerably.[13,18] Further the CLIF method is still often used. Comparable with the Farr assay, the CLIF assay also detects mostly high affinity anti-dsDNA.

Anti-dsDNA in serum is no surrogate marker of SLE, but may depict biological activity and have the ability to cause pathological changes typically for SLE. Considerable antigen affinity, complement activation and Fc-receptor engagement, make anti-dsDNA dangerous for multiple organs in SLE patients, characterized by a clearance deficiency of apoptotic cells.[19]

The SLE patients investigated in this study had already been included in a larger previous trial,[18] comparing different anti-dsDNA assays regarding their sensitivity and specificity for SLE. Besides the known analytical differences (assay conditions, source of antigen) seen between various anti-dsDNA assays, the laboratory parameters of the ACR criteria for SLE itself constitute a further problem,[20] since they influence the outcome of intermethod comparisons by the selection of possibly misdiagnosed patients and controls included in such studies. Considering the above mentioned factors, the main finding of our previous study was, that assays detecting predominantly high avidity anti-dsDNA are more specific for SLE and, in contrast to those detecting low avidity antibodies as well, are significantly linked to renal involvement.[19] Only recently, in a longitudinal study including 16 patients with new-onset biopsy-proven lupus nephritis, high avidity anti-dsDNA were again identified as best predictors for renal outcome.[21]

Role of anti-dsDNA in SLE and the associated lupus nephritis

Autoantibodies in SLE are characterized to be directed against nuclear constituents. Some of them are clinically and diagnostically relevant, whereas others are disease accompanying. In particular, autoantibodies to dsDNA and nucleosomes play pivotal roles and have overlapping diagnostic impact in SLE. Concerning the development of lupus nephritis, there is consensus that these two antibody species are pathogenic factors and may initiate nephritis. Though not all anti-dsDNA are involved in the evolution of this disease, there are different hypotheses on the features that make an anti-dsDNA antibody nephrotoxic.[22] Particularly conclusive is the affinity maturation model. Short-lived stimuli result first in low avidity anti-dsDNA, which in some of these cases could be regarded as transient epiphenomenons. High avidity antibodies usually result from sustained stimulation by immunogenic nuclear substrates (dsDNA, nucleosomes) and may have strong diagnostic and pathogenic impact.[23] However, in spite of persisting high levels of low avidity anti-dsDNA, some individual SLE patients never develop high avidity autoantibodies.

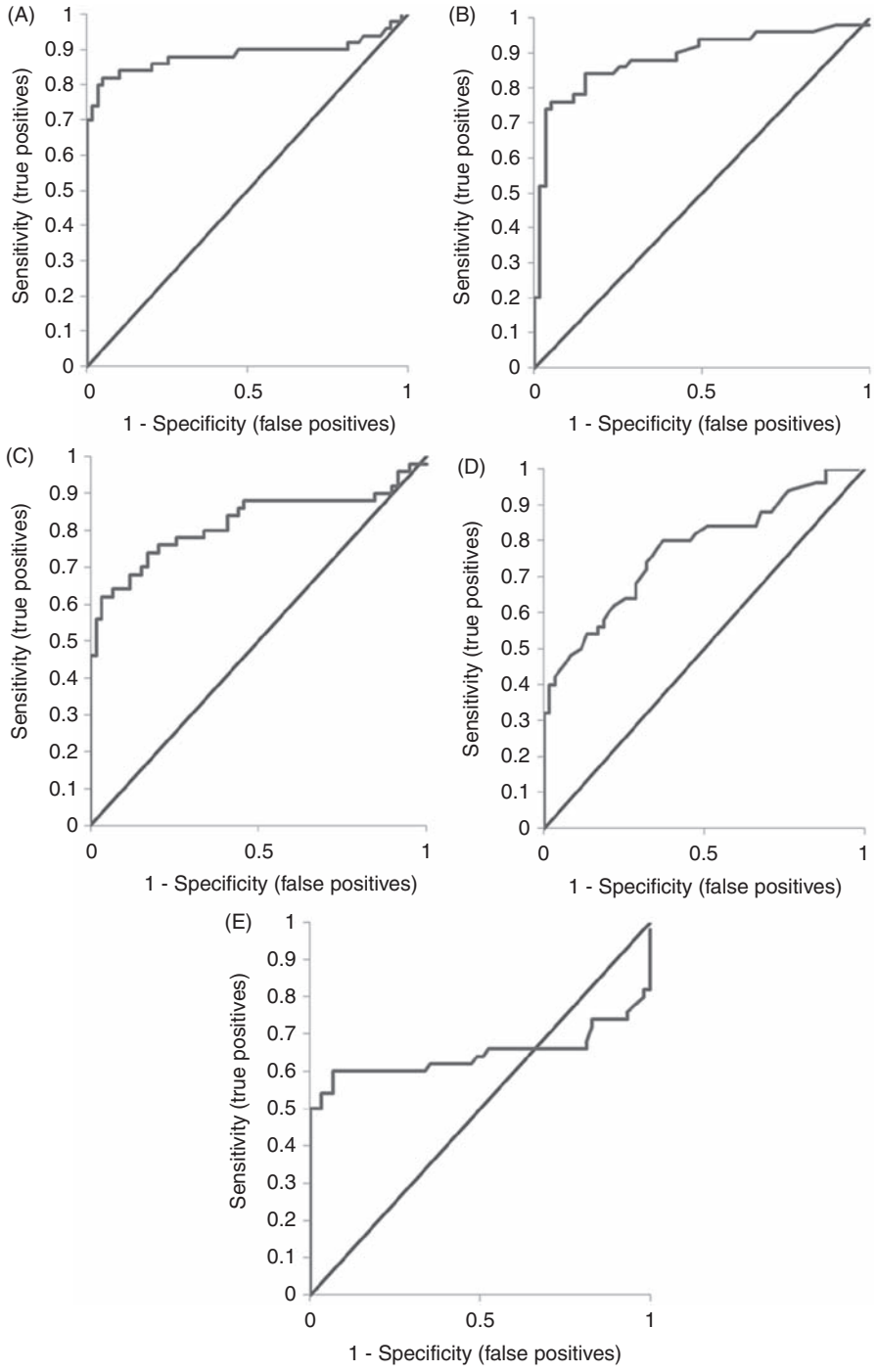

Figure 2 ROC curves of all investigated assay methods: A, Bindazyme; B, EliA; C, SPR biosensor; D, Farr; E, Farrzyme.

Anti-dsDNA SPR biosensor

In contrast to established analytical methods, the SPR technique is rarely used for autoantibody determinations. Our anti-dsDNA biosensor chip was evaluated in pilot experiments using sera from normal individuals, subjects suffering from non-SLE autoimmunopathies and patients, fulfilling the SLE-criteria.[10] It could be demonstrated, that using a cut-off value of 25 RU, the SPR biosensor assay offered an excellent specificity (98%) at a high sensitivity (83%). Thus, the novel system has proved its applicability for the laboratory diagnosis of SLE. In particular, the analytical sensitivity of the device is remarkable: The measurements are carried out in 90 µl of a 1 : 100 diluted serum samples and still result in signal heights of

approx. 100–300 RU in the respective sensorgrams of SLE patients. In this comparative study, however, there is no simple correlation between the ligand assay-derived concentration values and the respective binding values obtained in the biosensor due to the fundamentally different methodologies between immunoassay and SPR biosensor technologies.

Interpretation of the comparison of the five methods

According to Table 3, it is surprising that nonetheless the Spearman rank-order correlation coefficients for the comparison of the four methods were all significant ($r_s = 0.38$–0.71; two-tailed t-test $p < 0.01$).

Although the biosensor association levels depend not only on antibody concentrations, but also on their affinities, the investigated patient sera showed nearly uniform SPR sensorgrams. The reason for this seems to be that all patients, suffering already for longer periods from SLE, had predominately IgG isotype anti-dsDNA. Different curve shapes may be indicative for the predominance of either IgG or IgM isotype: Whereas IgM shows a delayed association phase and a weak dissociation correlated with a high residual binding, IgG depicts faster association and a variable dissociation phase. Due to this special patient group, we could only observe the latter type in the SPR sensorgrams.

In this study we investigated the assay performance characteristics of five different methods for the determination of anti-dsDNA in sera from SLE patients. For this purpose, clinically clearly defined patient and control groups were selected. The SLE patients fulfilled at least four of the ACR criteria, the collective being composed of subjects with active and inactive, treated disease states. The ROC analyses for the four ligand assays and the SPR biosensor showed that the overall diagnostic accuracy was found satisfactory in all assays. There were, however, three methods with DE values >0.8 (Bindazyme, EliA and SPR), and two with DE values <0.8 (Farrzyme and Farr RIA).

In conclusion, our study shows that the overall DE of the investigated ligand assays and the SPR biosensor method were found satisfactory. Best data were found for the Bindazyme and the EliA assays. Our in-house prototype SPR biosensor device for anti-dsDNA which monitors antigen/antibody interactions in real-time also produces good AUC and DE values and adds a new analytical quality to the existing methods, potentially beneficial in diagnosis and clinical monitoring of SLE. A more sophisticated biosensor device will probably provide even better AUC and DE data.

Based on presently available data concerning other SLE autoantibodies, such as anti-nucleosome, anti-C1q or anti-poly(ADP) ribose, the application of anti-dsDNA as a criterion for the diagnosis of SLE is still appropriate.[7] The remaining analytical problems, primarily the assay calibration and unification of the DNA source as antigen, however, have still to be solved.

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