Evaluation of a New Automated Enzyme Fluoroimmunoassay Using Recombinant Plasmid dsDNA for the Detection of Anti-dsDNA Antibodies in SLE

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ELISA methods to detect anti-double-stranded DNA (anti-dsDNA) antibodies are highly sensitive, but are less specific for the diagnosis of SLE than the immunofluorescence test on *Crithidia luciliae* (CLIFT) and the Farr assay because they also detect low-avidity antibodies. This study evaluated the specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) of a new automated fluoroimmunoassay (EliA dsDNA; Pharmacia, Freiburg, Germany). We compared the results with those obtained using a commercial CLIFT and an in-house anti-dsDNA IgG ELISA method, and verified its putative ability to detect only high-avidity anti-dsDNA antibodies. Sera from 100 SLE patients and 120 controls were studied. The control group included 20 healthy donors, 70 patients with other rheumatic diseases (32 systemic sclerosis (SSc); 18 primary Sjögren syndrome (pSS), 20 rheumatoid arthritis (RA)), and 30 patients with various infectious diseases (ID). Anti-dsDNA avidity was estimated using an ELISA method based upon the law of

mass action, and a simplified Scatchard plot analysis for data elaboration; the apparent affinity constant (K_{aa}) was calculated and expressed as arbitrary units (L/U). Sensitivity, specificity, PPV, and NPV for SLE were 64%, 95.8%, 93.8% and 72.7%, respectively, for the EliA anti-dsDNA assay; 55%, 99.2%, 98.5%, and 68.8%, respectively, for the CLIFT; and 64%, 93.3%, 90.6%, and 72.3%, respectively, for the in-house ELISA. Although EliA anti-dsDNA was positive mainly in SLE patients with high- ($K_{aa} > 80$ L/U) and intermediate- ($K_{aa} 30\text{--}80$ L/U) avidity antibodies (45.3% and 49.9%, respectively), it was also positive in five (7.8%) SLE patients with low-avidity anti-dsDNA antibodies, and five controls (three SSc, one pSS, and one ID) (mean $K_{aa} = 16.4 \pm 9.04$ L/U). In conclusion, EliA anti-dsDNA assay showed a higher sensitivity than the CLIFT, and a good specificity and PPV for SLE. Its putative ability to detect only high-avidity anti-dsDNA antibodies remains questionable. *J. Clin. Lab. Anal.* 16:227–232, 2002. © 2002 Wiley-Liss, Inc.

Key words: autoimmune rheumatic disease; avidity; assay sensitivity; assay specificity; ELISA; systemic lupus erythematosus

INTRODUCTION

The measurement of anti-double-stranded DNA (anti-dsDNA) antibodies is a useful tool for the diagnosis of systemic lupus erythematosus (SLE), and constitutes one of the 11 criteria of the American College of Rheumatology (ACR) for the classification of SLE (1,2). Moreover, several reports have demonstrated a correlation between disease activity and anti-dsDNA antibody levels in SLE, especially in patients with renal involvement (3–6).

However, in reference to immunoglobulin class, avidity, complement-fixing ability, antigenic specificity, and cross-reactive patterns, anti-dsDNA antibodies are

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Received 8 March 2002; Accepted 4 June 2002

DOI: 10.1002/jcla.10045

Published online in Wiley InterScience (www.interscience.wiley.com).

heterogeneous (7) and their detection depends, in part, on the type of assay used. The radioimmunoassay (Farr technique) predominantly detects high-avidity anti-dsDNA antibodies, which are highly specific for SLE and more strictly associated with renal involvement. Unfortunately, the Farr assay has limited use in clinical laboratories because it cannot be automated and it requires the use of radioactive material. The enzyme-linked immunosorbent assay (ELISA) also recognizes low-avidity anti-dsDNA antibodies; however, in this case a positive result does not always indicate SLE, because low titers of these antibodies may occur in other diseases (8). The *Crithidia luciliae* immunofluorescence test (CLIFT) is specific for anti-dsDNA antibodies of intermediate to high avidity (9), but since it is not a quantitative assay it is not an optimal method for monitoring SLE patients.

The aims of this study were to 1) evaluate the EliA dsDNA assay, a new automated fluoroimmunoassay, in terms of sensitivity and specificity, and positive and negative predictive values (PPV and NPV); 2) compare the results with those obtained using a CLIFT and an in-house anti-dsDNA IgG ELISA method; and 3) verify its putative ability to detect mainly high-avidity anti-dsDNA antibodies.

MATERIALS AND METHODS

Patients and Sera

Serum samples from 100 SLE patients were studied. All patients met at least four ACR criteria for SLE classification (1,2). The M/F ratio was 1/9, mean age was 29.8 ± 9.0 years, and mean disease duration was 80.2 ± 69 months; the patients' clinical features are described in Table 1. SLE disease activity was estimated using the European Consensus Lupus Activity Measure (ECLAM) score (10). The mean value was 2.1 ± 1.4 (range 0–7), indicating that most patients were in clinical remission ($ECLAM < 2$) when blood was collected.

As controls, we also assayed sera from 20 healthy subjects, 70 patients with other rheumatic diseases (32 systemic sclerosis (SSc), 18 Sjögren's syndrome (SS), and 20 rheumatoid arthritis (RA)), and 30 patients with viral infection (ID) (20 hepatitis C, and 10 infectious

mononucleosis). All sera were stored at -80°C until tested.

In-house anti-dsDNA ELISA assay

Serum samples were diluted 1:101 in phosphate-buffered saline (PBS) (0.14 M NaCl, 0.1 M sodium phosphate, 2% bovine serum albumin, pH 7.4). Then 100 μL of the diluted samples and the standard solutions (0, 10, 50, 150, and 300 IU/mL in PBS, calibrated against the WHO Wo/80 reference preparation) was added to microtiterplates coated with 100 μL of 3 $\mu\text{g/mL}$ purified calf thymus DNA (Sigma, St. Louis, MO). The plates were incubated for 60 min at room temperature and washed to remove the unbound material. Then 100 μL of horseradish peroxidase-labeled mouse anti-human IgG monoclonal antibodies (Bouty Laboratories, Milan, Italy), diluted to 0.6 $\mu\text{g/mL}$ with peroxidase stabilizing buffer (Dako, Mississauga, ON) with 5% fetal calf serum, was added to the plates, which were incubated for 30 min at room temperature, and washed again to remove unbound conjugate. Next, 100 μL of chromogen-substrate (TMB, H_2O_2) solution was added and the plates were incubated for 15 min at room temperature; the reaction was stopped with 100 μL of 1 N sulphuric acid, and absorbances were detected photometrically at 450 nm. IgG anti-dsDNA antibody concentrations (IU/mL) were calculated using the absorbance value of standard solution and an unweighted four-parameter logistic model (ElisaAid software, Bouty) for the interpolation. The optimal cut-off level, as determined by receiver operating characteristic (ROC) curves, was set at 30 IU/L.

CLIFT

The CLIFT assay was performed using a commercial test (Bio-Diagnostic, Ltd., Worcestershire, UK). In this method fluorescein isothiocyanate-labeled polyvalent anti-human immunoglobulin (IgG, IgM, and IgA) is used, and the starting sample dilution is 1:10 in PBS.

EliA dsDNA assay

EliA dsDNA (Pharmacia, Freiburg, Germany), a newly developed fluorescence enzyme immunoassay for dsDNA antibody measurement, uses a recombinant circular plasmid dsDNA, purified from *E. coli*, coated on irradiated polystyrene wells by adsorption to the plastic material. Antibodies to dsDNA are detected fluorometrically using a mouse monoclonal β -galactosidase conjugated antibody directed against the heavy chain of human IgG, and 4-methylumbelliferyl- β -D-galactoside (0.01%) as substrate. Unbound antibodies

TABLE 1. Clinical findings in the 100 SLE patients studied

Organ involvement	Number of cases
Renal involvement	26
CNS involvement	3
Skin involvement	15
Arthritis	13
Serositis	1
Cytopenia (anemia, leucopenia, thrombocytopenia)	62

are removed by a stringent washing buffer that also removes the binding of low-avidity antibodies.

Six different concentrations (0–600 µg/L) of purified human IgG preparations, calibrated against the WHO preparation IRP 67/86, were used for quantitative expression of antibody concentration.

All the assay procedures (sample dilution of 1:10; sample, conjugate, and substrate distribution; washings) are automatically performed on the UniCAP 100 instrument (Pharmacia) at 37°C. EliA dsDNA is calibrated against the WHO standard for dsDNA antibodies (Wo80), and the results are expressed as IU/mL.

Anti-dsDNA antibody avidity

Anti-dsDNA IgG antibody avidity was estimated using an ELISA method based on the law of mass action, and a simplified Scatchard plot analysis (two points) for data elaboration, as previously described (11). Briefly, the binding of the anti-dsDNA antibodies to the coated antigen (0.3 µg of purified calf thymus dsDNA/well) was measured simultaneously in two coated wells (one with 50 µL of buffer solution added, and one with 50 µL of dsDNA solution (10 µg/mL) added). After 120 min incubation at room temperature and a washing step to remove the unbound material, 100 µL of horseradish peroxidase-labeled mouse anti-human IgG was added, and the samples were incubated for 30 min at room temperature. Following a second washing step to remove the unbound conjugate, and measurement of optical density (OD) at 450 nm, a two-point Scatchard analysis was performed and the apparent affinity constant, expressed as arbitrary units (L/U), was calculated as follows:

Kaa =

$$\frac{(OD_2 - OD_1) \times [OD_t]^2}{(OD_t - OD_1) \times (OD_t - OD_2) \times [(OD_2 \times Ag_2) - (OD_1 - Ag_1)]}$$

where Kaa = apparent affinity constant; Ag₁ = coated dsDNA; Ag₂ = coated dsDNA + dsDNA in buffer solution; OD₁ = optical density of the well containing Ag₁; OD₂ = optical density of the well containing Ag₂; OD_t = total optical density.

For classification purposes, the antibody avidity results were arbitrarily divided into three groups: low avidity (Kaa < 30 L/U), intermediate avidity (Kaa 30–80 L/U), and high avidity (Kaa > 80 L/U).

Precision study

To determine intra- and interassay precision of the EliA dsDNA test, three samples with low (4.1 IU/mL),

high (56.7 IU/mL), and close to cut-off (17.3 IU/mL) antibody concentrations were arbitrarily chosen among the panel sera. The antibody levels in each sample were determined seven times in each run, and in seven independent runs.

Statistical analysis

Statistical analysis was performed using the SPSS 6.0 for Windows statistical package (SPSS, Chicago, IL). Mean, SD, and coefficients of variation (CVs) were used where appropriate.

An unpaired *t*-test was used to compare the data obtained in patients with and without renal involvement; significance was set at a *P*-value of <0.05.

Specificity and sensitivity for EliA dsDNA were calculated at different cut-off values, and the results were graphically presented by using ROC curves (12).

RESULTS

Sensitivity, Specificity, PPV, NPV, and Analytical Precision

With the cut-off set at 15 IU/mL, as determined by ROC curves (Fig. 1), the sensitivity, specificity, PPV and NPV for anti-dsDNA antibodies measured by the EliA dsDNA assay were 64%, 95.8%, 93.8%, and 72.7%, respectively. As shown in Table 2, these results are similar to those obtained with the in-house ELISA method, although the EliA dsDNA was slightly more specific. A marked difference in sensitivity was observed compared to CLIFT (64% vs. 55%); however, CLIFT showed an almost absolute specificity (99.2%). The distribution of anti-dsDNA antibody levels detected in

Fig. 1. ROC curve showing that a cut-off value set at 15 IU/mL provides the best compromise between sensitivity and specificity. AUC, area under the curve.

SLE patients and controls with the EliA dsDNA assay is shown in Fig. 2; five of 120 control sera (4.1%) had values above the cut-off, and were obtained from three patients with SSc (mean 27.5 ± 9.3 IU/mL), one with pSS (27.7 IU/mL), and one with HCV infection (62.7 IU/mL). This last patient, however, was also positive with the in-house ELISA method and CLIFT. Positive values were not found in any of the healthy subjects.

The intra- and interassay CVs ranged from 8.5% to 13.5% and from 8.8% to 16.4%, respectively. For values near the cut-off (17.1 IU/mL), the CVs were 8.8% and 9.4%, respectively.

Fig. 2. Log scale distribution of anti-dsDNA values obtained with the EliA dsDNA assay in the different patient groups (SLE, systemic lupus erythematosus; SSc, systemic sclerosis; pSS, primary Sjögren syndrome; RA, rheumatoid arthritis; ID, infectious diseases; HC, healthy controls).

Anti-dsDNA Antibody and Renal Involvement

With the EliA dsDNA assay, anti-dsDNA antibodies were more frequently detected in patients with renal involvement (76%) than in patients without renal involvement (58%). No such differences emerged using the in-house ELISA (65.4% and 63.5%, respectively) and CLIFT (both 55%) methods.

The mean antibody levels detected by EliA dsDNA were significantly higher ($P < 0.05$) in patients with renal involvement (87.6 ± 141.2 IU/mL) in comparison to patients without renal involvement (46.6 ± 74.3 IU/mL). In contrast, no significant differences were found with the in-house ELISA method (133.2 ± 258 vs. 100 ± 133 , respectively) (Fig. 3).

Avidity of Anti-dsDNA Antibodies

To verify the putative ability of EliA dsDNA to detect only high-avidity IgG antibodies, we determined the Kaa of anti-dsDNA antibodies in the sera of 64 EliA anti-dsDNA positive SLE patients: five (7.8%) had low-avidity ($Kaa < 30$ L/U), 30 (46.9%) had intermediate-avidity ($Kaa 30-80$ L/U), and 29 (45.3%) had high-avidity ($Kaa > 80$ L/U) anti-dsDNA antibodies. These data demonstrate that EliA dsDNA reveals mainly, but not only, high-avidity antibodies.

DISCUSSION

The measurement of anti-dsDNA antibodies is largely used for the diagnosis and monitoring of SLE, but the type of assay used crucially influences the results in terms of sensitivity and specificity. The ELISA is

Fig. 3. Mean anti-dsDNA concentration in SLE patients with renal involvement (RI) and without renal involvement (NRI) as measured by the in-house ELISA method (gray column) and EliA dsDNA (white column). A significant difference emerged only with the EliA dsDNA method.

becoming the prevalent method used in routine laboratory practice because it is automated and expresses a quantitative result; however, it tends to produce more false positives compared to the Farr assay and CLIFT, which reduce the PPV of the test (13). The reasons for these false positives include insufficient purification of the antigen employed; DNA molecule alteration during the coating of the microtiter plates, resulting in the exposure of binding sites for anti-single-stranded DNA (anti-ssDNA) antibodies (8,14); and even the possible presence of aspecific bonds with the plastic itself (15,16). Moreover, compared to other methods, the ELISA techniques also evidence low-avidity anti-dsDNA antibodies. On the one hand, this may contribute to their higher sensitivity, but on the other hand it reduces their clinical specificity, because low titers of low-avidity anti-dsDNA antibodies may also be present in clinical conditions other than SLE (8). However, despite recent remarkable advances in the accuracy of anti-dsDNA antibody assay due to improvements in antigen purification techniques, the use of plasmid DNA, and optimization of the coating procedures (9,17,18), little has been done regarding the ability of these techniques to detect only intermediate- and high-avidity antibodies.

We evaluated the analytical and clinical performance of ELiA dsDNA, a new fluoroimmunoenzymatic automated system for the assay of anti-dsDNA antibodies. Because it employs recombinant plasmid dsDNA bound directly to the plastic surface, it should guarantee a greater analytical specificity than the traditional ELISA method. Moreover, to increase clinical specificity and limit the formation of bonds by low-avidity antibodies, the antigen-antibody reaction is performed at 37°C in the presence of buffer with high ionic strength. Our findings showed that while the ELiA dsDNA test had the same sensitivity as our in-house ELISA method, it was more specific (95.8% vs. 93.3%). It was less specific than CLIFT (99.2%), which was confirmed to have low sensitivity (55%). Nonetheless, in the control subjects who were positive with ELiA dsDNA and negative with CLIFT, anti-dsDNA antibody titers were consistently low and close to the threshold value. This means that

the greater specificity of the CLIFT assay derives from a lesser analytical sensitivity, rather than a different ability to discriminate between antibodies of diverse avidity.

The qualitative and quantitative findings obtained in patients with renal involvement are also interesting. Indeed, compared to the other methods utilized in this study, the ELiA dsDNA assay detected anti-dsDNA antibodies in patients with renal involvement more frequently and with a significantly higher mean titer compared to patients without this complication. If the high-avidity antibodies correlate better with SLE nephropathy, as described by several authors (19–22), then the above observations may constitute indirect proof that the ELiA dsDNA assay is able to detect mainly high-avidity antibodies even if they are present in low titer.

In any case, the measurement of antibody avidity in patients who tested positive with the ELiA dsDNA method demonstrated that high-avidity antibodies were mostly, but not exclusively, detected. For this reason, the ELiA dsDNA test results are not completely similar to those obtained with the Farr assay. However, in clinical laboratories in which the Farr assay cannot be employed, the good sensitivity, high specificity, analytical accuracy, high degree of automation, and simplicity of execution of the ELiA dsDNA test make it particularly appropriate for both the diagnosis and the monitoring of SLE patients.

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TABLE 2. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of anti-dsDNA antibody measurement as obtained with the three different methods

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
EliA dsDNA	64	95.8	93.8	72.7
ELISA in house	64	93.3	90.6	72.3
CLIFT	55	99.2	98.5	68.8

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