Anti-dsDNA Antibody Assay: High Specificity and Sensitivity with a Filtration Radioassay in Comparison to Low Specificity with the Standard ELISA

LIPING YU, JIAN WANG, JAMES R. O'DELL, JAMES OATES, WILLIAM P. AREND, and GEORGE S. EISENBARTH

ABSTRACT. ***Objective.*** To evaluate whether a new fluid-phase filtration radioassay possesses both high sensitivity and specificity compared with the currently used ELISA and Farr assays.

Methods. Sequential sera (25 samples) from 9 patients with systemic lupus erythematosus (SLE), sera from 20 patients with SLE possessing anti-dsDNA antibodies by the Crithidia assay, 75 patients with rheumatoid arthritis possessing rheumatoid factors, 50 healthy control subjects, 767 from patients with type 1 diabetes, and a commercial standard serum sample were tested for anti-dsDNA antibodies with the 3 different assays.

Results. Of serial dilutions of a standard anti-dsDNA antibody sample, only the highest positive sample (50 IU/ml) in the ELISA and the highest 2 positive samples (50 and 25 IU/ml) in the Farr assay were above the normal range. In contrast, all dilutions (to 2.5 IU/ml) of the standard anti-dsDNA antibody sample were above the normal range in the filtration radioassay. Using the values of 50 healthy control subjects in each assay to define the normal range, all 25 sequential sera from 9 patients with SLE were positive. In addition, 20/20 of the SLE individual sera, 2/75 (2.7%) of the RA sera, and 12/767 (1.6%) of the diabetes sera were positive (signal above normal range) in the filtration radioassay. The SLE sera were further examined in 2 additional assays, ELISA and Farr assay, and both assays were less sensitive and specific compared with the filtration radioassay.

Conclusion. The fluid-phase filtration radioassay demonstrated high sensitivity and specificity for the detection of anti-dsDNA antibodies in SLE, with the standard ELISA exhibiting lower specificity. We suggest that testing for anti-dsDNA antibodies can be improved using a fluid-phase filtration radioassay in comparison to commercial assays. (J Rheumatol 2007;34:734–9)

Key Indexing Terms:
ANTI-dsDNA SYSTEMIC LUPUS ERYTHEMATOSUS RADIOASSAY
FARR ASSAY ENZYME LINKED IMMUNOSORBENT ASSAY

Systemic lupus erythematosus (SLE) is the prototypical autoimmune disease, affecting almost every organ of the body. Many autoantibodies are found in the circulation of patients with SLE, particularly autoantibodies to nucleic acids and to proteins associated with nucleic acids[1,2]. Of these autoantibodies, those against dsDNA have been found to be strongly associated with SLE. Even individuals without SLE at the time that high avidity anti-dsDNA antibodies are detected in their circulation are prone to subsequently develop SLE[3]. Fluctuations in the level of anti-dsDNA antibodies in an individual patient may give important information on the clinical status. Therefore, accurate determination of the presence of anti-dsDNA antibodies is of considerable help to the clinician and is included in the classification criteria for SLE[4].

Anti-dsDNA antibodies are commonly measured by commercially available kits such as an enzyme linked immunosorbent assay (ELISA) or a radioimmunoassay kit (RIA; the Farr technique). However, the prognostic value of anti-dsDNA antibodies is controversial and discrepancies exist between the various assays[3,5-8]. A series of workshops evaluating assays to detect autoantibodies to islet cell antigens in patients with type 1A diabetes aided the development of fluid-phase high-throughput filtration radioassays in the field of endocrinology[9-11]. These Protein A/G Sepharose filtration radioassays are usually performed in 96-well filtration plates and are both more sensitive and more specific compared to ELISA and to other assays utilizing nonspecific precipitants

From the Barbara Davis Center for Childhood Diabetes and the Division of Rheumatology, School of Medicine, University of Colorado Health Sciences Center, Denver, Colorado; the Division of Rheumatology, University of Nebraska Medical Center, Omaha, Nebraska; and the School of Medicine, University of South Carolina, Charleston, South Carolina, USA.

Supported by the Immune Tolerance Network as autoantibody core laboratory (N01-AI-95380).

L. Yu, MD, Research Associate; J. Wang, MD, Post-doctoral Fellow; G.S. Eisenbarth, MD, PhD, Professor, Barbara Davis Center for Childhood Diseases, University of Colorado Health Sciences Center; W.P. Arend, MD, Professor, Division of Rheumatology, University of Colorado Health Sciences Center; J.R. O'Dell, MD, Professor, Division of Rheumatology, University of Nebraska Medical Center; J. Oates, MD, Professor, Department of Medicine, Medical University of South Carolina.

Address reprint requests to Dr. G.S. Eisenbarth, Barbara Davis Center for Childhood Diabetes, UCHSC, B-140, PO Box 6511, Aurora, CO 80045, USA. E-mail: george.eisenbarth@uchsc.edu

Accepted for publication December 6, 2006.

such as polyethylene glycol. We adapted this high-throughput fluid-phase filtration radioassay format for anti-dsDNA antibody testing and compared it with 2 available commercial kits, ELISA and RIA (Farr assay).

MATERIALS AND METHODS

Subjects. Twenty serum samples of patients with SLE were kindly provided by Dr. M. Reichlin (University of Oklahoma Medical Center, Oklahoma City, OK, USA) from an anonymous coded serum bank of SLE sera. These 20 SLE sera were from patients with an age range of 14 to 48 years, 17 female and 3 male. All 20 sera were positive for anti-dsDNA antibodies by the Crithidia assay. In addition, 25 sequential serum samples of 9 patients with SLE were obtained from the University of South Carolina with anonymous codes, all women aged 18–54 years, disease duration 1–25 years, and sequential followup 0.5–2 years. Fifty serum samples were obtained from healthy individuals with an age range of 11–25 years, 30 female and 20 male. Seventy-five serum samples were from patients with rheumatoid arthritis (RA), all rheumatoid factor (RF)-positive by nephelometry. The patients with RA were aged 24-79 years, 58 female and 17 male, and had disease duration of 1.5–504 mo. We obtained 767 serum samples from patients with type 1A diabetes at the Barbara Davis Center, with informed consent and institutional review board approval. In addition, 6 commercial serial serum calibrators (Diagnostic Products Co., Los Angeles, CA, USA) containing 0, 2.5, 5, 12.5, 25, and 50 IU/ml of anti-dsDNA antibodies by the Farr assay were used in each assay.

ELISA. The ELISA was performed using the Kallestad anti-dsDNA antibody microplate kit (Bio-Rad Laboratories, Hercules, CA, USA). This kit uses 96-well microplates coated with calf thymus DNA with 10 μl of a 1:1000 dilution of the test serum, and 4 calibrators containing 10, 50, 150, and 300 IU/ml of anti-dsDNA antibodies. The ELISA is developed with an alkaline phosphatase-conjugated murine monoclonal antibody to human IgG and IgM.

Farr assay. The Farr assay was performed with a commercial kit (Diagnostic Products Co., Los Angeles, CA, USA) following the standard protocol. In brief, 25 μl of serum was mixed with 200 μl of ^{125}I-DNA, incubated for 2 h at 37°C in a water bath. One milliliter of cold ammonium sulfate was added and thoroughly mixed by vortexing. The mixture was centrifuged at 2000 × g for 15 min at 4°C, the supernatant was removed, and the precipitate was counted in a gamma-counter.

Filtration radioassay. A 96-well plate (Corning) was precoated with washing buffer. Fifteen microliters of serum were incubated with 100 μl of ^{125}I-DNA from the above Farr assay kit for 2 h at 37°C. One hundred microliters of 50%/8% (v/v) Protein A/G-Sepharose (Pharmacia) in washing buffer [20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.1% BSA, 0.15% Tween-20, and 0.1% sodium azide] was added to each well. The plate was shaken at a low speed for 45 min at 4°C, followed by 2 cycles of 4 washes per cycle with cold washing buffer using the Millipore vacuum-operated 96-well plate washer. After washing, 50 μl of scintillation liquid (Microscint-20; Packard) was added to each well, and radioactivity was determined directly in the 96-well plate with a Top-Counter (96-well plate ß-counter; Packard) scintillation counter. The assay procedure is detailed in Figure 1. The result was calculated using a positive control (the highest standard sample from the Farr assay kit) and a negative control, and was expressed as an index: index = (sample_cpm – negative control_cpm)/(positive control_cpm – negative control_cpm). The mean and standard deviation (SD) of 50 normals were 0.007 ± 0.007. The limit of normal (0.028) was chosen as the 100th percentile of 50 healthy control subjects and it is also equal to mean + 3 SD.

RESULTS

Filtration radioassay. Twenty serum samples from patients with SLE possessing anti-dsDNA antibodies by the Crithidia assay, 50 serum samples from healthy control subjects, 75 serum samples from RF-positive patients with RA, 767 serum samples from patients with type 1A diabetes, and standard calibrators from an RIA anti-dsDNA antibody assay kit (Diagnostic Products Co.) were analyzed for anti-dsDNA antibodies using the filtration radioassay. As shown in Figure 2, 100% (20/20) of the SLE sera were positive, above the highest value (index of 0.028, or 3 SD above the mean value) of 50 healthy control subjects. Only 2.7% (2/75) of the RA sera and 1.6% (12/767) of the diabetes sera were positive (> 0.028), all at low levels. In addition, 25 longitudinal followup samples from 9 patients with SLE were analyzed and the data were plotted in Figure 3. All clinical visits (25/25) from 9 patients were positive for anti-dsDNA antibodies using the filtration radioassay, and the levels of anti-dsDNA antibodies varied more or less among the different patients.

To compare the filtration radioassay to currently used methods, these same samples were analyzed in parallel using 2 commercial kits: an ELISA kit from Bio-Rad and a Farr assay kit from Diagnostic Products Co. The test results for these 2 methods are plotted in Figure 4. Using either the ELISA kit or the Farr assay kit, 19/20 of the SLE sera were positive, while 1/75 and 0/75 of the RA sera were positive in the ELISA and Farr assay, respectively. Of the 12 diabetes sera positive in the filtration radioassay, only 1 was positive in the ELISA and 1 in the Farr assay. For 25 longitudinal followup samples from 9 patients with SLE, as shown in Figure 3, the pattern of variation of antibody levels was comparable between 3 assays (Figure 3A), but 7/25 sera were negative for ELISA (Figure 3B) including the first 2 visits of Patient 2, first visit of Patient 5, and all 4 visits of Patient 8, while 4/25 were negative for Farr assay (4 visits of Patient 8). In both ELISA and Farr assays, only the least diluted (50 IU/ml) or next to least diluted (25 IU/ml) standard was positive (above the normal range) of the 5 serial samples for the respective assay. The dilution curve from standard samples is illustrated in Figure 5, with the defined normal ranges using the highest value from the same 50 healthy control subjects in each assay, respectively. The lowest positive sample (2.5 IU/ml) was clearly positive with the filtration radioassay. Using the cutoff values for positivity provided with each kit rather than the normal range of the 50 control subjects, the ELISA (normal range ≤ 30 IU/ml) was positive with 10/50 healthy control subjects, while the Farr assay (normal range ≤ 15 IU/ml) had no false positives (Figure 4).

The levels of anti-dsDNA antibodies detected with the filtration radioassay and the Farr assay were well correlated (R^2 = 0.52) among the 45 SLE sera, but correlations between filtration assay and ELISA and between Farr assay and ELISA were poor (R^2 = 0.27, 0.33, respectively). The correlations of autoantibody levels for SLE sera and normals between these 3 assays are plotted in Figure 6.

DISCUSSION

We evaluated a new Protein A/G high-throughput filtration radioassay for measurement of anti-dsDNA antibodies. In order to compare the results of the filtration radioassay with

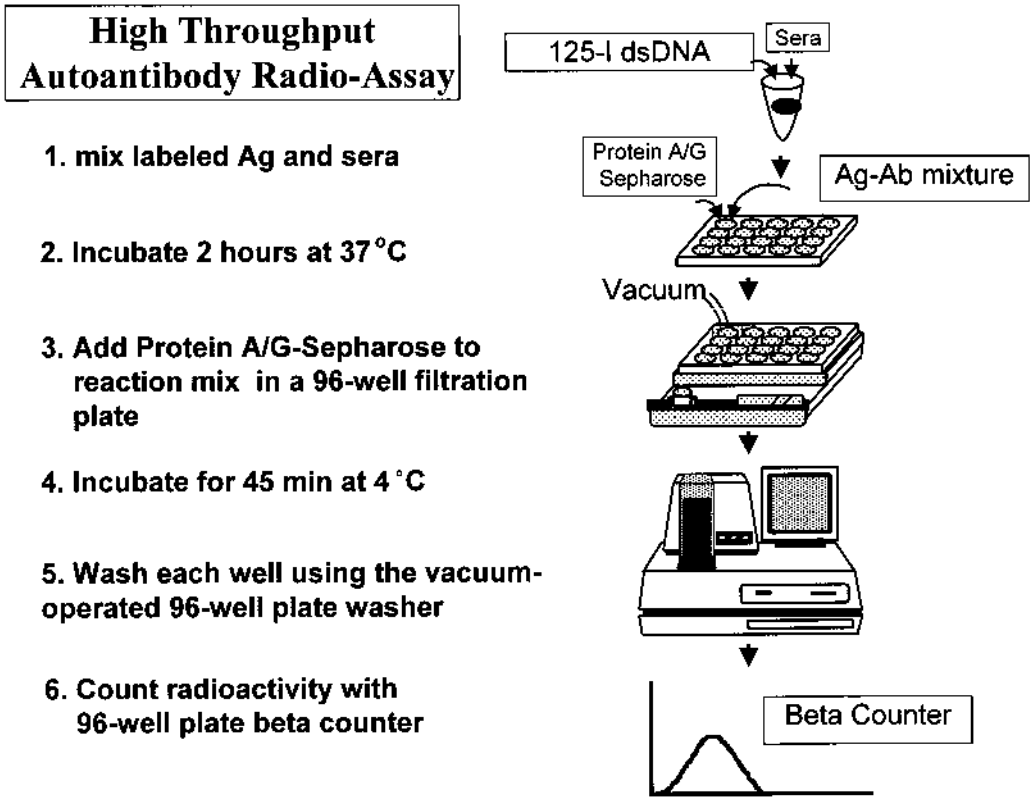

Figure 1. Procedure of the fluid-phase filtration radioassay.

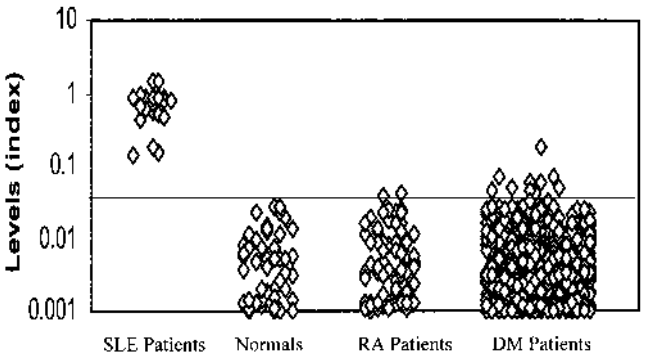

Figure 2. The results of filtration radioassay for anti-dsDNA antibodies for patients with SLE (n = 20), healthy controls (n = 50), patients with RA (n = 75), and patients with type 1A diabetes (DM; n = 767).

the conventional Farr assays and ELISA, 2 sets of SLE serum samples were studied, i.e., 25 sequential followup samples from 9 patients with SLE and 20 individual SLE sera possessing anti-dsDNA antibodies by the specific Crithidia assay. The filtration radioassay detected as positive 100% (25/25 and 20/20) of the patients with SLE at levels above the highest index of 50 healthy normal controls. The Farr assay and the ELISA detected 21/25 and 18/25 of sequential followup samples, respectively, and both detected 19/20 of individual SLE samples. With serial dilution of a standard positive sample, the filtration radioassay could detect the lowest titer of 2.5 IU/ml as above the 50 controls, while the ELISA detected only the least diluted standard (50 IU/ml) as above the upper limit of 50 normal controls, and the Farr assay detected only 50 and 25 IU/ml. The ELISA had an unacceptable false-positive rate in normal sera (20%) using the kit cutoff.

To confirm the specificity of the anti-dsDNA filtration radioassay, we analyzed 75 serum samples from RF-positive patients with RA. We also analyzed 767 serum samples from patients with type 1A diabetes, among whom there were 30% (234/767) with thyroid autoantibodies and 10% (78/767) with transglutaminase autoantibodies (data not shown). The filtration radioassay demonstrated low levels of positivity in the RA sera (2/75 or 2.7%) and in the sera from patients with type 1 diabetes mellitus (12/767 or 1.6%). One sample from a diabetic patient was also positive in both the ELISA and Farr assay. The positivity of anti-dsDNA antibodies was not directly related to patient age or to disease duration, and was not related to thyroid autoantibodies or to tissue transglutaminase autoantibodies among these patients (of 12 anti-dsDNA antibody-positive, only 5 had the other antibodies; data not shown).

Major discrepancies have been described between several currently used assays for anti-dsDNA antibodies. A study with a panel of monoclonal antibodies to dsDNA found a high percentage of false positives in ELISA assays[12,13]. It was sug-

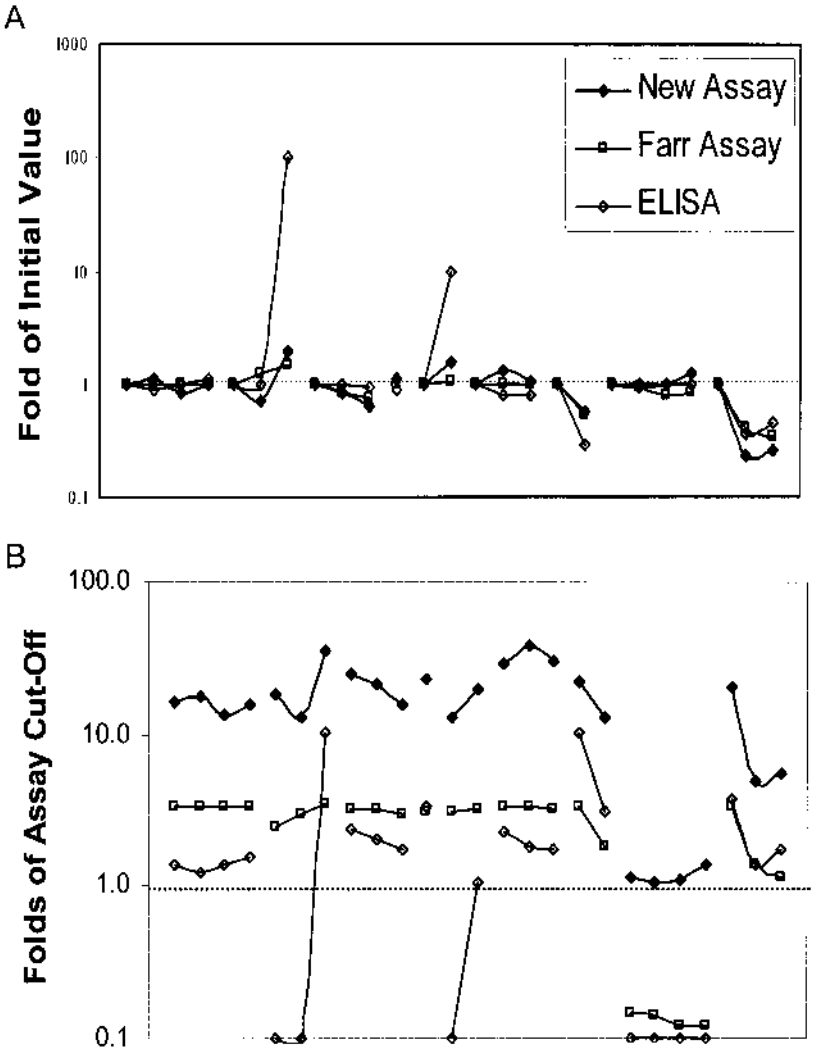

Figure 3. The results of filtration radioassay, Farr assay, and ELISA for anti-dsDNA antibodies on 25 longitudinal followup samples from 9 patients with SLE. The unit of Y-axis is expressed as the fold of the value of first sample in Figure 3A to show the pattern of variation of antibody levels, and the unit of Y-axis in Figure 3B is the fold of assay cutoff to show the antibody levels from each assay.

gested that reactivity detected in an anti-dsDNA antibody ELISA should be confirmed in another anti-dsDNA assay before being reported[3]. The Farr assay has been known to be more specific but less sensitive. In order to achieve a better sensitivity and specificity, some laboratories have relied on combinations of 2 or more of those different assays, with one assay used for screening and another for confirmation[3]. In our study, we demonstrated that a filtration radioassay achieves both high sensitivity and high specificity with high throughput efficiency using 96-well filtration plates. However, the filtration radioassay has the disadvantage of using low levels of radioactivity. In addition, the presence of rheumatoid factors may confound the results of some ELISA, but did not influence the results for anti-dsDNA antibodies detected by the filtration radioassay.

In previous studies, discrepancies observed between the various assays could only be explained in part by differences in assay sensitivity. A major determinant for these differences appears to be the isotype and avidity of the autoantibodies[14-16]. The Farr assay detects primarily high avidity IgG antibodies, while the ELISA detects both low avidity IgM antibodies and low and high avidity IgG antibodies. High avidity IgG anti-dsDNA antibodies are more specifically related to the pathogenesis of disease manifestations in SLE. Low avidity IgM anti-dsDNA antibodies are found in many normals, and patients possessing only low avidity IgG anti-dsDNA antibodies exhibit a mild disease course with absence of renal involvement[17,18]. Low avidity anti-dsDNA antibodies also occur in autoimmune diseases other than SLE. However, patients with only low avidity anti-dsDNA antibodies may still have or develop SLE. The fluid-phase filtration radioassay described in our report

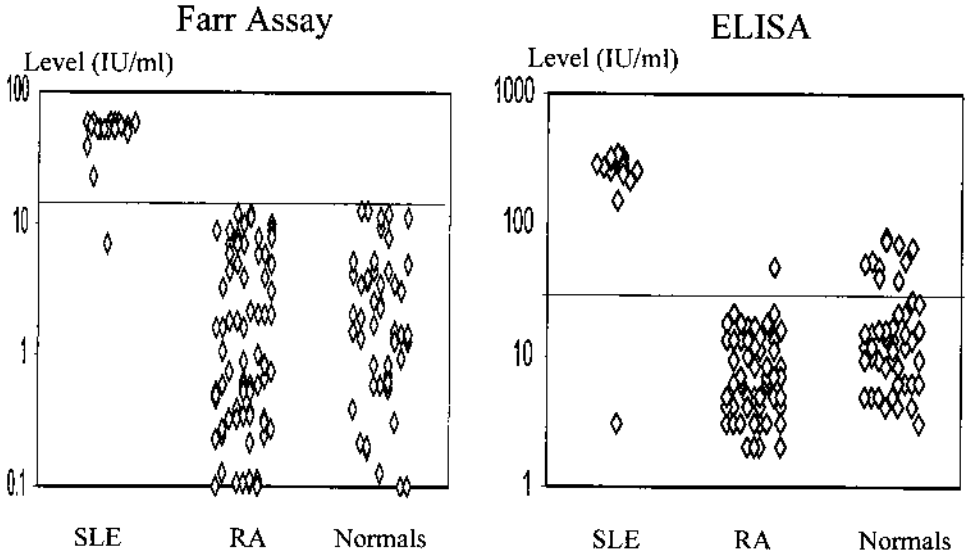

Figure 4. The results of Farr assay (left) and ELISA (right) for anti-dsDNA antibodies for patients with SLE (n = 20), patients with RA (n = 75), and healthy controls (n = 50).

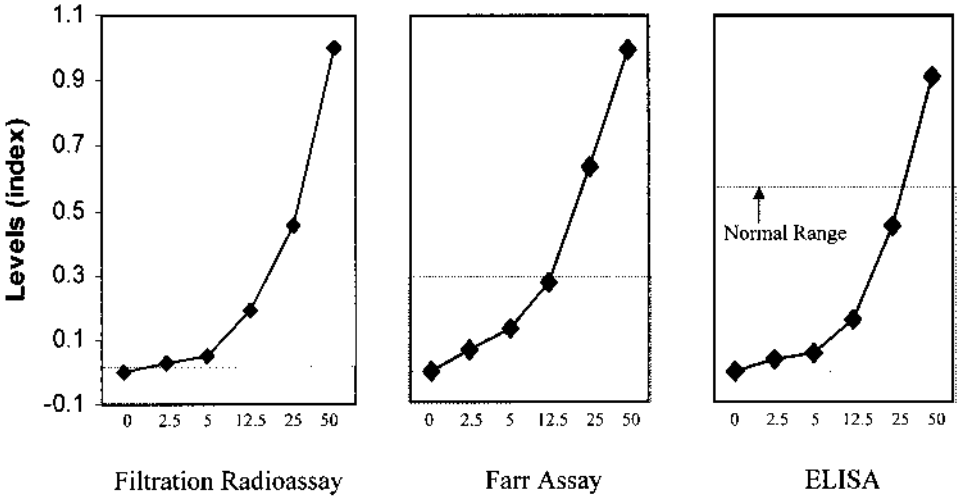

Figure 5. The dilution curves of 6 standard samples (0, 2.5, 5.0, 12.5, 25, 50 IU/ml) for anti-dsDNA antibodies from 3 different assays in this study: filtration (protein A/G) radioassay, Farr assay, and ELISA. The upper limit of normal range of 50 healthy controls for each assay is marked with a broken line.

probably detects both high and low avidity IgG antibodies to dsDNA. It is suggested[15] that a broad spectrum method with high sensitivity should be used for screening, followed by the determination of low/high avidity of autoantibodies to help define the likely disease course or to estimate the risk of developing disease.

The levels of anti-dsDNA antibodies detected with this new filtration radioassay were correlated with levels from the Farr assay. The apparent superior results with the fluid-phase filtration radioassay were not unexpected compared to the ELISA, given the results of multiple anti-islet autoantibody workshops, where the preferred assay methodology is analogous to that which we utilized (ELISA and polyethylene glycol based precipitation radioassays are not commonly used to measure antibodies to islet cell antigens[11,19]). Similar workshops with analysis of large sets of blinded sera from well characterized patients are essential to more completely evaluate new assays for anti-dsDNA antibodies such as we have described. Additional assays for SLE associated autoantibodies have been adapted to similar filtration radioassays[20], and a combination of such assays may enhance the diagnosis and management of SLE.

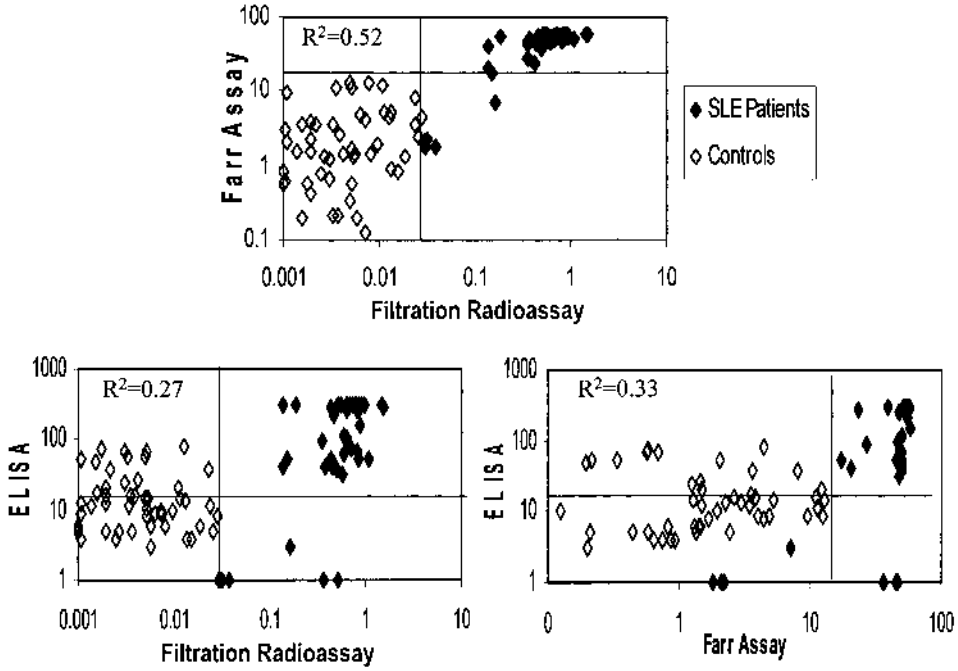

Figure 6. Correlations of anti-dsDNA autoantibodies in patients with SLE and controls between the 3 assays. The correlation coefficients for these 3 comparisons were: (top panel) Farr assay versus filtration radioassay, $R^2 = 0.52$; (left panel) ELISA versus filtration radioassay, $R^2 = 0.27$; and (right panel) ELISA versus Farr assay, $R^2 = 0.33$.

REFERENCES

1. Smeenk RJT, Westgeest AAA, Swaak AJG. Antinuclear antibody determination: the present state of diagnostic and clinical relevance. Scand J Rheumatol Suppl 1985;56:78-92.
2. Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol 1989;44:93-152.
3. Smeenk RJT, Brink HG, Brinkman K, Termaat RM, Berden JHM, Swaak AJG. Anti-dsDNA: Choice of assay in relation to clinical value. Rheumatol Int 1991;11:101-7.
4. Tan EM, Cohen AS, Freis JF, et al. The 1982 American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
5. Ward MM, Pisetsky DS, Christensen VD. Anti-double stranded DNA antibody assays in systemic lupus erythematosus: Correlations of longitudinal antibody measurements. J Rheumatol 1989;16:609-13.
6. Esdaile JM, Abrahamowicz M, Joseph L, MacKenzie T, Li Y, Danoff D. Laboratory tests as predictors of disease exacerbations in systemic lupus erythematosus. Why some tests fail. Arthritis Rheum 1996;39:370-8.
7. Esdaile JM, Joseph L, Abrahamowicz M, Li Y, Danoff D, Clarke AE. Routine immunologic tests in systemic lupus erythematosus: Is there a need for more studies? J Rheumatol 1996;23:1891-6.
8. Swaak AJG, Smeenk RJT. Following the disease course in systemic lupus erythematosus: Are serologic variables of any use? J Rheumatol 1996;23:1842-4.
9. Verge CF, Stenger D, Bonifacio E, et al. Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combined islet autoantibody workshop. Diabetes 1998;47:1857-66.
10. Bonifacio E, Atkinson M, Eisenbarth G, et al. International workshop on lessons from animal models for human type 1 diabetes. Diabetes 2001;50:2451-8.
11. Bingley PJ, Bonifacio E, Mueller Pw. Diabetes Antibody Standardization Program: First assay proficiency evaluation. Diabetes 2003;52:1128-36.
12. Smeenk RJT, Brinkman K, Brink HG, Westgeest AAA. Reaction patterns of monoclonal antibodies to DNA. J Immunol 1988;140:3786-92.
13. Brinkman K, Termaat RM, Berden JHM, Smeenk RJT. The specificity of the anti-DNA ELISA: a closer look. J Immunol Methods 1991;139:91-100.
14. Smeenk R, Aarden LA. The use of polyethylene glycol precipitation to detect low avidity anti-DNA antibodies in systemic lupus erythematosus. J Immunol Methods 1980;39:165-80.
15. Smeenk R, Lelij G, Aarden LA. Avidity of antibodies to dsDNA. Comparison of IFT on Crithidia luciliae, Farr-assay and PEG-assay. J Immunol 1982;128:73-8.
16. Smeenk R, Lelij G, Aarden LA. Measurements of low avidity anti-dsDNA by the Crithidia luciliae test and the PEG-assay. Clin Exp Immunol 1982;50:603-10.
17. Smeenk R, Rooyen A, Swaak AJG. Dissociation studies of DNA/anti-DNA complexes in relation to anti-DNA avidity. J Immunol Methods 1988;109:27-35.
18. Winfield JB, Faiferman I, Koffler D. Avidity of anti-DNA antibodies in serum and IgG glomerular eluates from patients with systemic lupus erythematosus. Association of high avidity antinative DNA antibody with glomerulonephritis. J Clin Invest 1977;59:90-6.
19. Yu L, Eisenbarth GS, Bonifacio E, Thomas J, Atkinson MA, Wasserfall C. The second murine autoantibody workshop: Remarkable interlaboratory concordance for radiobinding assays to identify insulin autoantibodies in nonobese diabetic mice. Ann NY Acad Sci 2003;1005:1-12.
20. Yamamoto AM, Amoura Z, Johannet C, et al. Quantitative radioligand assays using de novo-synthesized recombinant autoantigens in connective tissue diseases: new tools to approach the pathogenic significance of anti-RNP antibodies in rheumatic diseases. Arthritis Rheum 2005;43:689-98.