A Critical Evaluation of Enzyme Immunoassay Kits for Detection of Antinuclear Autoantibodies of Defined Specificities. II. Potential for Quantitation of Antibody Content

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ABSTRACT. ***Objective.*** To analyze the performance of different commercial enzyme immunoassay (EIA) kits for measuring antibody levels of antinuclear antibodies (ANA) specific for double stranded (ds) DNA, SSB/La, Sm, and Scl-70.

Methods. Twenty companies that were known major purveyors of EIA kits for detection of ANA were approached to determine their interest and willingness to participate in this study. The manufacturers were advised that they would be sent coded sera containing mixtures of the Arthritis Foundation/Centers for Disease Control reference reagents, and that they were to use their own test kits to analyze the antibody specificities of these sera and to report the data, in optical density (OD) units, or their equivalent. The analysts were blinded to the concentration of the antibodies and the specificities.

Results. Initially, 11 manufacturers out of 20 agreed to participate, but 2 subsequently withdrew. The commercial EIA kits have the potential of being able to quantitate specific autoantibody content to ds-DNA, SSB/La, Sm, and Scl-70. However, certain deficiencies in these kits were also detected, the most obvious being lack of uniformly good performance, with kits of certain manufacturers showing exceptional accuracy in 3 out of 4 of their antibody-specific kits and poor accuracy for a 4th kit.

Conclusion. It is important for clinicians to appreciate that there is marked inter-manufacturer variation in the performance of EIA kits used as an aid in the diagnosis of systemic rheumatic diseases. Manufacturers need to exercise constant surveillance of kit performance and to provide assurance that such is being done. Improved EIA kits would lend themselves to reliable quantitation of antibody levels in human sera and help to determine whether serial measurement of antibody levels might be useful in monitoring disease activity. (J Rheumatol 2002;29:68–74)

Key Indexing Terms:
AUTOANTIBODIES AUTOIMMUNITY DIAGNOSIS DIAGNOSTIC KITS

A question of great importance to clinicians and investigators in the field of rheumatology is whether autoantibodies are involved in pathogenesis and might therefore be related to exacerbations and remissions of disease activity. Attention to this issue was raised by the initial observation that in systemic lupus erythematosus (SLE) patients' antibodies to DNA fluctuated with disease activity and, in some patients, DNA antigen and antibody appeared in sequence in the circulation, suggesting that immune complexes of DNA antigen and antibody were formed and might be involved in disease pathogenesis[1]. It was soon reported that the nephritic kidneys of patients with SLE contained antibodies to DNA in manifold higher concentration than antibodies in the blood[2]. This observation was followed by reports showing that SLE patients with high levels of antibodies to DNA and low levels of complement were more likely to have active disease, especially renal involvement, and it was suggested that serial immunochemical observations of these factors may be useful in management[3,4]. A major problem encountered in the further pursuit of these initial observations was that it was difficult to determine the

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level of specific autoantibodies in blood or serum, and reliable quantitation of antibody levels was clearly required to determine whether there was any relationship between fluctuations in antibody levels and disease activity.

The quantitation of antibodies to DNA was addressed by the development of an ammonium sulfate based immunoprecipitation assay (sometimes called the Farr assay), in which isotope labelled DNA was mixed with serum, and immune complexes forming between the labelled DNA and immunoglobulin were precipitated with ammonium sulfate[5]. The use of this assay or modifications of it resulted in many publications reporting the association of changing levels of DNA antibody with disease activity in SLE[6-9]. Interest in trying to equate antibody levels with disease activity has extended to antinuclear antibodies (ANA) of other specificities, including anti-Sm, anti-SS-A/Ro, and anti-SSB/La. In some of these studies, antibodies to purified native or recombinant protein antigens were detected by enzyme immunoassays (EIA), but in many earlier studies, methods such as immunoblotting and immunoprecipitation were used. The data have been conflicting, some studies reporting a good correlation between antibody levels and disease activity[10-18], and other studies did not find such a correlation[19-21].

This study was designed to determine whether the EIA-ANA kits produced by some of the major manufacturers in this field could be used for quantitation of antibody content. As described earlier[22], sera from the Centers for Disease Control and Prevention (CDC) in Atlanta that were relatively monospecific and rigorously defined in terms of antibody specificities were sent in a coded fashion to the manufacturers. The manufacturers were not informed that the set of sera they received would contain individual antibodies at different dilutions. The data received from these manufacturers were analyzed using standard statistical methods, described below.

MATERIALS AND METHODS

Twenty companies that were known major purveyors of EIA kits for detection of ANA were approached to determine their interest and willingness to participate in this study. The manufacturers were advised that they would be sent coded sera containing mixtures of the Arthritis Foundation/CDC reference reagents, and that they were to use their own test kits to analyze the antibody specificities of these sera and to report the data in optical density (OD) units or their equivalent. The ANA Subcommittee of the International Union of Immunological Societies Standardization Committee had previously analyzed the CDC reference sera and the consensus antibody specificities of the reference sera were reported[23,24]. The manufacturers were informed that this study was designed to critically evaluate the performance of EIA based methods for detection of autoantibodies, and that the data would be published as a comprehensive evaluation of this methodology without divulgence of the specific performance of any individual manufacturer, since the specific aims of the study were to evaluate performance of currently available EIA test kits for accuracy and potential for quantitation and not to score the performance of individual companies. A previous analysis concerning sensitivity and specificity has been published[2]. Initially, 11 manufacturers out of 20 agreed to participate, but 2 manufacturers subsequently withdrew. The 9 participating manufacturers were (in alphabetical order): Cambridge Life Sciences (Cambridge, UK), Elias (Freiburg, Germany), Helix Diagnostics (Sacramento, CA, USA), ImmunoConcepts (Sacramento, CA, USA), Imtec Immunodiagnostika (Zepernick, Germany), Incstar (Stillwater, MN, USA), Inova Diagnostics (San Diego, CA, USA), MBL (Nagoya, Japan), and Shield Diagnostics (Dundee, Scotland).

Design of test samples. Serum samples (Table 1) were prepared by a research technologist (M. Byrd, CDC) who possessed the only key to the coded samples. For each antibody specificity, for example anti-double stranded (ds) DNA in column 2, specimens were prepared that contained concentrations of anti-Sm, SSA/Ro, SSB/La, and Scl-70 of 4-fold, 2-fold, and 1-fold (4×, 2×, 1×). To achieve these dilutions, the specimens were mixed with CDC reference sera of other specificities. For example, the starting relative concentration of the anti-dsDNA serum was 8× and the anti-SSB/La serum was 4×. Sample A was obtained by combining equal volumes of anti-dsDNA and anti-SSB/La to achieve a mixture with final relative concentrations of 4× and 2×, respectively. Similarly, sample B was prepared to achieve final relative concentrations of 2× anti-dsDNA and 1× anti-Sm. CDC2 reference serum containing anti-SSB/La and CDC5 reference serum containing anti-Sm were formulated in various doubling dilutions and various mixtures with other sera. Samples containing anti-Scl-70 (column 5) were also mixed with other CDC standards containing anti-SSA/Ro and anti-nucleolar antibodies (not shown in this abbreviated table, but see Table 1 in reference 22). A concern whether antibody of one specificity might interfere with detection of antibody of another specificity was addressed in the previous analysis concerning accuracy of detection[22], which showed there was no interference of this kind, at least for the mixtures of antibody specificities reported in this study.

Report forms were prepared for the participants. For each test sample, manufacturers' laboratory personnel were requested to determine (in duplicate) optical densities at serum dilutions of 1:100, 1:400, 1:1600, and at the manufacturer's recommended dilution (if different). In addition, manufacturers were asked to indicate whether their kits gave positive or negative results for each antibody at the recommended dilution.

Statistical considerations: linear regression model. A common method employed for quantitation of levels of ANA found in serum samples is calibration, a kind of inverse prediction. The calibration process consists of 2 stages. First, using reference sera with known antibody concentrations, a standard curve is established, defining the relationship between OD and dilution (usually taken on a log scale). This relationship is often modeled by a 4-parameter logistic function, a very flexible model for data following a sigmoidal shaped curve[25,26]. In the second stage, serum samples with unknown concen-

Table 1. Key ot serum samples containing antinuclear antibodies.

Sample	CDC1 (dsDNA)	CDC2 (SSB/La)	CDC5 (Sm)	CDC9 (Scl-70)
A	4×*	2×	—	—
B	2×	—	1×	—
C	1×	—	—	—
D	—	4×	1×	—
E	—	2×	4×	—
F	2×	1×	—	—
G	—	1×	2×	—
H	—	—	4×	—
I	1×	—	4×	—
J	—	2×	1×	—
K	—	—	—	4×
L	—	—	—	2×
M	—	—	—	1×
O	—	—	—	1×
P	—	—	—	2×

* 4×, etc: Each column shows the relative concentration of the designated antibody. For example, Sample A contains 4 times and Sample B 2 times the concentration of anti-dsDNA relative to Sample C. CDC: Centers for Disease Control and Prevention, Bethesda, MD, USA.

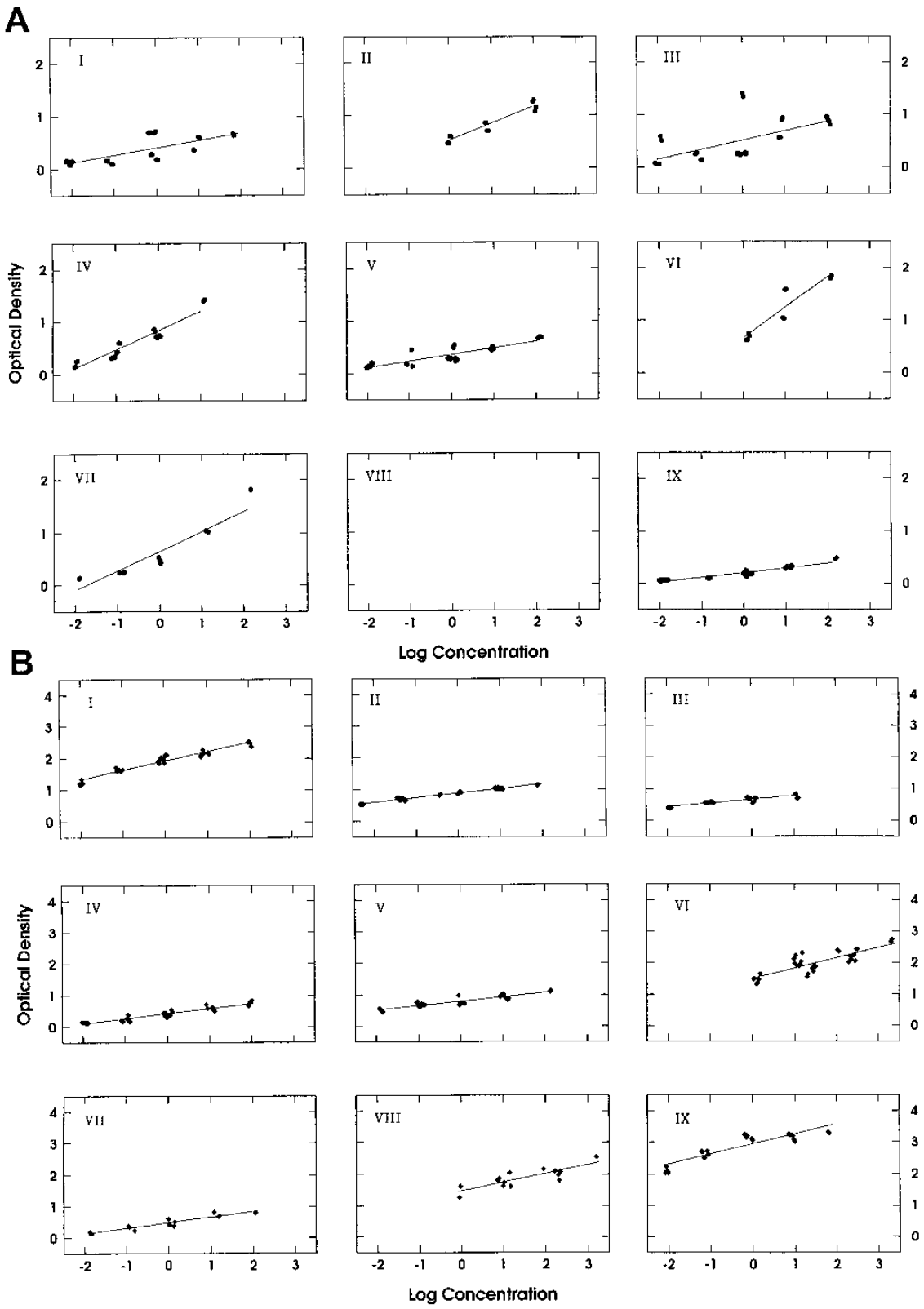

Figure 1. Observed data (dots), and fitted linear regressions (straight lines) of optical densities on log concentrations for the 9 manufacturers. Data for each manufacturer were chosen as detailed in the text. OD are untransformed. Logs of the antibody concentrations are to base 2, the standard being log concentration 0 for 1× sera (Table 1) at 1:100 dilution. Thus at 1:100 dilution, log concentration is 1 for 2× sera, and 2 at 4× sera. Log concentration is also 0 at 1:400 dilution for 4× sera, –1 for 2× sera, and –2 for 1× sera. The 9 manufacturers are denoted I through IX, as in Part I[22]. Manufacturer VIII did not report data for anti-dsDNA. A. Anti-dsDNA. B. Anti-SSB/La. C. Anti-Sm. D. Anti-Scl-70. To determine precision of an assay, both deviation about the standard regression line and slope of the line have to be considered as depicted in Figure 2.

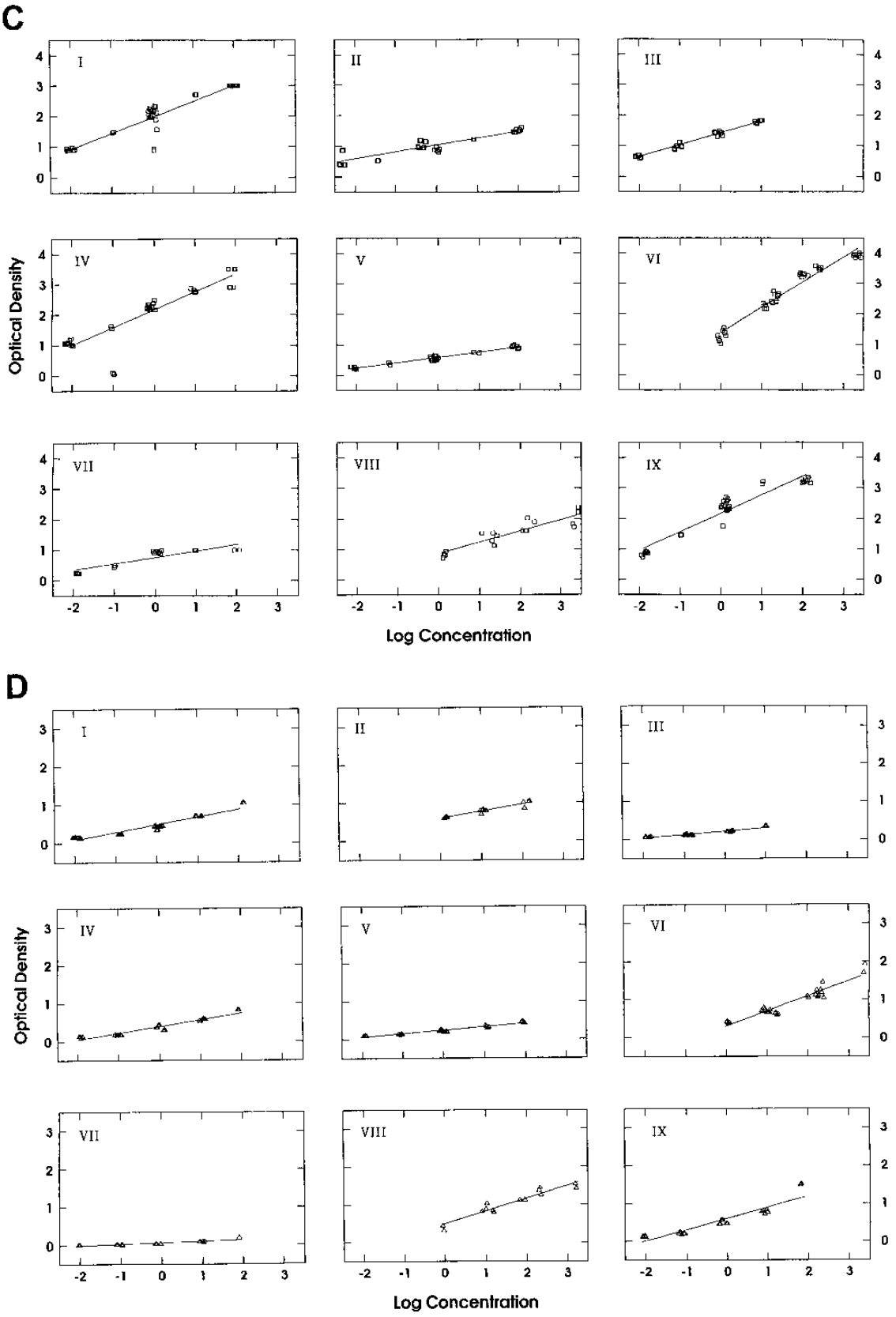

Figure 1 continued. C. Anti-Sm. D. Anti-Scl-70.

trations of antibodies are analyzed by the test method, and the antibody level of a test serum is estimated on the basis of its observed OD, using the standard curve (or its translation) as calibrator.

This estimation is somewhat simplified if the observed OD corresponds to the linear portion of the standard curve. The equation Y = aX + b is used, where Y = observed OD, X = (log) dilution of the test sample, a = slope, and b = intercept (where the slope crosses the Y axis), with the parameters a and b determined from the reference. For calibration, this equation is used to predict X (dilution for the test sample) from an observed value of Y with the test serum.

Precision factor f. The precision of this inverse prediction can be calculated by constructing a confidence interval (CI) for the predicted dilution X, given an observed OD (Y) of the test serum. The width of this CI is a measure of the precision of the inverse prediction, with a smaller (narrower) CI connoting increased precision of the estimated dilution X. The width of this CI is directly proportional to a factor called f, f being the ratio of the standard deviation about the regression line over the slope of that line[27]. That is, the precision of the predicted dilution is increased if the residual deviation about the standard linear regression line is decreased, or if the slope of the regression line is increased. Conversely, the precision of the estimate is decreased if the regression line fits poorly (large residual deviation), or if the slope is shallow. A value of f closer to zero denotes better precision and a value of f further from zero would denote poorer precision.

Analysis of test sera. The method for assessment of quantitated ELISA findings from the 9 commercial kits is based on the precision of calibration via linear regression described above. The analysis was restricted to anti-dsDNA, anti-SSB/La, anti-Sm, and anti-Scl-70. Data relating to other ANA specificities were either not consistently recorded by all manufacturers, or were not available for a suitably broad range of dilutions. The data were analyzed at the serum dilutions corresponding to the manufacturers' recommendations and the next closest dilution level from 1:100, 1:400, and 1:1600. For example, if (as was most commonly the case) the manufacturer's recommended dilution was 1:100, data were taken from 1:100 and 1:400 dilutions; if the manufacturer's recommended dilution was 1:40, data were taken from 1:40 and 1:100 dilutions, and so on. In the case of a particular antibody specificity, some laboratories by random allocation received replicate samples such as duplicates of samples C and I (containing anti-dsDNA) for laboratory IX. This would result in more data points for laboratory IX than for some other laboratories (see Figure 1, anti-dsDNA), and accounts for the different numbers of data points for each laboratory.

Anti-dsDNA, anti-SSB/La, anti-Sm, and anti-Scl-70. Concentrations of anti-dsDNA relative to CDC1, anti-SSB/La relative to CDC2, anti-Sm relative to CDC5, and anti-Scl-70 relative to CDC9 for all the serum samples containing these antibodies (Table 1) were analyzed. The following example describes the data analysis for a manufacturer whose recommended dilution level was 1:100. In this instance, we use this manufacturer's reported data from 1:100 and 1:400 dilutions. Relative to CDC1 (Table 1), anti-dsDNA is present at 4× concentration in Sample A, 2× concentration in Samples B and F, and 1× concentration in Samples C and I. We therefore set anti-dsDNA concentration at 1:100 dilution to be 4 in Sample A, 2 in Samples B and F, and 1 in Samples C and I. Correspondingly, anti-dsDNA concentration at 1:400 dilution was 1 in Sample A, 0.5 in Samples B and F, and 0.25 in Samples C and I. We next took logs to the base 2 of these concentrations. At 1:100 dilution, log concentration was 0 for 1× sera (C and I), 1 for 2× sera (B and F), and 2 for 4× sera (A). At dilution 1:400, log concentration was –2 at 1×, –1 at 2× sera, and 0 at 4× sera. These log concentration values (the Xs) were taken, together with the corresponding OD (the Ys) reported by the manufacturer, to fit the standard linear regression (Y = aX + b) as described above. Thus the anti-dsDNA data accrued solely in this instance from the manufacturer's results for Samples A, B, C, F, and I at dilution levels 1:100 and 1:400. Taking log concentrations almost invariably produced better fits to the data than the untransformed concentrations in terms of normalized residuals and reduced residual variation. This methodology was followed for each manufacturer and each of the 4 ANA, the standard being 1× sera at 1:100 dilution corresponding to log concentration 0. The precision factor f (standard deviation/slope) was calculated to quantitate the precision of the calibration for each manufacturer/ANA combination.

RESULTS

Figure 1A–D depicts the linear regressions of optical density on log concentration for each of the 9 manufacturers (denoted I through IX, as previously[22]) and for each of the 4 ANA. Clearly, there are differences between manufacturers, as well as between ANA, in terms of the goodness of fit of the individual regressions. Qualitatively, a good fit would be characterized by points clustered closely about the regression line, with little scatter, and no systematic deviations from the line. Kit III, for example, showed wide scatter of the observed points about the estimated line with anti-dsDNA (poor fit) shown in Figure 1A, yet little scatter about the lines with anti-SSB/La, anti-Sm, and anti-Scl-70 (good fit) shown in Figures 1B–D.

The calibration process was to estimate (log) concentration of antibody in a test serum from the observed OD, using these standard regressions. The precision of this estimation will be influenced not only by the goodness of fit of the linear regression (less scatter is better), but also by the slope of the regression (steeper is better). As described in Materials and Methods, this precision in quantitation can be measured by the factor f (SD about regression line/slope), and this determination is shown in Figure 2, which affords simultaneous comparison of the individual kits with each of the 4 ANA. It is clear, for example, that quantitation with kit III was good, with the notable exception of anti-dsDNA; indeed, quantitation of anti-dsDNA seemed problematic also with kit I. From Figure 2 it can be ascertained that quantitation of anti-dsDNA was least precise for 3 kits (I, III, and V), since these kits showed higher f values than others. Anti-Sm was less precise for 3 kits (II, IV, VII) and more precise with 4 other kits III, V, VI, and IX. Anti-SSB/La was most precise with 3 kits I, II, and VII. Generally, quantitation of anti-Scl-70 seemed uniformly good with all the kits.

DISCUSSION

Similar to the result in analysis of the sensitivity and specificity of the EIA-ANA test kits[22], the performance of the different test kits for antibody quantitation varied from one manufacturer to another. However, there were no statistically significant differences among the manufacturers in terms of overall precision of calibration, in contrast to the subgroupings that were established in terms of reproducibility, sensitivity, and specificity in the previous analysis[22]. It is possible that some of the variations in performance by certain manufacturers may not be related to common underlying factors. For example, manufacturer III showed good precision of calibration when antibodies to SSB/La, Sm, and Scl-70 were measured, but unusually poor precision with antibodies to dsDNA. Somewhat similarly, but to a lesser extent, the same held true for manufacturer I (Figure 2). These aberrations

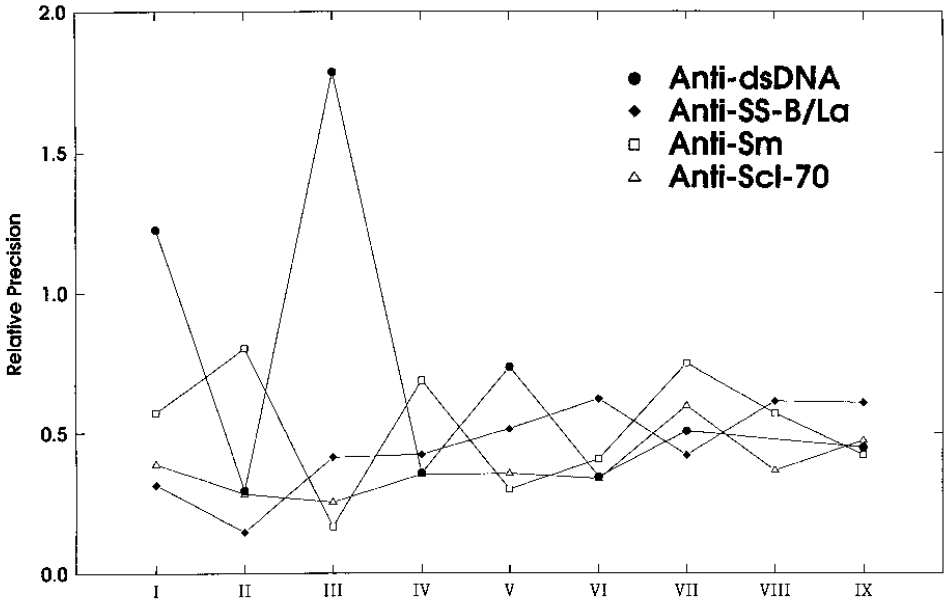

Figure 2. Precision factor f (SD about the standard regression/slope of the standard regression line) from each standard regression line in Figure 1. The precision factors are given separately by manufacturer — I through IX, as in Part I[22] — and by antibody. Factors f closer to zero connote more precise calibration than factors f further from zero. Points are connected so it is easier to visualize the degree of precision of different EIA kits for a particular antibody.

could be related to faulty kits on the one hand, which would constitute an important defect in manufacturing. On the other hand, these aberrations could be "outliers" and if not a defect in manufacturing, it could be related to operator inexperience. Whether it is the one or the other factor, and possibly even others, might be answered by subsequent analysis of the performance of these same kits in the hands of other operators. It is worthwhile to point out that 5 manufacturers (II, IV, V, VII, and IX) produced data with their test kits that showed relatively good precision for quantitation of anti-dsDNA antibody.

Evidence of variances of this nature was also detected in the quantitation of antibodies to Sm antigen and to a somewhat lesser extent for antibodies to SSB/La. With perhaps one exception (manufacturer VII), quantitation of antibodies to Scl-70 was remarkably good, with very little variation in relative precision from one manufacturer to another.

The data indicate that with the enzyme immunoassay method, quantitation of antibody content in sera can be precise, because for any one of the 4 antibodies, there were several manufacturers showing acceptable precision. Together with analysis of sensitivity and specificity of these same EIA kits[22], it appears that several of the currently available commercial kits have attained satisfactory levels of performance. This conclusion was also reached by a recent study comparing EIA kits with other immunoassays[28], with the added observation that a continuing problem might be the high number of inconclusive results because of problems determining the borderline between positive and negative tests. It should be noted that borderline test results present a dilemma for the clinical laboratory and clinician alike. To err on the side of caution, many consider the test positive until proven otherwise or until the clinical features of the patient clearly suggest a specific diagnosis.

This study shows that some EIA kits could also be used to quantitate antibody content and therefore, serial determinations of a particular antibody can be carried out on patients with the aim of determining whether there is a relationship between antibody fluctuation and disease activity. The availability of such assay systems was heretofore restricted to some research laboratories that could set up their own immunoassay systems. The availability of commercial EIA kits for measuring ANA levels would permit studies of this kind to be carried out by more clinical centers and hopefully arrive at more definitive conclusions regarding relationship of antibody levels to disease activity and help resolve some of the conflicting data discussed in the introduction.

Several important caveats should be carefully considered by potential users of EIA-ANA tests for this purpose. These include data supplied by the manufacturers themselves, that their kits are performing accurately and that the performance of one batch of kits is comparable to previous batches, since studies of this kind will involve serial samples of sera collected over time. It is clear that the manufacturers will need to exercise surveillance of kit performance and maintain high standards of quality control. As suggested in the previous study[22], and reiterated here, manufacturers should be ready to provide information attesting that their kits have met acceptable performance standards and they have made improve-

ments in the kits that have been shown to have deficiencies. One very positive outcome of this study is that after the data were communicated to the manufacturers, responses were received indicating that deficiencies were subsequently corrected. Such information should be made available to purchasers of these kits.

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