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Precise Quantitation of Antinuclear Antibodies on HEp-2 Cells without the Need for Serial Dilution

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Using HEp-2 cells as a substrate, we developed a method to quantitate antinuclear antibodies (ANA) by comparing the green fluorescence intensity of unknown samples with that of calibrated standards. Intensity was then converted to international units per milliliter by reference to a standard curve. This method is accurate and precise around the cutoff for positivity (5 to 10 IU/ml) and therefore provides a reliable screening test for active, untreated systemic lupus erythematosus. Furthermore, the method can identify sera likely to contain autoantibodies commonly detected in ANA-positive sera (SS-A, SS-B, Sm, small nuclear ribonucleoprotein, Scl-70, and double-stranded DNA).

Although the test for antinuclear antibodies (ANA) is widely used and listed as one of the American College of Rheumatology criteria for the diagnosis of systemic lupus erythematosus (SLE) (11), its exact diagnostic role is not defined. We previously argued that it is best used as a screening test for systemic lupus erythematosus (SLE) (1, 5). Precision of measurement, particularly around the decision threshold of 7.5 World Health Organization (WHO) IU/ml (determined by the finding that <3% of a normal healthy population had ANA levels of >7.5 IU/ml [4]) is critical, and small changes in ANA measurement will make a large difference in sensitivity, specificity, and predictive values.

Numerous studies and quality control programs demonstrate that laboratories vary greatly in their capacity to detect and measure ANA (2, 5, 7, 8, 15). There are differences in the threshold of detection, the range measured, precision, and accuracy. Improvement of interlaboratory comparisons for the homogeneous pattern is achieved when a common reference standard, such as the WHO homogeneous standard, is used (2). Surprisingly, few laboratories report results in international units based on the WHO standard or Centers for Disease Control and Prevention (CDC) reference preparations. Some argue that it is impractical and uneconomical to include quantitative standards for every variety of ANA in every routine assay. Others point out that the WHO standard is of a homogeneous pattern and that international units are not yet assigned to the CDC reference sera for other patterns (speckled and nucleolar).

We previously showed (by using a composite block of rodent tissue as a substrate and standards calibrated against the WHO international standard for homogeneous-pattern ANA) that homogeneous ANA can be estimated accurately and precisely over the range from 2.5 to 10 IU/ml and with acceptable precision within the range from 10 to 30 IU/ml without the need for titration by serial dilution (1). Use of a standard curve defined by secondary standards based on (but not using) the WHO standard for homogeneous ANA improves precision and assists in comparing results from different laboratories (5).

Some laboratories measure ANA by comparison with a single control after endpoint determination with doubling dilutions. Our previous studies show that endpoint determination is imprecise and error prone (1, 5). Measurement on a continuous scale above and below the decision threshold (7.5 IU/ml) is preferred over a simple dichotomy into positive and negative, because it recognizes the potential for classification of samples within the 5- to 10-IU/ml range. Further, it provides the clinical advantage of a continuum of specificity and predictive value related to the amount of ANA present. Quantitation of ANA by visual estimation is not, however, commonly used because of several putative shortcomings, including expected (but not demonstrated) problems in assessing ANAs of different patterns and mixtures, potential problems with differences in antibody affinities, and the presumed imprecision of visual estimation of fluorescence intensity by humans.

By using HEp-2 cells as a substrate (these cells give better quantitative interlaboratory agreement than rat liver or cultured fibroblasts [2]), we developed a method for estimating ANA activity by judgement of green fluorescence intensity (green fluorescence units) directed by a standard curve and supplemented by appropriate controls. We examined the accuracy and precision of this assay and its value as a screening test for SLE as well as for the presence of a variety of autoantibodies relevant to the diagnosis of connective tissue diseases.

MATERIALS AND METHODS

Preparation of primary and secondary standards. The WHO ANA homogeneous (WHO ANA H) primary standard was provided by the WHO in Geneva, Switzerland.

The Specialty Laboratories, Inc. (Santa Monica, Calif.), ANA homogeneous (SLI ANA H) secondary standard, a serum containing a high-titer homogeneous-pattern ANA (175 WHO ANA H IU/ml by endpoint titration and parallel curves) and double-stranded DNA (dsDNA) antibodies at a concentration of 240 IU/ml (Farr assay; Amersham Corporation, Arlington Heights, Ill.), was prepared by dilution in serum from an ANA-negative unit of plasma purchased from the American Red Cross. The ANA-negative plasma was clotted by recalcification (16) and thrombin, repeatedly precipitated and cleared by centrifugation, and sterilized by filtration and addition of 0.1% Kathon (a microbicide).

The Royal Perth Hospital (RPH [Perth, Western Australia, Australia]) ANA homogeneous (RPH ANA H) secondary standard was prepared from sera of SLE patients and calibrated against the WHO ANA H primary standard by using rodent tissue as substrate (1).

CDC preparations for ANA patterns and antibody reactivities. Arthritis Foundation (AF)-CDC reference preparations 1 to 9 (Table 1) were provided by the CDC (Atlanta, Ga.) (12, 13). These preparations were diluted in ANA-negative serum until each gave a green fluorescence intensity equivalent to 7.5 IU/ml (plus/minus positive or very subdued fluorescence) as defined by the WHO ANA

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TABLE 1. Patterns and specificities of AF-CDC reference preparations

Reagent	Immunologic feature(s)
AF-CDC1	Homogeneous-pattern ANA (dsDNA)
AF-CDC2	Anti-SS-B
AF-CDC3	Speckled-pattern ANA
AF-CDC4	Anti-U1 RNP (U1 snRNP)
AF-CDC5	Anti-Sm (U1, U2, U5, U4/6 snRNP)
AF-CDC6	Nucleolar-pattern ANA
AF-CDC7	Anti-SS-A
AF-CDC8	Centromere pattern ANA
AF-CDC9	Anti-Scl-70 (DNA topoisomerase I)

H primary standard. Concentrations were estimated by endpoint titration and the method of parallel curves.

ANA-negative and ANA-positive patient sera. Patient sera for ANA and ANA-related antibody testing were obtained from a store of consecutively received specimens submitted for ANA and autoantibody testing to Specialty Laboratories, Inc. (1,031 each of ANA-negative and ANA-positive serum specimens).

Controls. Negative controls were selected from units of donor plasma purchased from the American Red Cross. Remnant and donated serum samples served as positive controls.

ANA assay. Standards were diluted 1:40 with ANA-negative plasma. Patient sera are typically screened at a 1:40 dilution and read against the diluted standards. Hence, the reading obtained from the standards directly corresponds to the concentration of the patient sera, and no calculation is involved. Sera and standards can also be diluted in 1% goat serum in phosphate-buffered saline (PBS). Fixed monolayers of HEp-2 cells obtained from several commercial sources, fluorescein isothiocyanate-conjugated anti-human immunoglobulins, PBS at pH 7.6, and mounting medium were used according to the manufacturers' instructions.

Fluorescence microscopy employed a 100-mW mercury vapor lamp, an epi-illumination 350- to 380-nm excitation filter and a 440-nm barrier filter, 10× eye pieces, and a 20× objective and a 40× par objective, each with a numerical aperture of 0.85. Microscopy was undertaken under red-safety-light conditions after at least 10 min of dark adaptation. The microscope stage was modified to hold two slides at once so that it was convenient to move from wells exposed to patient sera on one slide to wells exposed to standard sera on the other multiwell slide. The 20× objective was used for quantitation and initial assessment of ANA pattern, and the 40× objective was used to confirm the pattern. Readers were instructed to estimate the green fluorescence intensity to the nearest unit on a scale from 0 to 30 IU/ml and >30 IU/ml, referring as often as necessary to the standards.

Assays for other autoantibodies. Antibodies to the small nuclear ribonucleoprotein (snRNP), Sm, SS-B, SS-A, and Scl-70 were assayed by enzyme immunoassay (3, 6, 9, 10, 14). Antibodies to dsDNA were assayed by the Farr assay (Amersham Corporation).

Validation of SLI and RPH ANA H secondary standards. The WHO ANA H primary standard and the SLI ANA H and RPH ANA H secondary standards were diluted in ANA-negative serum so as to span the range of 1.25 to 25 WHO IU/ml in increments of 2.5 IU/ml. Dilutions of the WHO ANA H standard were used as primary standards. Dilutions of the SLI ANA H and RPH ANA H secondary standards were masked, scrambled, and assayed three times by two observers. The dilution curves obtained for the WHO ANA H primary standard and SLI ANA H and RPH ANA H secondary standards are parallel and practically superimposable (Fig. 1). Increments of ≤5 IU/ml could be discriminated in the dilutions of all three standard sera in all three assays by both observers. These results with HEp-2 cells validate the SLI ANA H and RPH ANA H secondary standards and confirm the original calibration of the RPH ANA H secondary standard, which employed rodent tissue as substrate.

Quantitation of ANA by estimation of fluorescence intensity. In the first stage of development, SLI ANA H secondary standards were set up in ascending order and not masked. Observers estimated the fluorescence intensities of these standards in international units per milliliter with increments of 1 U in the range from 0 to 30 IU/ml or as >30 IU/ml. All observers, including those experienced and inexperienced in fluorescence microscopy, could readily detect the incremental differences between the standards.

In the second stage, the standards were scrambled and masked. Within one training session, observers learned to correctly identify the standards in terms of increasing intensity of fluorescence.

In the third stage, additional standards (this time used as controls) were scrambled, masked, and interspersed with approximately 75 routine diagnostic samples selected from the pool of consecutively received patient specimens in 50 consecutive routine assays. A curve was plotted from the estimated (observed) concentration of these controls (in this case masked standards) against the known (expected) concentration of these standards. The resulting curves for the first 20 consecutive assays were parallel and reproducible (data not shown). In all

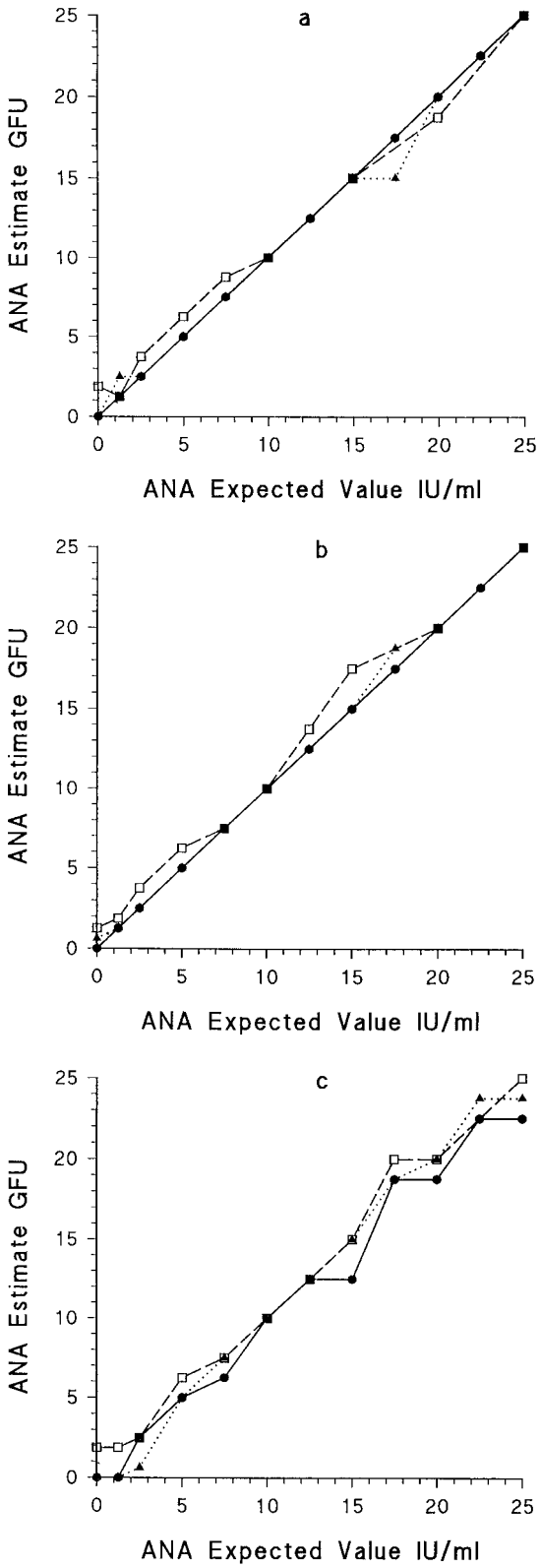

FIG. 1. Dilution curves for the WHO ANA H primary standard and the SLI ANA H and RPH ANA H secondary standards in a blind standardization run. The same dilutions of WHO ANA H were used as calibrators, and observers were instructed to select the calibrator closest in activity to each unknown sample. Mean estimates of two observers in three separate assays (a, b, and c) are plotted. GFU, green fluorescence units. Symbols: ●, WHO ANA H; □, RPH ANA H; △, SLI ANA H.

TABLE 2. Precision of uncorrected estimates of ANA activity in controls included in 49 consecutive routine assays

Control (IU/ml)	Uncorrected estimate (IU/ml)		CV[a] (%)
	Mean	SD	
7.5	8.0	1.0	13
15	15.6	1.7	11
20	19.6	1.4	7

[a] CV, coefficient of variation.

cases, observers were allowed to compare the fluorescence of the routine diagnostic samples as well as the blinded controls to that of the standards present on the other slide.

The method was also performed at the RPH Laboratories to demonstrate its transferability.

RESULTS

Precision. Dilutions of the SLI ANA H secondary standard were included as masked, scrambled controls in 49 consecutive routine assays. For control values of 7.5 (usual cutoff for the reference range), 15, and 20 IU/ml, the standard deviations (SDs) ranged from 1.0 to 1.7, and the coefficients of variation ranged from 7 to 13% (Table 2).

Masked duplicates of four samples selected at random were included in each routine assay. For 124 duplicates, the linear regression equation was $y = 1.02x + 5.6$ ($r = 0.99$; $P < 0.01$). The linear regression equation was $y = 0.97x + 0.79$ ($r = 0.99$; $P < 0.01$) for agreement between two readers.

Relationship of fluorescence intensity based on the SLI ANA H secondary standard to titers of CDC preparations for ANA patterns and antibody reactivities. In all cases, the dilution curves for the CDC preparations were parallel to that of the SLI ANA H secondary standard for ANA (data not shown), indicating that ANA of a variety of patterns and specificities can be reliably quantitated by use of standard curves. The dilution curves for standards with homogeneous, speckled, and nucleolar patterns were comparable; however, when the centromere pattern curve was used, the WHO ANA H standards had values higher than expected (data not shown). It was determined that centromere patterns could not be read from the other three curves, and other patterns could not be read from the centromere calibration curve.

Comparison of results in international units per milliliter with traditional titer results. There was overlap of the traditional titer ranges of 1:40 to 1:80, 1:80 to 1:320, and 1:160 to 1:640 for the 7.5-, 15-, and 30-IU/ml standards, respectively. This overlap reflected the imprecision of the method of doubling dilutions.

Robustness and transferability of the method. This method was easily introduced into the RPH Laboratories, where it proved similarly simple, accurate, and precise, as is evident from 50 consecutive, routine diagnostic ANA assays (Fig. 2).

Does a negative ANA test exclude antibodies to dsDNA, snRNP, Sm, SS-A, SS-B, and Scl-70? From our store of ANA-negative sera (≤7.5 IU/ml), consecutively received at Specialty Laboratories, Inc., for testing for ANA-related antibodies, 1,031 were available. Of these sera, ≤1% had antibodies against other nuclear antigens including SS-A, SS-B, snRNP, Sm, Scl-70, and dsDNA. Although five samples (0.5%) had dsDNA antibodies (cutoff of <8 IU/ml), two were borderline positive (8 IU/ml), two were weakly positive (15 and 27 IU/ml), and only one was strongly positive (184 IU/ml). Thus, the frequency of these six autoantibodies as determined by enzyme immunoassay (SS-A, SS-B, snRNP, Sm, and Scl-70) and the Farr assay (dsDNA) in ANA-negative sera approximated what would be expected with a cutoff for the upper limit of normal at 3 SD above the mean for a normal population.

Frequency of antibodies to dsDNA, snRNP, Sm, SS-A, SS-B, and Scl-70 in ANA-positive sera. In contrast to the ANA-negative sera, of 1,031 consecutively received ANA-positive serum samples (>7.5 IU/ml) which were tested for the presence of these six autoantibodies, 37 (3.6%) had only SS-A antibodies, 3 (0.3%) had only SS-B antibodies, and 38 (3.7%) contained both SS-A and SS-B antibodies. Twenty-six serum samples (2.5%) contained only snRNP antibodies, 3 samples (0.3%) contained Sm antibodies alone, and 59 (5.7%) had both snRNP and Sm antibodies. Nine samples (0.9%) had Scl-70 antibodies, and 94 (9%) had dsDNA antibodies. Thus, with the exception of SS-B antibodies (in the absence of SS-A antibodies), the sera positive for ANA by this method contained, as expected, other relevant autoantibodies much more often than ANA-negative sera.

DISCUSSION

The quantitation of ANA by estimation of fluorescent intensity assisted by controls and corrected by standards, including the WHO ANA H primary standard and locally prepared (SLI and RPH) ANA H secondary standards, is accurate and precise within the range of 5 to 25 IU/ml. Indeed, the method yields accuracy and reproducibility unparalleled and previously unanticipated for immunofluorescence microscopy. The analytical sensitivity provides a reliable screening test for active, untreated SLE, which is widely agreed to be ANA positive in over 95% of patients.

The method described herein also provides an efficient screening test for six relevant autoantibodies (SS-A, SS-B, Sm, snRNP, Scl-70, and dsDNA). This method for detection and quantitation of ANA has unprecedented reproducibility as a screening test for ANA in general and for specific autoantibodies against HEp-2 antigens. We have experience with numbers in excess of 10,000 assays for ANA by this method with simultaneous assays by enzyme immunoassay and the Farr assay for the six autoantibody specificities. With the exception of antibodies to SS-A (which is very soluble and can migrate to

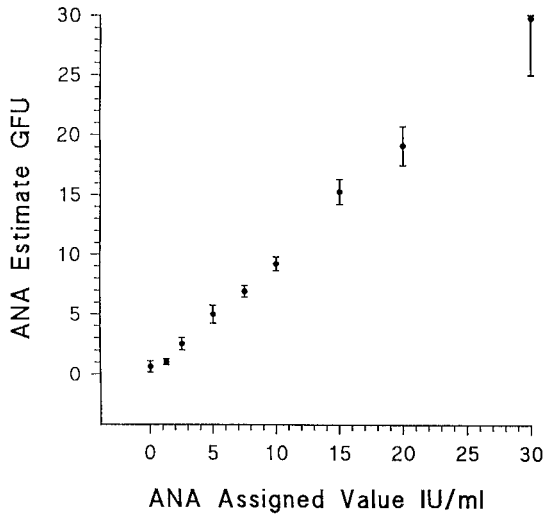

FIG. 2. Precision of estimates. Each ANA standard serum with assigned values of 0, 1.25, 2.5, 5, 7.5, 10, 15, 20, and 30 IU/ml was read 50 times under blinded conditions. Means and 1 SD are shown.

the cytoplasm, thereby causing problems in immunofluorescent ANA studies), the method typically allows one to rule out the presence of the other five autoantibodies in sera shown to be ANA negative by this method. Our current efforts are directed to optimizing the efficiency of this method for the detection of SS-A antibodies by careful attention to cytoplasmic fluorescence which characterizes the AF-CDC reference preparation 7 for SS-A antibodies. That preparation yields an ANA result of 15 IU/ml with the SLI ANA H method on HEp-2 cells.

Our results negate the contentions that humans cannot make precise judgements of immunofluorescence intensity and that standards with ANA of one pattern are not useful for quantitation of ANA of another pattern or mixtures of patterns. Because these two beliefs were previously accepted (without supporting data), inclusion of standards in routine ANA assays and resultant improvements in accuracy and precision were delayed.

The method for the assay of ANA as described herein is simple, economical, and easily learned by inexperienced as well as experienced observers. Compared with titration by serial dilution (which is almost universally employed for ANA assays), the method yields a two-thirds reduction in the use of HEp-2 cells as a substrate, saves technician time, and improves turnaround time by avoiding the necessity to identify, locate, and retest positive sera which require titration by dilution.

This method provides a convenient means for international standardization of ANA measurement. Currently, international units are defined only for the ANA H and dsDNA antibody standards. In the future, it will be possible to use this method to relate green fluorescence intensity for a variety of patterns and antibody reactivities to secondary standards (e.g., SLI, RPH, and other local standards) and ultimately to international units.

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