



## Antinuclear antibody testing

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In the clinical laboratory, the most commonly performed auto-antibody test is the one to detect antinuclear antibody (ANA). It provides the best single test to rule out the diagnosis of systemic lupus erythematosus (SLE) in children or adults, but it is also one of the most overordered tests in the laboratory [1,2]. Even though it has excellent sensitivity and reasonable specificity, its positive predictive value in many clinical settings is awful because of its overuse in low-risk populations. Furthermore, the evolution of the technique from the subjective (yet reasonably specific) lupus erythematosus (LE) cell preparation, to immunofluorescence ANA (ANA-IFA) performed on a variety of substrates, to the automated objective enzyme immunoassay (ANA-EIA) has added further confusion. Which of these assays do clinicians receive when they order the ANA test that is listed on the laboratory requisition? In the past 5 years there has been a notable shift in the deployment of ANA tests in the diagnosis of SLE. There are two main features to this shift. The first change involves an evolution from subjective to objective data, and the second change follows from the industry-wide effort to improve efficiency by switching from manual to automated procedures. Benefits of these changes are the lowered costs for ANA tests and the potential for improving the consistency of performance of the ANA test by providing objective data. The ready availability of this testing, however, may also encourage its overuse in low-risk populations, thereby diminishing its positive predictive value. This article provides background on the current state of ANA screening, reviews the ongoing shift in technology for ANA serology, illustrates how overuse of ANA testing reduces its value, and emphasizes the use of patient selection to improve its positive predictive value.

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## ANA testing

### *LE cell test*

The first laboratory test to detect SLE was the LE cell phenomenon described by Hargraves et al [3] in 1948. The assay may be performed directly on peripheral blood from a patient suspected of having SLE, or indirectly using control blood that is mixed with the serum from a patient suspected of having SLE. The assay itself is laborious and the interpretation is subjective. For direct assays there are several versions. Most typically, the patient's blood is mixed with glass beads, incubated for an hour at 37°C, and then the buffy coat is stained with a Wright-Giemsa stain [4]. During the incubation, the LE cell factor reacts with damaged lymphocyte nuclei and activates complement, which together opsonizes the nuclei for phagocytosis by normal, viable neutrophils in the preparation [5]. Recently, Schett et al [6] presented strong evidence that a major component of LE cell factor is antihistone H1. Absorption of the antihistone H1 reactivity in serum from SLE patients was able to abrogate the LE cell phenomenon, whereas other histone fractions failed to remove the LE cell factor. The indirect LE cell assay is performed in a similar manner, but requires preincubation of control peripheral blood cells with serum from the patient. In vivo formation of LE cells that may be detected in effusions from pleural, pericardial, or synovial fluids from patients with SLE are considered to be strong evidence for the disease [7–9]. There is an impression among clinicians and laboratorians alike that the presence of an LE cell in such fluids is specific for SLE. It is not. Although they are strong evidence for the disease, LE cells even in these locations can be found in conditions other than SLE [10].

The interpretation of the LE cell preparations requires distinction between “Tart cells” and “LE cells” (Fig. 1). Tart cells (named for the surname of the first individual in whom they were found) are those where the ingested nuclei have not reacted with LE cell factor and complement and retain some of their chromatin pattern and stain blue [4]. LE cells have homogeneous, purple-pink colored masses that do not have chromatin patterns. The distinction between Tart cells and LE cells is important because Tart cells may be found in individuals who lack the LE cell factor and who do not have SLE. The subjectivity of the LE cells assay remains problematic. In a recent Care Record of the Massachusetts General Hospital, it was reported that the laboratory did not record the presence of LE cells in a joint fluid, whereas the physician discussing the case detected them [11]. Indeed, because of the expense and subjectivity of this test and the presence of more readily obtained tests of high specificity, such as anti-double-stranded (ds) DNA or anti-Sm, the LE cell test from peripheral blood is rarely used today and it is not recommended by the author. Still, the LE cell phenomenon remains one of the criteria in the American College of Rheumatology classification criteria for SLE and is still ordered on these patients [12,13]. The

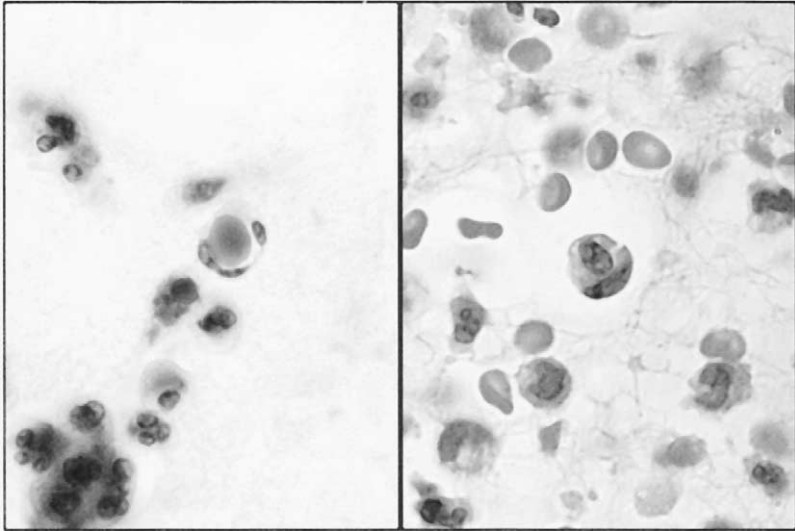


Fig. 1. Comparison of LE cell (*left*) with homogeneous engulfed material with the Tart cell that contains recognizable chromatin (*right*). (From Bibbo M, editor. *Comprehensive cytopathology*. 2nd edition. Philadelphia: WB Saunders Co; 1999. p. 578–9; with permission.)

recent suggestion by Schett et al [14] to use specific antibodies against histone H1 to detect the antibody associated with the LE cell phenomenon may offer an objective and reproducible assay to accommodate those criteria. Indeed, the presence of anti-H1 may offer more insight into the patient's clinical course because these antibodies are associated with a strong immune response to other nuclear antigens and with active clinical disease [14].

#### *Indirect ANA-IFA test*

The next major development in ANA screening tests was the development of the indirect ANA-IFA in the 1950s [15,16]. A variety of substrates have been used including frozen sections of rodent kidney, frozen sections of rodent liver, and tissue culture cell lines. The HEP-2 tissue culture cell line has been one of the most popular substrates in the past decade. HEP-2 cells are easier to read than the earlier substrates. The larger size of the HEP-2 cells than the rodent substrates enhances the ability to detect patterns in a consistent manner. Furthermore, some antigens, like centromere, are extremely difficult to detect on frozen sections of rodent liver and kidney, yet are readily evident on HEP-2 cells. These cells can be fixed with acetone to improve preservation of the readily water-soluble SSA/Ro antigen from that achieved by ethanol fixation. Formerly, the most common cause of a false-negative ANA test was an individual who produced mainly anti-SSA/Ro (the antigen that was often washed away because of inadequate fixation)

[17,18]. The characteristics of ANA-negative lupus with positive SSA/Ro define a subset of patients with subacute cutaneous lupus erythematosus [17,19]. The more recent versions of the ANA-IFA that use HEp-2 cells that are fixed to preserve the SSA/Ro antigen are able to detect up to 98% of cases of SLE giving the ANA-IFA test a high negative predictive value [1,2]. Other manufacturers have transfected HEp-2 cells (HEp-2000) to allow specific expression of the 60-kd antigen of SSA/Ro [20].

The use of these substrates quickly resulted in the recognition of several patterns of nuclear staining that could be distinguished. Further, the strength of ANA activity could be quantified by determining the titer. Although several patterns can be seen, the patterns on HEp-2 cell lines that are the most useful are homogeneous-rim, speckled, nucleolar, and centromere. Many patients with SLE have more than one type of pattern. The patterns often are better distinguished when different dilutions of the serum are examined. For instance, the presence of a homogeneous pattern may obscure the presence of a nucleolar pattern that can become apparent on use of a greater dilution of the patient's serum.

The peripheral-rim pattern on frozen section rodent substrates has fluorescence mainly around the periphery of the nucleus. It was the pattern most closely associated with the diagnosis of SLE. This pattern is consistent with the presence of antibodies against dsDNA or against single-stranded DNA (ssDNA). Unfortunately, antibodies against the nuclear membrane itself give a similar pattern on frozen sections substrates and are not specific for SLE (Fig. 2). A variety of antigens are associated with antinuclear membrane (nuclear envelope antibodies). Antigenic determinants of nuclear membrane include gp210, p62, lamina, and lamin B. Antibodies against these determinants have been associated with primary biliary cirrhosis [21,22]. With HEp-2 substrates, however, this pattern can be distinguished from the homogeneous-rim pattern because there is no staining of the mitotic figures with antinuclear membrane (Fig. 3). The homogeneous pattern on rodent substrates was distinguished from the peripheral-rim pattern. Antibodies responsible for the homogeneous pattern on frozen section substrates included anti-dsDNA, antihistone, and anti-ssDNA. In contrast, on HEp-2 substrates, the peripheral-rim pattern often is not distinguished from the homogeneous-rim pattern.

The homogeneous-rim pattern on the HEp-2 substrate requires that the nuclei stain in a homogeneous manner, occasionally with emphasis toward the periphery of the nucleus, and the mitotic figures must also stain (Fig. 4). This type of pattern can result from anti-dsDNA, anti-ssDNA, and antihistone [23]. Whereas antihistone antibodies and anti-ssDNA antibodies are often present in SLE, they lack the specificity for the disease provided by anti-dsDNA [1]. As mentioned previously, some antihistone antibodies (anti-H1) are associated with the LE cell phenomenon. Other antihistone antibodies, however, are associated with drug-induced lupus. Drugs, such as procainamide and hydralazine, may cause a disease reminiscent of SLE

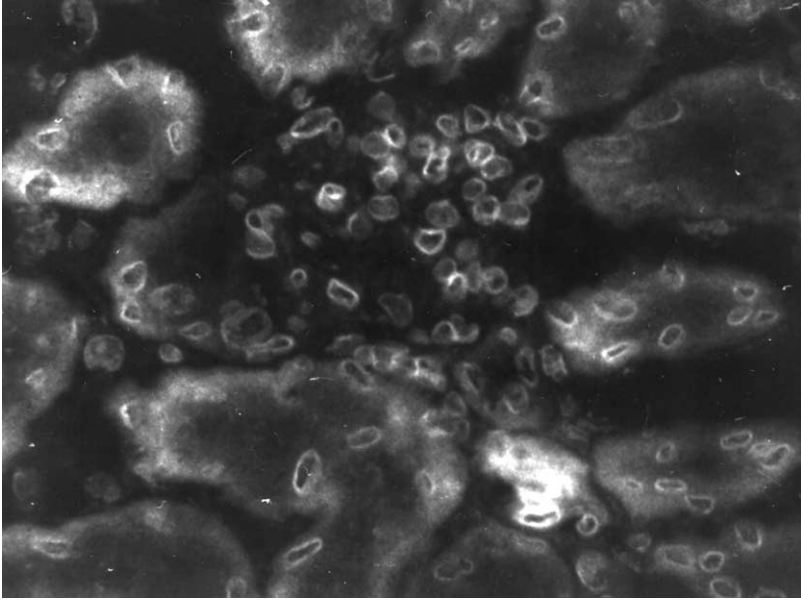


Fig. 2. Nuclear membrane pattern is indistinguishable from a peripheral-rim pattern on this frozen section substrate of rodent kidney.

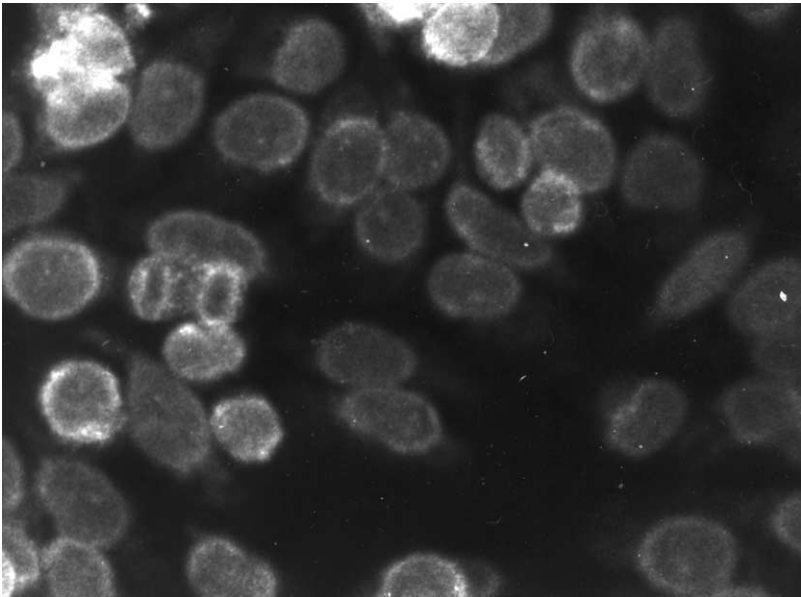


Fig. 3. Nuclear membrane pattern is evident on this HEP-2 substrate because of the lack of central staining in the mitotic figure located at 9 o'clock.

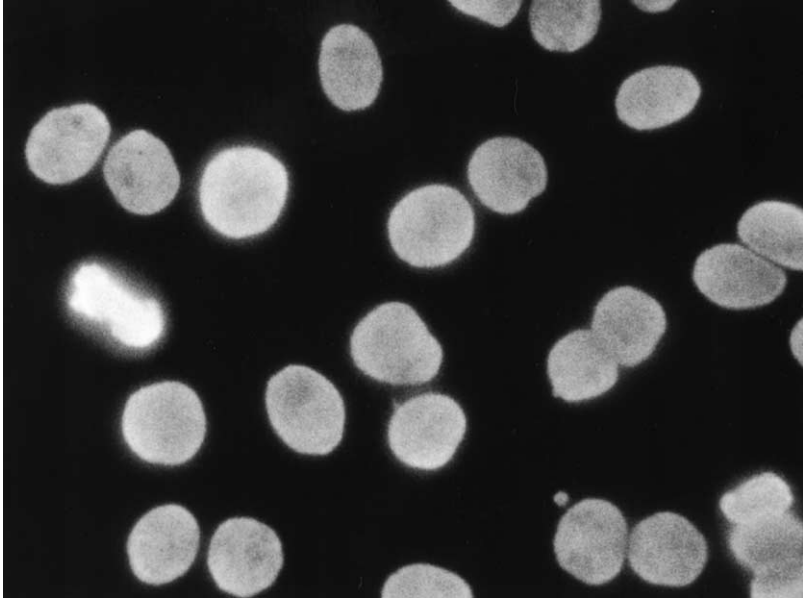


Fig. 4. Homogenous pattern is demonstrated on HEp-2 substrate with the strong staining of the mitotic figure at 9 o'clock.

that can be reversed by discontinuing the medication. Procainamide is associated with antibodies against two of the antigenic types of histones, H2A and H2B [6,24,25]. Hydralazine has been associated with antigenic determinants formed from a complex of H3 and H4 [24,26]. Often such antihistone antibodies are transient and of the IgM subclass [27].

Distinction of specific anti-dsDNA antibodies from the less specific anti-ssDNA or antihistone antibodies was originally performed by the Farr assay [28]. This assay mixed a radiolabeled preparation of dsDNA with the patient's serum. The immunoglobulin fraction was precipitated with ammonium sulfate and the amount of radioactivity adhering to the immunoglobulin fraction was measured. By using normal serum as a control, a cutoff point was obtained above which only patients with antibodies against dsDNA react. Careful control of the reaction conditions was required to avoid false-positive results. Binding to dsDNA under conditions of high salt concentration is found preferentially in SLE patients who have nephrotic syndrome and anemia [29]. Other methods are more commonly used today to determine anti-dsDNA. One method involves the use of *Crithidia luciliae*, a small hemoflagellate that contains a structure called the kinetoplast. The kinetoplast contains dsDNA but not ssDNA. Consequently, an indirect immunofluorescence test may be performed using the *Crithidia luciliae* as the substrate for the patient's serum. In addition to detecting the anti-dsDNA with this technique, the strength of reactivity may be estimated

by performing titration. It is not clear, however, that titration provides the same information as measurement of the anti-dsDNA by the Farr assay [30]. In the Farr assay, occasionally other serum proteins are able to bind to the radiolabeled dsDNA to give a false-positive and in the *Crithidea luciliae* assay antihistone may give false-positive results [31–33]. Most recently, anti-dsDNA assays have become available by EIA technology. This readily automated testing system has become popular; however, there may be considerable differences in results obtained both between different systems, such as EIA and *Crithidea luciliae* assays, and also between different commercially available EIA products [34]. In a recent evaluation of available EIA systems, Tan et al [35] found no single manufacturer clearly superior to others, especially in the area of antibodies to dsDNA and Sm antigen.

The speckled pattern is the least specific ANA pattern because it denotes the presence of antibodies against a wide variety of nuclear and cytoplasmic antigens (Fig. 5). With the exception of the centromere pattern (discussed later) and the sensitivity of some antigens to enzyme digestion, the speckled pattern itself is not able to distinguish between the many antigens that are included in this pattern. An older term for many of the antigens that result in the speckled pattern is *extractable nuclear antigens* (ENA). The author does not recommend use of this term; however, the reader may still find it in the medical literature. Originally, ENA referred to two specific antigens: Smith (for the first person who had this antibody reactivity demonstrated [Sm]), and ribonucleoprotein (RNP). These both give a typical speckled pattern on

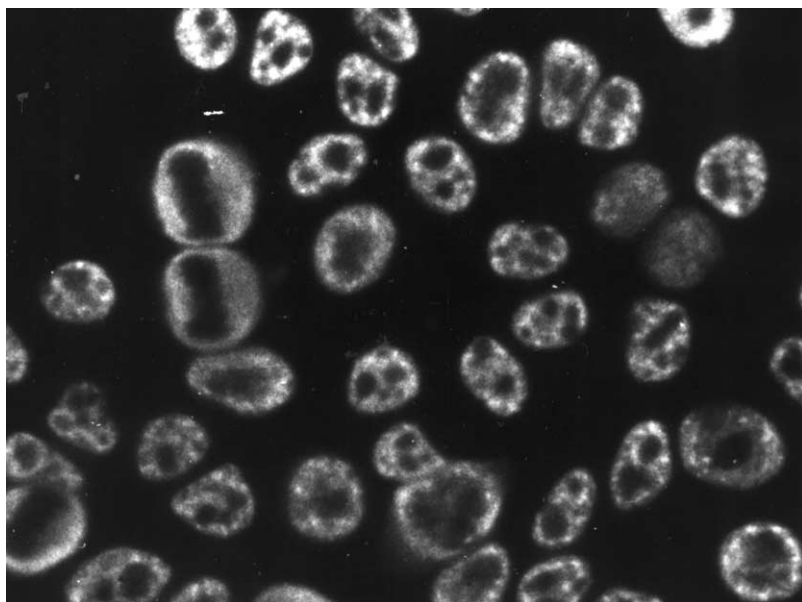


Fig. 5. Speckled pattern is demonstrated on HEP-2 substrate with lack of nucleoli in all cells and lack of central staining in the mitotic figures.

frozen section substrates, but when the substrates are predigested with ribonuclease, Sm is still demonstrable, whereas RNP is not. Sm was referred to as the *ribonuclease resistant antigen* and RNP was *ribonuclease sensitive*.

The Sm is a 95-kd protein that exists as a protein-RNA complex as part of the same molecular complex with RNP antigen. It functions to splice transcriptional mRNA. The antibodies against Sm react with several U1RNP antigens: B, B', D1, and E [36]. About 15% to 30% of patients with SLE have this antibody. When it is present, it is highly specific for SLE [37]. The older ribonuclease digestion method is no longer used to distinguish Sm from the other antigens that give a speckled pattern. Anti-Sm is commonly detected by gel diffusion, counter immunoelectrophoresis, or EIA [38,39]. Although the EIA methods are more sensitive than either the immunofluorescence or gel diffusion techniques, a recent study by Pan et al [40] confirmed that it is still a highly specific assay with a specificity of 98.6% in their population. Bloch reports that his laboratory's experience with the sensitive EIAs to detect anti-Sm suggests some individuals with anti-Sm do not have SLE and he retests positive samples obtained by EIA with a gel diffusion technique [11]. He notes some patients with SLE have the EIA against Sm demonstrable by EIA, however, but not by gel diffusion technique [11].

The RNP is the other antigen that was originally included by the designation ENA. Existing on the same molecular complex as Sm, it functions to splice transcriptional mRNA. RNP exists as a 70-kd nuclear matrix antigen. Its main antigenic determinants are A and C on U1 RNP. Anti-RNP is found in high titer in 95% to 100% of patients with mixed connective tissue disease (MCTD) [41,42]. Indeed, their presence was part of the original definition of MCTD by Sharp et al [43]. Clinically, patients with MCTD usually have a milder course than those with SLE. EIA are typically used to detect these antibodies, although other techniques, such as gel diffusion, immunoblotting, and counterimmunoelectrophoresis, are also available [21,44]. Levels of these antibodies, although present in about 30% of patients with SLE, do not correlate with activity of SLE [45,46].

The other two antigens that have been included under the term ENA are SSA/Ro (anti-52 and 60-kd) and SSB/La (anti-48-kd). Antibodies against these antigens were first detected in patients with Sjögren's syndrome, SLE, and rheumatoid arthritis [47]. Anti-SSA/Ro is the most common type of antibody detected in patients with subacute cutaneous lupus erythematosus being found in about 70% of cases, whereas anti-dsDNA is found in less than 10% of such cases [19]. For patients with SLE, only about 25% are positive for antibodies to SSA/Ro by specific immunoassays. The two proteins recognized by anti-SSA/Ro are a 52-kd and a 60-kd subunit. The presence of only or mainly antibodies to SSA/Ro, however, is the most common cause of SLE with a negative ANA screen using ANA-IFA. Maddison et al [48] found that two of three SLE patients who were negative by ANA-IFA testing using frozen section substrates had antibodies against SSA/Ro detected by agar gel diffusion. They recommended the use of KB tissue culture cell



lines to detect most of these cases. EIA and Western blot kits have good performance to detect anti-SSA/Ro antibodies [18,49]. There is a new HEp-2 substrate, the HEp-2000, cells transfected with Ro60 cDNA (the DNA that codes for the 60-kd antigen of SSA/Ro [20]). Using this substrate, Morozzi et al [50] were able to detect 89% of known positive anti-SSA/Ro antisera on HEp-2 tissue culture substrate. They recommended that when SLE associated with SSA/Ro is suspected (subacute cutaneous lupus erythematosus or neonatal lupus), a combination of two methods (eg, HEp-2 and a specific anti-SSA/Ro assay) should be used to rule out the presence of this auto-antibody. It is uncommon to detect anti-SSB/La when SSA/Ro is absent, whereas anti-SSA/Ro commonly occurs without anti-SSB/La. SLE patients who have anti-SSA/Ro but no SSB/La may have more severe renal involvement than those who also have anti-SSB/La [51].

A major area of importance in detecting anti-SSA/Ro antibodies is in patients with neonatal lupus. Neonatal lupus is an acquired condition that occurs when auto-antibodies pass from the mother through the placenta. Clinically, the babies develop erythemaform rash and cardiac conduction disturbances that may cause fatal heart block. Although the rash is transient, disappearing along with the decay of maternal IgG in about 6 months [52], the cardiac injury is generally irreversible [53]. The heart block with bradyarrhythmia has been detected before 30 weeks of gestation in 82% of fetuses studied who had congenital heart block, and there is a mortality rate of 14% before 3 months of age [54]. Furthermore, 63% of live-born children with congenital heart block eventually require pacemakers [54]. A study of serology in 60 Japanese infants with neonatal lupus by Kaneko et al [55] reported that almost 80% have antibodies against SSA/Ro. Monitoring babies from mothers with these antibodies, or those neonates who develop erythemaform rashes consistent with neonatal lupus for cardiac irregularities, is the key to preventing cardiac deaths. Eftekhari et al [56] suggested that antibodies to the 52-kd RNP may cross-react with the cardiac 5-HT<sub>4</sub> serotonergic receptor and this may antagonize the serotonin-induced L-type calcium channel activation on human atrial cells accounting for the electrophysiologic abnormalities. Interestingly, neonatal lupus has been reported in both twins and triplets [57].

Another important extractable acidic nuclear antigen is anti-Jo-1. By the HEp-2 cell ANA-IFA, these give predominately cytoplasmic speckles with faint nuclear staining. This antibody is found in about one third of patients with polymyositis-dermatomyositis, especially those who develop interstitial pulmonary fibrosis [58,59]. It is directed against histidyl-tRNA synthetase [60]. Jo-1 is not usually present in patients with SLE, but may give a positive ANA-IFA test. A negative ANA-IFA does not rule out the presence of anti-Jo-1, however, which should be sought for by specific immunoassays in patients suspected of having polymyositis-dermatomyositis or individuals with interstitial lung fibrosis [61]. The Jo-1 antigen is usually included in ANA-EIA screening tests.

Two antibodies giving speckled nuclear patterns by ANA-IFA are associated with scleroderma. Anticentromere antibodies are useful to detect the variant of scleroderma characterized by the presence of calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST variant) [62,63]. This auto-antibody usually is present in patients with a limited form of the disease as opposed to patients who are positive for anti-Scl-70 (antitopoisomerase) who more likely have systemic scleroderma. Anticentromere antibodies are readily recognized on HEp-2 substrates by the characteristic discrete speckling that lines up with chromosomes in the mitotic figures (Fig. 6). About 5% of patients with SLE have anticentromere antibodies. Although these SLE patients had an older age at onset and a higher incidence of Raynaud's phenomenon than those lacking these antibodies, the long-term significance of this subset of SLE patients is not yet known [64]. Several antigens, designated centromere protein A-E, have been demonstrated that correspond to the anticentromere antibodies demonstrated by ANA-IFA [65]. In contrast, anti-Scl 70 (antitopoisomerase) is present in about 40% of patients with diffuse cutaneous involvement with scleroderma and those who are likely to develop interstitial pulmonary fibrosis [1].

The nucleolar pattern is the one most closely associated with the presence of scleroderma (Fig. 7). In scleroderma, the specific autoantigens that have been identified as specific targets include Scl-70, centromere proteins, RNA polymerase I, U3 RNP-associated fibrillarin, PM-Scl, and 7-2 RNP [66,67].

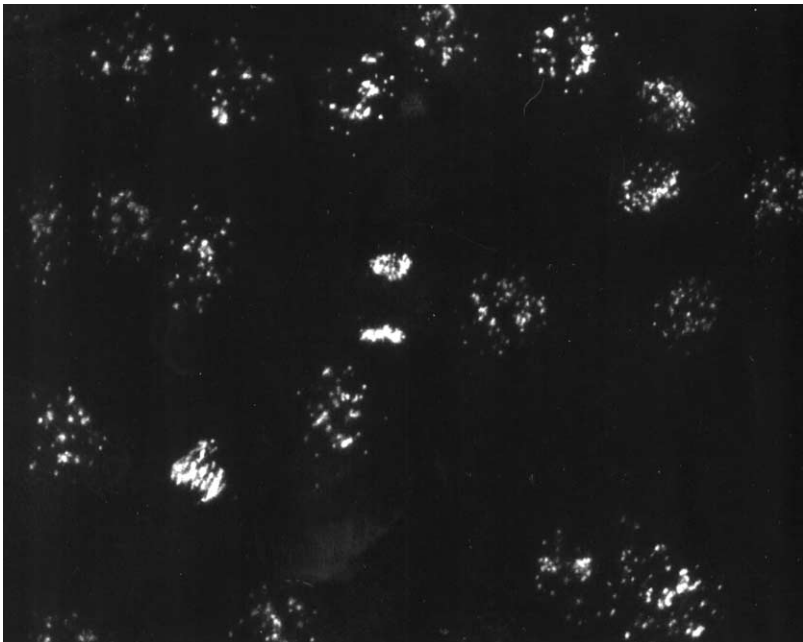


Fig. 6. Centromere pattern is demonstrated on HEp-2 substrate.

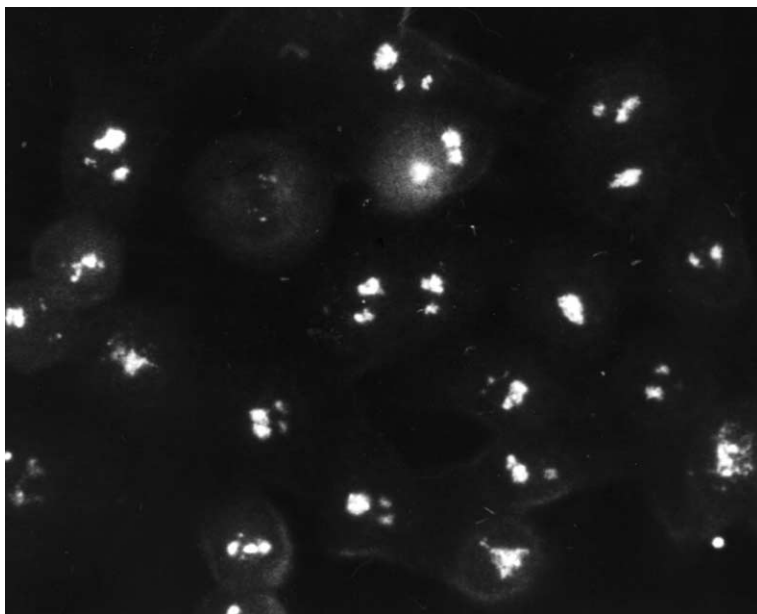


Fig. 7. The large nucleoli in HEp-2 cells are evident in this nucleolar pattern.

On a substrate of hamster liver imprints, anti-PM-Scl gives a homogeneous nucleolar fluorescence pattern on ANA-IFA as opposed to anti-Scl-70, which has a mixed fine diffuse nuclear and nucleolar pattern [68]. Anti-RNA polymerase I gives punctuate nucleolar staining. These antibodies define overlapping subsets of scleroderma. Anti-RNA polymerase I is associated with diffuse scleroderma with severe renal disease, whereas anti-U3 RNP-associated fibrillarin occurs most commonly in men with less joint involvement than in ANA-negative patients [67]. Exclusive or predominantly nucleolar pattern can occur in SLE, but is present in less than 1% of those patients [69].

Bloch has pointed out that certain patterns would likely not be detected by some of the newer ANA-EIA assays. These include oligodot pattern (0 to 3 dots) caused by anticoilin; oligodot pattern (7 to 10 dots) found in primary biliary cirrhosis; antinuclear envelope antibodies; anticentromere (unless centromere protein antigens are included); pseudocentromere; proliferating cell nuclear antigen; antiribosomal P; antispindle apparatus; anti-centriole-centrosome; and NuMA-1 and NuMA-2 antibody (KJ Bloch, personal communication, 2001).

#### *Variables with ANA-IFA testing*

Although the ANA-IFA patterns discussed offer subgroups of the diseases associated with a positive ANA, there are several variables with the ANA-IFA test that complicate its use in clinical laboratories. The laboratory needs

to decide which of the many commercially available kits they wish to use. As mentioned previously, commercially available kits use a variety of substrate from mouse and rat kidney and liver to HEp-2 cells. These substrates have different antigens at different concentrations. Consequently, the sensitivity of detection of ANA varies with the substrate used. Also, each kit has a unique fluorescein-conjugated antihuman IgG. The affinity of the antihuman IgG and the ratio of fluorescein to protein in the conjugate (a ratio of about 3 is preferred) are other important variables [1]. If the ratio is higher than 3, there may be non-specific staining that could lead to false-positive results.

The laboratory needs to determine the cutoff dilution of the patient's serum that is used. The cutoff varies with several factors: the kit used; the type of microscopy (darkfield versus epifluorescence); the light source of the microscope; the numerical aperture of the lens in the microscope; the type of filter; and the type of condenser (if darkfield microscopy is used). Originally, because of the insensitivity of rodent frozen section substrates and the optics of microscopes available at the time, cutoffs of 1:10 and 1:20 were typical. Data reported by Kavanaugh et al [1] from a review of the literature indicate the frequency of positive ANA results among normal persons as reported in their literature review as part of the guideline process discussed later (Table 1). To avoid some of the variables associated with immunofluorescence, some commercially available kits use an immunohistochemical approach [70]. This adds a substrate step, however, and has not gained widespread use.

If a high enough concentration of serum is used, most normal people give a positive ANA screen. This is why determination of the cutoff titer is a critical issue. Patients with SLE and some other connective tissue diseases discussed later often have ANA reactivity at significantly increased titers. Malleson et al [2] suggested that a screening serum dilution of 1:160 or even higher increases the usefulness of the ANA-IFA. Because of the numerous variables described, each laboratory is advised to determine their own cutoff on their own population [71]. Feltkamp [72] recommends that about 200 sera from known healthy controls with broadly represented ages and equally distributed by sex should be used to determine the cutoff. Feltkamp [72] indicates that the dilution used should give negative results in at least 95%

Table 1  
Effect of cutoff dilution of positive ANA results in normal persons

Cutoff dilution	Percent of normal persons positive
≥1:40	20–30
≥1:80	10–12
≥1:160	5
≥1:320	3

*Data from* Kavanaugh A, Tomar R, Reveille J, Solomon DH, Homburger HA. Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantibodies to nuclear antigens. Arch Pathol Lab Med 2000;124:71–81.

of healthy normal controls, yet it should be able to detect nearly all of the patients with SLE. A panel of approximately 100 known SLE patients also needs to be studied. By creating receiver operator characteristics (ROC), an optimal dilution can be chosen.

That this careful evaluation of cutoff is not generally performed is suggested by the dilutions actually being used as listed in a self-reporting survey of the College of American Pathologists subscribers in 2000. They found that most laboratories (59.6%) use a cutoff of 1:40 (Table 2) [73]. If the Kavanaugh et al [1] data are correct, this means that most laboratories that perform ANA may have a false-positive rate of about 25%. Years ago, Fritzler and Tan [24] reported that at a 1:20 screening dilution about a third of healthy adult women give a positive ANA using the tissue culture substrates. Of course, the actual situation is more complex because the cutoffs depend on all of the factors mentioned.

Another issue with the ANA-IFA test is the variability of titer from one laboratory to another. This has improved considerably in recent years with the institution of proficiency testing programs that allow comparison of the same sample from one laboratory to another; yet, it is still important that clinicians recognize the normal variation (result plus or minus one dilution) that occurs in titers both within laboratories and between laboratories. In Fig. 8, the results of the manufacturer with the largest numbers of laboratories reporting specimen ANA-08 for the S-B year 2000 CAP survey is shown. All laboratories using this method correctly reported the specimen as positive. Although the titers varied from 1:20 to greater than 1:2560, 70% of reporting laboratories using that method reported either 1:320 or 1:640 and 89% reported from 1:320 to 1:1280. Because titers are widely accepted to be accurate plus or minus one dilution, the data show agreement of almost 90% at the titer of 1:640. This demonstrates relatively good reproducibility of that method between laboratories. Further data from that same survey indicate that most methods had this same pattern of modest titer variation.

The most recent development in ANA-IFA testing uses of an automatic fluorescent image analyzer both to detect and quantify the ANA on one standard screening dilution of the patient's serum [74]. Using the Image Titer (Tripath Imaging, Burlington, NC), Nakabayashi et al [74] was able to evaluate a single slide per patient for both pattern and titer. The instrument is

Table 2  
2001 CAP survey of ANA testing

Cutoff dilution used	Percent of laboratories using that cutoff
≥1:40	59.6
≥1:80	23.1
≥1:160	14
Other	3.3

*Data from CAP Summary Report. Diagnostic immunology S-A 2001. Chicago: College of American Pathologists; 2001. p. 14.*

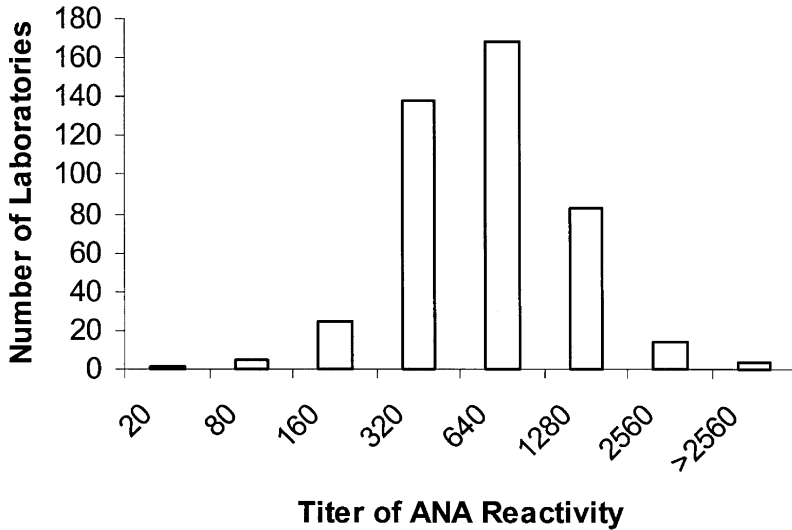


Fig. 8. Distribution of ANA titer results from several hundred laboratories on the same sample from one manufacturer.

able to transform the intensity of the fluorescent signal into images at various dilution points on a monitor, thereby eliminating the work for multiple dilutions and allowing inspection of the images by multiple viewers simultaneously. This eliminates the need for titrating the strength of the reactivity with multiple dilutions and decreases the workload for technologists. As shown in Fig. 9, there was an excellent correlation of major screening pattern titers by this method with a manual titer dilution method. Although the patterns were interpreted by individual readers off the monitor, one may speculate on the ability to train the computer to recognize the various patterns and provide a system that is objective, consistent, and with the potential for automation.

#### *ANA-EIA test*

In the past 10 years, several manufacturers developed EIA tests to screen for the presence of ANA [75,76]. For the ANA-EIA, the antigens discussed previously that have relatively good specificity and sensitivity for detecting patients with SLE are coated to microtiter plates or beads. Both purified and recombinant antigens have been used in the various systems. The concentration and specific preparation of each antigen is determined by the manufacturer and is not currently standardized from one manufacturer to the other. Early on, this resulted in significant difference from one system to the other in detection of ANAs [77]. Because of the variation of the antigens, the reader is advised to ask the manufacturer how consistency of antigen preparation is ensured from one lot of plates to another. For instance, some

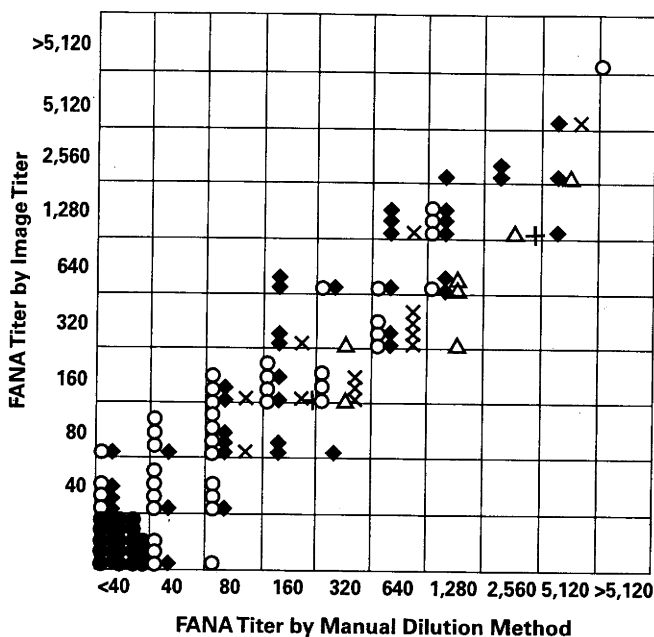


Fig. 9. Comparison of indirect immunofluorescence by manual methods using multiple tubes for dilution (FANA) with image titer using one dilution. Closed circle = negative; x = nucleolar; triangle = discrete speckled; open circle = homogeneous; diamond = speckled; + = proliferating cell nuclear antigen. (From Nakabayashi T, Kumagai T, Yamauchi K, Sugano M, Kuramoto A, et al. Evaluation of the automatic fluorescent image analyzer, image titer, for quantitative analysis of antinuclear antibodies. *Am J Clin Pathol* 2001;115:424-9; with permission.)

companies compare the reactivity of their lots with standardized sera from the Centers for Disease Control and Prevention. The recent development of antinuclear and anticytoplasmic antibody consensus sera by the Association of Medical Laboratory Immunologists may prove to be a helpful reagent to perform such lot-to-lot or even kit-to-kit comparisons [78].

To perform the assay, the unknown sera and controls are diluted with buffer provided in the kit and an aliquot of this is added to the well (or wells, because some kits use a two-well system, although most have a one-well screen). Both the initial dilution and subsequent incubation and washing steps are commonly automated on one instrument. Following incubation for 20 to 30 minutes, the wells are washed with buffer and an enzyme-conjugated antibody against human IgG is added for a second incubation of 20 to 30 minutes. After another wash in buffer, the substrate is added and the enzyme-substrate reaction proceeds typically for about 10 minutes. The optical density of the substrate reaction is graded against a cutoff that is specific to each assay system.

Despite several early attempts at ANA-EIA, it was not until a brief paper by Gniewek et al [79] was published in 1997 that this technique began to be

deployed by clinical laboratories. In their study, Gniewek et al [79] used the patient's clinical diagnosis rather than direct comparison with the traditional ANA-IFA testing as the gold standard. They used ROCs that compare the sensitivity of detection versus 1-specificity (Fig. 10). The patients included 283 serum samples from the Arthritis Center of Nebraska that were routine samples that had been ordered for traditional ANA-IFA testing. Criteria of the American Rheumatism Association [80] were used to establish the diagnosis. They also processed 98 serum samples that had been stored frozen at the National Institute of Dental Research from patients diagnosed with primary Sjögren's syndrome using standard criteria for the diagnosis [81]. The EIA test was the ANA-EIA from Bio-Rad Laboratories (Hercules, CA) on an automated analyzer, the RADIUS (Bio-Rad). In their study, the microtiter wells in that assay were coated with an extract from Hep-2 cells that contained the following antigens: dsDNA, SSA/Ro, SSB/La, Sm, RNP, Jo-1, and Scl-70. Their definition of disease-positive required the presence of one or more of the following diseases: SLE, discoid lupus erythematosus, scleroderma-CREST, Raynaud's syndrome, Sjogren's syndrome, MCTD, overlap connective tissue disease syndromes, polymyositis, and dermatomyositis. All other diagnoses were considered disease-negative. Note that this excludes autoimmune hepatitis and drug-induced lupus, which usually require the presence of an ANA for diagnosis [1], although a subset of autoimmune hepatitis exists that are ANA-negative [82]. They found 39 of the 283 individuals were positive for at least one of the diagnoses

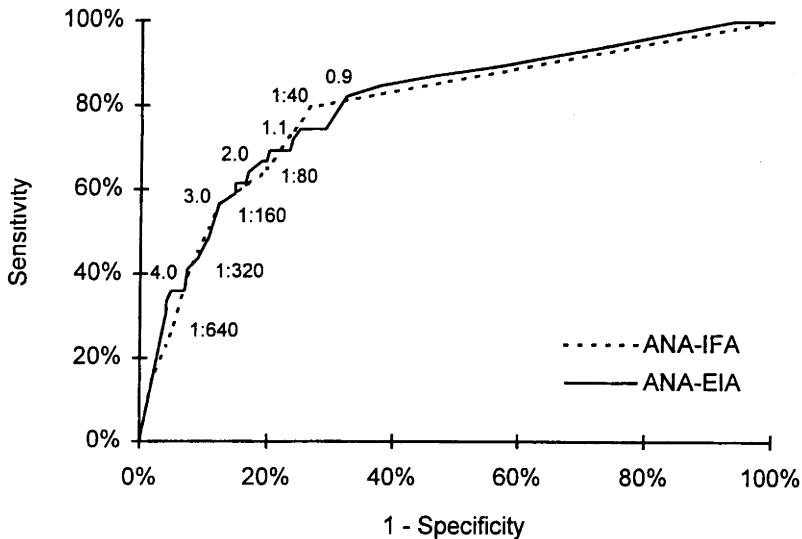


Fig. 10. Receiver operator characteristics compare the sensitivity of detection with 1-specificity for ANA-EIA with ANA-IFA. (From Gniewek RA, Sandbulte C, Fox PC. Comparison of antinuclear antibody testing methods by ROC analysis with reference to disease diagnosis. Clin Chem 1997;43:1987-9; with permission.)



Table 3  
Diagnoses of individuals included as disease-positive

Diagnosis <sup>a</sup>	Number of patients
Systemic lupus erythematosus	16
Raynaud's disease	6
Sjögren's syndrome	6
Scleroderma = CREST	5
Mixed connective tissue disease	3
Discoid lupus erythematosus	2
Dermatomyositis = polymyositis	2
Connective tissue disease (undefined)	9

<sup>a</sup> Some of these patients had more than one connective tissue disease diagnosis.

Data from Gniewek RA, Sandbulte C, Fox PC. Comparison of antinuclear antibody testing methods by Roc analysis with reference to disease diagnosis. Clin Chem 1997;43:1987–9.

listed in Table 3. The category of “Connective Tissue Disease Undefined” is vague and difficult for this writer to judge. For instance, does that include such conditions as fibromyalgia, which are not recommended for ANA screening? Nonetheless, overall the categorization of the findings by a diagnosis-based method provided a useful comparison to the standard ANA-IFA. The ROC of their data shown in Fig. 10 demonstrates no significant difference ( $P > 0.05$ ) between the areas under the ANA-EIA and the ANA-IFA curves. There was no difference in the sensitivity and specificity ( $P > 0.05$ ) (Table 4). Of the 98 samples tested separately from patients with Sjögren's syndrome, 88.8% were positive by ANA-IFA and 92.9% were positive by ANA-EIA, although the latter used a different cutoff from the other data.

This demonstration that the negative predictive value was equivalent and the sensitivity at least as good in the ANA-EIA compared with the ANA-IFA gave impetus to the use of the ANA-EIA test in screening patients suspected of having diseases discussed previously. This screening method, however, necessarily loses information, such as pattern and titer that have been traditionally used to help sub-classify patients with connective tissue diseases.

The most recent versions of ANA-EIA use recombinant, or purified antigens to provide consistent quality as a screening test. By including only a

Table 4  
Sensitivity and specificity of ANA-IFA and ANA-EIA

Parameter	ANA-IFA (at <1:80)	ANA-EIA (at <1.2) <sup>a</sup>
Sensitivity	64.1	71.8
Specificity	80.7	76.2
Positive predictive value	34.7	32.6
Negative predictive value	93.4	94.4

<sup>a</sup> Data expressed as percentage.

Data from Gniewek RA, Sandbulte C, Fox PC. Comparison of antinuclear antibody testing methods by ROC analysis with reference to disease diagnosis. Clin chem 1997;43:1987–9.

selected menu of antigens, the ANA-EIA can eliminate nonspecific false-positives that provide confusing information [83]. The use of ANA-EIA is increasing. The ANA Survey from the College of American Pathologists 2001 (Survey 1 samples ANA-01, 02) reported that ANA-EIA is used in 151 sites that subscribe to their proficiency testing survey.

Most recent evaluations of the ANA-EIA by authors who are not associated with instrument and reagent manufacturers concur with the strong negative predictive value of this assay. One of the largest independent studies was published by Reisner et al [84] from the University of Texas Medical Branch in Galveston. They compared the ANA-EIA with the ANA-IFA results on 808 serum samples that had been submitted for routine ANA testing. The screening dilution of the ANA-IFA HEP-2 cells used was 1:40; however, in their laboratory, they required a 1:160 or greater result to be considered screen-positive. Clinical diagnoses were determined by review of medical records by a rheumatologist. As shown in Table 5, of the 143 samples positive by the ANA-EIA, only 52 were positive by ANA-IFA at a titer of greater than or equal to 1:160. Three samples were negative by EIA and seven had borderline reactivity by ANA-EIA while demonstrating a positive ANA-IFA at that titer. They excluded from their calculations of predictive value the borderline results of 101 patients because follow-up is required for those individuals and this was not part of their study protocol. The ANA-EIA had a sensitivity of 94.6%, specificity of 86%, the negative predictive value of an ANA-EIA result was 99.5%, and the positive predictive value was 36.4%. Of the three samples that were positive by ANA-IFA and negative by ANA-EIA, one with a titer of 1:1280 had cytoplasmic staining and was interpreted as negative for ANA. Clinically, the patient did not have an autoimmune disease. Of the other two, one with an ANA-IFA titer of 1:160 was negative for autoimmune disease, whereas the other had an 8-month history of scleroderma and an ANA-IFA titer of 1:320. Because of the high negative predictive value and ease of automation of the ANA-EIA, Reisner et al [84] recommended a strategy of screening with their ANA-EIA and reflexing all positive samples for confirmation by the ANA-IFA. This strategy has the advantage of streamlining workflow while still providing titer and pattern information by the ANA-IFA. They calculated labor savings with this approach of 140 to 170 hours per year (using the College of

Table 5  
Comparison of ANA-EIA with ANA-IFA

ANA-EIA Result	ANA-IFA $\geq$ 1:160	ANA-IFA <1:160
Positive	52	91
Negative	3	561
Borderline	7	94

*Data from* Reisner BS, D. Blasi J, Goel N. Comparison of an enzyme immunoassay to an indirect fluorescent immunoassay for the detection of antinuclear antibodies. *Am J Clin Pathol* 1999;111:503–6.

American Pathologists work units [85]). In the author's comparison, an 86% agreement was found between ANA-EIA and ANA-IFA in 203 consecutive samples [86]. The discrepant results in the evaluation were almost always cases of low titer (<320) or weak EIA reactivity. As a result, with the ease of automation of EIA ANA tests, several larger laboratories are using them as the screening procedure. Some laboratories use the recommendation of Reisner et al [84] and reflex positive results to classic IIF ANA tests to provide titers and patterns for those clinicians who prefer them. Others offer only further testing with more specific single-antigen coated wells.

An impressive negative predictive value also was reported by Homburger et al [87]. They studied serum samples stored at  $-70^{\circ}\text{C}$  that had been previously found to be positive by ANA-IFA testing at 1:40 on mouse liver substrate. All of the 197 samples known to be from patients with systemic rheumatic disease were positive by ANA-EIA. Their results confirm that the negative predictive value in this subset of such patients at least matches the former gold standard (frozen section substrates) used in their laboratory and was slightly superior to the Hep-2 ANA-IFA that was performed simultaneously with the ANA-EIA. In healthy control subjects, both the ANA-EIA and the ANA-IFA had a positive rate of 15.6%. These were samples that had relatively low titers or low EIA units of ANA activity. Other workers [88,100] have reported similar strong sensitivity in the ANA-EIA as compared with the ANA-IFA. Indeed, Kern et al [89] noted that their comparison found 98% of sera negative by the ANA-EIA but positive by the use of ANA-IFA on rat liver and Hep-2 were from controls. On the other hand, a recent comparison between two automated ANA-EIA methods and a 1:160 cutoff of a Hep-2 ANA-IFA method by Bossuyt [90] did not confirm the high sensitivity of the other recent ANA-EIA studies. Clearly, all manufacturers are not the same and each laboratory should compare the performance of the kits with their own populations before adopting any new technique.

In its present state of development, however, the ANA-EIA does not seem to be superior in specificity to the ANA-IFA. The data of Homburger et al [87] indicate that patients who lacked diseases known to be associated with strong titers of ANA, but who had positive ANA-IFA methods, still were positive for ANA-EIA. Just as with the ANA-IFA where a higher titer is more likely to be associated with systemic rheumatic diseases, a high ANA-EIA in the data of Homburger et al [87] (>3) was more likely to be associated with these diseases than a positive value between 1 and 3 EIA units. Yet, even with improvements in both the ANA-IFA and ANA-EIA method, clinicians express frustration with the large number of false-positive ANA results that they receive. Because with either test the stronger reactivity correlates with a higher chance of having SLE, some workers have examined if use of the strength of ANA reactivity alone could improve the specificity of the ANA as a screening test. Vaile et al [91] performed a retrospective chart review on 320 individuals with high titer ANA screening test

results. They found that almost two thirds did not have a diagnosis of connective tissue disease and concluded that a higher cutoff for the screening ANA did not allow the clinician to abandon subsequent specific antibody testing.

In a recent paper, Rondeel et al [92] explored three strategies to optimize ANA screening. They compared the use of ANA-IFA with immunodiffusion (strategy 1); ANA-EIA (strategy 2); and a combination of ANA-IFA with ANA-EIA (strategy 3). Although they found the ANA-IFA with immunodiffusion superior to the ANA-EIA, they concluded that the poor performance of ANA testing related to the lack of selective test ordering by clinicians. A similar conclusion was recently reached by the recently published findings from the Consensus Conference on ANA testing [1]. One key problem with unselective ANA testing is that most normal individuals have some antibody reactivity with DNA. Typically, this is very low-level reactivity and is not detected by the usual screening dilutions used in ANA testing. It has been speculated that these types of antibodies may be stimulated by responses to foreign, for instance microbial, DNA, or perhaps when host cells are damaged and the DNA released may serve as a source of stimulation for these antibodies [93,94].

### **Patient selection and ANA tests**

The initial selection of patients is the first critical decision point that determines the predictive value of the ANA test [1,92]. All tests have a false-positive rate. When a test is performed of serum from patients selected to have a reasonably high likelihood of having the particular disease, the predictive value of a positive result of the test is usually high. In contrast, when the same test is performed in a low-incidence population, the chances of a false-positive result may become much greater than a true-positive result [71]. As discussed previously, the ANA test has a false-positive rate reported as being as high as 20% or more (depending on the cutoff, assay used, and interpretation of borderline staining) [1]. Because of this, the impressive sensitivity of the ANA test, greater than 95% for detecting patients with SLE, is diminished by its apparently poor performance when a low-incidence population is selected for testing. In the survey by Rondeel et al [92], the most frequent reasons for ordering an ANA test were joint symptoms (37%); follow-up (30%); or abnormal laboratory result (7%). Although arthritis is one of the classification criteria for SLE, the American College of Rheumatology recommends that 4 of the 11 criteria in Table 6 be present to confer a diagnosis of SLE on a patient [95,96]. This produces 95% specificity with a sensitivity of 85% for the diagnosis of SLE [95]. When there are less than four criteria present, the patient still may have SLE, but clinical judgment and different weight for each criterion may be applied. For instance, if the ANA is negative, it is unlikely that the patient has SLE because the test has a relatively high negative predictive value. On the other

Table 6

1997 Update criteria for the classification of systemic lupus erythematosus

## Clinical and laboratory features

- 
1. Malar rash
  2. Discoid rash
  3. Photosensitivity
  4. Oral or nasal ulcers (usually patches)
  5. Nonerosive arthritis (two or more joints)
  6. Pleuritis or pericarditis
  7. Renal disorder
  8. Neurologic disorder
    - Seizures or psychosis
  9. Hematologic disorder
    - Hemolytic anemia leukopenia, thrombocytopenia
  10. Immunologic disorder
    - Anti-DNA
    - Anti-Sm
    - Antiphospholipid antibody
      - False = positive test for syphilis  
(*confirmed*) or
      - Anti-cardiolipin antibody  
(*IgG or IgM*) or
      - Anti-lupus coagulant test
  11. Antinuclear antibody (in the absence of drug)
- 

*Modified from* Hochberg MC. Updating The American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus [letter]. *Arthritis Rheum* 1997;40:1725; with permission.

hand, a positive ANA result in a patient with no or minimal features of SLE is highly unlikely to have significance and may lead to unnecessary tests, an erroneous diagnosis, or inappropriate therapy. Patients with vague joint symptoms who lack any other criteria are unlikely to benefit from an ANA test.

Because the prevalence of SLE is only 1 in 1000 individuals, the chance of a false-positive in subjects who do not have SLE is considerably greater than a true-positive if patients are not selected appropriately for testing [95]. Even in the best circumstances 5% of normal individuals give a positive-ANA screening result [1]. If one tested all individuals among 100,000 unselected subjects one would detect 5000 positive results. Clearly, this number dwarfs the 100 true cases of SLE that would be expected. Because many normal individuals occasionally have minor joint pain, when joint pain alone is used as a reason to perform an ANA screening test, the likelihood of a positive result indicating disease is low. This is why pretest selection of patients with appropriate symptoms is so important.

In their guidelines for ANA testing, Kavanaugh et al [1] stratified the use of ANA testing as very useful for the diagnosis of SLE and scleroderma, and somewhat useful for the diagnosis of Sjögren's syndrome and dermatomyositis (Table 7). The ANA test also needs to be used under other circumstances. The presence of a positive ANA test is part of the diagnostic criteria for

Table 7  
Diseases where ANA testing is very or somewhat useful for diagnosis

Disease	Percent ANA positive
Systemic lupus erythematosus	95–100
Scleroderma	60–80
Sjögren's syndrome	40–70
Dermatomyositis or polymyositis	30–80

From Kavanaugh A, Tomar R, Reveille J, Solomon DH, Homburger HA. Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantibodies to nuclear antigens. Arch Pathol Lab Med 2000;124:71–81; with permission.

drug-induced lupus, autoimmune hepatitis, and MCTD [1]. In juvenile chronic arthritis and Raynaud's phenomenon, the ANA test may provide information that is useful to monitor diseases that are diagnosed clinically. In patients with juvenile chronic arthritis, the presence of a positive ANA test identifies those who are most likely to develop uveitis and benefit from more detailed ophthalmologic examination. Similarly, the presence of a positive ANA test in individuals who have the clinical diagnosis of Raynaud's phenomenon increases from about 19% to 30% the likelihood that a specific systemic rheumatic disease will develop. A negative ANA decreases the likelihood from 19% to 7% [1].

Avoidance of overuse is the key to improving the positive predictive value of the ANA test. ANA tests are commonly performed but not considered useful for diagnosis in rheumatoid arthritis, multiple sclerosis, idiopathic thrombocytopenic purpura, thyroid disease, discoid lupus, infectious diseases, malignancies, patients with silicone breast implants, fibromyalgia, and relatives of patients with autoimmune diseases (including SLE and scleroderma) [1].

Lastly, after the ANA test is positive under appropriate screening conditions, subsequent determination of which specific auto-antibody is present should take into consideration the specific symptoms of each patient. When the ANA is positive, it confirms that some antigen within the nucleus of the cell reacts with the patient's serum. There is a considerable diagnostic difference, however, when that something is Scl-70 (topoisomerase 1), which is relatively specific for progressive systemic sclerosis (scleroderma), versus Sm, which is highly specific for SLE. Reviewing the many specific autoantibody tests available to evaluate a positive ANA screen is beyond the scope of this article. Some methods use an immunoblot structure that allows ready detection of several autoantibodies at once [97]. Others are counterimmunoelectrophoresis, immunodiffusion, specific EIA, or radioimmunoassay techniques [98,99]. There are many specific tests generally available including anti-dsDNA; anti-Sm; anti-nRNP; anti-Ro (SS-A); anti-La (SS-B); anti-centromere; anti-Scl-70; and anti-Jo-1 (Table 8). Although some workers offer large batteries of tests for specific ANA antigens, the Guidelines panel does not recommend this type of shotgun testing [1]. The panel provided a

Table 8  
Guide to follow-up testing when the ANA is positive

Clinical suspicion	Further specific testing
Systemic Lupus erythematosus	Anti-dsDNA, anti-Sm
Scleroderma	Anti-Scl-70, anti-centromere <sup>a</sup>
Sjögren's syndrome	Anti-Ro (SS-A), anti-La (SS-B)
Polymyositis or dermatomyositis	Anti-Jo
Drug-induced lupus	No further testing
Mixed connective tissue disease	Anti-nRNP

<sup>a</sup> Anti-centromere is included when ANA-IFA on HEp-2 cells are used for screening. It may need to be added if ANA-IFA using frozen section substrates or ANA-EIA is the screening test.

Data from Kavanaugh A, Tomar R, Reveille J, Solomon DH, Homburger HA. Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantibodies to nuclear antigens. Arch Pathol Lab Med 2000;124:71–81.

flow chart using the clinical differential diagnosis to guide testing, briefly summarized in Table 8.

To help with improving the ordering of ANA tests, the laboratory should play a proactive role. The laboratory should select its menu of tests carefully, avoiding fads and unproved recent tests. An algorithm relevant to the local physician referrals should provide a logical sequence of screening and subsequent testing. The laboratory should produce a regular newsletter reviewing and updating the developments in autoimmune testing. These should be readable, brief, and up-to-date. Lastly, the laboratory should have a representative on local committees that determine the testing standards for a local hospital or community.

## Summary

The ANA test is an excellent screening test for patients with SLE and a few other connective tissue diseases. The LE cell preparation is an assay that is subjective and costly. Because of the presence of a superior screening test (the ANA) and superior specific auto-antibody tests, the author recommends that the use of LE cell preparations be discontinued. ANA screening tests may be performed either by indirect microscopic serology (usually IFA) or EIA. The latter technique is readily automated and many new products for this screening test have appeared in the past decade. The products differ, however, and laboratories are cautioned to test each in the context of the clinical needs of their clinicians. Proper use of the ANA test requires each laboratory to determine the cutoff used under their conditions of assay. Although either ANA screening test has a high negative predictive value in numerous studies, proper selection of patients to be tested is key to improving the predictive value of a positive result. The American College of Rheumatism criteria are reviewed and recommended as part of the patient selection process for this testing.

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