Screening for Antinuclear Antibodies by Enzyme Immunoassay

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Indirect fluorescent antibody (IFA) is the most widely used method in clinical laboratories to screen for autoantibodies against a wide variety of nuclear antigens. Recently, a number of antinuclear antibody (ANA) enzyme immunoassay (EIA) screens have become commercially available and claim to be an alternative method to screen for ANAs. Given the subjectivity of technical interpretation of IFA and the high number of ANA negative samples, a suitable EIA method for ANA screening would be beneficial to clinical laboratories with large sample volumes. Five ANA EIA screens were compared (Elias, Helix, Sanofi, TheraTest and Zeus) to IFA using a human epithelial cell line (HEp-2). Sera from 601 patients submitted to our reference laboratory for autoimmune testing, and from 202 normal healthy blood donors, were included in this study. Samples with discordant results between IFA and EIA were further analyzed using single antigen EIAs for SSA, SSB, Sm, RNP, Scl-70, histones, dsDNA, and ssDNA. Analyses were based on clinically significant IFA titers of ≥1:160 as positive and <1:40 as negative.

When compared to IFA, agreement, sensitivity and specificity for each ANA EIA screen were as follows: Elias: 87.0%, 69.5% and 97.9%; Helix: 94.6%, 90.2%, and 97.3%; Sanofi: 95.0%, 93.7%, and 95.9%; TheraTest: 95.3%, 97.7%, and 93.5%; Zeus: 87.1%, 96.2%, and 81.4%, respectively. In conclusion, screening for ANAs by EIA using several commercial assays was both sensitive and specific when compared to IFA. Moreover, the EIA is objective and much less labor intensive when screening a large number of clinical specimens. None of the EIAs were 100% sensitive and, thus, may fail to detect a few of the nonspecific ANAs that demonstrate atypical as well as classical IFA patterns. The advantages of employing these nonsubjective assays to screen out the vast majority of ANA negative sera is clear. The authors still recommend confirming titers and patterns of sera with positive EIA screens using classical IFA methods employing HEp-2 cells. (Key words: Antinuclear antibodies; Indirect fluorescent antibody; Enzyme immunoassay; Autoantibodies) Am J Clin Pathol 1996;105:468–473.

Detection of antinuclear antibody (ANA) is widely accepted as an important aid in the diagnosis of many rheumatic and connective tissue diseases such as systemic lupus erythematosus (SLE), scleroderma, Sjögren's syndrome (SS), and polymyositis.[1,2] Antinuclear antibody testing by indirect fluorescent antibody (IFA) techniques using human epithelial cells (HEp-2) as an antigen substrate is the preferred method when screening for ANAs in patient sera. Screening for ANAs by IFA requires highly trained technical personnel is time consuming and subjective. Recently, a number of ANA EIA screens have become commercially available and claim to be an alternative method to screen for ANAs. If suitable, an EIA method could be very useful in the clinical laboratory for screening large numbers of specimens for ANAs. Our objective in this study was not to replace IFA with EIA, but rather to determine if current EIAs could be used to screen out ANA negative sera without significant numbers of false negatives. Subsequently, IFA would be used to confirm a pattern and titer for sera with positive screens by EIA.

MATERIALS AND METHODS

Clinical Samples

Sera from 601 patients submitted to our reference laboratory for autoimmune testing and from 202 normal healthy blood donors were included in this study. Clinical specimens were chosen from our serum bank based on observed IFA patterns and titers to insure a variety of autoantibodies as well as sera with negative IFA results. Sera that were positive for ANA demonstrated the classical IFA patterns of speckled, homogeneous, nucleolar and centromere as well as mixed and atypical patterns with titers ranging from 1:40 to 1:20,480. Sera positive for anticytoplasmic antibodies (ACA) demonstrated appropriate IFA cytoplasmic patterns of coarse speckled, homogeneous, and fine speckled for antimitochondrial

antibody (AMA), anti-histidyl-tRNA synthetase (Jo-1), and anti-ribosomal RNP (rRNP), respectively with titers ranging from 1:40 to 1:1,280. Because these samples were sent to us from other laboratories, clinical information was not available to us.

All specimens were stored at −20 °C until assayed. Samples that required repeat testing were stored at 4 °C, avoiding freeze/thaw cycles.

Anti-Nuclear Antibodies by IFA

All ANA IFA testing was performed using kits purchased from INOVA Diagnostics (San Diego, CA). These kits use optimally fixed human epithelial (HEp-2) cells as the substrate and affinity purified, fluorescein labeled anti-human immunoglobulin (Ig) type G conjugate (from goat) for the detection of autoantibodies in patient sera.

Samples were diluted 1:40 in phosphate buffered saline (PBS). Approximately 50 μL of diluted sample and prediluted controls were added to wells and incubated at room temperature for 30 minutes in a moist chamber. Slides were then rinsed and submerged in PBS for 5 minutes. Excess PBS was shaken off and approximately 50 μL of fluorescein-labeled IgG conjugate was immediately applied to each well and allowed to incubate at room temperature for 30 minutes in a moist chamber. Slides were washed in the same manner as above then mounted on coverslips using the provided mounting medium. Slides were viewed at 400× (numerical aperture of 0.85 mm) using an Olympus (Tokyo, Japan) BH-2 transmitting fluorescent microscope with a 100 Watts mercury lamp.

Samples showing fluorescence greater than the negative control were considered positive for ANA or ACA. All sera demonstrating antinuclear or anticytoplasmic patterns were titered to endpoint (last titer showing 1+ fluorescence). Antinuclear antibody testing is performed routinely in our laboratory and all procedures are followed precisely as stated in the product insert.

ANA Screening by EIA

The EIAs included in this evaluation were provided by the following manufacturers/distributors: Elias USA (Osceola, WI), Helix Diagnostics (West Sacramento, CA), Sanofi Diagnostics Pasteur (Chaska, MN), TheraTest Laboratories (Chicago, IL), and Zeus Scientific (Raritan, NJ). All kits employed antigens only from natural sources (HEp-2 extracts, bovine spleen, and thymus) with the exception of the Elias screen that used a combination of natural and recombinant antigens.

These EIAs claim to detect autoantibodies responsible for homogeneous, peripheral, speckled, centromere, and nucleolar IFA patterns. Claims of detecting specific ANAs (SSA, SSB, Sm, RNP, SCL-70/DNA-topoisomerase I, histones, dsDNA) are also stated in the inserts. All but the Sanofi screen claimed to detect antibody to Jo-1. In addition, Helix claimed to detect autoantibodies against rRNP, TheraTest against ssDNA, and Zeus against mitotic spindle apparatus. All protocols were performed as stated in the package inserts. Other than ANA EIA kits, no other funds were derived from the manufacturers for these studies.

Single Antigen EIAs for Specific ANAs and ACAs

Single antigen EIAs for SSA, SSB, Sm, RNP, SCL-70, histones, dsDNA, AMA (M2 specific), and Jo-1 were provided by or purchased from INOVA Diagnostics. Enzyme immunoassays for ssDNA were purchased from TheraTest Laboratories. All single antigen EIAs were performed as stated in the package inserts.

EIAs for SSA, SSB, Sm, RNP, SCL-70, histones, dsDNA, and ssDNA were incorporated into the study in an attempt to resolve discrepant results between IFA and the EIA ANA screens. Indirect fluorescent antibody-negative (<1:40) sera giving positive EIA screen results (by at least 3 EIAs) and IFA clinically significant positive (≥1:160) sera with negative EIA screen results (by at least 2 EIAs) were assayed using the eight previously mentioned single antigen EIAs. EIAs for Jo-1 and AMA M2 were used to semiquantitate and confirm the presence of these autoantibodies that demonstrated appropriate IFA patterns on HEp-2. The INOVA EIAs (SSA, SSB, Sm, RNP, SCL-70, histones, dsDNA, Jo-1, and AMA M2) are semiquantitative (IU/mL for dsDNA) for the detection of IgG autoantibodies in human sera. They use antigens affinity purified from calf thymus coated in polystyrene microwells. All assays were performed as stated in the product inserts.

The TheraTest anti-ssDNA is a semiquantitative EIA for the detection of IgG autoantibody in human sera. This assay employs purified denatured DNA coated in polystyrene microwells as the antigen substrate. The protocol for this assay is identical to the TheraTest ANA screen included in this study and was performed as stated in the product insert.

Washing steps for all EIA assays were accomplished by using a Wellwash 4 automated EIA plate washer from Denley Instruments (Durham, NC). Optical densities for all EIAs were measured using a Thermomax bichromatic microplate reader from Molecular Devices (Menlo Park, CA).

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TABLE 1. AGREEMENT, SENSITIVITY, AND SPECIFICITY OF FIVE EIA ANA SCREENS WHEN COMPARED WITH HEp-2 IFA ANA RESULTS

	IFA			IFA			IFA			IFA			IFA	
	+	−		+	−		+	−		+	−		+	−
Elias			Helix			Sanofi			TheraTest			Zeus		
+	148	7	+	193	9	+	192	14	+	208	22	+	205	63
−	65	332	−	21	330	−	13	325	−	5	317	−	8	276
Agreement (%)	87.0			94.6			95.0			95.3			87.1	
Sensitivity (%)	69.5			90.2			93.7			97.7			96.2	
Specificity (%)	97.9			97.3			95.9			93.5			81.4	

EIA = enzyme immunoassay; ANA = antinuclear antibody; IFA = indirect fluorescent antibody.

Anti-Jo-1 by Ouchterlony Double Diffusion

Sera that demonstrated homogeneous cytoplasmic staining by IFA were assayed using the anti-Jo-1 EIA listed previously. Ouchterlony double diffusion kits (purchased from INOVA Diagnostics) were used to confirm the presence of Jo-1 antibody in samples with positive EIA results.

This assay uses Jo-1 antigen partially purified from calf thymus for the detection of precipitating anti-Jo-1 antibody in human sera using Ouchterlony double diffusion techniques. Reactions of identity, partial identity, or nonidentity were interpreted for each patient sample using a back-lighted, magnified viewing box (Behring Diagnostics). Samples with no reaction after 24 hours were further incubated (room temperature in moist chamber) and reexamined at 48 hours for any newly developed line of precipitation. Ouchterlony testing is performed routinely in our laboratory as specified in INOVA product inserts.

RESULTS

When compared to HEp-2 ANA by IFA, agreement, sensitivity and specificity for the five EIA ANA screens (Table 1) in clinical and blood bank sera combined were as follows: Elias: 87.0%, 69.5%. and 97.9%; Helix: 94.6%, 90.2% and 97.3%; Sanofi: 95.0%, 93.7%, and 95.9%; TheraTest: 95.3%, 97.7%, and 93.5%; Zeus: 87.1%, 96.2%, and 81.4%, respectively. Sera positive for Jo-1 antibodies (n = 8) were omitted from Sanofi's analyses. The sample positive for rRNP antibodies was included only in the analyses for Helix.

Analysis of sera with discrepant results between IFA and EIA screens are shown in Table 2. Four of 7 sera that were IFA negative/EIA screen positive (by at least 3 EIAs) showed 3 sera (samples 1, 3 and 4) contained low levels of antibody to histones and low to moderate levels of antibody to dsDNA, whereas one serum (sample 2) gave a result that was near the cutoff for SSA. The re-

maining three sera (not shown) gave negative results for the specific ANAs. Results for samples that were IFA positive/EIA screen negative (by at least 2 EIAs; n = 20) indicated that 2 sera (samples 5 and 9) contained low levels of antibody to histones, 1 serum (sample 8) had a moderate level of antibody to dsDNA, and 2 sera (samples 6 and 7) possessed high levels of antibody to ssDNA only. The remaining 15 sera (not shown) demonstrated no ANA specificity. Thus, only 3 of 20 sera (15%) which were IFA positive/EIA negative were found to contain sufficient antibody with an ANA specificity (2 high ssDNA/1 moderate dsDNA) to be clinically significant.

In the 202 sera from normal healthy blood donors, there was detectable autoantibody by IFA in 12.4% of samples, 1.5% of which possessed clinically significant titers by IFA (speckled 1:160, n = 2; speckled 1:2560, n = 1). Most of the EIA screens demonstrated good performance in screening out sera from normal individuals. Agreement, sensitivity, and specificity for the EIA screens were 99.4%, 100.0%, 99.4% for Elias; 99.4%, 100.0%, 99.4% for Helix; 99.4%, 100.0%, 99.4% for Sanofi; 97.8%, 100.0%, 97.2% for TheraTest; and 79.4%, 100.0%, 79.1% for Zeus, respectively, when compared to IFA with these blood bank samples. The following blood bank sera with equivocal IFA results were excluded from these analyses: speckled 1:40 (n = 18), speckled 1:80 (n = 2), nucleolar 1:40 (n = 1) and cytoplasmic 1:40 (n = 1).

When screening sera with IFA titers of 1:40 (n = 145), the percent positive for the EIAs were as follows: 15% (Elias), 14% (Helix), 32% (Sanofi), 29% (TheraTest), and 50% (Zeus). In contrast, the percent positive in sera with IFA titers of 1:80 (n = 87) increased significantly for most of the EIAs (Elias 22%, Helix 48%, Sanofi 54%, TheraTest 57%, and Zeus 71%) as was expected. The majority of these 232 samples demonstrated speckled or homogeneous patterns by IFA. These samples have been omitted from the statistical analyses shown in Table 1 because titers of 1:40 and 1:80 are of equivocal clinical significance in our opinion.

TABLE 2. SERA WITH DISCORDANT RESULTS BETWEEN EIA ANA SCREENS AND HEp-2 IFA ANA THAT CONTAINED DETECTABLE LEVELS OF AUTOANTIBODY BY SINGLE ANTIGEN EIAs

No.	IFA Result	Results from EIA ANA Screens					Results from Single Antigen EIAs*							
		Elias	Helix	Sanofi	TheraTest	Zeus	SSA	SSB	Sm	RNP	SCL-70	Histone	dsDNA	ssDNA
1	<1:40	−	+	+	+	+	5	5	7	8	11	1.3†	209†	33
2	<1:40	+	+	+	−	−	18	5	4	6	5	0.6	120	0
3	<1:40	+	−	+	+	+	7	8	5	8	8	1.4†	342†	13
4	<1:40	−	+	+	+	+	5	6	6	7	8	1.2†	204†	20
5	Spk 1:160	−	−	+	+	+	6	6	5	7	11	1.3†	150	29
6	Spk 1:640	+	−	−	+	+	4	4	6	4	6	0.9	198	514†
7	Hom 1:160	−	−	−	+	−	6	4	4	5	5	0.8	111	409†
8	Nuc 1:160	−	−	−	−	+	6	4	3	5	4	0.4	381†	0
9	Nuc 1:160, Spk 1:80	+	−	−	+	+	5	5	5	6	7	1.2†	185	0

EIA = enzyme immunoassay; ANA = antinuclear antibody; IFA = indirect fluorescent antibody; Spk = speckled; Hom = homogenous; Nuc = nucleolar.

* Cut-off values for single antigen EIAs are as follows: SSA, SSB, Sm, RNP, and SCL-70 = 20 U/mL; Histone = 1.0 U/mL; dsDNA = 200 IU/mL; ssDNA = 99 U/mL.

† Positive antibody level.

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Because of the low sensitivity observed using the Elias screen (Table 1), we selected 13 sera for further evaluation. These sera demonstrated classical IFA patterns with clinically significant titers (≥1:160) and were found to be negative only by the Elias screen. Further analyses showed variable levels of antibody (low-to-high for histones, low-to-moderate for dsDNA) to dsDNA and/or histones in 7 of the 13 sera.

The data for anti-Jo-1 positive sera (n = 8) indicated a decrease in sensitivity for this autoantibody when using the Helix and Zeus screens. These EIAs failed to detect five (Helix) and two (Zeus) of the eight sera positive for Jo-1 antibody. The presence of antibody to Jo-1 was determined by both Ouchterlony double diffusion and EIA methods. The Elias and TheraTest screens demonstrated 100% sensitivity for Jo-1 antibody. Even though the Sanofi screen did not claim to detect antibody to Jo-1, it also detected all Jo-1 positive sera.

Although none of these screens claimed to detect AMA, the TheraTest and Zeus EIAs detected all AMA positive sera (n = 7). These sera demonstrated coarse cytoplasmic staining of HEp-2 cells (1:160–1:1280) typical of AMA and all were highly positive for the M2 antigen by EIA.

We obtained one serum sample positive for rRNP antibody from INOVA diagnostics to include in our study. This sample demonstrated fine smooth speckling of the cytoplasm with staining of the nucleoli by IFA to a titer of 1:320. All EIA screens gave positive results for this serum except the Elias screen, but only Helix claimed to detect this rare cytoplasmic autoantibody.

Because most of these EIA screens begin with a HEp-2 cellular extract, one can assume the presence of mitochondrial and rRNP antigens.

Sera positive for autoantibodies that demonstrated a discrete atypical coarse speckling by IFA (n = 5; 1:320–1:2560) referred to as the "pseudo-centromere" or "nuclear dot" pattern gave variable results between the EIA screens. None of the screens detected all five sera, indicating decreased sensitivity of all the EIAs for this atypical autoantibody. These sera were also omitted from the statistical analyses in Table 1 because the clinical significance of this autoantibody has not yet been established and no claims were made for detecting atypical ANAs.

DISCUSSION

This is the first comprehensive study in which ANA screening by EIA, employing the majority of newly developed EIA ANA screens that are commercially available has been compared to the standard IFA method and to each other. Moncé and colleagues[3] compared the He-

lix EIA ANA screen to IFA ANA using HEp-2 cells in which similar findings were noted as in our study for the Helix screen, but data for anti-cytoplasmic autoantibodies, particularly Jo-1, are absent from that study.

Most of these EIA ANA screens demonstrated good correlation with IFA. Claims of sensitivity and specificity in some product inserts were lower than what we have determined mainly because the manufacturers have used titers of 1:40 or 1:80 as the positive cut-off for IFA. Low titers such as these are often found in the normal population and usually do not contain specific autoantibodies.[4-6] Therefore, one would expect these EIAs to sometimes disagree with IFA titers of 1:40 or 1:80. In contrast, we chose to use 1:160 as the positive cut-off for the IFA. Thus, some assays performed better with this more realistic cut-off than claimed in the inserts. Our data on sera with IFA titers of 1:40 and 1:80 showed average positive rates of 28% and 50%, respectively, using these EIA screens.

Some screens missed few sera with IFA titers ≥ 1:160. Results obtained from the analyses of discordant sera showed that the majority did not possess autoantibodies of known clinical significance. In fact, only one sample (sample 8; Table 2) contained significant levels of an autoantibody (dsDNA) specific for a rheumatic disease (SLE). Thus, the true false-negative rate for the better EIA screens was <0.5% in the 213 IFA-positive sera. However, there is evidence for sample 8 as well as others in Table 2 (samples 1, 3, and 4) that suggests false detection of antibody to dsDNA because homogenous patterns were absent and there was little or no cross-reactivity with the ssDNA EIA using these sera.

Our main interests were to determine the overall correlation between these EIA screens with IFA and to each other whether a specific ANA was present or not. The TheraTest and Zeus screens had better agreement with IFA in the discordant sera (Table 2) that were shown to possess specific ANAs. Moreover, TheraTest was the only screen to detect the high level of ssDNA antibody found in sample 7 (Table 2). However, antibodies to ssDNA are not readily detected by IFA since interphase and metaphase HEp-2 cells lack this antigen.[7] Therefore, the homogeneous pattern of 1:160 for sample 7 may have been the cause of some nonspecific ANA since EIAs for histones and dsDNA gave negative results. There were other sera in the study (sample 8, Table 2; others not shown) in which data from further analyses did not correlate with the IFA patterns and will be investigated further.

The sera positive for antibodies to Jo-1 and rRNP were included in this study to challenge specific claims for ACA(s) made by the manufacturers. Antibodies to Jo-1

are found in a sub-group of myositis patients with interstitial pulmonary fibrosis (68%) with poor prognosis[7,8] and antibodies to rRNP are found in 12% to 15% of SLE patients and have been strongly associated with the neuropsychiatric manifestations in some lupus patients (90%).[9,10] Both of these ACAs rarely occur in the clinical laboratory and specific testing is usually requested for their detection. Therefore, screens that showed low sensitivity for Jo-1 or rRNP may not be a concern for some reference laboratories who perform these specific tests separately from their regular ANA workload. We expected a substantial cost savings if approximately 70% of our IFA ANA workload could be eliminated by using an EIA screen followed by confirmation of positive results by IFA. According to our current IFA ANA workload, the data from this study, current list prices for ANA IFA HEp-2 kits, and the average list price for ANA EIA screens, we calculated a 32% increase in total cost (labor and reagents) if this format of ANA testing (using manual methods for EIA) were to be implemented in our laboratory. With our discount on IFA reagents (due to volume) and the savings in labor costs, we expect an actual cost savings of 16% by automating the EIA ANA screen. These figures will vary between laboratories depending on workload, reagent/labor costs and method of testing: manual *versus* automated. Most of the 32% increase is due to the high cost of EIA ANA screens. These are figures that must be reduced in this era of health care reform if the manufacturers of these assays expect acceptance of their product.

Anyone routinely performing IFA ANA testing knows how time consuming and subjective the method can be, especially when sample volumes are high. Because 70% of our IFA ANA workload is negative for ANAs, a more efficient, objective method such as EIA to screen for ANAs seemed to be a practical alternative. From this study, we conclude that some of these EIA ANA screens have proved to be specific and sensitive enough to assist in the screening portion of ANA testing. However, none of the EIAs in this study demonstrated 100% sensitivity when compared to IFA, and thus may fail to detect a few of the nonspecific ANAs that present atypical as well as classical IFA patterns on HEp-2 cells.

Further details on test performances are available per request from the author.

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