Evaluation of a Line Immunoblot Assay for Detection of Antibodies Recognizing Extractable Nuclear Antigens

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Sera ($n = 90$) giving positive results in a screening test for antibodies to extractable nuclear antigens (ENAs) were tested in a line immunoblot assay that measures antibody reactivity with individual ENAs in a single test field. Results were then compared to those obtained in monospecific ENA antibody enzyme immunoassays (EIAs). Discordant results were resolved by immunodiffusion. Of 540 result pairs (90 sera tested for 6 ENAs [Sm/RNP, Sm, SSA, SSB, Scl-70, Jo-1]), 509 (94%) showed concordance. Immunodiffusion resolved 28 of 31 discordant result pairs in favor of the

immunoblot result. After resolution of discordant data, the immunoblot assay exhibited 100% sensitivity for all ENA antibodies except those recognizing Scl-70, for which the sensitivity was 89%; specificity was over 96% for all 6 ENA antibodies. These findings show that a line immunoblot assay for the characterization of ENA antibodies yields results comparable to those obtained using monospecific ENA antibody EIAs. The immunoblot assay is easier and less expensive to perform due to its utilization of a single test field. *J. Clin. Lab. Anal.* 12:320–324, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

Identification of antibodies recognizing extractable nuclear antigens (ENAs) is useful in the diagnosis and characterization of a variety of connective tissue diseases. SSA and SSB antibodies are found in association with systemic lupus erythematosus (SLE) and Sjogren's syndrome, and Sm antibodies are highly specific for SLE (1–3). Ribonucleoprotein (RNP) antibodies are found in various collagen vascular diseases; high titers of RNP antibodies are associated with mixed connective tissue disease (3,4). Scl-70 antibodies are found in some patients with systemic sclerosis, and Jo-1 antibodies are associated with polymyositis, often complicated by pulmonary fibrosis (5–7).

Laboratory testing for ENA antibodies routinely involves initially performing a screening enzyme immunoassay (EIA) to detect antibodies recognizing a mixture of ENAs in a single microtiter well (8). A second phase of testing is then required to determine which ENAs are actually recognized by the reactive antibodies. Although immunodiffusion (ID) is considered the gold-standard assay for identifying ENA antibody specificities, many laboratories now utilize EIAs in which a single purified ENA is bound to the solid phase (8–12). Thus, a serum specimen reactive in the screening ENA antibody EIA must be further tested in six monospecific ENA antibody EIAs.

An alternative approach for identifying ENA antibody

specificities is the line immunoblot assay developed by Euroimmun (Lubeck, Germany). This assay employs microscope slides with five test fields per slide; each test field contains nitrocellulose paper coated with parallel lines of individual purified ENAs. Thus, antibody reactivity to all six individual ENAs can be visually assessed in a single field. The aim of this study was to compare the ENA line immunoblot assay to monospecific ENA antibody EIAs for the characterization of sera positive in the screening ENA antibody EIA.

MATERIALS AND METHODS

Serum Specimens

The main study panel consisted of 90 sera (each from a different patient) giving positive results in a screening EIA for ENA antibodies (ENA Screen 6 Kit manufactured by Hemagen, Waltham, MA). The core of the panel consisted of all consecutive screen-positive specimens identified during a five-week period ($n = 75$). Additional screen-positive sera were selected from a frozen serum bank based on prior positive results in ID assays for Sm antibody ($n = 5$), Scl-70 anti-

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body ($n = 5$), or Jo-1 antibody ($n = 5$). A secondary panel consisted of 30 consecutive screen-negative specimens selected for testing in the line immunoblot assay only.

Monospecific ENA Antibody EIAs

Monospecific ENA antibody EIA kits were manufactured and generously supplied by Hemagen. The antigenic specificities were Sm/RNP, Sm, SSA, SSB, Scl-70, and Jo-1. All six kits were used according to the manufacturer's instructions. Patient sera giving optical density (OD) values greater than the cutoff OD value were considered positive for the relevant ENA antibody.

Line Immunoblot Assay

The line immunoblot assay (ProfilPlus Euroblot, manufactured by Euroimmun) was performed according to the manufacturer's instructions. Each test kit contains microscope slides with two nitrocellulose strips fixed in each test field (five test fields per slide). The strips are coated with parallel lines of native affinity-purified antigens; one strip contains Sm/RNP, Sm, SSA, and SSB, and the other strip contains Scl-70, Jo-1, and a serum control (antihuman IgG). The slides were used with a reagent support system (Titerplane, Euroimmun), allowing all five test fields on the slide to come into contact with specimen at exactly the same time. Kit control sera (a negative control and a positive control recognizing all ENAs) and patient sera were diluted 1:100 in sample buffer, and 0.05 mL of each diluted serum was applied to a reaction area of the reagent support (five reaction areas per reagent support module, spaced to match the five slide test fields). Slides were then inverted and placed into recesses of the reagent support, allowing each slide test field to contact its relevant serum specimen. Slides were incubated for 30 min at room temperature on a circulatory shaker at 300 revolutions per min (rpm). Slides were then removed from the reagent support, and following a flush with wash buffer, were placed in a slide wash basin containing wash buffer. After 15 min, the slides were removed from the wash basin and placed on a reagent support containing 0.05 mL of conjugate (alkaline phosphatase-labeled goat antihuman IgG) per reaction

area. The slides were then incubated and washed as previously described. Each slide was then placed on a reagent support containing 0.05 mL of substrate solution (nitroblue tetrazolium/bromochloroindolyl-phosphate) per reaction area, and incubated for 10 min. Slides were then rinsed with deionized water and air-dried. A positive result was defined as a clearly visible blue/purple line within the area of the nitrocellulose strips defining a given ENA.

ID

Sera with discordant EIA versus immunoblot results for a given ENA specificity were tested by ID for the discordant antibody specificity. As a quality check, ID was also performed on subsets of sera with concordant positive EIA/immunoblot results for a given antibody specificity ($n = 8$ for Sm/RNP, Sm, SS-A, and SS-B, $n = 4$ for Scl-70 and Jo-1). Sera with concordant negative results for all six ENA antibodies ($n = 8$) were also tested by ID for all six antibody specificities. ID kits were purchased from Inova Diagnostics, Inc. (San Diego, CA), and used according to the manufacturer's instructions.

RESULTS

A comparison of monospecific ENA antibody EIA results and line immunoblot results obtained for the primary serum panel is shown in Table 1. Of 540 sets of results (90 sera tested for 6 ENA antibody specificities), concordance was observed for 509 (94%). Most discordant result sets (27/31 = 87%) showed a positive result in the monospecific ENA antibody EIA but a negative result by immunoblot. To better characterize this EIA-positive/blot-negative discordant group, the relationship between EIA OD values and blot reactivity was assessed for all specimens positive in monospecific EIAs. As shown in Table 2, negative blot results were strongly associated with EIA OD values in the low-positive range (defined as OD values between the cutoff OD (range = 0.093–0.214) and 0.3 in the relevant ENA antibody EIA). Of the 27 EIA-positive/blot-negative results pairs, 24 (89%) showed low-positive OD values by EIA. For all ENA specificities but SSA, the low-positive OD group showed a higher percent-

TABLE 1. Immunoblot Reactivity in Relation to Monospecific ENA Antibody EIA Reactivity for 90 Sera Testing Positive in a Screening Test for ENA Antibody

Antibody	Concordant results		Discordant results		% discordant
	EIA negative Blot negative	EIA positive Blot positive	EIA positive Blot negative	EIA negative Blot positive	
Sm/RNP	62	23	5	0	6
Sm	71	12	7	0	8
SSA	33	55	1	1	2
SSB	64	15	9	2	12
Scl-70	81	7	1	1	2
Jo-1	78	8	4	0	4
Totals	389	120	27	4	6

TABLE 2. Frequency of Negative Immunoblot Results in Relation to OD Values for EIA Positive Specimens

Antibody	Low-positive OD group ^a			High-positive OD group ^b		
	N	Blot negative	%	N	Blot negative	%
Sm/RNP	9	4	44	19	1	5
Sm	8	7	87	11	0	0
SSA	5	0	0	51	1	2
SSB	14	9	64	10	0	0
Scl-70	4	1	25	4	0	0
Jo-1	4	3	75	8	1	12
Totals	44	24	55	103	3	3

^aLow positive defined as OD values between the cutoff OD (range = 0.093–0.214) and 0.3 in the relevant ENA antibody EIA.

^bHigh positive defined as OD values of 0.3 or greater in the relevant ENA antibody EIA.

age of negative blot results compared to the high-positive OD group.

Specimens with discordant results were further tested by ID. As shown in Table 3, ID resolved 28 of 31 (90%) discordant results in favor of the immunoblot result; 26 of 27 EIA-positive/blot-negative specimens were ID negative, and 2 of 4 EIA-negative/blot-positive specimens were ID positive.

As a quality check to support the use of ID for resolving discordant EIA/immunoblot results, subsets of sera with concordant EIA/immunoblot results were tested by ID. All sera with concordant positive results for a given antibody specificity were also positive by ID ($n = 8$ for Sm/RNP, Sm, SS-A, and SS-B, $n = 4$ for Scl-70 and Jo-1). Likewise, eight sera giving concordant negative results for all antibody specificities were also negative by ID for all antibody specificities.

The performance characteristics of the immunoblot for detection of antibodies to individual ENAs, after resolution of discordant EIA/blot results by ID, are summarized in Table 4. Of 540 analyses, only 3 immunoblot results appeared to be incorrect (one false negative result for Scl-70 antibody, one false positive result for SSA antibody, and one false positive result for SSB antibody). The efficiency of the immunoblot (true negative plus true positive / total) was thus $537/540 = 99.4\%$.

The number of ENA specific antibodies identified per individual specimen is shown in Table 5. Of the 75 consecutive sera with positive results by screening EIA, 17 (23%) showed no immunoblot reactivity and 58 (77%) contained 1–3 specific ENA antibodies. As expected, all 15 sera selected from a serum bank due to prior detection of uncommon ENA anti-

bodies (Sm, Scl-70, or Jo-1) exhibited the expected ENA antibody; 11 of these 15 sera also contained additional ENA antibodies. Two specimens contained antibodies recognizing all six ENA specificities. None of 30 sera with negative results in the routine screening ENA antibody EIA showed immunoblot reactivity, further demonstrating the high specificity of the line immunoblot assay.

DISCUSSION

The findings presented here demonstrate that a commercially available line immunoblot assay for characterizing ENA antibodies yields results that are comparable to those obtained using monospecific ENA antibody EIAs. Only 6% of immunoblot results differed from the comparable EIA results, and most of these discrepancies reflected an EIA-positive (low OD)/blot-negative result set. Further testing by ID resolved 26 of 27 EIA-positive/blot-negative discrepancies in favor of the blot result; thus, when using ID as the gold standard, the immunoblot in fact appeared to be slightly more specific than individual EIAs.

Other investigators who have compared monospecific ENA antibody EIA results and ID results also reported a small percentage of discordant EIA-positive/ID-negative result sets (8,10–12). Because some patients with discordant results exhibited clinical characteristics consistent with connective tissue diseases (10,12) it has been suggested that EIA may be more sensitive than ID, and should replace ID as the gold standard for ENA antibody detection. Unfortunately, this issue could not be addressed in this study, since all specimens

TABLE 3. Resolution of Discordant Results by Immunodiffusion (ID)

Antibody	EIA-positive/Blot-negative			EIA-negative/Blot-positive		
	N	ID negative	ID positive	N	ID negative	ID positive
RNP	5	5	0	0	0	0
Sm	7	7	0	0	0	0
SSA	1	1	0	1	1	0
SSB	9	9	0	2	1	1
Scl-70	1	0	1	1	0	1
Jo-1	4	4	0	0	0	0
Totals	27	26	1	4	2	2

TABLE 4. Immunoblot Performance After Resolution of Discordant Results^a

Antibody	True negative	True positive	False negative	False positive	Sensitivity	Specificity
RNP	67	23	0	0	100%	100%
Sm	78	12	0	0	100%	100%
SSA	34	55	0	1	100%	97%
SSB	73	16	0	1	100%	99%
Scl-70	81	8	1	0	89%	100%
Jo-1	82	8	0	0	100%	100%

^aTrue negative (TN) = concordant EIA-negative/blot-negative results plus discordant EIA-positive/blot-negative results resolved as negative by ID. True positive (TP) = concordant EIA-positive/blot-positive results plus discordant EIA-negative/blot positive results resolved as positive by ID. False negative (FN) = discordant EIA-positive/blot-negative results resolved as positive by ID. False positive (FP) = discordant EIA-negative/blot-positive results resolved as negative by ID. Sensitivity = TP/(TP+FN). Specificity = TN/(TN+FP).

were referred to MRL from other laboratories, and complete clinical information was not available. Because EIA has not yet become widely accepted as the laboratory standard for ENA antibody measurements, ID was used as the gold standard here.

Utilization of ID for resolution of discordant findings was based on the assumption that concordant EIA/immunoblot results mirrored the ID result. Although testing of all 90 sera for all 6 ENA specificities by ID was financially infeasible, ID results for subsets of sera with concordant EIA/immunoblot results fully supported this assumption.

After resolution of discordant results by ID, only three apparently incorrect results (one false negative, two false positives) were obtained in the immunoblot assay. The false negative result (for Scl-70 antibody) was the lone discrepancy preventing a finding of 100% sensitivity for the immunoblot across the entire study (Table 4). This inconsistent result, along with the false positive results, may reflect subtle differences in the antigenic epitopes expressed by ENAs purified by different manufacturers. Additional studies are required to characterize these rare sera with inconsistent ENA antibody reactivities to different preparations of antigen.

In agreement with published findings (10,12), many screen-positive sera (29/90) contained antibodies recognizing multiple ENA specificities when tested by immunoblot (Table 5). As predicted from the structural relationship between Sm and RNP antigens (3–5), all 12 sera containing Sm antibody also exhibited Sm/RNP reactivity; further, 10 of these 12 sera also contained additional ENA antibodies (data not shown). Likewise consistent with published findings (1–3), 15 of 16 true positive SSB antibody-positive sera also

contained SSA antibody, and 4 of 16 contained other ENA antibodies as well.

The line immunoblot assay described in this report should not be confused with the Western immunoblot assay for detection of ENA antibodies (12–14). In the Western blot procedure, proteins in cell extracts are separated by electrophoresis in polyacrylamide gels, and then electrophoretically transferred to nitrocellulose paper. Thus, ENA specificities that are composed of multiple proteins (SSA, Sm/RNP) are separated into their individual components, each present in a different area of the paper. In addition to ENAs, many other cellular proteins are also present on Western blot strips, and antibodies reactive with these proteins may interfere with ENA antibody interpretation. In the line immunoblot assay, however, the paper contains only affinity-purified ENAs applied directly to a defined location; no other proteins are present that could be recognized by non-ENA antibodies in sera.

The advantages of the immunoblot over monospecific ENA antibody EIAs are mainly linked to its utilization of a single test field per specimen, rather than six test fields per specimen. This innovation reduces the required number of applications of diluted specimen, dramatically lowers the number of fields that must be devoted to controls, and enables rapid visual interpretation of results for all six ENAs in a single test field. In our experience, utilization of the immunoblot assay has resulted in savings of time and materials costs. As an additional advantage, immunoblot slides can be saved as a permanent record after test completion, and directly reviewed if questions arise.

Taken together, these data indicate that the line immunoblot

TABLE 5. Number of ENA Specific Antibodies Detected in Individual Sera by the Immunoblot Assay

Specimen group	N	Number of ENA specific antibodies per specimen:						
		0	1	2	3	4	5	6
Consecutive screen-positive	75	17	40 ^a	14	4	0	0	0
Serum bank	15	0	4	4	3	0	2 ^b	2
Consecutive screen-negative	30	30	0	0	0	0	0	0

^aIncludes 1 false positive SSA antibody.

^bIncludes 1 false positive SSB antibody.

assay is a reliable alternative to monospecific ENA antibody EIAs for defining which ENAs are recognized by antibodies in screen-positive sera. However, an extensive comparison with current ENA antibody characterization methods should precede implementation of the immunoblot assay to ensure comparability of results.

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