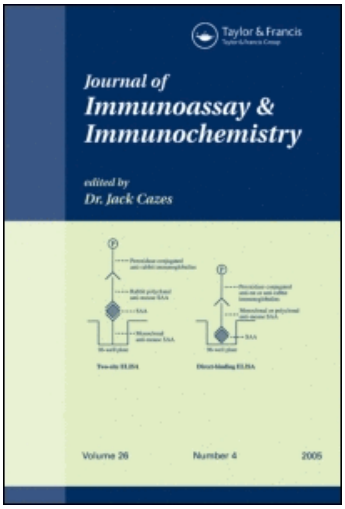

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Use of Flagellin-Enriched Antigens in a Rapid, Simple and Specific Quantitative Enzyme Immunoassay for Lyme Disease Antibodies in Human Serum Samples

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USE OF FLAGELLIN-ENRICHED ANTIGENS IN A RAPID, SIMPLE AND SPECIFIC QUANTITATIVE ENZYME IMMUNOASSAY FOR LYME DISEASE ANTIBODIES IN HUMAN SERUM SAMPLES.

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ABSTRACT

An enzyme immunoassay (EIA, ELISA) using microwells coated with a flagellin-enriched fraction of *B. burgdorferi* and absorbent-containing sample diluent for the quantitative determination of Lyme disease (LD) IgG and IgM antibodies in human serum samples was described. This LD EIA required three 15-minute incubations at room temperature, followed by a 1-step normalization of photometer readings to EIA units (EU/ml). Compared with tests using the whole bacterial extract as antigens and a sample diluent containing 6 % BSA, this new LD EIA revealed lower values for 20 syphilis (SS) and 21 normal serum samples (NS) but about the same for 21 Lyme disease (LD) samples, allowing lower cut-off points which would place almost all these SS and NS samples below while almost all LD samples above the positive cut-off point. The LD EIA results of larger numbers (67 to 291) of mixed samples correlated with results of four reference EIA. However, the LD EIA gave lower (2 to 4 fold) reactivities (index values) with SS and NS samples but higher values with positive serum samples than reference EIA. Thus, this LD EIA showed improvements in both specificity and sensitivity over other tests compared.
(KEY WORDS: Lyme disease antibodies, ELISA, flagellin-enriched antigens)

INTRODUCTION

Lyme disease (LD) was first discovered in the United States in 1975 and its cause by a spirochete species *Borrelia burgdorgferi* was elucidated by 1984 (1-4).

Some of clinical manifestations of LD have been reported in Europe for at least a century since 1883 (3). As in syphilis, another well known spirochetal disease, LD has three stages and can last for years if not treated. The signs and symptoms of LD are complex and are often clinically mistaken for many other diseases such as rheumatoid arthritis, brain tumors, and influenza. Serologic tests have therefore played an indispensable role in the diagnosis of LD, which is curable with antibiotics especially during the early stages of disease (3-6). Both immunofluorescence (IFA) and enzyme immunoassay (EIA) procedures have been used by clinical laboratories for detection of serum antibodies to *B. burgdorferi*, with EIA considered to be easier to do and superior in performance (3, 7-9). Both IFA and EIA as reported in the literature as well as in commercially available test kits still need to be improved (10, 11).

Numerous studies by Western blot have indicated that the major early immune responses are most frequently against 41 kd flagellin antigens, followed by antibodies to many other antigens such as outer envelope antigens 31 kd (OspA) and 34 kd (OspB) (7, 12, 13). Serologic tests using antigens rich in flagellin would show stronger reactions than otherwise. However, these antigens have or may have antigenic determinants shared with other spirochetes such as *Treponema pallidum* and even non-spirochetal bacteria (12-20). These cross-reactions could mask some weak LD-specific reactions thus reducing the sensitivity of the serologic tests. Reactions with syphilitic serum samples may be stronger than with weak LD serum samples. Therefore, the use of flagellin-enriched antigens in combination with a mechanism to remove cross-reactions would be expected to achieve higher specificity and sensitivity of serologic testing for LD. This assumption led us to develope an enzyme immunoassay for LD antibodies, and its performance characteristic is reported here.

MATERIALS AND METHODS

Antigens

Borrelia burgdorferi (B31 strain) (Bb) was grown in the BSK II medium (5), harvested by centrifugation, washed 4x with phosphate-buffered saline, pH 7.4 (PBS), and stored at - 70 C until antigen extraction. Flagellin-enriched antigens were prepared according to Coleman and Benach (13). Briefly, the bacteria were sedimented by cold centrifugation (12,000 g for 30 min) and then suspended in 0.03 % SDS (1 billion bacteria / ml) for 10 minutes at room temperature, followed by re-sedimentation (25,000 g, 90 minuntes) to remove solubilized outer envelope materials. After re-suspension in PBS, the organisms were sheared with a Waring blender for 10 minutes in an ice bath and centrifuged at high speed (25,000 g, 60 minutes). The supernatant was harvested as the flagellin-enriched antigens and stored at -20 C. Their concentrations were based on dry weight after lyophilization, corrected for salt contents.

For comparison studies, aliquots of the same batch of PBS-washed *B. burgdorferi* organisms without pretreatment with SDS were sonicated with six 20-second bursts at 30 second intervals in an ice bath and centrifuged at 10,000 g for 30 minutes, with the supernatant harvested as crude antigens.

Human Serum Samples

Normal serum samples were obtained from apparently healthy blood donors living in the South Florida (Group I, 97 samples), Missouri (Group II, 60 samples), Michigan (Group III, 100 samples), Wisconsin (Group IV, 454 samples) and several other states (Connecticut, New York and Nevada, Group V, 5 samples). A total of 282 clinical serum samples from patients suspected, but not necessarily proven, to have Lyme disease were acquired from four sources: Group A from 84 patients in Germany with major clinical features of LD equally divided

among the three clinical stages of LD; Group B from 162 patients in the New York and New Jersey States; Group C from 16 patients in Connecticut which were serologically positive for LD by IFA and/or EIA; and Group D from 20 patients in several other states (Massachessetts, Nevada and California). All Group A, most Group C and a number of Groups B and D patients meet the Centers for Disease Control 1982 Lyme disease case definition. These LD suspected clinical serum samples were used for comparisons among various serologic tests for Lyme disease antibodies. Twenty-one of these clinical samples were from patients with Lyme disease symptoms (ECM, Bell's Palsy, acrodermatitis chronica atrophicans, polyradiculitis, polyneuritis, carditis, lymphoma, arthritis, and/or others) and were also positive for Lyme antibodies tested by the laboratories that sent the samples to us; these 21 samples were identified as "documented LD serum samples " when applicable. Thirty-six syphilis RPR-positive serum samples were obtained either from a local county venereal disease clinic or from a blood supply center in Miami, Florida; these "syphilis" samples were further tested positive by the FTA-ABS, PHA and CORDIA Sy for treponemal antibodies in our laboratories (21). Serum samples from patients with infectious mononucleosis, systemic lupus erythematosus and rheumatoid factor were positive by commercial EIA for their specific antibodies as described elsewhere (22).

Lyme Disease Enzyme Immunoassay (LD EIA) Reagents

Antigen solution (100 µl per well) at a concentration of 100 µg/ml was placed in Immunolon 1 Removawells (Dynatech, VA) and incubated at 4 C overnight. The antigen solution was then replaced with 125 µl post-coat solution (0.6 % BSA in PBS). After a 30-minute incubation at room temperature, the postcoat was removed by shaking and drained as thoroughly as possible by inverting the wells

and tapping on paper towels. The antigen-coated wells were then dried and stored in a moisture-proof bag with desiccant at 4 C until assay.

Four different sample diluents were studied. Sample Diluent A contained 6 % BSA and 0.05 % Tween 20 in PBS as used elsewhere (22). Sample Diluent B was a modification of the one used in the syphilis FTA-ABS test, containing autoclaved *Treponema phagedenis* Reiter strain, rabbit serum and rabbit testis extract, as described elsewhere (21). Sample Diluent C was the same as Diluent B except the organisms ($5x10^7$/ml) were not autoclaved, not filtered and devoid of the rabbit testis extract. Sample Diluent D is from the Cambridge Bioscience Human Lyme EIA test kit (Cambridge, MA).

Four pools of 4 to 6 serum samples each, which were selected from the documented LD serum samples, were prepared according to their immunoflourescence antibody titers (64 to 1024) and, after extensive studies, one of them was chosen as Calibrator and the rest as Controls (two stronger and one weaker than the Calibrator). They were subdivided into 0.3 ml aliquots for short term storage (< 1 year) at 4 C and long term storage at -20 C when not in use. A pool of three negative serum samples served as the Negative Control. Goat anti-human IgG and IgM antibodies labeled with calf intestinal alkaline phosphatase (conjugate, 10 μg protein/ml), *p*-nitrophenyl phosphate (substrate, 1 mg/ml), wash and stop solutions (3M sodium phosphate, pH 12) have been described elsewhere (23). All reagents were ready-to-use and stable for at least 1 year at 4 C as described previously (23).

LD EIA Procedure

The reagent concentrations and assay conditions were optimized as described elsewhere (23), with the following procedure finally chosen. Test samples,

calibrator, one positive control and one negative control were diluted 1:101 with sample diluent (2 µl / 200 µl) in uncoated microwells for convenience. After mixing well, 100 µl of diluted sample was transferred into antigen-coated wells and incubated at room temperature for 15 minutes. At the end of the incubation, the fluid was shaken off and wells were washed 3 times quickly with wash solution. The wells were then drained thoroughly by inverting and tapping the wells vigorously on layers of paper towels. The wells were filled with 100 µl of conjugate and incubated 15 minutes at room temperature, washed as above and then filled with 100 µl of substrate. After another 15 minute incubation at room temperature, 100 µl of stop solution was added to stop the enzyme-substrate reaction and the hydrolyzed substrate product was read with a photometer at 405 nm. The absorbance values were divided by that of Calibrator and multiplied by 100 to obtain EIA units (EU/ml) for interpretation. Test samples with less than 15 EU/ml were considered negative for Lyme disease antibody; those with greater than 25 EU/ml, positive; and those between the two values, equivocal. The determination of these cut-off points are described in the Results.

Other Tests

The immunofluorescence assay for Lyme disease IgG and IgM antibodies was performed with reagents purchased from a commercial source (Zeus, NJ) and/or prepared according to reported procedures (8, 9). Commercial EIA test kits for Lyme disease IgG and IgM antibodies from four manufacturers were used according to the manufacturer's instructions (Hillcrest Biologicals, CA; Zeus, NJ; MarDx, NJ; Cambridge Biosciences, MA). The test results of these four reference and LD EIA were also compared after conversion to index values. The latter are the ratios of test sample absorbance to the positive cut-off point absorbance. Any test sample with an index value greater than 1 is positive for the test. The Hillcrest kit

used crude sonicate of *B. burgdorferi* as antigens and a sample diluent without specific absorbent.

The SDS-PAGE and Western blot procedure followed those described elsewhere, using commerical reagents and equipment (BioRad, CA) (13, 15). The electrophoresed antigens were stained with Coomassie blue or transferred to nitrocellulose paper for Western blot (sequential incubations in 1:100 dilution of Calibrator, conjugate and BCIP-NBT as substrate)

Correlation coefficient of Lee and Pearson was used to analyze correlation between test results, with a probability of 0.05 or less as significant (24).

RESULTS

Comparison between the Crude and Flagellin-Enriched Antigens

Three groups (21 normal, 20 syphilis and 21 documented LD) of serum samples were diluted with Sample Diluents A and C and assayed with wells either coated with crude or flagellin-rich antigens; 7 samples of each group were also diluted with Sample Diluents B and D and similarly assayed. The test results are summarized in Fig. 1. When crude antigens and Diluent A were used, most syphilis (16/20) and some normal sera (9/21) showed EU values as high as some LD sera; Diluent C slightly reduced some of these values for syphilis and normal sera. When flagellin-enriched antigens and Diluent C were used, the EU values for almost all syphilis and normal sera dropped to below the negative cut-off point while the values for LD sera remained as high as those with crude antigens. Diluent D rendered some LD serum reactions lower than some of other groups. The absorbances of the Calibrator were highest with Diluent A and lowest with Diluent D. The combination of flagellin-rich antigens and Sample Diluent C gave the best performance and were therefore chosen for the LD EIA.

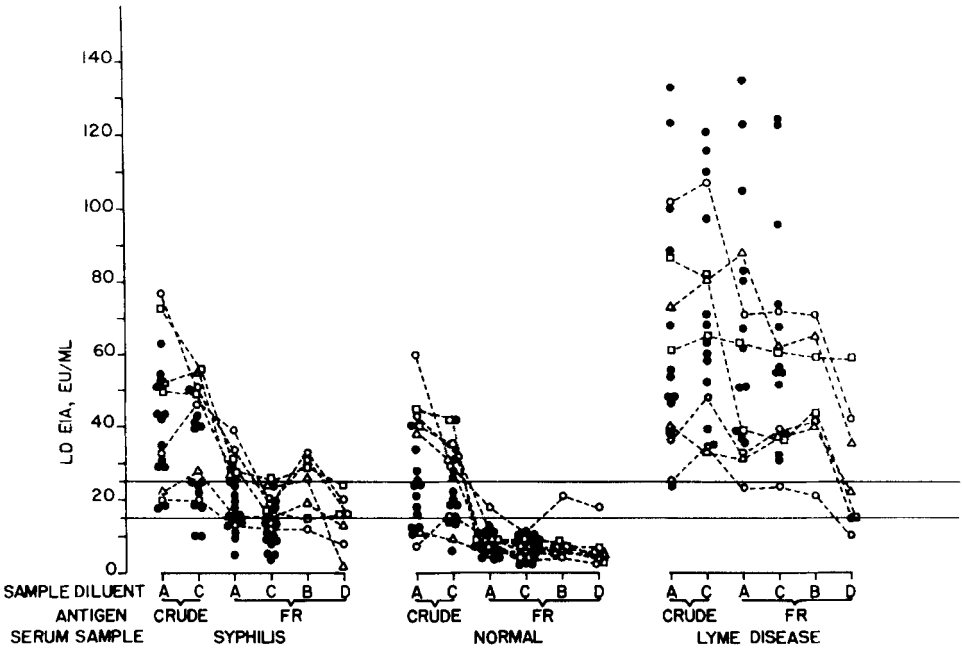

FIGURE 1. Comparison between crude and flagellin-enriched (FR) antigens of *B. burgdorferi* and among four sample diluents by the LD EIA. Borken lines connect the symbols for the same test samples; open symbols are for those tested with all four sample diluents; solid symbols are those tested with only two sample diluents. The two horizontal lines show the cut-off points for positive and negative interpretations. Sample Diluent A contained 6 % BSA; B, autoclaved *T. phagedenis*, normal rabbit serum and rabbit testis extract; C, *T. phagedenis* sonicate and normal rabbit serum; D, sample diluent from the Cambridge Bioscience Human Lyme EIA kit.

Fig. 2 shows the SDS-PAGE and Western immunoblot patterns of the flagellin-enriched and crude antigens at the same concentration. The Western immunoblot of the flagellin-enriched antigens with the Calibrator revealed strong bands at 41 kd, 34 kd, 31 kd, 15-20 kd and several other thinner bands around 60 kd.

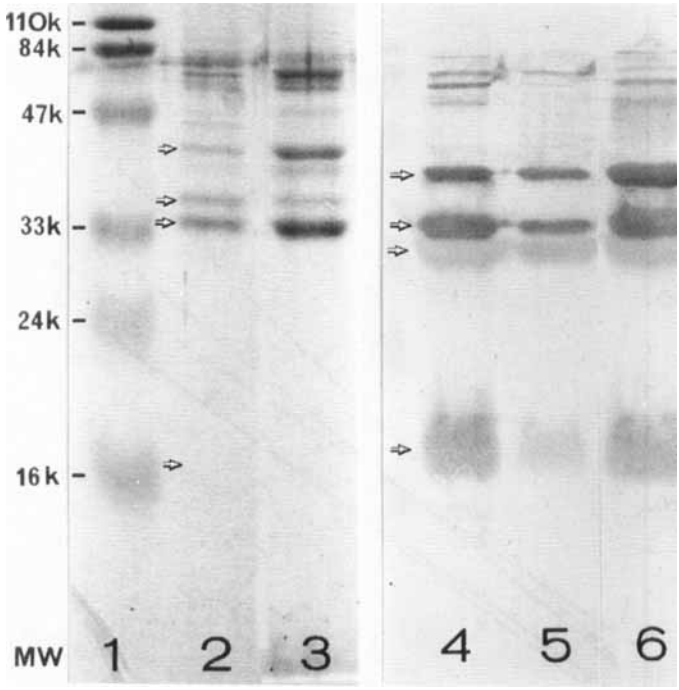

FIGURE 2. SDS-PAGE (lanes 2 & 3) and Western immunoblot patterns (lanes 4 to 6) of *Borrelia burgdorferi* crude antigen (lanes 2 & 5), flagellin-enriched fraction (lanes 3 & 6) and sonicates (not clarified, lane 4). Molecular weight (MW) markers are shown in lane 1. Arrows point to the flagellin (41 kd), OspB (34 kd), OspA (31 kd) and small molecules (15 - 20 kd). The Western blot was developed with Calibrator.

Precision

Assays of 2-fold serial dilutions of the Calibrator and other serum samples revealed the linearity of this LD EIA up to 100 EU/ml and a gradual leveling off beyond. Shown in Fig. 3 are the titration curves of the Calibrator assayed with three different sets of antigen wells and conjugates and, for one set, on different days after several months of storage at 4 C. The reproducibility of this LD EIA was very good, as revealed in assays of several LD and normal serum samples in 6

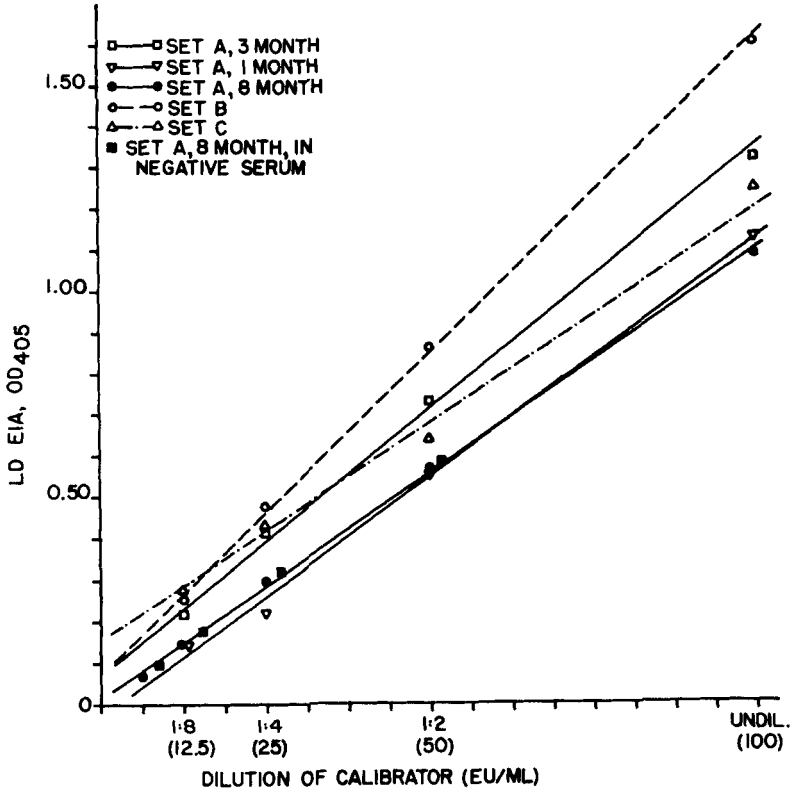

FIGURE 3. Titration curves of the calibrator for the LD EIA. The calibrator was serially diluted in the sample diluent C or, in one run, a normal serum. Three different sets of reagents differing in antigen wells and conjugates were used. One set of these reagents was used after storage for 1, 3 and 8 months at 4 C.

replicates and in six different runs, with 6.8 % and 8.0 % average within-run and run-to-run coefficients of variation, respectively (Table 1).

Assays of Normal and Clinical Serum Samples

A total of 711 serum samples from apparently healthy blood donors in 4 USA states were assayed with the LD EIA, as summarized in Fig. 4. The mean EU/ml

TABLE 1

Precision of the LD EIA. Seven and 6 serum samples were assayed in 6 replicates and in 6 runs, respectively.

Experiment design	Test sample	LD EIA results, EU/ml Range	Mean	SD	% CV
Within-run	A	144 - 182	157	13.3	8.4
	B	86 - 110	97	9.4	9.7
	C	94 - 104	100	4.2	4.2
	D	26 - 30	28	1.4	4.8
	E,F,G	All ≤1	---	---	---
	Average				6.8
Run-to-run	A	133 - 171	145	13.3	9.2
	B	74 - 96	87	7.8	8.9
	D	26 - 30	28	1.6	5.9
	E,F,G	All < 4	---	---	---
	Average				8.0

* ND, not done.

values (2.4 to 6.3) and positive rates (0 to 2.6 %) were higher in Wisconsin than Michigan, Florida and Missouri. The highest EU values for Florida, Missouri, Michigan and Wisconsin groups were 16, 26, 31 and 38 EU/ml, respectively. The mean and standard deviation (SD) for 257 samples of the first three groups combined were 3.2 and 4.2, respectively, with mean plus 3 SD equal to 15.8. The mean and SD for the Wisconsin group were 6.3 and 6.5, respectively, with mean plus 3 SD equal to 25.8. Wisconsin, not the other three states, is in one of the few well-known Lyme disease endemic areas (31) and therefore analyzed separately. Based on these data, 15 and 25 were selected as the negative and positive cut-off points.

Of the 107 suspected LD clinical serum samples from American patients (Groups B,C and D), 41 (58 %) were positive, 7 equivocal and 23 negative, with a

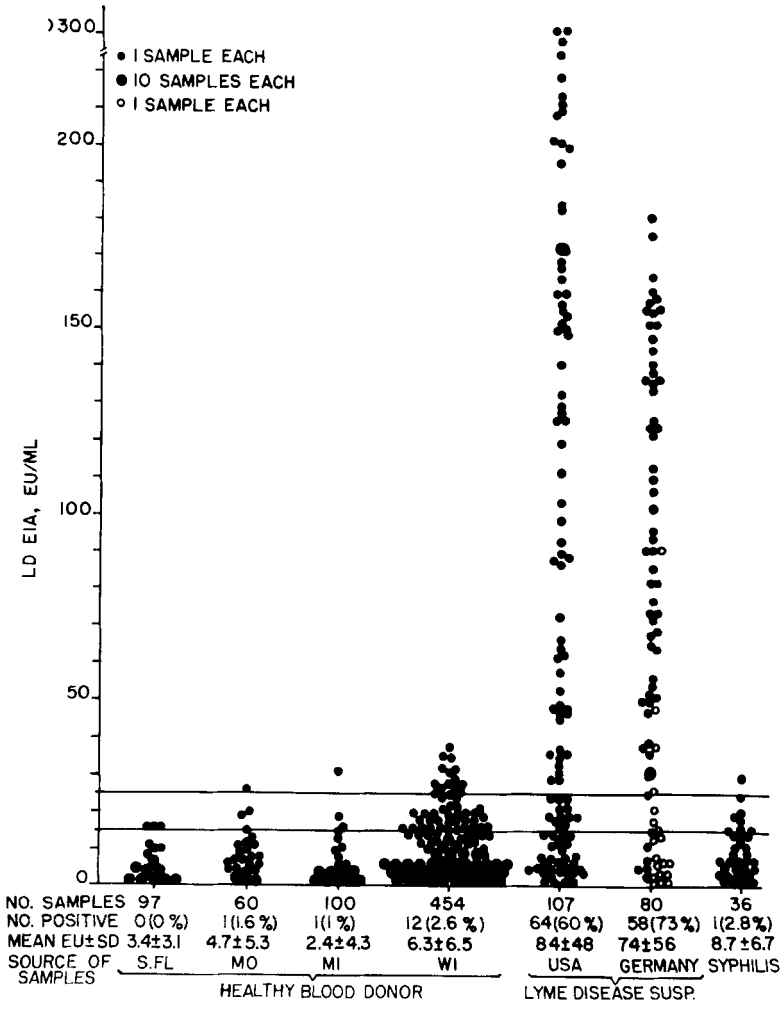

FIGURE 4. The LD EIA results for normal serum samples from healthy subjects in South Florida, Missouri, Michigan and Wisconsin; for clinical serum samples from U.S.A. and Germany patients suspected to have Lyme disease; and for syphilis serum samples from South Florida patients. The open circles were negative by the Hillcrest Lyme EIA.

TABLE 2

Comparisons of test results between the LD EIA and four reference EIA for Lyme disease IgG and IgM antibodies.

Reference test (No. of samples)		LD EIA results				
		No. of samples*			% agree-ment	Correlation coefficient
		Neg.	Equiv.	Pos.		
Hillcrest EIA (291)	Neg.	94	7	9	82	0.847
	Equiv.	14	2	6		
	Pos.	38	7	114		
Zeus EIA (167)	Neg.	86	5	4	94	0.876
	Equiv.	11	2	2		
	Pos.	4	0	53		
MarDx EIA (67)	Neg.	20	2	2	94	0.835
	Pos.	4	3	36		
Camb. Biosc. EIA (157)	Neg.	108	13	3	97	0.928
	Pos.	1	2	30		

* Neg., negative; Equiv., equivocal; Pos., positive; equivocal results were excluded from calculation of agreement.

mean EU value of 84. A few of these samples showed activity greater than 300 EU/ml. Of the 80 suspected LD clinical serum samples (Group A) from Germany, 58 were positive (73 %), 4 equivocal and 18 negative, with a mean EU value of 74. Most of these equivocal and negative samples were also negative by the Hillcrest EIA. Thus, the EU values for the suspected LD clinical samples were at least 10 times those for normal samples.

For comparison, Fig. 4 also shows the LD EIA values of 36 syphilis serum samples, of which 21 were used in the above study shown in Fig. 1. One (< 3 %) was weakly positive; 7 equivocal and 28 negative. The group mean was 8.7 EU/ml and the highest EU value was 29; both values are not much different from those for normal groups.

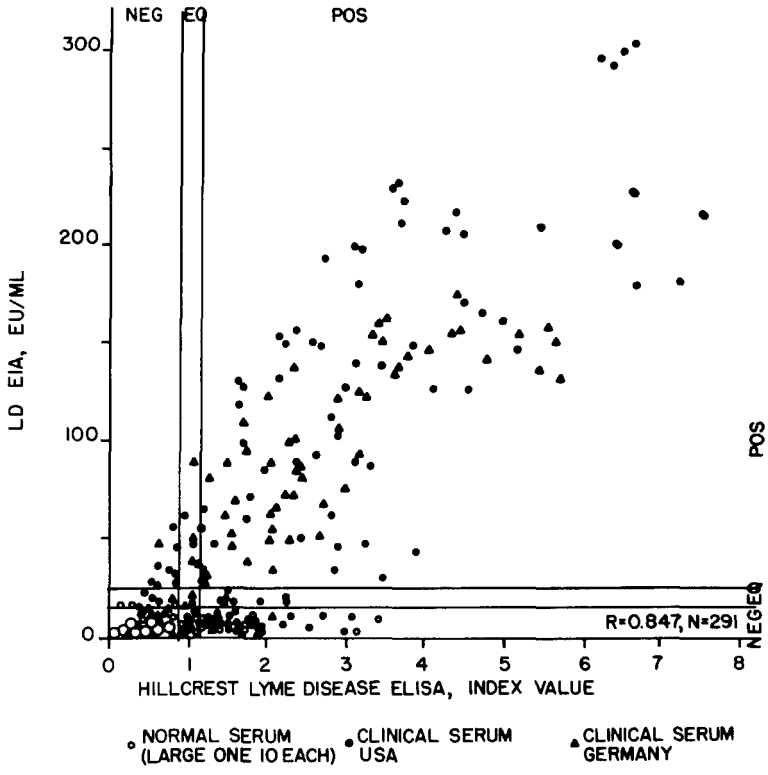

FIGURE 5. A scatter diagram showing correlation between the LD EIA and Hillcrest Biologicals Lyme Disease ELISA results for 291 serum samples: 100 normal samples (1 sample per small circle and 10 per large circle) from Groups I (96) and V (4); 84 Germany clinical samples from Group A (solid trangle); and 107 USA clinical samples (solid dot) from Groups B (71), C (16) and D(20).

The LD EIA did not reveal any positive reaction among the 18 samples from lupus patients and/or positive for antibodies to DNA, 19 serum samples with rheumatoid factor and 9 serum samples from infectious mononucleosis patients.

Comparisons with Other Tests for Lyme Disease IgG and IgM Antibodies

In this study, the LD EIA and four reference EIA results of larger number of serum samples were compared. As summarized in Table 2, the LD EIA results

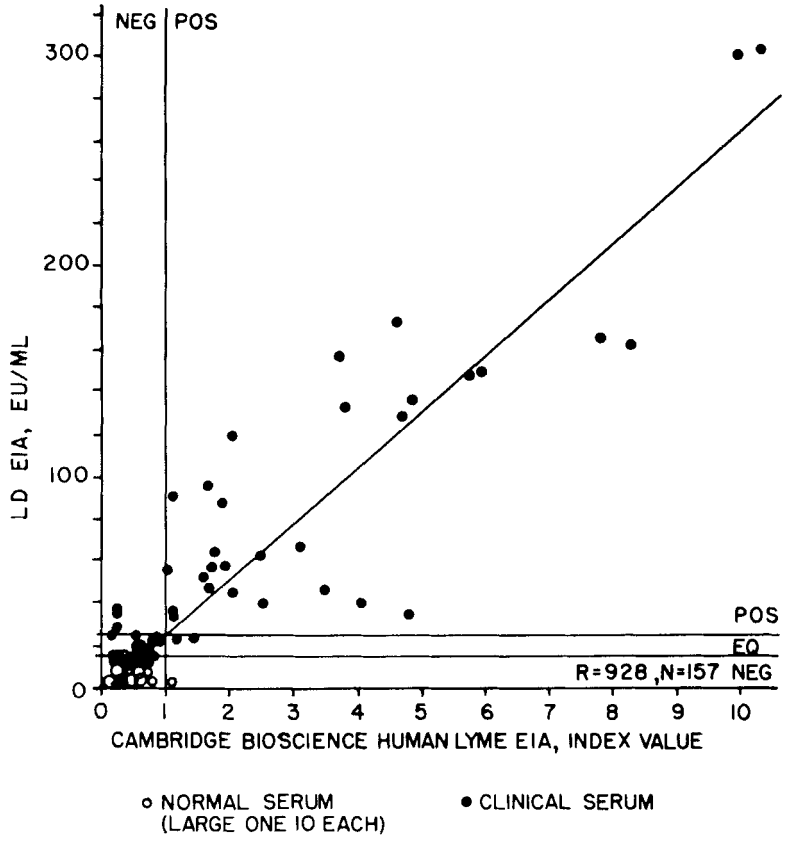

FIGURE 6. A scatter diagram showing correlation between the LD EIA and Cambridge Bioscience Human EIA results for 157 serum samples: 74 normal samples (1 per small circle and 10 per large one) from Group I; and 83 clinical samples (dot) from Groups A (43), B (30) and C (10).

significantly correlated (correlation coefficients = 0.835 to 0.928) and agreed well with each of the four reference EIA (agreements = 82 % to 97 %). Figs. 5 and 6 are scatter diagrams of two of these four comparisons. Discrepant samples had low values in all these EIA. From these four comparisons, the index values for four points on the regression lines and four representative positive samples were chosen

TABLE 3

Comparison among index values of Lyme disease IgG and IgM antibodies for positive serum samples as determined by five enzyme immunoassays.

Test system	Index values of Lyme disease IgG and IgM antibodies							
	From regression lines**				Four clinical serum samples			
	I	II	III	IV	A	B	C	D
	(25)*	(50)	(100)	(200)	(47)	(64)	(88)	(149)
LD EIA	1.0	2.0	4.0	8.0	1.9	2.6	3.5	6.0
Hillcrest EIA	1.0	1.7	3.0	5.0	0.9	3.1	3.1	4.9
Zeus EIA	1.0	1.3	1.5	2.3	1.1	1.6	1.5	2.1
MarDx EIA	1.0	1.3	2.0	3.5	1.4	1.5	1.6	2.8
Cambridge EIA	1.0	1.9	3.9	7.6	1.7	1.8	1.9	5.7

** From the regression lines comparing between Diamedix LD EIA and each of other four EIA's, as shown in Table 2 and Figs. 5 - 6.

* LD EIA values in EU/ml in parenthesis.

for further study. As shown in Table 3, the index values for the positive serum samples were highest with the LD EIA, followed by the Cambridge test. The differences were greater for those with higher index values.

When only the test results of Florida normal and syphilis serum samples were compared in index value, the LD EIA showed the lowest values and the Hillcrest test the highest, in parallel with their positive rates (Table 4). The average index values by the reference EIA were 2 to 4 fold those by the LD EIA. These and above data on positive samples based on index values indicate that the LD EIA shows greater differentiation between samples with different LD specific antibody activities as well as between samples with and without these activities than all the reference EIA.

The reactivity of antigen wells of the LD EIA and four reference EIA tests were compared by assaying the antigen wells with the same samples and reagents

TABLE 4

Mean Lyme disease antibody index values and positive rates of South Florida normal and syphilis serum samples measeured with five EIA for Lyme disease IgG and IgM antibodies.

Test system	Index value					
	Normal sera			Syphilis sera		
	Mean	SD	No. pos. / No. tested	Mean	SD	No.pos. / No. tested
LD EIA	0.12	0.12	0/97 (0 %)	0.35	0.27	1/36(3 %)
Hillcrest EIA	0.51	0.42	8/96 (9 %)	1.88	1.09	26/36(72 %)
Zeus EIA	0.26	0.19	0/65 (0 %)	0.92	0.40	14/36(39 %)
MarDx EIA	0.50	0.17	0/18 (0 %)	1.52	0.57	17/22(77 %)
Cambridge EIA*	0.39	0.13	1/74 (1 %)	0.51	0.20	2/36(6 %)

* Unlike other four EIA which have absorbance read against reagent blank, the Cambridge EIA uses air as blank for absorbance readings.

TABLE 5

Potency of antigen wells used in the LD EIA in comparison with those of reference Lyme disease EIA.

Antigen well compared	Serum sample tested	Test procedure and reagents*	Results (Absorbance): LD EIA vs other EIA
Hillcrest EIA	LD EIA Calibrator	LD EIA	0.949 vs 0.252
	Hillcrest Low Positive	Hillcrest EIA	1.459 vs 0.464
Zeus EIA	LD EIA Calibrator	Zeus EIA	1.500 vs 0.752
	LD EIA Calibrator	LD EIA	1.359 vs 0.452
MarDx EIA	LD EIA Calibrator	LD EIA	1.031 vs 0.483
	MarDx Positive Control	LD EIA	0.704 vs 0.302
Cambridge	LD EIA Calibrator	LD EIA	0.949 vs 0.243
Bioscience	LD EIA Calibrator	CB EIA	2.290 vs 0.424
(CB) EIA	CB EIA Positive Control	CB EIA	> 3.35 vs 1.030
3M Test**	LD EIA Calibrator	LD EIA	1.613 vs 0.773
	Hillcrest Low Positive	Hillcrest EIA	1.459 vs 0.745

* Reagents other than antigen well and serum sample tested, i.e., sample dileunt, conjugate, substrate and stop solution.

** 3M IgG/IgM FASTLYME Test, 3M Diagnostic Systems, Inc., Santa Clara, California.

(i.e., sample diluent, conjugate and substrate). The absorbance readings obtained with the antigen wells of the LD EIA were 2 times those of the Zeus EIA, MarDx EIA and 3M IgG/IgM FASTLYME Test (Santa Clara, CA), 3 times those of the Hillcrest EIA and 5 times those of Cambridge EIA (Table 5).

DISCUSSION

The early immune response in Lyme disease is against the 41 kd flagellin antigen (12, 13). The usefulness of this antigen in serologic tests for early detection of Lyme disease has been well studied (13, 25). In one of these studies, purified flagellum was used and shown to react with serum samples from patients with diseases other than Lyme disease (25). Some patients, especially in Europe, may lack antibody to this flagellin antigen (26, 27). Antibodies to the outer envelope antigens, especially the 31 kd OSP-A and 34 kd OSP-B antigens, which appear in later stages of Lyme disease but not in all patients, are other major immune responses. A large number of additional antigens are present in the sonicate or crude extract of *B. burgdorferi* and many of these are shared with other micro-organisms (12-20). The flagellin-enriched fraction contains more flagellin, OSP-A and OSP-B than the sonicate or crude extract and therefore proportionally less of the other substances, since the bacterial membrane and cytoplasm components were removed in the preparation procedure. A serologic test using the flagellin-rich antigens would be expected to give stronger specific reactions than assays using whole sonicate. Several reports have shown that the specificity of Lyme disease serology can be improved by using absorbents to remove non-specific reactions (7, 14, 28, 29). The absorption reduces the non-specific reactions and thus improves the sensitivity of the tests (29). It was further demonstrated in this study that the LD EIA showed lower index values for the negative and higher values for the positive samples than other reference EIA . In

certain areas, such as South Florida, a rather large number of patients with current or past syphilis may be included in clinics for testing Lyme disease (30). Elimination from the Lyme disease serology of false positive reactions due to syphilis, as accomplished with this LD EIA, offers a significant advantage. We did not test this LD EIA with serum samples from patients with other infectious diseases such as HIV infection and relapsing fever, which had been reported to show stronger or different false positive reactions in Lyme disease serologies than syphilis (2, 7, 17, 20). As stated again and again in the literature, relapasing fever can be differentiated from Lyme disease by clinical features and epidemiological data (12, 17).

Test results of normal sera from 4 states agreed with the Lyme disease epidemiological data. The serum samples from Wisconsin, which is in one of the most well known Lyme disease endemic areas, showed a greater positive rate and a higher mean value in the LD EIA. These data suggested the need for the cut-off values for positive and negative interpretation to be established according to the population to be studied. Equivocal results of the LD EIA could be interpretated as negative in one population, but positive in others.

The LD EIA gave lower reactions (index values) with normal and syphilis sera and higher reactions with positive clinical sera than the other four reference EIA tested. With comparable assay precision, the LD EIA would allow a greater margin of differentiation between the negative and positive results than reference EIA.

Selection of serum samples for evaluation of serologic tests is always difficult and often subjective. No official or generally accepted panel of serum samples for Lyme disease antibody testing is available. Recently, the Centers for Disease Control (CDC) and Association of State and Teritorial Public Health Laboratory

Directors conducted a program evaluating commercially available test kits for Lyme disease antibodies with more than 150 well-documented serum samples. After months of testing and evaluation by best qualified state public health laboratories in four states, the program was completed, but the data were inconclusive. One of the major faults in this program was the panel of serum samples used (32). The CDC is now preparing a new panel of serum samples from patients with clinically documented and microbiologically proven Lyme disease to repeat the evaluation program. We plan to evaluate the LD EIA further with this new panel of serum samples as soon as it become available.

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