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Comparison of Four Different Serological Methods for Detection of Antibodies to *Borrelia burgdorferi* in Erythema Migrans

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Three different enzyme-linked immunosorbent assays (ELISA) and Western blot were compared in regard to the detection of antibodies to *Borrelia burgdorferi* in sera from 100 patients with erythema migrans and from 100 controls. For IgG detection, a commercial indirect ELISA kit with flagellum antigen (flagellum ELISA) was significantly more sensitive than the routinely-used indirect ELISA with sonicated whole-cell antigen (sonicate ELISA) ($p=0.008$). The difference in positivity in the IgM test was of borderline significance ($p=0.058$). An IgM antibody-capture ELISA with sonicated whole-cell antigen (capture ELISA) was significantly more sensitive than either the IgM sonicate ELISA ($p<0.001$) or IgM flagellum ELISA ($p<0.001$). With the Western blot pattern chosen as the criterion for positivity, IgM Western blot was at least equal to IgM capture ELISA in terms of the number of positive erythema migrans sera, but a frequent discrepancy between these two tests was noted as to positivity in individual sera. IgG Western blot was considered to be of less value for the diagnosis of current disease due to a high occurrence of positivity among controls. *Key words: Lyme; Borreliosis.*

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The disease spectrum of Lyme borreliosis, caused by the spirochete *Borrelia burgdorferi*, becomes continuously more complex and most of the various clinical manifestations are not pathognomonic (1). This accentuates the need for reliable diagnostic aids. Since the first demonstration, by indirect immunofluorescence, of specific antibodies in Lyme disease (2), serology has been one of the cornerstones of the diagnostics, and continuous efforts have been made to refine the serological methods. Enzyme-linked immunosorbent assay (ELISA) was soon evaluated (3, 4, 5) and has beed judged to be superior or equal to immunofluorescence. These tests have mainly been performed using whole cells or sonicated whole cells as antigen. Performed in this manner, both have often been insufficient for diagnosing early disease and have been impaired by problems with false positive results.

Attempts have been made to improve ELISA by replacing the antigen used so far by fractions of spirochetes, with concentration on the *Borrelia burgdorferi* flagellum antigen (6–10). The partly diverging results might be explained by differences in the antigen preparations. Where a purified flagellum antigen was used (8, 9, 10), it was reported to increase the sensitivity and specificity to some degree compared to a sonicate antigen.

Analyses of the components of the antibody response have been performed by Western blot (WB) (6,7,11–15). Among the findings is an early and commonly occuring antibody response to the 41 kilodalton (kDa) flagellin protein. WB has also been used as a diagnostic tool. With varying criteria for positivity in the different investigations, it has been reported to be more sensitive than sonicate indirect ELISA (7,15). A gain in specificity was found in only one of these studies (7).

IgM antibody-capture ELISA has been reported to be considerably (16) or slightly (17) more sensitive than the standard indirect ELISA as well as superior to immunoblotting (16). A further contribution to serological diagnostics is the recent demonstration of specific antibodies sequestered in immune complexes in seronegative Lyme borreliosis (18).

In the present study, we have investigated the antibody response in patients with erythema migrans (EM), the primary stage skin manifestation of Lyme borreliosis, and in controls. The aims of the study were to characterize the WB pattern of the antibody response in patients with EM in an attempt to find

practicable diagnostic criteria and to compare the diagnostic sensitivity of WB and three different ELISAs with the four methods all applied to the same sera.

MATERIALS AND METHODS

Patients

Sera from 100 untreated patients with EM without major extracutaneous manifestations were investigated. The sera were not from consecutive patients but were chosen from a bank of frozen sera. The patients had all been diagnosed at the Department of Dermatology, Södersjukhuset, by the authors. There were 74 women and 26 men, ant the mean age was 49 years (range 2–83 years). The median duration of the EM lesion was 4 weeks, ranging from one day to one year. Twelve patients had multiple lesions.

Controls

Control sera were obtained from 100 individuals seen at the Department of Dermatology, Södersjukhuset, without a history or present symptoms of borrelial infection. There were 62 women and 38 men, and the mean age was 42 years (range 15–82 years).

Preparation of whole-cell sonicate antigen

Spirochetes from the Swedish *Borrelia burgdorferi* ACA 1 strain, isolated from the skin of a patient with acrodermatitis chronica atrophicans (19), were grown for five days at 32°C in BSK medium. The cells were harvested by centrifugation (7000×g, 20 min) and washed three times in phosphate buffered saline (PBS; pH 7.4) containing 5 mM $MgCl_2$. The pellet was resuspended in PBS and sonicated on ice with 6×30 sec. bursts with a MSE 150 W ultrasonic disintegrator (Manor Royal, Crawley, England). The sonic extract was centrifuged (10.000×g, 20 min.) and the supernatant was used as antigen in the sonicate indirect ELISA, in the IgM capture ELISA and in Western blot.

Preparation of rabbit antiserum and HRP-conjugate

Antiserum to the sonicate antigen of the ACA I strain of *Borrelia burgdorferi* was raised in rabbits. An amount of 0.5 ml sonicate antigen (3 mg protein/ml) emulsified in Freund's complete adjuvant was injected intracutanously, followed by two injections with the antigen in Freund's incomplete adjuvant at 14-day intervals. Fourteen days after the final injection, the rabbits were injected intravenously with 0.2 ml sonicate antigen twice with a 14-day interval. The antiserum collected 7 days after the final injection was precipitated in 33% saturated ammonium sulfate and dialyzed and then separated on a Sephacryl S-300 column (Pharmacia, Sweden). The IgG fraction was conjugated with horseradish peroxidase (HRP) according to the method of Wilson & Nakane (20).

Sonicate indirect ELISA (sonicate ELISA)

Microtiter plates Immulon 2 (Dynatech laboratories, Chantilly, Virginia) were coated overnight at 20°C with 100 µl of whole cell sonicate antigen (approximately 7 µg protein/ml) diluted in PBS. The coated plated were stored at 4°C prior to use. The wells were washed four times in PBS (10 mM Na-phosphate, 0.15 M NaCl, pH 7.4) with 0.5% (wt/vol) Tween 20 (PBS-Tween) before 100 µl of serum diluted in 1:1000 in PBS-Tween was added. The plates were incubated at 20°C for 1 h for IgG and 2 h for IgM determinations. After washing four times with PBS-Tween, 100 µl alkaline phophatase conjugate was added, either swine antihuman IgG (Orion, Helsinki, Finland) diluted 1:500 in PBS-Tween or antihuman IgM (Sigma Chemical Co., St. Louis, Mo) diluted 1:1000. The plates were incubated at 20°C overnight and then washed four times in PBS-Tween before the addition of 100 µl substrate solution. The plates were incubated for 30 min with the substrate solution which contained 1 mg/ml p-nitrophenyl phosphate and 0.5 mM $MgCl_2$ in 1 M diethanolamine, pH 9.8. The reaction was stopped by adding 50 µl 3 M NaOH and the absorbance value was read at 405 nm (Titertec Multiscan, Flow Laboratories, UK).

Flagellum indirect ELISA (flagellum ELISA)

The commercial Lyme borreliosis kit from Dakopatts, Copenhagen, was used for testing IgG and IgM antibodies to flagella. The tests were performed according to the recommendations of the manufacturer.

IgM capture ELISA

Microtiter plates (Immulon 2) were coated overnight with 100 µl rabbit antihuman IgM (Dakopatts) diluted 1:3000 in PBS (the plates could be stored for several weeks at 4°C). After washing three times in PBS with 0.5% Tween (PBS-Tween), 0.1 ml test serum diluted 1:100 in dilution buffer (PBS-Tween containing 2% fat free milk powder) was added. The plates were incubated for 2 h at 20°C. After washing four times in PBS-Tween, the plates were incubated with 100 µl sonicate antigen (5 µg protein/ml) in dilution buffer overnight at 20°C. The plates were washed four times in PBS-Tween and incubated with rabbit IgG anti-Borrelia HRP conjugate for 1 h at 20°C. The plates were washed four times and incubated with 100 µl substrate solution for 30 min at 20°C. The substrate solution was prepared immediately before use by dissolving 0.5 mg of o-phenylenediamine per ml in 0.1 M citrate-phosphate buffer at pH 5.0. 1 µl of 30% H_2O_2 per ml substrate solution was added. The reaction was stopped by adding 50 µl 3 M H_2SO_4, and the absorbance value was read at 392 nm.

Western blot

The protein content of the whole cell sonicated antigen was determined by Biorad protein assay (Bio-Rad, Richmond, California). The antigen was diluted with an equal volume of sample buffer (0.125 M Tris-HCl buffer, pH 6.8) containing 20% glycerol, 4% sodium dodecyl sulphate (SDS), 0.2 M dithiothreitol and 0.003% bromphenol blue and boiled on a water bath for 5 min. The antigen was subjected to SDS-polyacrylamide gel electroforesis (SDS-PAGE) according to the method of Laemmli (21). 330 µg protein was separated in a 0.75 mm thick, 16 cm wide, and 12% polyacrylamide gel (acrylamide:bisacrylamide 37,5:1, Bio-Rad).

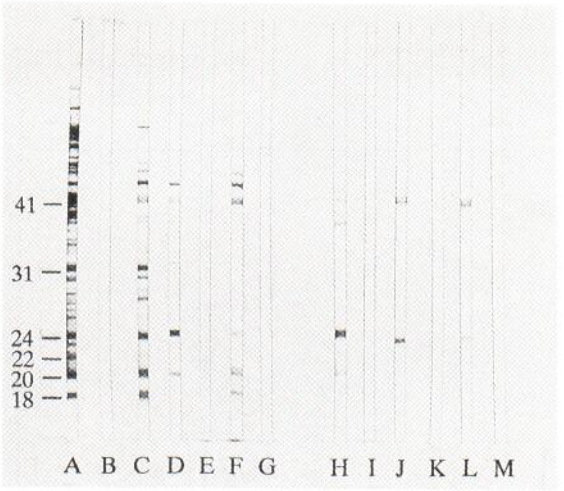

Fig. 1. Examples of Western blots of sera from patients with erythema migrans (EM) and controls (Co) with strain ACA 1 of *Borrelia burgdorferi* as antigen. Molecular weights are indicated to the left. (+) indicates that the immunoblot pattern was considered positive and (−) that it was considered negative according to chosen criteria. A-G are IgG tests. A = reference serum from a patient with acrodermatitis chronica atrophicans, B = reference serum from a healthy individual, C = EM (+), D = EM (+), E = EM (−), F = Co (+), G = Co (−). H-M are IgM tests. H = reference serum from a patient with EM, I = reference serum from a healthy individual, J = EM (+), K = EM (−), L = Co (+), M = Co (−).

After separation in SDS-PAGE, the antigen was transferred electroforetically to a nitrocellulose sheet (0.45 µm, Bio-Rad) by semi-dry blotting (Sartoblot II, Sartorius, GmbH, W. Germany) using 1.5 mA/cm^2 for 50 min. The sheet was cut into strips which were blocked by immersion in PBS (10 mM Na-phosphate, 0.15 M NaCl, pH 7.4) containing 5% fat free milk for 2 h at room temperature. The blocked strips were incubated overnight at 4°C with patient serums diluted 1:50 in blocking buffer containing 0.1% Tween 20. After five washings (10 mM Na-phosphate, 0.5 M NaCl, 0.3% Tween 20, pH 7.4), the strips were incubated at room temperature for 1 h with alkaline phosphatase conjugated rabbit antihuman IgG (Dakopatts) diluted 1:1000 or goat anti-human IgM (Sigma) diluted 1:800. After five more washings, the immunocomplexes were detected by reaction with alpha-naphtyl-phosphate (Sigma, 2 mg) and Fast Red TR-salt (Sigma, 5 mg) in 10 ml Tris buffer (5mM, pH 8.6) containing $MgCl_2$ (2 mM) for 30 min. Low molecular weight standard 14−97 kD (Bio-Rad) was used for identification of the Western blot bands.

All strips were read by one of the investigators without knowledge of the ELISA values. The intensity of the bands were subjectively graded, but when calculating the total results, all noticed bands were counted, irrespective of intensity.

Statistical analyses

The diagnostic sensitivities of the serological tests for one immunoglobulin class were compared by using Mc Nemar test. For comparing non-paired data, the ordinary chi-square test was used.

RESULTS

Western blot

Representative Western blots with sera from EM patients and controls are shown in Fig. 1.

In IgG WB, all but one of the EM sera as well as all control sera showed reactivity and often with a considerable number of protein bands. Counting all bands on the strips, five bands or more were recognized by 95% of the EM sera and 85% of the control sera and 15 bands or more by 26% and 3%, respectively. In IgM WB, blank strips were found in 8% of the EM sera and 26% of the control sera and reactivity with five bands or more in 19% and 6%, respectively.

The major reactivities of the sera with protein bands in the regions 16−41 kDa are shown in Table I. In IgG WB, with weak bands included, most of the EM sera as well as the control sera bound to the 41 kDa region. Reactivities in the 31−36 kDa re-

Table I. *Western blot (WB) reactivity of sera from 100 patients with erythema migrans (EM) and from 100 controls (Co) with proteins of Borrelia burgdorferi. The strain used is ACA 1.*

	No. of sera reactive with the proteins			
	IgG-WB		IgM-WB	
Proteins (kDa)	EM	Co	EM	Co
41−40	97	94	81	58*
24−23	51	15	51	7*
22	13	3	0	0
20	57	15	16	0
18	46	20	5	0
16	18	14	0	0
41−40 + at least one out of 24−23,22,20,18	75	38	51	2
41−40 + at least one out of 24−23,22,20	73	25	51	2
41−40 + at least two out of 24−23,22,20,18	54	9		
41−40 + at least two out of 24−23,22,20	41	7		

*Weak bands in the majority of the cases

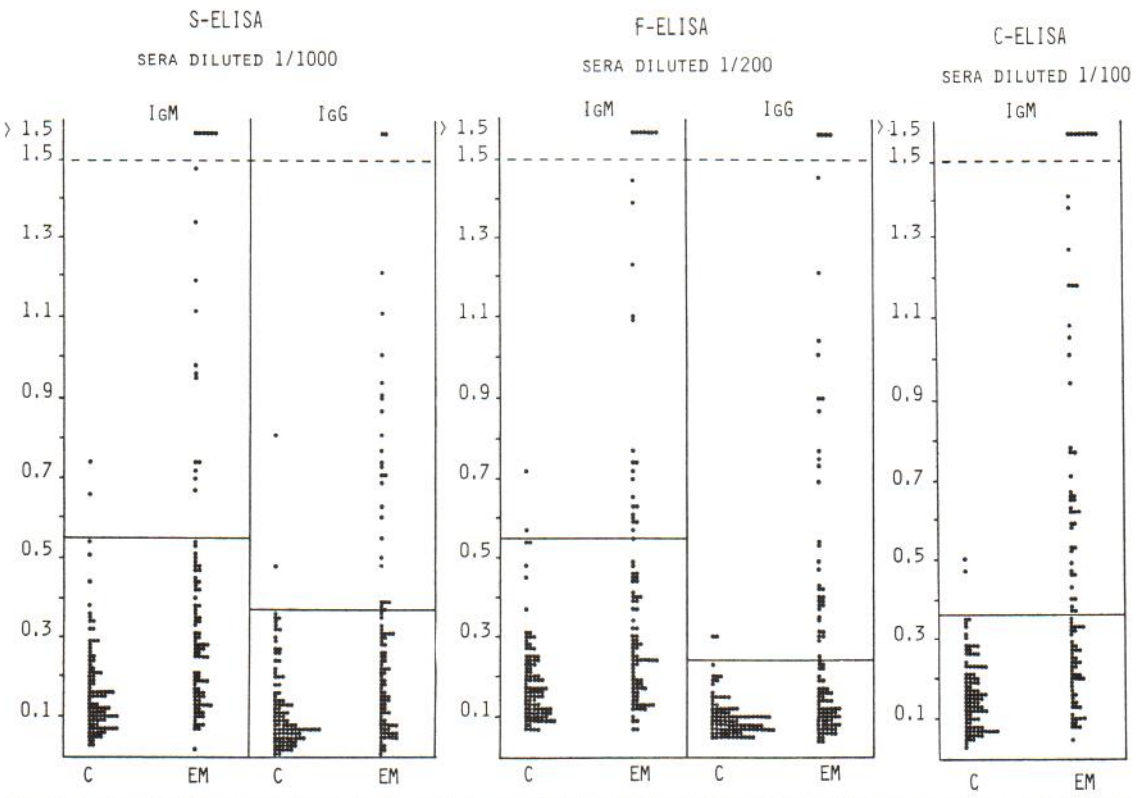

Fig. 2. IgG and IgM antibody levels to *Borrelia burgdorferi* in sera from 100 patients with erythema migrans (EM) and from 100 controls (C) measured with different ELISAs. Antibody levels are given as absorbance values. Horizontal lines indicate cut-off levels at the 98th percentile of the controls. S-ELISA = indirect ELISA with sonicated whole-cell antigen, F-ELISA = indirect ELISA with flagellum antigen, C-ELISA = antibody-capture ELISA with sonicated whole-cell antigen.

gion, not included in the table, were also common and found in more than half of both patients and controls. The low molecular proteins of 18, 20, 22 and 23–24 kDa were more frequently recognized by the EM sera than by the control sera, the difference being least pronounced for the 18 kDa protein. None of these reactivities, however, was specific for the EM sera and they were not considered to be of diagnostic value in and of themselves. The occurrence of reactivity in the 41 kDa region in various combinations with one or two low molecular bands was calculated, as exemplified in Table I. Reactivity in the 41 kDa region combined with at least two bands in the regions 20, 22, and 23–24 kDa was chosen as the criterion for a positive IgG WB. This immunoblot pattern was found in 41% of the EM sera and 7% of the control sera. In the IgM immunoblots, the majority of the reactivities found in the control sera were weak. Of the seven control sera that bound to the 24 kDa protein, five were not reactive in the 41 kDa region. Reactivity in the 41 kDa region combined with reactivity to at least one of the low molecular proteins of 20, 22, and 23–24 kDa was chosen as the criterion for a positive IgM WB. This criterion corresponded to the immunoblot pattern in 51% of the EM sera and 2% of the controls.

The ELISAs

The absorbance values obtained with sera from EM patients and controls in the different ELISAs are shown in Fig. 2. The 98th percentile of the controls' absorbance values was used as the cut-off level. In sonicate ELISA, a positive value was thus defined as ≥ 0.370 for IgG and ≥ 0.550 for IgM. In flagellum ELISA, a positive value was ≥ 0.240 for IgG and ≥ 0.550 for IgM. In the IgM capture ELISA, a positive value was defined as ≥ 0.360. Due to the fact that different dilutions of sera and different methods were used, the cut-off levels per se should not be compared.

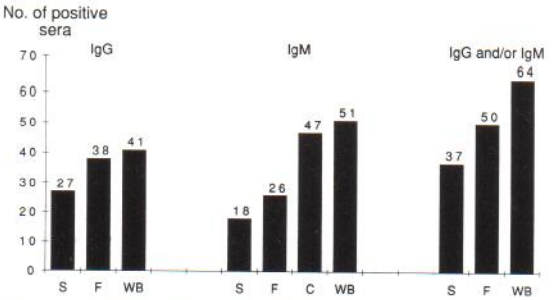

Fig. 3. Comparison of different serological methods as regards the detection of antibodies to *Borrelia burgdorferi* in sera from 100 patients with erythema migrans. S = indirect ELISA with sonicated whole-cell antigen, F = indirect ELISA with flagellum antigen, C = IgM antibody-capture ELISA with sonicated whole-cell antigen, WB = Western blot. Positive ELISA values are defined as values above the 98th percentile of 100 controls. WB patterns defined as positive were found in 2% of the controls in the IgM test and in 7% of the controls in the IgG test.

Comparison of the serological methods

The number of EM sera that were positive in the different ELISAs and WB are compared in Fig. 3.

The flagellum ELISA was found to be significantly more sensitive than sonicate ELISA in the IgG test ($p=0.008$), while the gain in the number of positive sera in the IgM test was not significant ($p=0.058$). IgG WB, with the criterion chosen for positivity, was about equal to IgG flagellum ELISA in terms of the number of positive EM sera, but was also positive in 7% of the control sera. The results are thus not readibly comparable. IgM WB and IgM capture ELISA were about equal in the number of positive sera and both were significantly more sensitive than IgM sonicate ELISA ($p<0.001$) and also than IgM flagellum ELISA ($p<0.001$).

The concordance of the different ELISAs and WB as regards positivity is illustrated in Fig. 4.

Comparison of solitary and multiple EM lesions as regards test results

In 12 patients with multiple EM lesions, an increased percentage of positive sera was found compared with 88 patients with solitary skin lesions. The difference was significant in the IgG ($p=0.01$) and IgM ($p<0.01$) sonicate ELISA and in the IgM flagellum ELISA ($p<0.05$) but not in the other tests.

DISCUSSION

The purpose of this study was to compare the sensitivity of different serological methods in sera from patients with a clinically reliable diagnosis of early Lyme borreliosis. As the sera were not obtained from consecutive patients, the results of the study must not be interpreted as charting the frequency of seropositivity among EM patients. What is preformed is a comparison of the serological methods.

By using WB, antibodies to a number of proteins have been found in Lyme borreliosis. The major interest has, however, concerned reactivities to proteins between 16 and 41 kDa (12–15), and we have concentrated our efforts on finding a diagnostic WB pattern to this region. An additional reason for this limitation is the fact that the SDS-PAGE method used involves difficulties in more exactly identifying the high molecular weight proteins. Found in the 16–41 kDa protein range are a 21–22 kDa protein designated pC, outer surface proteins of approximately 31 kDa (OspA) and 34 kDa (OspB), and the 41 kDa flagellar antigen. 17–18 and 23 kDa proteins have also been specifically observed. The reactivities

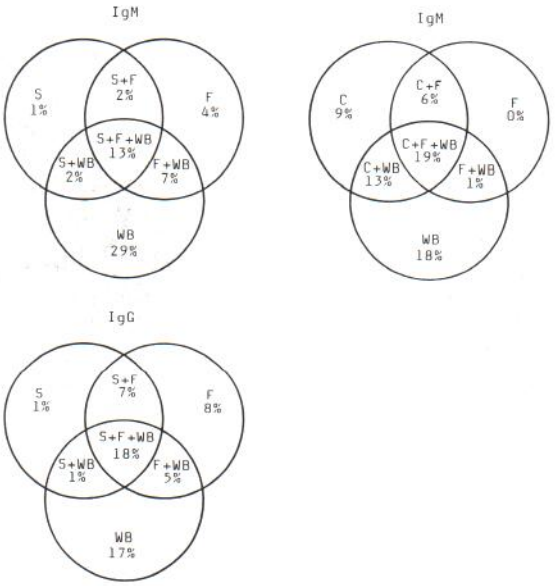

Fig. 4. Concordance of Western blot and three different ELISAs in the detection of antibodies to *Borrelia burgdorferi* in sera from 100 patients with erythema migrans. The numbers indicate the percentage of positive sera. Positive ELISA values are defined as a value above the 98th percentile of 100 controls. Western blot patterns defined as positive were found in 2% of the controls in the IgM test and in 7% of the controls in the IgG test. S = indirect ELISA with sonicated whole-cell antigen, F = indirect ELISA with flagellum antigen, C = IgM antibody-capture ELISA with sonicated whole-cell antigen, WB = Western blot.

18 sera positive in IgM S-ELISA were all positive in C-ELISA.

of our sera corresponded well to these regions. Reactivities within the region 31–36 kDa were common both in patients and controls. However, these bands were often weak and of indistinct location within the region. These findings indicate that they do not mainly represent antibodies to OspA or OspB, which are considered to be specific to Borrelia. There have been reports of commonly occurring cross-reactivities to a protein between OspA and OspB (22) and also, in natural canine Lyme borreliosis, on antibodies in the 31 kDa region probably directed to a protein other than OspA but of similar molecular mass (23). This may be in accordance with our impression that this region is difficult to evaluate for routine analyses, and we did not include it in the diagnostic criteria. It has been pointed out that different laboratories will probably be compelled to find their own definitions of a positive WB due to variations in the *Borrelia burgdorferi* strains which are used as antigen as well as in the immunoblot methods applied. A considerable heterogeneity of major proteins and antigenic variability in different *Borrelia burgdorferi* strains have been shown (12, 24).

In accordance with other investigators, we found no single band in WB specific to the borrelial sera. As criterion for a positive WB, we chose the pattern least common among the control sera with an appreciable occurrence among the EM sera. In the IgG test, the thus positive pattern was yet found in as many as 7% of the control sera. Lyme borreliosis is a common infection in Sweden, and some or all of these positive control sera might represent specific reactions from previous exposure to Borrelia. This high occurrence of positivity among controls makes the IgG WB of doubtful value for diagnosis of a current infection, at least in our geographical area. Moreover, comparisons with the other methods used in this study are not readibly made. We found IgM WB more valuable. The pattern which was used here as the criterion for positivity was found in only two control sera. This is comparable to the 98% cut-off levels used in the ELISAs, and the sensitivity was significantly increased compared with both IgM sonicate and IgM flagellum ELISA.

The commercial flagellum ELISA kit that was investigated was significantly more sensitive than the sonicate ELISA in the IgG test, while the difference in the IgM test was of borderline significance. This is mainly in agreement with previous studies where a pure flagellum antigen was used (8, 9, 10), and in two of them (8, 9), the increase in IgM detection was significant as well. The gain in sensitivity is most likely the result of the elimination of a number of antigens eliciting cross-reactions included in the whole-cell preparation. However, as also shown in previous WB studies, reactivities in the 41 kDa region may also be found in many control sera. Cross-reacting antibodies to oral or gastrointestinal spirochetes have been speculatively incriminated, but ultimately, the nature of these reactivities is not known. The usefulness of the flagellum ELISA in spite of this cross-reactivity indicates that the antibodies in the 41 kDa region in the WB differ between EM sera and control sera in quantity or quality or both.

For IgM detection, the capture ELISA was superior to both the sonicate and the flagellum ELISA. A gain in sensitivity by IgM capture ELISA compared to indirect ELISA has been partly attributed to the fact that the antibody-capture ELISA does not measure the specific antibody level itself but rather the proportion of specific to total antibodies of a given immunoglobulin class. Moreover, the captured specific IgM antibodies can bind the antigen without competition from antibodies of other immunoglobulin classes. An additional advantage of the capture ELISA is that it diminshes or eliminates the effect of rheumatoid factor IgM.

IgM capture ELISA and IgM WB were about equal in the number of positive sera, but there was a frequent discrepancy between them as regards positivity in individual sera. Thus, 32 EM sera were positive in both tests but another 19 were positive in IgM WB but not in IgM capture ELISA, and 15 were positive in IgM capture ELISA alone (Fig. 4). WB may visualize antibodies below the cut-off level of the ELISA. A positive capture ELISA value and negative WB could be partly explained by the fact that a WB pattern considered as positive may include only part of the antibody response, partly by the possibility that some antigenic determinants are lost during the pretreatment of the antigen for immunoblotting. Although the disagreement between the two tests is so far not fully explained, it may thus be that they detect different parts of the antibody response. It is then likely that they should be used together and not interchangeably.

Thus, as regards sensitivity, IgG flagellum ELISA and IgM capture ELISA offer advantages over the routinely used sonicate ELISA. We also found IgM WB to be a sensitive method, but it has the disad-

vantages of being time comsuming and non-quantitative. It is therefore probably primarily useful as a complementary test, for example in early suspected cases of Lyme borreliosis negative in capture ELISA, or for qualitative analysis of the components of an antibody response.

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