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Interpretation Criteria for Standardized Western Blots for Three European Species of *Borrelia burgdorferi* Sensu Lato

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Western blots (WBs; immunoblots) are a widely used tool for the serodiagnosis of Lyme borreliosis, but so far, no defined criteria for performance, analysis, and interpretation have been established in Europe. For the current study WBs were produced with strains PKa2 (*Borrelia burgdorferi* sensu stricto), PKo (*Borrelia afzelii*), and PBi (*Borrelia garinii*). To improve resolution we used gels of 17 cm in length. In a first step, 13 immunodominant proteins were identified with monoclonal antibodies. Then, the apparent molecular masses of all visually distinguishable bands were determined densitometrically. Approximately 40 bands of between 14 and 100 kDa were differentiated for each strain. From a study with 330 serum samples (from 189 patients with Lyme borreliosis and 141 controls), all observed bands were documented. To establish criteria for a positive WB result, the discriminating ability of a series of band combinations (interpretation rules) were evaluated separately for each strain (for immunoglobulin G [IgG] WB, >40 combinations; for IgM WB, >15 combinations). The following interpretation criteria resulting in specificities of greater than 96% were recommended: for IgG WB, at least one band of p83/100, p58, p56, OspC, p21, and p17a for PKa2; at least two bands of p83/100, p58, p43, p39, p30, OspC, p21, p17, and p14 for PKo; and at least one band of p83/100, p39, OspC, p21, and p17b for PBi; for IgM WB, at least one band of p39, OspC, and p17a or a strong p41 band for PKa2; at least one band of p39, OspC, and p17 or a strong p41 band for PKo; and at least one band of p39 and OspC or a strong p41 band for PBi. The overall sensitivity was the highest for PKo WB, followed by PBi and PKa2 WB, in decreasing order. Standardization of WB assays is necessary for comparison of results from different laboratories.

Lyme borreliosis (LB) is a global tick-borne disease caused by infection with *Borrelia burgdorferi* sensu lato. The disorder develops in stages and with different manifestations involving mainly the skin, the nervous system, and the joints. The diagnosis of Lyme disease is based on the recognition of typical clinical signs and is assisted by laboratory tests, especially if the clinical picture is not clear. Routine testing comprises mostly serological methods including screening tests like the enzyme immunoassay (EIA) and confirmation tests like indirect immunofluorescence assays and Western blots (WBs; immunoblots). In part, these tests are hampered by the occurrence of cross-reacting antibodies (7), leading to false-positive results, and patients may still be seronegative in early stages of infection. Furthermore, serological assays for LB have not been standardized so far, resulting in tests with various levels of sensitivity and specificity. In Europe, three species pathogenic for humans (3) and at least eight different serotypes of *B. burgdorferi* sensu lato (43, 45) are known. This heterogeneity further complicates the comparability and standardization of assay systems.

Immunological and molecular biological investigations have revealed and characterized a variety of borrelial antigens including p83/100 (also referred to as p93) (21, 26, 32, 34), p66 (8), p41 (Fla) (19, 40), p39 (BmpA) (36, 37), and p17 (47) and the outer surface proteins OspA (5, 45), OspB (4), OspC (18, 22, 46, 49), OspD (31), OspE, and OspF (25). Heat shock proteins of the HSP60 and HSP70 families (homologs of *Escherichia coli* GroEL and DNaK) have also been analyzed (20, 27, 38, 39).

In the United States the Centers for Disease Control and Prevention (CDC) recommend a two-step protocol for the evaluation of sera. An EIA should be performed for screening, and positive results should be confirmed by WB assay (11). The following interpretation criteria have been recommended: at least five bands of 18, 21 (OspC), 28, 30, 39 (BmpA), 41 (Fla), 45, 58, 66, and 93 kDa for immunoglobulin G (IgG) tests (16) and at least two reactive bands of OspC, 39 kDa (BmpA), and 41 kDa (Fla) for IgM tests (17).

In Europe, generally accepted criteria have not been established so far. Various studies for the evaluation of WBs have been performed, but the comparability of the results is limited since different selections of patients and a variety of strains of *B. burgdorferi* sensu lato with varying or missing expressions of certain proteins (or lipoproteins) such as OspA and OspC and different WB protocols have been used (2, 9, 14, 15, 28, 30, 51). Furthermore, the description of bands is different in various studies, and only a few investigators have used monoclonal antibodies (MAbs) to identify particular proteins (13, 14, 17, 30).

The purpose of the current study was to compare WBs of three different strains representing the species in Europe pathogenic for humans under standardized conditions in terms of the following criteria. All strains used for antigen preparations abundantly expressed OspA and OspC. To achieve a high resolution of bands, gels of 17 cm in length were used. A series of immunodominant proteins was identified with MAbs, and subsequently, proper identification of all immunoreactive bands (about 40 per strain) was established in the first part of the study.

Various interpretation criteria were evaluated for each strain, and the specificities and sensitivities for the most favorable criteria were compared for sera from patients at all stages of the disease. Finally, certain proteins expressed only by in-

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dividual strains were evaluated for their significance for diagnosis.

MATERIALS AND METHODS

Sera. Sera from the following study groups comprising patients with LB (n = 189) and controls (n = 141) were investigated.

Sixty-six serum samples from unselected, untreated patients with erythema migrans (EM) which were collected for a former therapy study (41) were obtained from a dermatologist. The median time period between the appearance of EM and the collection of serum samples was 3 weeks (range, 1 day to 31 weeks). The neuroborreliosis (NB) group (n = 83) included 39 patients (designated group NB I) from whom *B. burgdorferi* sensu lato was isolated from the cerebrospinal fluid (CSF) and 44 other patients (designated group NB II) with typical signs of acute NB, CSF pleocytosis, and CSF/serum antibody indices of ≥2.0 (42). Cultures of CSF from 20 patients of the NB II group were negative. All sera were obtained on the same day that the CSF samples were obtained. The median duration of neurological symptoms was 3 weeks (range, 3 days to 1 year) in group NB I and 4 weeks (range, 10 days to 6 months) in group NB II. These data were obtained from 16 of 39 patients in group NB I and 40 of 44 patients in group NB II. The group with late LB (n = 40) comprised 30 patients with acrodermatitis chronica atrophicans (ACA) diagnosed by a dermatologist and 10 patients with Lyme arthritis. Possible differential diagnoses had been excluded. Serum samples from 120 healthy blood donors, 11 patients with syphilis in stage II or III, and 10 patients with rheumatoid factor (RF) levels of ≥45 IE/ml served as control group. The healthy blood donors had no history of frequent tick bites, erythemas, neurological symptoms, or joint disorders.

MAbs. The following MAbs raised in our laboratory were used to identify proteins: L100 22G3 against p83/100 (34), L41 1C11 against p41 (46), L32 1F11 against OspA (45), L22 1F8 against OspC (46), L75 3G10 against p75, L60 E6 against p60, L39 B1 against p39, L35 1F4 against p35 (strain PKo only), L34 1G8 against OspB (strain PKa2 only), L34 A4 against OspB (strain PBi only), L30 1B10 against p30, and L19 A11 against p19 (41a). MAb 8D5 against p66 (10) and CB625 against p21 (12) were kindly provided by A. G. Barbour (University of California, Irvine, College of Medicine) and B. J. B. Johnson (CDC, Fort Collins, Colo.), respectively.

Preparation of antigens. Borrelial strains PKa2 (*B. burgdorferi* sensu stricto, OspA serotype 1), PKo (*Borrelia afzelii*, OspA serotype 2), and PBi (*Borrelia garinii*, OspA serotype 4) (45) were used for antigen preparations. All strains were isolated from German patients; strain PKo was isolated from skin (EM lesion), and strains PKa2 and PBi were isolated from CSF. Low-passage strains (approximately 25 passages) were grown in modified Kelly medium (33) at 33°C for 4 to 5 days to a cell density of 10^7/ml. Cells were harvested by centrifugation at 12,000 × g for 20 min at 15°C and were washed four times with phosphate-buffered saline (pH 7.4)–5 mM $MgCl_2$. The protein concentrations of the final suspensions were estimated by the Bradford (6) protein assay (Bio-Rad, Munich, Germany). The preparations were stored at 20°C.

Only passages of strains abundantly expressing OspA and OspC were taken for this study. From each strain, two antigen preparations produced in large amounts were used. Prior to Western blotting all antigen preparations were adjusted to contain equal amounts of p41, as determined by serial dilutions by using Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and WBs. For preparative gels, cell lysates (350 to 400 μg of protein/gel) of each strain were electrophoresed separately by the protocol of Laemmli (24). Polyacrylamide gels (12.5%; acrylamide/bisacrylamide ratio, 37.5:1; 17 cm by 16 cm by 0.75 mm) were run at 4.0 to 4.5 mA for 24 h at room temperature. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by semidry blotting (23) for 1 h at 24 V and 200 mA. Staining with Ponceau S served to control efficient and homogeneous protein transfer. After blocking of unspecific binding sites with 50 mM Tris-OH/HCl (pH 7.4)–200 mM NaCl–0.1% Tween 20–5% nonfat dried milk for 1 h at 37°C, strips of 3 mm in width were cut, dried, numbered, and stored for up to 6 weeks at 4°C.

Prior to testing for IgM, sera were pretreated with RF absorbent (Behringwerke AG, Marburg, Germany) for 15 min. Strips were incubated overnight at room temperature in sera diluted 1:200 for IgG WB and 1:100 for IgM WB by using dilution buffer for Recomblots (Mikrogen, Munich, Germany), washed four times for 10 min each time (with 0.9% NaCl, 10 mM Tris-OH/HCl, 0.2% Tween 20), and incubated with horseradish peroxidase-conjugated rabbit anti-human IgG and IgM antibodies, respectively (Dakopatts, Copenhagen, Denmark) (dilutions, 1:1,000 for IgG and 1:500 for IgM in the same diluent as the sera). After four washes of 10 min each, color was developed by adding diaminobenzidine and H_2O_2. Development was stopped when the strips of the reference serum sample and the IgM-positive control serum sample reached a defined intensity (after approximately 5 min).

To avoid biases due to limitations of reproducibility, WB strips obtained from the same gel were always incubated with sera from subjects in the different study groups. Incubation was always done in parallel with one strip of each antigen in the same well. For high accuracy, strips were always fixed in the original position (as they were cut from the membrane) for documentation and analysis.

Control sera. The first and last strip of each batch (strips obtained from one gel) and every 5th to 10th strip were incubated with a broadly reactive IgG-positive reference serum sample (laboratory sample 93-19400, obtained from a patient with arthritis; see further description in Analysis of WBs). One IgM-positive, one IgG-negative, and one IgM-negative control serum sample were added to each test. The IgM-positive serum sample was obtained from a patient with acute NB and reacted with p41 and OspC of all strains and with OspA of strains PKo and PBi.

Analysis of WBs. The positions of bands (apparent molecular masses [apparent MMs]) were calculated by interpolation between MM marker lanes. Therefore, the R_f values (relative migration fronts) of the proteins were determined densitometrically by using a desktop scanner and the software Image Master, version 1.0 (Pharmacia Biotech, Freiburg, Germany). In our 12.5% polyacrylamide gel system, a log-linear relationship between R_f values and MMs was proven in the range of 14 to 70 kDa by analysis of lanes with a commercially available MM marker (range, 14.3 to 200 kDa; Gibco BRL, Gaithersburg, Md.).

For gels and WBs of this size (17 by 16 cm) several marker lanes are necessary. Since these lanes cannot be easily included in preparative gels, another strategy was used. WB strips incubated in a well-defined, broadly IgG-reactive reference serum (serum sample 93-19400, which also served as an IgG-positive control) were used as MM markers. The apparent MMs of seven prominent bands recognized by this serum had been determined previously.

For this purpose analytical gels with different strains and MM markers (Gibco BRL) in every fourth lane were blotted, the membranes were stained with Ponceau S, and the positions of the marker proteins were traced with a ballpoint pen. After incubation with serum sample 93-19400, the apparent MMs of seven prominent bands were calculated separately for each strain. This protocol was repeated with 10 different blots, and the geometric means of the calculated MMs were taken as the internal MM standard for the following assays.

After reproducible identification of all bands was established, blots were analyzed only visually (blots were assessed blindly, always by the same person). Band intensities were determined semiquantitatively by comparison with defined control sera (very faint [interpreted as negative], weak [interpreted as positive], strong, and very strong). Data were imported into a data bank for further analyses (see Definition of interpretation criteria for a positive WB result in the Results section).

Statistics. Where appropriate, results were analyzed by Fisher's exact test (independent proportions) or McNemar's χ^2 test (paired proportions) (50). All analyses were performed two-sided.

RESULTS

Identification of bands. The following bands were identified with MAbs (Fig. 1): p83/100, p75 (presumably DNaK), p66, p60 (GroEL), p41 (Fla), p39 (BmpA), p35 (strain PKo only), OspB (strains PKa2 and PBi), OspA, p30, OspC, p21 (strains PKa2 and PBi), and p19. In all strains p39 could clearly be differentiated from p41, and p30 could clearly be differentiated from OspA. There was also a clear distinction between p60 and a protein with a slightly smaller MM which was recognized by the reference serum. This antigen was designated p58.

Establishing an unequivocal description of all bands was a prerequisite for all further analyses. The WB patterns of different strains varied considerably, and homologous antigens of different strains migrated differently (Fig. 2B to E). Therefore, identification and designation of bands had to be performed separately for each strain. In the 58-kDa range we observed a most frequently detected band common to all strains which was also recognized by the reference serum (Fig. 2E). The clear discrimination from p60 could be demonstrated with WB strips from preparative gels (see Fig. 1 and Fig. 5B for examples). A certain serum sample reacted with no other bands in this region, and therefore, it was assumed that these proteins recognized in all three strains are homologous and were designated p58 (Fig. 2D). A similar approach was used for p21 (MAb CB625 only recognized p21 of strains PKa2 and PBi; Fig. 1). The apparent MMs of p83/100, p75, p58, p39, p30, p21, and p17 (p17, p17a, and p17b; see below) of each strain were determined (included in Fig. 3) and were used as internal standard MMs for further analyses of WBs from preparative gels.

Next, WB strips probed with 40 serum samples from patients with late-stage LB (IgG tests only) and 50 serum samples from patients with early-stage LB (IgG and IgM tests) were analyzed densitometrically, and the apparent MMs of all visually distin-

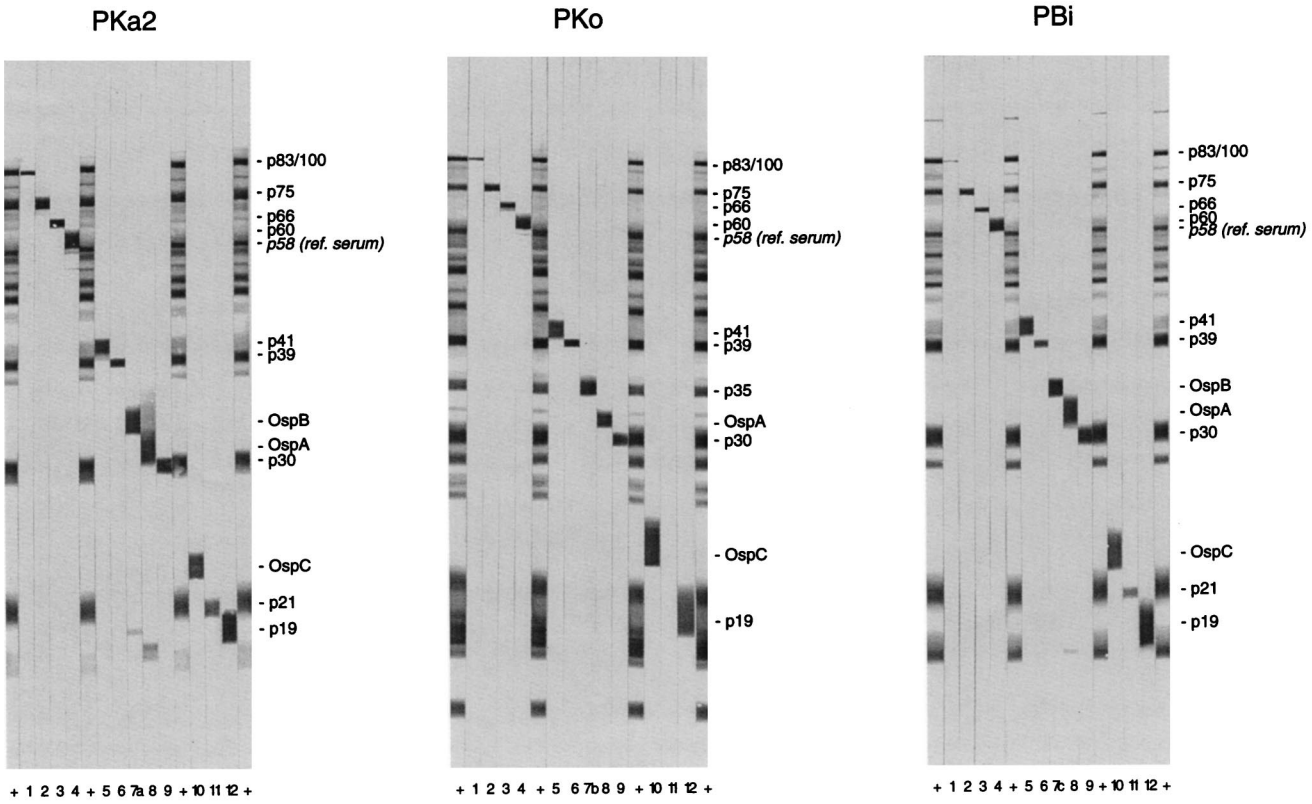

FIG. 1. Identification of antigens with MAbs. WB strips with the indicated strains were incubated with the following MAbs: lanes 1, L100 22G3; lanes 2, L75 3G10; lanes 3, 8D5; lanes 4, L60 E6; lanes 5, L41 1C11; lanes 6, L39 B1; lane 7a (PKa2 only), L34 1G8; lane 7b (PKo only), L35 1F4; lane 7c (PBi only), L34 A4; lanes 8, L32 1F11; lanes 9, L30 1B10; lanes 10, L22 1F8; lanes 11, CB625; lanes 12, L19 A11; lanes +, IgG-positive reference serum 93-19400. The identified proteins are indicated on the right; a certain protein recognized by the reference serum is indicated in italics (further specification is given in the text).

guishable bands were determined. If bands recognized by certain sera could not be identified properly, repeat incubation of the strips with the serum sample in question and the reference serum was performed and these strips were analyzed by direct comparison. Reproducibility was confirmed by repeat probing of WB strips from different gels with the same sera. For each strain, WBs from seven gels were analyzed by this protocol, and the median values of the calculated MMs were determined for each band. A summary is given in Fig. 3, which shows examples of WB strips incubated in broadly reactive sera. A total of 45 bands were distinguished in WBs of PKa2, 42 bands were observed in WBs of PKo, and 39 bands were observed in WBs of PBi. All bands ever observed in this study are included in this summary. The positions of the bands identified with MAbs are also indicated. When different antigen preparations of the same strain were used, minor displacements of some bands occurred in the region between p30 and p39. This was caused by moderate variations in the levels of expression of OspA and OspB. At the position of OspB, a narrower band sometimes occurred; this band was designated separately from OspB as 32.6 kDa (PKa2) and 33.2 kDa (PBi). OspB of strain PKo could not be identified on the WB since no MAb was reactive. For convenience, prominent bands (mostly identified with MAbs) were designated according to their approximate MMs as p83/100, p58, etc. Some of these bands are important for interpretation criteria and will be discussed later. Several homologous proteins of different strains have considerably different sizes, for example, p83/100, p39, OspA, OspC, and p19. In all three strains immunodominant bands with an MM of approximately 17 kDa were found. Due to the different reactivities of various sera with these bands, they do not seem to represent homologous proteins and therefore were designated p17 (PKo), p17a (PKa2), and p17b (PBi). The designation p17a for both the 18.0- and the 17.3-kDa bands of PKa2 was useful since these bands were difficult to discriminate, and both were important for interpretation criteria, as will be shown later. p17 (PKo) is by far the most immunogenic of these antigens. Furthermore, strain PKo expresses three other very immunogenic proteins (p43, p35, and p14) which could not be identified in strains PKa2 and PBi.

Registration of all bands from the study with clinically defined sera. The reactivities of all tested sera in all three WBs were documented semiquantitatively in tables which served as a database for all further analyses.

Table 1 summarizes the frequency of occurrence of individual bands detected by sera from subjects in the respective study groups. The results for strain PKo are presented in Table 1; similar tables were established for strains PKa2 and PBi. The frequencies of band recognition for each study group with LB in comparison with those for the total control group (blood donors, patients with syphilis stage II or III, and patients with elevated RF levels) were analyzed statistically, and bands showing highly significant differences between patients and controls ($P < 0.001$) are boxed. For the group with late LB, the results for most bands even reached this (arbitrarily chosen) high level of significance. On the other hand, by probing sera from patients with early stages of LB, the results for only a few bands were highly significant.

Definition of interpretation criteria for a positive WB result. By means of database queries in which a subset of data is

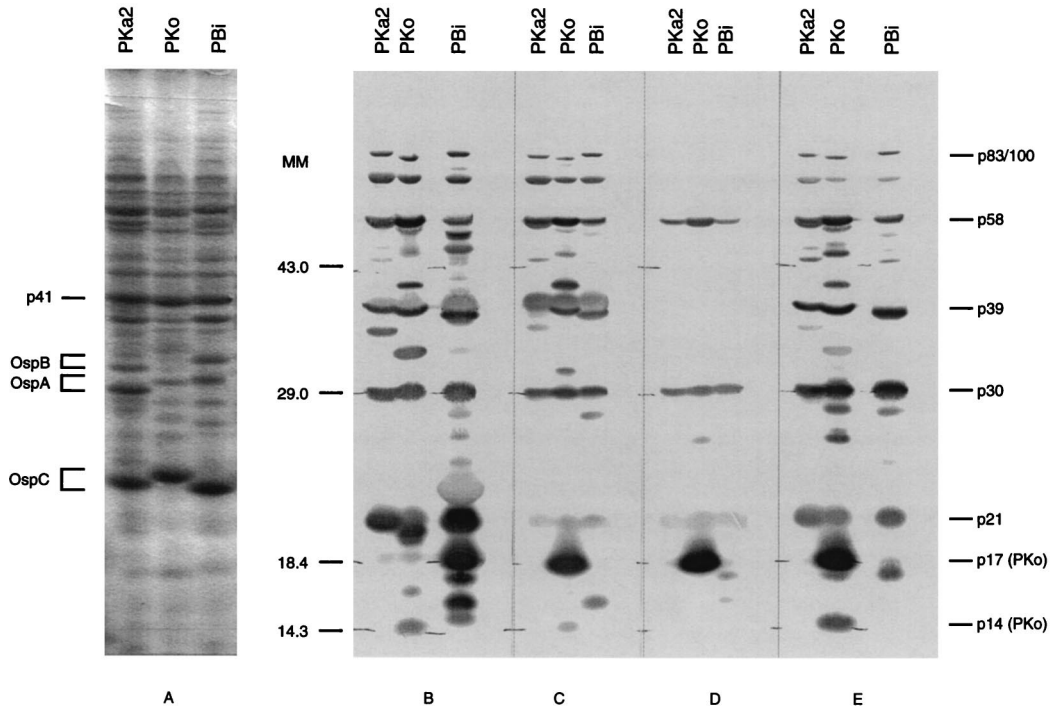

FIG. 2. (A) Coomassie brilliant blue-stained SDS-polyacrylamide gel of the antigen preparations used for this study. Proteins critical with regard to standardization of the antigen preparations are indicated on the left. Antigen loads of the three cell lysates were adjusted so that they had comparable amounts of p41. All strains abundantly expressed OspA and OspC. (B to E) IgG WB of the indicated strains probed with four different sera from patients with different manifestations of LB: chronic NB (B), arthritis (C), acute NB (D), and arthritis (E) (serum sample E served as IgG-positive reference serum [93-19400]). Numbers on the left are sizes of the proteins of the MM marker (in kilodaltons). Several immunodominant proteins are indicated on the right. Antigens p17 and p14 were observed only in strain PKo.

selected from the database, criteria consisting of combinations of reactive bands were evaluated systematically for their discriminating abilities. Since it is not possible to prove all conceivable combinations, the following strategy was used. Combinations of bands showing no false-positive reactions were evaluated first. Then, significant bands ($P < 0.05$ by testing the results for patients with EM or NB versus the results for the whole control group) were added stepwise to the putative criteria. For each strain more than 40 band combinations were evaluated for IgG tests and more than 15 band combinations were evaluated for IgM tests. These putative criteria were sorted according to the resulting overall sensitivities (for all sera from patients with LB) and specificities (determined with the sera from the total control group). From all band combinations resulting in the same specificity, only those with the highest sensitivities were extracted (Table 2; for IgM WBs, several interpretation rules with equal specificities have also been shown to enable comparisons with previous studies). The use of several bands which had good discriminatory abilities as individual bands showed no further benefit in combination with other bands. Since WBs are used for confirmatory testing, a specificity of at least 95% was required. Under this prerequisite the criteria boxed in Table 2 seemed to be most useful. All interpretation rules listed in Table 2 resulted in extremely significant discriminations between the sera from patients with LB and those from the control group ($P < 0.001$). From the results of the PKa2 WBs, it was evident that both the 18.0- and the 17.3-kDa bands were important for interpretation. Since these two bands were difficult to discriminate, it was reasonable to use the designation p17a for both of them. As a consequence, "reactive p17a" means that at least one of the two bands must be present.

Reactivity to p41 in general did not give good discrimination. However, since a number of serum samples from patients with LB but none of the control serum samples were strongly reactive with p41 in the IgM WB, strong reactivity with p41 was included in the criteria for a positive IgM WB result.

Sensitivities and specificities of WBs with different strains. Using the best interpretation criteria (boxed in Table 2), sensitivities and rates of false-positive results were determined for all strains and study groups (Table 3). In the EM group in both the IgG and the IgM tests, PKo WBs were the most sensitive, followed by PBi and PKa2 WBs. For the sera from patients with NB, sensitivities were determined separately for the patients from whom isolates were obtained from CSF (group NB I) and those selected by clinical signs and CSF/serum antibody indices of >2 (group NB II). The sensitivities of the IgG WBs were significantly higher for the latter group ($P < 0.05$ for all strains), and for both groups the highest sensitivities were obtained with PBi. In IgM tests of all sera from patients with NB, the PKo and PBi WBs achieved the highest sensitivities. The sensitivity of the IgM PBi WB was higher for sera from patients in group NB II than that for sera from patients in group NB I, but the difference was not significant. One serum sample from a patient with ACA was negative in the IgG PKa2 WB, and one serum sample from a patient with arthritis was negative in the IgG PBi WB; otherwise, all late-stage sera were positive by all IgG WBs of all strains. The rate of false-positive results (determined by the total control group) was quite similar for all strains by both IgG and IgM WBs.

Within the study groups, differences in WB results were analyzed by pairs by using McNemar's χ^2 test. None of these differences was significant except for the sensitivities of the

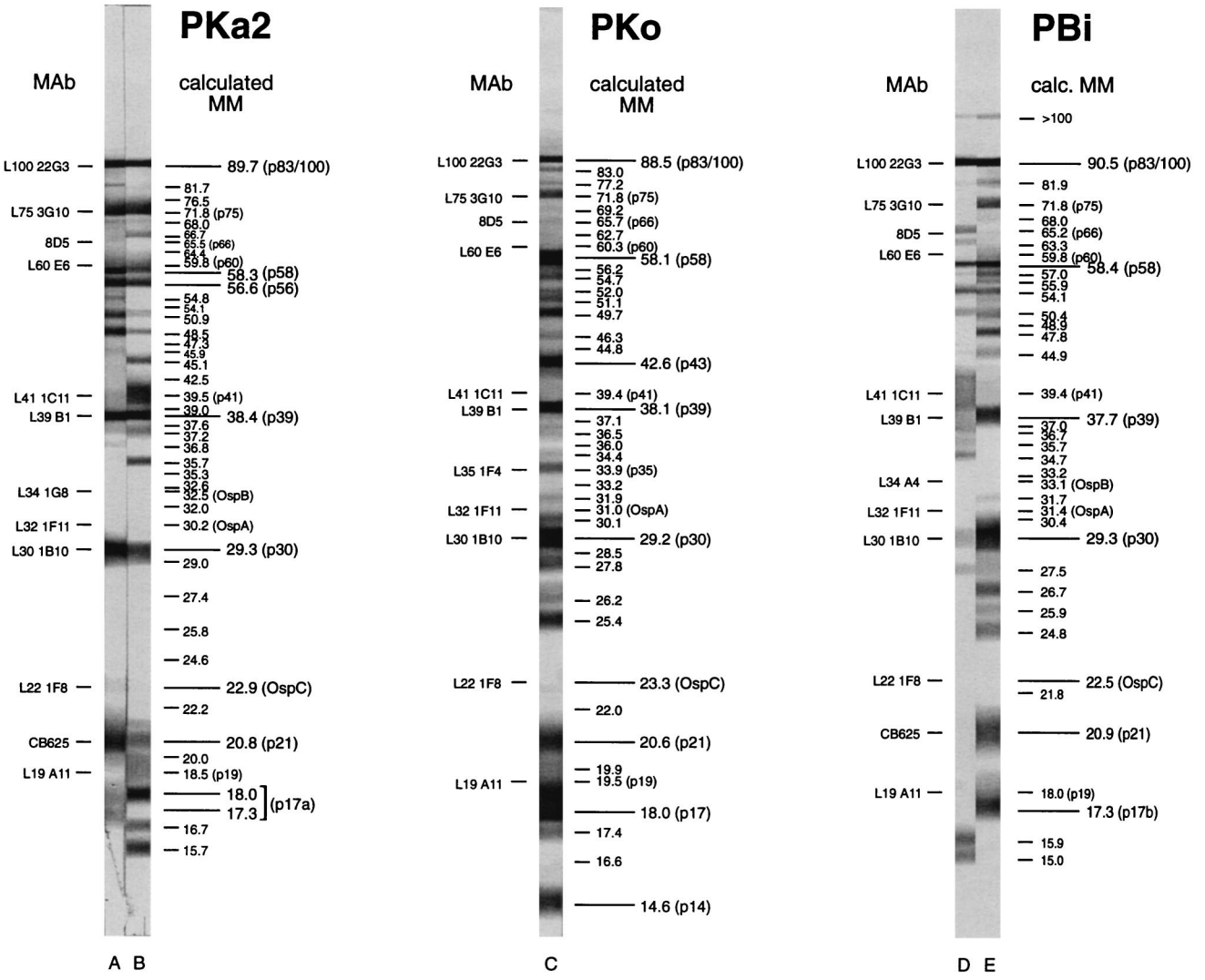

FIG. 3. WB strips of the indicated strains incubated in broadly reactive sera (all sera were from patients with arthritis). Strips A, C, and E, reference serum (93-19400); strips B and D, two other serum samples. The apparent MMs (in kilodaltons) of all visually distinguishable bands are indicated to the right of each strip. Familiar designations, with approximate MMs for prominent bands are indicated in parentheses. Designations farther to the right indicate bands important for interpretation criteria for a positive IgG WB result. MAbs which identified the respective bands are indicated on the left. Note that labelling includes all bands ever observed in this study. A complete pattern of all bands could never be shown by incubation with just one serum sample or a pool of sera (diluting effects). Therefore, the WB strips shown here are only for demonstration and orientation.

PKo IgG WB and the PKa2 IgG WB for the EM group (36.4 versus 18.2%, respectively; $P < 0.01$).

Numbers of recognized significant bands. As an example, the numbers of recognized bands among the nine significant bands necessary for interpretation of IgG PKo WB results (p83/100, p58, p43, p39, p30, OspC, p21, p17, and p14) were determined for all sera (Fig. 4). In general, this number increases with the duration of infection. All sera from patients with late-stage LB were reactive with at least four of these bands. However, increasing the stringency of interpretation criteria from two of nine bands to three of nine bands would have resulted in a considerable loss of sensitivity for patients with early-stage LB. For instance, in the NB I and NB II groups of patients, sensitivities would have been reduced from 41 to 23% and 59 to 43%, respectively.

Comparison of reactivities with selected bands of different strains. Summaries for direct comparison of the reactivities of significant immunodominant homologous proteins of the three strains are given in Tables 4 and 5 for IgG and IgM WBs, respectively. If homologous proteins were not known, data were shown only for the strain with the respective band.

For the group of patients with EM, several bands on IgG PKo WB were recognized by relatively high percentages of sera, namely, p58, p43, p17, and p14. When results for the two groups of patients with NB were compared, the most striking findings were that p43 and p14 were recognized by only 5 and 3% of the sera from patients in group NB I, respectively, but by 30 and 27% of the sera from patients in group NB II, respectively ($P < 0.01$). For the reactivity with p17, the same observation was made ($P < 0.01$), in contrast to the recognition of p39, which occurred more frequently when sera from patients in group NB I were probed (49% versus 34% for patients in group NB II). The band most frequently recognized by sera from patients in group NB II was OspC of PBi. OspC of all strains was recognized significantly more frequently by sera from patients in group NB II than by sera from patients in

TABLE 1. Frequency of recognition of proteins of strain PKo (*B. afzelii*)

Protein band		% Sera with IgG reactivity to protein band[a]						% Sera with IgM reactivity to protein band[a]				
		LB sera			Control group sera			LB sera[b]		Control group sera		
Desig-nation	Apparent MM (kDa)	EM (n = 66)	Neuro-borreliosis (n = 83)	Late LB (n = 40)	Blood donors (n = 120)	Syphilis II/III (n = 11)	Positive for RF (n = 10)	EM (n = 66)	Neuro-borreliosis (n = 83)	Blood donors (n = 120)	Syphilis II/III (n = 11)	Positive for RF (n = 10)
p83/100	88.5	8	18	83					4			
	83.0	2	2	23								
	77.2	3	2	3								
p75	71.8	6	14	50	8	9				1		
	69.2		1	5								
p66	65.7		1	35	5			2				
	62.7	6	2	5	9			2		1		
p60	60.3	6	6	13	4	9		2	3	5		
p58	58.1	26	39	98	3				1			
	56.2	2	10	53				2	1			
	54.7	2	16	70	2				1			
	52.0	2	4	65								
	51.1	2	2	8								
	49.7	5	5	75	1							
	46.3	3	7	60								
	44.8	3	4	5					4			
p43	42.6	32	18	93	5	9	20					
p41	39.4	33	41	75	22	9	30	19	19	7		10
p39	38.1	6	41	83	1		10	3	8			
	37.1			30					4			
	36.5	2		28					3			
	36.0	5	1	15				3	4			
	34.4			15								
p35	33.9	6	2	45	3			13	5	4		
	33.2	2		5						1		
	31.9	3	2	58								
OspA	31.0	3		5	3				3			
	30.1	8	1	58			20					
p30	29.2	5	19	78	1							
	28.5			5								
	27.8		2	28								
	26.2		5	43								
	25.4	3	6	40								
OspC	23.3	6	17	20	1			41	27	1		
	22.0			3								
p21	20.6	2	19	70								
	19.9		5	25								
	19.5	26	10	53	1		20		3			
p17	18.0	27	27	95	4			6	7			
	17.4		5	38								
	16.6	2	1	45								
p14	14.6	18	16	78	1							

[a] Frequencies of band recognition of sera from patients with LB versus those of sera from total control group (n = 141) were analyzed by Fisher's exact test. If $P < 0.001$, frequencies were boxed. Zeros were not shown to improve clarity.

[b] Sera from patients with late-stage LB were not tested for IgM.

TABLE 2. Evaluation of various interpretation criteria for positive WB result[a]

Ig class	Strain	Bands required	Specificity (%)[b]	Sensitivity (%)[c]
IgG	PKa2	≥2 of p83/100, p58, p56, 45.1 kDa, p39, p30, OspC, p21, 18.0 kDa, and 17.3 kDa	100.0	42.9
		≥1 of p83/100, p58, p56, p21, 18.0 kDa, and 17.3 kDa	99.3	45.5
		≥1 of p83/100, p56, OspC, p21, 18.0 kDa, and 17.3 kDa	97.2	47.1
		≥1 of p83/100, p58, p56, OspC, p21, 18.0 kDa, and 17.3 kDa	96.5	50.8
		≥1 of p83/100, p58, p56, p39, OspC, p21, 18.0 kDa, and 17.3 kDa	95.7	51.3
		≥1 of p83/100, p58, p56, p39, p30, OspC, p21, 18.0 kDa, and 17.3 kDa	92.2	52.9
	PKo	≥2 of p83/100, p58, p39, p30, OspC, and p21	100.0	43.4
		≥2 of p83/100, p58, p39, p30, OspC, p21, and p17	99.3	49.2
		≥2 of p83/100, p58, p43, p39, p30, OspC, p21, p17, and p14	97.9	56.1
		≥1 of p83/100, p58, p39, p30, OspC, and p21	94.3	56.6
		≥1 of p83/100, p39, p30, OspC, p21, and p17	93.6	58.2
		≥1 of p83/100, p58, p39, p30, OspC, p21, and p17	91.5	61.9
		≥1 of p83/100, p58, p39, p30, OspC, p21, 19.5 kDa, p17, and p14	90.1	63.0
	PBi	≥1 of p39, p21, and p17b	100.0	42.3
		≥1 of p83/100, p39, p21, and p17b	99.3	46.0
		≥1 of p39, OspC, p21, and p17b	97.9	54.0
		≥1 of p83/100, p39, OspC, p21, and p17b	97.2	56.1
		≥1 of p83/100, p58, p39, OspC, p21, and p17b	96.5	57.1
		≥1 of p83/100, p58, p39, p30, OspC, p21, and p17b	94.3	59.3
IgM	PKa2	≥2 of p41, p39, and OspC	99.3	16.1
		OspC	97.2	31.5
		≥1 of p39, and OspC	97.2	33.6
		≥1 of strong p41[d], p39, and OspC	97.2	35.6
		≥1 of strong p41[d], p39, OspC, 18.0 kDa, and 17.3 kDa	97.2	36.9
		Any 2 or more bands	93.6	19.5
	PKo	≥2 of p41, p39, and OspC	100.0	14.1
		OspC	98.6	32.9
		≥1 of p39, and OspC	98.6	37.6
		≥1 of strong p41[d], p39, and OspC	98.6	39.6
		≥1 of strong p41[d], p39, OspC, and p17	98.6	42.3
		≥1 of strong p41[d], p39, p35, OspC, and p17	95.0	44.3
		Any 2 or more bands	97.2	22.1
	PBi	≥2 of p41, p39, and OspC	100.0	22.1
		OspC	97.9	38.3
		≥1 of p39, and OspC	97.9	38.9
		≥1 of strong p41[d], p39, and OspC	97.9	40.3
		Any 2 or more bands	92.2	30.9

[a] Recommended interpretation criteria are boxed.
[b] Specificity was determined by testing 120 serum samples from healthy blood donors, 11 serum samples from patients with syphilis stage II or III, and 10 RF-positive serum samples.
[c] Total sensitivity for all sera from patient with LB tested (n = 189 for IgG WBs; n = 149 for IgM WBs).
[d] Band intensity equal to or greater than band intensity of a strongly reactive control serum sample.

group NB I ($P < 0.01$ for PKa2 and PBi; $P < 0.05$ for PKo). The sera from patients with late-stage LB reacted most frequently with p58 of all strains and p17 of strain PKo. Twenty percent of sera from patients in this group were still reactive to OspC of PKo. Sera from the control group rarely reacted (less than 6%) with all of these bands.

In IgM tests (Table 5) of all sera from patients with LB, OspC (of all strains) was the most frequently recognized protein. Reactivity with p41 also occurred frequently, but since only strong band intensities were evaluated for positive IgM WBs, the overall p41 reactivity does not appear in Table 5. None of the differences between patients in groups NB I and NB II was significant. In sera from patients in both NB groups, the reactivity with OspC of PBi was prominent. The rate of false-positive reactions was less than 3% for all of these proteins.

The results summarized in Tables 4 and 5 are further illustrated in Fig. 5, which shows representative WBs for strain PKo. On the IgG WBs with sera from patients in group NB I, no reactivity with p43 or p14 but recognition of p39 was observed (Fig. 5B), whereas the opposite situation was true on the WBs with sera from patients with EM (Fig. 5C).

TABLE 3. Frequency of positive WBs of different strains

Ig class	Study group	No. of serum samples tested	% Sera with positive WB result for strain[a]:		
			PKa2	PKo	PBi
IgG	EM	66	18	33	23
	NB I	39	33	41	46
	NB II	44	66	64	71
	ACA	30	97	100	100
	Arthritis	10	100	100	90
	Blood donors	120	4	2	3
	Syphilis stage II or III	11	0	0	9
	Positive for RF	10	0	10	0
IgM	EM	66	41	46	42
	NB I	39	31	41	33
	NB II	44	36	39	43
	Blood donors	120	4	2	2
	Syphilis II/III	11	0	0	0
	Positive for RF	10	0	0	10

[a] Criteria for positive WB results are indicated in Table 2 (in boxes).

DISCUSSION

Performance and analysis of WB assays. The first aim of this study was to optimize our WB assay in terms of reproducibility and resolution. By the use of 12.5% polyacrylamide gels of 17 cm in length, approximately 40 bands per strain could be differentiated reproducibly. Actually, there might even be many more antigens which could probably be separated only by other methods such as two-dimensional gel electrophoresis. Furthermore, antigens with a size of less than 14 kDa which were observed by others (29, 51) might have been missed by our gel system. For this study, other gel systems, including gradient gels (7.5 to 17.5% and 10 to 15% polyacrylamide), which should have increased resolution, were of no advantage (data not shown). In comparison with standard dilution buffers for sera (for instance, 150 mM NaCl, 10 mM Tris-OH/HCl [pH 7.4], 1% nonfat dried milk, 0.2% Tween 20), the buffer that we used led to decreased binding of antibodies with a low affinity. Thus, nonspecific reactions could be decreased, but weakly binding antibodies produced very early in LB could possibly be missed also. The intra-assay reproducibility of band intensities was very good, but blots from different gels showed considerable variations. Therefore, intensities must be referred to the intensities of control sera, and several weakly positive control sera with different complementary reactivities should be used. The definition of cutoff intensities is especially critical and should ideally be done separately for each significant band. For the present study, this was not completely possible since not all significant bands were known at the beginning of the study.

Densitometry is an objective method, but it harbored several problems for our application: Faint but clearly visible bands which occur frequently in WBs of sera from patients with early-stage LB could not be detected by the scanner. Furthermore, bands like OspC that appeared to be rather broad on the gels were often read as two bands. Since the differences in apparent MMs of some bands were very small, these bands could not be reliably detected. Therefore, repeat incubation of adjacent strips with the serum sample in question and the reference serum had to be performed. The specified values

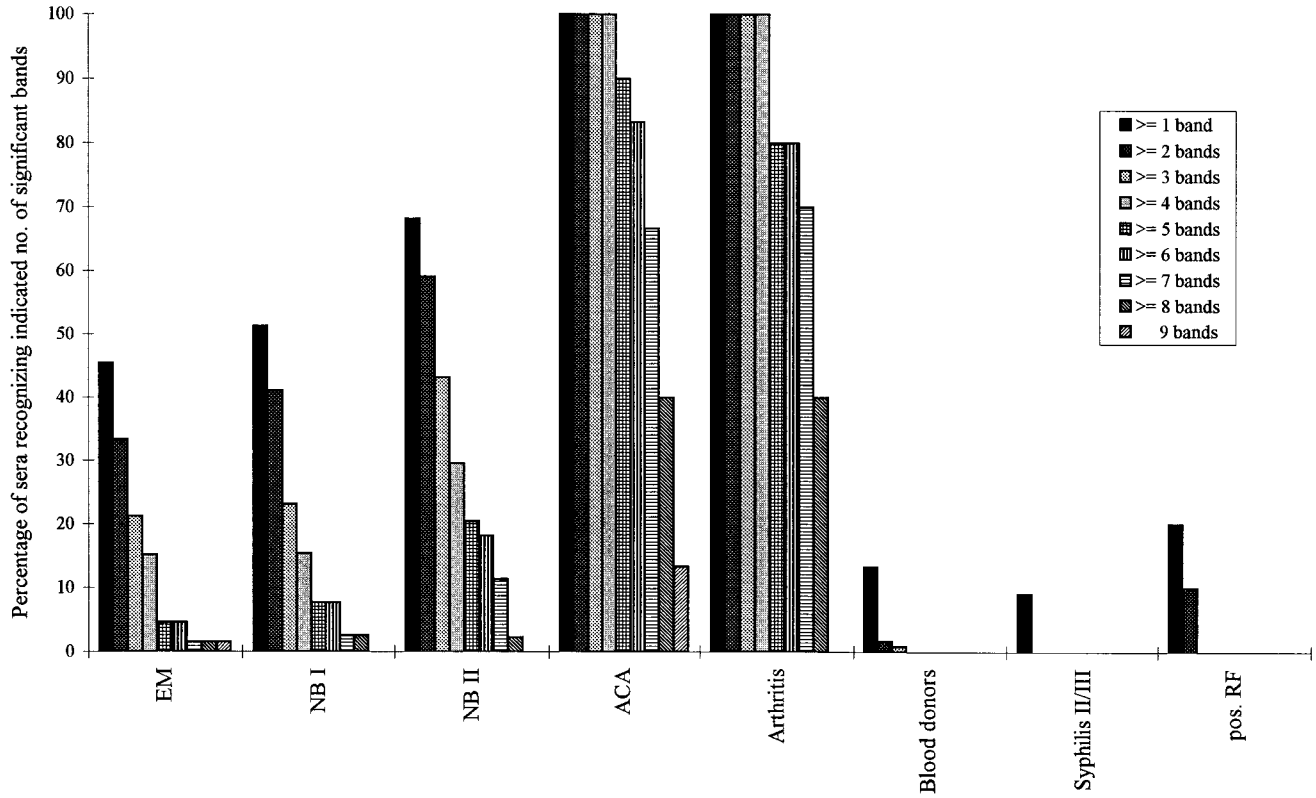

FIG. 4. Percentage of sera recognizing certain numbers of significant bands in IgG WBs with strain PKo. Bands p83/100, p58, p43, p39, p30, OspC, p21, p17, and p14 were evaluated.

TABLE 4. Frequency of recognition of selected proteins of different strains in IgG WBs

Study group	Strain	% Sera with reactivity to the following protein band:										
		p83/100	p58	p56[a]	p43[b]	p39	p30	OspC	p21	p17[b]	p17a[a,c]	p17b[d]	p14[b]
EM (n = 66)	PKa2	5	5	3		2	9	8	6		8		
	PKo	8	26		32	6	5	6	2	27			18
	PBi	9	8			6	8	12	5			3	
NB I (n = 39)	PKa2	13	26	23		15	15	8	13		5		
	PKo	13	33		5	49	21	8	10	10			3
	PBi	15	18			33	18	18	13			10	
NB II (n = 44)	PKa2	25	45	27		14	20	34	34		0		
	PKo	23	45		30	34	18	25	27	41			27
	PBi	36	36			32	25	48	36			23	
Late LB (n = 40)	PKa2	90	98	83		73	80	10	63		58		
	PKo	83	98		93	83	78	20	70	95			78
	PBi	88	95			90	80	8	78			35	
Control group (n = 141)	PKa2	0	1	0		1	4	3	0		0		
	PKo	0	3		6	1	1	1	0	4			1
	PBi	1	2			0	2	2	0			1	

[a] Protein(s) expressed by strain PKa2 only.
[b] Protein expressed by strain PKo only.
[c] The 18.0- and 17.3-kDa proteins of strain PKa2 were combined in the designation p17a.
[d] Protein expressed by strain PBi only.

serve as designations for visually distinguishable bands and should not be taken as real MMs.

Identification of bands. Since an unselected evaluation of all bands was the aim, a complete documentation of all bands throughout the study had to be achieved. Therefore, all visually distinguishable bands had to be identified and designated in a first step.

There were clear discriminations between p60 and p58, between OspA and p30, and between p41 and p39, as well as between p39 and a weakly reacting band of almost the same apparent MM; this last band might be a degradation product of p41 since it is weakly recognized by MAb L41 1C11. Misinterpretations concerning these differentiations have occurred frequently, especially the common antigen p60, which is frequently recognized by cross-reacting antibodies, and the highly specific and most sensitive antigen, p58, on the other hand, have never been differentiated in any other study.

Strains from different species are rather heterogeneous (45, 46), leading to differences in reactivities on WBs (2, 28, 44, 48, 49, 51). As shown in Fig. 2, only certain proteins seem to be relatively well conserved; otherwise, the reactivity patterns of the different strains vary considerably.

Interpretation criteria. First, we tried to define interpretation criteria valid for all three strains used in this study. In comparison to the finally established criteria, these common criteria would have led to a considerable loss of discriminatory ability. Next, combinations of bands were evaluated separately for each strain. By systematically calculating the outcomes for all reasonable combinations and further sorting according to specificities and sensitivities, the recommended interpretation criteria were finally established.

A different approach was used by Dressler et al. (16). They constructed receiver operating characteristic (ROC) curves for the most frequently occurring bands and determined the amounts of bands to be considered to obtain the greatest ROC areas. For these optimal ROC curves, the minimum number of IgM or IgG bands needed to obtain 99% specificity was selected.

In the United States, CDC recommends the interpretation criteria defined by Dressler et al. (16) for IgG (five bands of 18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa; strain *B. burgdorferi* sensu stricto G39/40 was used) and by Engstrom et al. (17) for IgM (two bands of OspC, p39, and p41; low-passage strain *B. burgdorferi* sensu stricto 297 was used). However, in our study the use of such strict criteria would result in very low sensitivities (see Fig. 4 for IgG and Table 2 for IgM). Many of our sera from patients with LB reacted with no band other than OspC in IgM WBs. Furthermore, comparison of our band definitions

TABLE 5. Frequency of recognition of selected proteins of different strains in IgM WBs

Study group	Strain	% Sera with reactivity to the following protein band:				
		Strong p41[a]	p39	OspC	p17[b]	p17a[c]
EM (n = 66)	PKa2	5	2	38		5
	PKo	6	3	41	6	
	PBi	3	0	41		
NB I (n = 39)	PKa2	10	5	23		5
	PKo	13	13	26	3	
	PBi	13	8	31		
NB II (n = 44)	PKa2	8	0	31		3
	PKo	8	3	28	11	
	PBi	11	0	42		
Control group (n = 141)	PKa2	0	0	3		1
	PKo	0	0	1	0	
	PBi	0	0	2		

[a] Band intensity equal to or greater than band intensity of a strongly reactive control serum sample.
[b] Protein expressed by strain PKo only.
[c] The 18.0- and 17.3-kDa proteins of strain PKa2 were combined in the designation p17a.

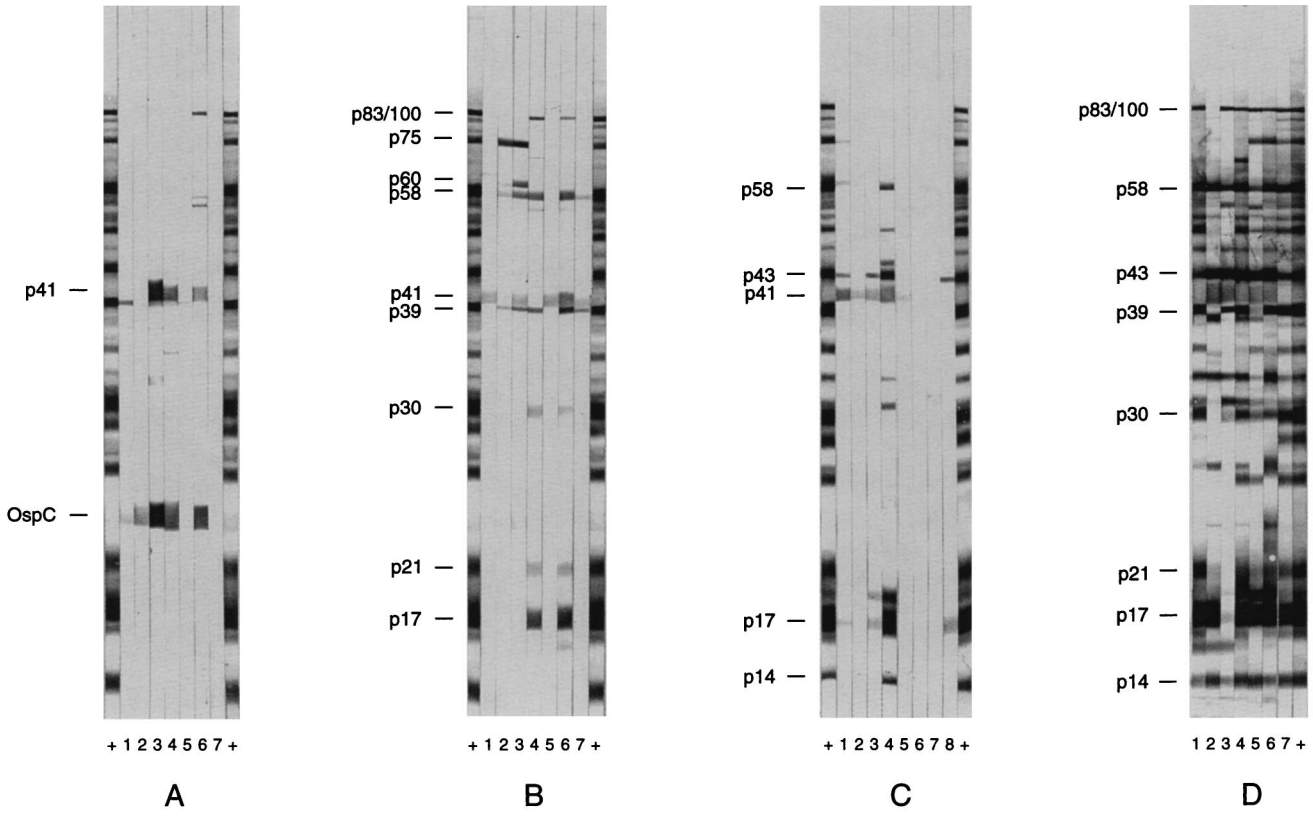

FIG. 5. Representative WBs of strain PKo with sera from patients with various manifestations of LB. (A) Acute NB (group NB I), IgM WB; (B) acute NB (group NB I), IgG WB; (C) EM, IgG WB; (D) ACA, IgG WB. Lanes 1 to 8, sera from patients in the respective study groups; lanes +, reference serum (93-19400). The most frequently reacting bands in these examples are indicated.

to the band descriptions of Dressler et al. (16) was only partially possible.

In a further study with European strains and German sera, Dressler et al. (15) also found that the antibody response in European patients with LB seems to be more restricted than that in U.S. patients with the disease.

Sensitivities and specificities. In our study, the sensitivities for sera from patients with EM ranged from 18% (PKa2) to 33% (PKo) in IgG WBs, whereas the IgM WBs were positive for 41 to 46% of serum samples (Table 3). By testing sera from patients with EM in the United States, Engstrom et al. (17) reported sensitivities of 43.6% for both IgG and IgM WBs, whereas Dressler et al. (16) found 0 and 40% positive sera in IgG and IgM WBs, respectively. The outcomes obtained by using the very different interpretation criteria for IgG WBs for sera from U.S. patients with LB recommended by these two groups is clearly demonstrated by these results, even if one considers the fact that the patients were not identically selected.

A total of 64 and 80% of the U.S. patients with meningitis had positive IgG and IgM WB results, respectively, in the study of Dressler et al. (16). In our groups of patients with NB, the sensitivities of the IgG WB ranged from 33 to 71%; however, the IgM WB was considerably less frequently positive (31 to 43%). No significant differences were observed between our IgG WB results for patients with late-stage LB and the results of Dressler et al. (16).

Antigens detected by IgG antibodies. p83/100, p58, p39, p30, OspC, and p21 were highly sensitive and specific for IgG tests and could be identified in all three strains. By comparison of WBs with different strains, however, there were remarkable differences in the frequency of recognition of certain homologous proteins (Table 4). These differences are reflected in the three different proposed interpretation criteria for positive IgG WB results. The OspC of PBi showed a higher sensitivity than OspC of PKa2 and OspC of PKo for sera from patients with EM and patients with NB. In part, the high sensitivity of the OspC of PBi accounts for the relatively simple interpretation criteria for positive PBi IgG WB results. p58 of PBi seemed to be slightly less sensitive than the homologs of PKa2 and PKo, whereas p39 of PKa2 was the least sensitive of the p39 homologs, and p21 of PKo was the least sensitive of the p21 homologs. The unique PKo antigens p43, p17, and p14 were considerably more frequently recognized by sera from patients with EM than other immunodominant proteins. The p41 of all strains was rather unspecific, which has already been reported by others (16, 17, 28, 51). In our investigation, p58 of all strains and p17 (PKo) turned out to be the most sensitive and also very specific antigens. A 58-kDa antigen was also the most sensitive band in the study by Dressler et al. (16), but 7% of their control sera were also reactive with this band. Furthermore, Engstrom et al. (17) reported that 45% of their control sera recognized a 58-kDa protein on their IgG WB. We suggest that p60 and p58 had not been discriminated in those studies. p39 was the protein giving the best discrimination in the study by Ma et al. (28), whereas Dressler et al. (16) reported that 10% of their control sera were also reactive with p39.

OspA and OspB were only rarely recognized by sera from our patients with LB, which is in accordance with other European studies (15, 29, 47, 48). Furthermore, OspB is difficult to

identify since it comigrates with a variety of other weakly immunogenic proteins. OspD could not be identified with MAb H1C8 (35) provided by B. J. B. Johnson (CDC) (data not shown). OspE, OspF, and pG could also not be identified on our WBs since no appropriate MAbs were available.

Antigens detected by IgM antibodies. In the current study, OspC (of all strains) was by far the antigen most frequently recognized by IgM antibodies. p41 was also observed frequently, but since up to 10% of the control sera also reacted with p41 (depending on the strain), the usefulness of this antigen was limited. However, applying an increased intensity cutoff led to a good discrimination, and although the sensitivity under these premises was low, evaluation of strong p41 bands in addition to OspC and p39 turned out to increase the overall sensitivity. The predominance of OspC and p41 for IgM reactivity as well as frequent unspecific reactions with p41 were also reported by others (1, 16, 28, 48, 51).

p39 was recognized much less frequently than OspC and p41 in the IgM WBs in our study, as well as in the studies by Dressler et al. (15, 16). On the other hand, Engstrom et al. (17) reported that p39 was the most sensitive antigen and also gave the best discrimination in their IgM WBs.

Unexpected findings in patients with NB. The two groups of patients with NB in our study showed unexpected differences in WB reactivity. The selection criterion for group NB I was isolation of *B. burgdorferi* sensu lato from the CSF, whereas the selection criterion for group NB II was a CSF/serum antibody index greater than or equal to 2.0. Cultures were negative for all 20 patients in group NB II from whom CSF was cultured. The duration of neurological symptoms was not significantly different (median, 3 and 4 weeks in groups NB I and NB II, respectively). Sera from patients in group NB II were significantly more frequently positive in IgG WBs ($P < 0.05$ for all strains). This result was analyzed further by comparing the frequencies of recognition of individual proteins (Table 4). The three immunodominant bands p43, p17, and p14 which are unique for strain PKo (at least among the strains used in this study) were recognized significantly less frequently by the sera from patients in group NB I ($P < 0.01$). A suggestion to explain this result could be that borrelial strains lacking these antigens might be in favor to escape the immune response and therefore survive in culturable amounts.

Comparison of species. It was repeatedly shown that sera from European patients with NB tend to react more frequently with *B. garinii* strains, whereas disorders related to the skin can be detected more frequently by *B. afzelii*-based tests (2, 30, 44). This observation was confirmed by the current study. However, none of the differences between the sensitivities of the WBs with the different strains were statistically significant except for the results of the PKo and PKa2 IgG WBs with sera from patients with EM (33 versus 18%, respectively).

In our study the overall sensitivity and specificity of WBs with strain PKo were slightly better than those with PKa2 and PBi, and therefore, we recommend that this strain should be used for WBs (at least in our region). Since strain PBi was the most sensitive for detection of antibodies in sera from patients with NB, additional testing of sera with PBi WBs might be valuable for the diagnosis of NB. Other *B. afzelii* strains might show a similar antigenicity as strain PKo, and other *B. garinii* strains might be similar to strain PBi. Since strains of the same species, however, can also differ in their immunoreactivities, the MMs of diagnostically important proteins might be different (47), and the different levels of expression of some proteins might be critical, results of studies should only be referred to the strains used. In a study by Norman et al. (30) WBs of eight different European strains were probed with sera from different European regions. Those investigators also found that *B. afzelii* and *B. garinii* strains were more suitable for WBs used for diagnostic testing in Europe than *B. burgdorferi* sensu stricto strains. Besides the localization of manifestations, differences in the geographical distributions of *B. burgdorferi* strains in Europe seem to influence the preferential reactivities (9, 30).

Conclusions. The difficulties in comparing the results of the leading studies concerning the evaluation of WBs for the serodiagnosis of LB demonstrate the need for standardization. Identification of significant proteins with MAbs is a prerequisite for comparison of WBs from different laboratories. Likewise, additional probing with well-defined sera available in sufficient amounts might be valuable. Strains should express the diagnostically most important antigens (for instance, OspC) in sufficient amounts. Furthermore, important specific proteins must clearly be differentiated from nonspecific antigens in the same MM range. The use of longer gels for SDS-PAGE can be helpful in achieving this.

Finally, the selection of one strain would be desirable, but to date this is not yet possible. Further comparison of the proficiencies of different strains, in Europe mainly involving *B. afzelii* and *B. garinii* strains, should be performed.

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