JOURNAL OF CLINICAL MICROBIOLOGY, June 1990, p. 1362–1366
0095-1137/90/061362-05$02.00/0

Vol. 28, No. 6

Characterization of a Tick Isolate of *Borrelia burgdorferi* That Possesses a Major Low-Molecular-Weight Surface Protein

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Received 27 November 1989/Accepted 21 February 1990

An unusual strain of *Borrelia burgdorferi* (DN 127 cl 9-2) that was isolated from an *Ixodes pacificus* tick did not react with monoclonal antibodies (MAbs) to OspA and OspB surface proteins, which are found in most U.S. strains. The strain exhibited an abundant protein with an apparent molecular weight of 25,000 (25K protein). A MAb, 86 DN-1, that was prepared to the 25K protein was used in studies on the effect of proteases on the intact spirochetes, immune electron microscopy, and Western blot (immunoblot) analyses; the results indicated that the low-molecular-weight protein was an apparent surface protein that was loosely attached to the spirochete. Five tick isolates from California possessed low-molecular-weight proteins in the 20,000- to 25,000-molecular-weight range that reacted with the 86 DN-1 MAb. The 25K protein of DN 127 cl 9-2 was unaffected by prolonged in vitro passage of cultures in BSK II medium, while the low-molecular-weight proteins of the other strains of *B. burgdorferi* from California either decreased in quantity or became undetectable on long-term in vitro passage.

The molecular and immunochemical characterization of strains of *Borrelia burgdorferi* has led to a better understanding of the spirochete that is the etiologic agent of Lyme disease (for a review see reference 4). Two major surface proteins with relative molecular weights of 31,000 (31K protein; OspA) and 34,000 (34K protein; OspB) have been described, and monoclonal antibodies (MAbs) against epitopes of these proteins have been used in the characterization of strains from the United States and Europe (7, 8, 20, 21). The majority of strains isolated in the United States appear to be homogeneous in their reactions to the OspA MAb, but considerable heterogeneity exists in the reactions to the OspB MAb (7). Strains that do not react with the OspA or the OspB MAb and that possess a major protein with a molecular weight of 20,000 to 24,000 have been reported from Europe but not from North America (7, 8, 20, 21). We recently reported the isolation of a strain of *B. burgdorferi* from an adult *Ixodes pacificus* tick that differed from any previously reported North American isolate but that was similar to strains isolated in Europe (12, 13). The strain, DN 127 cl 9-2, which was cloned by limiting dilution, did not react with the MAb to the OspA or the OspB protein, but it possessed a major protein with a molecular weight of about 25,000.

In this report we describe results of further analyses of proteins of strain DN 127 cl 9-2 and results of studies with a MAb that was produced to the 25K protein that indicate that this low-molecular-weight protein is located on the surface of the spirochete.

MATERIALS AND METHODS

Strains. *B. burgdorferi* DN 127, DN 127 cl 9-2, SON 188, SON 2110, SON 328, SON 335, and MEN 2523 were isolated from *I. pacificus* in California (13). *Borrelia burgdorferi* B-31 (ATCC 35210) was used as a control strain. See Table 2 for sources of strains of *B. hermsii*, *Leptospira* spp., and *Treponema pallidum*.

MAbs. The procedure for the production of MAb 86 DN-1 to the 25K protein band of strain DN 127 cl 9-2 was described previously (12). Briefly, the MAb was prepared by excising the band from a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel, electroluting the protein from the band, and inoculating the resultant material into BALB/c mice. Hybridomas were produced by fusion of spleen cells with P3x63-Ag8.653 myeloma cells. The hybridoma culture fluids were screened for specific antibody on day 14 of growth by a dot immunobinding assay. The antibody class was determined by immunodiffusion to be immunoglobulin G3.

The MAb to OspA protein (H5332) (10) and that to periplasmic flagella (H9724) (6) were kindly supplied by Alan Barbour.

IFA test. Suspensions of organisms for indirect immunofluorescent-antibody (IFA) tests were prepared as described previously (13), with one exception. It was necessary to use 0.5% Formalin in phosphate-buffered saline (PBS) (PBS-F) in place of M/15 PBS with 5 mM magnesium chloride to wash the spirochetes before they were fixed to slides to obtain satisfactory IFA test results with MAb 86 DN-1. The adjusted suspension of formalinized spirochetes was applied to slides, air dried, fixed for 10 min in 10% methanol, air dried, and stored frozen at −70°C until use. If the suspensions of formalinized spirochetes were stored for more than 24 h at 3°C before preparation of the slides, the surface of stained spirochetes became distorted and "ragged." Approximately 500 to 700 spirochetes were screened to determine the percentage of organisms that reacted with each MAb in the IFA tests.

Sodium dodecyl sulfate-PAGE. The procedure used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (13). Low-molecular-weight or prestained standards (Bio-Rad Laboratories, Richmond, Calif.) were run with each gel. Water (Milli-Q water) that was used in all reagents and procedures was processed through a water system (Milli-Q; Millipore Corp., Bedford, Mass.).

Western blots. Procedures for the Western blots (WBs)

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(immunoblots) were performed as described previously (12, 13). The protein antigen-MAb test was performed at room temperature, as follows. First, the blot was immersed in Tween-PBS and incubated for 30 min. The blot was incubated for 1 h in a dilution of MAb prepared in Tween-PBS. Determinations of dilutions for MAbs and conjugates were made by titration on blots (data not shown). After three washings with Tween-PBS (10 min per wash), the blot was incubated for 30 min with a 1:4,000 dilution of horseradish peroxidase-protein A conjugate (Bio-Rad) prepared in PBS. After three washings with PBS (10 min per wash), the blot was incubated for 15 min in a peroxidase substrate solution for color development. The blot was washed in Milli-Q water for 10 min and allowed to air dry. All incubations and washings were performed with gentle agitation.

Protease treatment of spirochetes. The procedure used for protease treatment was a combination of procedures described by Barbour et al. (9) and Benach et al. (11). A BSK II culture of DN 127 cl 9-2 passage 4 was centrifuged and washed once in PBS-MgCl, and the cells were suspended in 0.1 ml of Milli-Q water. A protein determination (14) was performed; the cell suspension was diluted in PBS to a concentration of 1.28 mg of protein per ml, and this suspension was used in the protease treatment. To 425 μl of the cell suspension in each of four microfuge tubes, 25 μl of one of the following was added: PBS, pH 7.2 (control); 10^{-3} M hydrochloric acid (control); proteinase K (8 mg/ml) (Sigma Chemical Co., St. Louis, Mo.); or trypsin (1 mg in 10^{-3} HCl; Sigma). The tube containing proteinase K was incubated at 53°C for 2 h. The tubes containing PBS, HCl, and trypsin were incubated at 37°C for 2 h. During incubation, all the tubes were gently vortexed every 10 min. After incubation, 10 μl of phenylmethylsulfonyl fluoride (50 mg/ml in methanol), a protease inhibitor, was added to all the tubes. The cell suspensions were centrifuged at 7,000 × *g* for 15 min in a Microfuge 12 (Beckman Instruments, Inc., Palo Alto, Calif.) and washed twice with PBS-MgCl. The cells were suspended in 425 μl of PBS-MgCl, and a 50-μl volume was removed for IFA smears. The spirochete suspension was centrifuged, and the pellet was suspended in 375 μl of Milli-Q water and 187 μl of sample buffer containing 70 μl of phenylmethylsulfonyl fluoride per ml. This suspension was used as the antigen for the sodium dodecyl sulfate-PAGE.

Immune electron microscopy. A 1.0-ml volume of a BSK II culture of DN 127 cl 9-2 was centrifuged at 7,000 × *g* for 5 min in a Microfuge 12, washed once with PBS-MgCl, and suspended in 0.1 ml of PBS-MgCl. Then, 0.1 ml of 10% Formalin in PBS-MgCl was added to make a final concentration of 5% Formalin. The suspension was allowed to stand for 1 h at room temperature. A copper grid (carbon-coated Formvar on 400-mesh grids) was placed on a 30-μl drop of the formalinized suspension and allowed to absorb for 30 min at room temperature and then gently washed in PBS-MgCl by sequential transfers into three large drops of PBS-MgCl. The washed grid was transferred onto a 30-μl drop of undiluted MAb 86 DN-1 and incubated for 30 min at room temperature. The grid and the drop of MAb were gently flooded with PBS-MgCl and then washed as described above. The washed grid was transferred onto a 25-μl drop of undiluted protein A coated with 15-nm-diameter particles of colloidal gold (protein A 15; Auroprobe EM; Janssen Life Sciences, Redding, Calif.) and incubated for 30 min at room temperature. The grid was washed as described above with Milli-Q water instead of PBS-MgCl and stained with 3% phosphotungstic acid for electron microscopic examination.

TABLE 1. IFA and WB reactions of MAb 86 DN-1 against six strains of *B. burgdorferi* isolated from ticks in California

Strain	No. of passages	IFA		WB[a]
		% of organisms reacting	Reaction[b]	
B-31[c]	Many		−	−
DN 127 cl 9-2	4	100	4+	+
	38	100	4+	+
SON 188	6	20	3+	+
	21	<1	3+	−
SON 2110	5	20	3+	+
	64	10	4+	+
SON 328	7	100	4+	+
	63	5	4+	+
SON 335	7	50	4+	+
	62	<1	4+	−
MEN 2523	8	90	2+	+
	40	<1	3+	−

[a] Symbols: −, no binding of antibody detected; +, appearance of bands indicating binding of antibody.
[b] Symbols: −, negative; +, positive (4+, 3+, and 2+ fluorescence).
[c] Control strain B-31 is the type strain of *B. burgdorferi*.

RESULTS

MAb. The 86 DN-1 MAb reacted in IFA and WB analyses with the DN 127 and DN 127 cl 9-2 strains and with five other strains of *B. burgdorferi* isolated from adult *I. pacificus* ticks that were collected in California (Table 1). The number of passages of the organisms in BSK II medium appeared to affect the level of reactivity of some strains to the 86 DN-1 MAb. MAb 86 DN-1 did not react with strains of *B. hermsii*, *Leptospira* spp., or *T. pallidum* (Table 2).

Location of the 25K protein on the spirochete. A major protein band of DN 127 cl 9-2 with an M_r of 25,000 reacted with the 86 DN-1 MAb in WBs (Fig. 1), and the spirochetes were brightly fluorescent in IFA tests. When IFA tests were performed without prior washing of DN 127 cl 9-2 with

TABLE 2. Sources of the *B. hermsii*, *Leptospira* spp., and *T. pallidum* strains used in this study[a]

Strain	Source[b]
Borrelia hermsii HS1	ATCC 35209
Borrelia hermsii MAN-1	Patient isolate
Borrelia hermsii CON-1	Patient isolate
Borrelia hermsii YOR-1	Patient isolate
Leptospira interrogans serotype pomona	CDC
Leptospira interrogans serotype canicola	CDC
Leptospira interrogans serotype icterohemorrhagiae	CDC
Leptospira interrogans serotype andamona	CDC
Leptospira interrogans serotype sejroe	CDC
Leptospira interrogans serotype patoc	CDC
Leptospira interrogans serotype grippotyphosa	WR
Leptospira interrogans serotype autumnalis	WR
Treponema pallidum	ZS

[a] None of the strains fluoresced by the IFA test.
[b] Sources: Patient isolates, *B. hermsii* isolated from blood of patients with relapsing fever in California; CDC, Centers for Disease Control, Atlanta, Ga.; WR, Walter Reed Hospital, Washington, D.C.; ZS, Zeus Scientific Inc., Raritan, N.J.

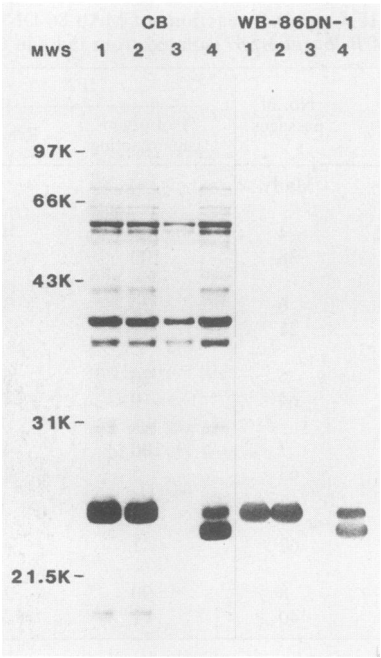

FIG. 1. Coomassie blue-stained polyacrylamide gel and WB (probed with MAb 86 DN-1) analyses of proteinase K- and trypsin-treated intact cells of DN 127 cl 9-2. Lanes 1, PBS-treated cell control; lanes 2, HCl-treated cell control; lanes 3, proteinase K-treated cells; lanes 4, trypsin-treated cells. The M_rs (10^3 [K]) of the molecular weight standards (MWS) are indicated on the left.

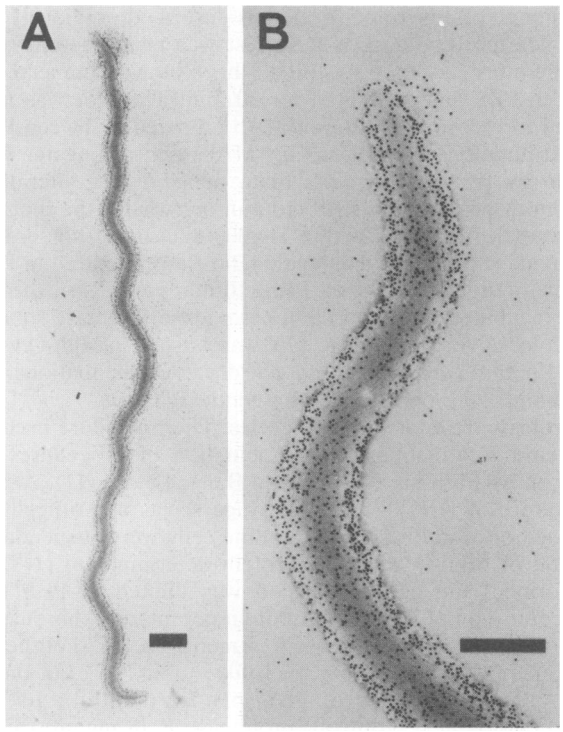

FIG. 2. Negatively stained *B. burgdorferi* DN 127 cl 9-2 tagged with MAb 86 DN-1 and gold-labeled anti-protein A antibody. (A) Bar, 0.5 μm; (B) bar, 1.0 μm.

PBS-F, fluorescence occurred over the entire area where the organisms had been fixed. The spirochetes, which were washed in PBS-F prior to fixation, fluoresced specifically in IFA tests with MAb 86 DN-1. The fluorescence was bright (4+) and occurred over the entire surface of individual organisms, or in some organisms, the fluorescence was intermittent, giving a beaded effect. Occasionally, there was a fluorescent haze surrounding the brightly fluorescent spirochetes, suggesting the loss of specific antigen from the surface of the organism. Enzyme experiments were performed to determine whether the antigen reacting with MAb 86 DN-1 was susceptible to the action of proteases. Intact DN 127 cl 9-2 spirochetes were treated with proteinase K and trypsin. After the reaction was halted with phenylmethylsulfonyl fluoride, antigens for PAGE and smears for IFA tests were prepared from the suspension of enzyme-treated and untreated spirochetes. Proteinase K treatment removed the 25K protein band in the gel, and no reaction with this band was observed in WB analyses with MAb 86 DN-1 (Fig. 1). The 25K protein was incompletely digested by trypsin treatment. The protein was clipped into two observable bands, both of which reacted with MAb 86 DN-1 in the WB. The smaller cleavage product of this digestion probably migrated off the end of the gel. No change was observed in either PAGE or WB analyses of untreated or HCl-treated DN 127 cl 9-2 spirochetes. IFA test results with MAb 86 DN-1 were similar, producing fluorescence of the untreated and HCl- and trypsin-treated spirochetes and no fluorescence of proteinase K-treated spirochetes.

Immune electron microscopy. Figures 2A and B illustrate the attachment of the colloidal gold complex onto the surface of spirochetes which were exposed to MAb 86 DN-1. It was necessary to treat the organisms with 5% Formalin prior to exposure to the antibody-protein A-colloidal gold complex to maintain the determinants on the surface of the spirochetes. Extreme care in the washing procedure was also necessary. Less vigorous washing procedures were achieved by attaching the Formalin-treated spirochetes to grids prior to exposure to the MAb and the protein A-colloidal gold complex. When these precautions were observed, the colloidal gold complex attached homogeneously to the surface of the spirochetes, suggesting that the 86 DN-1 MAb reacted with determinants which were associated with a loose outer surface membrane of the spirochetes.

Effects of passage in BSK II medium. Passage of strain DN 127 cl 9-2 in BSK II medium appeared to affect the presence and quantity of the protein detected by OspA MAb, as was noted previously with other strains (9, 18, 20). To study this effect of transfer further, strain DN 127 cl 9-2 was passaged weekly for a period of over 50 weeks. No change was observed in the quantity or the reactivity of the 25K protein with the 86 DN-1 MAb at any passage level (Fig. 3). However, there was a change in the amount of protein detected by the OspA MAb H5332. There was a faint band observable in the 32K protein region of the WB at the third passage. The reactive protein increased at passage 8 and remained the same at passages 14 and 27, but at passages 45 and 50, the quantity diminished to the amount observed at 3 weeks. Results of the IFA test showed the same pattern (Table 3).

Studies of the effect of passage on other strains of tick isolates which exhibited reactivity with the 86 DN-1 MAb showed variability in the amount of low-molecular-weight protein detected (Table 1). In the WB analyses with the 86 DN-1 MAb, some strains at high passage levels (SON 188, SON 335, and MEN 2523) apparently lost detectable low-molecular-weight protein (Fig. 4). Strain SON 328 showed a

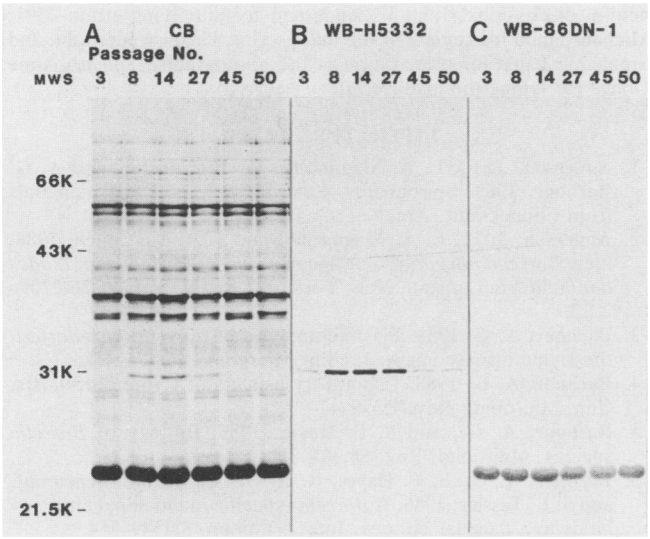

FIG. 3. Coomassie blue-stained polyacrylamide gel and WB with MAb H5332 and 86 DN-1 of weekly passages of strain DN 127 cl 9-2 in BSK II medium. The passage numbers across the top indicate the number of weekly passages. (A) Coomassie blue-stained PAGE results at each passage level. (B) WB with MAb H5322 bound to the 31K protein. (C) WB with MAb 86 DN-1 bound to the 25K protein. Molecular weight standards (MWS; 10^3 [K]) are indicated on the left.

much diminished reaction at passage 63, while strain SON 2110 appeared to have essentially the same reaction at passage 64 as it did at passage 5. All five of the strains showed decreased percentages of organisms reacting with MAb 86 DN-1 in IFA tests of strains at high passage levels (Table 1).

DISCUSSION

Characterization of surface proteins of disease-causing organisms contribute to the understanding of the epidemiology and pathogenesis of disease. These proteins have been shown to play an important role in the invasive, virulent, and antigenic properties of organisms (15). The study of surface proteins of *Borrelia* spp. have been important in elucidating the pathogenesis of relapsing fever and have contributed to the characterization of the etiologic agent of Lyme disease, *B. burgdorferi* (for a review, see reference 5).

In 1987 we reported the isolation of a strain of *B. burgdorferi* that did not react with MAbs to OspA (outer surface protein with an M_r of 31,000) or OspB (M_r, 34,000) but did possess a major low-molecular-weight protein of 25,000 (13).

TABLE 3. Effect of passage of DN 127 cl 9-2 on reactivity of FTA tests by using OspA MAb H5332

DN 127 cl 9-2 passage no.	% of organisms that reacted	Level of reactivity[a]
3	5	3+
8	10	3+
14	50	2+
27	50	3+
45	0	−
50	0	−

[a] Levels of fluorescence: 3+, brightly fluorescing organisms; 2+, fluorescing organisms; −, no fluorescence.

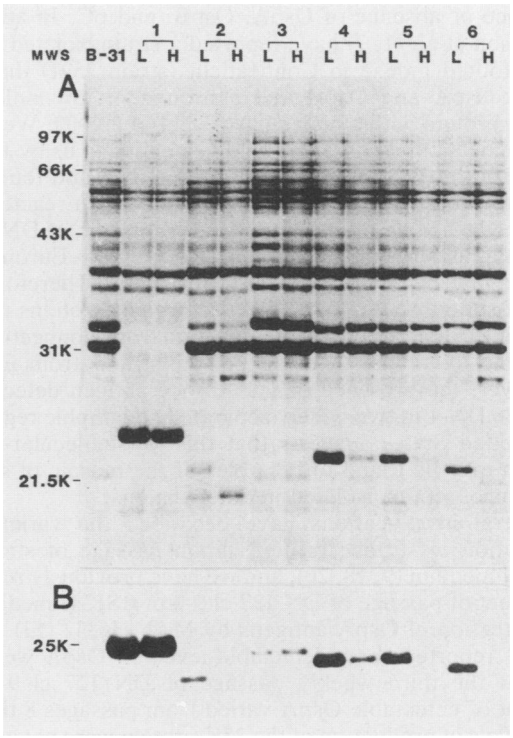

FIG. 4. (A) Coomassie blue-stained polyacrylamide gel of whole-cell lysates of six strains of *B. burgdorferi* from California at low (L) and high (H) number of passages in BSK II medium and control strain B-31. The isolates and number of passages (Tr) are as follows: lane 1, DN 127 cl 9-2; L Tr, 4; H Tr, 38; lane 2, SON 188; L Tr, 6; H Tr, 21; lane 3, SON 2110; L Tr, 5; H Tr, 64; lane 4, SON 328; L Tr, 7; H Tr, 63; lane 5, SON 335; L Tr, 7; H Tr, 62; lane 6, MEN 2523; L Tr, 8; H Tr, 40. (B) WB of the polyacrylamide gel shown in panel A probed with 86 DN-1 monoclonal antibody. Positions of the molecular weight standards and of the 25K protein are indicated on the left.

A MAb (86 DN-1) was prepared to the 25K protein and was used in studies to characterize this protein.

Enzyme studies on intact spirochetes of the strain showed that the low-molecular-weight protein was removed by the action of proteinase K and was clipped into two closely migrating antigens by the action of trypsin. Immune electron microscopy revealed that the protein was located on the outer surface of the organism (Fig. 2A and B). The need for extreme caution in the manipulation of the organisms prior to and during immune electron microscopic procedures, the requirement for Formalin fixation of the spirochete prior to IFA and immune electron microscopic tests, the apparent leaching of antigen that produced a haze of specific fluorescence around the spirochete in IFA tests, and the results of the enzyme and immune electron microscopic studies suggest that this protein is located on the outermost surface of the organism.

Of 11 *B. burgdorferi* isolates from California, 5 also possessed low-molecular-weight (20,000 to 24,000) proteins which bound MAb 86 DN-1 in WB and IFA tests (Table 1 and Fig. 4). Strains with similar prominent low-molecular-weight proteins have been reported from Europe (7, 20) and from Connecticut (2) and New York State (2). Wilske et al. (21) isolated strains in Europe with no detectable OspA or OspB proteins but with a major protein with a molecular weight of 20,000 to 24,000 (designated pC). A serotyping scheme was devised by those investigators based on the

presence or absence of OspA, OspB, and pC. In addition, Anderson et al. (1, 2) have reported a strain isolated from a white-footed mouse in Connecticut (strain 2591) that possessed OspA and OspB and a prominent low-molecular-weight protein with a molecular weight of 22,000. We tested the Connecticut strain 2591 (kindly supplied to us by John F. Anderson) in WB tests using MAb 86 DN-1 and found that the low-molecular-weight protein of this strain reacted with the 86 DN-1 MAb prepared to the 25K protein of DN 127 cl 9-2 (data not shown). The strains with pC from Europe have not been tested with the 86 DN-1 MAb. Therefore, we cannot state that the low-molecular-weight proteins present in strains from California and the strain from Connecticut are antigenically similar to the pC of the strains from Europe. However, the presence of the surface antigen detected by MAb 86 DN-1 in two widely separated geographic regions of the United States suggests that this low-molecular-weight protein may be found to be a part of the mosaic of surface proteins of strains isolated in other regions.

Several investigations have described the variation in production of surface proteins upon passage of strains in BSK II medium (9, 18, 20), and we have previously reported the effect of passage of DN 127 cl 9-2 in BSK II medium on the detection of OspA antigens by MAb H5332 (13). In the studies reported here, detectable levels of OspA were evident at the third weekly passage of DN 127 cl 9-2; the amount of detectable OspA varied from passages 8 through 50. Levels of production of the 25K protein were not affected by passage and remained remarkedly stable throughout the 50 weekly passages. This was not found in studies of low and high passage levels of the other strains from California that produced proteins that were reactive with MAb 86 DN-1 (Table 1 and Fig. 4). The amount and number of organisms producing low-molecular-weight proteins detected in WB and IFA tests with MAb 86 DN-1 decreased at high passage levels. In other studies, both the gain and loss of surface protein after passage in vitro has been reported (9, 13, 18, 20). Loss of infectivity in laboratory animals and loss of plasmids from the organism are associated with continuous passage of the organism in culture (3, 16, 17, 19). It is not known whether the events that occur during prolonged cultivation of *B. burgdorferi* also occur in the infectious process of Lyme disease. Further studies with a suitable animal model for this disease may provide the answer to this question.

In this study we attempted to characterize further an unusual isolate of *B. burgdorferi* and showed that a low-molecular-weight protein produced by this strain is located on the surface of the organism. The variability of antigen production in different strains of *B. burgdorferi* may be indicative of a method of escaping the response of the patient's immune system, similar to that observed in the spirochete that causes relapsing fever. Studies of strains from other geographic locations to assess the presence and variability of production of the low-molecular-weight protein detected by MAb 86 DN-1 should assist investigators in determining the importance of this surface protein in the epidemiology and pathogenesis of Lyme disease.

ACKNOWLEDGMENTS

We acknowledge and thank Will Probert for preparation of the 86 DN-1 MAb, Paul Duffey and June Kani for propagation of the clone, Alan Barbour for supplying other MAbs, Mary Struthers and Melinda Daugherty for expert technical assistance, Robert Lane for helpful discussions, John F. Anderson for supplying strain 2591, Michael Janda for review of the manuscript, Hazel Anderholm and Stephanie Paula for preparation of the photographs, and Lu-Anne Dodge for typing the manuscript.

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