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Variation in a Major Surface Protein of Lyme Disease Spirochetes

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Two monoclonal antibodies (H6831 and H5TS) differed in their indirect immunofluorescence reactivity when tested against 14 strains of Lyme disease spirochetes. Strains were bound by both antibodies, by H6831 or H5TS alone, or by neither. Western blot and immunoprecipitation studies revealed that the determinants of both antibodies were associated with abundant proteins with apparent subunit molecular weights of ca. 34,000 (34K-range proteins). The following results indicated that the 34K-range proteins were exposed on the surface of the spirochetes. (i) Antibody H6831 agglutinated the spirochetes; (ii) immune electron microscopy showed that the H6831 determinant was associated with the outer membrane; (iii) radiolabeled H6831 bound to live organisms; and (iv) proteases effectively removed the 34K-range proteins from intact cells. With their demonstrated variability and exposure on the surface, the 34K-range proteins may contribute to the serotype specificity of Lyme disease spirochetes.

A spirochete which was first discovered in *Ixodes dammini* ticks (12) is the etiological agent of Lyme disease (LD) (8, 9, 21) and probably of the related disorders erythema chronicum migrans (1, 13; K. Weber, G. Schierz, B. Wilske, and V. Preac-Mursic, Yale J. Biol. Med., in press) and tick-associated meningoradiculitis (Bannwarth's syndrome) (1, 17, 18) as well. The LD complex of syndromes has been reported from the continents of Europe, North America, and Australia (2, 22, 23). These LD spirochetes have to date been isolated from ticks, mammals, and humans in the United States (3, 8–10, 12, 21) and from ticks collected in areas of Europe where erythema chronicum migrans and tick-associated meningoradiculitis are endemic (5; R. Ackerman and B. Sköldenberg, personal communications).

Although these spirochete isolates have shown considerable homogeneity in their polyacrylamide gel electrophoresis (PAGE) protein profiles (5, 7) and in the reactivity of a common 31,000-molecular-weight protein with a monoclonal antibody (H5332 [7]), there also were indications of significant differences between strains. One characteristic that seemed to distinguish strains was the electrophoretic migration of proteins with apparent molecular weights (M_rs) of 34,000 to 35,000 (34K to 35K proteins) (5, 7). Although members of this family of proteins were abundant in whole-cell lysates, as determined by Coomassie brilliant blue staining of gels (5, 7), Western blot analyses of sera showed that few patients with LD had detectable antibody to the 34K protein of strain B31 of LD spirochetes (4; A. G. Barbour 1, Yale J. Biol. Med., in press). These findings suggested to us that the proteins in the 34,000-M_r range may confer some degree of serotype specificity to LD spirochetes (Barbour 1, in press).

We now report a closer examination of the 34K class of proteins. Using monoclonal antibodies, we found that these proteins are exposed on the surface of the spirochetes and that LD spirochetes can differ in their expression of antigenic determinants associated with these proteins.

MATERIALS AND METHODS

Organisms and culture conditions. The sources for 7 (strains B31, IRS, HB4, 50-2, 39/40, 2535, and HB19) of the 14 LD spirochete isolates studied were described previously (7). Strains B31 (ATCC 35210) and HB19 were cloned by limiting dilution (5). We also examined the original Shelter Island LD spirochete isolate (IDS [12]) which was frozen (6) before strain B31 was subsequently derived from it (5). The IDS strain was passed only three times before being harvested for this study.

The six remaining isolates were TLO-017, which was isolated by the Centers for Disease Control from *Ixodes dammini* ticks collected in New Jersey and provided to us by George Schmid, Centers for Disease Control, Atlanta, Ga.; TLO-030 and TLO-031, LD spirochetes isolated from a cerebrospinal fluid sample and a skin sample, respectively, from LD patients in Connecticut (21); SK1 and SK15, which were isolated from biopsied skin samples from patients with erythema chronicum migrans in New York and provided by Mark Kaplan, North Shore University Hospital, Manhasset, N.Y.; and G25, a spirochete isolated from *Ixodes ricinus* ticks in Sweden and provided by Birgit Sköldenberg, University Clinic for Infectious Diseases, Danderyd Hospital, Danderyd, Sweden. The 14 strains were bound by monoclonal antibody H5332 (7) in indirect immunofluorescence assays (IFA; see below).

The LD spirochetes were grown in BSK II medium (A. G. Barbour 2, Yale J. Biol. Med., in press) under conditions described previously (7). BSK II medium is BSK medium (5) modified by the addition of Yeastolate (Difco Laboratories, Detroit, Mich.) at a final concentration of 0.2% and by the replacement of 10X CMRL 1066 with glutamine (GIBCO Laboratories, Grand Island, N.Y.) by 10X CMRL 1066 without glutamine (GIBCO Laboratories).

The other spirochete genera and species used in this study, with the exception of the *Leptospira* strains, and the growth conditions used for them have been described previously (7). *Borrelia hermsii* HS1 serotype C (ATCC 35209) was used several times in the study and was also grown in BSK II medium. The *Leptospira interrogans* serotypes *icterohaemorrhagiae*, *canicola*, and *pomona* were provided by Russell Johnson, University of Minnesota, Minneapolis.

The technique for enumeration of spirochetes in culture or suspension and the procedures for harvesting and freezing cells were described previously (6). Medium components were removed from freshly harvested cells or from thawed cell preparations by centrifugation and two washes with 0.15

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M phosphate-buffered saline (PBS; pH 7.5)–5 mM $MgCl_2$ (PBS-Mg). In this procedure and in others described below, unless otherwise indicated, centrifugation was done for 3 min in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and cells were washed by suspension in 1 ml of the designated buffer.

IFA. All spirochetes were prepared for immunofluorescence as described previously (6, 7), except that PBS-washed sheep erythrocytes (50% [vol/vol] in fetal calf serum) replaced rat erythrocytes in the production of smears. The procedures for the IFA itself were also those previously described (6, 7).

Production of monoclonal antibodies. One monoclonal antibody, H6831, was obtained by immunizing BALB/c mice with strain B31 organisms and using the fusion, screening, cloning, and hybridoma expansion protocols described previously (6, 7). Additional hybridomas were generated in the same way, except that live strain HB19 organisms were injected intravenously into adult female New Zealand black mice on days 1, 20, and 65. Fusions of the mouse spleen cells with NS1 myeloma cells were performed on day 68. Hybridoma culture supernatants were screened by IFA (6, 7). Hybridomas were tested for their cloning adequacy and secreted immunoglobulin subtype by methods published previously (6).

Protein A-binding monoclonal antibodies were purified by passing hybridoma culture supernatants over a protein A–Sepharose CL-4B column (Sigma Chemical Co., St. Louis, Mo.) and elution with citrate buffer (15). The eluted immunoglobulins were then dialyzed against PBS (15). The protein concentration of the purified antibodies was adjusted to 0.35 mg/ml, as determined by the method of Bradford (11). Mouse immunoglobulin (Sigma) was used as the protein standard.

Purified monoclonal antibodies were radioiodinated in the presence of chloramine T to a specific activity of 4 to 5 μCi/μg (15).

Immune electron microscopy. Complexes of protein A and colloidal gold were prepared as described previously (7). Cultures of spirochetes in late exponential phase were centrifuged for 5 min in a Microfuge, and the pellets were suspended in a 1/10 volume of PBS. The cell suspension (3 ml per grid) was placed on carbon-coated, Parlodion-filmed, 300-mesh copper grids. The spirochetes were allowed to adsorb to the carbon-coated films for 30 min at room temperature. Excess fluid was removed with a micropipette, and the grids were washed with PBS. The grids were then overlaid with 3 μl of hybridoma supernatant and incubated at room temperature for 30 min. The fluid was removed, and the grids were again washed with PBS. A suspension of protein A-colloidal gold complexes in PBS with 0.1 mg of polyethylene glycol (molecular weight, 20,000) per ml was applied in a 3-μl volume and allowed to react with bound antibodies at room temperature for 30 min. After the excess fluid was removed, the grids were rapidly rinsed with distilled water and then negatively stained for 20 s with 2% ammonium molybdate, pH 6.5. The stained samples were then air dried and examined with a Hitachi 11E-1 electron microscope. Images were recorded on Kodak SO-163 film (Eastman Kodak Co., Rochester, N.Y.) at 75 kV.

Agglutination assay. Freshly harvested spirochetes were suspended at a concentration of ca. 2×10^9 cells per ml in buffer B (BSK medium without gelatin, Neopeptone, and, except for glucose and the inorganic salts, any of the components of CMRL 1066). The spirochete suspension (100 μl) and 100 μl of purified monoclonal antibody, which had been twofold serially diluted in buffer B, were added to polyvinyl chloride microtiter plates with V-shaped wells. The plates were incubated at 4°C and examined after 2 h for agglutination at the bottom of the wells.

Binding of labeled antibodies to spirochetes. Radioiodinated monoclonal antibodies were mixed with unlabeled antibody to give specific activities of 1 μCi/μg. These preparations were in turn diluted in PBS with 1% bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) to give 5×10^6 cpm/ml. Cultures of spirochetes in the late exponential phase (ca. 10^8 cells per ml) were distributed in 850-μl volumes to 1.5-ml Microfuge tubes. Unlabeled antibody (100 μl [35 μg of protein]) was added to the culture fluids. The spirochetes and the unlabeled antibody were incubated together for 10 min at room temperature. Labeled antibody (50 μl) was added, and incubation was continued for another 10 min. A 50-μl volume of the sheep erythrocyte suspension was added to each tube, and 200 μl of the spirochete-erythrocyte suspension was dispensed to each of four 300-μl Microfuge tubes. Three tubes were centrifuged in a Microfuge for 4 min. After the supernatants had been aspirated by vacuum, the radioactivity in each pellet and in the uncentrifuged suspension was counted in a gamma counter (Beckman).

Protease treatment of spirochetes. Freshly harvested spirochetes were centrifuged and washed once with PBS-Mg. They were resuspended in this buffer at a concentration of ca. 2×10^9 cells per ml. To 950 μl of the cell suspension in a Microfuge tube was added one of the following: 50 μl of distilled water, 10^{-3} M HCl, proteinase K (4 mg/ml in water; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), or trypsin (1 mg/ml in 10^{-3} M HCl; Sigma). After the protease-treated and control solutions had been incubated for 40 min at 20°C, 10 μl of phenylmethylsulfonyl fluoride (PMSF; 50 mg of isopropanol per ml of PMSF; Sigma) was added to all tubes. The cells were centrifuged and washed twice with PBS-Mg. After the second suspension, a 50-μl volume was removed and mixed with an equal volume of the sheep erythrocyte suspension. Smears for IFA were made with this preparation. The remaining 950 μl of the spirochete suspension was centrifuged, and the pellet was suspended in PAGE sample buffer (see below) containing, in addition, 70 μg of PMSF per ml.

PAGE and Western blotting. Spirochete lysates and other samples were subjected to PAGE under conditions previously described (6, 7). Except where indicated, DL-dithiothreitol (50 mM final concentration; Sigma) was substituted for 2-mercaptoethanol in the sample.

The Western blot procedure was also essentially that previously described (7). In the present study, however, the solvent used for incubating all antibody and radioiodinated protein A preparations, as well as for the blocking step and all washes, was TSEA (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.05% sodium azide) with 0.05% Tween 20. When a second antibody was needed, the blots were incubated in a 1:400 dilution of the immunoglobulin G (IgG) fraction of rabbit anti-mouse immunoglobulin (Miles) in TSEA plus 0.05% Tween 20 for 1 h; they were then washed three times, and labeled protein A was applied (6).

Immunoprecipitation. Frozen spirochetes (1 ml; ca. 5×10^9 cells) were thawed, placed in a Microfuge tube, centrifuged, washed twice with PBS-Mg, and, after the last centrifugation, lysed by suspension in 1 ml of TSEA with 1% Nonidet P-40 and 50 μg of PMSF per ml and subsequent incubation in a 37°C water bath. The lysate was centrifuged to remove large particles. The resultant Microfuge supernatant was in turn centrifuged in an Airfuge (Beckman) at

TABLE 1. IFA reactions

Strain	Reaction with monoclonal antibody:	
	H6831	H5TS
IDS	+	+
B31	+	−
IRS	+	+
HB4	+	+
50-2	+	+
SK1	+	+
39/40	−	+
2535	−	+
HB19	−	+
TLO-031	−	+
TLO-017	−	+
SK15	−	+
TLO-030	−	−
G25	−	−

148,000 × *g* for 30 min. To 200 μl of the final supernatant was added 60 μl (21 μg) of purified monoclonal antibody in PBS-bovine serum albumin. The reaction mixture was incubated for 1 h at 37°C. Antibodies and any antigen-antibody complexes were precipitated by the addition of 70 μl of a 10% suspension of Formalin-fixed cells of *Staphylococcus aureus* Cowan (7). After a 45-min incubation at room temperature, the immunoabsorbent cells were pelleted by centrifugation and washed three times with TSEA plus 0.05% Tween 20. The final pellet was suspended in PAGE sample buffer, boiled for 5 min, and subjected to electrophoresis. After separation of the immunoglobulin chains and any immunoprecipitated components in the gel, Western blotting was performed.

RESULTS

Monoclonal antibodies. One monoclonal antibody, H6831, an IgG2A, was the eventual product of a fusion of BALB/c mouse spleen cells with myeloma cells. This antibody reacted by IFA with some strains of LD spirochetes but not others. H6831 bound to protein A–Sepharose CL-4B columns and was removed from the culture medium by affinity chromatography.

A hybridoma that produced a second antibody with differential strain specificity was subsequently isolated from a fusion cell line incorporating New Zealand black mouse spleen cells. This second antibody, H5TS, an IgG2B, had little affinity for protein A. Therefore, in the detection of H5TS in Western blots, a rabbit anti-mouse immunoglobulin serum was used before protein A was applied.

Monoclonal antibodies H6831 and H5TS were used in the expanded studies of strain specificities and in the experiments characterizing their determinants. A third monoclonal antibody, H4825, an IgG2A, was used in these experiments as a control. This antibody was isolated as part of a study of surface components of *B. hermsii* and was found to have specificity for an outer membrane protein of this organism (A. G. Barbour, submitted for publication). Like H6831, H4825 and the previously described H5332 (7) bound to protein A and were recovered from hybridoma supernatants by affinity chromatography.

Neither H6831 nor H5TS bound, as assessed by IFA, to *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. recurrentis*, *Treponema pallidum*, *T. phagedenis*, or the three serotypes of *L. interrogans* examined.

H6831 and H5TS each reacted by IFA with several strains of LD spirochetes. However, the pattern of their specificities differed (Table 1). Of the 14 strains examined, 5 were recognized by both monoclonal antibodies, 6 were recognized by H5TS alone, 1 was recognized by H6831 alone, and 2 were recognized by neither. Four strains (B31, IDS, HB19, and TLO-030), which exemplified the four reaction patterns, were used to identify the antigenic determinants recognized by H6831 and H5TS.

PAGE and Western blotting. The Coomassie blue-stained proteins of strains B31, IDS, HB19, and TLO-030 and the Western blot reactions of H6831 and H5TS against those strains are shown in Fig. 1. The stained gel revealed that the 34K-range proteins of three strains (B31, IDS, and HB19) migrated with slight differences. The protein of strain B31 migrated in this and other gels consistently ahead of the protein of strain IDS. The M_r of the protein of strain HB19 appeared to be intermediate between that of strain B31 protein and that of strain IDS protein. Strain TLO-030 either lacked a 34K-range protein or had much less of it than did the other three strains.

The migration of the 34K-range proteins in Coomassie blue-stained gels was not altered by deletion of DL-dithiothreitol, addition of 2-mercaptoethanol in concentrations up to 10%, or reduction of the sample incubation temperature to 45 or 20°C.

The results of the Western blot analyses, in terms of positive or negative reaction, for the four strains were the same as those found by IFA (Table 1). The Western blots also helped to identify the antigenic determinants. For both

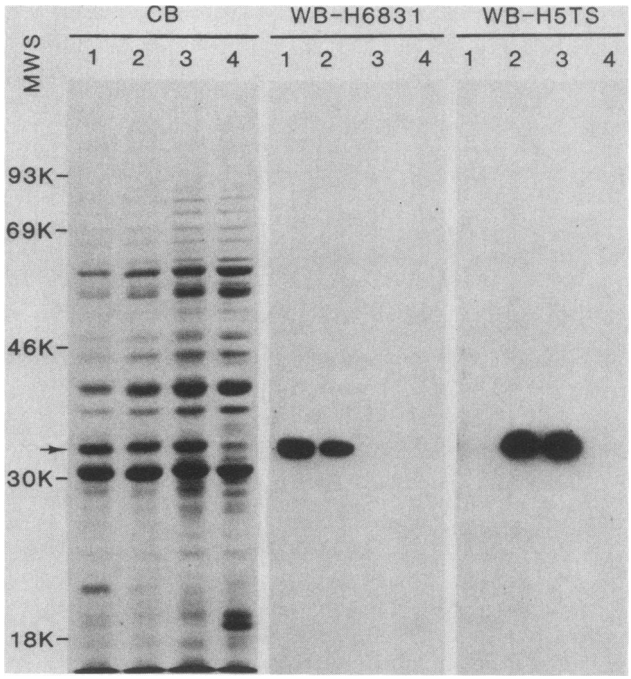

FIG. 1. Coomassie blue (CB)-stained proteins and Western blots (WB) of lysates of four LD spirochete isolates. Components were separated by PAGE and either stained with Coomassie blue (left) or transferred to nitrocellulose for Western blot analyses. The blots were reacted with monoclonal antibody H6831 (center) or H5TS (right); hybridoma supernatants were diluted 1:10. Bound antibody was detected with ^{125}I-protein A and subsequent radioautography. Isolates: lane 1, B31; lane 2, IDS; lane 3, HB19; and lane 4, TLO-030. The ^{14}C-labeled molecular weight standards (MWS) were phosphorylase *b* (93,000 [93K]), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K), and beta-lactoglobulin (18K) (New England Nuclear Corp., Boston, Mass.). The arrow indicates the position of the 34K-range proteins.

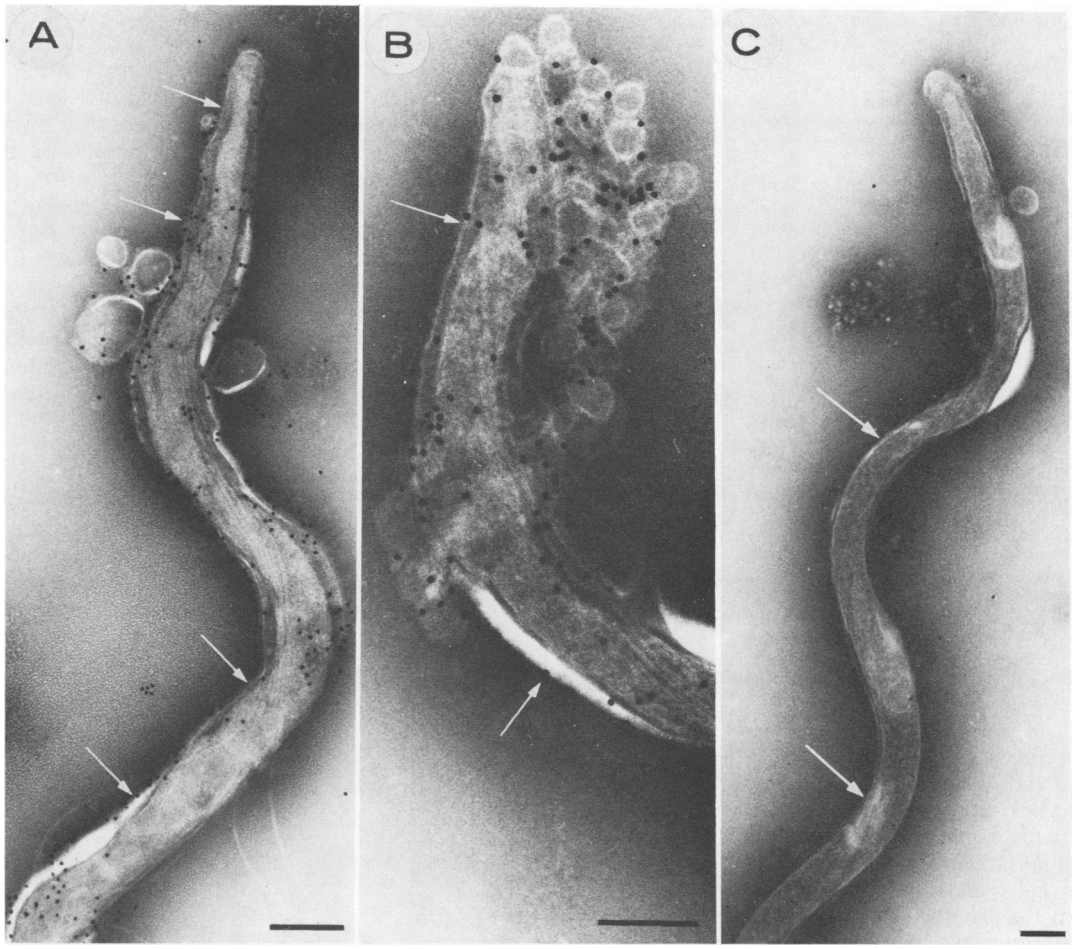

FIG. 2. Labeling of LD spirochete strain B31 with monoclonal antibody and protein A-coated colloidal gold. Arrows, Outer membrane; bars, 0.2 μm. The monoclonal antibody used was H6831 (A and B) or H4825 (C). Outer membrane blebs are seen in all frames but are prominent in B.

antibodies, the determinants were associated with components that had M_rs of ca. 34,000 as assessed by comparison of their migration with that of the molecular weight standards. When the nitrocellulose membranes were stained with amido black (24), the blot bands were seen to overlay those of the 34K-range proteins (data not shown). Western blots containing lysates of strains IRS, HB4, 50-2, 39/40, and 2535 showed the same pattern of reactivity as was found by IFA and confirmed that the reactive spirochete components had M_rs of ca. 34,000 (data not shown). As a control for both the binding of antibodies in the rabbit antiserum to spirochete components and the nonspecific binding of protein A to components, we used hybridoma culture fluid (6) without antibody for Western blots with and without rabbit antiserum. In the resultant radioautographs, there were only faint bands, similar to those demonstrated previously (6).

Studies of surface exposure of 34K-range proteins. Although the Western blot studies identified the likely proteins with which the determinants of the strain-specific antibodies were associated, we did not know the location of the proteins (and thus the determinants) in the cell. We used four techniques to determine whether the proteins were on the surface of the spirochetes. Each technique was designed to approach this question from a different view and to involve greater or lesser manipulation of the spirochete. The techniques were (i) an agglutination assay, (ii) immune electron microscopy with protein A-colloidal gold complexes used as the second ligand, (iii) binding of radiolabeled antibodies to live spirochetes, and (iv) protease treatment of intact spirochetes. Because these experiments were best performed with a concentrated monoclonal antibody that bound protein A, H6831 was used instead of H5TS.

(i) Agglutination assay. H6831 and H4825, the control antibody, were assayed for the ability to agglutinate strain B31 LD spirochetes and strain HS1 *B. hermsii* spirochetes. The minimum concentration of antibody which produced macroagglutination of B31 cells was 2.8 μg of H6831 per ml of buffer B. H4825 did not agglutinate B31 cells at a concentration of ≤175 μg/ml, but it did agglutinate *B. hermsii* cells at 1.4 μg/ml. H6831 did not agglutinate *B. hermsii* cells at ≤175 μg/ml.

(ii) Immune electron microscopy. To minimize the number of cell washes and to eliminate prior fixation of the spirochetes, we performed all antibody and protein A-colloidal gold complex incubations while the spirochetes were attached to grids. The electron photomicrographs (Fig. 2A and B) demonstrate the binding of H6831 to strain B31 organisms. The determinants appeared to be associated with the outer membrane. In the center frame, the gold particles are seen to be attached to the prominent outer membrane blebs at the end of the cell. The reduced binding of gold complexes, and thus by inference of H4825, to B31 cells is shown in

TABLE 2. Binding of radioiodinated monoclonal antibodies to live spirochetes

^{125}I-labeled antibody	Excess unlabeled antibody	Spirochete strain[a]	Labeled antibody bound (cpm ± SEM)[b]	Net binding[c] (cpm)
H6831	H4825	B31	5,307 ± 157	4,175
		HS1	1,113 ± 56	−19
		None	1,132 ± 91	
	H6831	B31	2,507 ± 167	1,311
		HS1	1,205 ± 40	9
		None	1,196 ± 49	
H4825	H6831	B31	1,060 ± 46	8
		HS1	46,190 ± 931	45,138
		None	1,052 ± 9	
	H4825	B31	1,119 ± 124	−24
		HS1	13,614 ± 148	12,471
		None	1,143 ± 70	

[a] Spirochetes were either Lyme disease spirochete strain B31 or *B. hermsii* HS1 grown in BSK medium. None, Medium alone was used.

[b] Bound to spirochetes in pellet after centrifugation. Values are means of three determinations.

[c] Mean counts per minute in pellets without spirochetes were subtracted from the counts per minute in pellets with strain B31 or *B. hermsii* spirochetes for each labeled-unlabeled antibody pair.

Fig. 2C. In similar experiments, we found that H6831 did not bind to *B. hermsii* cells.

(iii) Binding of radiolabeled antibody to live spirochetes. To eliminate any washing or centrifugation steps before the application of antibodies, we used radioiodinated H6831 and H4825 antibodies and measured the amount of each that bound to spirochetes in the culture medium. The specificities of the reactions were assessed by using, in addition to heterologous cells and antibodies, unlabeled antibody to compete with the labeled ligands for binding sites. In the assay, sheep erythrocytes were added to produce a readily detectable pellet for the aspiration step. The results of a representative experiment are given in Table 2. H6831 bound to B31 cells but not to *B. hermsii* spirochetes; the opposite results were obtained with labeled H4825. Unlabeled heterologous antibody did not compete with labeled homologous antibody for binding sites, but unlabeled homologous antibody reduced the binding of both H6831 and H4825 by about 70%.

(iv) Protease treatment of intact spirochetes. Strain B31 and HB19 spirochetes were treated with trypsin or proteinase K. Proteolysis was stopped with PMSF, and the cells were washed before IFA, PAGE, or Western blot analyses were made. The IFA results for H6831, H5TS, and H5332 with protease-treated and untreated cells are given in Table 3. We found that IFA and Western blot (see below) reactions with the untreated cells were the same irrespective of whether water or dilute HCl was added to the cells. The effects of in situ proteolysis by trypsin of the 34K protein and proteolysis by proteinase K of both the 34K and 31K proteins of HB19 cells are shown by the PAGE analysis in Fig. 3. The same PAGE results were obtained with protease-treated B31 cells (data not shown). A minor protein with an M_r of 66,000, (called component 8 in reference 4), was also affected by the proteases. Western blot analyses demonstrated, as did IFA (Table 3), that whereas the determinants recognized by H6831 and H5TS were eliminated by both trypsin and proteinase K, the determinant recognized by H5332 was apparently only affected by proteinase K at the given concentration (Fig. 4).

TABLE 3. IFA reactions of monoclonal antibodies H6831, H5TS, and H5332 against protease-treated spirochetes

Strain	Protease	Reaction with monoclonal antibody:		
		H6831	H5TS	H5332
B31	None	+	−	+
	Trypsin	−	−	+
	Proteinase K	−	−	−
HB19	None	−	+	+
	Trypsin	−	−	+
	Proteinase K	−	−	−

Immunoprecipitation. The preceding experiments indicated that the strain-specific monoclonal antibodies bound to 34K-range proteins that were surface exposed and protease susceptible. But with a strain such as IDS that was bound by both antibodies, were the two determinants on or associated with the same protein or, alternatively, were there perhaps two 34K proteins present either on the same cell or on different cells in a mixed population? To resolve this question, we immunoprecipitated the detergent-soluble components of IDS with one monoclonal antibody, H6831, and used the immunoprecipitated material for Western blots with

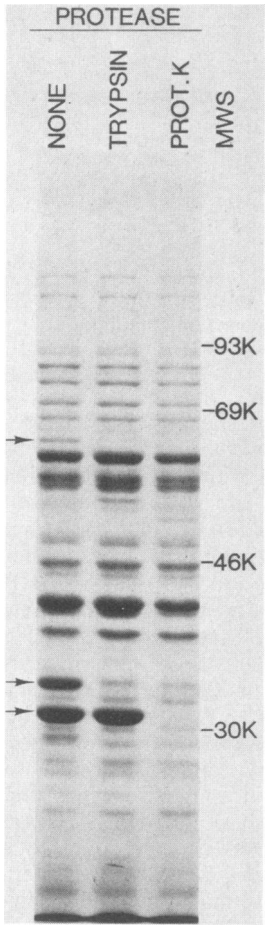

FIG. 3. Effect of proteases on LD spirochete HB19. Whole cells were incubated with buffer alone, trypsin (50 μg/ml), or proteinase K (200 μg/ml). Reactions were stopped with PMSF, and the cells were washed before PAGE. The gel was stained with Coomassie blue. Arrows indicate the positions of the 31K, 34K, and 66K proteins. Molecular weight standards (MWS) are shown (see the legend to Fig. 1).

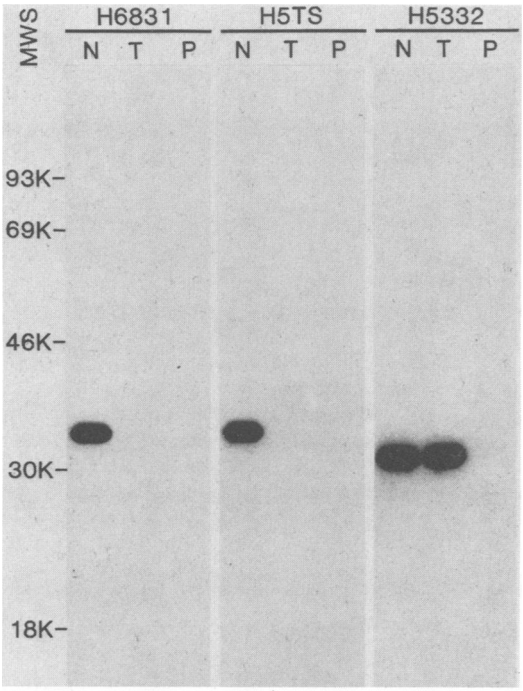

FIG. 4. Western blots of monoclonal antibodies versus protease-treated LD spirochetes. Strains B31 (left) and HB19 (center and right) were treated with buffer alone (N), trypsin (T), or proteinase K (P) as described in the legend to Fig. 3 and in the text. After PAGE and transfer to nitrocellulose, the spirochete components were reacted with monoclonal antibody H6831, H5TS, or H5332; the hybridoma supernatants were diluted 1:10. Molecular weight standards (MWS) are shown (see the legend to Fig. 1).

the second antibody, H5TS. Two concentrated control antibodies were also used: H4825, which we assumed would not bind to any LD spirochete component and thus would help in the assessment of any nonspecific binding of the 34K protein to IgG or *S. aureus* cells; and H5332, which would aid in the determination of whether a 34K protein could be precipitated as a result of direct or indirect interaction with the 31K protein, another probable outer membrane protein (7). The Western blots showed that H5TS bound to the component immunoprecipitated with H6831 but not detectably to any component immunoprecipitated with H4825 or H5332 (Fig. 5). When strain B31 was used in place of IDS, H5TS was not seen, in the Western blots, to bind to anything in the H6831 immunoprecipitate (data not shown).

DISCUSSION

Epidemiological and clinical studies of LD spirochetes indicate that these bacteria proliferate in the blood and tissues of several types of mammals (3, 8–10, 21) and are transmitted from one vertebrate host to another by *Ixodes* spp. ticks (5, 12, 13, 21). The spirochetes can also produce a generalized infection of the tick vector (13). Although parasitism by LD spirochetes may not result in significant disease in a natural host such as deer (10), the pathogenic potential of these spirochetes for humans, who may be inadvertent hosts, is not in doubt (8, 19–21).

The ecological niche occupied by LD spirochetes is similar to that of the relapsing fever borreliae (14), and LD spirochetes are indeed closely related to borreliae by the criterion of DNA-DNA hybridization (F. W. Hyde and R. C. Johnson, J. Clin. Microbiol., in press; G. P. Schmid, A. G. Steigerwatt, S. Johnson, A. G. Barbour, A. C. Steere, I. M. Robinson, and D. J. Brenner, J. Clin. Microbiol., in press). The relapsing fever borreliae probably owe their persistence in mammalian hosts to an antigenic variation mediated by differential expression of their outer membrane proteins (6). Could antigenic variation explain, in part, the chronicity of many cases of LD (16, 20)? Or if an extensive antigenic repertoire is not available to an LD spirochete, are there other surface properties of these organisms that aid tissue invasion and then thwart clearance by the immune system?

In a previous report, we described a surface antigenic determinant that was common to all LD spirochetes which we examined and that was associated with an abundant protein with an M_r of 31,000 (7). In the present study, we examined surface components that were not common to all strains. Before conducting the experiments reported here, we knew that there were slight variations between strains in the migration of major proteins with M_rs of ca. 34,000 (5, 7) and that few LD patients had detectable antibodies to the one example of these proteins that had been examined (4; A. G. Barbour 1, in press). We therefore selected for monoclonal antibodies that possessed strain specificity as shown

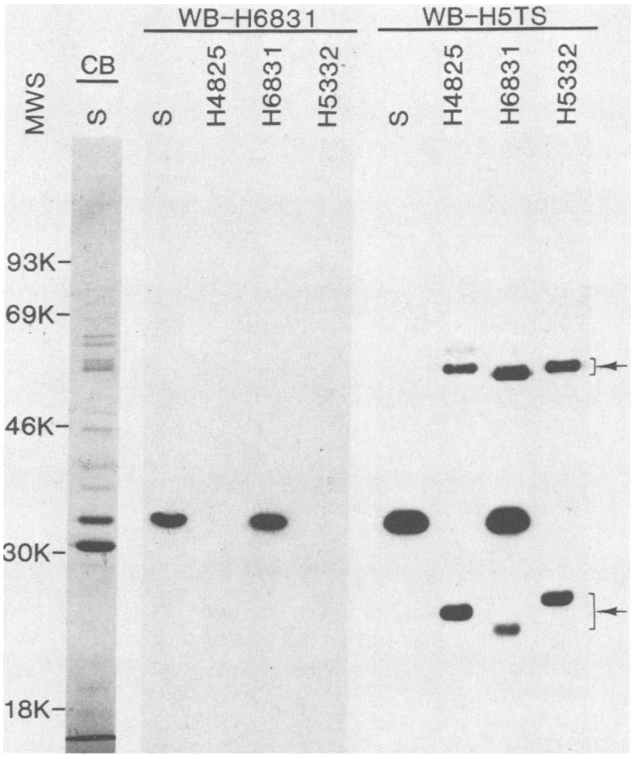

FIG. 5. Immunoprecipitation of strain IDS LD spirochetes. Non-idet P-40-soluble components (S) of the spirochetes were incubated with monoclonal antibody H4825, H6831, or H5332 as described in the text. Antigen-antibody complexes were recovered with protein A-bearing staphylococci. The immunoprecipitated and soluble components were separated by PAGE, transferred to nitrocellulose, and reacted in Western blots (WB) with monoclonal antibody H6831 or H5TS. The hybridoma supernatants were diluted 1:10. The additional bands, identified by brackets and arrows, in the H5TS blot are the heavy (top) and light (bottom) chains of the monoclonal antibodies which were bound by the rabbit anti-mouse immunoglobulin used with this blot. Left, Coomassie blue (CB)-stained proteins of the soluble components and molecular weight standards (MWS) (see the legend to Fig. 1).

by IFA. We then sought, using Western blots, the antigenic determinants against which the monoclonal antibodies were directed. These determinants were, in fact, associated with the variable 34K proteins. We assume that the specificities shown by the antibodies in Western blots reflect the actual epitopes in or on whole live cells.

Several lines of evidence indicate that the 34K proteins are on the surface of the spirochetes. (Because the strains had been grown in vitro before examination, we cannot exclude the possibility that a more exterior layer exists in vivo.) The supporting findings include (i) agglutination of live organisms by antibody; (ii) the electron microscopic visualization of an immunoglobulin G-specific ligand, i.e., protein A-coated gold particles, bound to spirochetes that had been incubated with a monoclonal antibody specific for a 34K protein; (iii) the binding of radiolabeled antibody to LD spirochetes that had not been washed and centrifuged; and (iv) the effective removal from the cell of the 34K proteins, as well as their associated antigenic determinants, by proteases. Whereas the 34K proteins in situ were susceptible to both trypsin and proteinase K at the concentrations used, the 31K protein was susceptible only to proteinase K.

If our interpretation of the immunoprecipitation-Western blot experiment (Fig. 5) is correct, i.e., that both antibodies bound to the same 34K protein, then there are at least three different 34K-range proteins to be found among the LD spirochetes. Two proteins are recognized by one monoclonal antibody but not the other, and a third protein is recognized by both antibodies. There may be other strains, e.g., TLO-030, which do not have the equivalent of a 34K protein and, as a consequence, are nonreactive with both antibodies.

Can a population of LD spirochetes manifest more than one 34K protein over time and even turn off the expression of these proteins? The difference between the 34K protein of the newly isolated IDS strain and that of the derivative, multipassaged B31 strain suggests that LD spirochete populations can undergo change in their surface proteins. However, this question, as well as those posed above, cannot be answered until adequate animal models of infection are established and the population of spirochetes can be sampled at various times before and after the immune response begins.

Although in vivo antigenic shifts by LD spirochetes remain to be demonstrated, the results in this report do indicate that LD spirochete isolates differ serologically and that the basis for serotype distinctions may be the 34K-range proteins. The paucity of LD patients with detectable antibody to the 34K protein of strain B31 (4; A. G. Barbour 1, in press) may occur for reasons other than or in addition to a hypothetical poor immunogenicity of these proteins. LD patients lacking those particular antibodies may have been infected by an LD spirochete of a serotype different from that of strain B31.

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