INFECTION AND IMMUNITY, Aug. 1983, p. 795–804
0019-9567/83/080795-10$02.00/0
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Vol. 41, No. 2

Lyme Disease Spirochetes and Ixodid Tick Spirochetes Share a Common Surface Antigenic Determinant Defined by a Monoclonal Antibody

ALAN G. BARBOUR,[1]* SANDRA L. TESSIER,[1] AND WILLIAM J. TODD[2]

Laboratory of Microbial Structure and Function,[1] and Electron Microscopy Section, Operations Branch,[2] Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana 59840

Received 3 March 1983/Accepted 10 May 1983

Ixodid tick-associated spirochetes have been implicated as the etiological agents of Lyme disease. We raised a murine monoclonal antibody (H5332) against a spirochete, strain B31, isolated from *Ixodes dammini* ticks. In indirect immunofluorescence assays and western blot analyses, H5332 reacted with whole cells or isolated components of not only strain B31 but also spirochetes isolated from *Ixodes ricinus* ticks, a field mouse, a raccoon, and patients with Lyme disease. In contrast, H5332 did not bind to representative borreliae, treponemes, and leptospires. Using indirect immunofluorescence assays and immune electron microscopy, we found the H5332 determinant to be diffusely distributed over the surface of prefixed spirochetes but to be aggregated in patches when the organisms were incubated with H5332 and a second ligand before fixation. Radioimmunoprecipitation and western blot studies revealed the H5332 determinant to be either on or tightly associated with an abundant outer membrane protein with an apparent subunit molecular weight of 31,000.

Lyme disease is a multisystem disorder that follows the bite of ixodid ticks of North America, Europe, and Australia (15, 27, 31). The most characteristic manifestation of Lyme disease is erythema chronicum migrans (ECM; 28). ECM may be accompanied or followed by an acute arthritis (28), cranial neuritis and meningoencephalitis (21), or cardiac involvement (24). Some patients develop a chronic arthritis which has features of rheumatoid arthritis (25).

Several findings (presented in historical order) indicated that the cause of Lyme disease is an infectious agent and that the agent is a tick-borne spirochete: (i) temporal and geographic clustering of cases (30); (ii) amelioration of the disease by antibiotics (29); (iii) demonstration of antibodies in patients with Lyme disease to hitherto unknown spirochetes of *Ixodes dammini* and *Ixodes ricinus* ticks (1, 9); and finally, (iv) isolation of these spirochetes from the blood, cerebrospinal fluid, and skin of Lyme disease patients (4, 26).

The ixodid tick-borne or Lyme disease-associated spirochetes can be cultivated in cell-free medium (1, 9). They appear to be more closely related to borreliae and treponemes than to leptospires and free-living spirochetes (9). Erythema chronicum migrans-like skin lesions have been observed on rabbits that have been fed upon by infected *I. dammini* or *I. ricinus* ticks (9; 10) and spirochetes have been recovered from the blood of mice, deer, and raccoons (6; J. F. Anderson. L. A. Magnarelli, W. Burgdorfer, and A. G. Barbour, Am. J. Trop. Med. Hyg., in press). Nevertheless, we know little of the biology of these spirochetes in mammals, let alone in ticks. It is also not known whether the pathology observed in Lyme disease is solely a result of tissue invasion or toxin elaboration by spirochetes or whether host factors, e.g., the immune response, are also critical.

An appropriate structure to scrutinize in a study of Lyme disease pathogenesis is the surface of the implicated spirochete. Presumably it is the surface of the spirochete that first interacts with the host during infection. Accordingly, we generated and selected monoclonal antibodies on the basis of their immunofluorescent staining of spirochetes. The majority of monoclonal antibodies selected in this way were directed against determinants that were part of or associated with an abundant outer envelope protein. Using one of these antibodies, we found that one determinant occurred in all strains examined and that the determinant aggregated in patches when bound by antibody and a second ligand.

MATERIALS AND METHODS

Organisms and culture conditions. Seven isolates of ixodid tick-associated or Lyme disease-associated spi-

rochetes were studied. The strain designations and the sources are listed in Table 1. All isolates were grown in BSK broth medium at 35°C (1) and had undergone at least three passages in this medium after their original isolations. The concentration of spirochetes was estimated by direct count (32). The spirochetes were harvested by centrifugation (8,000 × *g* for 20 min at 20°C); the pellet was suspended in one-fiftieth volume of M/15 phosphate-buffered saline with 5 mM $MgCl_2$ (PBS/Mg) and 16% (vol/vol) glycerol. The suspensions were kept frozen at −76°C until use.

Other spirochete species were used in the study. The origin of *Borrelia hermsii* HS1 serotypes C, 7, and 21 has been described previously (2, 32); they were grown in BSK medium. *Borrelia turicatae* and *Borrelia parkeri* were obtained from W. Burgdorfer and H. G. Stoenner (Rocky Mountain Laboratories). They also were grown in BSK medium. *Borrelia recurrentis* was provided by P. Perrine (University of Washington, Seattle). This strain was present in the blood of a patient with relapsing fever in Ethiopia. It was not cultivable; instead, we used the borreliae present in the infected plasma. *Treponema pallidum* was provided by the Centers for Disease Control, Atlanta, Ga. It was in a lyophilized preparation of rabbit testicle and was suspended in PBS/MG before making smears. *Treponema phagedenis* Kazan 8 (ATCC 27087) was likewise lyophilized and suspended in PBS/Mg before making smears. A *Leptospira interrogans* strain, which was isolated from a resident of a Lyme disease endemic area, was provided by G. Schmid (Centers for Disease Control, Atlanta, Ga.). The isolate was untypable when tested with the battery of antisera at the Centers for Disease Control. The leptospire was cultivated in Leptospira medium EMJH (Difco Laboratories, Detroit, Mich.) and washed with PBS/Mg before use.

For ^{14}C labeling, *I. dammini* spirochete strain B31 was grown in BSK medium until the population was in late-log phase. The cells were harvested by centrifugation and washed twice with PBS/Mg. In this procedure and in others described below, unless otherwise indicated, centrifuged means a 3-min centrifugation in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and washed means suspension in 1 ml of the designated buffer. The final pellet, which contained approximately 5×10^8 spirochetes, was suspended in 3 ml of a medium formulated as follows. To 100 ml of RPMI 1640 tissue culture medium lacking leucine and glutamine (Flow Laboratories, McLean, Va.) was added 3 g of bovine serum albumin, fraction V (BSA; Miles Laboratories, Elkhart, Ind.); 0.6 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) acid (Sigma Chemical Co., St. Louis, Mo.); 0.07 g of sodium citrate; 0.5 g of glucose; 0.08 g of sodium pyruvate; and 0.2 g of sodium bicarbonate. The pH was adjusted with 1 N NaOH to 7.5, and 11 ml of dialyzed fetal calf serum (Flow Laboratories) was added. The medium was sterilized by filtration. [^{14}C]leucine (30 μCi; 10 μg; New England Nuclear Corp., Boston, Mass.) was added to the medium before the addition of the spirochetes. The suspension was incubated in a 35°C static water bath for 4 h. The labeled spirochetes were harvested by centrifugation, washed twice with PBS/Mg with 10 mM NaN_3 (PBS/Mg/A), and resuspended in 1 ml of this buffer.

TABLE 1. Spirochete strains

Strain	Source (location)	Reference
B31	*I. dammini* (New York)	9
39/40	*I. dammini* (Connecticut)	26
IRS	*I. ricinus* (Switzerland)	1
HB4	Human blood (New York)	4
HB19	Human blood (Connecticut)	26
50-2	White-footed mouse blood (New York)	6
2535	Raccoon blood (Connecticut)	Anderson et al., in press

Production of hybridomas. Frozen suspensions of *I. dammini* spirochete B31 were thawed, centrifuged, and resuspended in PBS/Mg. BALB/c mice (6 weeks old; Rocky Mountain Laboratories breeding colony substrain) were each inoculated intravenously with about 10^8 spirochetes on days 1 and 21. On day 24, the mice were sacrificed and their spleens were removed. The remaining steps in the production of hybridomas through fusions of the spleen cells with cells of the P3-NS-1 Ag-4/1 derivative of BALB/c myeloma MOPC 21 have been described previously (2). The hybridomas were cloned by limiting dilution, and the resulting culture supernatants were examined as to immunoglobulin class by immunodiffusion and to success of cloning by methods described previously (2). The supernatant fluids of the hybridomas were screened by indirect immunofluorescence assay (IFA; see below) and then by solid-phase radioimmunoassay. The latter procedure was that previously described (A. G. Barbour, W. Burgdorfer, E. Grunwaldt, and A. C. Steere, J. Clin. Invest., in press). Briefly, sodium dodecyl sulfate (SDS)-soluble components of strain B31 were covalently bound to paper through activated diazonium groups. The paper was then reacted with culture supernatants and probed for bound immunoglobulin G (IgG) with ^{125}I-labeled protein A.

Immunofluorescence. Thin smears were made of the strains listed in Table 1 and the additional spirochetal species listed above; they had been washed with PBS/Mg and mixed with a suspension of washed rat erythrocytes in 50% PBS–50% fetal calf serum. The slides were fixed in 100% methanol, air dried, and kept in a desiccator at −20°C until use. The procedure for the IFA has been described previously (2). Hybridoma culture supernatants were used undiluted.

We also examined the binding of monoclonal antibody to unfixed B31 spirochetes. Fresh, washed organisms were suspended in 1% BSA in PBS/Mg/A (BSA/PBS/Mg/A). An equal volume of monoclonal antibody in PBS was added, and the suspension was incubated on ice for 60 min. The cells were centrifuged, washed twice with PBS/Mg/A, and suspended in a 1:200 dilution of fluorescein-coupled, goat anti-mouse immunoglobulin (Becton Dickinson Research Center, Research Triangle Park, N.C.) diluted in BSA/PBS/Mg/A. The suspension was incubated for 20 min on ice. After centrifugation, one wash with BSA/PBS/Mg/A, and resuspension in one-fifth volume of this buffer, the spirochetes were immobilized under a cover slip and immediately examined for fluorescence (Zeiss Photomicroscope III; Carl Zeiss, Inc., N.Y.).

Immune electron microscopy. Colloidal gold was generated by the reduction of gold chloride with

sodium citrate (14). Protein A (Sigma; no. P-6650) was complexed to colloidal gold as described previously by Slot and Geuze (23) except that the pH of the colloidal gold was raised to 9.0 with 0.1 M K_2CO_3 before the addition of protein A and then dropped to pH 4.0 with 0.1 M HCl. After complexing the colloidal gold with protein A, we adjusted the pH to 6.8 with Hanks balanced salt solution lacking calcium and magnesium and containing 0.1 mg of polyethylene glycol per ml (molecule weight, 20,000). Uncomplexed protein A was removed by ultrafiltration with an XM 300 filter (Amicon Corp., Danvers, Mass.) and washing with Hanks balanced salt solution with polyethylene glycol. The specificity of binding of the complexes was assessed by incubation of the complexes with rabbit IgG bound to Immunobeads (Bio-Rad Laboratories, Richmond, Calif.). Binding of the prepared complexes to the beads was blocked by prior incubation with free protein A.

A suspension of washed strain B31 spirochetes in PBS/Mg/A ($\sim 5 \times 10^8$ in 2 ml) was divided in half. Formalin was added to a final concentration of 0.5% to one sample. The cells were fixed for 5 min. This sample and the second, unfixed sample were both centrifuged and washed with first PBS/Mg/A and then BSA/PBS/Mg/A. The final pellets were resuspended in 0.2 ml of the latter buffer. To this was added 0.2 ml of hybridoma culture supernatant. Antibody and suspensions were incubated for 30 min on ice. After centrifugation, the cells were washed twice with PBS/Mg/A. The two samples were resuspended in 0.4 ml of this buffer, and to this was added 60 μl of protein A-colloidal gold complexes at various concentrations. The suspensions were incubated for 30 min on ice. The cells were centrifuged and washed twice with PBS/Mg/A. They were resuspended in this buffer. The second sample, i.e., the one not originally fixed in Formalin, was fixed in 0.5% Formalin in PBS/Mg/A for 5 min and then rinsed again to remove the Formalin.

The spirochetes were applied to grids, air dried, and then stained for 30 min with vapors of osmium tetroxide. The grids were examined in a Hitachi HU-11 electron microscope.

Polyacrylamide electrophoresis. Frozen spirochetes were thawed, centrifuged, and washed twice with PBS/Mg. Cells were suspended in a volume of distilled water and sample buffer to give a protein concentration of 0.85 mg/ml as determined by the Bradford method (7). The method for the SDS-polyacrylamide gel electrophoresis (PAGE) system has been described previously (2). The pH of the separating gel buffer was 8.7, and the acrylamide concentration was 10%. The gels were stained with Coomassie brilliant blue R-250. Molecular weight standards labeled with ^{14}C were phosphorylase *b* (93,000 [93K]), BSA (69K), ovalbumin (46K), carbonic anhydrase (30K), and beta-lactoglobulin (18K) (New England Nuclear Corp.). One lysate preparation was treated with proteinase K (0.5 mg/ml, final concentration; type XI; Sigma) for 1 h at 60°C after it had been boiled in sample buffer for 5 min. Proteolysis was stopped by addition of phenylmethylsulfonyl fluoride (0.7 mg/ml, final concentration; Sigma).

Immunoprecipitation. Surface-exposed protein antigens were immunoprecipitated by a modification of the method of Swanson (33). A portion (50 μl) of the final suspension of ^{14}C-labeled spirochetes (see above; ca. 70 μg of protein) was incubated with 1 ml of hybridoma culture supernatant for 1 h at 20°C. The suspensions were centrifuged and washed twice with PBS/Mg/A. The cells were suspended in 200 μl of 1% Zwittergent 3-14 (dipolar ionic detergent; Calbiochem-Behring Corp., LaJolla, Calif.) in Dulbecco PBS (13). The spirochetes lysed during incubation in this detergent for 12 h at 20°C. The lysate was centrifuged in a Microfuge for 5 min. The supernatant was removed and mixed with 40 μl of a 10% suspension of Formalin-fixed *Staphylococcus aureus* (ATCC 12598) Cowan in 50 mM Tris (pH 7.4)–150 mM NaCl–5 mM EDTA (TSE) with 0.05% Nonidet P-40 (19). This suspension was incubated for 30 min at 20°C. It was then centrifuged for 2 min and washed twice with TSE. The pellet was suspended in 30 μl of sample buffer (1% SDS, 10% glycerol, 63 mM Tris, pH 6.8). 2-mercaptoethanol (2-ME; Sigma) (3 μl) was added. The samples were boiled (98°C) for 5 min and centrifuged. A 20-μl sample was subjected to SDS-PAGE. The gel was stained, destained, and impregnated with 2,5-diphenyloxazole in the presence of dimethyl sulfoxide by the method of Bonner and Laskey (5), dried, and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) for 56 h at −76°C.

Western blotting. The procedure used for performing transfer of proteins from SDS-PAGE gels to nitrocellulose and incubation of blots with antibody was a modification of the methods of Towbin et al. (34), Renart et al. (22), and Batteiger et al. (3). The proteins in a gel were transferred to nitrocellulose (HAHY; Millipore Corp., Bedford, Mass.) in a Trans-Blot cell (Bio-Rad) containing 192 mM glycine, 25 mM Tris base, and 20% (vol/vol) methanol in distilled water. The cell was kept at 25°C with a cooling coil during electrophoresis (60 V for 3 h). After electrophoresis, the blots were blocked by overnight incubation without agitation in TSE with 0.05% Tween 20 (TSE/Tween). We incubated the blots with 1:10 dilutions of hybridoma culture supernatants in TSE/Tween with 2% BSA in heat-sealable plastic bags on a rocking platform for 2 h. The blots were washed three times with TSE/Tween and incubated with ^{125}I-labeled protein A (2) at 50,000 cpm/ml in TSE/Tween for 1 h. These were washed four times with TSE/Tween, rinsed several times with water, dried, and exposed to AR film.

RESULTS

Monoclonal antibodies. Hybridomas were produced by injecting mice with live spirochetes and boosting 3 weeks later. Hybridoma culture supernatants were screened first with IFA. A typical positive reaction is shown in Fig. 1A. Only fusions producing antibodies that bound to outer membrane blebs as depicted in the figure were selected and cloned.

We further screened with radioimmunoassay those antibodies that appeared to bind to surface components by IFA. Positive reactions by radioimmunoassay identified protein A-binding IgG antibodies that recognized determinants still intact after solubilization in SDS. Six monoclonal antibodies were selected in this way. In prelimi-

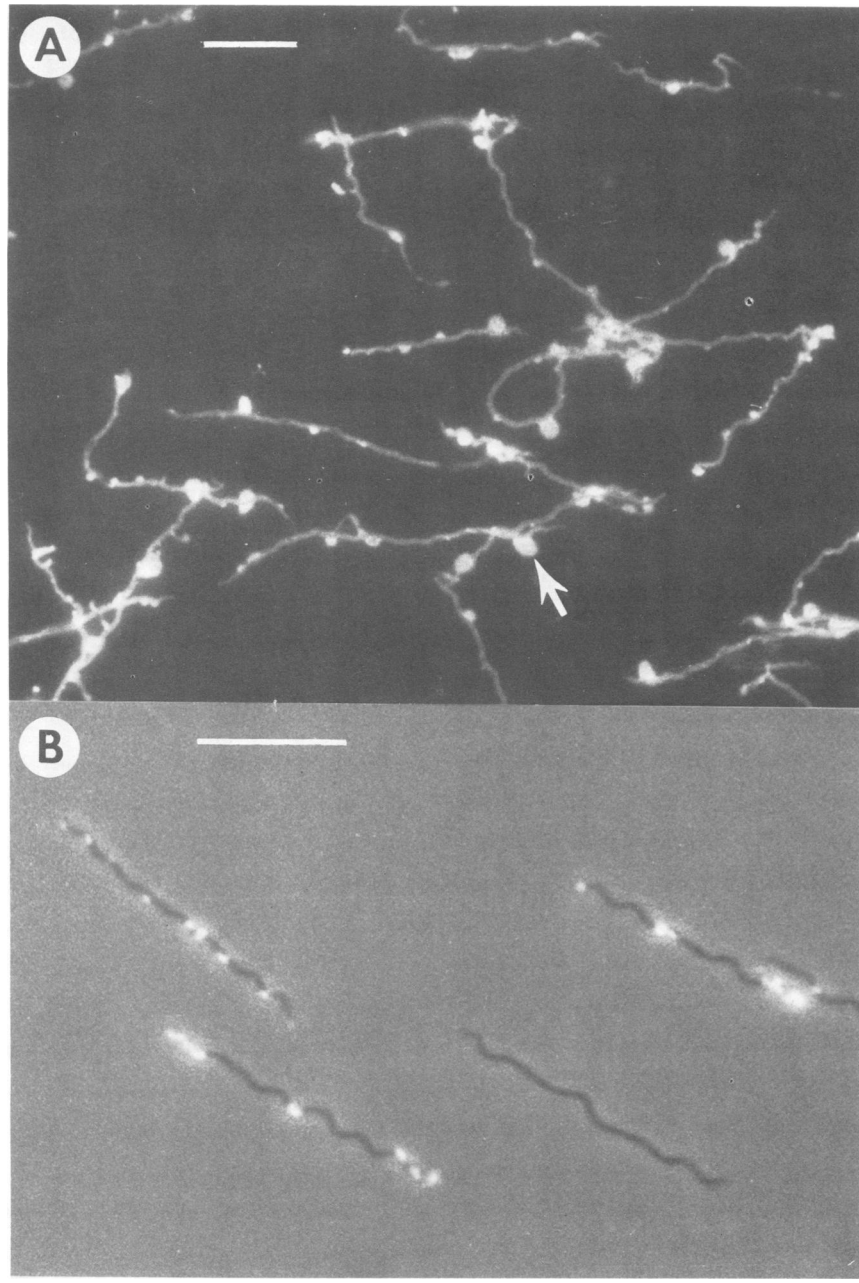

FIG. 1. Indirect immunofluorescence of fixed and unfixed spirochetes. (A) Ixodid tick spirochete strain B31 organisms on slides were fixed in methanol as described in the text. The slides were incubated with monoclonal antibody H5332, washed, incubated with fluorescein-conjugated goat anti-mouse immunoglobulin, washed, dried, and examined for fluorescence. Arrow indicates an outer membrane bleb. Bar, 5 μm. (B) Ixodid spirochete strain B31 organisms in suspension were incubated with monoclonal antibody, centrifuged, washed, incubated with fluorescein-conjugated goat anti-mouse immunoglobulin, washed, and examined for fluorescence. Slides were illuminated by both epifluorescence and low-intensity transmitted light. Phase-contrast photomicrographs were then taken. Bar, 5 μm.

nary IFA studies, five of the six antibodies bound to all strains of ixodid tick and Lyme disease spirochetes examined. The sixth monoclonal antibody, which bound to a subset of the strains tested, will be the subject of another investigation.

The five monoclonal antibodies lacking strain specificity were finally screened by western blot analysis. Each of the five bound, to a greater or lesser extent, a strain B31 component or components with the same mobility in SDS-PAGE gels. One of the monoclonal antibodies (H5332) consistently produced the highest counts in the radioimmunoassay and the most intense bands in radioautographs. This hybridoma, which produced an IgG1 antibody, was successfully expanded and used in other investigations.

Immunofluorescence and immune electron microscopy. The specificity of H5332 for ixodid tick and Lyme disease spirochetes is shown in Table 2. The brilliance of fluorescence was not altered by replacing methanol in the slide fixation step with acetone, 0.5% Formalin, or by heating slides at 42°C for 60 min. However, fixation in 2% Formalin or glutaraldehyde considerably diminished the intensity.

H5332 and the fluorescein isothiocyanate-coupled second antibody produced homogeneous staining of fixed strain B31 spirochetes (Fig. 1A). A similar pattern was seen on organisms fixed in 0.5% Formalin, acetone, or with heat. However, with unfixed spirochetes in suspension a different pattern was seen (Fig. 1B). The binding of antibody and the fluoresceinated second ligand was not homogeneous. Instead, we observed patches of fluorescence. Some organisms did not fluoresce at all or had only a small fluorescent tip at one end. The figure was taken with both epifluorescence and low-intensity phase-contrast illumination to show both the fluorescent patches and the remainder of an organism.

TABLE 2. Indirect immunofluorescence reactions

Strain or species	Reaction
Tick and mammal isolates[a]	
B31	+
IRS	+
HB19	+
HB4	+
50-2	+
39/40	+
2535	+
Other spirochete species[b]	
B. hermsii	−
B. parkeri	−
B. turicatae	−
B. recurrentis	−
T. pallidum	−
T. phagedenis	−
L. interrogans	−

[a] See Table 1.
[b] See text.

Because it was possible that the determinant recognized by H5332 was a culture medium component that remained with the spirochetes through the washings, we used crushed *I. dammini* ticks containing spirochetes in the IFA. H5332 bound to in situ spirochetes, which had never been exposed to medium components, as well.

We used immune electron microscopy to determine whether the patchy pattern was due to a loss of outer membrane from some portions of the cell surface. Strain B31 spirochetes were incubated with hybridoma supernatant containing either H5332 or, as a control, a protein A-binding monoclonal antibody directed against a *B. hermsii* outer membrane protein (H4825; A. G. Barbour and S. L. Tessier, manuscript in preparation). Protein A complexed with colloidal gold was the probe for bound antibody. Spirochetes fixed in Formalin before exposure to antibody had a fairly homogeneous distribution of gold particles over the outer envelope surface (Fig. 2A). In contrast, spirochetes fixed in Formalin after exposure to the two ligands demonstrated aggregations—usually at or near one end—of gold particles (Fig. 2B). The intactness of the outer envelope in areas without particles and the density of label in the patches indicated that the patching was not due to stripping off of the outer envelope during preparation of the samples. We did not see gold particles attached to spirochetes when H4825 was used in place of H5332.

Immunoprecipitation and western blotting. We employed immunoprecipitation and western blotting to identify the H5332 determinant. Strain B31 organisms, their proteins ^{14}C labeled, were incubated with either H5332 or H4825 supernatant. The cells were then washed to remove unbound antibody and lysed with a dipolar ionic detergent. Antibody that had bound to whole cells was precipitated with protein A, and the antibody-antigen complex was dissociated with SDS.

Fig. 3 shows the Coomassie blue-stained proteins (lane a) and [^{14}C]leucine-labeled proteins (lane d) of strain B31. By both methods, the most abundant protein had an apparent subunit molecular weight of 31K as assessed by comparison with molecular weight standards.

A protein of identical molecular weight by SDS-PAGE was bound and precipitated by H5332 but not by H4825 (Fig. 3, lanes b, c, e, and f). The other two bands in lane b were also seen when H5332 alone was incubated with *S. aureus* cells and are, therefore, presumably the heavy and light chain of H5332. Longer expo-

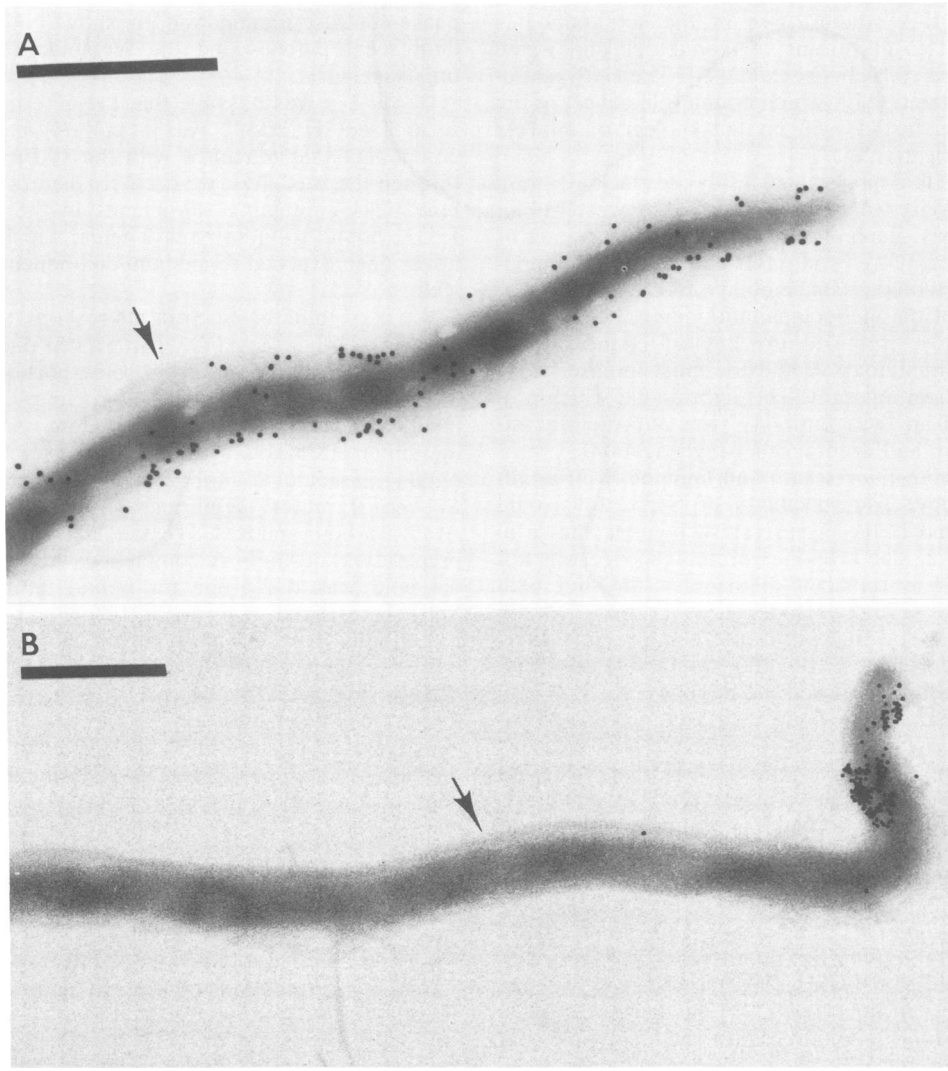

FIG. 2. Labeling of Lyme disease spirochetes with monoclonal antibody H5332 and protein A-coated colloidal gold under two different experimental conditions. Arrows indicate outer membrane. Bars, 0.5 μm. (A) Spirochetes fixed before reactions with antibody and protein A-coated colloidal gold show a fairly homogeneous distribution of gold particles. An axial filament is also seen. (B) Spirochetes fixed after reactions with antibody and protein A-coated colloidal gold show a patchy distribution of gold particles; the patches are usually located at or near one end of the spirochete.

sures of the fluorogram to film did not reveal any other labeled proteins precipitated by H5332 or H4825.

We found abundant 31K proteins in each of the other six isolates examined by SDS-PAGE and western blots (Fig. 4). The only conspicuous difference among isolates by Coomassie blue staining of gels was in proteins of about 35K molecular weight (Fig. 4). We have observed that migration of proteins in this area can vary even among the progeny of a single organism. Note that whereas there are two proteins in the 35K region of strain B31 lysate in Fig. 4, there is only one in Fig. 3.

H5332 bound to 31K proteins in the western blot of the seven isolates (Fig. 4). There were also remarkable trails of the determinant above the 31K band in the blot. "Negative" bands were interspersed in the trails of each isolate. The molecular weights of many of these negative bands did not correspond to those of major proteins in the Coomassie blue-stained gel.

To investigate the trailing phenomenon, we altered conditions under which strain B31 ly-

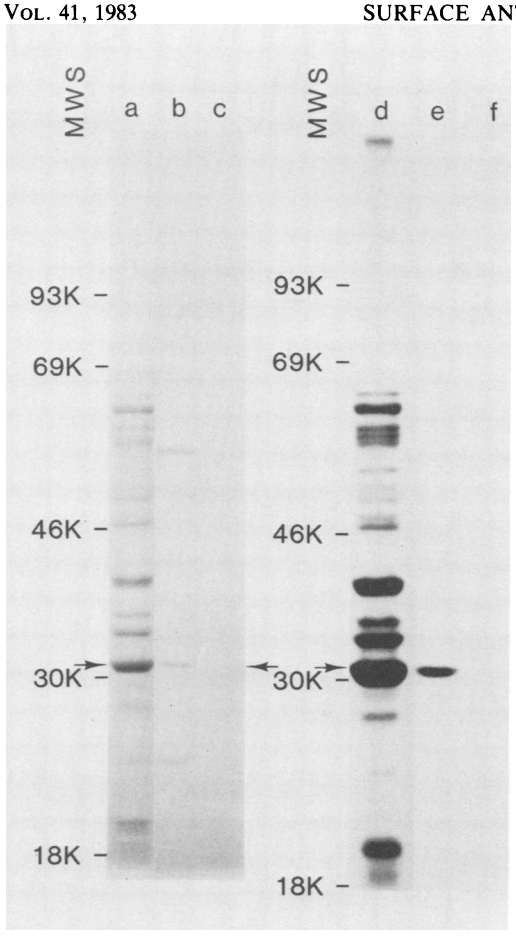

FIG. 3. Immunoprecipitation of [^{14}C]leucine-labeled strain B31 ixodid tick spirochetes. Lane a, Coomassie blue-stained proteins of whole cell lysate. Lanes b and c, Coomassie blue-stained proteins bound by monoclonal antibodies H5332 (lane b) and H4825 (lane c) in immunoprecipitation experiment (see text). Lane d, fluorogram of ^{14}C-labeled proteins in whole cell lysate. Lanes e and f, fluorogram of ^{14}C-labeled proteins bound by monoclonal antibodies H5332 (lane e) and H4825 (lane f) in immunoprecipitation experiment. Arrows indicate the position of the 31K protein. The positions of molecular weight standards (MWS) are shown.

sates were prepared for SDS-PAGE. Fig. 5 shows the pertinent results of three experiments. Trailing was undetectable in H5332 blots when the 2-ME concentration was 2% (290 mM) or less and when DL-dithiothreitol (DTT; Sigma) replaced 2-ME as the reducing agent. The trailing was considerably diminished when samples containing 10% 2-ME (1.4 M) were not boiled before electrophoresis and when 100 mM (but not 10 mM) $NaAsO_4$, a trivalent arsenical, was added to the 10% 2-ME sample buffer. Trailing was unaltered by addition of DTT (1, 10, or 100 mM) to samples already containing 10% 2-ME. DTT and 2-ME together, in fact, appeared to increase the amount of trailing (Fig. 5, lane 2d). The apparent subunit molecular weight of the protein associated with the H5332 determinant was 31K under all of the conditions we employed.

In the third experiment (Fig. 5, lanes 3a and 3b), we examined the effect on the determinant of exposure to a undiscriminating proteolytic enzyme. Treatment of the sample with proteinase K before electrophoresis eliminated both the trailing and the 31K band itself in the blot.

DISCUSSION

We isolated a hybridoma producing an antibody that recognized spirochetes recovered from patients with Lyme disease, a field mouse, a raccoon, and ixodid ticks from North America and Europe. The H5332 epitope was also detected by IFA on three other *Peromyscus* isolates from New York (unpublished observations), two additional human isolates from Connecticut (26), and a spirochete isolated from *I. dammini* ticks of New Jersey (G. P. Schmid, personal communication). This determinant has not been found on other spirochete species that we have examined (26; this study). The close relatedness of the Lyme disease-, ixodid tick-, and small mammal-associated isolates in their morphology (S. F. Hayes, W. Burgdorfer, and A. G. Barbour, manuscript in preparation), SDS-PAGE profiles (1; this study), and antigens (26; Anderson et al., in press; this study) suggests that these spirochetes belong to the same species and that mammals, both large and small, become spirochetemic via ixodid tick bites.

The H5332 determinant is either part of the 31K protein itself or is tightly associated with this protein. We cannot at this time exclude the possibility that a non-proteinaceous epitope, such as a carbohydrate or glycolipid, is bound to the protein.

The 31K protein has the same apparent molecular weight as protein 4, which was identified in western blot analyses of sera from patients with Lyme disease and rabbits with erythema chronicum migrans-like lesions (Barbour et al., in press). About 40% of all patient sera tested and six of nine sera from chronically arthritic patients contained detectable IgG to this protein. In that study, we also found the 31K protein (or protein 4) to be poorly iodinated in the presence of Iodogen (Pierce Chemical Co., Rockford, Ill.) (Barbour et al., in press). One explanation of this poor labeling is that the 31K protein is not exposed on the cell surface. However, the evidence from immunofluorescence studies, immune electron microscopy, and immunoprecipi-

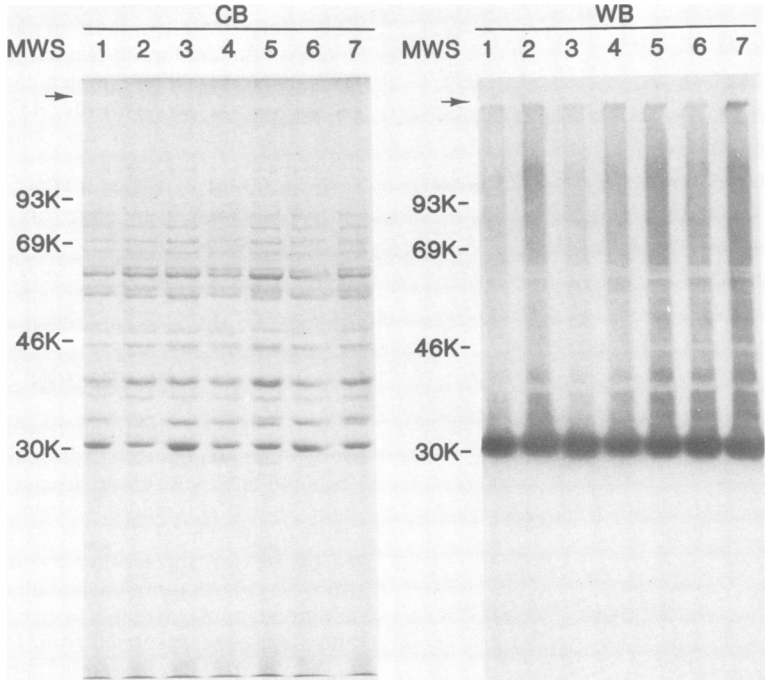

FIG. 4. Coomassie blue (CB)-stained proteins and western blot (WB) analyses of whole cell lysates of seven spirochete isolates. Lysates contained 10% 2-ME and were boiled for 5 min before SDS-PAGE. The components in the gel were either stained with Coomassie blue or transferred to nitrocellulose for western blot analysis and radioautography. The isolates were as follows: lane 1, 50-2; lane 2, 39/40; lane 3, 2535; lane 4, HB19; lane 5, B31; lane 6, IRS; and lane 7, HB4. Arrows indicate the top of the separating gel. The positions of molecular weight standards (MWS) are shown.

tation with whole cells counters this proposal. Explanations invoking a paucity of accessible tyrosyl residues or a shielding of these residues by a non-proteinaceous substance, perhaps the H5332 determinant, are, therefore, more likely.

Incubation of unfixed organisms first with H5332 and then with either fluorescein-coupled anti-mouse immunoglobulin or protein A-colloidal gold complexes resulted in a patchy arrangement of label. In contrast, when the spirochetes were first fixed on glass with methanol, acetone, or heat or were in suspension with dilute Formalin, the labels were more diffusely distributed over the outer envelope. Determinant aggregation in the presence of azide at 4°C would seem to make this phenomenon not a capping analogous to the active process in mammalian cells (12). Rather, the phenomenon seems to more closely resemble "patching," the passive two-dimensional aggregation of molecules in a fluid membrane, described by DePetris and Raff (12) and by Braun and colleagues (8). Charon et al. (11) have, in fact, demonstrated, through use of antibody-coated beads, the longitudinal movement of antigens through the outer envelope of a leptospire. Although final classification of the aggregation of the epitope awaits additional investigations, such as on the role of the second ligand and on the effect of other metabolic inhibitors, the fact that spirochetal outer membranes are only loosely associated with the more rigid protoplasmic cylinder (17, 20) would seem to rule out a capping of surface components directed by the underlying cytoskeleton. Whether this patching might have a role in pathogenesis, for example, in making the phagocytosis of opsonized spirochetes as difficult as that of capped lymphocytes (16), also remains to be determined.

The trailing exhibited by the 31K protein in western blots was not seen in Coomassie blue-stained gels or in radioautographs of ^{14}C-labeled proteins. The sensitivity of the western blotting technique may have permitted this demonstration (34). The trailing was eliminated or much diminished by steps that presumably reduced the amount of 2-ME associated with the protein. These steps included the use of less 2-ME and the addition of sodium arsenite to the buffer. 2-ME forms mercaptides with trivalent arsenicals (18). Such complex formation may have effectively removed excess 2-ME from the protein.

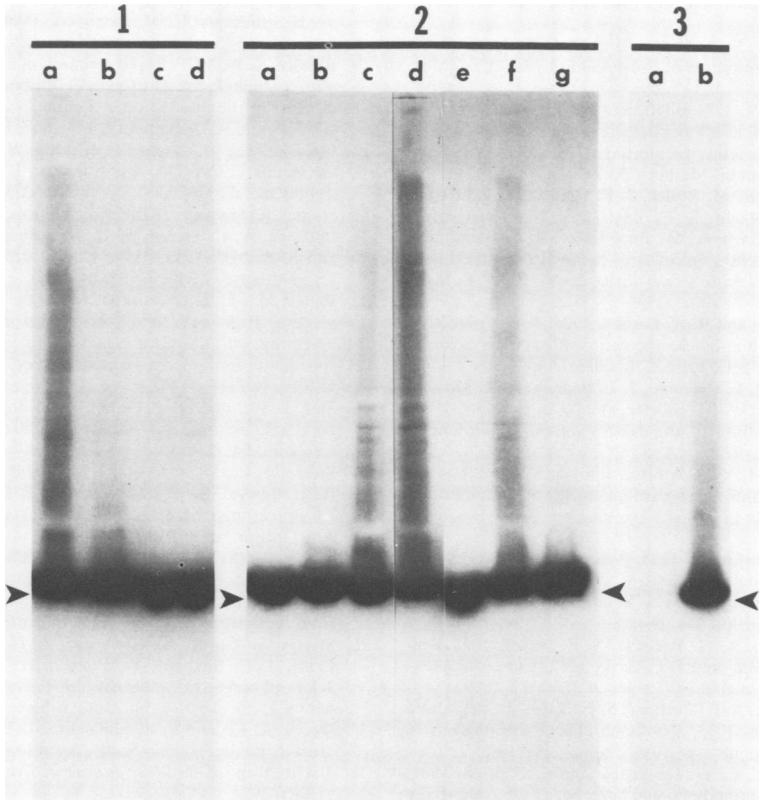

FIG. 5. Western blot reactions of monoclonal antibody H5332 with strain B31 cell lysates that were prepared for SDS-PAGE under different conditions. All samples contained 1% SDS, 10% glycerol, and 63 mM Tris (pH 6.8). 2-ME, DTT, sodium arsenite ($NaAsO_4$), or proteinase K (see text) or some combination of these was added to those samples identified below. Unless otherwise indicated in parentheses, samples were heated to 98°C for 5 min. Three experiments are shown. Arrowheads indicate the location of the 30K molecular weight standard in each gel. Experiment 1: lane a, 10% 2-ME; lane b, 10% 2-ME (22°C for 30 min); lane c, 1% 2-ME; lane d, 1% 2-ME (22°C for 30 min). Experiment 2: lane a, 2% 2-ME; lane b, 5% 2-ME; lane c, 10% 2-ME; lane d, 10% 2-ME and 100 mM DTT; lane e, 100 mM DTT; lane f, 10% 2-ME and 10 mM $NaAsO_2$; lane g, 10% 2-ME and 100 mM $NaAsO_2$. Experiment 3: lane a, proteinase K (0.5 mg/ml) and 10% 2-ME; lane b, 10% 2-ME.

Use of a lower temperature in sample preparation may have prevented access of 2-ME to buried regions of the protein. Although trailing was associated with 2-ME, it was not associated with another reducing agent, DTT. At the concentrations (1.4 M) at which we observed this phenomenon, 2-ME may exert effects additional to reduction of protein sulfhydryl groups.

In summary, we can define the H5332 determinant as follows. It is part of or associated with a 31K protein. This protein is located in or on the outer membrane, is aggregated by antibody and a second ligand, is immunogenic in infected humans and laboratory animals, and has occurred in all isolates we have examined to date. We do not imply that there are not other surface components which are perhaps more important in terms of pathogenesis or immunity elicitation. At the very least, though, additional study of the 31K protein and the H5332 determinant should help in revealing the architecture of the outer envelope. This determinant, and possibly related determinants in other strains, may also be useful for immunodiagnosis of Lyme disease, epidemiological investigations, and taxonomic decisions.

ACKNOWLEDGMENTS

We thank J. Anderson, J. Benach, P. Perrine, G. Schmid, and A. Steere for sending strains; P. Barstad, W. Burgdorfer, H. Caldwell, and J. Swanson for advice; R. Evans and C. Taylor for photographic assistance; and S. Smaus for preparation of the manuscript.

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