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A *Borrelia*-Specific Monoclonal Antibody Binds to a Flagellar Epitope

ALAN G. BARBOUR,[1,2] STANLEY F. HAYES,[1] RAMONA A. HEILAND,[2] MERRY E. SCHRUMPF,[1,2] AND SANDRA L. TESSIER[1,2]

Laboratories of Pathobiology[1] and Microbial Structure and Function,[2] National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840

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In immunofluorescence assays monoclonal antibody H9724 recognized eight species of the spirochetal genus *Borrelia* but not representatives of the genera *Treponema*, *Leptospira*, and *Spirochaeta*. We examined the reactivity of H9724 against subcellular components of *Borrelia hermsii*, an agent of relapsing fever, and *B. burgdorferi*, the cause of Lyme disease. H9724 bound to isolated periplasmic flagella of the two borreliae. In Western blots the antibody reacted with the predominant protein in flagellar preparations from *B. hermsii* and *B. burgdorferi*; the apparent molecular weights of these flagellins were 39,000 and 41,000, respectively.

The genus *Borrelia* contains three major groups of organisms that are pathogenic for humans or other animals: (i) the several species that cause relapsing fever; (ii) *Borrelia burgdorferi*, the etiologic agent of Lyme arthritis, erythema migrans, tick-borne meningoradiculitis, and acrodermatitis chronica atrophicans; and (iii) *B. anserina*, the cause of avian spirochetosis.

During the screening of monoclonal antibodies to various serotypes of the relapsing fever species *B. hermsii*, we found antibodies that reacted with all serotypes examined (4). In a preliminary study of these cross-serotype-reactive antibodies, some also bound to *Borrelia* species other than *B. hermsii*. We now report on the specificities of one of these cross-reactive monoclonal antibodies (H9724) and on the borrelial antigen that the antibody is directed against.

MATERIALS AND METHODS

Monoclonal antibody. Antibody H9724 was the product of a previously described fusion of a mouse spleen with NS1 myeloma cells (9). The mouse had been immunized with washed, whole cells of *B. hermsii* HS1. The production, cloning, and assessment of the hybridomas have been described previously (8, 9). The antibody was determined to be of the immunoglobulin G2a isotype by immunodiffusion (8). A control monoclonal antibody, H4825, was also of the immunoglobulin G2a isotype; this antibody is directed against an outer membrane protein of *B. hermsii* (7).

Spirochete strains. *B. hermsii* HS1, serotype C (ATCC 35209), and *B. burgdorferi* B31 (ATCC 35210) were used for periplasmic flagellar isolations and biochemical studies as well as for immunofluorescence assays (see below).

Other *Borrelia* isolates (and their sources) were as follows: a serologically distinct *B. burgdorferi* isolate from *Ixodes ricinus* ticks in Sweden (G. Stiernstedt, Danderyd Hospital, Stockholm, Sweden) (6); *B. anserina* and *B. crocidurae* (R. Johnson, University of Minnesota); *B. parkeri* and *B. turicatae* (Rocky Mountain Laboratories); *B. duttonii* (D. Wright, Charing Cross Hospital, London, England); and *B. recurrentis* (P. Perine, University of Washington). The *B. recurrentis* isolate was from the plasma of an Ethiopian patient with relapsing fever; the isolate was passed once in BSK II medium (2) containing 10% pooled human serum in place of 6% rabbit serum. The remaining borrelial isolates were grown in unmodified BSK II broth medium. The borreliae were harvested, stored frozen at −75°C, and washed after being thawed by previously described procedures (7, 8). For immunofluorescence studies, smears of spirochetes on glass slides were air dried and then fixed in methanol (7, 8). Fixed smears were kept in a desiccator at −20°C.

Other genera of spirochetes were sent to us either as frozen cells or as methanol-fixed smears on slides. (Preliminary studies had shown no difference in the reactivity of H9724 against never-frozen cells of *B. hermsii* or cells of the same strain that had been frozen and then thawed. We had also found that the reactivity of H9724 against *B. hermsii* cells was not detectably altered even after storage of fixed smears for 3 years). These other spirochetes (and their sources) were as follows: *Treponema pallidum* Nichols (S. Norris, University of Texas, Houston); *T. denticola* and *T. vincentii* (R. Nauman, University of Maryland); *T. phagedenis* Kazan (ATCC 27987); *T. hyodysenteriae* (T. Stanton, U. S. Department of Agriculture, Ames, Iowa); *Leptospira interrogans* serovars pomona and *icterohaemorrhagiae* (R. Johnson, University of Minnesota); and *Spirochaeta aurantia* (P. Greenberg, Cornell University).

IFA. The indirect immunofluorescence assay (IFA) has been described previously (7, 8). The hybridoma supernatants were used undiluted.

Isolation of periplasmic flagella. A modification of the method of Hardy et al. for the isolation of treponemal flagella was used (17). Half-liter cultures (approximately 5×10^{10} cells) of *B. hermsii* HS1 or *B. burgdorferi* B31 were harvested by centrifugation and washed twice with phosphate-buffered saline–5 mM $MgCl_2$ (8). To the final pellet was added 20 ml of 2% *N*-lauroylsarcosine (sarcosyl; Sigma Chemical Co., St. Louis, Mo.) in 10 mM Tris (pH 8.0)–1 mM EDTA (TE). The cell suspension was incubated at 37°C for 45 min, during which time the spirochetes lysed. The lysate was centrifuged at 48,000 × *g* for 45 min at 25°C in a fixed-angle rotor. After the supernatant was discarded, the pellet was suspended in 10 ml of 2% sarcosyl–TE and incubated at 37°C for 10 min. The suspension was again centrifuged at 48,000 × *g* for 45 min at 25°C. The pellet was taken up in 10 ml of 150 mM NaCl, and the suspended material was sheared in a Waring blender for 10 min. The

* Corresponding author.

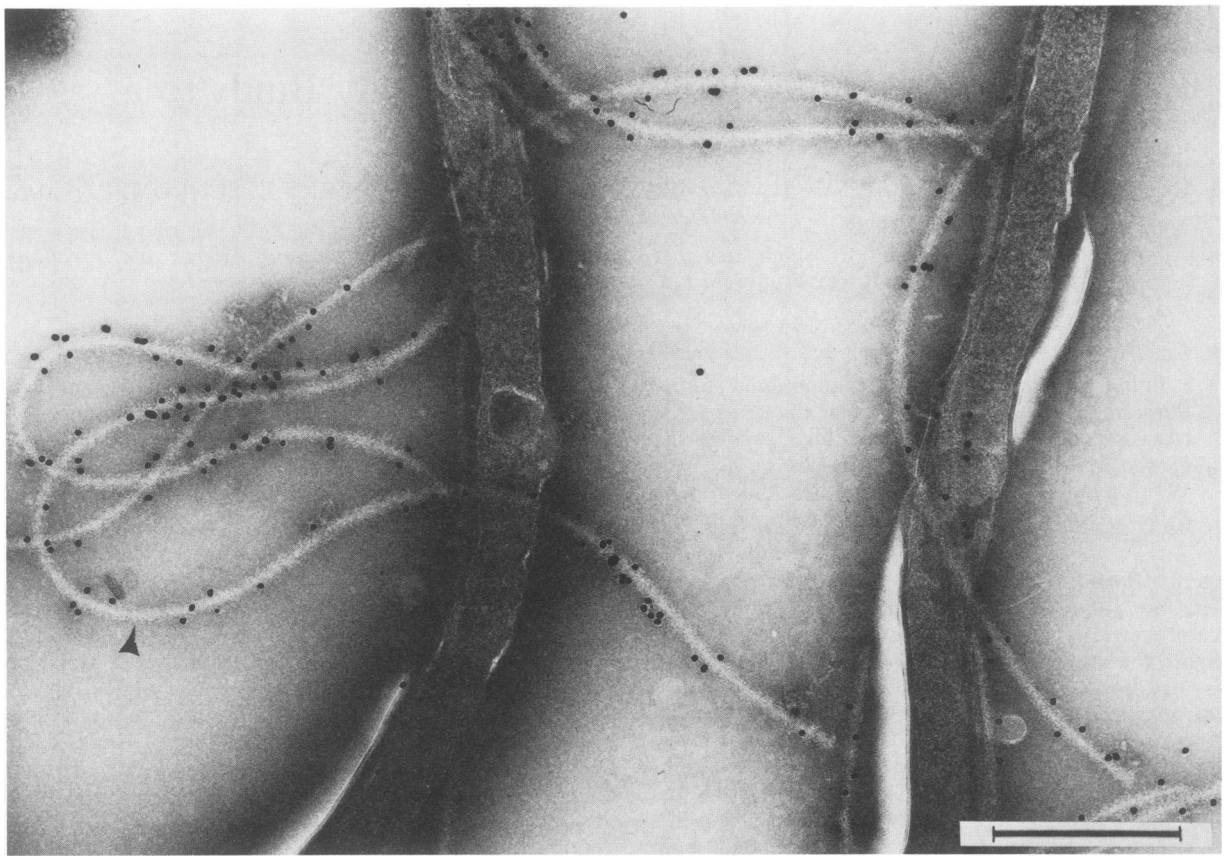

FIG. 1. Labeling of partially disrupted *B. burgdorferi* cells with monoclonal antibody H9724 and protein A-coated colloidal gold. The stain was 2% ammonium molybdate. The arrow indicates a released flagellum. Bar, 0.5 μm.

resultant suspension was centrifuged at 220,000 × *g* for 35 min at 15°C in a fixed-angle rotor. The pellet was suspended in 2 ml of 2% sarcosyl–TE. This suspension was loaded on top of a CsCl step gradient formed 4 to 6 h previously. The steps were 40, 35, 30, 25, and 20% CsCl in 0.2% sarcosyl–TE. The gradient was centrifuged at 175,000 × *g* for 15 h at 25°C in a swinging bucket rotor. Visible bands in the gradient tubes were collected and examined by electron microscopy (see below). Bands containing flagella were pooled, diluted 1:1 in TE, and centrifuged at 190,000 × *g* for 3 h at 25°C in a swinging bucket rotor. The pellet was first suspended in and then dialyzed against distilled water.

Electron microscopy. (i) Negative staining. Three-microliter volumes of suspected or confirmed flagellar suspensions were placed on 3% Parlodion-coated, 300 mesh copper grids (Pelco; Ted Pella, Inc., Tustin, Calif.) prepared by the method of Garon (16). Samples were allowed to adsorb to the grids for 30 min at room temperature. Excess fluid was removed with a micropipette, and the grids were washed with distilled water. The preparations were stained for 20 s with 0.1% uranyl acetate (pH 3.9) or 2% ammonium molybdate (pH 6.5). The stained samples were then air dried and examined with a Hitachi 11E-1 electron microscope.

(ii) Immune electron microscopy. When whole borreliae were to be examined, they were washed first in a microcentrifuge tube with phosphate-buffered saline–5 mM $MgCl_2$. The washing was repeated three times; the cell pellets were suspended with a 200-μl micropipette tip and then pelleted by a 3-min centrifugation in a microcentrifuge (model B; Beckman Instruments, Inc., Fullerton, Calif.). These washed cells or the periplasmic flagella were adsorbed onto grids. The method for detection of antibody bound to subcellular structures has been described previously (7). The second ligand was 10- to 12-nm complexes of protein A and colloidal gold (15). The hybridoma supernatants were undiluted. The negative stain was 2% ammonium molybdate (pH 6.5).

Polyacrylamide gel electrophoresis and Western blotting. The methods used for polyacrylamide gel electrophoresis and Western blotting have been described previously (6, 8).

TABLE 1. Reactivity of monoclonal antibody H9724 against various species of spirochetes

Species	IFA reaction
Borrelia hermsii	+
B. turicatae	+
B. parkeri	+
B. duttonii	+
B. crocidurae	+
B. recurrentis	+
B. anserina	+
B. burgdorferi	+
Treponema pallidum	−
T. phagedenis	−
T. denticola	−
T. vincentii	−
T. hyodysenteriae	−
Leptospira interrogans	−
Spirochaeta aurantia	−

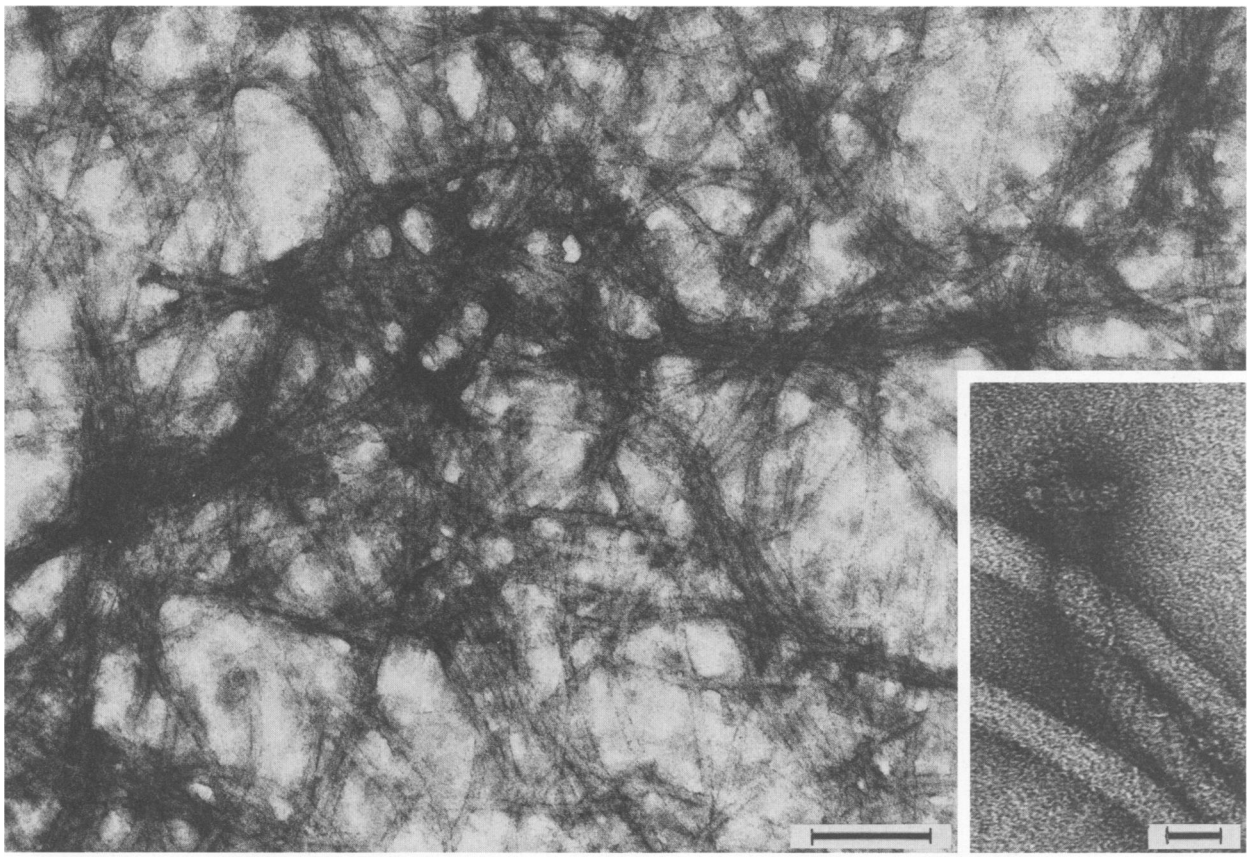

FIG. 2. Electron micrographs of a *B. hermsii* fraction enriched for periplasmic flagella. The stain was 0.1% uranyl acetate. Bar, 0.1 μm. The insert (lower right-hand corner) shows a proximal hook and an insertion disk of one flagellum. Bar, 0.01 μm.

Hybridoma supernatants were diluted 10-fold in 2% bovine serum albumin in 50 mM Tris (pH 7.6)–150 mM NaCl–5 mM EDTA–0.05% sodium azide. Bound antibody was detected with radioiodinated protein A and subsequent radioautography (8).

RESULTS

Immunofluorescence. Antibody H9724 originally drew our attention because it reacted with several serotypes of *B. hermsii* HS1, which came from Washington (25), and subsequently with *B. hermsii* strains isolated from relapsing fever patients living in Idaho and California. The immunofluorescence reaction seen with H9724 against fixed *B. hermsii* spirochetes was not as intense (a score of 2 out of a possible score of 3) as that seen with membrane protein-specific monoclonal antibodies, which usually give a score of 3 (4, 8). The type of cell staining seen was consistent with the heterologous reaction shown by Stoenner et al. (25).

The IFA specificity of H9724 was tested against a variety of spirochetes (Table 1). The positive reactions had equivalent degrees of fluorescence in the assays; therefore, the results are shown without gradations.

H9724 bound to all *Borrelia* species examined. The collection included representatives for the three major groups of borreliae. Among the relapsing fever spirochetes in the collecton were tick- and louse-borne species from both the Old World and the New World. In addition to strain B31, the *B. burgdorferi* isolate from Europe was bound by H9724.

The binding of H9724 to *B. burgdorferi* cells was not altered by prior treatment of intact cells with trypsin or proteinase K by methods previously described (7). Neither was the fluorescence in the IFA appreciably diminished by fixation of *B. burgdorferi* cells with 4% Formalin or 2% glutaraldehyde instead of methanol (data not shown).

None of the other genera of spirochetes were recognized by H9724 (Table 1). With the exception of the free-living *S. aurantia*, all of the represented spirochetes obligately or facultatively parasitize humans or other animals to some degree. The only spirochete genus not included was *Cristispira*, which has only been found in molluscs and has never been grown in a pure culture (21).

Periplasmic flagella. To identify the sites of binding of H9724 to borreliae, we incubated *B. burgdorferi* cells adsorbed to a grid first with H9724 or an irrelevant monoclonal antibody and then with protein A-colloidal gold complexes. During the course of three washings and three resuspensions, approximately 10% of the spirochetes were disrupted and released their periplasmic flagella from the confines of the outer membrane. In these preparations, we found that H9724 bound to the released flagella (Fig. 1). The antibody did not associate with the outer membrane. The control monoclonal antibody did not bind to the flagella (data not shown).

Having localized an epitope for H9724 to a particular subcellular structure, we proceeded to recover from cells of *B. hermsii* and *B. burgdorferi* fractions that were enriched for periplasmic flagella. This was accomplished by first lysing the cells with sarcosyl, a detergent that did not disaggregate the flagella. Electron microscopy revealed that the crude sarcosyl-insoluble material contained flagella but

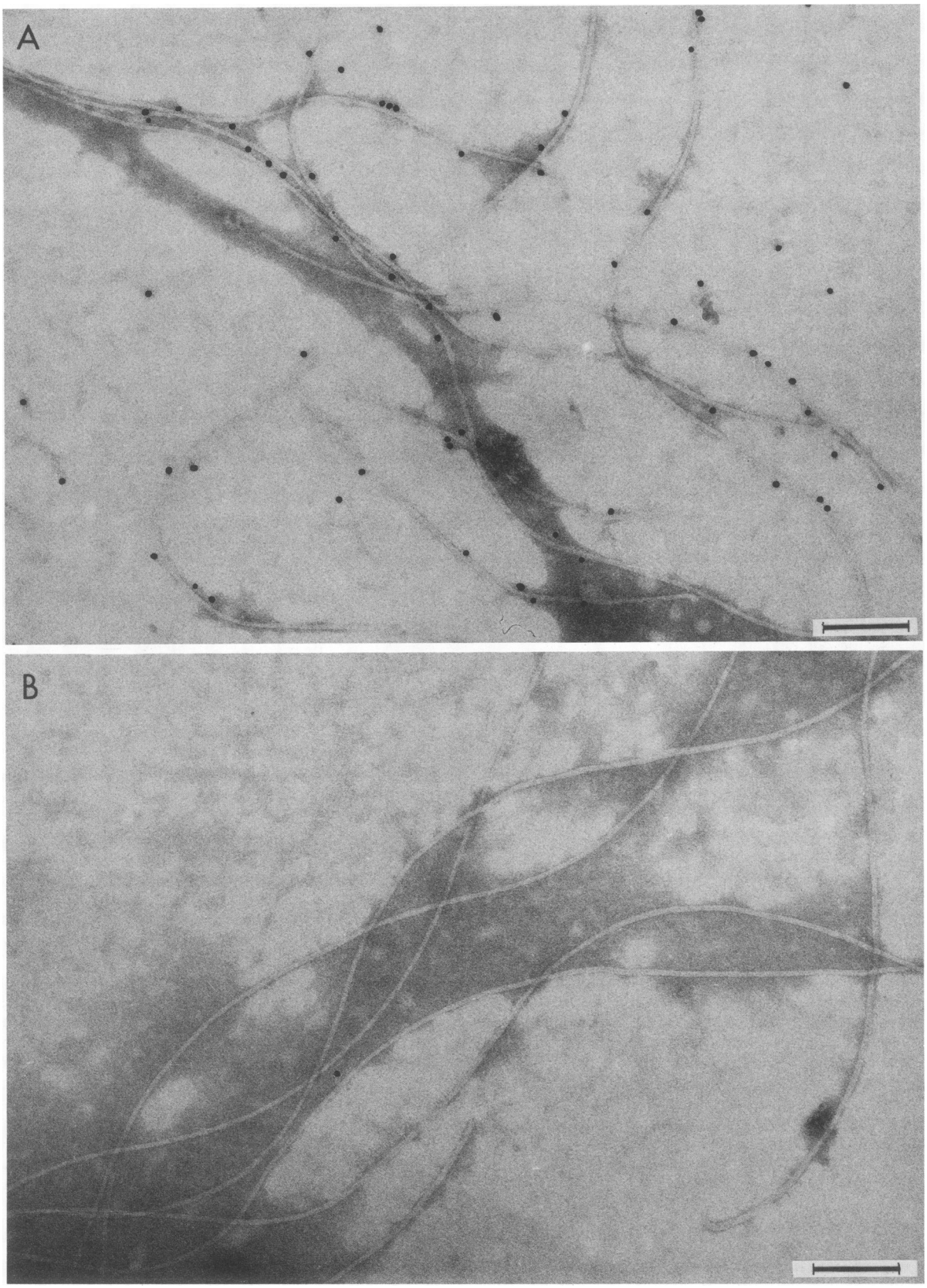

FIG. 3. Labeling of isolated periplasmic flagella from *B. hermsii* with monoclonal antibodies and protein A-coated colloidal gold. The monoclonal antibodies used were H9724 (A) or a monoclonal antibody directed against an outer membrane protein of this strain (B). The stain was 2% ammonium molybdate. Bars, 0.1 μm.

also many structures consistent with peptidoglycan walls (data not shown). These two components were separated by a shearing step, followed by density gradient centrifugation in an adaption of the method of Hardy et al. (17). The final preparation from *B. hermsii* is shown in Fig. 2. An insertion disk and a proximal hook of one of the flagella are also shown.

The fractions enriched for flagella were placed on grids and incubated with H9724 or a monoclonal antibody that was specific for an outer membrane protein of the particular strain under study. Bound antibody was detected with protein A-coated colloidal gold. An examination of *B. hermsii* flagella is shown in Fig. 3. (Results with *B. burgdorferi* were comparable.) H9724 bound to the isolated flagella, but the outer membrane protein-specific antibody did not.

Analyses by polyacrylamide gel electrophoresis and Western blotting served to identify the subunit size of the antigen against which H9724 was directed. Figure 4A shows the Coomassie blue-stained proteins in whole-cell lysates of *B. hermsii* and *B. burgdorferi* and in the flagellum-enriched fractions from these two species. The apparent molecular weights of the major proteins in the flagellar fractions were 39,000 and 41,000 for *B. hermsii* and *B. burgdorferi*, respectively. Note that two major bands of almost the same molecular weight can be distinguished in the flagellar fraction from *B. burgdorferi*. Abundant proteins with identical molecular weights can be seen in whole-cell lysates of the two strains. The Western blot (Fig. 4B) showed that the epitope for H9724 was associated with the major proteins in the flagellar preparations and that these proteins comigrated with the abundant proteins found in the lysates. H9724 reacted with both of the closely migrating proteins in the *B. burgdorferi* flagellar preparation.

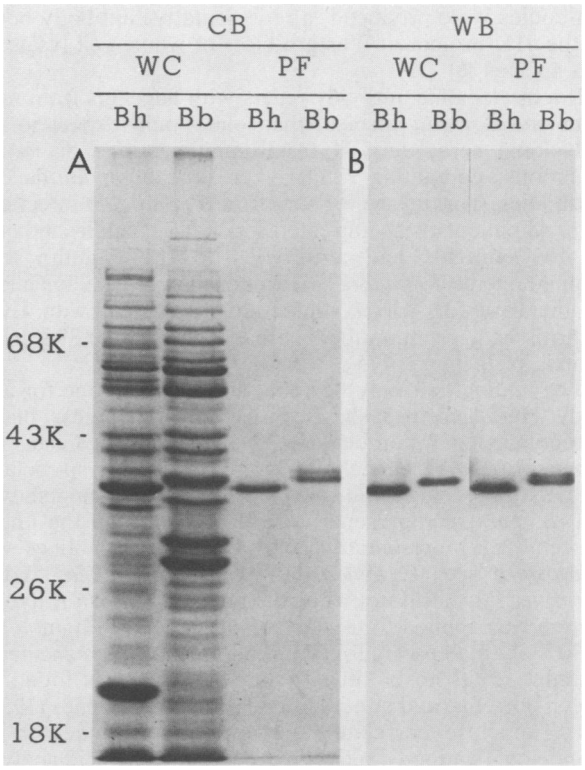

FIG. 4. Coomassie blue (CB)-stained proteins and Western blots (WB) of whole-cell lysates (WC) of or isolated periplasmic flagella (PF) from *B. hermsii* (Bh) or *B. burgdorferi* (Bb). The monoclonal antibody in the Western blots was H9724; bound antibody was detected with ^{125}I-protein A and subsequent radioautography. The acrylamide concentration in the polyacrylamide gels was 10%. The migrations of prestained molecular weight standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) are shown on the left.

DISCUSSION

Spirochetes have been distinguished from other gram-negative-staining bacteria only at the relatively low taxonomic level of order (*Spirochaetales*; 21). However, when the criteria of rRNA homology and oligonucleotide cataloging are applied in the discrimination, it appears that spirochetes occupy their own phylum among the eubacteria (14, 23). One morphologic feature that unequivocally distinguishes spirochetes from all other bacteria is the possession by spirochetes of flagella that are entirely periplasmic in location (reviewed in reference 18). (Periplasmic flagella have also been known as axial filaments or fibrils [18].)

In the present study, we showed that several species of the pathogenic genus *Borrelia* share an antigen that either is closely associated with or is a constituent of their periplasmic flagella. Representatives of the spirochetal genera *Treponema, Spirochaeta,* and *Leptospira* did not have a similarly cross-reactive antigen. Monoclonal antibody H9724, which is directed against that flagellar antigen, can be said, therefore, to be genus specific.

The immune electron microscopy studies of whole cells showed that monoclonal antibody H9724 bound only to released flagella. The positive IFA reactions we saw were probably the result, therefore, of the inevitable disruption of the outer membranes of borreliae during drying and fixation on glass. Antibody H9724 did not agglutinate or immobilize live cells of *B. hermsii* (A. G. Barbour, unpublished results), as can monoclonal antibodies directed against borrelial surface proteins (7). The lack of an effect of proteases on the epitope for H9724 in intact cells was additional evidence that, as would be expected for a periplasmic constituent, it is not surface exposed.

The epitope for H9724 was associated with the most abundant proteins in borrelial fractions enriched for flagella. In Western blots H9724 bound to proteins with apparent molecular weights of 39,000 and 41,000 for *B. hermsii* and *B. burgdorferi*, respectively. These apparent molecular weights were close to the estimated weights of the major flagellar proteins of not only other spirochetes but also gram-positive and gram-negative bacteria (10–12). In keeping with convention, we propose then that the 39K and 41K proteins in the flagellar preparation from *B. hermsii* and *B. burgdorferi* be called flagellins.

On the basis of its molecular weight, the 39K flagellin of *B. hermsii* HS1 appears to be the pII protein of this strain (1, 8). Earlier studies had shown that there were antibodies in anti-*B. hermsii* polyclonal sera that bound to the pII proteins of different serotypes in Western blots (8). Antiflagellin antibodies may be mediators of these heterologous IFA reactions (25).

It is also possible now to identify the 41K antigen of *B. burgdorferi* as probably being a flagellin (3, 5). This antigen is one that Lyme disease patients, as assessed by Western blotting, commonly form antibody against during the course of their illnesses (3, 5, 13). In regard to this 41K protein, the present study complements a previous report: when mice were immunized with *B. burgdorferi* flagella and monoclonal

antibodies were produced, a representative antibody bound to the 41K protein in Western blots of whole-cell lysates of this species (6).

If a monoclonal antibody reacts with flagellins from more than one *Borrelia* species, than one would expect to find polyclonal sera, derived from immunizations or natural infections, containing similarly cross-reactive antiflagellin antibodies. This is true for sera from *T. pallidum* infections: 45 of 45 patients with untreated secondary syphilis and none of 106 controls had antibodies to the flagellin from nonpathogenic *T. phagedenis* Reiter (22). It also appears to be the case for *Borrelia* infections: a patient with Lyme arthritis had serum antibodies that bound to the 39K major protein of *B. hermsii* (5).

The finding that only borreliae have the epitope for antibody H9724 further signifies the appropriateness of the taxonomic separation between this group of organisms and other spirochetes. The characterization of many spirochetes by rRNA homology and oligonucleotide cataloging showed that *B. hermsii* represents a deep branching in the line of descent (23); representatives of the other main sublines were *Leptospira* sp., *T. hyodysenteriae*, *T. denticola*, and *S. aurantia*. Furthermore, when the technique of DNA hybridization was applied to taxonomic studies, DNA homologies of 30% or greater were found among various *Borrelia* species examined, but no or little DNA homology was found between borreliae and either leptospires or treponemes (19, 20, 24). The close correlation between the results with the monoclonal antibody and with the two nucleic acid analyses suggest that H9427 (or other antibodies with similar specificities) will be useful for the characterization of newly discovered spirochetes, especially those that move between arthropod and vertebrate hosts.

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