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Mapping the Major Antigenic Domains of the Native Flagellar Antigen of *Borrelia burgdorferi*

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Purified flagellar protein (p41) of *Borrelia burgdorferi* (strain B31) was subjected to chemical cleavage with hydroxylamine or proteolysis with V8 protease, endoproteinase Asp-N, or α-chymotrypsin. The resulting polypeptides were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and their positions in the published DNA sequence of the p41 protein were determined by amino-terminal sequencing and amino acid analysis. Epitope specificities of antibody binding by a monoclonal antibody raised by immunization of mice with purified flagella and pooled sera from patients with multiple erythema migrans, late Lyme borreliosis, or secondary syphilis were analyzed by Western blots (immunoblots) of peptides transferred to Immobilon polyvinylidene difluoride filters. The major epitope binding one murine monoclonal antibody (158) was localized to a carboxy-terminal domain that includes residues 300 to 336. The dominant epitopes binding human polyclonal antibodies are in the central portion of the molecule (residues 182 to 218) that is not conserved compared with other bacterial flagellins. Additional reactive epitopes were identified in the amino-terminal domain of the protein. Sera from patients with syphilis bound strongly to the amino-terminal conserved domain, providing a structural basis for cross-reactivity seen in standard enzyme-linked immunosorbent assays, but not to the central part of the molecule. Specific and cross-reactive antigenic determinants need to be considered in the design of improved immunodiagnostics for spirochetal diseases.

Similar to other spirochetes, *Borrelia burgdorferi*, the etiologic agent of Lyme disease, has an outer envelope and protoplasmic cylinder complex, between which lie 7 to 11 flagella. The flagellin of *B. burgdorferi* is a single molecule with a mass of 41 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1); furthermore, the molecular weight and isofocusing point of this protein do not vary significantly when different strains of *B. burgdorferi* are compared. Flagellin genes cloned from several *B. burgdorferi* strains (B31, CA12, and GeHo) have been shown to be highly conserved (8, 9, 25).

The exact role of the flagellin in the pathogenesis of *B. burgdorferi* infection is unclear. Recently, a flagellumless mutant of *B. burgdorferi* was found to have reduced ability to penetrate human endothelial cell layers in vitro, suggesting a role in invasion or attachment to host cells which might be crucial in dissemination of the organism from the skin (21). However, monoclonal antibodies (MAbs) to recombinant p41 protein did not afford protection to severe combined immunodeficient (SCID) mice challenged with *B. burgdorferi* (23). In patients with *B. burgdorferi* infection, the first detectable immune responses are directed to the flagellin. The fact that specific immunoglobulin M (IgM) and IgG responses are essentially restricted to this protein in early human disease makes this antigen important for serodiagnosis. The IgG response persists with prolonged illness (7). Purified flagella or flagellum-enriched fractions, rather than sonicated whole spirochetes, have been used as antigens for testing sera from patients with early Lyme disease and to increase sensitivity of enzyme-linked immunosorbent assays (ELISAs) (5, 11). In previous studies, recombinant p41 expressed in *Escherichia coli* has been used to characterize the B- and T-cell responses to this protein in infected persons and immunized animals (2, 6, 8, 14). Since it is not clear whether posttranslational modifications may be important in determining antigenicity, we used purified flagellar protein (p41) isolated from cultures of *B. burgdorferi* to define the important antigenic domains of this protein binding to mouse and human antibodies.

MATERIALS AND METHODS

Isolation of native flagellin from *B. burgdorferi*. The method used to isolate native flagellin was that described previously by Barbour et al. (1), with minor modifications. Briefly, *B. burgdorferi* (strain B31) cells were grown at 37°C in BSK-II medium until a density of 10^8 cells per ml was reached. The organisms were harvested by centrifugation at 8,000 × *g* for 30 min at 25°C. Cells were washed three times with phosphate-buffered saline–5 mM $MgCl_2$. To the final pellet, 10 ml of 2% *N*-lauroylsarcosine–10 mM Tris-HCl–1 mM EDTA (pH 8.0) (2% STE) was added. The cell suspension was incubated at 37°C for 1 h and then centrifuged at 48,000 × *g* for 1 h at 25°C in a fixed-angle rotor. The resulting pellet was resuspended in 10 ml of 2% STE, incubated at 37°C for 10 min, and then centrifuged at 48,000 × *g* for 45 min at 25°C. The pellet was resuspended in 0.5 ml of 2% STE and vortexed repeatedly at 4°C for 10 min. This suspension was layered on top of a 20, 25, 30, and 40% (wt/vol) CsCl gradient with 0.2% STE and centrifuged at 175,000 × *g* overnight (17 h) at 25°C in a swinging bucket rotor. Two bands, each of which was collected separately, were visualized in the gradient. The suspensions were diluted to equal volume with 10 mM Tris-HCl–1 mM EDTA (pH 8.0) and centrifuged at 190,000 × *g* for 3 h at 25°C in a swinging bucket rotor, following which pellets were resuspended in water and dialyzed overnight against water at 4°C.

Chemical cleavage and Cleveland digestion. Hydroxylamine-HCl and α-chymotrypsin were purchased from Sigma

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(St. Louis, Mo.). Endoproteinase Asp-N and Glu-C (protease V8) were from Boehringer Mannheim. Hydroxylamine cleavage was carried out as described elsewhere (3). Flagellar protein (25 μg) in 1 M hydroxylamine-HCl–0.1 M K_2CO_3 (pH 10.5) was incubated at 35°C for 1.5 h, and cleaved peptides were loaded onto SDS–15% PAGE gels. Cleveland digestion (4) with Asp-N, α-chymotrypsin, and protease V8 was carried out on the same gel for comparison. The sample buffer was 0.125 M Tris-HCl (pH 6.8), 0.1% glycerol, and 0.001% bromophenol blue. The flagellum-to-enzyme ratio was 10:1 (wt/wt), mixed with an equal volume of sample buffer. Gel electrophoresis was performed as described previously (13), with current turned off for 30 min when the bromophenol blue dye neared the interface of stacking and separating gels.

Sera and antibodies. Serum samples from 5 patients with acute Lyme disease characterized by multiple erythema migrans (EM) lesions, 5 patients with chronic Lyme disease characterized by neurological symptoms and/or arthritis, and 10 patients with secondary syphilis living in an area in which Lyme disease is not endemic were each pooled separately for Western blot (immunoblot) analysis (24). Each individual serum specimen was diluted with 20 mM Tris-HCl (pH 8.0)–0.15 M NaCl–0.1% Tween 20. The final dilution when all sera were combined was 1:100. Sera from Lyme disease patients were selected on the basis of high-titer antiborrelia antibodies by ELISA and apparent anti-41-kDa flagellin immunoreactivity on blots run against whole sonicates; similarly, all sera from syphilis patients were positive according to the standards of the Venereal Disease Research Laboratory and reacted with *B. burgdorferi* p41 on Western blots. To produce MAbs, BALB/c mice were immunized by two intraperitoneal injections of purified *B. burgdorferi* flagella (100 μg per mouse) separated by 2 weeks. Hybridomas were produced as previously described (12). The supernatants of hybridoma cells were applied to Western blots.

Western blots. Proteins and peptides were separated by SDS-PAGE and electroblotted onto Immobilon polyvinylidene difluoride (PVDF) (Millipore) membranes as described elsewhere (24). Blotted proteins and peptides were detected by amido black staining (20). Immunoreactions were carried out with diluted sera of Lyme disease patients or supernatants of murine hybridomas as the primary antibodies. The secondary antibodies were alkaline phosphatase-conjugated goat anti-human or anti-mouse IgM or IgG, and specific binding was detected with a 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium developer system (KPL, Inc., Gaithersburg, Md.).

Amino acid sequencing. Proteins and peptides were processed for amino-terminal amino acid sequencing as described previously (15). Amido black-stained bands were excised from Immobilon PVDF blots and applied to a Biosystems model 475A protein sequencer with a model 120A PTH analyzer and model 900A control-data analyzer.

RESULTS

Purification of flagella from *B. burgdorferi* B31. Following extraction of whole *B. burgdorferi* organisms with 2% STE, the p41 flagellin was found in both the insoluble and soluble fractions (Fig. 1, lanes 2 and 3). The insoluble fraction was vortexed vigorously and layered over a CsCl step gradient. Two visible bands were observed following gradient centrifugation. The upper band was sharp and contained pure 41-kDa protein (Fig. 1, lane 4), whereas the lower band was wide and contained a mixture of 93-, 73-, 66-, and 41-kDa proteins (Fig. 1, lane 5). The identity of the 93-, 73-, and 66-kDa proteins as immunogenic epitopes of *B. burgdorferi* (15) was confirmed by immunoblot analysis with MAbs (data not shown). The yield of pure flagellar protein obtained from the upper band and used for further studies was 1 to 2 mg from 2.5×10^{10} cells.

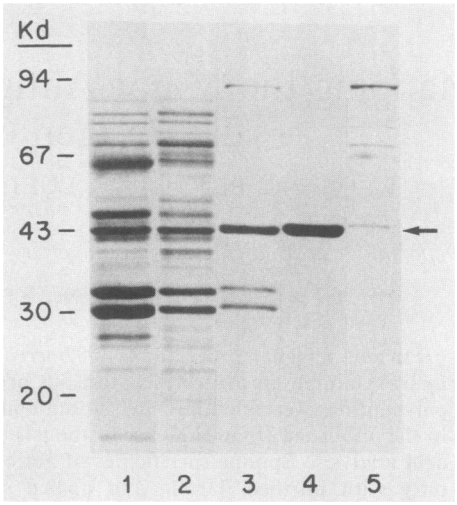

FIG. 1. An SDS–10% polyacrylamide gel was loaded with 20 μg of proteins solubilized from whole B31 spirochetes (lane 1). Lanes 2 and 3, Sarkosyl-soluble and insoluble fractions, respectively, obtained from whole cells treated as described in Materials and Methods; lanes 4 and 5, upper and lower bands, respectively, of the Sarkosyl-insoluble pellet fractionated on a CsCl gradient. The gel was stained with 0.25% Coomassie blue–50% ethanol–10% acetic acid.

Western blot of MAbs and sera. Nineteen hybridomas were found to have antiflagellum reactivity by ELISA run as previously described (12) against purified p41 protein. The hybridomas producing these antibodies were cloned and then subcloned by limiting dilution and used for subsequent studies. Antiflagellin MAb 158 was isotyped as IgG1. As can be seen in Fig. 2, MAb 158 reacted exclusively with p41. In comparison of the sera from patients with early and late Lyme disease, only the latter contain antibodies that bind to OspA and OspB, the major immunogenic outer surface proteins of *B. burgdorferi* (7) (lane 1); however, the flagellar antigen was recognized by sera from both types of patients (lanes 4 and 5, arrows). Control sera, negative for antibody binding to *B. burgdorferi* by ELISA and obtained from uninfected patients, did not react with p41 or its fragments by immunoblot analysis (data not shown).

Peptide mapping of p41. In order to define which domains of the flagellin were immunogenic, purified flagella were chemically or enzymatically cleaved with hydroxylamine-HCl (specificity for Asn-Gly bonds), chymotrypsin (for Tyr-X), Asp-N (for X-Asp), and V8 protease (for Glu-X or Asp-X). Peptides were separated by SDS-PAGE, transferred onto PVDF filters, stained with amido black, and numbered in sequence. For example, as seen in Fig. 3A, lane HA shows uncleaved p41 and the two expected cleavage products, HA1 and HA2, produced by hydroxylamine. Each band was excised from the blot, and amino-terminal sequencing was performed to place the peptide by comparison with the amino acid sequence of the flagellin derived from DNA studies (9). The location of the carboxy terminus of

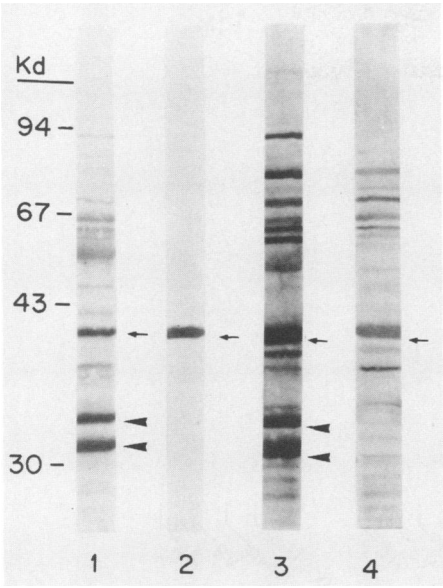

FIG. 2. An SDS–10% polyacrylamide gel was loaded with 20 μg of strain B31 proteins per lane. After electroblotting onto Immobilon PVDF membranes, the antigens were stained with amido black (lane 1) or reacted with MAb 158 (lane 2), sera from patients with late Lyme disease (lane 3), or sera from patients with multiple EM lesions (lane 4). Antibody binding was detected with alkaline phosphatase-conjugated goat anti-mouse or anti-human IgG and the 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium developer system.

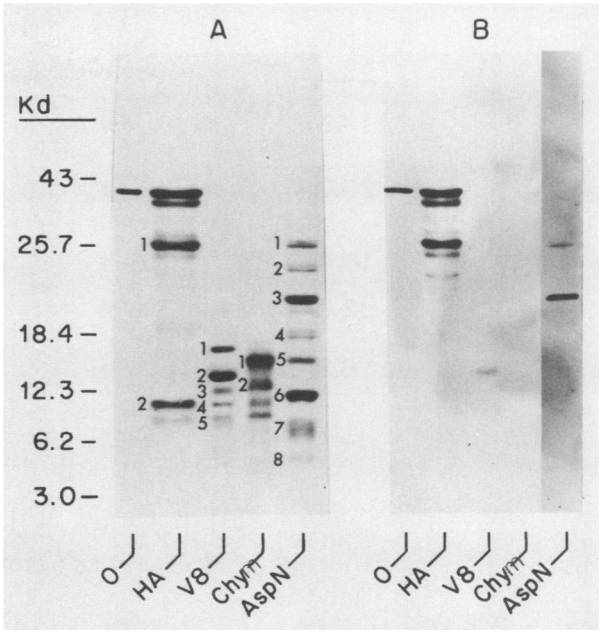

FIG. 3. Blots of an SDS–15% polyacrylamide gel showing uncleaved p41 (lanes O) and p41 cleaved by hydroxylamine (lanes HA), V8 protease (lanes V8), α-chymotrypsin (lanes Chym), or endoproteinase Asp-N (lanes AspN). Panel A is stained with amido black; panel B is a Western blot reacted with MAb 158. The secondary antibodies are the same as those used for Fig. 2.

each peptide was deduced from the molecular weight on gels and known cleavage sites in the p41 sequence for the particular chemical or enzymes. Thus, HA2 peptide has the amino terminus of the native flagellin and an apparent molecular mass of 10.6 kDa on gels, consistent with the expected cleavage of p41 between Asn-99 and Gly-100. Since Asp-N-6, V8-2, and V8-1 have higher apparent molecular weights than HA2 on gels (Fig. 3A) but share the identical amino-terminal sequence, we deduced the carboxy-terminal residues of Asp-N-6, V8-1, and V8-2 to be positions 105, 113, and 120, respectively. Cleavage by Asp-N yielded two peptides, designated Asp-N-3, both of which had a molecular mass of 16.5 kDa (Fig. 3A, lane Asp-N). When this band was subjected to sequential Edman degradation, two residues were obtained at each position, and two sequences could be placed by comparison with the published DNA sequence of p41 (8, 9). Sequence analysis indicated that one of the peptides could be placed between residues 182 to 336. Although the other had an amino terminus identical to that of p41, there is no suitable cleavage site which would give a molecular mass of 16.5 kDa. Therefore, the carboxy terminus of this peptide may represent a unique cleavage site or be due to contamination by an exogenous enzyme activity. The locations of each peptide and the amino-terminal sequences obtained are summarized in Fig. 5.

Antigenic domains of p41. The epitope specificity of the anti-p41 hybridoma was defined by Western blot of the Cleveland digests (Fig. 3B). As seen in panel B, MAb 158 bound to Asp-N-1 but not to Asp-N-2. Since Asp-N-2 lacks positions 297 to 336 of p41 (see Fig. 5), this suggests that the important epitope recognized by MAb 158 is located in the carboxy-terminal domain of the flagellin.

Pooled sera obtained from patients with multiple EM were then tested for antibody binding to the chemically or proteolytically cleaved fragments of p41 (Fig. 4C and D). As seen in panel C, IgG anti-p41 antibodies recognized Asp-N-1, -2, -3, and -4 but did not bind to V8-1, V8-2, or Asp-N-5, -6, and -7. As noted, Asp-N-3 was found to contain two peptides; however, given the fact that Asp-N-6 was nonreactive, these antibodies are most likely binding to the peptide that begins at residue 182 (Fig. 5). Similarly, since the V8-2 band was found by sequencing studies to contain a peptide that begins at residue 219 (Fig. 5) and this band was not reactive with sera from patients with EM, we conclude that the domain inclusive of residues 182 to 218 is immunogenic for EM IgG antibodies. As seen in Fig. 4D, IgM anti-p41 antibodies in sera from EM patients bound to Asp-N-1, -2, -3, and -4, suggesting that the relevant epitope(s) was located in same domain (residues 182 to 218) as the IgG antibodies. However, IgM anti-p41 also bound to Asp-N-6 but not to Asp-N-5, indicating that there is at least one determinant present at the amino terminus of p41. These data suggest that the important epitopes for both IgG and IgM antiflagellin antibodies are located between residues 182 and 218 (Fig. 5). When pooled sera obtained from chronic Lyme disease patients were tested (Fig. 4B), the binding pattern was similar. However, in addition to the reactivity found with the sera from EM patients, we found that Asp-N-6 was also positive and Asp-N-5 was weakly positive, suggesting reactivity to an additional epitope located in the amino-terminal domain (Fig. 5). These data are summarized in Table 1. Pooled sera from 10 syphilis patients bound p41 as expected; however, when tested on proteinase-cleaved peptides, Asp-N-1, corresponding to the region from residues 126 to 336, did not significantly bind antibody (Fig. 6).

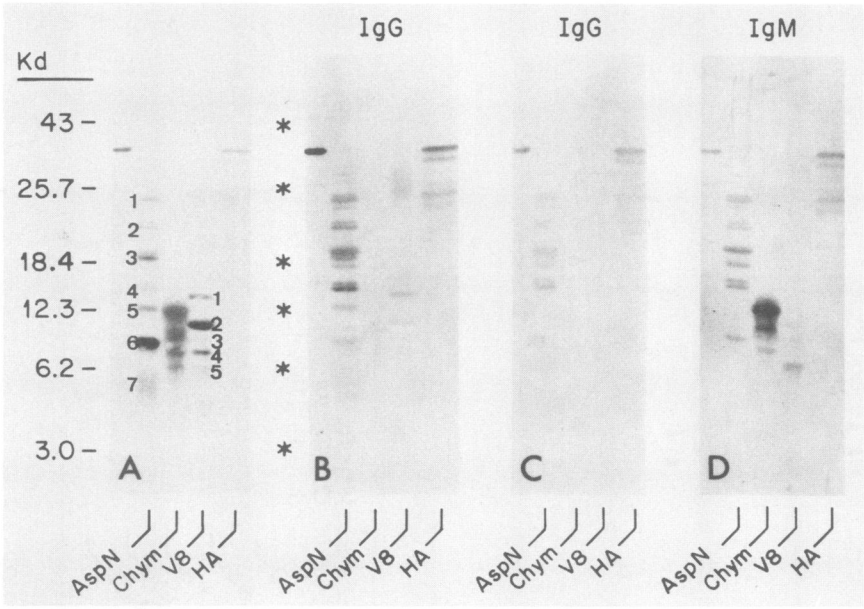

FIG. 4. Blots of an SDS–15% polyacrylamide gel, showing p41 (unlabeled lanes) and p41 cleaved with endoproteinase Asp-N (lanes AspN), α-chymotrypsin (lanes Chym), V8 protease (lanes V8), or hydroxylamine (lanes HA). The panels were stained with amido black (A) or reacted with pooled sera from patients with late Lyme disease (B) or EM (C and D). Antibody binding was detected with goat anti-human IgG (B and C) or goat anti-human IgM (D).

DISCUSSION

Definition of the relevant immunogenic domains of important microbial antigens has become a major concern in the design of specific diagnostic reagents and possibly for immunotherapy. We used peptide mapping combined with Western blot analysis to identify the major primary sequence epitopes of the native flagellin of *B. burgdorferi* that are reactive with mouse and human antibodies. The method we employed complements previous studies that have used synthetic peptides, recombinant p41 proteins, and deletion constructs. These approaches are limited, however, by the fact that discontinuous epitopes dependent on the secondary or tertiary structure of the protein may be destroyed by proteinase cleavage or removal of a relevant part of the molecule. We deduced the carboxy-terminal position of each peptide by determining the apparent molecular weight by SDS-PAGE and by noting predicted cleavage sites for that particular endoproteinase or for hydroxylamine from the published DNA sequence of p41. As has been noted by

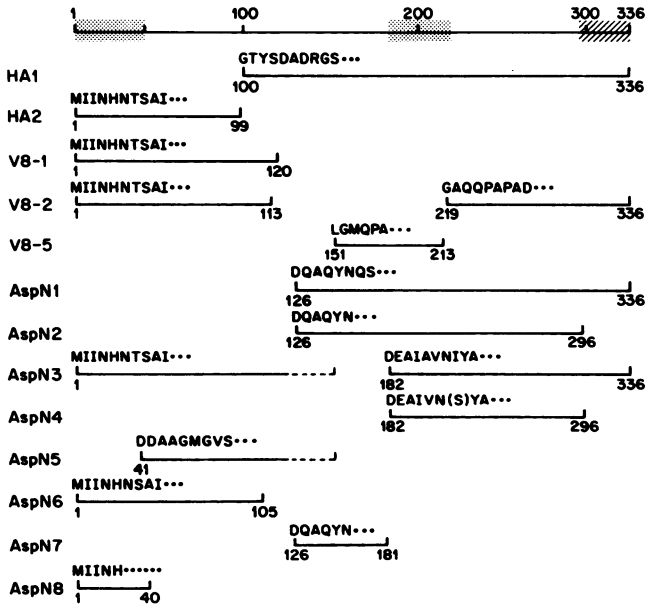

FIG. 5. Amino-terminal peptide sequences and epitopes mapped for the flagellin of *B. burgdorferi* B31. Amino-terminal sequences of each peptide are shown on the left, and the numbers under each line indicate the N- and C-terminal residues.

TABLE 1. Antibody binding to p41 and its cleavage products

Class	Binding[a] by:				
	IgG MAb 158	IgM EM patient sera	IgG from:		Syphilis patient sera
			EM patient sera	Late lyme disease patient sera	
Flagellum	+	+	+	+	+
HA1	+	+	+	+	ND
HA2	−	−	−	±	ND
V8-1	−	−	−	+	ND
V8-2	+	±	−	+	ND
V8-5	−	+	−	−	ND
Asp-N-1	+	+	+	+	−
Asp-N-2	−	+	+	+	*
Asp-N-3	+	+	+	+	+
Asp-N-4	−	+	+	+	*
Asp-N-5	−	−	−	+	+
Asp-N-6	−	+	−	+	+
Asp-N-7	−	−	−	−	*
Asp-N-8	−	−	−	−	*

[a] +, binding present; −, no binding; ±, weak binding; *, not reliably visualized on gels; ND, not determined.

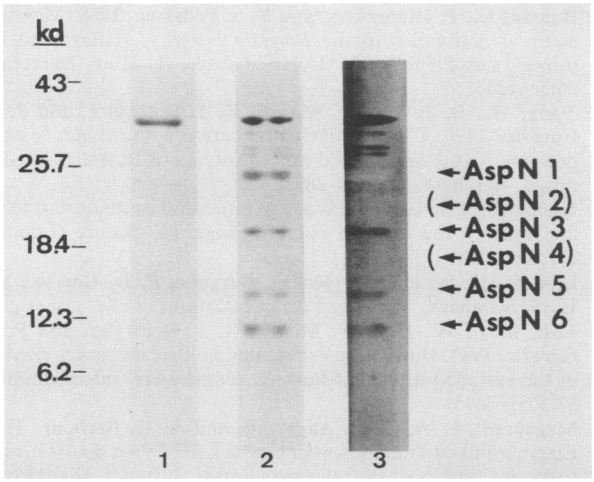

FIG. 6. Lane 1, purified p41; lane 2, digest obtained with endoproteinase Asp-N. The latter was separated on an SDS–15% polyacrylamide gel and electroblotted to Immobilon PVDF membranes. Membranes were stained by amido black (lanes 1 and 2) or reacted with pooled sera from 10 patients with secondary syphilis (lane 3). Antibody binding was detected with alkaline phosphatase-conjugated goat anti-human IgG.

others (8), *B. burgdorferi* flagellin migrates at 41 kDa during SDS-PAGE, whereas its calculated molecular mass is only 35.7 kDa, a difference that has been attributed to posttranslational modification. In contrast to the other peptides examined, we found the amino-terminal third (residues 1 to 125) of this molecule to consistently migrate on gels at a higher apparent molecular weight than would be expected, thus suggesting that this part of the molecule may be responsible for the anomalous migration.

Using recombinant flagellar proteins, Collins and Peltz (6) found that a murine MAb (H9724) recognized an epitope within residues 90 to 266 of p41, the so-called nonconserved region of flagellins that is distinct among species. This MAb is of particular interest because it has been utilized by two groups to demonstrate cross-reactivity with an antigenic determinant on myelinated fibers of peripheral nerve, thus providing a mechanism that may induce autoimmune responses that may contribute to the pathogenesis of neuroborreliosis (22). Gassman et al. (8) synthesized a series of overlapping octapeptides with a commercial epitope-scanning kit to screen rabbit polyclonal antisera made to whole *B. burgdorferi*, sonicates of the organism, and gel-purified p41; these studies identified a number of relevant epitopes spanning the nonconserved region but also extending into the conserved sequences at the ends of the molecule. Two MAbs specific for p41 were notable for reactivity directed to a specific epitope in the conserved region at the N terminus (8). Peptide mapping of the epitope bound by our MAb 158 demonstrates that the carboxy-terminal domain (residues 300 to 336) is another potential binding site for murine MAbs.

Recombinant p41 has also been used to map the relevant epitopes binding sera of patients infected with *B. burgdorferi*. Of note, Gassman et al. (8) found reactivity of 35% of sera from patients with Lyme borreliosis by Western blot analysis to a p41-TrpE fusion protein that contained the central part of the protein. Collins and Peltz (6) studied 15 serum specimens, including 5 from our area in which Lyme borreliosis is endemic, and found that all bound recombinant flagellin by immunoblot analysis, with specific reactivity directed to an epitope within the first 90 N-terminal residues of the molecule. Our results, from pooled sera from five patients with early disease and another five with late disease, are more in agreement with those recently reported by Berland et al., who studied 18 serum specimens, including specimens from both patients with EM and those with chronic disease, against a larger series of glutathione-S-transferase p41 proteins and found the immunodominant domain to be centered in amino acids 197 to 241 in the part of the molecule that is not homologous to other flagellins (2).

Comparing IgM and IgG anti-p41 responses in pooled sera of EM patients, we found the IgM response to be stronger on the basis of the intensity of antibody binding to Western blots. This is consistent with the fact that the IgM response to p41 occurs prior to the IgG response in early Lyme disease (5, 7). Our data show that the specific primary IgM response to the flagellin is directed to the same domains (on both amino-terminal and the middle domains) as the IgG response. Others (8) have reported that this domain was recognized by rabbit antibodies raised against *B. burgdorferi*. Although Lyme disease can usually be distinguished clinically from syphilis (*Treponema pallidum*) and relapsing fever (*Borrelia hermsii*), false-positive reactions to these and other spirochetes have complicated development of reliable serologic tests (10, 17). Pooled sera from 10 syphilis patients bound p41 when tested by Western blot analysis against both native and recombinant proteins. However, when the sera were tested with proteinase-cleaved p41 peptides, we found that the region from residues 126 to 336 did not bind. Similar results were found with proteinase-cleaved recombinant p41 (unpublished observations). Thus, the use of peptides that include the sequence from 126 to 336 as antigens in standard ELISAs might be predicted to avoid false-positive reactions present in the sera from patients with syphilis. The published DNA sequences of the major endoflagellar protein of *B. burgdorferi* show overall more than 30% identical residues in the variable regions of the molecule in comparison with the sequence of the FlaB2 flagellin of *T. pallidum* cloned by Palleson and Hindersson (19). No continuing identical sequence longer than 6 residues is apparent in this portion of p41 or FlaB2. This suggests in turn that false-positive reactions may be limited or nonexistent in this variable domain. Nevertheless, because of difficulty in obtaining sufficient native flagella to assess a large number of individual serum specimens, it remains possible that future studies will demonstrate greater variability in the anti-p41 antibody response in infected persons.

Ultrastructural studies have shown that the purified flagellum is a tubular structure (1). X-ray fiber analysis of the flagellum of *Salmonella typhimurium* (18) demonstrated that flagellin monomers fold side by side in a packing arrangement to form the filament structure. In these studies, three domains were identified: domains 1 and 2 are the amino and carboxy termini of the flagellin, and domain 3, which is exposed to the outside of the filament, represents the central and more variable portion of the molecule. Domains 1 and 2 contain predicted coiled-coil segments indicative of functional importance and are felt to be necessary for quaternary interactions of subunits and for export through a specific pathway. In other species, domain 3 may be variable in length and structure and can even be replaced or deleted without a loss of function. Since domain 3 corresponds to residues 130 to 250 of the flagellin of *B. burgdorferi* (14), it will be important to determine whether the *B. burgdorferi* flagellum conforms to a similar ultrastructural pattern, and

ultimately determination of epitopes on the native protein that are involved in antibody binding may require three-dimensional analysis of immune complexes.

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ADDENDUM

Since submission of our manuscript, Schneider et al. (21a) have reported the reactivity of sera from patients with late Lyme disease, but not syphilis, with an epitope in the central region of the flagellar protein.

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