Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 183–187, Janaury 1993
Immunology

Serologic response to the *Borrelia burgdorferi* flagellin demonstrates an epitope common to a neuroblastoma cell line

EROL FIKRIG*†, ROBERT BERLAND*¶, MANCHUAN CHEN*, STEPHANIE WILLIAMS‡, LEONARD H. SIGAL‡, AND RICHARD A. FLAVELL*§

*Section of Immunobiology and §Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510; and ‡Departments of Medicine and Microbiology–Immunology, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, New Brunswick, NJ 08903-0019

Communicated by Edward A. Adelberg, August 12, 1992

ABSTRACT **Antibodies in sera of 7 patients with neurologic manifestations of Lyme borreliosis and a monoclonal antibody (mAb H9724) to the flagellin of *Borrelia burgdorferi* have been shown to bind neural tissue. To identify the antibody binding site common to the *B. burgdorferi* flagellin and the neural tissue, we made recombinant fusion proteins expressing epitopes of flagellin. Antibodies in patients' sera and mAb H9724 bound within an 18-amino acid epitope (residues 208–225) in the central region of flagellin, whereas two other mAbs bound to epitopes mapping elsewhere in the protein. Antibodies in patients sera and mAb H9724 also bound to a human neuroblastoma cell line. Absorption of patients sera with a peptide, EGVQQEGAQQPA, corresponding to amino acids 213–224 of flagellin, inhibited binding to the neuroblastoma cell line. The data suggest that the immune response to a specific B-cell epitope within flagellin, shared by a human neuroblastoma cell line, may be involved in the pathogenesis of neuroborreliosis.**

Lyme borreliosis, a multisystem infection caused by the spirochete *Borrelia burgdorferi* (Bb), can cause neurologic disease (1). Acute neuroborreliosis includes meningitis, cranial neuritis, and radiculoneuritis. Chronic neurologic involvement includes encephelopathy, polyneuropathy, and leukoencephalitis (1, 2). The pathogenesis of neuroborreliosis, however, is unclear (3). Examination of the cerebrospinal fluid (CSF) of patients with neuroborreliosis shows a mononuclear pleocytosis, the presence of antigen-specific T-cells, and intrathecal production of Bb-specific antibody, suggesting that a local immune response may be involved in the disease (4–6). In some cases, spirochetes have been cultured from the CSF of patients with neurologic symptoms, implicating local infection as a cause of tissue destruction (7). However, the organism has never been seen in or grown from biopsy specimens of affected nervous tissue. Nevertheless, experiments using mice have suggested that spirochete virulence may play a role in the development of neurologic infection (8). The disease, therefore, may be due to a direct effect of spirochetes at the site of infection, the host response to Bb, or the host response to tissue antigens that may mimic those of Bb.

Antibody may play a role in the development of neurologic Lyme disease. Antibodies to the Bb flagellin are prominent in serum during infection, and local CSF antibody production to spirochetal antigens, including flagellin, occurs (6, 9, 10). Furthermore, sera from patients with neurologic manifestations of Lyme disease have IgM antibodies that bind human axons (11). Binding has been shown to be weak or absent in patients without neurologic disease. Similar results occur with a mouse monoclonal antibody (mAb H9724) to flagellin (11). The same human antibodies that bind to human axons, and mAb H9724, bind to human neuroblastoma cell lines SK-N-SH, SK-N-HC, and IMR-32, as well as rat neuroblastoma cell lines B103-6 and B104, rat phenochromocytoma cell line PC12, and calf adrenal tissue, but not to mouse neuroblastoma cells (L.H.S., unpublished data). mAb H9724 also binds to myelinated fibers of peripheral nerve and other human tissues, including epithelial cells in the joint synovium and on heart muscles (13). This suggests that the immune response to an epitope shared by the Bb flagellin and human tissue may play a role in the pathogenesis of Lyme borreliosis.

We used a polymerase chain reaction (PCR)-based molecular mapping approach to show that antibodies in sera from patients with late Lyme disease strongly bind epitopes in the central region of flagellin (14). Other regions of flagellin are recognized with less frequency. Our data correlate with epitope mapping studies of flagellin that used animal sera and synthetic decapeptides (15). Both these results differ from the work of Collins and Peltz (16), who showed that antibodies in patients' sera bind to epitopes only within the N-terminal region of flagellin. Here we show that antibodies in the sera of patients with neuroborreliosis and mAb H9724 bind to a 12-amino acid (aa) sequence, residues 213–224, in the central portion of flagellin. Moreover, incubation of patients' sera or mAb H9724 with a peptide corresponding to this antibody binding site eliminated binding to neuroblastoma cells. This suggests that the immune response to a B-cell epitope common to the Bb flagellin and neural tissue may be involved in the pathogenesis of neuroborreliosis.

MATERIALS AND METHODS

Plasmid Construction and Recombinant Fusion Protein Expression. Plasmids expressing 11 overlapping regions of flagellin were prepared by using the sequence of flagellin from Bb B31 and the pMX vector (14, 17, 18). Eleven fragments of flagellin, designated A–K, were expressed as fusion proteins with glutathione transferase. The fusion proteins contained the following amino acid sequences in flagellin: A, 1–37; B, 1–86; C, 29–104; D, 96–173; E, 165–241; F, 197–241; G, 197–273; H, 228–273; I, 228–311; J, 260–311; K, 290–336.

In addition, eight plasmids, designated F1–F8, including overlapping epitopes within fragment F were constructed. Oligonucleotides corresponding to nucleotides 583–621 (aa 195–207) (F1), 595–633 (aa 199–211) (F2), 610–648 (aa 204–

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: aa, amino acid(s); Bb, *Borrelia burgdorferi*; CSF, cerebrospinal fluid; mAb, monoclonal antibody.

†Present address: Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06510.

¶Present address: Department of Hematology/Oncology, Division of Medicine, New England Medical Center, 750 Washington Street, Boston, MA 02111.

216) (F3), 622–660 (aa 208–220) (F4), 634–675 (aa 212–225) (F5), 646–684 (aa 216–228) (F6), 664–702 (aa 222–234) (F7), and 682–720 (aa 228–240) (F8) were synthesized at the Yale Oligonucleotide Synthesis Center. Oligonucleotides corresponding to both DNA strands were flanked by overhanging *Eco*RI and *Bam*HI restriction enzyme sites, 5′ and 3′ respectively, to facilitate subcloning. Individual plus- and minus-strand oligonucleotides, in equimolar amounts (1 mM), were mixed together in water. The mixture was heated to 90°C for 5 min and cooled to room temperature overnight. The annealed oligonucleotides were cloned into pMX, and the recombinant plasmids were used to transform *Escherichia coli* strain DH5α. The flagellin epitopes were expressed in *E. coli* as glutathione transferase fusion proteins and purified by passing a lysate of *E. coli* over a glutathione column (14).

Serum Collection. With the exception of two samples, patients' sera were collected at the Yale Lyme Disease Clinic in the Section of Rheumatology, Yale University School of Medicine. All were positive in a standard enzyme-linked immunosorbent assay for Lyme disease. Patients 1–7 had erythema migrans. Patients 8–12 had neuroborreliosis (meningoencephalitis, peripheral neuritis, cranial nerve palsy, or weakness and radiculoneuritis). All the other patients seen at Yale had Lyme arthritis. Sera were drawn from the patients when the characteristic symptoms were present. Sera from two patients with neuroborreliosis were collected at the Robert Wood Johnson Medical School.

mAbs and Synthetic Peptides. mAbs H9724 (IgG2a) and H604 (IgG2a) were kindly provided by Alan G. Barbour, University of Texas Health Science Center at San Antonio. mAb X1161B (IgG2b) was produced at Yale from a hybridoma prepared against Bb N40 antigen by standard techniques. Peptides were synthesized at the Yale Protein Synthesis Center.

Neuroblastoma Cell Culture. Neuroblastoma cell line SH-K-SH was obtained from the American Type Culture Collection (no. HTB 11). Cells were grown to confluence in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine (300 μg/ml), penicillin (0.2 unit/ml), and streptomycin (200 μg/ml).

Immunoblots. Recombinant fusion proteins were boiled for 5 min in SDS sample buffer containing 2% SDS, 100 mM dithiothreitol, and 50 mM Tris (pH 7.0). Five hundred nanograms of recombinant antigen was electrophoresed in an SDS/12.5% polyacrylamide gel and electroblotted onto nitrocellulose. Filters were incubated for 1 hr with 5% nonfat dry milk in phosphate-buffered saline (PBS) to block nonspecific sites and then were allowed to react with patients' sera or mAbs diluted 1:100 in blocking buffer. The filters were washed with PBS three times and then incubated for 1 hr with 1 mCi (37 MBq) of I^{125}-labeled goat anti-mouse or anti-human immunoglobulin (Amersham) in blocking solution. The filters were washed three times and air-dried. Antibody binding was visualized by autoradiography.

Indirect Immunofluorescence. Coverslips were treated overnight with 0.1 M HCl, autoclaved, then placed into 35-mm Petri dishes. Two milliliters of culture medium containing 2×10^5 cells was placed in each plate. Cells were allowed to attach for 24 hr, then the medium was aspirated and coverslips were washed three times with PBS. The coverslips with cells were incubated in 3.7% formaldehyde in PBS for 15 min and washed in PBS three times. Coverslips with cells were incubated with serum for 30 min at 37°C, washed three times, incubated with fluorescein-labeled goat anti-human gamma globulin (diluted 1:30) for 30 min at 37°C, and again washed three times. Each coverslip, with a drop of mounting oil, was placed on a slide and examined by fluorescence microscopy.

Serum Absorption. Peptide was prepared at 4 mg/ml in PBS. Patients' sera and mAbs were diluted 1:640. Equal volumes of the peptide and the patients' sera or mAbs were mixed and incubated for 1 hr at room temperature. The samples were centrifuged for 10 min at 8000 × *g* and the supernatants were used for immunofluorescence assays.

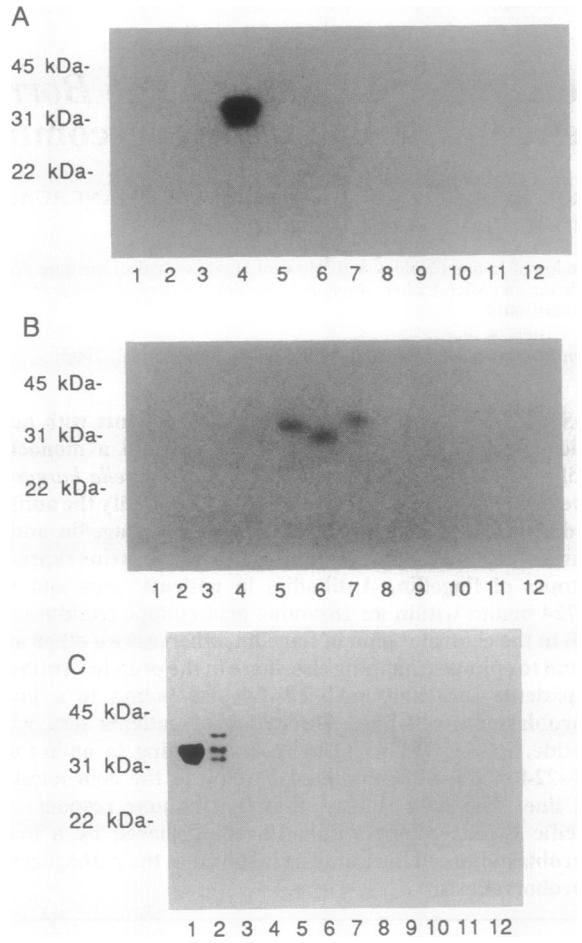

FIG. 1. Immunoblots of recombinant glutathione transferase fusion proteins containing fragments of the Bb flagellin, probed with X1161B (*A*), H9724 (*B*), and H604 (*C*) mAbs to flagellin. Lanes: 1, fragment A; 2, fragment B; 3, fragment C; 4, fragment D; 5, fragment E; 6, fragment F; 7, fragment G; 8, fragment H; 9, fragment I; 10, fragment J; 11, fragment J; lane 12, glutathione transferase (control).

RESULTS

We used 13 recombinant fusion proteins expressing regions of flagellin to identify the epitopes which bind to three monoclonal antibodies. mAb H9724 bound within the central region of flagellin fragment F (aa 197–241) (Fig. 1*B*). As

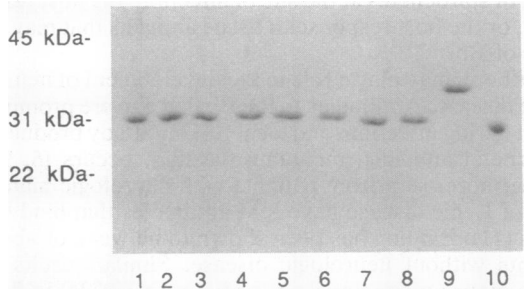

FIG. 2. Coomassie blue-stained SDS/polyacrylamide gel of eight recombinant glutathione transferase fusion proteins expressing epitopes of flagellin within fragment F. Lanes 1–8, F1–F8; lane 9, fragment F; lane 10, glutathione transferase (control).

expected, binding to two fragments (E and G) that encompass F was apparent. mAb X1161B bound closer within the N-terminal region of flagellin fragment D (aa 96–173) (Fig. 1*A*). A third mAb, H604, bound to an epitope within the first 37 aa of flagellin (Fig. 1*C*).

Since mAb H9724 has been demonstrated to bind to neural tissue, additional recombinant fusion proteins (F1–F8) were produced that expressed epitopes within fragment F (Fig. 2). Immunoblots with these fusion proteins localized mAb H9724 binding to F4 and F5, which encompassed aa 208–225 (Fig. 3). The overlapping sequences contain aa 212–220. mAb H9724, therefore, most likely binds to an epitope including aa 212–220.

To determine the fine specificity of the human serologic response to epitopes within fragment F, we tested 39 patients' sera by immunoblotting using fragments F1–F8. Representative immunoblots are shown in Fig. 3 *B–D*. Normal human sera showed no reactivity (data not shown). Table 1 indicates that 37 of 39 patients showed reactivity with fragment F. Of the 37 sera which bound fragment F, 29 sera bound to F4 or F5. Specifically, 24 patients' sera bound to F4 and 21 bound to F5. Patients 8–12 had neurologic symptoms. All 5 sera bound F5 and sera from patients 8, 9, 11, and 12 bound F4. In addition, sera from 5 of 7 patients with erythema migrans bound F4 and F5. Sera from 15 of 27 patients with arthritis bound F4, and sera from 11 of 27 patients with arthritis bound F5. Sera from patients with erythema migrans, arthritis, or neuroborreliosis also bound epitopes of flagellin, in addition to the central region, as described by Berland *et al.* (14). Furthermore, the quantitative antibody response, determined by the intensity of binding to fragment F on immunoblots, was similar in patients with erythema migrans, neuroborreliosis, or arthritis.

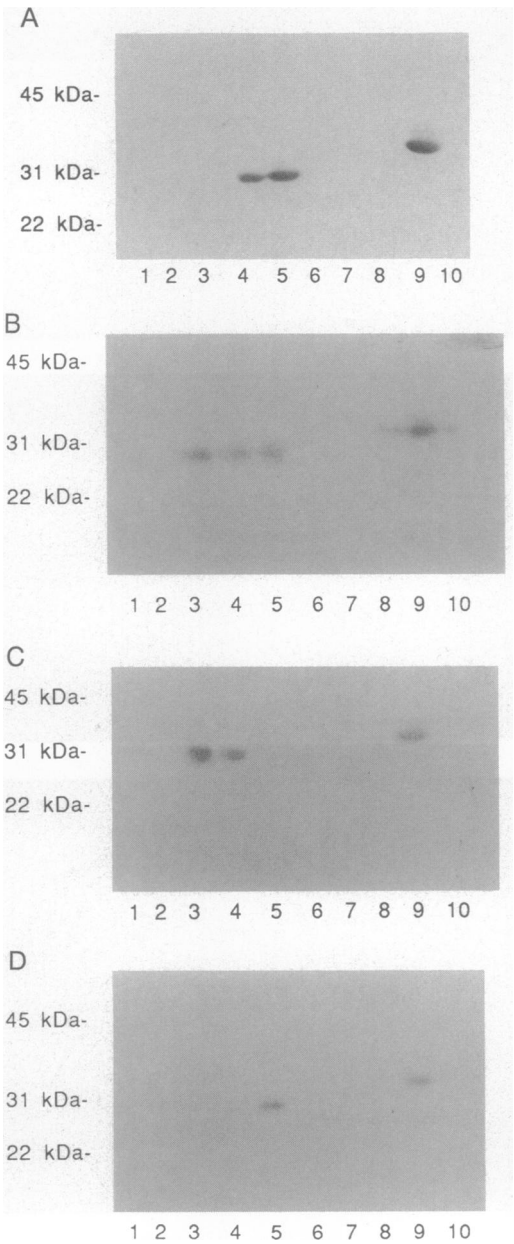

FIG. 3. Immunoblots of glutathione transferase fusion proteins expressing epitopes of fragment F, probed with mAb H9724 (*A*) and sera from patients with Lyme borreliosis (*B–D*). Lanes 1–8, F1–F8; lane 9, fragment F; lane 10, glutathione transferase (control).

Table 1. Binding of sera from patients with Lyme borreliosis to epitopes F1–F8 within the central region of fragment F of the Bb flagellin

Disease manifestation/ patient number	Flagellin epitope								
	1	2	3	4	5	6	7	8	9
Erythema migrans									
1		+	+	+					+
2			+	+	+				+
3				+	+	+			+
4			+	+					+
5				+	+				+
6					+	+			+
7					+				+
Neuroborreliosis									
8			+	++	++	+			++
9			++	++	++				++
10					++	++	+		++
11				+	+				++
12				+	++				++
Arthritis									
13	+	+	++	++	++	++			++
14		+	+	+	+				++
15			+	++	++	++			++
16			+	++	++	++			++
17				+	++	++	+		++
18	+			+	+				++
19			++	++	++				++
20				+	+	+			+
21	+			+					++
22			++	+					+
23			+	+					+
24				+	+				++
25					+	++			++
26						+	+		++
27			++						++
28			+						+
29				++					++
30				+					+
31				+					+
32					+				+
33									+
34									+
35									+
36									+
37									+
38									
39									

Patients are categorized by disease manifestation. Weak to moderate binding, +; moderate to strong binding, ++. Columns 1, F1 (aa 95–207); 2, F2 (aa 199–211); 3, F3 (aa 204–216); 4, F4 (aa 208–220); 5, F5 (aa 212–225); 6, F6 (aa 216–228); 7, F7 (aa 222–234); 8, F8 (aa 228–240); 9, fragment F (aa 197–241).

Immunoblots with absorbed sera showed that antibodies in sera from patients with neuroborreliosis specifically bound to an epitope within aa 213–224 of flagellin. Immunoblots of the flagellar fragments A–K and fragments F1–F8, as described for Figs. 1 and 2, respectively, probed with serum from patient 11, showed a binding pattern identical to mAb H9724. Sera from patients with Lyme arthritis that bound to F4 and F5 also bound other epitopes within the central region of flagellin (Fig. 3*B*) with the exception of patient 24 (Table 1). In addition, sera from patients with arthritis bound other regions of flagellin outside the central region, as previously described (14). Serum from patient 11 was then incubated with a peptide corresponding to aa 213–224. Immunoblots with the absorbed serum showed that binding to flagellin (fragments F, F1–F8) was eliminated (Fig. 4). Binding to flagellin fragments A–K was also eliminated. Absorption experiments with sera from two other patients with neuroborreliosis, and mAb H9724, gave similar results.

To determine whether sera from patients with neurologic Lyme disease and mAb H9724 bound to a neuroblastoma cell line, we performed indirect immunofluorescence studies (Fig. 5). The results, in agreement with previous work indicating that mAb H9724 and antibodies in patients' sera bind to human tissue, showed that mAb H9724 and sera from patients with neurologic disease bound to neuroblastoma

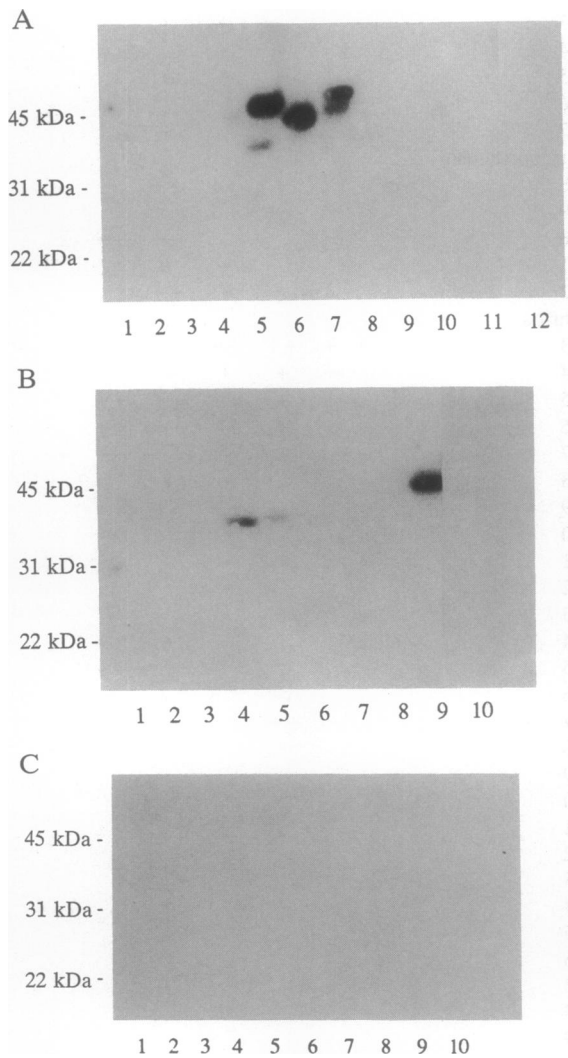

FIG. 4. Immunoblots of flagellin fragments A–K and F1–F8 probed with sera from patient 11 (Table 1). Lanes with fragments A–K (*A*) and F1–F8 (*B* and *C*) are described in Figs. 1 and 2. Patient serum was incubated with peptide EGVQQEGAQQPA for *C*.

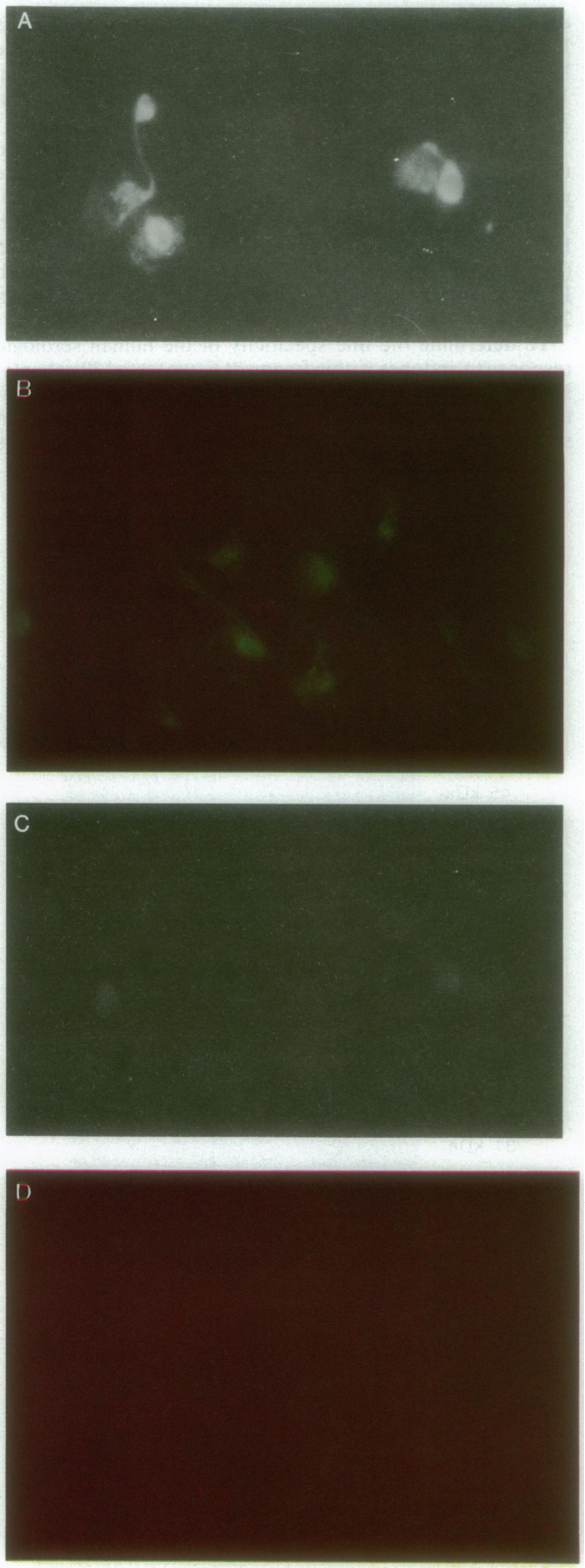

FIG. 5. Indirect immunofluorescence of a human neuroblastoma cell line, stained with serum from a patient with neuroborreliosis (*A* and *C*) or mAb H9724 (*B* and *D*) without (*A* and *B*) or with (*C* and *D*) preabsorption with a peptide corresponding to amino acids 213–224 of the Bb flagellin.

cells (Fig. 5 *A* and *B*). Sera from several patients with Lyme arthritis did not show significant binding to the neuroblastoma cell line (data not shown), as expected from previous studies (11). mAb H604, which binds to a flagellar epitope near the N terminus, and which is the same isotype (IgG2a) as H9724, did not bind to neuroblastoma cells. In addition, both fixed and unfixed neuroblastoma cells probed with mAb H9724 showed staining. This suggests that the crossreactive epitope is expressed, at least in part, on the cell surface (data not shown).

To determine whether binding in patients' sera was specific for this epitope, patient serum was first incubated with a peptide corresponding to aa 213–224 of flagellin. Immunofluorescence studies using the absorbed serum showed that binding to the neuroblastoma cell line was eliminated (Fig. 5*C*). Incubation of the mAb H9724 with peptide also eliminated binding to the cell line (Fig. 5*D*).

DISCUSSION

In this study we identified an antibody binding site shared by the Bb flagellin and neural tissue. This epitope of flagellin (aa 213–224) was also recognized by mAb H9724. This study is in agreement with our previous work showing that the central region of flagellin is immunodominant (14). Our work also extends the results of Schneider *et al.* (19), showing that antibodies in sera from patients with late Lyme disease bind to epitopes on various regions of flagellin, including aa 121–148, 205–226, and 253–280. The central region (aa 205–226) was heterologous to the amino acid sequences of other bacterial flagellin, and mAb H9724 binds within this region. Since mAb H9724 binds to human tissue, we evaluated the humoral response to the central region of flagellin (aa 197–241) in 39 patients with Lyme disease. Thirty-seven sera showed binding, and the majority showed binding to aa 208–225. Sera from all five patients with neuroborreliosis showed binding within the region identified by mAb H9724.

Sera from patients with neuroborreliosis and mAb H9724 bound to a neuroblastoma cell line. Absorption of the sera or mAb H9724 with the dodecapeptide corresponding to aa 213–224 of flagellin eliminated the binding. These data indicate that antibody to the epitope containing aa 213–224 accounted specifically for the crossreactivity seen between antibodies to flagellin and neural tissue.

The role of antibody in the pathogenesis of infectious diseases is not fully understood. A 12-amino acid epitope on the Fl-160 antigen of *Trypanosome cruzi* trypomastigotes mimics a binding site on neural tissue (20), and this crossreactivity may cause pathologic damage in chronic Chagas disease. Previous work has shown that Bb flagellin mAb H9724 binds to a human axonal antigen (11). Whether direct antibody-mediated damage is part of the immunopathogenesis of Lyme disease is uncertain. However, the presence of high antibody titers in the CSF correlates with clinical signs of neuroborreliosis (4). Interestingly, while C3H mice infected with a cloned strain of Bb N40 develop severe arthritis and carditis, the animals do not develop nervous system infection, symptoms, or pathology (8). Furthermore, neural tissue from mice does not react with mAb H9724, suggesting that murine neural tissues do not contain the crossreacting epitope (L.H.S., unpublished data). This suggests that antibody that binds to a specific epitope of the Bb flagellin and crossreacts with human neural tissue may play a role in the pathogenesis of disease.

It has been suggested that the peripheral nerve lesions in Lyme borreliosis and experimental allergic neuritis, a T cell-mediated disorder, are similar (13). While T-cell clones have been isolated from patients with Lyme borreliosis, their role in the pathogenesis of disease is not known (21). It is interesting, however, that some patients with Lyme central nervous system disease do have antigen-specific T cells in the CSF (5).

The pathogenesis of Lyme borreliosis is just beginning to be elucidated. Three working hypotheses can be advanced. One is that differences in the spirochete, including the production of specific virulence factors, may account for differences in disease. Other studies indicate that host susceptibility may be a factor. Third, autoreactivity due to the host immune response and possibly predicated on molecular mimicry may be involved (12). This study identified the epitope of flagellin which is bound to mAb H9724. This epitope is recognized by antibodies from patients with neuroborreliosis, and binding of patients' sera and H9724 to a human neuroblastoma cell line can be eliminated by absorption with peptide. The data suggest a role for human antibodies that crossreact with the Bb flagellin and neural tissue in the pathogenesis of neuroborreliosis.

We thank Fred S. Kantor for providing mAb X1161B; Dan Rahn, John A. Hardin, and Stephen E. Malawista for providing patients' sera; Alan G. Barbour for providing mAbs H9724 and H604; and Hong Tao, Nancy Marcantonio, and Kathleen Deponte for assistance. This work was supported by National Institutes of Health Grants P01-AI30548 and R01-AR40452; a Lederle Young Investigator Award from the Infectious Disease Society of America; a Dean's Faculty Award, Centers for Disease Control Grant U5-CCU-106581; the Mathers Foundation; a grant from the Arthritis Foundation, New Jersey chapter; the Foundation of the University of Medicine and Dentistry of New Jersey; and the Morris L. Sigal Medical Research Foundation. R.A.F. is an Investigator of the Howard Hughes Medical Institute.

1. Steere, A. C. (1989) *N. Engl. J. Med.* **321,** 586–596.
2. Pachner, A. R., Duray, P. & Steere, A. C. (1989) *Arch. Neurol.* **46,** 790–795.
3. Sigal, L. H. (1989) *Semin. Arthritis Rheum.* **18,** 151–167.
4. Baig, S., Olsson, T., Hansen, K. & Link, H. (1991) *Infect. Immun.* **59,** 1050–1056.
5. Pachner, A. R., Steere, A. C., Sigal, L. H. & Johnson, C. J. (1985) *Neurology* **35,** 1642–1644.
6. Hansen, K., Cruz, M. & Link, H. (1990) *J. Infect. Dis.* **161,** 1194–1202.
7. Preac-Mursic, V., Wilske, B., Schierz, G., Pfister, H. & Einhaupl, K. (1984) *Eur. J. Microbiol.* **3,** 564–565.
8. Pachner, A. R. & Itano, A. (1990) *Neurology* **40,** 1535–1540.
9. Craft, J. E., Fischer, D. K., Shimamoto, G. T. & Steere, A. C. (1986) *J. Clin. Invest.* **78,** 934–939.
10. Baig, S., Olsson, T. & Link, H. (1989) *Lancet* **ii,** 71–74.
11. Sigal, L. H. & Tatum, A. H. (1988) *Neurology* **38,** 1439–1442.
12. Sigal, L. H. (1989) *Semin. Arthritis Rheum.* **18,** 151–159.
13. Aberer, E., Brunner, C., Suchanek, G., Klade, H., Barbour, A., Stanek, G. & Lassmann, H. (1989) *Ann. Neurol.* **26,** 732–737.
14. Berland, R., Fikrig, E., Rahn, D., Hardin, J. & Flavell, R. A. (1991) *Infect. Immun.* **59,** 3531–3535.
15. Gassman, G. S., Jacobs, E., Deutzmann, R. & Gobel, U. B. (1991) *J. Bacteriol.* **173,** 1452–1459.
16. Collins, C. & Peltz, G. (1991) *Infect. Immun.* **59,** 514–520.
17. Gassman, G. S., Kramer, M., Gobel, U. B. & Wallich, R. (1989) *Nucleic Acids Res.* **16,** 3590.
18. Sears, J. E., Fikrig, E., Marcantonio, N., Deponte, K., Kantor, F. & Flavell, R. A. (1991) *J. Immunol.* **147,** 1995–2001.
19. Schneider, T., Lange, R., Ronspeck, W., Weigelt, W. & Kolmel, H. W. (1992) *Infect. Immun.* **60,** 316–319.
20. VanVoorhis, W. C. V., Schlekewy, L. & Trong, H. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5993–5997.
21. Martin, R., Ortlauf, J., Sticht-Groh, V., Bogdahn, U., Goldmann, S. F. & Mertens, H. G. (1988) *Ann. Neurol.* **24,** 509–516.