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Molecular and Immunological Analysis of a Polymorphic Periplasmic Protein of *Borrelia burgdorferi*

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***Borrelia burgdorferi* is the causative agent of Lyme disease, a tick-borne spirochetosis with a worldwide prevalence. To assist the categorization and typing of fresh isolates from global foci, we have identified a unique species-specific periplasmic protein (P22-A) conserved among all North American and European isolates examined. The gene encoding this antigen was cloned, and the recombinant was used to screen serum collected from experimentally infected animals. Although antibodies were detected in all infected animals at 21 days after inoculation with live, low-passage spirochetes, the response was stronger in other animals that were inoculated with inactivated and lysed bacteria. This result, along with the immune electron microscopy data, suggests P22-A is concentrated in the periplasmic space. The P22-A antigens exhibited size heterogeneity among different isolates, ranging between 20 and 23 kDa, but as a group the P22-A antigens appeared to retain antigenic homogeneity. Thus, P22-A can serve as a structural marker for characterizing new isolates of *B. burgdorferi* and may prove useful in future serological assays with a mixture of *B. burgdorferi*-specific antigens.**

Lyme borreliosis is a worldwide disease caused by the spirochete *Borrelia burgdorferi* (10, 11, 22, 28, 33), the prevalence of which is apparently increasing within the United States (11). Unfortunately, as with many diseases that have an infectious origin, the clinical picture can mimic other maladies, at times making a definitive diagnosis of Lyme disease difficult. Although this disease is reported to have a global distribution, the causative agent has not been clearly defined or characterized for all the presumed endemic foci throughout the world. It remains possible that the disease reported in some countries (or parts of countries) is caused by a related but distinctly different organism. Furthermore, the pathogenesis of the disease may be influenced by putative differences between different isolates that still represent the same species. Thus, antigenic components of *B. burgdorferi* that can be shown to be species specific and conserved among well-described isolates will be useful in identifying and typing isolates from different geographic areas or from patients with different clinical manifestations. In addition, antigens that are specific for *B. burgdorferi* may also serve as components of serological tests or prove responsible for host immunity and thus may be suitable candidates for vaccine development.

Many components of *B. burgdorferi* are immunogenic, as evidenced by the numerous immunoreactive bands observed in gels immunoblotted with sera from infected humans (15, 19, 22, 24) and other animals (6, 14, 27, 29). Of these, however, only a few have actually been characterized with specific antibodies. The use of such antibodies ensures that the reactive bands associated with different cell lysates have the same antigen. It is necessary to exclude the possibilities that a single band observed in stained gels or in immunoblots with nonspecific sera represents several unrelated proteins and that the composition of a band corresponding to a specific molecular weight differs among strains. The outer surface proteins A (OspA) and B (OspB) (4, 5) are major constituents of the outer membrane, but their ability to elicit antibodies during infections is variable (7, 14), making them less than optimal serological antigens. Nevertheless, monoclonal antibodies specific for these lipoproteins (9) have been useful as markers for *B. burgdorferi*. Flagellin varies in its reactivity with immune sera (9, 29, 32), and polyclonal antibodies reactive with this antigen are not even genus specific. These facts indicate that the flagella of other *Borrelia* and non-*Borrelia* spirochetes share cross-reactive epitopes (3, 8, 12, 13, 32), a potential problem when flagellin is used as a diagnostic antigen.

A 39-kDa protein, designated P39, was recently identified, and its gene was cloned in *Escherichia coli* (32). Although its function is unknown, this antigen is specific for *B. burgdorferi*, and it appears to be conserved among both North American and European isolates. In addition to being a potential marker for *B. burgdorferi*, it elicits a reliable and specific antibody response in naturally and experimentally infected animals (32). The genes encoding 60-kDa (16) and 83-kDa (20) antigens have also been cloned. The 60-kDa antigen, however, is believed to be the common antigen which has cross-reactive epitopes with an analogous antigen produced by many other bacteria. The 83-kDa antigen appears to elicit antibodies during infection, but it is not clear if this antigen is immunogenically conserved among all isolates or if it is species specific. Like P39, however, this antigen may prove useful as a diagnostic antigen once additional human serological studies have been done with the recombinant or purified proteins. Wilske et al. (35, 36) described a 20- to 22-kDa protein (pC) from *B. burgdorferi* which is also species specific. pC, however, appears to vary antigenically (35), with some isolates failing to react with antiserum specific for pC. Although it is probably not suitable as a specific marker for *B. burgdorferi* because of this variation, pC appears to react to a significant number of human Lyme disease sera, indicating that this antigen could help to improve, in combination with other selected antigens, the specificity of laboratory tests. Jiang et al. (18) and Luft et al. (21) have also described immunoreactive antigens of *B. burgdorferi* of approximately 20 and 22 kDa.

In the present study, anti-*B. burgdorferi* serum was used

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to screen a recombinant library to identify proteins that could be used as specific markers. We describe a periplasmic protein that varies in molecular weight among isolates but is immunologically conserved and specific for *B. burgdorferi*. This antigen should, therefore, be a useful biological marker for characterizing new isolates of this spirochete.

MATERIALS AND METHODS

Bacterial strains. All *B. burgdorferi* strains, including cloned low- and high-passage variants of strain Sh-2-82 and the five additional species *B. hermsii* (HS1), *B. coriaceae* (Co53), *B. parkeri*, *B. turicatae*, and *B. anserina*, have been described previously (29, 31). *Borrelia* strains were cultured at 32°C in BSK-II medium as previously described (1).

Animal inoculations. The white-footed mice (*Peromyscus leucopus*) used were females between 8 and 12 weeks of age at the time of inoculation and were from a colony maintained at Rocky Mountain Laboratories (Hamilton, Mont.). Mice were bled from the retro-orbital sinus 1 day prior to inoculation and at 21 days postinoculation.

Mice were inoculated with viable or heat-inactivated spirochetes as described previously (29). Infections were confirmed by culturing the spleen and urinary bladder from animals killed at 21 days postinoculation as described previously (29).

Immunoblot procedures. Rabbit anti-P22-A serum (anti-pSPR37) was prepared in rabbits inoculated with an equal mixture of whole-cell lysates of *E. coli* (strain XL1-Blue; Stratagene) carrying plasmid pSPR29 and *E. coli* cells carrying pSPR37. The bacteria were cultured, the lysates were prepared for immunization, and the inoculation schedule was as described for the preparation of anti-pSPR33 serum (32). Anti-*E. coli* serum was raised in rabbits against whole-cell lysates of *E. coli* carrying only the cloning vector and has been described previously (32).

Mouse anti-P22-A serum was pooled from six white-footed mice inoculated with whole-cell lysates of *E. coli* carrying pSPR37. Lysates were prepared as described above for rabbit anti-P22-A serum, and mice were inoculated (without adjuvant) intramuscularly with 1 ml of the cell extract (0.5 ml per hind leg) and boosted with the same immunogen at 21 and 40 days after the primary immunization. Sera were collected 2 weeks after the last inoculation, pooled, and absorbed with *E. coli* cells as described previously (32).

Rabbit anti-*B. burgdorferi* serum was prepared as follows. Bacterial cells were recovered from a 500-ml stationary-phase BSK-II culture, washed once in phosphate-buffered saline (pH 7.0) (PBS), and resuspended in PBS at 10^8 cells per ml. The cells were killed by incubation at 56°C for 30 min and disrupted by sonication on ice (2 min at an output of 4; Sonifier cell disrupter model 185; Branson Sonic Power Co., Danbury, Conn.). A New Zealand White rabbit was immunized intramuscularly with 1.5 ml of a sonic cell extract that had been emulsified with an equal volume of complete Freund's adjuvant and was boosted with the same immunogen (without adjuvant) at 21 and 42 days after primary exposure. Rabbit serum was collected every 2 weeks thereafter for 3 months, pooled, and stored at −20°C. Immunoblot analyses of whole-cell lysates and animal sera or monoclonal antibodies were done as described previously (29).

DNA methodologies. The *B. burgdorferi* library and screening procedures were as reported previously (32), except that clones were screened with anti-*B. burgdorferi* serum. Total DNA was purified from 500-ml stationary-phase *Borrelia* cultures, and recombinant plasmids were isolated from *E. coli* as reported previously (32). The cloning vector pBluescript SK was obtained from Stratagene, and subcloning strategies were as recommended by the supplier.

Undigested DNA was electrophoresed in 0.4% agarose gels (12 V for 16 h) and Southern blotted as described previously (30, 32). Digested DNA was electrophoresed in 0.7% agarose gels (12 V for 16 h). The DNA probes were isolated from agarose gels with GeneClean (Bio 101, Inc., Gaithersburg, Md.) and labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by nick translation in accordance with the manufacturer's (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) directions. Probes were boiled for 4 min and cooled rapidly on ice immediately before addition to the hybridization solution. The prehybridization and hybridization solution was comprised of 50% formamide, 0.9 M sodium chloride, 0.9 M sodium citrate, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, 0.1% sodium PP_i, and 100 μg of denatured salmon sperm DNA per ml and was used as described previously (30, 32). Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used in accordance with the manufacturer's directions.

Immune electron microscopy. *B. burgdorferi* cells were routinely removed from BSK-II stationary-phase cultures by centrifugation at 15,000 rpm for 20 min in a TOMY MTX-150 apparatus (RM TMS 4 rotor and RB-MS415-04 buckets). Cells were sometimes recovered at 3,000 rpm for 30 min to reduce the loss of the slime layer or reduce damage to the outer membrane. Ultracentrifugation (100,000 rpm) was also performed in a Beckman TL-100 centrifuge with a TLA 100 rotor to concentrate P22-A from the supernatant. Cells and other pelleted material from culture supernatants were fixed either in a periodate-lysine-paraformaldehyde fixative (23) or in a water-soluble carbodiimide [1-ethyl-3 (3-dimethyl-aminopropyl) carbodiimide-HCl] fixative as described by Yamamoto and Yasuda (37). Some preparations fixed with the latter fixative contained ruthenium red at 0.1 mg/ml to aid in the stabilization of the slime layer. The fixative was removed, and each sample was placed in 0.1 M sodium phosphate–5 mM $MgCl_2$ buffer (pH 7.0) containing 2.3 M sucrose for infusion. Infusion was done for at least 1 h prior to sectioning. Samples were stored at room temperature in Eppendorf tubes with O-ring caps.

Cryosectioning was done with a Reichert FC4D cryoultramicrotome as described previously (34). A temperature differential of 2 to 3°C was maintained between the sample and the knife edge, and the phosphotungstic acid concentration was as described previously (17). Sections were 60 nm thick, and immunolabeling and negative staining were performed as described previously (2), except that staining was done with 0.3 to 0.5% phosphotungstic acid or ammonium molybdate.

RESULTS

Cloning of *B. burgdorferi* DNA. A DNA library of *B. burgdorferi* was constructed with *Eco*RI fragments and screened for the expression of immunoreactive antigens with a rabbit antiserum raised against a *B. burgdorferi* whole-cell lysate (anti-*B. burgdorferi* serum). Phagemid clones that expressed antigens immunoreactive for *B. burgdorferi* were detected at a frequency of 10%, two were selected at random, and the plasmid components, designated pSPR29 and pSPR30, were excised from the phage DNA constructs. Each clone contained a 2.5-kb *Eco*RI fragment. The 2.5-kb

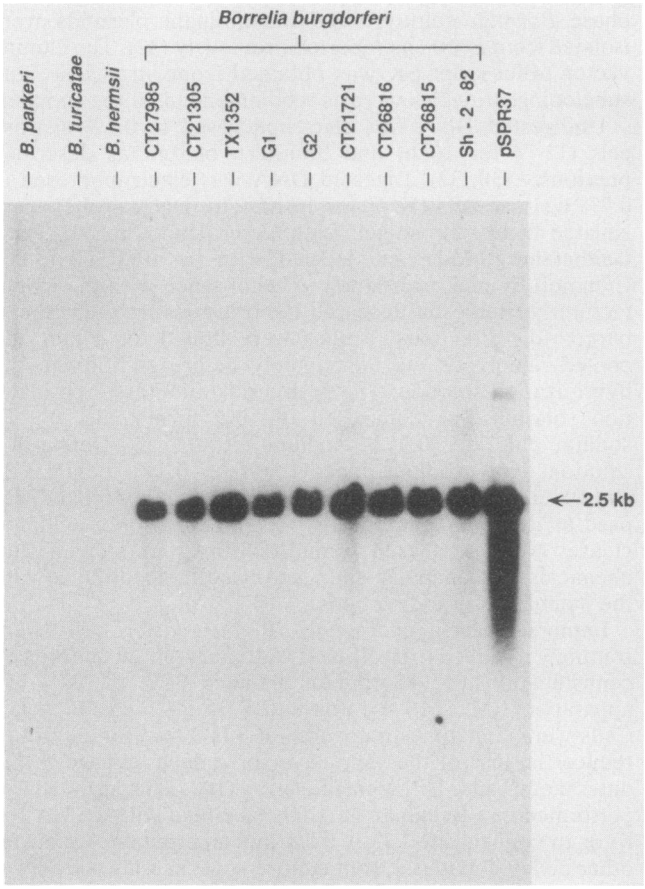

FIG. 1. Autoradiograph showing hybridization of the ^{32}P-labeled 2.5-kb *Eco*RI fragment from pSPR29 with total DNAs from nine isolates of *B. burgdorferi* and three other *Borrelia* species digested with *Eco*RI. Not all of the DNA tested is shown. The right-most lane contained DNA from pSPR37 digested with *Eco*RI.

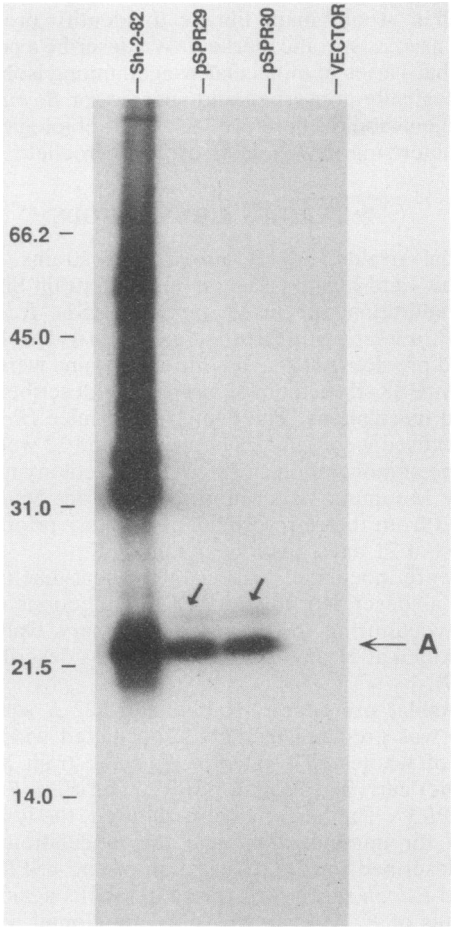

FIG. 2. Immunoblot analysis of proteins expressed by cells carrying pSPR29 and pSPR30. Whole-cell lysates of *B. burgdorferi* Sh-2-82 and *E. coli* carrying pSPR29, pSPR30, or the cloning vector were immunoblotted with rabbit anti-*B. burgdorferi* serum. The arrow labeled A denotes P22-A. Molecular weights (in thousands) of marker proteins (not shown) are indicated on the left.

fragments from both clones were recovered from agarose gels and shown by Southern blot analysis to hybridize to each other (data not shown) and to similarly sized fragments in *Eco*RI-digested total DNAs from 16 isolates of *B. burgdorferi*, of which 9 are shown in Fig. 1. Neither fragment hybridized with total DNAs from five additional *Borrelia* species, including *B. hermsii*, *B. parkeri*, and *B. turicatae* (Fig. 1) as well as *B. anserina* and *B. coriaceae* (data not shown). In addition to the 2.5-kb fragments, the inserts carried by pSPR29 and pSPR30 were composed of other *Eco*RI fragments. However, they did not hybridize to each other or to the 2.5-kb fragments (data not shown). Furthermore, Southern blot analysis of total DNA from *B. burgdorferi* indicated that they were not linked to the 2.5-kb segment in the spirochete (data not shown) and that each insert must have formed from random association of separate *Eco*RI fragments during library construction. All insert DNAs carried by pSPR29 and pSPR30 hybridized to the smeared band that migrated slower than the 49-kb linear plasmid from strain Sh-2-82, assumed to represent chromosomal fragments. Additional work is necessary, however, to confirm the chromosomal location of the P22-A gene of *B. burgdorferi*.

Immunoreactivity of P22-A. *E. coli* cells carrying pSPR29 or pSPR30 expressed a 22-kDa antigen that reacted with anti-*B. burgdorferi* serum (Fig. 2, arrow A). A larger band that reacted more weakly with the anti-*B. burgdorferi* serum was observed in both profiles (Fig. 2, unlabeled arrows). This serum did not react with antigens associated with lysates of *E. coli* carrying only the cloning vector. Because only the 2.5-kb fragment was shared between the *Borrelia* DNAs carried by pSPR29 and pSPR30, this fragment was subcloned into the pBluescript SK vector and the resulting construct was designated pSPR37. Immunoblot analysis confirmed that *E. coli* cells carrying pSPR37 expressed a 22-kDa antigen, designated P22-A, that reacted with the anti-*B. burgdorferi* serum (data not shown). Antiserum raised to whole-cell lysates of *E. coli* carrying only the vector (anti-*E. coli* serum) did not react with P22-A, indicating that this antigen is immunologically unrelated to native *E. coli* components (data not shown).

Anti-P22-A serum was raised in rabbits and used to screen *B. burgdorferi* isolates from various geographical origins to determine the prevalence of P22-A. All 3 European and 13 of the North American isolates of *B. burgdorferi* tested expressed in Western blots (immunoblots) a single immunoreactive band that bound anti-P22-A antibodies (Fig. 3). The size of the antigen was variable, however, ranging from 20 to

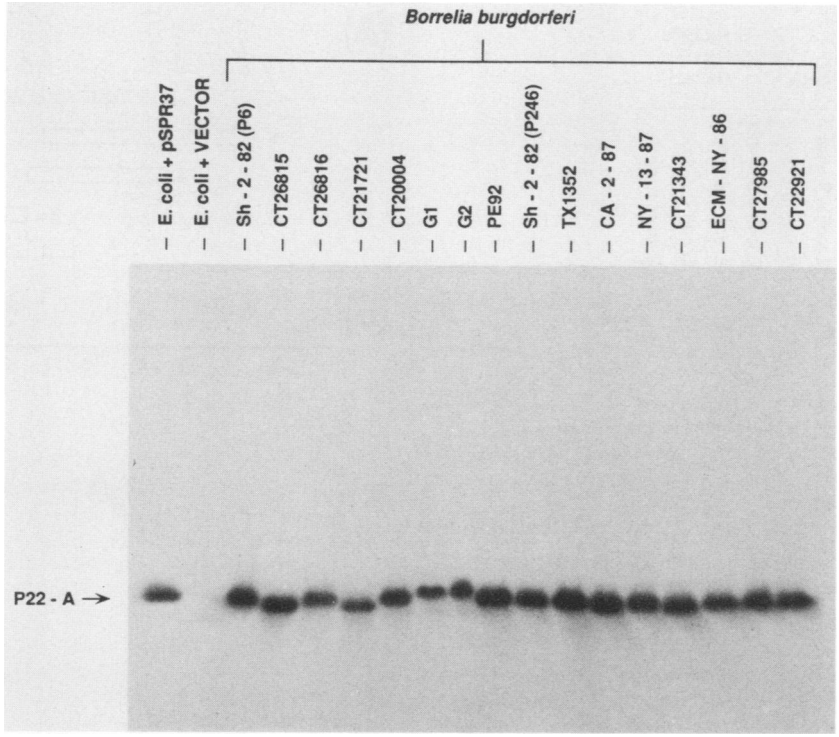

FIG. 3. Prevalence of P22-A among *B. burgdorferi* isolates. Whole-cell lysates of different *B. burgdorferi* isolates (including low [P6]- and high [P246]-passage variants of strain Sh-2-82) from North America and Europe (isolates G1, G2, and CT20004) were immunoblotted with rabbit anti-P22-A serum. Lysates of *E. coli* cells that expressed P22-A (*E. coli* + pSPR37) and that did not (*E. coli* + VECTOR) were positive and negative controls, respectively. Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 4 h.

23 kDa, indicating that although the expression of P22-A appears to be conserved among isolates, this antigen is heterogeneous with respect to its molecular weight. P22-A was not detected in whole-cell lysates of *B. hermsii*, *B. parkeri*, *B. anserina*, *B. turicatae*, and *B. coriaceae*, implying that this antigen is specific for *B. burgdorferi* (data not shown). Notably, a 39-kDa antigen which was detected only in *B. burgdorferi* lysates was weakly reactive with anti-P22-A serum (Fig. 4; note that the 39-kDa band is not visible in Fig. 3 for the exposure shown). Because cells carrying pSPR29 and pSPR37 do not express the 39-kDa antigen, it is assumed that the 39-kDa antigen is distinct from P22-A but shares cross-reactive epitopes.

P22-A did not react with monoclonal antibodies specific for OspA (H5332), OspB (H5TS), or flagellin (H9724) (Fig. 4). These data and the fact that polyclonal anti-P22-A serum did not react with any of these three *B. burgdorferi* antigens demonstrate that P22-A is apparently distinct from them. Monoclonal antibodies specific for a fourth, previously characterized antigen, P39 (32), also failed to react with P22-A (data not shown).

Southern blot analysis of the P22-A gene in *B. burgdorferi* isolates. To determine whether the difference in the molecular weights of P22-A expressed by various isolates was reflected by alterations detectable in the DNA, we Southern blotted total DNAs from representative isolates and probed them with the radiolabeled insert from pSPR37 (Fig. 5). Three isolates that expressed different sizes of P22-A appeared to have identical *Bam*HI and *Hin*dIII restriction profiles (Fig. 5, lanes 1, 2, 3, 7, 8, and 9). Two European isolates (G1 and G2; Fig. 5, lanes 4, 5, 10, and 11), however, had different profiles. Bands A and D were unique to these two isolates (Fig. 5). In addition, the G1 and G2 isolates exhibited signals weaker than those exhibited by the other isolates (Fig. 5, lanes 4, 5, 10, and 11), although this difference was not obvious when DNAs from these isolates were digested with *Eco*RI and probed with the insert from pSPR37 (Fig. 1).

Anti-P22-A antibodies in mice infected with *B. burgdorferi*. To determine whether P22-A may stimulate antibodies during an infection, we tested by Western blotting sera collected at 21 days postinfection from 12 white-footed mice for reactivity with recombinant P22-A. These animals were inoculated intraperitoneally with a cloned, low-passage (P6) variant of *B. burgdorferi* Sh-2-82, and at 21 days postinoculation spirochetes were cultured from the spleen or urinary bladder from each animal. Anti-P22-A antibodies were detected in all sera by comparison of immunoblot profiles of *E. coli* cells that contained P22-A with those that did not (Fig. 6). An additional six animals that were similarly inoculated with a cloned, high-passage (P246) variant of strain Sh-2-82 (noninfectious) and tested 21 days postinoculation also elicited anti-P22-A antibodies (Fig. 6), although spirochetes were not isolated from triturated spleen or urinary bladder from these animals. The strength of this response, however, was weaker than that seen in infected animals, suggesting that an active infection resulted in a better response. Both low- and high-passage inocula expressed equivalent amounts of P22-A in vitro (Fig. 3). A higher concentration of anti-P22-A antibodies, however, was detected in sera collected at 21 days postinoculation from six mice inoculated with a similar number of P6 cells that had been inactivated with

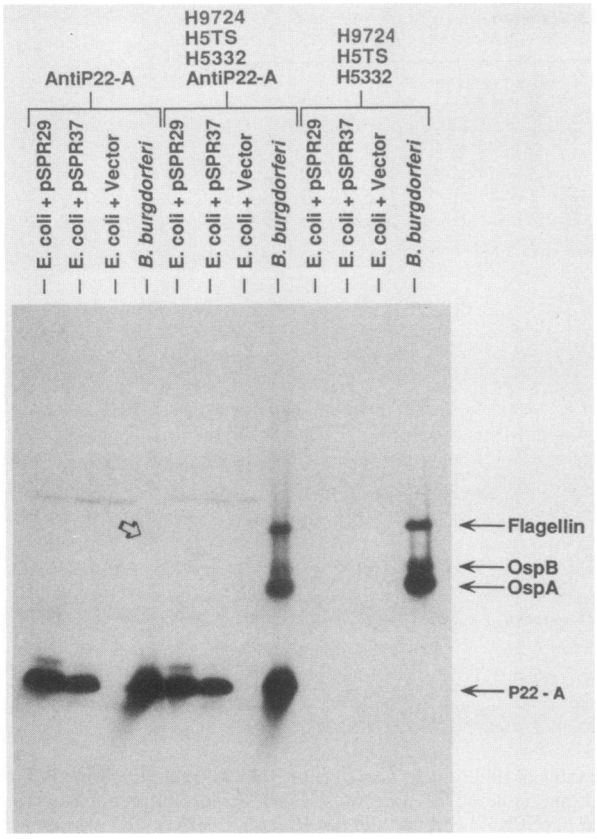

FIG. 4. Immunoblot showing a comparison of reactivities of anti-P22-A serum and monoclonal antibodies specific for flagellin (H9724), OspA (H5332), and OspB (H5TS). Whole-cell lysates of *B. burgdorferi* and *E. coli* carrying pSPR29, pSPR37, or only the cloning vector were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel components were transferred to nitrocellulose, which was then cut into three strips such that each portion contained a lane representing each of the four lysates. The three strips were incubated with anti-P22-A serum; anti-P22-A serum plus the three monoclonal antibodies H9724, H5TS, and H5332; or only the three monoclonal antibodies. Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 8 h. The relative positions of key proteins are indicated on the right. The arrow within the figure denotes the position of a 39-kDa reactive band in the *B. burgdorferi* profile during incubation with anti-P22-A serum.

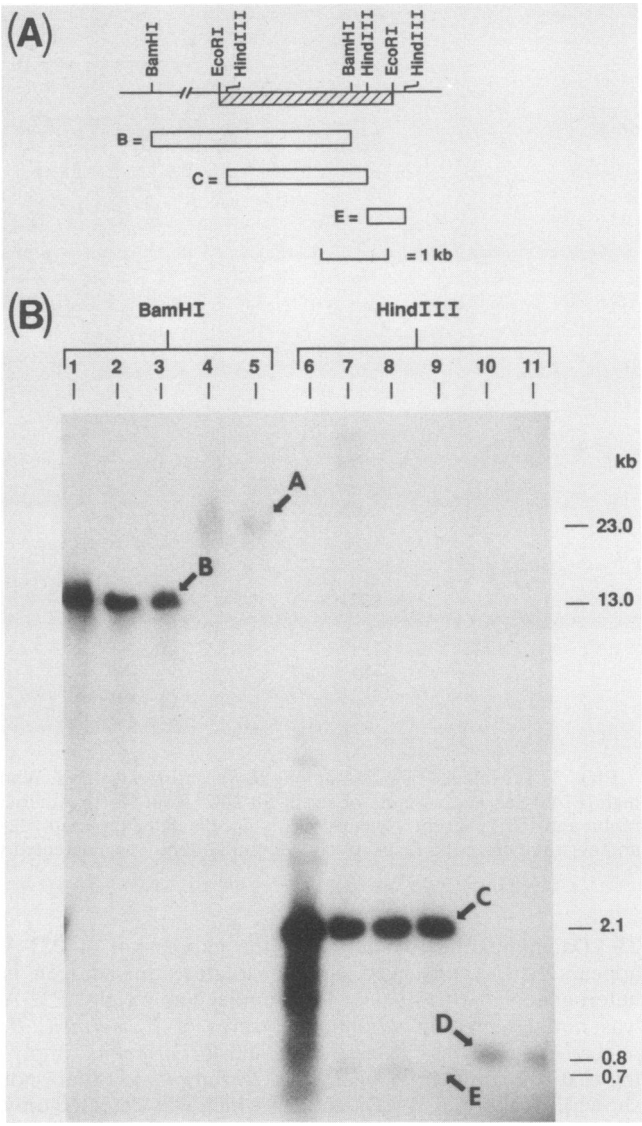

FIG. 5. Hybridization of the pSPR37 insert to *Bam*HI and *Hin*dIII digests of total DNAs from strains Sh-2-82 (lanes 1 and 7), NY-13-87 (lanes 2 and 8), CT26816 (lanes 3 and 9), G1 (lanes 4 and 10), and G2 (lanes 5 and 11). Lane 6 contained digested pSPR37 DNA. (A) Restriction map of the 2.5-kb *Eco*RI fragment in *Borrelia* DNA. The region corresponding to cloned *Borrelia* DNA in pSPR37 is shown as a hatched box. Open boxes below the map correspond to the fragments that have hybridized with the probe in panel B. (B) Autoradiograph of DNAs digested and probed with the plasmid pSPR37 insert. The positions of molecular size markers are indicated on the right. Bands A and D are unique to the isolates (G1 and G2) from Germany.

heat and disrupted by sonication (data not shown). Thus, disruption of cells presumably released more P22-A than was available to stimulate the immune system when the cells were intact. Therefore, infected animals may merely support the accumulation of enough lysed *B. burgdorferi* to elicit a better response, as opposed to rapidly cleared noninfectious organisms.

Localization of P22-A to the periplasmic space. Immune electron microscopy of *B. burgdorferi* cells with anti-P22-A antiserum demonstrated that P22-A is probably concentrated in the periplasmic space of *B. burgdorferi* and is only infrequently, if ever, naturally exposed on the bacterial surface. P22-A was only occasionally detected in the cytoplasm (Fig. 7B), inner membrane (which forms the outer boundary of the protoplasmic cylinder [Fig. 7B]), or outer membrane (Fig. 7D and E). When spirochetes were recovered by centrifugation so that the periplasmic cylinder remained intact but the outer membrane was damaged or removed, P22-A was detected as large aggregates associated with the cellular fraction (Fig. 7D and E, arrowheads). P22-A aggregates could also be detected in the supernatant after the bacteria had been washed with PBS (Fig. 7C). This latter procedure did not disrupt the inner membrane but removed the outer membrane and putative surface layer, as was shown by the extrusion of the periplasmic flagella. Cryosections revealed anti-P22-A antibodies concentrating along the periplasmic space (Fig. 7B). Bacteria that had an intact outer

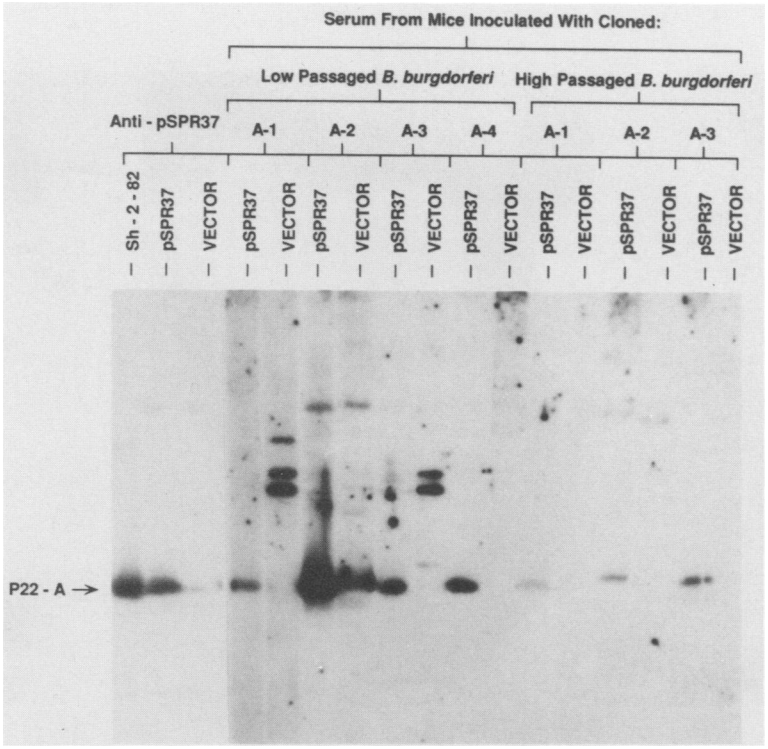

FIG. 6. Immunoblot showing the detection of anti-P22-A antibodies in serum specimens collected from mice inoculated with either cloned, low-passage (infectious) or cloned, high-passage (noninfectious) *B. burgdorferi* Sh-2-82. Western-blotted whole-cell lysates of *E. coli* carrying pSPR37 (pSPR37) or only the cloning vector (VECTOR) were incubated with serum specimens collected from white-footed mice at 21 days postinoculation. Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 6 h. The three leftmost lanes were incubated with polyclonal anti-P22-A serum (anti-pSPR37). A whole-cell lysate of *B. burgdorferi* Sh-2-82 (Sh-2-82) was included as a control. Not all of the animal sera tested are shown.

membrane did not bind anti-P22-A serum. Preimmune serum (data not shown) and serum collected from either mice (Fig. 7F) or rabbits (data not shown) immunized with *E. coli* cells carrying only the cloning vector did not bind to Sh-2-82 organisms, indicating that the material binding the anti-P22-A serum was P22-A and that it did not share reactive determinants with native *E. coli* components.

DISCUSSION

In the present study, a 20- to 23-kDa protein was identified and shown to be most likely concentrated in the periplasmic space along with, but not part of, the flagella. This antigen, designated P22-A, was readily released from this location when the outer membrane was removed. It appeared to aggregate once it was released, although it is not clear if this aggregation reflects affinity for itself or for other cellular material released at the same time.

Although all isolates of *B. burgdorferi* tested expressed P22-A, the sizes of the antigen varied. There was no evidence, however, to suggest that this size variation reflects antigenic variation, since antisera raised to the recombinant P22-A antigen, which was derived from strain Sh-2-82, reacted equally well to all sizes of P22-A. The species specificity of this antigen and its conservation among North American and European isolates are features which make P22-A a potentially useful marker for identifying isolates as *B. burgdorferi*.

Because this antigen appears not to be part of the flagella or the inner and outer membranes (Fig. 7), P22-A is probably a soluble periplasmic protein. Although this idea is only speculation, it is supported by the ease with which this antigen was released from cells which had lost or suffered damage to their outer membranes. Alternatively, some of the P22-A molecules found associated with a single bacterium may be exposed on the surface; however, in this case it would be necessary to explain why polyclonal anti-P22-A serum did not bind to intact bacteria or portions of the outer membrane. Several proteins of *B. burgdorferi*, including OspA and OspB, have been shown to be acylated (9), and this characteristic may be a feature of many *Borrelia* antigens that are associated with membranes. The molecular structures of P22-A and other antigens of *B. burgdorferi* not associated with membranes or axial filaments are still undetermined.

The different sizes of P22-A expressed by various isolates were not reflected universally at the DNA level. Southern blot analysis did not show any length polymorphisms among restriction fragments containing the gene and flanking sequences for three of the five isolates tested. Two European isolates, however, did show distinct patterns and weaker hybridization signals, although these results may not mean that there are differences between the P22-A genes. The size of the gene is estimated to be 0.7 kb and, conceivably, the differences in restriction sites may not be within the gene but may lie in the flanking regions. Therefore, while isolates G1 and G2 are generally less similar at the P22-A locus than the other isolates tested, all isolates tested may still contain nearly identical P22-A genes.

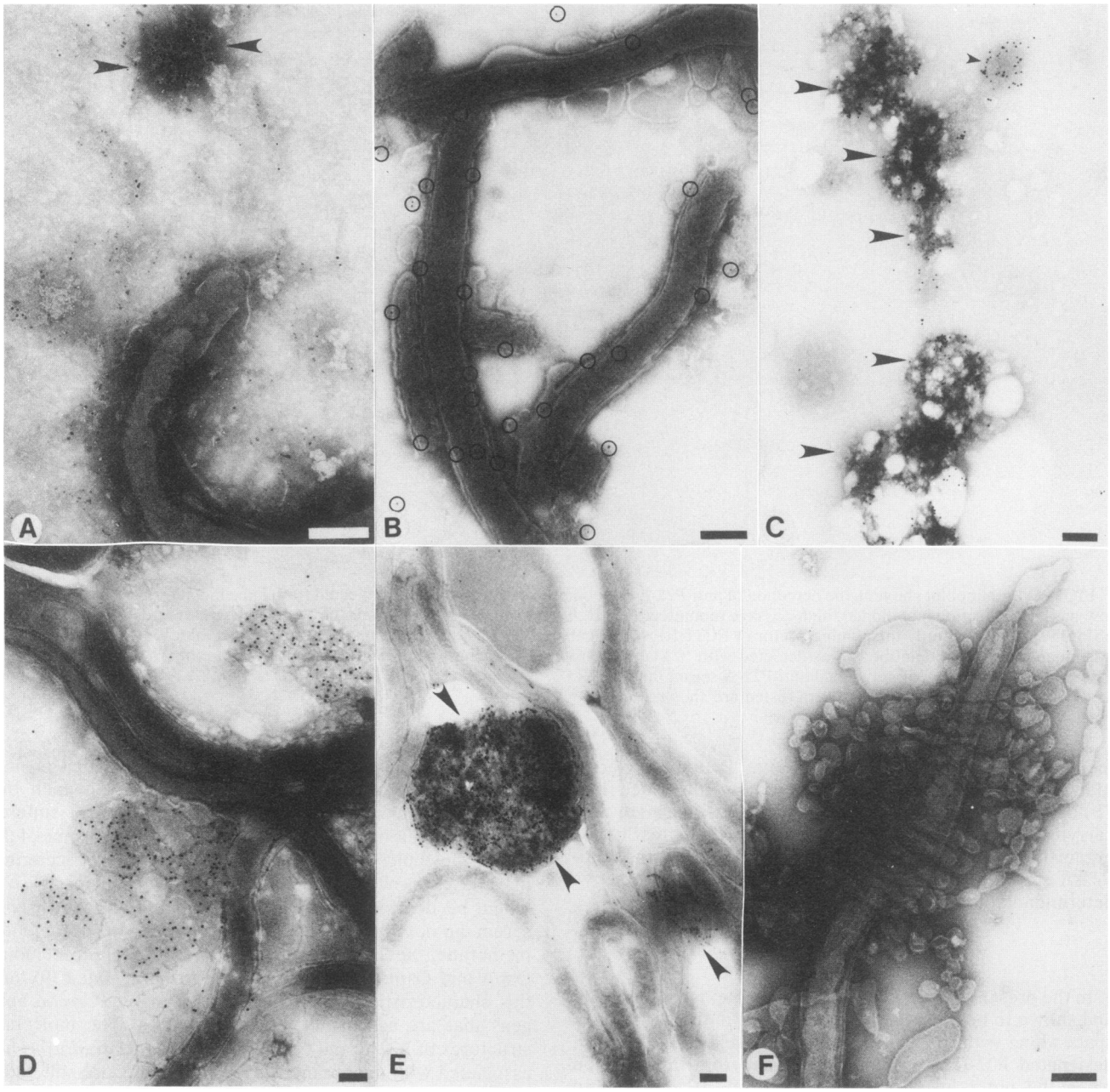

FIG. 7. Immune electron micrographs of *B. burgdorferi* cells or sections incubated with anti-P22-A sera. (A) *B. burgdorferi* Sh-2-82 (P6) treated with rabbit anti-P22-A (anti-pSPR37) antibody complexed to protein A-gold (8 nm). Arrowheads denote a large aggregate of material labeled with the antibody-gold complex and negatively stained with 1% ammonium molybdate. Bar, 0.2 μm. (B) Cryosections of *B. burgdorferi* Sh-2-82 (P6) recovered at 3,000 rpm for 30 min in a TOMY MTX-150 apparatus. Cells were fixed with periodate-lysine-paraformaldehyde fixative, infiltrated with sucrose, quickly frozen, cryosectioned (60 nm), treated with mouse anti-P22-A antibody complexed to protein G-gold (10 nm), and negatively stained with 0.5% ammonium molybdate. Circles show the locations of individual gold grains in the area of the periplasmic space. Bar, 0.2 μm. (C) Cellular pellet from a high-speed spin (100,000 rpm for 30 min in a Beckman TL-100 ultracentrifuge with a TLA 100 rotor) of the culture supernatant after cells had been removed by a low-speed spin (15,000 rpm for 30 min in a Beckman GSA rotor) incubated with rabbit anti-P22-A antibody (anti-pSPR37) complexed to protein A-gold (16 nm). Arrowheads denote aggregated material that was labeled, adherent to Parlodion film, and negatively stained with 0.5% ammonium molybdate. Bar, 0.2 μm. (D) *B. burgdorferi* B31 (ATCC 35210) collected by a gentle spin (3,000 rpm for 20 min) and associated material labeled with rabbit anti-P22-A antibody (anti-pSPR37) complexed to protein A-gold (10 nm). Bar, 0.2 μm. (E) Same as in panel D, but treated with mouse anti-P22-A serum. Arrowheads indicate an aggregate of P22-A. Bar, 0.2 μm. (F) Negative control. Serum from a mouse immunized with a lysate of *E. coli* carrying only the cloning vector was incubated with strain Sh-2-82 and processed as described in panel B. Bar, 0.2 μm.

The apparent dichotomy observed between isolates when a region of DNA containing the P22-A gene was used probably reflects the two groups of *B. burgdorferi* isolates reported recently (25, 26). In one of these studies (26), it was found by polymerase chain reaction typing with specific sets of primers that all North American isolates and a few European isolates constituted one category and that the other group comprised most of the European and Asian isolates. It remains to be determined whether these primers bind to any area of the P22-A locus or whether isolates representing these two groups at the genetic level are divergent enough that many areas of their genomes exhibit significant differences.

The weak immunoreactive 39-kDa band detected with anti-P22-A serum in *B. burgdorferi* lysates (Fig. 4) was shown not to be the P39 antigen reported previously (32). Although the nature of this presumably cross-reactive antigen is not known, the existence of multiple antigens (P39 and the second unidentified protein) in a single immunoreactive band emphasizes the importance of using purified or recombinant antigens when testing a specific protein for reactivity to sera from infected humans or other animals. Because *E. coli* cells carrying the cloned *Borrelia* DNA containing the gene for P22-A did not express the P39 antigen, it can be assumed that the putative gene encoding P39 is not part of the cloned DNA and, therefore, is not linked to the gene encoding P22-A. The possibility that *B. burgdorferi* has an undefined mechanism or condition that permits the aggregation of P22-A to itself or other components, thereby creating an aberrant migration, needs to be addressed. This possibility is supported by the fact that aggregates of P22-A were seen by immune electron microscopy (Fig. 7E).

The recombinant clones expressing P22-A also produced a slightly larger (approximately 25-kDa) protein that reacted weakly with the anti-*B. burgdorferi* serum. Whether this is a variant of P22-A or a cross-reactive antigen encoded by a distinct gene has yet to be determined. Notably, this second antigen was only barely detected in lysates of clones representing pSPR37 and not at all in lysates of *B. burgdorferi*, indicating that its expression is variable and therefore that it may be an artifact of expression due to the cloning system and, thus, an aberrant form of P22-A.

Immunoblot analysis demonstrated that P22-A is not related to OspA, OspB, flagellin, or P39. However, the 20- to 22-kDa antigen described by Wilske et al. (35, 36) and designated pC does exhibit many similarities to P22-A. Not only do these proteins have similar molecular weights, but also pC expressed by different isolates varies in size. Furthermore, like P22-A, pC appears to elicit an immune response during the course of an infection (22, 24). Other studies have also reported immunoreactive proteins of similar sizes (15, 18, 19, 21), but these have not been characterized further and, therefore, it is not known if they are P22-A, pC, or some other antigen. It will be helpful to use the recombinant P22-A antigen as a specific antigen to screen human sera from Lyme disease patients to determine whether, as our animal data suggest, this antigen will have diagnostic value for human Lyme disease.

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