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Biochemical and Immunological Characterization of the Surface Proteins of *Borrelia burgdorferi*

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The immunodominant proteins and glycoproteins of *Borrelia burgdorferi* were analyzed by one-dimensional (1D) and 2D gel electrophoresis. More than 100 polypeptide species could be detected on silver-stained 2D gels. Separation of sonic extracts of the organism by differential centrifugation (100,000 × *g*) revealed several of the major proteins to reside predominantly within the pellet fraction. The antigenicity of the individual polypeptides was determined by Western (immuno-) blot analysis with sera from humans with chronic Lyme disease and from rabbits immunized with *B. burgdorferi*. Surface proteins of viable *B. burgdorferi* labeled with ^{125}I or long-arm hydroxysuccinimide biotin were identified by gel analyses. Thirteen major surface proteins were apparent, including the highly immunogenic 41-kilodalton (kDa) endoflagellar antigen. Two of these proteins, with molecular masses of 22 and 41 kDa, were further characterized by electroblotting and microsequencing their amino termini. Significant (35%) homology between the first 20 amino acids of the 22-kDa protein and the deduced amino acid sequence of the 31-kDa (outer surface protein A) protein of *B. burgdorferi* may indicate that these proteins are processed similarly or are part of a gene family expressed at the surface of the organism. In addition, highly significant (88%) homology was found between the first nine amino acids of the 41-kDa protein of *B. burgdorferi* and the 33-kDa endoflagellar protein of *Treponema pallidum*, after which the sequences diverge. This observation provides in part a structural basis for the observed cross-reactivity between the two organisms and suggests alternative approaches to the development of specific immunodiagnostics.

Infection with *Borrelia burgdorferi*, the causative agent of Lyme disease, is the most common tick-borne infectious disease in the United States, accounting for more cases per year than all other specific diseases due to this vector combined. In endemic areas, between 30 and 90% of *Ixodes* ticks are infected with this organism. Since the geographic distribution of the *Ixodes* ticks extends along both coasts of the United States and in various parts of Europe, the Soviet Union, and China, it is not surprising that outbreaks of serious disease due to *B. burgdorferi* are being described throughout the world (B. J. Luft and R. J. Dattwyler, *in* J. S. Remington and M. N. Swartz, ed., *Current Clinical Topics in Infectious Disease*, in press). However, full definition of the prevalence of this disease and its protean clinical manifestations has been severely hampered by the lack of sensitive and specific diagnostic serological tests.

The major proteins of *B. burgdorferi* have been partially characterized on single-dimension gels of lysates prepared from solubilized whole organisms (2, 14, 42). Of note in these studies have been two major proteins with constant molecular masses of 60 and 41 kilodaltons (kDa) and variably expressed lower-molecular-mass proteins of 21 to 22, 30 to 32, and 34 to 36 kDa. One-dimensional (1D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies of partially purified flagellar (13, 19) and membrane (13) preparations, as well as immunoelectron microscopy using monoclonal antibodies, have shown the 41-kDa band to be a major endoflagellar protein (4) and the 30- to 32-kDa (outer surface protein [OSP] A) (7) and 34- to 36-kDa (OSP B) (6) proteins to be significant constituents of the outer membrane. OSP A and B are encoded by a plasmid (3). Immunoblot studies have shown that the flagellar protein is the major antigenic determinant to which infected individuals respond during the early stages of the disease, with chronic infection being characterized by an expansion of the immune response to multiple antigens, including 22- (pC), 31- (OSP A), 34- (OSP B), 55-, 58-, 60-, and 80- to 90-kDa proteins (2, 14, 42).

We utilized 2D electrophoresis to further resolve and characterize the major proteins and antigens of *B. burgdorferi*. Surface antigens were identified by labeling viable organisms either with ^{125}I or biotin. Specific proteins were further analyzed by Western (immuno-) blot analysis of 2D gels with sera from patients with late Lyme disease and from rabbits immunized with *B. burgdorferi*. The 22- and 41-kDa proteins were further characterized by electroblotting and microsequencing. Striking sequence homology was found at the amino terminus between the first nine amino acid residues of the 41-kDa protein of *B. burgdorferi* and the 33-kDa major endoflagellar antigen of another spirochetal pathogen, *Treponema pallidum* (9).

MATERIALS AND METHODS

B. burgdorferi. The B31 strain was grown in modified Barbour-Stoener-Kelly II (BSK II) medium (1) at 33°C for 3 days. Spirochetes were centrifuged at 10,000 × *g* at 10°C, washed three times in 200 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.2, and enumerated by dark-field microscopy. Organisms were then suspended in PBS containing 20 mM EDTA (Sigma Chemical Co., St. Louis, Mo.) and 10 mM D-phenylalanyl-L-arginine chloromethyl ketone (Sigma) and sonicated with a Biosonic sonicator 10 times (18,000 Hz) at 15-s intervals at 4°C. For single-dimension SDS-PAGE, samples from sonicated organisms (25 μg per lane) or subcellular preparations (10^8 organisms per lane) were dissolved in a sample buffer that contained Tris hydrochloride, 3% SDS, 5% glycerol, 10 mM EDTA, 0.001% bromophenol blue, and, for reducing gels, 2.5% mercaptoethanol (Sigma).

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For 2D gel electrophoresis, the spirochetes were suspended in 0.01 M Tris hydrochloride (pH 7.4)–5 mM $MgCl_2$ containing 50 μg of pancreatic RNase per ml (Sigma) (31) and then sonicated as described above. After sonication, 50 μg of DNase per ml (Sigma) was added, and the mixture was incubated at 4°C for 5 min. Sonicated preparations of whole organisms (40 to 100 μg per gel) or the subcellular preparation from 10^9 organisms was solubilized in a sample buffer containing 0.05 M cyclohexylaminoethane sulfonic acid (CHES) (Sigma), pH 9.5, 2% SDS, 1% dithiothreitol (Sigma), and 10% glycerol. The antigen was divided into aliquots and frozen at −70°C until solubilized. The protein concentration was assayed by the method of Bradford (12).

Subcellular fractions were separated by ultracentrifugation of *B. burgdorferi* sonic extracts. The sonic extracts were centrifuged at 10,000 × *g* at 10°C for 10 min to remove any whole borrelia, and the supernatant was then further separated by centrifugation at 100,000 × *g* for 2 h at 4°C. The pellets were washed twice in PBS before being solubilized in sample buffer, and the supernatant was used as a source of soluble cytoplasmic antigens (20, 33).

Gel electrophoresis. Single-dimension gels were made with the buffers of Laemmli (22) and electrophoresed in a discontinuous 0.1% SDS–10 to 12.5% polyacrylamide gel. Phosphorylase *b*, ovalbumin, chymotrypsinogen, β-lactoglobulin, and lysozyme were used as molecular mass markers (Amersham Corp., Arlington Heights, Ill.).

Methods for 2D gels were performed as described by O'Farrell (31). Isoelectric focusing was performed in polyacrylamide tube gels, pH 3.5 to 10.0 (ampholytes; LKB Pharmacia, Piscataway, N.J.). Immediately after focusing was completed, the pH gradient was measured directly with a flatbed pH electrode (Microelectrode, Inc., Londonderry, N.H.). Separation in the second dimension was done with 10 or 12.5% polyacrylamide SDS-PAGE slab gels. Gels were serially stained with Coomassie blue and silver stains (43).

Detection of lectin-binding proteins. Proteins were electrophoretically transferred from the gels to polyvinylidene difluoride membrane Immobilon-PVDF (Millipore Corp., Bedford, Mass.) paper, as described by Towbin et al. (40). Individual blots were incubated overnight with biotinylated concanavalin A (Vector Laboratories, South San Francisco, Calif.) diluted in PBS–2% bovine serum albumin at a concentration of 10 μg/ml (16, 17). To test the specificity of the concanavalin A for carbohydrate, we incubated the lectin with or without 100 mM alpha-methyl mannoside (Sigma). Blots were washed several times with Tris-saline, incubated for 2 h with avidin biotinylated to horseradish peroxidase, washed, and developed in a solution of 0.02% diaminobenzidine–0.1% hydrogen peroxide–0.01 M PBS (pH 7.2). Color development was stopped by washing the blots with Tris-saline after 10 min of incubation. Dried blots were photographed immediately.

Determination of surface protein. Surface proteins of *B. burgdorferi* were analyzed by two different methods. Live *B. burgdorferi* were ^{125}I surface labeled by a modification of the Iodogen (Pierce Chemical Co., Rockford, Ill.) technique as described by Markwell and Fox (27). A 4-ml glass vial was coated with 62.5 μg of Iodogen dissolved in 625 μl of methylene chloride. The solvent was dried under a stream of nitrogen. A 1-ml volume of PBS (pH 7.2) containing 5×10^{10} organisms was added to the vial along with 250 μCi of Na-^{125}I. The mixture was incubated for 2 to 3 min at room temperature with periodic agitation, after which the contents were gently aspirated. After labeling, >95% of organisms were viable, as assessed by characteristic motility. The organisms were washed 10 times in 1.5 ml of PBS (pH 7.2), each wash followed by centrifuging at 10,000 × *g* in an Eppendorf tabletop centrifuge for 10 min. Approximately 20 to 25 μg per lane was used for 1D PAGE, and 50 to 100 μg per gel was used for 2D gels. The final pellet was suspended and solubilized in sample buffer. By this method, virtually all the ^{125}I-labeled proteins were solubilized in the sample buffer. Individual gels were Coomassie blue or silver stained (43) and dried, and the presence of ^{125}I-labeled proteins was determined by autoradiography.

Surface proteins of live organisms were also labeled by biotinylation (21). A total of 5×10^{10} *B. burgdorferi* were suspended in 1 ml of 100 mM sodium bicarbonate (pH 8.0). Seven microliters of a solution of long-arm *N*-hydroxysuccinimide ester-biotin (Vector Laboratories), 25 mg/ml, dissolved in dimethyl sulfoxide was added to the spirochetes and incubated at room temperature for 30 min. The sample was checked for viability, centrifuged, washed three times in PBS, suspended in buffer, and stored at −70°C before use. Biotinylated protein preparations were electrophoretically transferred to Immobilon-PVDF as described above, blocked, and incubated with avidin-horseradish peroxidase (Vector Laboratories). The blot was washed in PBS (pH 7.2), developed in a solution of 0.1% tetrahydrochloride–0.2% hydrogen peroxide–0.1 M Tris-buffered saline (0.5 M NaCl, 20 mM Tris hydrochloride [pH 7.4]) (TBS) (pH 7.2), and photographed immediately.

Sera. Sera from patients with known late Lyme disease were stored at −70°C prior to study. New Zealand White rabbits were immunized with *B. burgdorferi* by suspending 2 mg of sonic extract in 1 ml of Freund complete adjuvant (Sigma) or by injecting 10^9 organisms that had been freeze (−70°C)-thawed. The rabbits were inoculated both intramuscularly (one injection) and subcutaneously (two injections). At 2 and 4 weeks after the initial injection, rabbits were given a booster injection of an additional 2 mg suspended in 1 ml of Freund incomplete adjuvant or 10^9 freeze-thawed organisms. Thereafter, rabbits were bled by phlebotomy of the lateral ear veins.

Immunoblot studies. Proteins separated by 1D or 2D gel electrophoresis were transferred to Immobilon-PVDF (40). Unreacted protein-binding sites were blocked with 2% bovine serum albumin in TBS for 2 h at room temperature. The blots were washed in TBS three times and incubated overnight with sera from humans or rabbits containing anti-*B. burgdorferi* antibody at dilutions of 1:200 and 1:1,000, respectively. They were then washed three times and incubated with 500,000 cpm of ^{125}I-labeled staphylococcal protein A (New England Nuclear Corp., Boston, Mass.) for 2 h at room temperature. Following washing, individual blots were autoradiographed for various periods of time.

Microsequencing. Proteins separated by 1D and 2D gel electrophoresis were electroblotted onto Immobilon-PVDF (Millipore) overnight at 30 V and then for 1 h at 70 V in a transfer buffer consisting of 25 mM Tris, 192 mM glycerine, and 200 ml of methanol per liter at a pH of 8.3 (28). The transblot was stained with amido black and then destained with several changes of methanol, followed by rinsing with deionized water. The transblot was air dried on filter paper under a fume hood, and the stained spots were excised and placed in Eppendorf tubes (28). Amino-terminal sequences were determined by using a gas phase microsequencer (model 470A; Applied Biosystems) coupled to an on-line high-performance liquid chromatograph (model 120A).

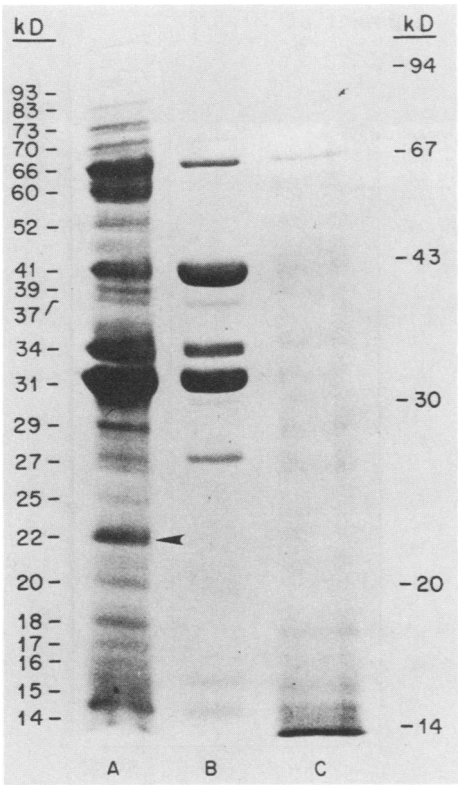

FIG. 1. *B. burgdorferi* proteins separated on 10% SDS-PAGE slab gels. (A) Whole-organism sonic extract; (B) pellet obtained after ultracentrifugation; (C) supernatant (cytosol fraction) from 10^8 organisms. Gels were Coomassie blue stained. Molecular masses of internal standards (in kilodaltons) are shown on the right, and the apparent molecular masses of individual *B. burgdorferi* proteins are shown on the left. The arrowhead indicates the 22-kDa band electroblotted for microsequencing studies.

RESULTS

Analysis of *B. burgdorferi* by 1D and 2D gel electrophoresis. The pattern obtained by Coomassie blue staining of a sonic extract of *B. burgdorferi* run on a single-dimension gel (Fig. 1A) resembled that described previously (2, 14, 42). Of note were major bands at molecular masses of 22, 31, 34, 41, 60, and 66 kDa, none of which was affected by reduction or pretreatment with nucleases. In order to define which molecular species were primarily membrane associated (i.e., enriched by ultracentrifugation of a crude fraction including cell membranes and organelles) and which were cytosolic proteins, the patterns obtained following differential centrifugation were also analyzed (Fig. 1B and C). These studies clearly show colocalization of these major molecular species, notably including the 31- and 34- (OSP A and B), 41- (flagellar), and 66-kDa antigens in the pellet.

B. burgdorferi proteins were further characterized by 2D gel electrophoresis. Figure 2A shows a Coomassie blue-stained sonic extract of whole organisms, demonstrating again major species of protein in the molecular-mass range of 20 to 100 kDa, consisting of 12 to 15 spots, including an acidic grouping in the pH range of 5.0 to 7.0 and the basic OSP A and B proteins at pH 8.0 to 9.0, as previously described (8). Additional resolution was achieved by silver staining the same gel (Fig. 2B), which showed several proteins to constitute charge trains, including the 41-kDa protein, which consisted of two to three contiguous spots. Basic higher-molecular-mass species were also noted, which could not be defined under equilibrium conditions. More than 100 spots were seen, most in the molecular-mass range of less than 100 kDa. Organisms that were solubilized without sonication gave a simpler 2D gel pattern when silver stained compared with the sonicated preparation; this simpler pattern facilitated localization following electroblotting for microsequencing studies (see below).

In order to determine which bands and spots may be glycoproteins, 1D and 2D gels were blotted onto Immobilon-PVDF paper and probed with biotinylated concanavalin A. Various dilutions of lectin were tested, and conditions were optimized so as to reduce to a minimum background staining and achieve complete inhibition following preincubation of lectin with alpha-methyl mannoside. Concanavalin A bound specifically to eight different proteins, both on 1D and 2D gels, including spots with apparent molecular masses of 41, 60, 66, 70, 83, and 91 kDa (Fig. 3). Under these conditions, minimal binding to the 31- and 34-kDa OSP A and B and other lower-molecular-mass proteins was found, although some nonspecific binding was noted if more concentrated solutions of lectin were employed and complete inhibition by sugar was not achieved.

Surface-labeling studies. Surface-exposed proteins were defined by labeling viable *B. burgdorferi* by two different methods in separate experiments and then analyzing single and 2D gels of whole organisms compared with pellet and cytosol fractions obtained after differential centrifugation. Cell surface labeling was achieved with ^{125}I, using the Iodogen technique, and with biotin, using a long-chain *N*-hydroxysuccinimide ester-biotin conjugate. For both experiments, selective labeling of surface proteins was established by checking (i) the viability of organisms after labeling, assessed by motility; and (ii) the subsequent absence of label (established by autoradiography or probing of blots with avidin-horseradish peroxidase) in the cytosol fraction.

2D gels of surface radioiodinated (Fig. 4A) or biotinylated (Fig. 4B) *B. burgdorferi* showed significant enrichment of several species of proteins compared with silver-stained gels of sonic extracts, with general agreement between the two methods. The autoradiogram of the ^{125}I-labeled preparation shows 13 distinct species, including spots at 22, 24, 29, 31, 34, 37, 39, 41, 52, 66, 70, 73, and 93 kDa. The corresponding 2D gels of surface-biotinylated (Fig. 4B) preparations confirmed selectivity of labeling. Additional studies demonstrated that in order to identify the surface-labeled 93-kDa protein, it was necessary to solubilize the whole organism. Even in the presence of proteolytic enzyme inhibitors, this protein degraded to lower-molecular-mass species with sonication (data not shown). It is also remarkable that the 2D analysis was superior to the 1D (data not shown) in demonstrating several well-defined proteins, including those with apparent molecular masses of 73, 70, 52, and 29 kDa, as strongly surface labeled.

Both radioiodination and biotinylation labeled basic and relatively acidic proteins. The corresponding autoradiogram and silver-stained gels are shown in Fig. 5A and 2B. Of note is a grouping of acidic spots at molecular masses of 66, 70, 73, and 93 kDa, as well as basic species at 22, 29, 31, 40, and 68 kDa. Although iodinated (Fig. 4A) and biotinylated (Fig. 4B) preparations were quantitatively similar, differences in intensity presumably reflect the groups labeled and the smaller amount of total protein (4 to 10 μg) used for biotinylation studies.

Immunoblot studies. In order to define which of the surface proteins elicited specific humoral immune responses in indi-

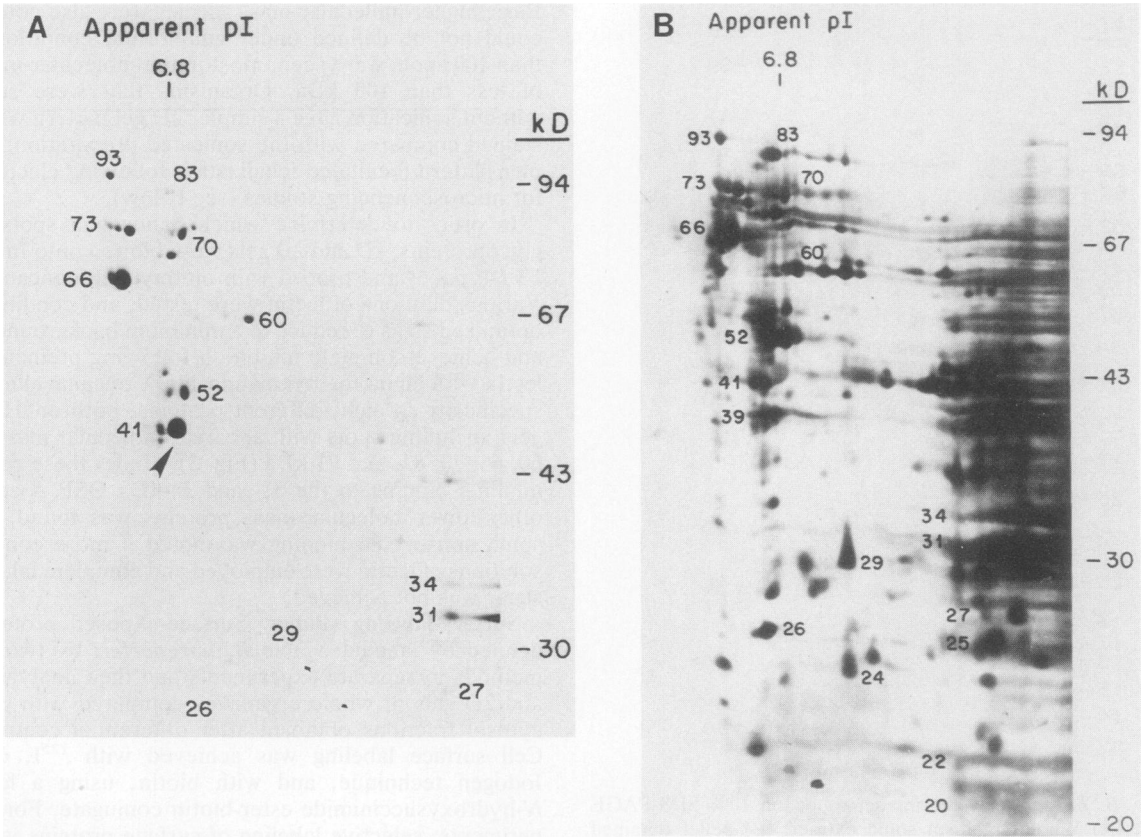

FIG. 2. 2D gels of *B. burgdorferi* (first dimension, pH 3.5 to 10.0; second dimension, 12.5% SDS-PAGE). Gels were either Coomassie blue (A) or silver (B) stained. Molecular masses (in kilodaltons) are indicated adjacent to each spot. The arrowhead indicates proteins electroblotted for microsequencing studies.

viduals with late Lyme borreliosis, *B. burgdorferi* antigens separated by 2D gel electrophoresis were studied by Western blot analysis, using radioiodinated staphylococcal protein A as a secondary probe. Figure 5 shows immunoblots for two patients (A and B) with late Lyme disease. These blots show consistent reactivity with the 41-kDa flagellar antigen, as well as 66-, 70-, 73-, and 93-kDa species, variable binding to the OSP antigens, and intense reactivity with a quantitatively minor group of spots at 20 and 22 kDa. In contrast, antisera from two rabbits immunized with intact organisms (Fig. 6A) or a sonic extract of whole organisms (Fig. 6B) both showed striking reactivity with the 41-kDa and OSP A and B determinants, as well as with higher-molecular-mass species.

Microsequencing studies. Individual spots that were surface exposed and immunodominant were then electroblotted from 2D gels onto Immobilon-PVDF for microsequencing. Figures 7 and 8 show amino-terminal sequences obtained with the 22- and 41-kDa proteins. A computer search (Protein Identification Resource Center, Georgetown University, Washington, D.C.) failed to disclose significant homologies for the N-terminal sequence of the 22-kDa protein (Fig. 7). However, the first nine amino acids of the 41-kDa protein were strikingly (88%) homologous to the N-terminal sequence of the 33-kDa major endoflagellar antigen of *T. pallidum* (Fig. 8) recently reported by Blanco et al. (9). In contrast, with the exception of residue 17, the amino acid sequences of the two flagellar antigens are completely divergent over residues 10 to 20. Furthermore, heterogeneity at residue 10 (alanine and isoleucine in equimolar ratios) sug-

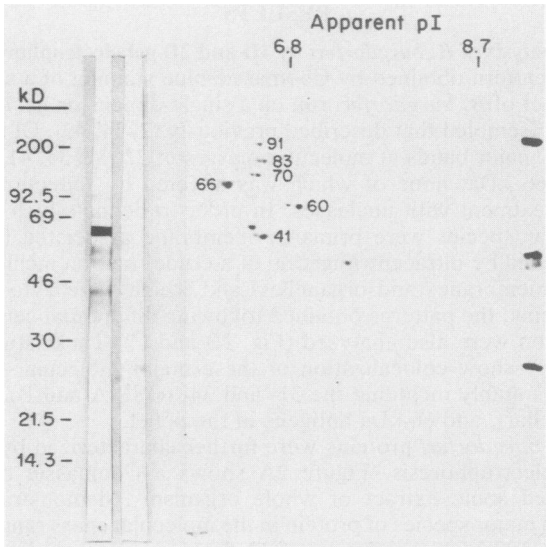

FIG. 3. Glycoproteins of *B. burgdorferi* analyzed by binding of concanavalin A on 1D and 2D gels. On the 1D 12.5% SDS-PAGE gel (left), concanavalin A binding without (left lane) or with (right lane) the specific sugar that inhibits the lectin binding (100 mM alpha-methyl mannoside) is shown. A 2D pattern of polypeptides that bound concanavalin A is shown on the right. Molecular masses (in kilodaltons) are indicated.

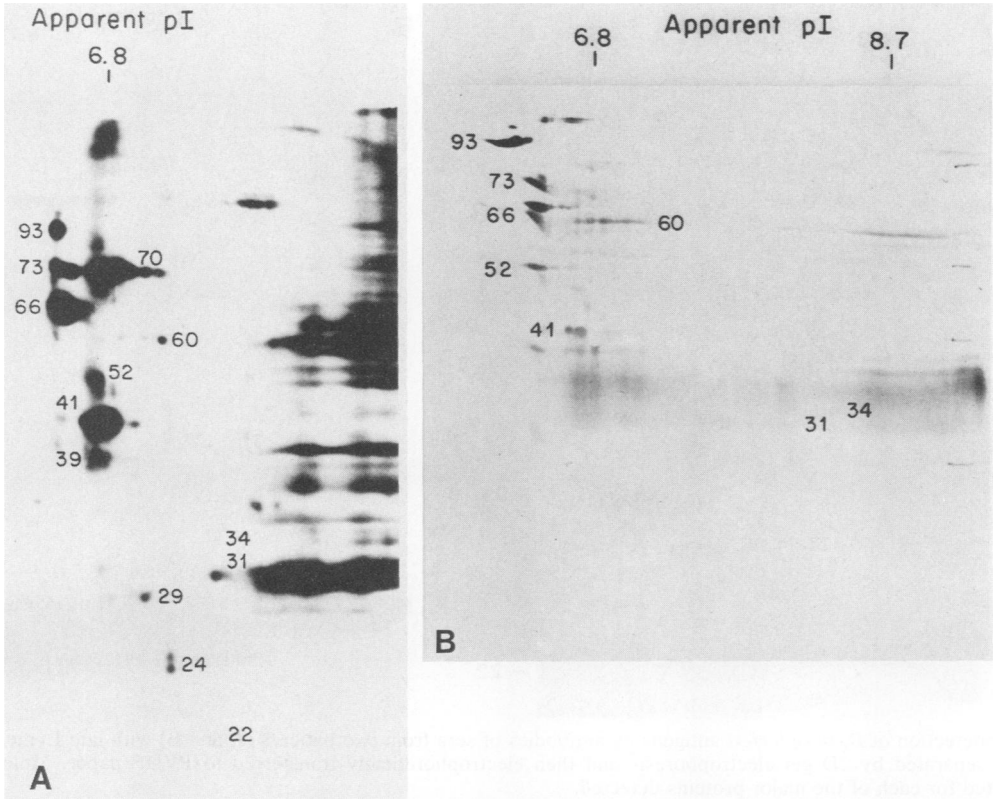

FIG. 4. Comparison of 2D gel-electrophoresed polypeptides of *B. burgdorferi* that were surface labeled with either ^{125}I (A) or *N*-hydroxysuccinimide ester-biotin (B). Molecular masses (in kilodaltons) are noted for each of the major proteins.

gests that more than one species of *B. burgdorferi* flagellar protein may have been sequenced.

Significant (31%) homology was also noted between the first 19 residues of the 22-kDa protein and the deduced N-terminal amino acid sequence of the 31-kDa (OSP A) protein of *B. burgdorferi* (8a); both molecules are significantly hydrophobic over the sequences obtained. Heterogeneity at several residues (Fig. 7) suggests that the 22-kDa protein may be polymorphic.

DISCUSSION

We used 2D gel analysis to probe and define the major immunodominant surface proteins of the B31 strain of *B. burgdorferi*. Resolution was optimized by the use of CHES buffer, pretreatment of samples with nucleases, and serial development of gels with Coomassie blue and silver stains. Furthermore, in order to minimize heterogeneity that has been noted by SDS-PAGE analysis of different strains of *B. burgdorferi* (5, 42) (some of which has been ascribed to conditions of culture and the number of passages), our studies were carried out on organisms grown in batches and studied only at the log phase of growth in samples taken from a single isolate of the B31 strain. Similar to other published 2D gels of procaryotic organisms, such as those of *Escherichia coli* (32) and *T. pallidum* (30), most proteins were in the molecular-mass range of 20 to 100 kDa, with silver staining revealing more than 100 distinct spots. This complicated pattern could be simplified somewhat by separate analysis of the pellet and cytosol fractions and may in part explain differences from previous analyses of *B. burgdorferi* proteins by 2D gel electrophoresis, which were limited to the soluble fraction of a centrifuged preparation (8). Although 2D gel electrophoresis offers the advantage of separation of proteins that comigrate on single-dimension gels, we felt it important to identify proteins found on the surface of the viable organism and correlate these findings with Western blots for infected humans and immunized rabbits. Surface labeling was achieved by biotinylation, using long-arm biotin, and by radioiodination, using the Iodogen technique. Thirteen surface-labeled proteins were clearly identifiable in the 2D gels. Surface proteins consisted of a group of very basic proteins of different molecular masses and another group of proteins, most of which had isofocusing points between five and seven and molecular masses (both under reducing and nonreducing conditions) between 20 and 100 kDa. Quantitative differences between the two methods may have reflected the amount of protein used for the gels, the relative availability of groups needed for labeling, or factors affecting the binding of avidin to biotinylated sites on the surface. Whereas ^{125}I labels the relatively rare tyrosine residues, biotin forms covalent bonds with more abundant lysine residues and free amino termini of acidic amino acids. Because the biotinylation method is highly sensitive, between 10- and 25-fold less protein was used in order to maintain a relatively low level of background needed to clearly discern the bands. Although the Iodogen technique is widely felt to be the mildest form of surface iodination (38, 39), subsurface membrane proteins have been noted to be labeled in some bacteria (24). Such studies raise the possibility that proteins belonging to structures below the outer membrane may be expressed at the surface or that the

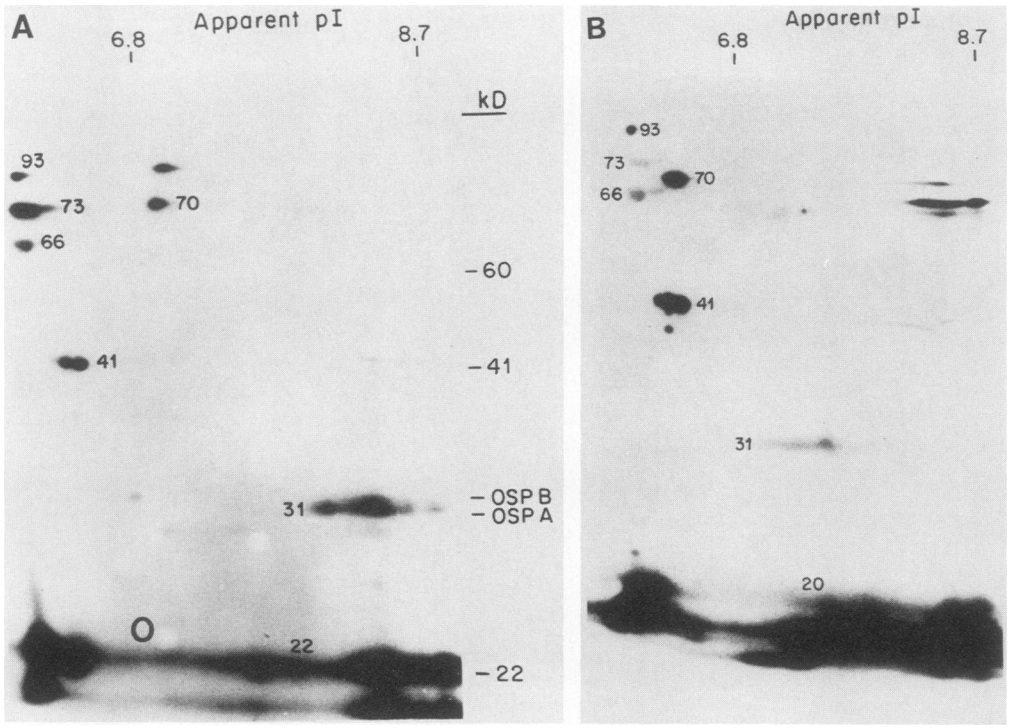

FIG. 5. Immunodetection of *B. burgdorferi* antigens by antibodies of sera from two patients (A and B) with late Lyme disease. Antigen preparations were separated by 2D gel electrophoresis and then electrophoretically transferred to PVDF paper. Molecular masses (in kilodaltons) are noted for each of the major proteins detected.

surface may break down or be modified during in vitro incubation or be labeled without a loss of viability.

Glycoconjugates play a prominent role in cell-cell recognition and are important immunogens in the host immune response to various microorganisms (36). In particular, the outermost component of the bacterial cell envelope, the S layer of many procaryotes, is composed of protein or glycoprotein subunits that have been implicated as important immunogens and sites for cell adhesion and surface recognition (36). It was therefore of interest to determine which proteins in our gels were lectin binding. For this purpose, we used biotinylated concanavalin A and confirmed specificity by the ability of alpha-methyl mannoside to inhibit the binding of lectin to *B. burgdorferi* proteins on electroblots (16, 23, 35). Eight distinct major bands bound concanavalin A, most prominently proteins with molecular masses of 41, 60, and 66 kDa. Only four surface proteins bound this lectin. Of note was the finding that the basic 31- and 34-kDa OSP A and B proteins did not bind concanavalin A, and the basic and highly immunogenic 22-kDa protein, which appears to share epitope(s) with OSP A (W. Jiang et al., submitted for publication), failed to bind any lectin tested. Further studies with additional lectin-binding ligands, as well as detailed carbohydrate analysis, will be necessary to establish the exact nature of the glycosylation.

We found relatively poor labeling of the 34-kDa surface protein by either the biotin or Iodogen technique. It would thus seem from our analysis that OSP A is a more prominent surface protein than OSP B, which may in fact be largely buried in the membrane rather than exposed to the external environment. Although immunohistochemical studies using monoclonal antibodies (6, 7) and limited solubilization of the surface have revealed these proteins to be abundant in the outer membrane of the organism (13), the humoral immune response to them was limited and in general seen only in patients with prolonged disease (2, 14), with some differences noted between infected individuals in the United States and in Europe (2, 42). Both proteins are encoded on a linear 49-kilobase extrachromosomal plasmid in the cell (3), and it has been suggested that they may modulate during the course of infection (14, 34) and thereby become immunogenic over time. This hypothesis is supported by the observation that OSP B can change in molecular mass and on immunoblots during the course of in vitro cultivation (34). Another possibility is that there is a delay in the expression of these proteins by the organism.

The surface proteins of *B. burgdorferi* were clearly defined by surface labeling with either ^{125}I or biotin. The organisms that were used for these analyses were uniformly grown for 3 days to ensure that they were at the same stage of growth for all experiments. Interestingly, we found striking differences in our ability to demonstrate surface labeling of the high-molecular-mass species of proteins, depending on whether the organisms were sonicated prior to solubilization in the sample buffer or whether the intact whole organisms were solubilized. Most likely, this result was due to degradation of these proteins, which surface label intensely but are present in relatively minor amounts compared with other membrane proteins. Microsequence analysis of the amino termini of two of the surface-labeled proteins revealed no known homology to albumin, the major protein found in BSK II medium. Since the constituents of BSK II are not fully defined, it remains possible that some of the proteins thus identified as surface proteins may be derived from the medium. However, this is unlikely, since all the surface proteins described thus far are constitutively labeled with [^{35}S]methionine (data not shown).

The 41-kDa flagellar antigen is the first antigen of *B.*

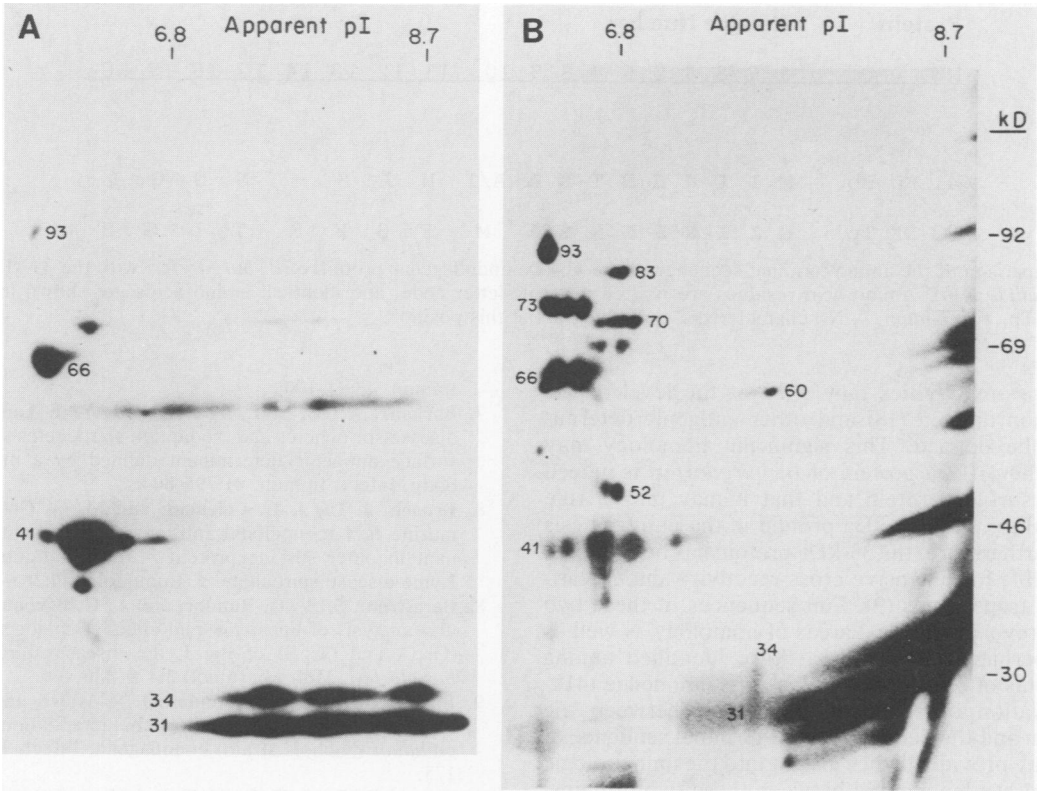

FIG. 6. Immunodetection of *B. burgdorferi* antigens by sera of rabbits immunized with the whole organism (A) or a sonic extract of *B. burgdorferi* (B). Antigen preparations were separated by 2D gel electrophoresis and electrophoretically transferred to PVDF paper. Molecular masses (in kilodaltons) are indicated adjacent to each spot.

burgdorferi to be recognized by rabbits or humans during natural infection or experimental immunization (2, 14). Barbour and colleagues developed a monoclonal antibody specific for the 41-kDa protein and demonstrated immunohistochemically that it recognized a periflagellar epitope (4). It was assumed in these studies that the highly immunogenic 41-kDa antigen was below the outer membrane and therefore cryptic. In contrast, we found that this protein can be clearly identified following surface labeling of viable organisms. Both biotin and ^{125}I bound avidly to give an intense signal. In fact, on 2D analysis, several proteins were identifiable in the 41-kDa range, but only two closely related proteins were surface labeled. It remains possible that the monoclonal antibody reported by Barbour et al. binds to one of these non-surface-labeled proteins with an apparent molecular mass of approximately 41 kDa. That subsurface proteins are labeled by these techniques seems less likely, since both the Iodogen and biotinylation methods labeled the same protein, with cells remaining viable throughout the procedure. More likely is that the 41-kDa protein is indeed a periflagellar protein that has exposure to the outer surface of the organism. This perplexing phenomenon of the periflagellar proteins being surface related has also been noted for *T. pallidum* (10, 11). Thus, antisera to endoflagella of treponemes are known to possess complement-dependent in vitro treponemicidal activity against *T. pallidum*. Stamm and colleagues (37) demonstrated that the endoflagellar proteins were surface labeled and could be immunoprecipitated by immune sera. In particular, the 33-kDa protein of *T. pallidum* has been identified as a periflagellar protein that is surface exposed (10, 11, 30, 37).

The remarkable and highly significant homology between the 41-kDa antigen of *B. burgdorferi* and the class B periflagellar proteins of *T. pallidum* (29) described by Blanco et al. (11) and the five periflagellar proteins described by Norris et al. (29), including the 33-kDa antigen of *T. pallidum*, provides a partial structural basis for the serological cross-reactivity between these two pathogens (25, 26). Significant sequence homology is also apparent between the 41-kDa antigen of *B. burgdorferi* and that of *Bacillus subtilis* (15).

Protein	Residue Number																	
MW	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
22KD	**M**	E	**K**	**Y**	**L**	S	Y	**I**	K	I	N	**L**	L	-	A	I	Q	L
31KD	**M**	K	**K**	**Y**	**L**	L	G	**I**	G	L	I	**L**	A	L	I	A	C	K

FIG. 7. Comparison of the amino-terminal sequence of the 22-kDa protein with the 31-kDa (OSP A) (8a) protein of *B. burgdorferi*. Amino acid residues are written in single-letter code, and identical amino acids are shown in boldface. –, No characteristic chromatogram at this position.

Protein MW	Residue Number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
41 KD Bb	**M**	**I**	**I**	**N**	**H**	**N**	**T**	**S**	**A**	A/I	N	A	S	-	N	N	**G**	I
33 DK Tp	**M**	**I**	**I**	**N**	**H**	**N**	**M**	**S**	**A**	M	F	S	**F**	R	T	L	**G**	H

FIG. 8. Comparison of the amino-terminal sequence of the 41-kDa endoflagellar protein of *B. burgdorferi* with the 33-kDa endoflagellar protein of *T. pallidum* (9). Amino acid residues are written in single-letter code, and identical amino acids are shown in boldface. Bb, *B. burgdorferi*; Tp, *T. pallidum*. –, No characteristic chromatogram at this position.

Additional cross-reactivities may exist at the level of the 60-kDa common antigen (18) and other antigenic determinants yet to be defined. This significant homology may indicate that the 41-kDa protein of *B. burgdorferi* is indeed an important surface protein and that it may play a role analogous to that of the 33-kDa protein in the pathogenesis of syphilis. Furthermore, the 33-kDa protein has been shown to be responsible for extensive cross-reactivity among various strains of treponemes (9). Full sequences of these two proteins may reveal additional areas of homology as well as divergence, perhaps analogous to those identified among flagellar proteins of different serotypes of salmonellae (41).

Our observation of significant homology between the 22-kDa protein and the deduced amino-terminal sequence of OSP A (8a) may provide further insight into the immunologic cross-reactivity we have noted between these two proteins, as well as between higher-molecular-mass surface proteins by direct amino acid sequence analysis (manuscripts in preparation). Similarly, homology between OSP A and B was most striking for the initial and terminal thirds of the deduced sequence, with preservation of a hydrophobic sequence at the N terminus that has the configuration of a signal peptide (8a). Further studies will be necessary to determine whether the OSP proteins, the 22-kDa protein, and other molecules are part of a larger family of proteins and whether preservation of N-terminal sequences with leader configurations may have significance in defining processing of these proteins and their localization in the membrane.

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