VARIABLE MAJOR PROTEINS OF *BORRELIA HERMSII*

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The reappearance of borreliae in a patient's blood during a second, third, or fourth febrile crisis of relapsing fever has suggested to students of this disease that these spirochetes undergo antigenic variation (1–4). Meleney summarized the state of knowledge of this phenemenon in 1928 (4): "At the time of the crisis which terminates the attack of fever, there is rapid agglutination and destruction of the spirochetes with the subsequent formation of immune bodies in the blood. These substances are specific for the strain of spirochetes which was present during the preceding attack, but have no influence on the spirochetes of the succeeding relapse. The spirochetes of the relapse give rise, in turn, to immune substances which are specific for them but not for the spirochetes of the first attack." Schuhardt and Wilkerson (5) showed in 1951 that these antigenically distinct relapse strains appeared after inoculations of rats with single organisms of *Borrelia turicatae*. Coffey and Eveland (6) subsequently identified by immunofluorescence three novel serotypes of *Borrelia hermsii* in the blood of rats experiencing relapses. One of us (H. G. Stoenner) has further defined this phenomenon of antigenic variation by studying *B. hermsii*, an agent of relapsing fever in North America, in mice. The use of serotype-specific antisera permitted identification of 24 different serotypes among the progeny of a single organism inoculated into a mouse (7).

We are attempting to identify and characterize the variable antigens of *B. hermsii*. Although there has been considerable interest among biologists in similar phenomena shown by the salivarian trypanosomes (8–12), relatively little is known of borrelial antigens, their locations in the cells, and the mechanism of antigenic variation. Using both polyclonal antisera and monoclonal antibodies, we have identified in whole cell lysates of *B. hermsii* an abundant protein that is serotype specific.

Materials and Methods

Organisms and Culture Conditions. The origin of the *B. hermsii* strain used in these studies has been described (13, 14). It was designated strain HS1. Details of the strategy and methods used for identification and isolation of relapse serotypes is described elsewhere (7). In summary, HS1 was cloned by limiting dilution in Swiss mice of the Rocky Mountain Laboratories stock (RML).[1] Spirochetes were enumerated by dark-field microscopy (14). Preliminary experiments had shown that one borrelia inoculated intraperitoneally was sufficient to produce spiroche-

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C, culture; FA, direct immunofluorescence assay; FCS, fetal calf serum; IFA, indirect immunofluorescence assay; NCP, nitrocellulose; PBS, phosphate-buffered saline, pH 7.4; PBS/Mg, phosphate-buffered saline with 5 mM $MgCl_2$; RML, Rocky Mountain Laboratories; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris-(hydroxy-methyl)aminomethane; TSGAN, 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% sodium azide, and 0.05% Nonidet P-40.

temia in a mouse. The source of *B. hermsii* for cloning was the plasma of a mouse that had been infected via the bite of an *Ornithodoros hermsi* tick. Mice infected with one organism from this original population were bled during their initial spirochetemia. A cloned population obtained in this way from one mouse was used both to produce fluorescinated antiserum (see below) and as an inoculum to infect other mice. Borrelial populations obtained during relapses of spirochetemia in these additional mice also were cloned by limiting dilution; these clones were subsequently used to produce other antisera. Eventually, 24 serotypes were identified in and cloned from the progeny of a single borrelia (7).

We chose three of these serotypes, 7, 14, and 21, for further study. Seed stocks of each serotype in mouse plasma with 10% (vol/vol) glycerol were kept frozen at $-68°C$. Before in vitro cultivation, a serotype was passed from a seed stock into cyclophosphamide-treated (300 mg/kg) 18- to 19-d-old RML mice. Cyclophosphamide at this dosage usually delayed the beginning of clearance of borreliae from the blood of the mouse until about 80 h post-inoculation. At 48- to 60-h post-inoculation, we bled the mice by cardiac puncture, inoculated glass tubes (Pyrex 9826; Corning Glass Works, Corning, NY) with fortified Kelly's medium (15) with 0.1 ml of citrated blood containing $\sim 5 \times 10^6$ borreliae, and then incubated the tightly capped tubes at $35°C$. Borreliae in the broth were enumerated by dark-field microscopy (14). When the density of borreliae was at least $10^7/ml$ (usually 3-6 d after inoculation), we aspirated the culture broth, taking care not to take up blood elements and fibrin at the bottom of the tube. The broth was centrifuged at 9,500 g for 20 min at $20°C$, and the pellet was suspended in $\frac{1}{30}$ volume of M/15 phosphate-buffered saline, pH 7.35 (PBS), with 5 mM $MgCl_2$ (PBS/Mg). This suspension was centrifuged for 10 s in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, CA) to remove remaining fibrin clots. Glycerol was added to a final concentration of 16% (vol/vol), and the suspension was frozen at $-76°C$. The homogeneity of the borreliae in the harvest was assessed by direct immunofluorescence assays (FA) with antisera against 24 different serotypes.

Another serotype that was studied had been passed 27 times in plain Kelly's medium (16) before being cloned by limiting dilution in fortified Kelly's medium. Stock cultures of this cloned population were frozen. This culture (C) serotype was not passed in mice before in vitro culture. Instead, a stock culture was thawed and passed directly to culture broth. Thereafter, it was handled as described above.

Antisera. Hyperimmune antisera against live whole cells of the different serotypes were produced in 6- to 7-mo-old RML mice; a portion of each serum was conjugated with fluorescein isothiocyanate (7). Briefly, mice received $\sim 2 \times 10^6$ borreliae intraperitoneally on day 0. Relapses were prevented by administering tetracycline in their drinking water beginning 36-40 h post-inoculation. On days 6, 9, 12, 16, and 19, the mice were inoculated intraperitoneally with $2 \times 10^6-10^7$ organisms of the same serotype. Mice were bled on day 25. Antisera were conjugated with fluorescein by a modification of the method of Peacock et al. (17). Table I summarizes the immunofluorescent reactions of antisera against serotypes C, 7, 14, and 21. We raised antisera in New Zealand rabbits by inoculating live, washed, in vitro cultivated organisms of serotype 7 into ear veins. The rabbits received an injection of $\sim 10^8$ borreliae on days 1, 3, 121, 124, and 125. They were bled on day 136.

Production of Hybridomas. Frozen suspensions of serotypes 7 and 21 were rapidly thawed in warm water, centrifuged for 4 min in a microfuge, and resuspended in an equal volume of PBS. 3- to 4-wk-old male and female BALB/c mice (RML breeding colony substrain) were each inoculated intraperitoneally with $\sim 10^8$ organisms. 48 h after inoculation, we examined by phase microscopy blood obtained from the tail vein for motile spirochetes. Mice with heavy burdens of spirochetes, i.e., $\sim 10^7$ borreliae/ml, were reexamined at 72 h. Mice that had spirochetemia at 48 h but no detectable spirochetes in the blood at 72 h were killed. The spleen cells were teased into single-cell suspensions and washed three times with RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) without serum. Hybrid clones were obtained essentially as described by Chesebro et al. (18). Briefly, spleen cells from borrelia-infected mice were fused with cells of the p3-NS-1-Ag-4/1 derivative of the BALB/c myeloma MOPC 21 at a ratio of 10:1 in 35% (wt/vol) polyethylene glycol 1540 (J. T. Baker Chemical Co., Phillipsburg, NJ) in RPMI 1640. After fusion, $\sim 5 \times 10^6$ cells in RPMI 1640 with 15% fetal calf serum (FCS) were added to each well of a 24-well Linbro culture plate (Flow Laboratories,

TABLE I

Summary of Immunofluorescence Reactions*

Antibody	B. hermsii serotype			
	C	7	14	21
Mouse antisera‡				
αC	+	−	−	−
α7	−	+	−	−
α14	−	−	+	−
α21	−	−	−	+
Hybridoma supernatant§				
H1826	−	+	−	−
H3326	−	−	−	+

* See Materials and Methods for preparation of borreliae on slides.

‡ Direct immunofluorescence assay using fluorescein-conjugate of polyclonal mouse antisera.

§ Indirect immunofluorescence assay. Second antibody was fluorescein-conjugated goat anti-mouse immunoglobulin.

Rockville, MD). The tray was placed in a 37°C, 10% CO_2 incubator. 24 h later, 1 ml of hypoxanthine-aminopterin-thymidine (HAT) medium (RPMI 1640 with 15% FCS and 13.6 μg/ml hypoxanthine, 0.18 μg/ml aminopterin, and 3.9 μg/ml thymidine) was added. HAT medium was replaced by HT medium (HAT medium without aminopterin) after 14 d. The HT medium was replaced in turn by RPMI medium supplemented with 15% FCS alone 28 d after the fusion.

Selecting and Cloning Hybridomas. Supernatants from wells containing proliferating hybridomas were assayed by indirect immunofluorescence (IFA). Smears were made of washed borreliae of serotype C, 7, 14, or 21 that had been suspended in citrated rat blood. The slides were fixed in 100% methanol for 30 min, air dried, and kept in a desiccator at −20°C until use. Hybridoma culture fluids were spotted onto the slides with a wire loop. The slides were incubated for 30 min at 37°C. After washing the slides for 10 min in PBS, we spotted onto the slides fluorescein-coupled, goat anti-mouse immunoglobulin (Becton Dickinson Research Center, Research Triangle Park, NC), that had been diluted 1:250 in PBS with 1% bovine serum albumin (BSA, Fraction V, Miles Laboratories, Elkhart, IN). Slides were incubated at 37°C for 30 min and washed again in PBS. We examined borreliae on the slide for fluorescence under a Zeiss Photomicroscope III (Carl Zeiss, Inc., New York). A positive assay was a hybridoma supernatant that reacted with borreliae of one serotype (7, 14, 21, or C) but not the other three.

Cells from antibody-positive wells were cloned by limiting dilution in Linbro 96-well trays containing 2.5×10^5 normal BALB/c spleen cells per well as a feeder layer. When a well was confluent with growth, the supernatant was assayed again by IFA. Clones were expanded in larger flasks. We confirmed the success of cloning by subjecting the light chains present in the tissue culture supernatants to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18).

The class of monoclonal antibody was determined by immunodiffusion (19). Class-specific rabbit antibodies to mouse γM, γG1, γG1b, γG2a, γG2b, γG3, and γA were obtained from Litton Bionetics, Kensington, MD.

Polyacrylamide Electrophoresis. Frozen, in vitro cultivated borreliae were thawed, centrifuged for 3 min in a microfuge, and washed twice with PBS/Mg. Cells were suspended in a volume of distilled water to give an OD_{595} of 0.2 when the cell suspension was analyzed for protein content by the Bradford method (20); preliminary experiments had shown that borreliae were lysed by the Bradford reagents. Sample buffer was added to the cells in water for final concentrations of 1% SDS (BDH Chemicals, Poole, England) and 10% 2-mercaptoethanol. The samples were boiled for 5 min, and 25 μl was subjected to SDS-PAGE as described by Laemmli and Favre (21). Acrylamide and N,N'-methylenebis-acrylamide (Sigma Chemical Co., St. Louis, MO) were in a ratio of 30:0.8 in the stock solution. The pH of the separating gel buffer

was either 8.8 or 8.6. Tris-(hydroxymethyl)aminomethane (Tris) was obtained from Research Organics, Inc., Cleveland, OH. Gels were stained with Coomassie brilliant blue R-250 or by the silver stain method of Poehling and Neuhoff (22). Molecular weight standards labeled with ^{14}C were phosphorylase B (93,000), bovine albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and beta-lactoglobulin (18,000) (New England Nuclear, Boston, MA).

   Electrophoretic Transfer of Separated Proteins from SDS-PAGE to Nitrocellulose and Application of Antibody to the Blots.   The procedure used for performing transfer of proteins from SDS-PAGE gels to nitrocellulose (NCP) and incubation of blots with antisera or antibody (Western blots) was a modification of the method described by Towbin et al. (23). The proteins in a gel were transferred to NCP (HAHY; Millipore Corp., Bedford, MA) in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA) containing 192 mM glycine, 25 mM Tris base, and 20% methanol in glass-distilled water. The cell was kept at 25°C with a cooling coil during the electrophoresis (60 V for 3 h). After electrophoresis, the NCP blots were blocked by overnight incubation in heat-sealable plastic bags containing 2% BSA in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin (Knox, Englewood Cliffs, NJ), 0.05% sodium azide, and 0.05% Nonidet P-40 (TSGAN) (24). We incubated the blots with either a 1:100 dilution of mouse antisera or a 1:4 dilution of hybridoma culture supernatant in 2% BSA/TSGAN for 2 h at room temperature. The strips were then washed three times with TSGAN. The blots exposed to monoclonal antibodies were incubated for 2 h at room temperature with the IgG fraction of rabbit anti-mouse immunoglobulin (Miles Laboratories) diluted 1:400 in 2% BSA/TSGAN. These were washed again three times with TSGAN. We incubated the antisera blots (no second antibody) and the monoclonal antibody blots with ^{125}I-labeled protein A (50,000 cpm/ml of TSGAN) for 2 h at room temperature. Protein A (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) had been labeled by a modification of the chloramine-T method of Hunter and Greenwood (25) to a specific activity of 5 μCi/μg. The blots were washed four times with TSGAN, rinsed several times with water, and dried between sponges in an incubator at 37°C. Autoradiography was performed with Kodak X-Omat AR or TL film (Eastman Kodak Co., Rochester, NY).

   Labeling of Borreliae with Radioactive Iodine.   Washed, intact borreliae were labeled with ^{125}I in the presence of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, IL) essentially as described by Markwell and Fox (26). Briefly, 10 μg of Iodogen in 10 μl of chloroform was placed in a glass tube, and the chloroform was evaporated with a nitrogen jet. Washed borreliae were suspended in a volume of PBS/Mg that gave a protein content of the suspension of 0.3 mg/ml by the Bradford assay (20). 1 ml of the suspension was centrifuged for 3 min in a microfuge. The cells were suspended in 50 μl of PBS/Mg. The cells and then 0.5 mCi of Na ^{125}I (ICN Chemical & Radioisotope Div., ICN Nutritional Biochemicals, Irvine, CA) were added to the tubes containing adsorbed Iodogen. The tubes were incubated for 5 min at room temperature. Reactions were stopped by removing the entire suspension from the tubes and adding it to 700 μl of PBS/Mg. The intact cells were washed three times with PBS/Mg and finally suspended in 600 μl of water. These labeled preparations were analyzed on SDS-PAGE. The gels were stained, blotted, or dried, and radioautography was performed on the dried gels or blots as described above. Stained gels and radioautographs were examined with a Joyce-Loebl microdensitometer (Joyce-Loebl, Div. of Vickers Ltd., Galeshead, England). Integration of peaks was performed with the aid of a Zeiss MOP 3 image analyzer (Carl Zeiss Inc.).

Results

   Borrelia Preparations.   In vitro cultivated preparations of serotypes 7, 21, and C were 100% homologous serotype by FA when 100–200 borreliae were examined. Two preparations of serotype 14 each contained 2% serotype C in addition to serotype 14.

   SDS-PAGE.   We analyzed whole cell lysates of the four serotypes by SDS-PAGE (Fig. 1). The Coomassie blue-stained and silver-stained protein patterns were similar among the different serotypes with few exceptions. The notable exception was a major protein species that we have designated as pI. The pI of each serotype, e.g., pI$_7$ in the
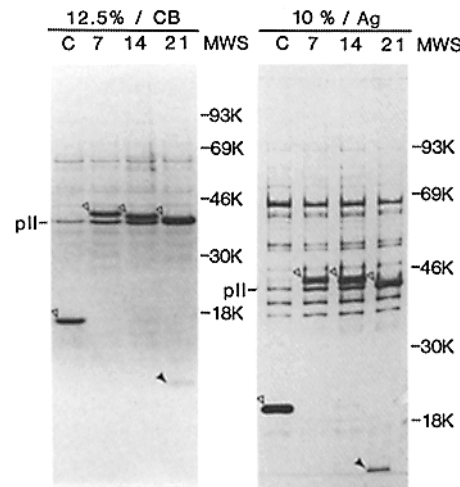
Fig. 1.   SDS-PAGE of whole cell lysates of *B. hermsii* serotypes C, 7, 14, and 21. Gels contained 12.5% (left) or 10% (right) acrylamide. The separating gel buffer was ph 8.8. Gels were stained with Coomassie blue (CB) or silver (Ag). The position of the pII proteins is shown. Open arrows indicate the pI proteins in each serotype. Closed arrows indicate a low molecular weight band in serotype 21. The positions of molecular weight standards (MWS) in each gel are shown.

case of serotype 7, was unique in its apparent subunit molecular weight. In a representative 12.5% gel with a pH 8.8 separating buffer, the apparent molecular weights were 42, 41, 40, and 19 × 10^3 for pI7, pI14, pI21, and pIc, respectively. Molecular weight was estimated by comparison with standards. We also noted that the SDS-PAGE protein profiles of borreliae recovered directly from the plasma of cyclophosphamide-treated or nontreated mice were identical to SDS-PAGE profiles or organisms cultivated in vitro (data not shown).

The other major protein in the lysates was pII. It had an identical molecular weight by SDS-PAGE in the four serotypes; it was 39 × 10^3 mol wt in the case of the 12.5% gel described above. Serotype 21 also had a minor, low-molecular weight band (~12 × 10^3) not seen in the other serotypes (Fig. 1). The electrophoretic migrations of pI and pII were not altered when we removed 2-mercaptoethanol from the sample buffer or heated the samples with and without 2-mercaptoethanol for 60°C for 45 min instead of boiling for 5 min before applying samples to SDS-PAGE.

Mouse and Rabbit Antisera.   Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes. We incubated the blots with mouse antisera and used ^{125}I-labeled protein A as a probe for detecting IgG bound to *B. hermsii* components. Antibody in homologous and heterologous antisera bound to several proteins shared by serotypes C, 7, 14, and 21 (Fig. 2). Preimmune mouse serum did not detectably bind to any borrelial protein (data not shown). Among the common proteins recognized by each of the antisera was a protein with the same apparent MW as pII. In contrast to pII, proteins of the same molecular weight as pIc and pI14 were only recognized by IgG in homologous antisera. The band in the lane of serotype 14, which had the same molecular weight as pIc, probably reflects the 2% contamination of this preparation with serotype C.

We suspected that pI21 also was bound by homologous antibody. Although pI21 and pII could not be easily distinguished in the Coomassie blue-stained gel, there was
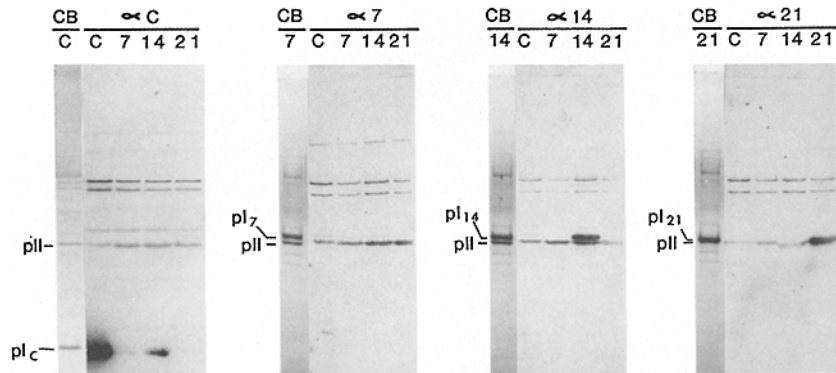
FIG. 2.   Western blot analysis of binding of polyclonal mouse antisera to *B. hermsii* proteins. Proteins of serotypes C, 7, 14, and 21 were separated by SDS-PAGE (10% acrylamide and pH 8.8 separating gel buffer) and transferred electrophoretically to nitrocellulose. The blots were incubated with antisera diluted 1:100 in TSGAN and 2% BSA (see Materials and Methods). After washing in TSGAN alone, we used ^{125}I-labeled protein A as a probe for binding of IgG to proteins. Radioautography was performed on Kodak X-Omat AR film. Along the left side of each blot are shown the Coomassie blue- (CB) stained proteins of the homologous serotype in the gels. The lanes containing lysates of each serotype are indicated at the top. The locations of the pIs and pII are shown. The SDS-PAGE and blot on the far left was performed at a different time than the other three.
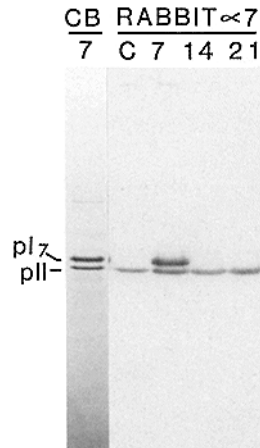

FIG. 3.   Western blot analysis of binding of rabbit anti-serotype 7 antiserum to components of serotypes C, 7, 14, and 21. See legend to Fig. 2 and Materials and Methods for details. The gel contained 12.5% acrylamide and pH 8.8 separating gel buffer. The Coomassie blue (CB) stained pI7 and pII proteins are shown on the left.

a broad band at the level of pI_{21}-pII of serotype 21 in the radioautograph. This broad band was not present at the pII levels in lanes of the other serotypes (Fig. 2).

In contrast to pI_C, pI_{14}, and probably pI_{21}, pI_7 was not detectable in Western blots which incorporated mouse anti-serotype 7 serum (Fig. 2). Anti-serum raised against whole cells of serotype 7 in a rabbit, however, did bind to proteins with the same electrophoretic mobilities as pI_7 and pII but not pI_C, pI_{14}, and pI_{21} in Western blots (Fig. 3).

Monoclonal Antibodies. For hybridoma fusions, spleens were removed from mice 3 d after inoculation with live organisms and 1 d after peak spirochetemia. We used either

serotype 7 or 21 to produce infections. In the case of serotype 7, five spleens yielded 11 cloned, productive hybridomas that were specific by IFA for serotype 7. The corresponding figures for serotype 21 were two spleens and six hybridomas. The IFA reactions of two monoclonal antibodies, H1826 and H3326, are summarized in Table I. A monoclonal antibody cross-reactive with two or more serotypes was not encountered during screening of numerous fusions derived from 3-d post-inoculation spleens.

A representative IFA reaction of homologous monoclonal antibodies against borreliae is shown in Fig. 4. The anti-serotype 7 antibody was H1826. Staining of the organisms was homogeneous. Large blebs of borrelial envelopes and thread-like extensions of spirochetes in the long axis also fluoresced brightly. Culture medium controls and heterologous antibody (e.g., H1826 vs. serotype 21) did not stain borreliae.

Nine of the anti-serotype 7 antibodies and the six anti-serotype 21 antibodies were further screened by Western blot analysis. Two anti-serotype 7 and two anti-serotype 21 antibodies did not appear to bind to any component in the blots. Seven anti-serotype 7 antibodies bound to a single protein, or at least to a single band, of the same apparent molecular weight in serotype 7. Four anti-serotype 21 antibodies similarly bound to a single band, which was of slightly lower molecular weight, in serotype 21 whole cell lysates. Monoclonal antibodies H1826 and H3326 (Table I) were representative of the Western blot-reactive antibodies. Both were IgM antibodies as determined by immunodiffusion. Fig. 5 shows that these antibodies only recognized components in the homologous serotypes, and that the apparent molecular weight of these components differed between serotypes 7 and 21. The faint bands seen in all lanes of the radioautographs in Fig. 5, including that of a culture medium control,
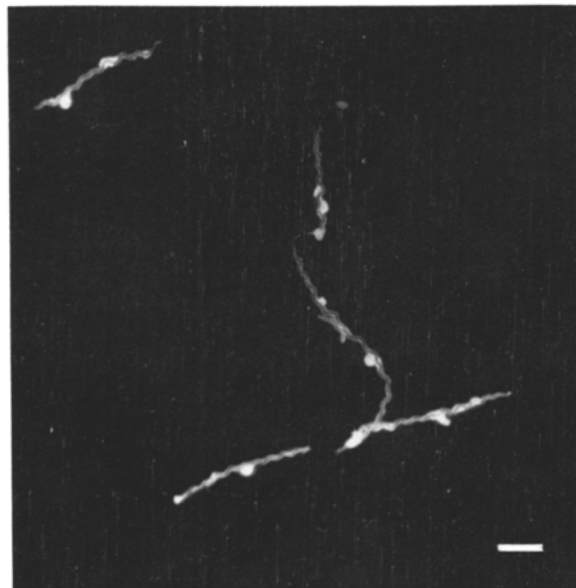

Fig. 4. Indirect immunofluorescence. *B. hermsii* serotype 7 organisms on slides were fixed with methanol as described in Materials and Methods. Slides were incubated with monoclonal antibody H1826, washed, incubated with fluorescein-conjugated goat anti-mouse immunoglobulin, washed, dried, and examined for fluorescence. Bar, 5 μm.
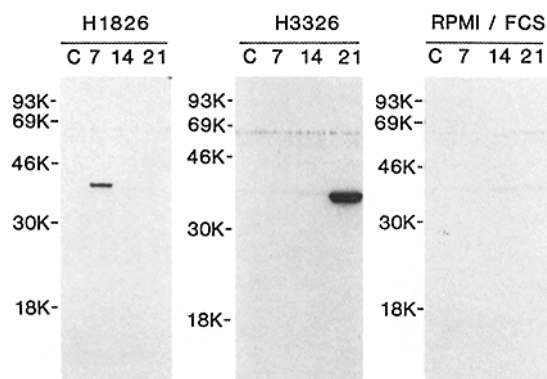
Fig. 5. Western blot analysis of binding of monoclonal antibodies to components of serotypes C, 7, 14, and 21. The gels contained 10% acrylamide and pH 8.8 separating gel buffer. The methods were the same as described in the legend to Fig. 2, except that rabbit anti-mouse immunoglobulin was used as a second antibody before exposure of blots to labeled protein A. The monoclonal antibodies were H1826 (anti-serotype 7) and H3326 (anti-serotype 21). The right-most blot shows the weak nonspecific reactions that were obtained with culture medium (RPMI/FCS) without monoclonal antibody. The locations of ^{14}C-labeled molecular weight standards are shown to the left of each blot.

were probably a result of binding by IgG in the FCS or, more likely, in the rabbit immunoglobulin used as a second antibody. No bands were seen when the blot was exposed only to labeled-protein A.

^{125}*Iodine Labeling of Borreliae.* Whole cells of serotypes 7 and 21 were labeled with ^{125}I in the presence of Iodogen. These preparations were subjected to SDS-PAGE. Fig. 6 A shows the Coomassie blue-stained proteins and the radioautograph of this gel. The pH of the separating gel buffer in this experiment was 8.6 instead of 8.8. We found that lowering the pH of the buffer from 8.8 to 8.6 resulted in altered electrophoretic migrations of pI and pII. This alteration was consistently more pronounced for the pI than pII and resulted in a lowering of the apparent molecular weights of the pI relative to pII but not to each other. Manipulation of the buffer pH permitted separation of pI$_{21}$ and pII in gels. This can be seen in Fig. 6 A, where pI$_{21}$ migrated ahead of pII, and pI$_7$ and pII of serotype 7 now co-migrate. Further separation of pI$_{21}$ from pII, as well as pI$_7$ from pII, was achieved using both an acrylamide concentration of 7.5% and pH 8.6 separating gel buffer. Fig. 6 B shows the Coomassie blue-stained proteins and the radioautograph of such a gel of lysates of ^{125}I-labeled borreliae. Densitometry of the Coomassie blue-stained band and radio-emitting bands demonstrated that pII of serotypes 7 and 21 was iodine-labeled to a lesser extent than pI$_7$ or pI$_{21}$. The peak in the radioautograph corresponding to pII was 2.5-fold smaller in area than expected from analysis of the Coomassie blue-stained pI$_{21}$ and pII peaks.

The probable identity of the two bands bound by H1826 or H3326 with pI$_7$ and pI$_{21}$, respectively, is demonstrated in Fig. 7. ^{125}I-labeled preparations of 7 or 21 were run in the same gel as unlabeled preparations of the serotypes. All lanes were electrophoretically transferred to NCP. The NCP with lanes of unlabeled components was incubated with antibody and processed as described above. The NCP with lanes of labeled components was washed and dried without exposure to antibody. The H1826- and H3326-reactive bands co-migrated with pI$_7$ and pI$_{21}$, respectively.
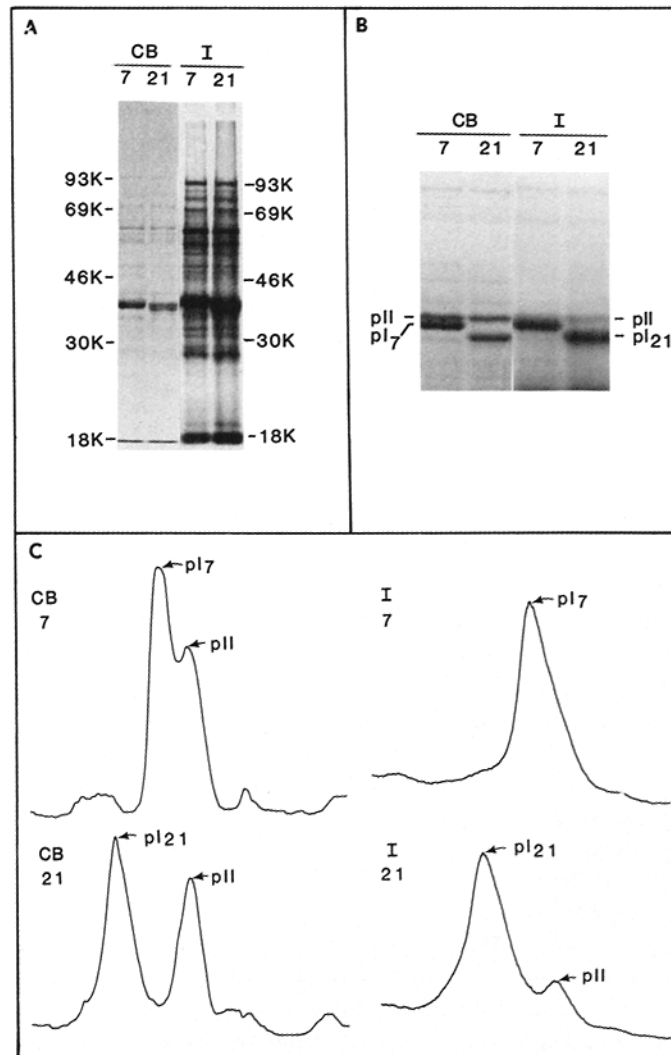
Fig. 6. ^{125}I-labeled *B. hermsii* serotypes 7 and 21. Borreliae were labeled with ^{125}I in the presence of Iodogen (see Materials and Methods). (A) Whole cell lysates of iodinated organisms were subjected to SDS-PAGE (10% acrylamide and pH 8.6 separating gel buffer). One-half of the gel was stained with Coomassie blue (CB). The borrelial proteins in the other half of the gel (I) were electrophoretically transferred to nitrocellulose, and radioautography was performed. The positions of the ^{14}C-labeled molecular weight standards are indicated on each side. (B) Stained gel (CB) and radioautograph of dried gel (I). The gel contained 7.5% acrylamide and pH 8.6 separating gel buffer. The locations of pI$_7$, pI$_{21}$, and pII are indicated. (C) Micro-densitometric tracings of stained gel (CB) and radioautographs of dried gel (I) that were shown in B. The SDS-PAGE anode is to the left.

Discussion

An inability to grow pathogenic borreliae in vitro and a lack of a battery of serotype-specific antisera limited past investigators who wished to study borrelial antigens and antigenic variation. It was clear to early workers (1–6), however, that relapse isolates showed a different antigenic "face" to the host than that of the original
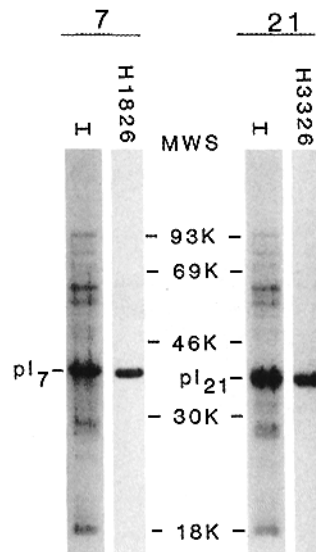
Fig. 7. Electrophoretic migrations of ^{125}I-labeled proteins (I) and of antibody-bound proteins (H1826 and H3326) of *B. hermsii* serotypes 7 and 21. SDS-PAGE gels contained 10% acrylamide and pH 8.6 separating gel buffer. Labeled (I) and unlabeled (H1826 and H3326) whole cell lysates of borreliae were run in the same gel and subsequently transferred to nitrocellulose. Strips containing unlabeled components were cut out and incubated with homologous monoclonal antibody (H1826 or H3326 for serotypes 7 and 21, respectively). The blots were processed as described for Figs. 2 and 5. Strips with labeled components were not exposed to antibody prior to radioautography. The location of ^{14}C-labeled molecular weight standards (MWS), pI_7, and pI_{21} are indicated.

infecting strain. Felsenfeld and co-workers (27) did identify two protein fractions of *B. turicata* which were original strain and relapse strain specific (27). However, these fractions were crude and were derived from rat-grown bacterial populations that had not been cloned.

In the present study, we also undoubtedly worked with mixtures of organisms. The frequency of appearance of new serotypes during both in vivo and in vitro cultivation is on the order of 10^{-4}–10^{-3} per cell per generation (7). Nevertheless, the in vitro cultivated preparations we used were relatively pure, i.e., ≥98% homogeneous. If "contamination" occurred, it was usually C serotype, the serotype that appears to have a selective advantage in fortified Kelly's medium.

SDS-PAGE of serotypes C, 7, 14, and 21 revealed that each serotype had an abundant protein of unique electrophoretic mobility. This was apparent by Coomassie blue and silver staining of the gels and in radioautographs of iodine-labeled cells. There was also one minor, low-molecular weight band present in serotype 21 only. The serotypes that we passaged in mice before in vitro culture, i.e., 7, 14, and 21, had pI estimated to be within 3,000 mol wt of each other. In contrast, C serotype had, in addition to pII, a major protein of ~19,000 apparent subunit mol wt. Interestingly, this is about half the weight of the pI in the other serotypes. Our designation of C serotype's 19,000 mol wt protein as a pI protein was supported by two-dimensional peptide maps that showed considerable homology between pI_7, pI_{14}, pI_{21}, and pI_C (R. C. Judd and A. G. Barbour, manuscript in preparation).

We found that the pI proteins were the serotype-specific antigens by Western blot analysis. It is possible, however, that other serotype-specific antigens exist. These may

have eluded detection because they did not transfer to NCP under the conditions used or because the epitopes were altered by detergent treatment. Examples of the latter circumstance may have been the lack of binding of the polyclonal anti-serotype 7 mouse serum to pI_7 and of the four monoclonal antibodies to a pI (or any other component) in Western blots.

The pI appear to be surface antigens from immunofluorescent studies using monoclonal antibodies. We suspect that the blebs highlighted by antibody were local separations of the outer envelope away from the underlying peptidoglycan-cytoplasmic membrane complex (28). The solid-phase radioiodination experiments, in which we found greater labeling of pI_7 and pI_{21} compared with the pII of those serotypes, also suggested a surface location for the pIs (26).

Protein pII was another major protein species seen by SDS-PAGE analysis. The apparent molecular weight was identical in all serotypes, and polyclonal antisera raised against one serotype bound to the pII of the heterologous serotypes. As evidenced by Iodogen iodine-labeling studies, protein pII does not appear to have as many accessible labeling sites as the pI. We presume that during infection borreliae lyse or are degraded by phagocytes upon appearance of neutralizing antibody, and that subsurface components are then exposed. This proposition was further suggested by results obtained with a monoclonal antibody that resulted from a fusion incorporating a spleen removed 7 d after infection. This monoclonal antibody reacted by immunofluorescence and by Western blot analysis with the SDS-insoluble sacculi of all four serotypes (A. G. Barbour, unpublished results). In addition to pII, other antigenically cross-reactive proteins, notably of relatively high molecular weight, occurred among the four serotypes.

The relapsing fever *Borrelia* species belong to a group of pathogenic bacteria that manifest antigenic variation (29). This group also includes, among others, *Salmonella* species with their flagellar antigens (30, 31) and *Escherichia coli* with their type 1 fimbriae (32, 33). *B. hermsii* stands apart, however, from these other bacteria by virtue of the degree of antigenic diversity found during a relapsing fever infection. The extensive antigenic repertoire and the sequential appearance of two or more of these serotypes during the course of an infection put us in mind of the antigenic variation shown by salivarian trypanosomes (8, 9). These also are vector-borne microorganisms that proliferate in the blood of the mammalian host. The selective pressures for mechanisms to avoid immune clearance appear to be the same for both the procaryotic borreliae and the eucaryotic trypanosomes. Nevertheless, it remains to be determined whether the genetic bases for variation that did evolve are the same in these phylogenetically very distant genera. Work on the mechanism of antigenic variation of *B. hermsii* is in progress.

Summary

Borrelia hermsii, a relapsing fever agent, manifests antigenic variation in vivo and in vitro. We studied three mouse-passaged serotypes of strain HS1 (7, 14, and 21) and a HS1 derivative obtained after multiple in vitro passages (C serotype). All four serotypes had two major proteins in whole cell lysates fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One major protein species (pII) had the same apparent subunit molecular weight ($\sim 3.9 \times 10^4$) in all the serotypes. In contrast, the other abundant protein in lysates, pI, had a different apparent molecular weight

in each serotype. In one gel the molecular weights of pI_C, pI_7, pI_{14}, and pI_{21} were 1.9, 4.2, 4.1, and 4.0 $\times$ 10^4, respectively. Serotype-specific mouse antisera bound to both hemologous and heterologous pIIs, to homologous pI, but not to heterologous pI in Western blots. Hybridomas were raised from spleens of mice infected with *B. hermsii*. Monoclonal antibodies were identifed by immunofluorescence assays using whole organisms. Monoclonal antibodies specific for serotype 7 (H1826) or for serotype 21 (H3326) bound only to pI_7 or pI_{21}, respectively, in Western blots. The surface location of the pI was suggested not only by the immunofluorescence studies but also by the labeling of pI_7 and pI_{21} when whole cells of serotypes 7 and 21 were incubated with ^{125}I in the presence of Iodogen. Under the same circumstances, pII was relatively poorly labeled. These studies have identified the variable pI proteins of *B. hermsii* as serotype-specific antigens. A change from one pI to another may be the basis of antigenic variation of *Borrelia* species during relapsing fever.

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