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Antigenic Variation among *Borrelia* spp. in Relapsing Fever

KAREN SUE C. KEHL,†* SILAS G. FARMER, RICHARD A. KOMOROWSKI, AND KONSTANCE K. KNOX

Department of Pathology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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Seven antigens of *Borrelia hermsii*, *B. parkeri*, and *B. turicatae* with isoelectric points in the range of 4.4 to 5.0 and molecular masses of 40 to 43 kilodaltons played a role in the relapse phenomenon of relapsing fever. Based upon location of the antigens in the outer envelope, the molecular weight, and Western blot analysis, the antigens from each phase of spirochetemia appeared to be a mixture of the serotype-specific antigens of cloned *B. hermsii*.

Borrelia hermsii, *B. parkeri*, and *B. turicatae* are the etiologic agents of tick-borne relapsing fever. The initial period of fever is terminated by a crisis phenomenon. After 1 to 2 weeks, a relapse with return of fever and other clinical manifestations follows (7). The relapse phenomenon is generally considered to be an immunologic phenomenon resulting from the inherent capacity of borrelias to undergo one or more antigenic variations during the course of the disease. With fluorescent antibody techniques 24 different serotypes of cloned *B. hermsii* were described by Stoenner et al. (19) and Barbour et al. (4). A single antigen was identified on three of these cloned serotypes, which they believe was responsible for the serotype specificity. This serotype-specific antigen had a molecular weight of ca. 40,000, which varied by 1,000 for each serotype studied. Based on immunofluorescence patterns, this antigen was believed to be located in the outer envelope of the organism.

In an attempt to correlate in vitro data with the disease, the antigens of *B. hermsii*, *B. parkeri*, and *B. turicatae* isolated from each phase of spirochetemia in experimental disease were examined. Isoelectric focusing (IEF)-crossed-immunoelectrophoresis was employed to demonstrate three genus-specific, two species-specific, and two phase-specific antigens. The antigens had pI's of 4.4 to 5.0 and molecular weights of 40,000 to 43,000. An outer envelope location for these antigens was confirmed by electron microscopy. They were identified as a mixture of serotype-specific antigens by preparative gel IEF, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analysis. The organisms isolated from each phase appear to be composed of a heterogeneous population of serotypes.

The strains selected for this study were *B. hermsii*, *B. parkeri*, and *B. turicatae* maintained in *Ornithodoros hermsi*, *O. parkeri*, and *O. turicata* ticks, respectively. Weanling male Swiss mice were infected with the borrelias via the bite of an infected tick. The mice were monitored daily by using dark-field examination of blood obtained from the retro-orbital sinus for evidence of spirochetemia. Since the spirochetemia lasts only 2 to 3 days, the mice were bled by cardiac puncture 24 h after the borrelias were first seen in the blood. The borrelias were enumerated (18) and passaged in cyclophosphamide-treated mice to enhance the spirochetemia (19).

BSK medium (3) was inoculated with 0.2 ml of citrated blood containing 10^6 to 10^7 *B. parkeri*, *B. hermsii*, or *B. turicatae* per ml in the attack, first-relapse, or second-relapse phase. The organisms were obtained from cyclophosphamide-treated mice. The tubes were incubated for 5 to 7 days at 37°C. Growth of the borrelias was monitored by using dark-field microscopy.

The borrelias were harvested by centrifugation and suspended in distilled water to a final concentration of 0.5 to 1.0 mg of protein per ml as determined by the method of Bradford (6). Whole-cell antigen was prepared by disrupting the organisms by sonication. The outer envelope preparations were prepared by using the method described by Klaviter and Johnson (11).

Whole-cell antigen was prepared by suspending the borrelias in 1% glutaraldehyde to a final concentration of 0.5 to 1.0 mg of protein per ml (6) and used to immunize adult male New Zealand White rabbits. Two rabbits were immunized with each borrelia strain by the procedure of Felsenfeld et al. (10). The serum was collected and then stored at −70°C. Before use, it was thawed and concentrated by ammonium sulfate precipitation (8) to yield antibody titers of 1:8 and 1:16 to the whole-cell sonicated antigen as determined by counterimmunoelectrophoresis (15).

Two-dimensional IEF-crossed-immunoelectrophoresis was performed by using a modification of the technique of Laurell (13). The whole-cell sonicated antigen (45-μl samples) was separated by using IEF in a pH 3.5 to 9.5, 4.0 to 6.5, or 4.0 to 5.0 gradient PAGplate (LKB Instruments, Inc., Rockville, Md.) at a constant power of 30 W. The IEF strip was placed on an agarose-coated glass lantern slide (8 by 10 cm), the "laying-on" technique of Soderholm and Smith (17). The second-dimension antiserum mixture consisted of a solution of the appropriate antiserum in 1.0% molten agarose suspended in barbital buffer (pH 8.6; ionic strength, 0.075) to give 1% of the optimum dilution as determined by counterimmunoelectrophoresis.

Homologous antigen-antibody systems were examined to determine the antigenic profiles of each organism. The peaks were numbered according to their pI. Several heterologous antigen-antibody systems were also examined to determine the presence of shared antigens.

IEF-crossed-tandem-immunoelectrophoresis was performed to confirm the identity of shared antigens. This was accomplished by placing a second IEF strip 2 cm to the right and above the first strip.

SDS-PAGE was performed by a modification of the procedure by Laemmli and Favre (12). A 12% separating gel (Tris [pH 8.8]) and a 6% stacking gel (Tris [pH 6.8]) were employed with acrylamide and *N,N'*-methylenebisacrylamide in a ratio of 30:0.8. Molecular weight markers

* Corresponding author.
† Present address: Mount Sinai Medical Center, Milwaukee, WI 53233.

TABLE 1. Borrelial antigens identified by IEF-crossed-immunoelectrophoresis

Spirochetemia phase and organism	Antiserum[a]	Antigen identified[b] at pI:						
		4.4	4.6	4.65	4.7	4.75	4.8	5.0
Attack *B. hermsii*	Anti-AH		mi		MA	mi	mi	mi
Relapse *B. hermsii*	Anti-AH		mi		MA	MA	mi	mi
	Anti-RT		mi		MA	MA		
	Anti-AP		mi		MA	MA		mi
Attack *B. parkeri*	Anti-AP		mi		MA	mi	mi	mi
	Anti-RP		MA		MA	MA	mi	
Relapse *B. parkeri*	Anti-AP		mi		MA	MA	mi	mi
	Anti-RP		mi		MA	MA		
	Anti-RT		mi		MA	mi		
Attack *B. turicatae*	Anti-AT		mi		MA	MA	mi	mi
	Anti-RT		mi		MA	MA	mi	
	Anti-AP		mi		MA	MA	mi	mi
Relapse *B. turicatae*	Anti-AT	mi	MA		MA	MA		
	Anti-RT	mi	mi	MA	MA	MA	mi	
	Anti-AH	mi	MA		MA	MA		
	Anti-AP	mi	MA		MA	MA		

[a] AH, Attack-phase *B. hermsii*; AT, atttack-phase *B. turicatae*; AP, attack-phase *B. parkeri*; RT, relapse-phase *B. turicatae*; RP, relapse-phase *B. parkeri*.
[b] MA, Major antigen; mi, minor antigen (peak height of <0.5 that of the MA); spaces indicate none found.

(Pharmacia Diagnostics, Piscataway, N.J.) were α-lactalbumin (14,000), soybean trypsin inhibitor (20,000), carbonic anhydrase (30,000), ovalbumin (45,000), albumin (67,000), and phosphorylase *b* (94,000).

Preparative IEF of whole-cell sonicated antigen was performed as described by Winter et al. (21), using the Ampholine Electrofocusing Kit for Granulated Gel (no. 2117-501; LKB) with a pH range of 3.5 to 5.5. The gel sections corresponding to pH 4.2 to 4.9 were transferred to their respective elution columns. The gels were suspended in 0.25 ml of SDS-PAGE sample buffer and eluted with 0.5 ml of the same buffer. SDS-PAGE was performed as described above.

The Electro-Blot System (E-C Apparatus Corp., St. Petersburg, Fla.) was used to transfer the borrelia proteins separated by SDS-PAGE to nitrocellulose paper by the procedure of Towbin et al. (20). The blot was blocked for 18 h in 2% bovine serum albumin in a modification of Barbour TSGAN (11) (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% sodium azide, 0.05% Triton X-100), incubated for 2 h with a 1:5 dilution of rabbit anti-borrelia antiserum in 2% bovine-serum albumin-TSGAN, and washed three times with TSGAN. The blot was incubated in a 1:25 dilution of fluorescein-conjugated goat anti-rabbit antiserum in 2% bovine serum albumin-TSGAN for 2 h at room temperature, washed again three times with TSGAN, and viewed under longwave UV light.

The antigenic profiles of the attack and relapse phases of all three species are given in Table 1. IEF-crossed-immunoelectrophoresis of the outer envelope preparations yielded antigenic peaks that corresponded to those seen with the whole-cell sonicated antigens.

All three species of borrelia possessed antigens with pI's of 4.7, 4.75, and 4.8. IEF-crossed-tandem-immunoelectrophoresis of attack- and relapse-phase *B. hermsii*, *B. turicatae*, and *B. parkeri* demonstrated the identity of "antigen 4.7" in these three species. Antigen 4.7 of relapse-phase *B. turicatae* is identical with antigen 4.7 of relapse-phase *B. parkeri* when electrophoresed into relapse-phase *B. turicatae* antiserum (Fig. 1). The same was true of antigens 4.75 and 4.8.

All borrelia species studied possessed an antigen with a pI of 4.6 (Table 1). However, based on IEF-crossed-tandem-immunoelectrophoresis data, this antigen was distinct for each species. There was a lack of identity between antigen 4.6 of relapse-phase *B. parkeri* and antigen 4.6 of relapse-phase *B. turicatae* (Fig. 1). Within each species, antigen 4.6 varied slightly from phase to phase. When the attack-phase antiserum was used in the second dimension, the attack- and

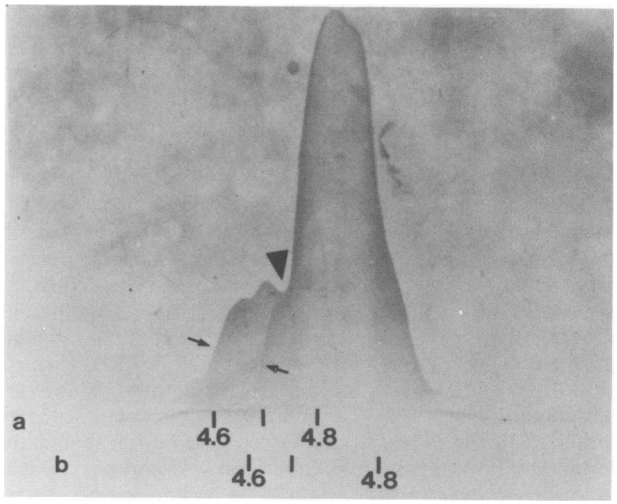

FIG. 1. IEF-crossed-tendem-immunoelectrophoresis of (a) relapse-phase *B. turicatae* whole-cell sonicated antigen (b) relapse-phase *B. parkeri* whole-cell sonicated antigen electrophoresed into agarose plus anti-relapse-phase *B. turicatae* serum. Antigen 4.7 of *B. turicatae* demonstrates identity (▼; fusion of the peaks) with antigen 4.7 of *B. parkeri*. The lack of identity between antigen 4.6 of relapse-phase *B. turicatae* and relapse-phase *B. parkeri* is also shown (arrows) as a lack of fusion of the precipitin peaks.

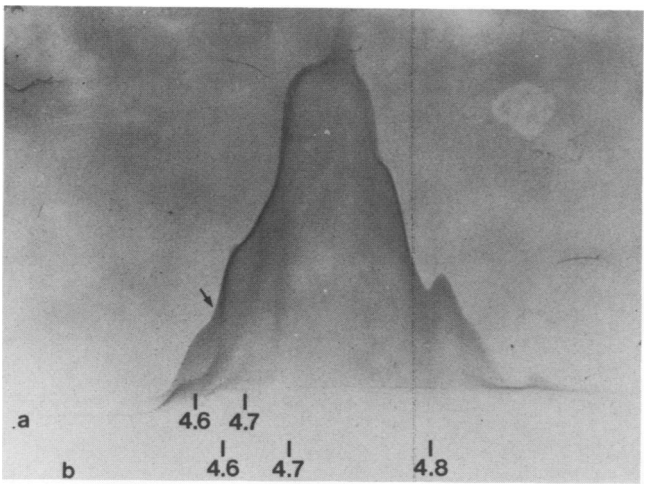

FIG. 2. IEF-crossed-immunoelectrophoresis of (a) relapse-phase *B. parkeri* whole-cell sonicated antigen and (b) attack-phase *B. parkeri* whole-cell sonicated antigen electrophoresed in the second dimension into agarose plus anti-relapse-phase *B. parkeri* serum. Partial identity of antigen 4.6 of the attack and relapse phases of the same species is shown (arrow).

relapse-phase antigens appeared identical. When relapse-phase antiserum was used in the second dimension, a partial identity of antigen 4.6 of the attack and relapse phases of the same species was observed (Fig. 2).

Species-specific antigens with pI's of 4.4 and 4.65 were found only on *B. turicatae*. These antigens could not be detected on any phase of *B. parkeri* or *B. hermsii* when anti-relapse-phase *B. turicatae* antiserum (which contains anti-4.4 and anti-4.65 antibodies) was used in the second dimension. An antigen with a pI of 5.0 was found only on the attack phase of each species. Relapse-phase organisms can be shown to possess this antigen by using anti-attack-phase antiserum. However, relapse-phase antiserum contains no detectable anti-5.0 antibody. This protein, though present in the relapse phase, does not evoke antibody production and was thus considered to be an attack-phase-specific antigen.

Whole-cell sonicated antigens of attack, first-relapse, and second-relapse phases of *B. hermsii*, *B. parkeri*, and *B. turicatae* were analyzed by using SDS-PAGE. Coomassie brilliant blue-stained patterns were similar. Figure 3 shows the patterns obtained with the attack and first-relapse phases of *B. turicatae*.

Western blot analysis of the SDS-PAGE of whole-cell sonicated antigens demonstrated an antigenic protein with a molecular weight of 40,000 to 43,000. Figure 3 shows the fluorescent bands obtained with attack, first-relapse, and second-relapse phases and the outer envelope preparation of *B. turicatae*.

To determine if this 40,000- to 43,000-molecular-weight protein was the same as those described with IEF-crossed-immunoelectrophoresis, the whole-cell sonicated antigens were first separated by using preparative gel IEF. Elution of the proteins within the pH 4.7 ± 0.1 grid with subsequent SDS-PAGE yielded a protein with molecular weight of 40,000 to 43,000 (Fig. 3). Western blot analysis of this protein and two-dimensional IEF-crossed-immunoelectrophoresis confirmed that these were the same protein.

Removal of the outer envelope, as confirmed by electron microscopy (Fig. 4), resulted in the elimination of the antigens as evidenced by IEF-crossed-immunoelectrophoresis. IEF-crossed-immunoelectrophoresis of the outer envelope preparations demonstrated the location of these antigens within the outer envelope. There were flagella on the outer surface of the organisms stripped of their outer envelopes. IEF-crossed-immunoelectrophoresis of these organisms demonstrated no antigenic peaks, thus eliminating the possibilities that the antisera contained antiflagellar antibodies or that the outer envelope preparations were contaminated with flagella.

In an attempt to describe the antigenic variation responsible for the relapse phenomenon seen in naturally occurring relapsing fever, we studied organisms isolated from each phase of spirochetemia in experimental disease. Within each phase of spirochetemia, there was a mixture of antigens on the organisms. These antigens had molecular weights of 40,000 to 43,000 and pI's of 4.4 to 5.0. They were located on the outer envelope of the organism. This location of the antigens responsible for the relapse phenomenon is consistent with observations by Barbour et al. (4), Stoenner et al. (19), and Djankov et al. (9) based upon immunofluorescence studies. An antigen of *B. burgdorferi* believed to play a role in Lyme disease (2) has also been found to be located in the outer envelope of the organism (5).

Techniques used in this study for the isolation of the organisms, cultivation of the organisms, and antibody production have been shown not to induce antigenic changes in the organisms (10, 19). Polyclonal rabbit antisera were used to demonstrate antibody response to a heterogeneous population of organisms. It is not possible to describe the antigenic variation seen in naturally occurring relapsing fever in a system employing cloned organisms and monoclonal antisera.

Based upon the outer envelope location, molecular weights, and Western blot analysis, the antigens described from each phase of spirochetemia have been shown to be a mixture of the serotype-specific antigens of cloned *B. hermsii* (1, 4, 19). Changes in the serotype-specific antigen are under genetic control (1, 14) and occur at high frequency (19). Conversion from one serotype to another occurs spon-

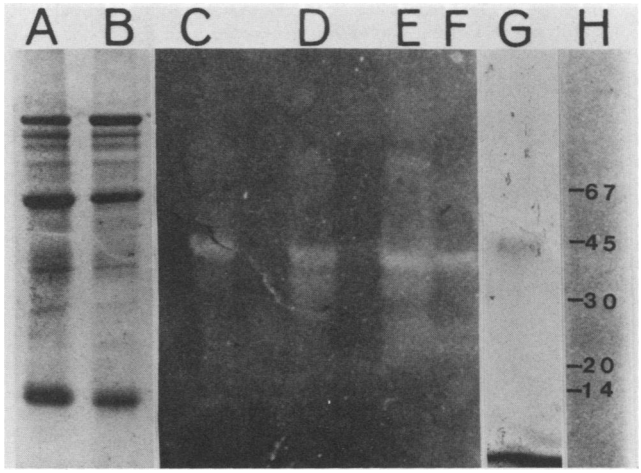

FIG. 3. Coomassie brilliant blue-stained SDS-PAGE of (A) attack and (B) first-relapse phases of *B. turicatae*. (C to F) Western blot analysis of SDS-PAGE of (C) attack (D) first-relapse (E), and second-relapse phases and (F) an outer envelope preparation of *B. turicatae* with anti-relapse-phase *B. turicatae* antiserum. (G) Coomassie brilliant blue-stained SDS-PAGE of the eluate from pH 4.7 ± 0.1 preparative gel IEF of *B. turicatae*. (H) Molecular weight (10^3) marker proteins.

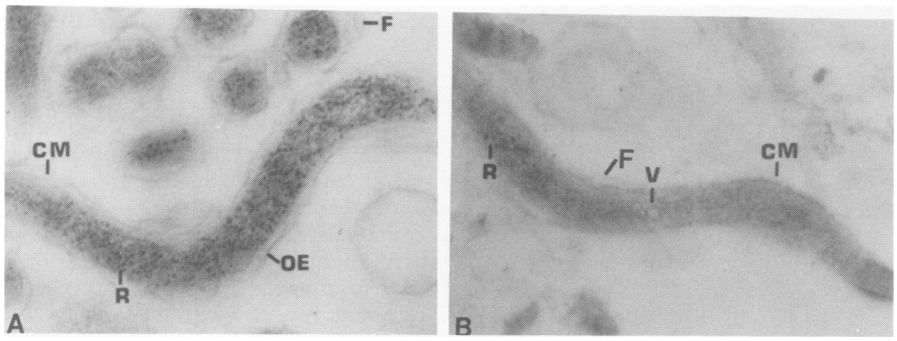

FIG. 4. Electron micrograph of second-relapse phase of *B. turicatae* (A) before and (B) after treatment with SDS. Note the removal of the outer envelope. OE, Outer envelope; F, flagella; CM, cell membrane; R, ribosome; V, vacuole.

taneously, giving rise to several other serotypes as well as reversion to the original type. Some serotypes are more common than others. Consequently, during each phase of spirochetemia some serotypes will be more common and appear as major protein antigens within a mixture of serotypes. Stoenner et al. (19) and Barbour et al. (4) used clones of a single organism to demonstrate a single antigen and hypothesized that each relapse is composed of a mixture of organisms. We employed organisms from each phase of spirochetemia to demonstrate the antigenic heterogeneity of the infecting population.

There is a similarity between borrelias and trypanosomes in the modulation of antigenic surface proteins. Rovis et al. (16) cloned *Trypanosoma congolense* and found that the protein coat had a pI of 6.4. Organisms cultured from the attack phase possess surface proteins with pI's in the range of 6.2 to 6.6. The attack-phase trypanosomes are a heterogeneous population of organisms, each possessing a single protein on its surface. Similarly, attack-phase borrelias are a heterogeneous population of serotypes, each possessing a serotype-specific antigen. Variation of these proteins allows the organism to elude host immune mechanisms, thus causing a cyclical, relapsing form of the disease.

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