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Spirochetal Non-Borrelia Microorganism Isolated from *Ixodes ricinus*

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ABSTRACT. Spirochetal microorganisms were isolated from female *Ixodes ricinus* in Slovakia. Morphological, immunochemical and molecular biological analysis showed that the microorganism shared several common antigens with *Borrelia* species while other genetic traits were distinct and not related to *Borrelia burgdorferi sensu lato*. Lyme disease patient's serum contained antibodies reacting with antigens of this microorganism. On the one hand the cross-reacting antigens represent a risk of false positive results in laboratory diagnostics, while on the other hand they have a certain potential for vaccine development against Lyme disease.

Ticks represent a vector transmitting a large number of microorganisms including viruses, bacteria, rickettsiæ and protozoa (Grist 1992; Gustafson 1994). The spirochetal microorganism *Borrelia burgdorferi* transmitted by ticks has been studied as an etiological agent of Lyme borreliosis (Burgdorfer *et al.* 1982; Johnson *et al.* 1984; Barbour and Hayes 1986). It can be isolated and cultured from the midgut of *Ixodes* ticks as well as from mammalian tissues. Such isolates have been used for studying their taxonomy, ultrastructure, physiology, cultivation and pathogenicity (Aberer and Duray 1991; Baranton *et al.* 1992; Wilske *et al.* 1986; Hovind-Hougen *et al.* 1986).

During the experimental work focused on the characterization of *Borrelia burgdorferi* isolated from *Ixodes ricinus* in Slovakia we have observed that, during the cultivation of tick's intestinal content, a spirochetal microorganism similar to *Borrelia* spp. but distinct in some properties appeared in the cultivation medium.

The modified BSK-II medium used in the experiments enabled us to cultivate the microorganism and to prepare it in a quantity suitable for immunochemical analysis. In this paper we describe some morphological, immunochemical and molecular biological characteristics of this spirochetal microorganism.

MATERIAL AND METHODS

Bacterial strains and cultivation. Adult ticks were collected in 1992 in the *Malacky region* in Slovakia. The internal organs from 15 *Ixodes ricinus* female ticks were cultivated in modified BSK-II medium (Schönberg *et al.* 1988; Kmety *et al.* 1990). The original BSK-II medium (Barbour 1984) was modified by replacing CMRL medium 1066 (*Gibco Laboratories*, USA) with Eagles minimal essential medium (E-MEM, *SEVAC*, Prague, Czech Republic), and no Yeastolate and rabbit serum were used. The midgut of the ticks was incubated in tightly capped culture tubes under microaerophilic conditions at 34 °C for 4 weeks. After one week the primary cultures were observed in a dark-field microscope (magnification 200×). Cultures without massive bacterial contamination by nonspiral forms were treated with 8–16 μg of kanamycin per mL (Johnson *et al.* 1984). The subcultures of spirochetal organisms were grown in triplicate for 6–7 d to a cell concentration of 10^8/mL at 34 °C and then stored at −70 °C. Strains *Borrelia garinii* K24 and K48 were isolated from *Ixodes ricinus* in Slovakia (Kmety *et al.* 1990), *B. burgdorferi sensu stricto* Son328 and *B. afzelii* VS461 were obtained from Prof. G. Baranton, *Institut Pasteur*, Paris (France). The strains *B. burgdorferi sensu stricto* TX32, TX1352 and TX358 were kindly provided by Dr. J. Rawlings, *Texas Health Department*, Austin (USA), and strains 51A-31 and 54A-33 by Dr. P.A. Rosa, *Rocky Mountain Labs.*, Hamilton (USA).

Electron microscopy. The isolates were examined by electron microscopy as described by Hovind-Hougen (1984) and Hulinská (1989). Ten to eleven day-old cultures were harvested by centrifugation at 6 000 *g*, washed in PBS (pH 7.2), fixed in glutaraldehyde, negatively stained for 3 min with

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2 % ammonium molybdate and examined in electron microscope (*EM Tesla* Brno, Czech Republic).

SDS–PAGE. The whole cell lysates were analyzed as described by Assous *et al.* (1993), using a mini-gel apparatus (*Bio-Rad Laboratories*, USA). The samples (4000 μg/mL) were subjected to electrophoresis in 12.5 % SDS–PAGE on 80 × 100 mm gels (Laemmli 1970). Proteins were separated by PAGE and either stained with Coomassie brilliant blue or transferred to nitrocellulose membrane for Western blot analysis.

Western blot analysis. After electrophoresis on 5–15 % gradient gel, the proteins were transferred to 0.45 μm nitrocellulose membranes (*Sigma–Aldrich*, Germany) after Towbin *et al.* (1979). The membrane strips were then saturated with 5 % dry milk in TBS buffer (50 mmol/L Tris, 2 mmol/L $CaCl_2$, 80 mmol/L NaCl, pH 8.0) for 1 h at room temperature and incubated overnight at 4 °C with polyclonal rabbit antisera to spirochetal microorganisms diluted in TBS buffer at a 1/100 dilution. After four washes, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-rabbit Ig (SwAR-Px) and the protein bands were visualized by addition of 4-chloro-1-naphtol (*Aldrich*, USA).

PCR. Heated bacterial suspensions in a volume of 5 μL were analyzed using two primers specific for *Borrelia burgdorferi sensu lato* (primer 1 [5'-CTGCGAGTTCGCGGGAGA-3'] and primer 2 [5'-TCCTAGGCATTCACCATA-3']) as described by Postic *et al.* (1994). The amplification reaction was carried out for 35 cycles with the following profile: denaturation at 93 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min.

Crossed immunoelectrophoresis (CIE). CIE standard and intermediate gel modification was performed as described by Weeke (1973) using 1 % agarose. Each well was loaded with 10 μL sample of a sonicated preparation containing 0.20 mg/mL of proteins. Protein staining of the gel after the electrophoresis of antigens was done with a Coomasie brilliant blue.

Protein determination. Protein content was measured by the Lowry method.

Immunogen preparations. Spirochetes from 1 L of culture (approximately 10^8 cells per mL) were harvested by centrifugation (10 000 ***g***, 30 min) and washed three times in phosphate-buffered saline (PBS, pH 7.4) containing 5 mmol/L $MgCl_2$. The final pellet was suspended in 1 mL distilled water and sonicated on ice (5 × 1 min) at 20 kHz in an Artek-model 300 sonic disintegrator (*Dynatech*, Switzerland). The sonic extract was lyophilized (*Lyolab ALSL Secfroid*, Switzerland). The lyophilization had no effect on the reactivity of antigens with specific antisera and this procedure was therefore used to store the antigens.

Immunization of rabbits. Polyclonal rabbit antisera to spirochetal microorganisms were raised by intravenous inoculation of washed and sonicated spirochetes to 2.5–3 kg rabbits. The immunization schedule included eleven doses from 0.25 to 1.0 mL of suspension (10^8/mL) applied at two-week intervals within a 6-month period. This scheme was found to be simple and effective in producing hyperimmune rabbit antisera suitable for immunodiffusion studies.

Preparation of spirochetal LPS-like component. The component was isolated from the spirochetal microorganism by a modification of the hot phenol–water extraction procedure (Beck *et al.* 1990). The separated LPS-like material was then dialyzed for 2 d in running tap water and for 1 d in distilled water and lyophilized.

Preparation of spirochete Osp. Osps were isolated by the 1-butanol extraction procedure (Gondolf *et al.* 1990).

Adsorption experiments. Isolated LPS and Osp were added to rabbit antiserum (0.2 mg of LPS or Osp per mL of antisera) and incubated for 1 h at 37 °C and 12 h at +4 °C. Then the mixture was centrifuged at 8 000 ***g*** and supernatant was used for CIE.

Human serum was obtained from adult patients with confirmed clinical Lyme borreliosis and positive laboratory tests (*ELISA-DAKOPATTS*, Denmark and *ImmunoDott-EPIGNOST*, Austria).

RESULTS

Electron microscopy. Electron microscopy of the spiral bacteria (Fig. 1 *left*) showed an amorphous matter attached to the surface layer of the cells. The cells were helically shaped. None of the 3 isolates was found to have flagella. Several small structures "gemmæ" or "blebs" (Fig. 1 *right*), similar to those visible also in borrelia cultures (Barbour 1986) and containing numerous small granules were observed at the outer envelope. The cells were elongated and less tightly coiled than *Borrelia* cells.

Cultivation. Three isolates of spirochetal organisms (SL1, SL2 and SL4) were obtained from 15 *Ixodes ricinus* females. The spirochetal cells were detected in the primary culture tubes after an incubation period of four weeks in BSK-II modified medium under microaerophilic conditions at 34 °C.

The modification of the medium composition included deletion of rabbit serum, Yeastolate and CMRL (replaced by E-MEM). Strains SL1, SL2 and SL4 cultivated in such medium were kept for three years in laboratory without any observable change in morphology or growth pattern. The cells examined by dark-field microscopy were less spiral and less motile than *Borrelia* cells (strain *B. garinii* K24). Typical for the three isolates was the production of gas in the tightly capped culture tubes.

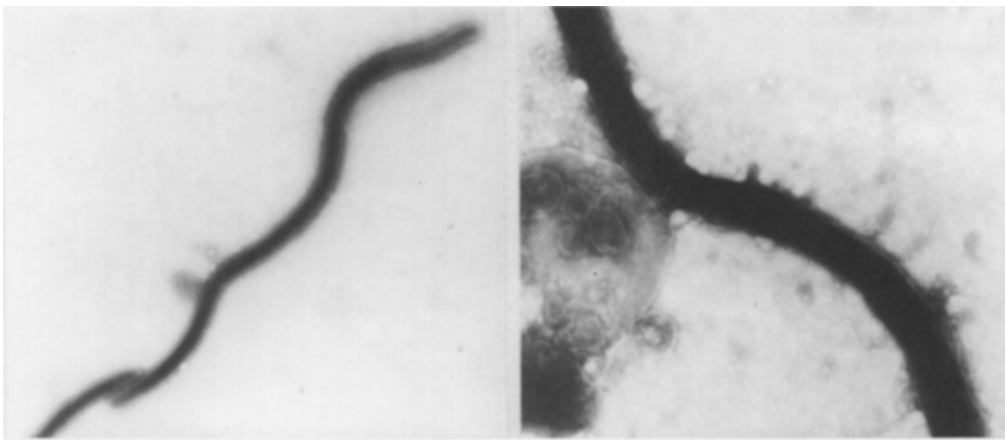

Fig. 1. *Left*: Electron microscopy of the spiral bacteria SL2 shows an amorphous matter attached to the surface layer of the cells. Negatively stained with 2 % ammonium molybdate. Magnification ×8000. *Right*: Several "gemmæ" or "blebs" containing numerous small granules were observed at the outer envelope. Magnification ×22000.

Experiments focused on the cultivation of the SL-strains in commercial BSK-H medium (Pollack *et al.* 1993) were not successful. The isolates survived only 1–2 subcultures, and then their morphology shifted to thinner and smaller cells that lost viability.

Crossed immunoelectrophoretic analysis of sonicated antigen preparations of spirochetal bacteria isolated from *I. ricinus* revealed the banding pattern shown on Fig. 2 *left*. It consisted of three major bands, one located at the start and two located close to the anode. Several other bands were also distributed from the start to the anode. All three strains revealed a similar banding pattern. When rabbit antiserum used in CIE was absorbed with a preparation of Osp the fast migrating bands disappeared from the picture. Similarly, when LPS-like material was used for adsorbtion of the rabbit antiserum and this was further used in CIE, the slow migrating band at the start either disappeared or was substantially reduced. These experiments showed that the spiral microorganism possessed structures similar to Osp and LPS of other Gram-negative bacteria.

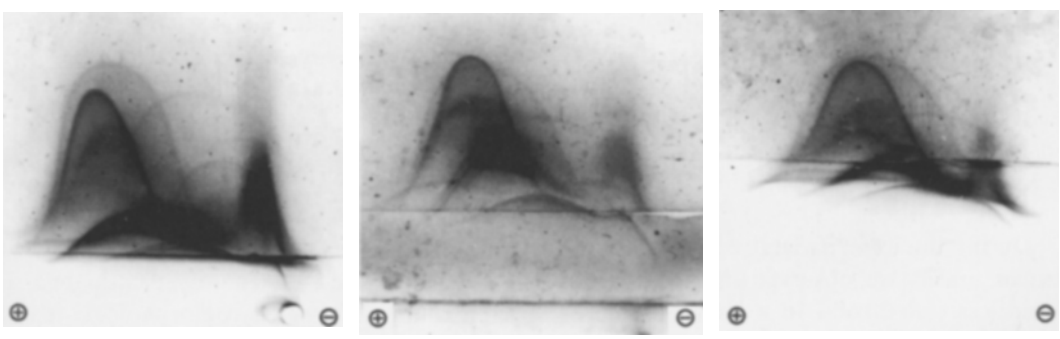

Fig. 2. *Left*: Precipitin bands in CIE formed by antigenic extract from SL2 and hyperimmune rabbit antiserum to SL2 strain. 1st dimension: 1 h, 11 V/cm, 2nd dimension: 18 h, 1 % agarose, 2 V/cm. *Staining*: Coomassie brilliant blue. *Middle*: CIE, intermediate gel modification with veronal buffer as a control. The rabbit serum (200 µL) contained antibodies reacting with antigens of SL2 strain. *Right*: CIE, intermediate gel modification with incorporated serum from a patient with clinically confirmed Lyme borreliosis. The patient's serum (300 µL) contained antibodies reacting with antigens of SL2 strain.

Cross reacting antigens of *B. garinii* strain K48 were detected by CIE using rabbit hyperimmune serum against the spirochetal form. Three bands were detected in CIE with whole cell lysate of *B. garinii* strain K48 and the rabbit anti-SL2 serum.

Crossed immunoelectrophoresis, intermediate gel modification with incorporated serum from a patient with clinically confirmed Lyme borreliosis showed that the patient's serum contained antibodies reacting with the antigens of the SL2 strain (Fig. 2 *middle* and *right*).

PAGE analysis of whole-cell lysates of SL1 spirochetal strain and *B. burgdorferi sensu lato* strains is shown in Fig. 3. Analysis of the tested strains revealed visible differences in the protein profile. While the protein profile of *B. burgdorferi sensu lato* strains showed a pattern with bands typical for Osp, the protein profile of SL strain did not show such banding pattern and did not correlate with any of the *B. burgdorferi* strains.

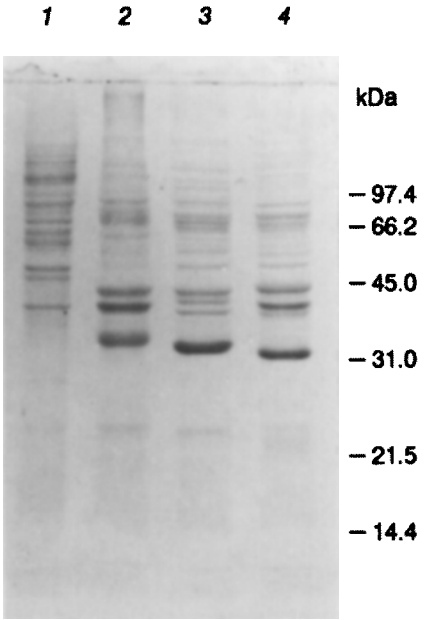

Fig. 3. Coomasie brilliant blue stained 12.5 % SDS-PAGE of whole-cell lysate of *Borrelia* and SL1-spirochetal microorganisms. Molar mass standards with the following proteins: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2), ovalbumin (45.0), carbonate dehydratase (31.0), soybean trypsin inhibitor (21.5), lysozyme (14.4); *lane 1*: SL1 spirochetal strain, *lane 2*: *B. burgdorferi sensu stricto* Son328, *lane 3*: *Borrelia garinii* K24, *lane 4*: *Borrelia afzelii* sp. nov. VS461.

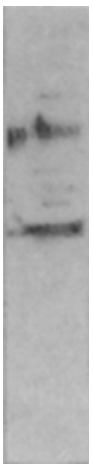

Fig. 4. Western-blot analysis of *Borrelia garinii* K48 isolate using rabbit antiserum against the tested spirochetal cells. Proteins were separated on 5-15 % gradient gel for SDS-PAGE and transferred on a nitrocellulose membrane.

On the other hand, the Western blot analysis showed that anti-spirochetal rabbit antiserum reacted with *B. garinii* K48 antigens. Fig. 4 shows two strong and four less intensive bands on a nitrocellulose membrane.

Results of the PCR analysis are shown in Fig. 5. DNA from a whole-cell lysate of the spirochetal cells was not amplified by the two primers used for specific identification of *Borrelia* strains. No amplification product was obtained from SL1, SL2 and SL4 strains, contrary to the reference strains *B. burgdorferi sensu stricto* 51A-34, 54A-33, TX32, TX1352, TX358 and *B. garinii* K24, as shown in Fig. 5.

DISCUSSION

During the experimental work focused on the isolation and characterization of *B. burgdorferi* from *Ixodes ricinus* we observed that, after a 6-d cultivation, the medium contained also long, less motile and less coiled cells. In a dark-field microscopy the cultures showed cytomorphologic features ranging from "unipolar taper" to "Medusa-like" colony forms, similar to those described by Aberer and Duray (1991). The cells multiplied to a high density in a BSK-II medium which was modified by replacing CMRL by E-MEM and by deleting rabbit serum and Yeastolate. The microorganisms grew optimally at 34 °C. On repeated freezing and thawing of the culture, the cells were surrounded by several blebs, in appearance very similar to those described by Barbour and Hayes (1986). No flagellar structures were observed in these cells.

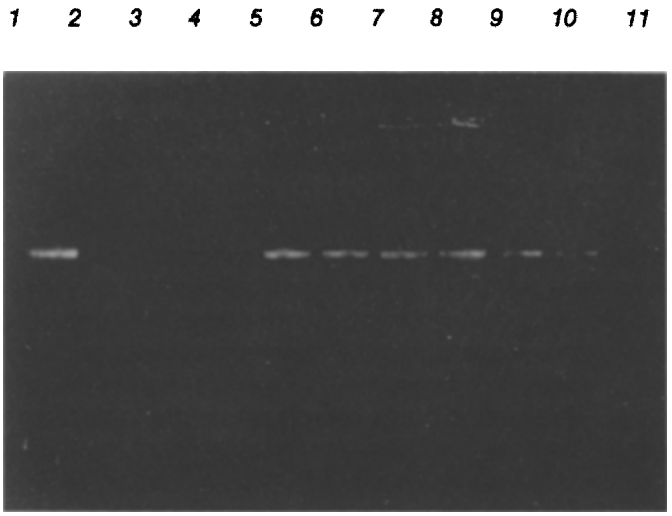

Fig. 5. PCR analysis of DNA from a whole-cell lysate of *Borrelia* and SL-spirochetal cells. *Lane 1: Borrelia garinii* K24, *lane 2–4:* SL1, SL2, SL4, *lane 5–9: Borrelia burgdorferi sensu stricto* 51A-31, 54A-33, TX32, TX1352, TX358, *lanes 10 and 11:* positive and negative controls.

SDS–PAGE of whole-cell *Borrelia* lysates shows a characteristic pattern of bands for Osp (Wilske *et al.* 1991). Though there is a variability in the molar mass of Osp (Wilske *et al.* 1993) the pattern revealed by SDS–PAGE analysis of spirochetal strains showed no sign of similarity with *Borrelia* strains.

Since these experiments did not clearly characterize the spirochetal cells isolated from *I. ricinus* as *B. burgdorferi sensu lato* isolates, we focused our attention on the immunochemical characterization of the culture. Polyclonal antisera were prepared by immunization of rabbits and the profile of the antigenic spectrum of the cells was analyzed by CIE. The banding pattern obtained by this method was very similar to the pattern produced by *B. burgdorferi sensu lato* (*to be published*). However, only three cross-reacting antigens forming three separate bands were found in reaction of *B. garinii* antigens with the rabbit antiserum specific for SL2 antigens. When a modification of CIE with intermediate gel containing the patient's serum was used, the shift of the bands in comparison with control clearly indicated that at least six bands were blocked in migration by the patient's serum. We concluded that six antigens of the spirochetal microorganism were cross-reacting with antibodies present in the patient's serum.

These findings were confirmed by Western blot analysis. *B. garinii* antigens reacted with rabbit antiserum against the spirochetal microorganism, forming two intensive and several weaker bands.

Since the phenotype analysis of the spirochetal isolates showed some similarities with *Borrelia sensu lato* strains we were interested also in genotype characteristics. However, PCR analysis with a primer set specific for *Borrelia* strains did not show any DNA homology and the strains thus could not be classified as *Borrelia* species.

Based on these experimental data we conclude that *I. ricinus* may carry spirochetal bacteria which cross-react with antibodies specific for *Borrelia burgdorferi sensu lato* and thus contribute to a spurious positive reaction when using such spirochetal isolates for laboratory diagnostic. On the other hand, the cross-reacting antigens, and particularly the presence of antibodies in a Lyme disease patient against this spirochetal cells opens the possibility to study this microorganism as a potential vaccine candidate. Its taxonomical classification requires further studies.

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