BACTERIAL PATHOGENICITY

A Portuguese isolate of *Borrelia lusitaniae* induces disease in C3H/HeN mice

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A low-passage, Portuguese isolate of *Borrelia lusitaniae*, strain PotiB2, was inoculated into C3H/HeN mice and disease was monitored by histopathology at 8 weeks after spirochaete challenge. Ear, heart, bladder, femoro-tibial joint, brain and spinal cord were examined. *B. lusitaniae* strain PotiB2 (6 of 10 mice) and *B. burgdorferi sensu stricto* strain N40 (9 of 10 mice) induced similar lesions in the bladder of infected mice characterised as a multifocal, lymphoid, interstitial cystitis. Moreover, both *B. lusitaniae* PotiB2 and *B. burgdorferi* N40 induced lesions in the heart of infected mice. The lesions induced by *B. lusitaniae* PotiB2 (2 of 10 mice) were characterised as a severe, necrotising endarteritis of the aorta, with a minimal, mixed inflammatory infiltrate (neutrophils, macrophages and lymphoid cells) extending into the adjacent myocardium. In contrast, *B. burgdorferi* N40 induced a periarteritis of the pulmonary artery (7 of 10 mice), with no involvement of the endothelium and more extensive inflammation and subsequent necrosis of the adjacent myocardium. This infiltrate was composed entirely of mononuclear cells, predominantly mature lymphocytes and plasma cells. No lesions were noted in the joints or central nervous system with inoculation of strains N40 or PotiB2, and co-inoculation of either strain with *Ixodes ricinus* salivary gland lysate did not affect the resulting pathology. Serology, examined 8 weeks after inoculation, indicated a different reactivity in mice infected with *B. lusitaniae* PotiB2 compared with *B. burgdorferi* N40. Immunoblot analysis demonstrated that mice with lesions resulting from infection with *B. lusitaniae* PotiB2 reacted only to the flagellin protein (41 kDa) or to flagellin and OspC, whereas mice infected with *B. burgdorferi* N40 reacted with multiple high and low mol. wt proteins, including flagellin, p93, p39, OspA, OspB and OspC. These results indicate that *B. lusitaniae* PotiB2 induced pathology similar to *B. burgdorferi* N40 when inoculated into susceptible mice. Moreover, these results establish the first animal model of disease with *B. lusitaniae*. This mouse model can be used to characterise the immunopathogenesis of *B. lusitaniae* infection and to delineate the proteins responsible for disease induction in susceptible mice.

Introduction

Lyme borreliosis occurs world-wide and encompasses diverse symptomatology in man, including the development of erythema migrans at the tick bite site and tertiary symptoms encompassing degenerative arthritis, lesions of the central and peripheral nervous system,

and myocarditis [1]. In Europe, the causative agents of disease in man are *Borrelia burgdorferi sensu stricto* and the genospecies *B. garinii* and *B. afzelii* [2, 3]. The pathogenic potential of other genospecies – *B. valaisiana*, *B. lusitaniae* and *B. bissettii* – is currently unknown, although isolates of *B. bissettii* have been obtained recently from the cerebrospinal fluid of neuroborreliosis patients in Slovakia [4].

B. lusitaniae has been cultured from *Ixodes ricinus* and detected by PCR amplification in *Hyalomma marginatum* ticks in Portugal [5–7], Tunisia [8], Spain [9],

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Moldavia, the Ukraine and the Czech Republic [10] and Slovakia [11]. Based on comparative RFLP and sequence analysis of the *rrf-rrl* intergenic spacer region, *B. lusitaniae*, specifically subspecies PotiB1, PotiB2 and PotiB3, form a clade distinct from other northern European isolates of *Borrelia* spp.[6].

Although cases of Lyme borreliosis have been diagnosed in Portugal [12], *B. lusitaniae* has not been isolated from human patients. Moreover, only one study has examined the pathogenic potential of *B. lusitaniae* in an animal model [9]. This isolate was obtained from *I. ricinus* ticks in Spain and, based on sequence analysis of the 16S rRNA gene, was closely related to *B. lusitaniae* PotiB2. No pathology was seen 4 weeks after needle inoculation of this isolate into the skin of C3H mice [9].

The current study examined the pathogenic potential of *B. lusitaniae* PotiB2, isolated originally from *I. ricinus* ticks collected in the Alentejo region of central Portugal [7].

Materials and methods

Mice

C3H/HeN mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and certified to be specific-pathogen free. All mice were housed and killed for tissue harvest and analysis according to the Animal Care and Use Committee of the Centers for Disease Control and Prevention.

Infection of mice with *B. burgdorferi* N40 and *B. lusitaniae* PotiB2 and recovery of tissues for histopathological analysis

Low passage (less than three passages *in vitro*) isolates of *B. burgdorferi sensu stricto* N40 (isolated from Westchester County, NY, USA) or *B. lusitaniae* PotiB2 (originally isolated from *I. ricinus* ticks collected in the Alentejo region of Portugal) were grown to log phase in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma) at 33°C and 10⁶ organisms in 1 ml of medium were inoculated subcutaneously, along the dorsal midline of mice. Mice (10 per group) were randomly assigned to receive *B. burgdorferi* N40, *B. burgdorferi* N40 + *I. ricinus* salivary gland lysate (SGL), *B. lusitaniae* PotiB2, *B. lusitaniae* PotiB2 + SGL, SGL + BSK-H or BSK-H medium alone. Four weeks after inoculation, an ear biopsy was taken and cultured to monitor individual mice for infection as described previously [13]. Eight weeks after inoculation, mice were killed and tissues (ear, bladder, heart, femoro-tibial joint, tibio-tarsal joint, brain and spinal cord) were placed in formaldehyde 10% (w/v) in PBS for histological analysis.

Preparation of *I. ricinus* salivary gland lysate for co-inoculation

I. ricinus nymphal ticks were placed on C3H/HeJ mice and allowed to feed for 3 days. Ticks (50) were then collected and dissected to remove individual pairs of salivary glands. Salivary glands were then prepared for lysates (SGL) according to the method described by Labuda *et al.* [14]. Briefly, glands (100) were pooled, sonicated in PBS on ice (pulsed three times for 10 s per 40% duty cycle) (Vibra Cell Sonics Materials, Danbury, CT, USA), and centrifuged to remove all non-specific connective tissue. The supernate was filtered through a 0.2-µm filter and the total protein of the filtrate was determined by the bicinchoninic acid method according to manufacturer's instructions (Pierce, Rockford, IL, USA). A total of 25 µg of SGL protein was then co-inoculated with either *B. burgdorferi* N40 or *B. lusitaniae* PotiB2 spirochaetes in a total volume of 1.0 ml of medium.

Tissue preparation for histological analysis

Formalin-fixed tissue samples were subjected to standard processing, embedded in paraffin, and sections of 3–5 µm were prepared. All femoro-tibial and tibio-tarsal joint samples were incubated for 1 h in rapid decalcifying solution (Shandon Corp., Pittsburgh, PA, USA) before initial sectioning. Individual tissues were then stained with haematoxylin and eosin for evaluation by light microscopy.

SDS-PAGE and immunoblotting

Low passage (less than three passages) *B. lusitaniae* PotiB2 strains and *B. burgdorferi* N40 were grown to log phase in BSK-H medium, centrifuged at 10 000 rpm for 3 min, resuspended in 1 ml of PBS, sonicated and fractionated at 125 V in SDS-PAGE gels containing acrylamide 12% w/v (Invitrogen, Carlsbad, CA, USA) as described previously [15]. Protein profiles were obtained by silver staining (BioRad Laboratories, Hercules, CA, USA).

For immunoblotting, 10 µg of *B. lusitaniae* PotiB2 or *B. burgdorferi* N40 lysate were fractionated in SDS-PAGE gels containing acrylamide 10% w/v (Invitrogen), transferred to a nitrocellulose membrane (Invitrogen) which was blocked for 1 h with dried milk 10% w/v in 10 mM Tris-buffered saline (TBS blotto, pH 8.0) [15]. The membrane was then cut into strips and incubated with a 1 in 100 dilution of mouse serum in TBS blotto overnight at 4°C. The strips were then washed five times in TBS blotto and treated for 1 h with goat anti-mouse IgG and IgM alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at a 1 in 1000 dilution in TBS blotto. After washing, the strips were incubated with BCIP/NBT substrate (Kirkegaard & Perry Laboratories) for 20

min. Subsequent colour development was stopped by extensive washing of strips with distilled water [15].

Results

Histopathological evaluation

All mice became infected with either *B. lusitaniae* PotiB2 or *B. burgdorferi* N40 as determined by ear biopsy and culture at 28 days after inoculation of spirochaetes (data not shown). Lesions of the bladder were noted after inoculation of both *B. burgdorferi* N40 (9 of 10 mice) and *B. lusitaniae* PotiB2 (6 of 10 mice). Lesions were characterised as a multifocal, lymphocytic, interstitial cystitis. Multifocal, perivascular accumulations of mature lymphocytes and plasma cells were seen within the connective tissue layers below the transitional epithelium (Fig. 1a and b) at 8 weeks after inoculation of either *B. burgdorferi* N40 or *B. lusitaniae* PotiB2. These nodular accumulations of small lymphocytes and plasma cells did not extend into either the transitional epithelial layer or into the surrounding smooth muscle. Attempts to localise either *B. lusitaniae* PotiB2 or *B. burgdorferi* N40 spirochaetes within these nodules with a Warthin-Starry (silver) stain were unsuccessful. No quantitative or qualitative difference in these lymphoid nodules was noted in groups receiving spirochaetes in BSK-H and spirochaetes plus SGL, or when comparing the two *Borrelia* strains.

Lesions in the heart were also demonstrated with both *Borrelia* strains. *B. burgdorferi* N40 induced lesions in 7 of 10 inoculated mice, whereas only 2 of 10 mice inoculated with *B. lusitaniae* PotiB2 developed pathology. Moreover, differences in pathology were noted between the *Borrelia* isolates. In mice inoculated with *B. burgdorferi* N40, a periarteritis of the pulmonary artery was noted (Fig. 2a), with extensive inflammation and some necrosis of adjacent myocardium (Fig. 3b). In contrast, injection of *B. lusitaniae* PotiB2 induced single-cell necrosis of the endothelium (endarteritis) of the aorta (Fig. 2b, Fig. 3a) which then extended through the wall of the vessel into the adjacent myocardial tissue. In both cases the inflammatory infiltrates consisted of a mixture of neutrophils, lymphocytes, plasma cells and macrophages. Individual myocardial cells within areas of inflammation demonstrated vacuolar change and necrosis, especially after inoculation with *B. burgdorferi* N40 (Fig. 3b). Co-inoculation of SGL with spirochaetes did not affect the extent or the number of myocardial lesions seen with either *B. burgdorferi* N40 or *B. lusitaniae* PotiB2.

No histopathological lesions were noted in the joints or central nervous system tissues in animals inoculated with either *B. burgdorferi* N40 or *B. lusitaniae* PotiB2, with or without SGL.

Protein profiles and humoral responses

Fig. 4 shows a comparison of the protein profiles of *B. lusitaniae* strains (lanes 2, 3 and 4) with that of the B31 strain of *B. burgdorferi* (lane 1) and the N40 strain of *B. burgdorferi sensu stricto* (lane 5). The culture of *B. lusitaniae* PotiB2 (lane 3) used for inoculation of mice did not express outer-surface protein A (OspA), nor did the other Portuguese isolates of *B. lusitaniae*, PotiB1 (lane 2) and PotiB3 (lane 4). Bands representing the outer-surface protein B (OspB), and the flagellin protein (Fla) were noted in each Poti strain as well as the *B. burgdorferi* N40 strain used for in-vivo studies.

The serological response to both *B. burgdorferi* N40 and *B. lusitaniae* PotiB2 was analysed at 8 weeks after inoculation of spirochaetes. As seen in the immunoblots in Fig. 5, reactivity to *B. lusitaniae* PotiB2 in all mice was restricted to the flagellin protein or outer-surface protein C (OspC), or both. Each of these mice had lesions in both the bladder and the heart. Reactions between reference monoclonal antibodies directed toward flagellin, OspA, OspB and OspC and *B. lusitaniae* PotiB2 indicated that the bands in the 41- and 23-kDa regions corresponded to flagellin and OspC (data not shown). The humoral response to *B. burgdorferi* N40 at 8 weeks after inoculation included reactivity to a range of large and small mol. wt proteins, including OspA, OspB, OspC and flagellin protein (Fig. 5). As with *B. lusitaniae* PotiB2, only representative serum samples, from animals that developed pathology of both the heart or bladder, are presented in Fig. 5.

Discussion

To our knowledge this is the first report of the pathogenic potential of *B. lusitaniae* in mice. This study indicated that at least one strain of *B. lusitaniae*, PotiB2, isolated originally from *I. ricinus* ticks in southern Portugal [7], induced both nodular interstitial cystitis and vasculitis of the great vessels of the heart. This contrasts with an earlier unsuccessful attempt to induce pathology by needle inoculation of a Spanish isolate of *B. lusitaniae* (PV8) in C3H mice [9]. The reasons for the differences between these studies are varied and include inoculum size (10^4 spirochaetes of *B. lusitaniae* PV8; 10^6 of *B. lusitaniae* PotiB2) and interval of time to demonstrate dissemination from the skin and resultant pathology of internal organs (4 weeks for *B. lusitaniae* PV8; 8 weeks for *B. lusitaniae* PotiB2). Although phylogenetic analysis revealed homology between the 16S genes of *B. lusitaniae* PV8 and PotiB2, the current study may indicate a difference in virulence between these two isolates.

In the present study, a higher percentage of mice infected with *B. burgdorferi* N40 developed lesions of the bladder and the heart, although the severity of inflammation was not qualitatively different to that seen

Fig. 1. Histopathological sections of bladder, 8 weeks after injection of spirochaetes (H&E 20×). **a**, *B. burgdorferi* N40; **b**, *B. lusitaniae* PotiB2; **c**, sham-inoculated control mouse. Arrows indicate perivascular lymphoid nodules within the interstitium.

with *B. lusitaniae* PotiB2. Interestingly, although the lesions in the bladder were similar histologically, lesions in the heart were noticeably different. *B. lusitaniae* PotiB2 induced an inflammatory response of the endothelium, with evidence of single-cell necrosis of endothelial cells, which occurred solely within the aorta. In contrast, *B. burgdorferi* N40 induced pathology within the pulmonary artery, characterised as

Fig. 2. Histopathological sections of heart, 8 weeks after injection of spirochaetes (H&E 20×). **a**, arrow indicates perivascularitis in the wall of the pulmonary artery after inoculation with *B. burgdorferi* N40; **b**, arrow indicates vasculitis of the aorta after inoculation with *B. lusitaniae* PotiB2; **c**, aorta of a sham-inoculated control mouse.

an inflammation of the lateral portion of the vascular tunic, which extended laterally causing necrosis of adjacent myocardium. A similar vasculitis of the great vessels has been demonstrated previously with needle inoculation of *B. burgdorferi* N40 [16]. Whether this difference in tissue tropism and resultant inflammatory pattern between *B. burgdorferi* N40 and *B. lusitaniae* PotiB2 is due to a difference in outer-surface protein expression of cultured spirochaetes remains to be

Fig. 3. Histopathological sections of heart, 8 weeks after injection of spirochaetes (H&E 40 $\times$). **a**, arrows indicate endarteritis of the aorta which extends through the vascular wall and into the adjacent myocardium, after inoculation with *B. lusitaniae* PotiB2; **b**, arrows indicate periarteritis of the pulmonary artery extending into the surrounding myocardium after inoculation with *B. burgdorferi* N40.

Fig. 4. Protein profile of Portuguese isolates of *B. lusitaniae* compared to *B. burgdorferi* strains B31 and N40. Low-passage isolates were fractionated by SDS-PAGE and stained with silver. Lane 1, *B. burgdorferi* B31; 2, *B. lusitaniae* strain PotiB1; 3, *B. lusitaniae* strain PotiB2; 4, *B. lusitaniae* strain PotiB3; 5, *B. burgdorferi* strain N40; 6 and 7, mol. wt standards.

investigated. Also, the co-inoculation of *I. ricinus* SGL with *B. lusitaniae* PotiB2 or *B. burgdorferi* N40 did not affect the extent of pathology described here. Whether this was due to the preparation of SGL inoculum, the amount of SGL inoculated, or the time point examined after spirochaete inoculation remains to be tested. Moreover, as noted earlier, neither *B. lusitaniae* PotiB2 nor *B. burgdorferi* N40 induced joint lesions. It is possible that in the examination of mice at 8 weeks after inoculation, the acute or chronic time point for evaluation of joint disease was missed. Earlier reports indicated that *B. burgdorferi* N40 induced an acute joint lesion at 3–4 weeks after spirochaete inoculation which had resolved by day 90 [16]. Studies in this laboratory have demonstrated joint pathology at

Fig. 5. Humoral responses to *B. lusitaniae* PotiB2 (**a**) and *B. burgdorferi* N40 (**b**) after needle inoculation with spirochaetes. Lanes 2, 4 and 6, reaction between individual sera from mice infected with *B. lusitaniae* and cultured *B. lusitaniae* PotiB2; 8, 10, and 12, reaction between individual sera from mice infected with *B. burgdorferi* N40 and the cultured *B. burgdorferi* spirochaetes; 1, 3, 5, 7, 9 and 11 (not labelled), represent pre-infection sera of individual mice. MAb, reaction between reference monoclonal antibodies directed toward flagellin, OspB, OspA and OspC and *B. burgdorferi* N40.

12 weeks after inoculation of *B. burgdorferi* N40 by nymphal ticks which persisted to 18 weeks [17].

As demonstrated here, *B. lusitaniae* PotiB2 cultured *in vitro* does not express OspA, and animals infected with this organism produce an antibody response to only the flagellin and OspC proteins at 8 weeks after inoculation. Moreover, at least one animal, which had lesions of both bladder and heart, demonstrated an antibody response that reacted with only the flagellin protein. In contrast, those animals infected with *B. burgdorferi* N40 produced an antibody response to several proteins, including OspA, OspB, OspC and flagellin. Further studies are needed to properly characterise both the humoral and T-cell responses to *B. lusitaniae* PotiB2 and to correlate these responses with resultant tissue pathology. What is apparent from this study is that pathology induced by *B. lusitaniae* PotiB2 in the bladder and heart is not dependent on either the humoral or T-cell response to OspA. However, this needs to be tested in a rigorous manner by the natural route of transmission by using *I. ricinus* nymphal ticks.

Since the first case of Lyme borreliosis was reported in Portugal in 1989 [18], all strains of *Borrelia* isolated from ticks in southern Portugal have been classified within a distinct clade of Poti strains of *B. lusitaniae* [5, 6, 19]. To date, no human isolates have been investigated in an animal model. The establishment of the pathogenic potential of *B. lusitaniae* PotiB2 in a mouse model affords the opportunity to investigate the pathogenesis of this unique isolate which lacks expression of OspA. Future studies will include the inoculation of *B. lusitaniae* PotiB2 by tick bite, a more detailed examination of outer-surface protein expression in affected tissues, and an examination of the adaptive immune responses that correlate with lesion development in this animal model.

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