HOST RESPONSE TO INFECTION

Cloning of the gene encoding the decorin-binding protein B (DbpB) in *Borrelia burgdorferi sensu lato* and characterisation of the antibody responses to DbpB in Lyme borreliosis

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A genome walking technique was applied to borrelial DNA to clone the gene encoding decorin-binding protein B (DbpB) in *Borrelia garinii* and *B. afzelii*. Sequence analysis showed 62–67% identity of the predicted amino acid sequences of DbpB between the *B. afzelii* and *B. garinii* strains and *B. burgdorferi sensu stricto*. Within subspecies, the sequences were 99–100% identical. The respective recombinant DbpBs (rDbpBs) were produced and tested as antigens in an enzyme-linked immunosorbent assay (ELISA) for Lyme borreliosis (LB). In IgG ELISA, with rDbpBs as antigens, 11 (73%) of 15 adult patients with Lyme arthritis and 9 (64%) of 14 with neuroborreliosis were positive. Of children with Lyme arthritis, 40 (77%) of 52 were positive. All adult and paediatric patients with disseminated LB had high titres of anti-flagellin IgG antibodies. Seropositivity against rDbpB from *B. garinii* predominated, 39 (65%) of 60 of the positive samples reacting with rDbpB from *B. garinii*. In patients with erythema migrans, IgM antibodies to rDbpB were detected in 1 (4%) of 23 and IgG antibodies in 6 (26%) of 23. These results indicate that DbpB may be a useful antigen in the IgG serology for disseminated LB. The high inter-species sequence heterogeneity observed indicates that a combination of the variant DbpBs should be included in the antigen set to cover all the relevant borrelial subspecies in the serodiagnosis of LB.

Introduction

Lyme borreliosis (LB) is the most frequent tick-borne infectious disease in North America and Europe. The disease is caused by spirochaetes belonging to the *Borrelia burgdorferi sensu lato* complex and is characterised by multi-stage skin, joint, neurological and cardiac manifestations [1]. In Europe, in contrast to the USA, all three pathogenic borrelial subspecies, *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii*, are known to be aetiological agents of LB [2].

In the absence of erythema migrans (EM), the clinical diagnosis of disseminated LB requires laboratory

confirmation. This depends primarily on detection of antibodies specific to *B. burgdorferi*, because direct demonstration of the organisms in clinical specimens by culture or by PCR has a low sensitivity. The serological methods most commonly used for LB are enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB), but these assays are not without limitations. These assays with whole-cell lysates (WCL) or flagellin as antigens are not standardised and their performance in different laboratories is highly variable [3]. To increase specificity, several recombinant borrelial proteins (e.g., OspA, OspB, OspC, OspE, OspF, p22, DbpA, BBK32, BBK50, VlsE, BmpA, FlaA and p41) [4–14] have been studied as antigens in serological assays. Some of these proteins have improved the sensitivity or specificity, or both, but none has proved superior to the current commercial serological assays.

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The decorin-binding proteins A and B (DbpA and DbpB) are borrelial surface lipoproteins that have been shown to elicit early antibody responses in experimental murine borreliosis [15–17]. A previous study cloned and sequenced *dbpA* genes from the three pathogenic subspecies of *B. burgdorferi sensu lato* and investigated the antigenic properties of the variant rDbpAs [9]. These studies showed substantial sequence heterogeneity of DbpA. In the serodiagnosis of LB, with a single rDbpA as antigen, the sensitivity was low, but with all the variant rDbpAs included in the antigen panel, the sensitivity was comparable to that of routine seroassays [9]. At present, the amino acid sequences of DbpB in *B. burgdorferi sensu lato* have not been studied in detail, possibly on account of sequence heterogeneity and difficulties in the cloning of *dbpB* genes from various borrelial subspecies. The *dbpB* sequences in isolates from *B. burgdorferi sensu stricto* may be highly conserved [18]. However, except for a single *dbpB* sequence from *B. garinii* available in the GenBank database (AF069263), information about *dbpB* sequences other than those of *B. burgdorferi sensu stricto* is lacking. Consequently, the antigenic potential of DbpB has not been thoroughly evaluated.

The purpose of the present study was to clone and sequence *dbpB* from the three European pathogenic borrelial species, *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii*. The respective recombinant DbpBs (rDbpBs) were then evaluated as antigens in the serodiagnosis of LB.

Materials and methods

Bacterial strains

Finnish borrelial strains were obtained from the National Public Health Institute, Turku, Finland. *B.*

burgdorferi sensu stricto strain ia (here referred to as Bbia) was isolated from the cerebrospinal fluid of a Finnish patient with neuroborreliosis (NB) and *B. afzelii* strains A91, 1082 and EM9 and *B. garinii* strains 40, 46 and 50 (referred to, respectively, as BaA91, Ba1082, BaEM9 and Bg40, Bg46 and Bg50) were obtained from skin biopsy samples of Finnish patients with LB. The strains were genotyped by PCR and sequencing, the target DNA being a fragment from the flagellin gene of *B. burgdorferi* [19]. *Borreliae* were cultivated in Barbour-Stoenner-Kelly (BSK-H) medium (Sigma, USA) at 33°C in air with CO₂ 5%. *Escherichia coli* host cells for cloning and expression of recombinant proteins were INFaF (Invitrogen, Groningen, The Netherlands) and M15 (Qiagen, Hilden, Germany), respectively.

DNA purification

Borrelial genomic DNA was purified with a Dneasy Tissue Kit (Qiagen). Purified DNA was used in PCR and cloning experiments. Plasmid DNA was purified with a QIAprep-spin plasmid kit (Qiagen).

PCR

A PCR-based approach was employed to amplify and sequence the *dbpB* from *B. burgdorferi sensu stricto* (Bbia). Primers for Bbia *dbpB* PCR amplification were designed on the basis of published *dbpB* sequences (Table 1). Several primer pairs were designed and tested to ensure that the entire coding sequence of the *dbpB* was obtained. Expression primers for strain Bbia, encoding the mature portion of the DbpB protein after cysteine at the site of post-translational acylation, were chosen from the sequence analysed. Approximately 1 ng of template DNA was used in standard PCR conditions: 30 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min and extension with

Table 1. Primers used for PCR amplification of *dbpB*

No.	Species	Primer	Location	Source
1	<i>B. burgdorferi sensu stricto</i>	5'-TGC ATA AAA CAA ATT CAC ACT-3'	–208 to –187	B31 (AF069269)
2		5'-CCG <u>GAT CCA</u> GTA TTG GAT TAG TAG AAA GAA C-3'	64–86	Bbia (AY083920)
3		5'-ATC ATT TTC GTT ATT TGG TTA T-3'	660–639	B31
4		5'-CCG <u>GTA CCT</u> TTT TAA TAT TTA TTT CTT TTT TTT GC-3'	628–604	Bbia
5	<i>B. garinii</i>	5'-CTT CTC TTT TAT TTT TAA GAC C-3'	–341 to –320	Bg40 (AY083917)
6		5'-CCG <u>GAT CCA</u> ATT TTG GAT TAA CAG GAG AAG-3'	58–79	Bg40
7		5'-GCT TCC TCT GAA ATG GAG CTT TTT ATT C-3'	416–389	Bg40
8		5'-CAT TAA ATC AAA CAT AGC CAA GAA TTG AC-3'	348–320	Bg40
9		5'-TAT CAG AAG ACG ATT CAA GC-3'	109–90	Bg40
10		5'-CCG <u>GTA CCG</u> TTT TTA GCC AAT TCT AAT TAC-3'	594–573	Bg40
11		5'-CAT GCT ACT AAC AGG CTA AC-3'	744–725	Bg40 (AF441832)
12	<i>B. afzelii</i>	5'-CCC CTG GCA AAA TAA AAT TC-3'	–458 to –439	BaA91 (AY083914)
13		5'-CCG <u>GAT CCA</u> ATT TTG GAT TAA TGG AAG AAA C-3'	58–80	BaA91
14		5'-CTG ATG ATT CCA ATC TAG CTT TTC CTG-3'	765–739	BaA91 (AF441833)
15		5'-TGC TGC TAA CAG GCT AGC AAG TAA AG-3'	728–703	BaA91 (AF441833)
16		5'-TTA ACC TCA ATT AAT CTT TCA G-3'	461–440	BaA91
17		5'-CCG <u>GTA CCT</u> TAT TTT TGA TTT TTA GTT TGT T-3'	513–491	BaA91
18	GenomWalkerKit	5'-GTA ATA CGA CTC ACT ATA GGG C-3'	Adapter primer 1	
19		5'-ACT ATA GGG CAC GCG TGG T-3'	Adapter primer 2	

Restriction enzyme sites of *Bam*HI and *Kpn*I in expression primers are underlined.

AmpliTaQGold DNA Polymerase (Perkin Elmer, USA) at 72°C for 1.5 min.

Genome walking

The genome walking methodology (Universal Genom-Walker Kit, Clontech, USA) was applied to borrelial DNA from *B. afzelii* and *B. garinii*. Firstly, borrelial DNA from *B. afzelii* strain BaA91 and *B. garinii* strain Bg40 was digested with four different restriction endonucleases (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I) separately. GenomWalker adapters were ligated to both ends of the cut DNA. Then DNA fragments were amplified by PCR, with downstream primers selected from known *dbpA* sequences downstream from *dbpB* [9] and the GenomWalker adapter primers as upstream primers (Table 1). Two PCR amplifications were performed, the second as a nested PCR, and to walk the sequence upstream, new primers were designed from the sequences obtained (Table 1). The *dbpB* sequences of the other *B. afzelii* and *B. garinii* strains (Ba1082, BaEM9, Bg46 and Bg50) were obtained by PCR, with primers chosen from analysed BaA91 and Bg40 sequences. To eliminate any errors possibly due to the use of Taq polymerase, the two strands of each *dbpB* were sequenced independently at least twice. These sequences were used to choose expression primers for the BaA91 and Bg40 strains encoding the mature portion of the DbpB protein after cysteine at the site of post-translational acylation.

DNA sequencing

The full-length or partial *dbpB* DNA strands obtained by PCR were cloned into the pCR 2.1-TOPO vector (Invitrogen) and sequenced at the Core Facility of the Haartman Institute, University of Helsinki, with a DyePrimer (T7, M13Rev) cycle sequencing kit (Applied Biosystems, USA). Sequencing reactions were run and analysed with the automated sequencing apparatus model 373A (Applied Biosystems). DNA and protein sequences were analysed with Lasergene software (DNASTAR, USA).

Cloning and expression of recombinant DbpB

For expression of the rDbpBs, six histidine-tagged protein constructs were generated as described previously [9].

ELISA

For ELISA assays measuring anti-DbpB antibodies, the wells in the microtitration plates were coated with 100 µl (2 µg/ml) of the variant recombinant DbpB proteins overnight. After washing, 100 µl of a diluted serum sample were added to each well and the wells were incubated overnight. Serum samples were diluted 1 in 10 (EM) or 1 in 100 (neuroborreliosis and Lyme arthritis) with bovine serum albumin (BSA) 5 mg/ml in

155 mM NaCl-Tween 20 0.04% buffer (BSA-NaCl-Tween). Mouse plasma samples were diluted 1 in 100 in 1 M PBS-Tween 20 0.01%-gelatine 0.25%. After washing, the wells were incubated with alkaline phosphatase-conjugated rabbit anti-human IgG or IgM (Jackson Immuno Research Laboratories, USA) diluted 1 in 5000 or alkaline phosphatase-conjugated rabbit anti-mouse IgG (Orion, Espoo, Finland) diluted 1 in 1000 in BSA-NaCl-Tween for 2 h. The reactions were visualised with 4-nitrophenylphosphate (Boehringer Mannheim GmbH, Mannheim, Germany) 1 mg/ml in diethanolamine buffer, pH 10.0. After 10–20 min, the optical density (OD) measurements were made with a Multiscan photometer (Thermo Labsystems, Helsinki, Finland) at 405 nm wavelength. The cut-off values for human and mouse samples were determined as the means +3 SD of the respective control samples.

Samples

For ELISA analyses serum samples were collected from 14 adult patients with NB, 15 with Lyme arthritis (LA) and 23 with culture- or PCR-positive EM (*B. afzelii* n = 17, *B. garinii* n = 4, and genotyping not feasible n = 2). In the patients with NB and LA, the clinical diagnosis was confirmed in ELISA by demonstrating serum antibodies (and CSF anti-flagellin antibodies in NB patients) against flagellin and *B. burgdorferi* WCL [20]. The EM patients provided serum samples at diagnosis (acute) and at 1–3 months after treatment (convalescent). Serum samples from patients with syphilis (SY), systemic lupus erythematosus (SLE) and Epstein–Barr virus (EBV) infection, and from patients positive for rheumatoid factor (RF) or anti-streptolysin antibodies (ASO), and from healthy blood donors were used as controls.

In a second set of samples, sera from 52 children with well characterised LA from Germany were analysed [21, 22]. Serum samples from 20 children with other inflammatory joint diseases and samples from 20 healthy blood donors were used as controls.

Plasma samples from mice infected with *B. garinii* strain Å218 were obtained from Matti Viljanen (National Public Health Institute, Turku, Finland). The infection was verified by culturing ear pinnae from each mouse at each time point when groups of five mice were killed. Plasma from individual infected mice was pooled at time points 2, 4, 8 and 16 weeks after infection. Plasma samples from an identical set of sham-infected mice were used as control samples in ELISA.

Nucleotide sequence accession nos

The nucleotide sequences for *dbpB* were submitted to GenBank under accession nos: BaA91, AY083914; Ba1082, AY083915; BaEM9, AY083916; Bg40, AY083917; Bg46, AY083918; Bg50, AY083919; and

Bbia, AY083920. Published *dbpB* sequences from *B. burgdorferi sensu stricto* strains 297 (U75867), LP4 (AF069264), LP5 (AF069261), LP7 (AF069255), NCH-1 (AF069259), FRED (AF069260), HB19 (AF069254), ZS7 (AF069251), B31 (AF069266) and N40 (AF069252) and *B. garinii* strain 20047 (AF069263) were obtained from GenBank.

Results

Analysis of *dbpB* sequences of the Finnish borrelial isolates

The open reading frames of the *dbpB* sequences analysed consisted of 552–564 nucleotides. The identity of the *dbpB* sequences between the borrelial subspecies was 78–82%. Within *B. garinii* strains, *dbpB* in Bg46 differed by one nucleotide (position 312) from the Bg40 and Bg50 sequences, but this did not change the amino acid composition. Similarly, *dbpB* in *B. afzelii* strain Ba1082 differed by one nucleotide (position 416) from BaA91 and BaEM9. The codon difference caused a putative translational change from alanine to valine.

DbpBs of the Finnish borrelial isolates

The deduced mature proteins DbpB_{BaA91,1082,EM9}, DbpB_{Bg40,46,50} and DbpB_{Bbia} contained 165, 166 and 167 residues, respectively (Fig. 1). Differences in the amino acid sequences were distributed along the entire sequence. The calculated molecular masses of the predicted mature proteins DbpB_{BaA91}, DbpB_{Bg40} and DbpB_{Bbia} (without lipid acylation) were 18.6, 18.1 and 18.2 kDa, respectively. Comparison of the deduced mature amino acid sequences of DbpB_{BaA91} and

DbpB_{Bbia}, DbpB_{BaA91} and DbpB_{Bg40}, and DbpB_{Bg40} and DbpB_{Bbia} showed 67%, 67% and 62% identity, respectively (Fig. 2). Protein analysis revealed differences in the frequencies of charged, polar and hydrophobic amino acid composition, yielding calculated iso-electric points of 8.61, 9.35 and 9.47 for DbpB_{BaA91}, DbpB_{Bg40} and DbpB_{Bbia}, respectively.

Analysis of *DbpB* of *B. burgdorferi sensu lato*

The amino acid sequences of DbpBs from the three *B. afzelii* isolates (BaA91, Ba1082 and BaEM9) were 99–100% identical. In the GenBank database there are no published *dbpB* sequences of *B. afzelii* strains. The identical DbpB_{Bg40}, DbpB_{Bg46} and DbpB_{Bg50} sequences were compared with the published *B. garinii* DbpB sequence from strain 20047 (AF069263). There was 62% identity. The sequence from strain 20047 was

	Bbia	297	B31	Bg40	BaA91	Ba1082
Bbia	-	98.8	99.4	<u>61.7</u>	<u>66.9</u>	<u>66.9</u>
297		-	99.4	61.7	68.1	68.1
B31			-	62.3	67.5	67.5
Bg40				-	<u>67.5</u>	<u>66.9</u>
BaA91					-	<u>99.4</u>
Ba1082						-

Fig. 2. Identities of deduced amino acid sequences of DbpB among isolates of *B. burgdorferi sensu lato*. The identities (%) were calculated without the sequence encoding the leader peptide by the Jotun Hein method with Lasergene software. Underlined figures represent identities of DbpB between the Finnish borrelial isolates. *B. garinii* strain Bg40 also represents the identical strains Bg46 and Bg50. *B. afzelii* strain BaA91 also represents the identical strain BaEM9.

1	C S I G L V E R T N A A L E S S S K D L K N K I L K I K K E A T G K G V L F E A F T G L K T G S K V	Bbia
1	C S I G L V E R T N A A L E S S S K D L K N K I L K I K K D A T G K G V L F E A F T G L K T G S K V	297
1	C S I G L V E R T N A A L E S S S K D L K N K I L K I K K E A T G K G V L F E A F T G L K T G S K V	B31
1	C N F G L T G E V K A M L E S S S D N V K N K I L Q I K E E A A K K G V N F K A F T G T A T G S K V	Bg40
1	C N F G L M E E T K I A L E S S S K D V K N K I L Q I K K D A E D K G V N F A A F T S S E T G S K V	BaA91
1	C N F G L M E E T K I A L E S S S K D V K N K I L Q I K K D A E D K G V N F A A F T S S E T G S K V	Ba1082
51	T S G G L A L R E A K V Q A I V E T G K F L K I I E E E A L K L K E T G N S G Q F L A M F D I M L E	Bbia
51	T S G G L A L R E A K V Q A I V E T G K F L K I I E E E A L K L K E T G N S G Q F L A M F D I M L E	297
51	T S G G L A L R E A K V Q A I V E T G K F L K I I E E E A L K L K E T G N S G Q F L A M F D I M L E	B31
51	A N G G S A L R E A K V Q A I N E V E K F L N T I E E E A L L L K K N G N S Q F L A M F D I M L E	Bg40
51	T N G G L A L R E A K I Q A I N E V E K F L K R I E E E A L K L K E H G N S G Q F L E L F D L L L E	BaA91
51	T N G G L A L R E A K I Q A I N E V E K F L K R I E E E A L K L K E H G N S G Q F L E L F D L L L E	Ba1082
101	V V E S L E D V G I I G L K A R V L E E S K N N P I N T A E R L L A A K A Q I E N Q L K V V K E K Q	Bbia
101	V V E S L E D V G I I G L K A R V L E E S K N N P I N T A E R L L A A K A Q I E N Q L K V V K E K Q	297
101	V V E S L E D V G I I G L K A R V L E E S K N N P I N T A E R L L A A K A Q I E N Q L K V V K E K Q	B31
101	V T G S L D E I G I K G I K S S I S E E A K S N P V N T A E R L V E V K A K I E N K L E G V K K R Q	Bg40
101	V L E S L E P I G I K G L K D F I S E E A K C N P I S T S E R L I E V K V Q I E N K M E E V K R K Q	BaA91
101	V L E S L E P I G I K G L K D F I S E E V K C N P I S T S E R L I E V K V Q I E N K M E E V K R K Q	Ba1082
151	N I E N G G E K K N N K S K K K K	Bbia
151	N I E N G G E K K N N K S K K K K	297
151	N I E N G G E K K N N K S K K K K	B31
151	K L D D - E E K K I S K S K K N K	Bg40
151	N L N K - E - R K S N K G K K K K	BaA91
151	N L N K - E - R K S N K G K K K K	Ba1082

Fig. 1. Alignment of predicted mature DbpB amino acid sequences of *B. burgdorferi sensu stricto* (Bbia, 297 and B31), *B. garinii* (Bg40) and *B. afzelii* (BaA91 and Ba1082). Residues differing from Bbia are boxed. The *B. garinii* strain Bg40 also represents the identical strains Bg46 and Bg50. The *B. afzelii* strain BaA91 also represents the identical strain BaEM9.

available for amino acid analysis from position 6 to the end of the DbpB protein. The DbpB_{Bbia} sequence was compared with published DbpB sequences of human *B. burgdorferi sensu stricto* strains 297 (U75867), LP4 (AF069264), LP5 (AF069261), LP7 (AF069255), NCH-1 (AF069259), FRED (AF069260), HB19 (AF069254) and tick isolates B31 (AF069266), ZS7 (AF069251) and N40 (AF069252) (Fig. 2). The sequence identity of the deduced mature amino acids ranged from 99% to 100%. The deduced amino acid sequences of DbpB in strains HB19, FRED, LP5, LP7, NCH-1, B31, N40 and ZS7 were identical and differed by only one amino acid from the Bbia sequence. The amino acid at position 127 was isoleucine in Bbia, but leucine in all the other sequences. In the identical DbpB sequences of strains 297 and LP4, glutamic acid at position 60 was substituted in the other sequences by aspartic acid. The DbpB sequence in *B. garinii* 20047 was identical with that of most strains of *B. burgdorferi sensu stricto*.

The DbpBs of all three subspecies had in common a C-terminal decapeptide that contained six or seven positively charged amino acids but no negative charges. Elsewhere in the sequences, there were marked differences in the position of the charged amino acids. The location of the hydrophobic amino acids also differed, e.g., at position 74 of the putative mature sequence, the isoleucine of *B. burgdorferi sensu stricto* was replaced by charged arginine in *B. afzelii*. In place of isoleucine at position 81 in *B. garinii* DbpB, in the other two species there was lysine.

ELISA

In IgG ELISA, 9 (64%) of 14 samples from patients with NB were positive. When rDbpB_{BaA91}, rDbpB_{Bg40} and rDbpB_{Bbia} were used as antigens, 3 (21%), 7 (50%) and 1 (7%) of 14, respectively, were positive (Fig. 3). Of the 15 samples from patients with LA, 11 (73%) were positive, 1 (7%), 9 (60%) and 6 (40%) of 15 with rDbpA_{BaA91}, rDbpA_{Bg40} and rDbpA_{Bbia}, respectively (Fig. 3). Of the nine positive samples from patients with NB, five were positive only with DbpB from *B. garinii*, two only with DbpB from *B. afzelii*, one with DbpB from *B. afzelii* and *B. garinii* and one with DbpB from *B. garinii* and *B. burgdorferi sensu stricto*. Of the 11 positive samples from LA patients, five were positive only with DbpB from *B. garinii*, one with DbpB from *B. afzelii* and *B. burgdorferi sensu stricto*, one only with DbpB from *B. burgdorferi sensu stricto* and four with DbpB from *B. garinii* and *B. burgdorferi sensu stricto*. Of the 20 positive samples, 13 reacted with one antigen only. Of the control samples, one from a patient with SLE was positive with all three DbpBs and a few others were low positives (Fig. 3). The calculated specificity was 98%, 88% and 92% when rDbpB_{BaA91}, rDbpB_{Bg40} and rDbpB_{Bbia} were used as antigens, and the total specificity was 93%.

Fig. 3. IgG ELISA OD₄₀₅ values with recombinant DbpB as an antigen from (a) *B. afzelii* (BaA91), (b) *B. garinii* (Bg40) and (c) *B. burgdorferi sensu stricto* (Bbia). Serum samples were from patients with neuroborreliosis (NB) and Lyme arthritis (LA) and control samples from patients with syphilis (SY), systemic lupus erythematosus (SLE), Epstein-Barr virus infection (EBV), from patients positive for rheumatoid factor (RF+) and for anti-streptolysin (ASO), and samples from healthy blood donors (BD). The cut-off level (mean + 3 SD of BD samples) is indicated by the horizontal line.

In the 52 paediatric LA patients, 20 (38%), 23 (44%) and 30 (58%) serum samples were positive when rDbpB_{BaA91}, rDbpB_{Bg40} and rDbpB_{Bbia}, respectively, were used as antigens (Fig. 4). In total, 40 (77%) of the 52 serum samples were positive in rDbpB IgG ELISA. Of the 40 positive samples, 18 reacted with one antigen only, 15 with two antigens and 7 reacted with all 3 antigens. In the control patients with other joint diseases, 2 (10%) of 20 had low positive reactions in ELISA with rDbpB_{Bbia} as antigen. No positive reactions were observed with the sera of healthy blood donors. The calculated overall specificity was 98%.

Fig. 4. IgG ELISA OD_{405} /cut-off values with recombinant DbpB as an antigen from *B. afzelii* (BaA91), *B. garinii* (Bg40), *B. burgdorferi sensu stricto* (Bbia) and the highest OD_{405} /cut-off value for a sample in any of the three tests (Total). Serum samples were from children with Lyme arthritis (LA) and control samples from children with other inflammatory joint disease (CO) and from healthy blood donors (BD). The OD_{405} /cut-off value 1 (cut-off = mean +3 SD of BD samples) is indicated by the horizontal line.

In IgM and IgG ELISA of samples from 23 early LB patients with culture- or PCR-positive EM, 1 (4%) and 6 (26%), respectively, were positive with the variant DbpB proteins used as antigens (data not shown).

IgG ELISA with mouse plasma

At 2 weeks after inoculation, plasma from mice infected with *B. garinii* already showed highly positive OD_{405} values with rDbpB from *B. garinii* as an antigen (Fig. 5). At 4 weeks, the immunoreactivity was further

Fig. 5. IgG ELISA OD_{405} /cut-off values with recombinant DbpB as an antigen from *B. afzelii* (BaA91, ■), *B. garinii* (Bg40, ●), and *B. burgdorferi sensu stricto* (Bbia, ○) in plasma samples from mice infected with *B. garinii* A218. Pooled plasma samples were collected at time points 2, 4, 8 and 16 weeks. Samples of plasma taken from sham-infected mice at the same time points were used as controls. The OD_{405} /cut-off value 1 (cut-off = mean +3 SD of samples from sham-infected mice) is indicated by the horizontal line.

increased and remained at high levels at 8 and 16 weeks after infection. When heterologous rDbpBs from *B. afzelii* and *B. burgdorferi sensu stricto* were used as antigens, the immunoreactivity in ELISA at 2 and 4 weeks was low. At 8 weeks the OD_{405} values were clearly positive, but at lower levels than the OD_{405} values against the homologous rDbpB from *B. garinii*.

Discussion

The present study describes, for the first time, the sequences of DbpB in *B. afzelii* and *B. garinii* strains, and characterises the antibody responses to the variant rDbpBs in the serodiagnosis of LB. The results indicate that DbpB may be a useful antigen in IgG serology for disseminated LB. The high heterogeneity observed in the sequences implies that, to cover all the relevant borrelial subspecies in the serodiagnosis of LB, a combination of the variant DbpBs should be included in the antigen set.

As the PCR-based approach for cloning of individual *dbpB* genes was feasible only for *B. burgdorferi sensu stricto*, the genome walking methodology was used to clone *dbpB* genes from *B. afzelii* and *B. garinii* isolates. This method relies on established sequences in close proximity to unknown DNA. The present study took advantage of the *dbpA* sequences from various borrelial subspecies analysed in a previous study [9]. The inter-species sequence identity of the deduced DbpB proteins varied from 62% to 68%. Interestingly, except for *B. garinii* 20047, in which the DbpB sequence was identical with that of most strains of *B.*

burgdorferi sensu stricto, the intra-species sequence identity within subspecies was 99–100%. This contrasted with the sequences of the closely related DbpA, which were highly heterogeneous both between and within subspecies [9, 18].

Both decorin-binding proteins have been shown to bind decorin and thus they possibly mediate attachment of borreliae to the extracellular matrix of the host [23, 24]. On the other hand, a recent study reported difference in binding specificity between DbpA and DbpB, suggesting that DbpB may not be as important as DbpA in mediating the tissue adherence of *B. burgdorferi* [24]. At present, it is not known whether DbpB from *B. afzelii* or *B. garinii* binds decorin. The heterogeneity of the charged and hydrophobic amino acids of this protein in the three borrelial subspecies implies that folding of the mature proteins may vary considerably, which would cause conformational and, consequently, functional changes. However, it can be speculated that the positively charged C-terminal decapeptide observed in all three subspecies might serve as a binding motif of this protein to negatively charged connective tissue structures.

To our knowledge, the antigenic potential of DbpB in the serodiagnosis of LB has not previously been evaluated in detail. In a recent study, 13% of EM and 16% of late LB patients from Italy were positive for DbpB in an IgG Western blot [25]. However, the origin of the DbpB was *B. burgdorferi sensu stricto* 297. In the light of the present results, it would seem that the use of a single DbpB variant would account for the low sensitivity of their analyses. The present study shows that DbpB does not seem to improve the serodiagnosis of early LB. However, at later stages of LB, the sensitivity of the IgG ELISA had risen to 77%, provided that all the variant DbpBs were used as antigens in parallel. With this approach, the DbpB ELISA seemed to give equally good results with serum samples from Western and Northern Europe. As an ELISA antigen, recombinant DbpB from *B. garinii* predominated. This is in line with European studies, in which either *B. garinii* or *B. afzelii* has been observed to be the most prevalent strain causing human LB [26–28]. In patients with LA from Germany, the DbpB from *B. burgdorferi sensu stricto* also performed well as an ELISA antigen. This finding is consistent with the known arthritogenic potential of *B. burgdorferi sensu stricto* [26] and the greater prevalence of this subspecies in Central Europe than in Scandinavia, especially in patients with disseminated LB [26, 29]. The present results suggest that variant DbpBs have specific antigenic epitopes with relatively low degrees of cross-reactivity. This hypothesis was strengthened by the stronger immune reactions against homologous as compared with heterologous DbpBs during experimental murine borreliosis.

The genes encoding DbpB and DbpA are located in a

bi-cistronic operon and have been shown *in vitro* to be transcribed together [16]. In experimental murine borreliosis, early and strong immune responses have been reported against both proteins [15–17]. Interestingly, in a previous study, rDbpA proved to be 100% sensitive as an antigen in the serodiagnosis of disseminated LB. In the present study, in contrast, the sensitivity of DbpB was lower. Furthermore, the immune reactivity to rDbpBs seemed to remain quantitatively at a lower level than that to rDbpAs originating from the same pathogenic isolates (data not shown). Given the functional and (secondary) structural similarities between DbpA and DbpB [24], it can be speculated that, during human LB, DbpB may be expressed at lower levels than DbpA. The mechanisms by which *B. burgdorferi* regulates its gene expression in various environments are insufficiently characterised. However, it has been shown that at least some environmental signals, such as pH and temperature, may influence the levels of expression of several borrelial proteins, including DbpA and DbpB [30–32].

In summary, the high sequence heterogeneity of DbpB implies that further studies are needed to elucidate the roles of the variant DbpBs in the tissue adherence of borreliae. Our hypothesis is that the heterogeneity of the amino acid sequences may have implications for the usefulness of a given antigen in the serodiagnosis of LB. Consistent with previous results for DbpA [9], the present study provides evidence that, in a European epidemiological setting, a combination of recombinant antigens derived from all the pathogenic borrelial species appears to be a promising approach for the development of new serodiagnostic tests with higher sensitivity.

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