Purification and characterization of a tryptic peptide of *Borrelia burgdorferi* flagellin, which reduces cross-reactivity in immunoblots and ELISA

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In man the early immune response in Lyme disease is primarily directed against the endoflagellin antigen. Isolated flagellar protein of *Borrelia burgdorferi* suggests itself as a suitable test antigen. However, cross-reactivity between flagellins of *B. burgdorferi*, *Escherichia coli*, *Bacillus subtilis*, *Proteus mirabilis* and *Salmonella typhimurium* was demonstrated by immunoblotting and ELISA with polyclonal rabbit-hyperimmune-sera. Tryptic cleavage of recombinant *B. burgdorferi* 41 kDa flagellin, expressed in *E. coli*, produced a peptide fragment which was recognized exclusively by antisera to *Borrelia* species. This peptide was designated as the 14 kDa fragment due to its migratory behaviour in SDS-PAGE. The fragment is part of the variable region of the flagellin, as proven by amino acid sequencing. The flagellin peptide was employed as an antigen in ELISA and immunoblot assays, testing the polyclonal sera mentioned above. The specificity was superior to that obtained with the intact recombinant flagellin.

Introduction

Lyme borreliosis is a chronic infectious disease caused by the tick-borne spirochaete *Borrelia burgdorferi* (Steere *et al.*, 1983). This multisystemic disorder starts typically with an erythematous skin lesion, erythema chronicum migrans (ECM), which in some patients progresses to acrodermatitis chronicum atrophicum (ACA). Other manifestations include neurological, cardiac and articular syndromes (Steere 1989). Isolation of *B. burgdorferi* is, due to the fastidious nature of this organism, difficult and time consuming. Diagnosis of Lyme disease therefore relies on serological assays. The early immune response of most patients is directed primarily against the *B. burgdorferi* flagellin, a 41 kDa polypeptide, which constitutes the flagella. Isolated flagellar protein suggests itself as a suitable test antigen. Hansen *et al.* (1988, 1991) were able to improve serodiagnosis by using either purified *B. burgdorferi* flagella in a direct ELISA or biotinylated flagella in a μ -capture ELISA. There are, however, pronounced amino acid sequence similarities between the flagellin of *B. burgdorferi* and those of other spirochaetes (*Borrelia hermsii*, 95%, *Treponema pallidum*, 60%), and other unrelated eubacteria (*Salmonella typhimurium*, 51%; *Escherichia coli*, 51%; *Serratia marcescens*, 50%; *Bacillus subtilis*, 49%) (Gassmann *et al.*, 1991). The

B. burgdorferi flagellin consists of 336 amino acids; conserved and semi-conserved regions are located exclusively at the N- and C-termini of the protein, whereas the central region (amino acids 131–266) exhibits variability. Epitopes cross-reacting with hyperimmune sera raised against the flagella of *Ba. subtilis* and *Treponema phagedenis* have recently been identified. These epitopes are located in the highly conserved regions at the N- and C-termini of the flagellin. Many *Borrelia*-specific epitopes were found in the variable central region (Gassmann *et al.*, 1991) and this part, therefore, should represent a suitable antigen. Here we report on the characterization and purification of a tryptic peptide of recombinant flagellin, expressed in *E. coli*. This peptide is part of the variable region of the flagellin and exhibits only *Borrelia*-specific epitopes within the limits of tested bacterial genera.

Methods

Bacteria and culture conditions. *Borrelia hermsii*, *Borrelia parkeri*, *Borrelia turicatae* (provided by Dr. Baranton, Institut Pasteur, Paris, France) and *Borrelia burgdorferi* GeHo (isolated from a skin biopsy by Dr. Pelz, Institut für Medizinische Mikrobiologie und Hygiene, Freiburg, FRG) were grown in modified Kelly's medium (Barbour *et al.*, 1983) to the late exponential phase. Microbes were harvested by centrifugation (5000 g, 30 min at room temperature) and washed twice

with PBS containing protease inhibitors (5 mM-EDTA, 5 mM-sodium tetrathionate, 5 mM-benzamidine, 0.5 mM-PMSF). Final bacterial pellets were resuspended in the same buffer and disintegrated by ultrasonication.

Expression and purification of recombinant *B. burgdorferi* flagellin. *Escherichia coli* HB101, containing pBb20 as a cloning vector (Gassmann *et al.*, 1991), was cultivated in a heart-brain bouillon containing ampicillin (100 µg ml⁻¹) for 24 h at 37 °C. The *B. burgdorferi* 41 kDa flagellin was expressed as a non-fusion protein.

The cells of a 5 l culture were harvested as follows: bacteria were pelleted by centrifugation at 10000 g for 30 min and washed twice with cold PBS: the resulting pellet (about 2 g l⁻¹) was resuspended in 20 mM-imidazole/HCl, pH 6.5, containing 8 M-urea (Gibco) (starting buffer for the following anion exchange chromatography, final vol. = tenfold vol. of bacterial pellet) and stirred for 2 h at room temperature. After centrifugation at 27000 g for 45 min the urea-extracted recombinant flagellin was found in the supernatant (5–7 mg ml⁻¹). As a first purification step, anion exchange chromatography (DEAE-Cellulose, Serva) was applied [column size; 4 cm (height) × 4.5 cm (diam.); starting buffer, 20 mM-imidazole/HCl, pH 6.5, plus 8 M-urea; final buffer, starting buffer plus 1 M-Na citrate; linear gradient from 0–100% of the final buffer; gradient volume, 100 ml; flow rate, 48 ml h⁻¹]. Fractions (fractions size, 8 ml) containing recombinant flagellin were pooled and dialysed against distilled water. Precipitated flagellin was resuspended in starting buffer for cation exchange chromatography [SP-Sephadex C-50, Pharmacia; column size; 4 cm (height) × 4.5 cm (diam.); starting buffer, 50 mM-MES, pH 6.5, plus 8 M-urea; final buffer, starting buffer plus 1 M-NaCl; linear gradient from 0–100% of the final buffer; gradient volume, 100 ml; flow rate, 48 ml h⁻¹]. Flagellin-containing fractions (fraction size, 8 ml) were pooled and dialysed against distilled water. Precipitated, purified recombinant flagellin was stored at –20 °C. We obtained 12–16 mg protein from a 5 l culture. Protein content was determined by a BCA-protein assay (Pierce). After each chromatographic step, fractions were analysed by SDS-PAGE (12.5% gel) according to the method of Laemmli (1970).

Tryptic cleavage of recombinant flagellin. Purified recombinant flagellin was resuspended in 0.1 M-Tris/HCl, pH 7.5, and digested with trypsin (sequencing grade, Boehringer Mannheim) at a ratio of 1 µg trypsin per 100 µg protein (2–3 mg ml⁻¹) for 4 h at 37 °C.

Purification of the 14 kDa fragment. For use as an antigen in ELISA and for amino acid analysis, the 14 kDa fragment was purified by gel filtration [Superdex 75 HR 10/30, Pharmacia; buffer, 0.1 M-Tris/HCl (pH 7.5); flow rate, 0.5 ml min⁻¹]. The column was calibrated according to the manufacturer's instructions. From a 5 l culture we obtained 3–6 mg of the 14 kDa fragment.

SDS-PAGE. Cleavage products were analysed in a Tricine-SDS-PAGE system previously described by Schägger & von Jagow (1987). Proteins were separated in a 16.5% T, 3% C separating gel (7 cm in length) overlaid by a 10% T spacer gel and a 4% stacking gel (both 2 cm in length). Electrophoresis was performed for 16 h at 15 mA. For molecular mass determination, a prestained low-molecular-mass marker (BRL) was used. After electrophoresis gels were fixed in 50% (v/v) methanol/10% (v/v) acetic acid and then stained with 0.025% (w/v) Coomassie G-250 (Sigma) in 10% (v/v) acetic acid. Destaining was done in 10% (v/v) acetic acid.

Immunoblotting. For immunodetection and N-terminal sequencing, proteins were transferred after electrophoresis to a polyvinylidene fluoride membrane (PVDF, Millipore) as described by Towbin *et al.* (1979). A 50 mM-borate buffer, pH 8.9, containing 10% (v/v) methanol and 0.02% (v/v) 2-mercaptoethanol was used as transfer buffer (transfer time, 4 h). After transfer, non-specific binding sites of the membrane were blocked with 10% (w/v) skimmed milk in PBS. Rabbit-hyperimmune-sera were diluted 1:1000 in 10% (w/v) skimmed milk in

Table 1. Polyclonal rabbit-hyperimmune-sera

Polyclonal sera to:	Rabbits immunized with:	Rabbit number
<i>B. burgdorferi</i>	Intact cells	K 248
<i>B. burgdorferi</i>	Whole-cell lysate	K 312
<i>B. burgdorferi</i>	41 kDa flagellin	K 17
<i>B. burgdorferi</i>	41 kDa flagellin	K 314
<i>B. burgdorferi</i>	Recombinant flagellin	K 297
<i>B. burgdorferi</i>	Recombinant flagellin	K 549
<i>B. burgdorferi</i>	14 kDa fragment	K 265
<i>B. burgdorferi</i>	14 kDa fragment	K 270
<i>B. parkeri</i>	Whole-cell lysate	K 1
<i>B. turicatae</i>	Whole-cell lysate	K 4
<i>B. hermsii</i>	Whole-cell lysate	K 567
<i>Ba. subtilis</i>	Flagella	K 306
<i>E. coli</i>	Flagella	K 215
<i>Proteus mirabilis</i>	Flagella	K 110
<i>S. typhimurium</i>	Flagella H1.2.	K 301
<i>S. typhimurium</i>	Flagella Hi	K 267
<i>T. phagedenis</i>	Whole-cell lysate	K 779

PBS and incubated for 2 h at room temperature. Immunodetection was done with a peroxidase-conjugated goat-anti-rabbit IgG (Dianova, diluted 1:3000 in 10% (w/v) skimmed milk in PBS and incubated for 2 h at room temperature) with diaminobenzidine (Sigma) as substrate. All washing steps were performed with 0.1% (v/v) Tween 20/PBS.

ELISA. ELISA was done in the usual way. Briefly, 96-well flat-bottomed microdilution plates (Greiner) were coated for 24 h with the previously determined optimal antigen concentration of (a) intact recombinant flagellin (0.025 µg per well) and (b) purified 14 kDa fragment (0.05 µg per well) diluted in coating buffer (0.05 M-carbonate buffer, pH 9.5). Coating with intact recombinant flagellin was performed starting from an 8 M-urea solution that was diluted in coating buffer to the appropriate concentration (final urea concentration was about 8 mM). After washing [0.1% (v/v) Tween 20/PBS] rabbit-hyperimmune-sera, diluted from 1:1600 to 1:204 000 [dilution buffer; 2% (v/v) foetal calf serum, 2% (v/v) Tween 20 in PBS], were incubated for 2 h at room temperature. Detection of antibodies was achieved with peroxidase-conjugated goat-anti-rabbit IgG (Dianova) at a dilution of 1:3000, incubated for 2 h at room temperature. After a third washing step, substrate solution [0.4 mg phenylenediamine ml⁻¹ (Sigma) in citrate buffer, 2 µl 30% (v/v) H₂O₂ added prior to use] was added and the mixture was left for 15 min in a dark chamber. The reaction was stopped with 2 M-H₂SO₄ and the optical density was read at 462 nm (Titertek Multiscan). A commercially available ELISA-test kit, employing intact flagella (Dakopatts), was used according to the manufacturer's instructions.

The cut-off was fixed at an OD₄₆₂ value of 0.2 for all three assays by comparison of non-immune rabbit sera, which showed mean OD₄₆₂ values of 0.060 (recombinant flagellin), 0.080 (14 kDa fragment) and 0.100 (ELISA-test kit) in the first dilution step.

Characterization of the 14 kDa fragment. For N-terminal sequencing of the 14 kDa fragment, the tryptic digest was transferred to a PVDF-membrane as described for immunoblotting. After transfer, proteins were stained with 0.1% (w/v) Coomassie R-250 (Sigma) in 50% (v/v) methanol. The 14 kDa band was excised and analysed by Edman degradation in a gas-phase sequencer (477A Protein Sequencer with on-line 120 A PTH-Analyzer, Applied Biosystems). To determine the C-terminal sequence, the purified fragment was cleaved with endoproteinase Asp-N (Boehringer Mannheim) at a ratio of 1 µg protease per 65 µg protein overnight at 37 °C (digestion of the 14 kDa fragment with carboxypeptidase A and B was not successful). Cleavage products were

separated by reversed-phase chromatography (HPLC column Pep-S C2/C18, Pharmacia) and analysed by N-terminal sequencing. Amino acid analysis after acid hydrolysis (Hill, 1965) of the purified 14 kDa fragment was done on a Biotronik amino acid analyser BT 6000 E. Additionally, some tryptic peptides of recombinant flagellin that had been purified by reversed-phase chromatography were N-terminally sequenced to provide further information about the C-terminal end of the fragment.

Polyclonal rabbit-hyperimmune-sera. Rabbits were immunized at 4-week-intervals by repeated subcutaneous injections of 50 µg purified recombinant *B. burgdorferi* flagellin (sera K297, K549) or 80 µg purified 14 kDa fragment (sera K265, K270) in Freund's Complete Adjuvant (FCA). Serum K312 was raised against whole-cell lysates of *B. burgdorferi* GeHo and serum K248 was obtained by intravenous injections of whole organisms (*B. burgdorferi* GeHo) without FCA (Gassmann *et al.*, 1991). Sera K17 and K314 are monospecific sera raised against flagellin of *B. burgdorferi*. Rabbits were immunized as described above with 41 kDa bands excised from preparative SDS-polyacrylamide gels containing about 50 µg protein. Hyperimmune-sera against whole cell lysates of *B. hermsii* (serum K567), *B. parkeri* (serum K1) and *B. turicatae* (serum K4) were obtained by immunizing the rabbits at 4-week-intervals by repeated subcutaneous injection of 100 µg protein in FCA. The hyperimmune-sera against purified flagella from *B. subtilis* (serum K306), *E. coli* (serum K215), *Proteus mirabilis* (serum K110), *Salmonella typhimurium* H1.2. and Hi (sera K301 and K267) were kindly provided by Torsten Schmidt (Freiburg). We thank Alexandra Otto (Freiburg) for kindly providing us with the hyperimmune-sera against whole-cell lysates of *Treponema phagedenis* (sera K38 and K779). A list of the antisera is given in Table 1.

Fig. 1. SDS-polyacrylamide gel (16.5%), stained with Coomassie G-250 showing kinetics of cleavage of recombinant *B. burgdorferi* 41 kDa flagellin with trypsin. Lanes: M, molecular mass markers; T, trypsin; Fl, flagellin (41 kDa); 1–8, aliquots taken at 30 min intervals from 30 min (1) to 4 h (8). The 14 kDa fragment is indicated by the arrowhead.

Fig. 2. Immunoblot of IgG-antibodies against recombinant *B. burgdorferi* 41 kDa flagellin of various polyclonal rabbit-hyperimmune-sera. Lanes: M, molecular mass markers; 1, control for the second antibody (peroxidase-conjugated goat-anti-rabbit IgG); 2, non-immune rabbit serum; 3, K248 (anti-intact cells of *B. burgdorferi* GeHo); 4, K312 (anti-whole-cell lysate of *B. burgdorferi* GeHo); 5, K17; 6, K314 (anti-41 kDa flagellin of *B. burgdorferi*); 7, K297; 8, K549 (anti-recombinant flagellin of *B. burgdorferi*); 9, K265; 10, K270 (anti-14 kDa fragment); 11, K1 (anti-whole-cell lysate of *B. turicatae*); 12, K4 (anti-whole-cell lysate of *B. parkeri*); 13, K567 (anti-whole-cell lysate of *B. hermsii*); 14, K38; 15, K779 (anti-whole-cell lysate of *T. phagedenis*); 16, K306 (anti-flagella of *Ba. subtilis*); 17, K215 (anti-flagella of *E. coli*); 18, K110 (anti-flagella of *P. mirabilis*); 19, K301 (anti-flagella of *S. typhimurium* H1.2.); 20, K267 (anti-flagella of *S. typhimurium* Hi).

Results

Purification of recombinant flagellin by combined anion and cation exchange chromatography resulted in a 41 kDa fraction revealing only weak contaminants in the

lower molecular mass range (< 30 kDa) when analysed in 16.5% SDS-PAGE (Fig. 1). Almost all higher molecular mass contaminants that could be seen in the crude urea extract, as well as DNA and RNA, were removed by these purification steps. Complete tryptic

Fig. 4. Immunoblot of IgG-antibodies of rabbit-hyperimmune-sera against (a) whole-cell lysate of *T. phagedenis* (kindly provided by A. Otto, Freiburg, FRG), purified flagella of (b) *E. coli* and (c) *Ba. subtilis* (kindly provided by T. Schmidt, Freiburg, FRG), and whole-cell lysate of (d) *B. hermsii* (e) *B. parkeri* and (f) *B. turicatae*. Lanes: 1, K297 (anti-recombinant flagellin of *B. burgdorferi*); 2, K270 (anti-14 kDa fragment).

Table 2. Cross-reactivity of various polyclonal rabbit-hyperimmune-sera with intact flagella, recombinant 41 kDa flagellin and 14 kDa fragment as antigens in immunoblot and ELISA

The assays were carried out as described in Methods; the cut-off point being fixed at an OD₄₆₂ value of 0.2. The degree of cross-reactivity in the immunoblot assays is indicated: (–), no cross-reactivity; (+)–(+++), weak–strong cross-reactivity.

Polyclonal sera to:	Immunoblot <i>B. burgdorferi</i>		ELISA <i>B. burgdorferi</i>		
	Recombinant flagellin	14 kDa fragment	Flagella	Recombinant flagellin	14 kDa fragment
<i>B. burgdorferi</i> *	+++	+++	1:204800	1:204800	1:51200
<i>B. hermsii</i> *	+++	+	1: 51200	1: 51200	1:12800
<i>B. parkeri</i> *	+++	+	> 1:204800	1:204800	1:25600
<i>B. turicatae</i> *	+++	++	> 1:204800	> 1:204800	1:25600
<i>B. phagedenis</i> *	–	–	1: 1600	1: 1600	<1: 1600
<i>Ba. subtilis</i> †	+++	–	1: 51200	1: 51200	<1: 1600
<i>E. coli</i> †	+++	–	1: 51200	1: 51200	<1: 1600
<i>P. mirabilis</i> †	+	–	1: 1600	1: 1600	<1: 1600
<i>S. typhimurium</i> H1.2.†	++	–	1: 6400	1: 1600	<1: 1600
<i>S. typhimurium</i> Hi†	–	–	1: 1600	<1: 1600	<1: 1600

* Sera raised against whole-cell lysate.

† Sera raised against flagella.

	10	20	30	40	50
1	MIINHNTSAI	NASRNNGINA	ANLSKTQEKL	SSGYRINRAS	DDAAGMGVSG
51	KINAQIRGLS	QASRNTSKAI	NFIQTTEGNL	NEVEKVLVRM	KELAVQSGNG
101	TYSDADRGSI	QIEIEQLTDE	INRIADQAQY	NQMHLNSNKS	ASQNVRTAEE
151	LGMQPAKINT	PASLSGSQAS	WTLRVHVGAN	QDEAIAVNIY	AANVANLFSG
201	EGAQTAQAAP	VQEGVQQEGA	QQPAPATAPS	QGGVNSPVNV	TTTVDANTSL
251	AKIENAIRMI	SDQRANLGAF	QNRLESIKDS	TEYAIENLKA	SYAQIKDATM
301	TDEVVAATTN	SILTQSAMAM	IAQANQVPQY	VLSLLR	

Fig. 5. Amino acid sequence of the *B. burgdorferi* GeHo flagellin (Gassmann *et al.*, 1991). Bold letters, 14 kDa fragment (amino acids 175–252); underlined, Asp-N peptides of the 14 kDa fragment, identified by N-terminal sequencing; variable part, amino acids 131–266.

cleavage of purified recombinant flagellin produced two peptides with apparent molecular masses of 14 and 4 kDa in SDS-PAGE (Fig. 1).

Figs. 2 and 3 show the results of immunoblotting using recombinant *B. burgdorferi* flagellin and the tryptic digest as antigens. Strong cross-reactions with intact *B. burgdorferi* flagellin were observed using sera raised against the flagella of *B. subtilis* (serum K306), *E. coli* (serum K215), *S. typhimurium* H1.2. (serum K301) and sera raised against whole-cell lysates of *B. hermsii*, *B. parkeri* and *B. turicatae* (sera K567, K4, K1). With sera raised against the flagella of *P. mirabilis* (serum K110) and *S. typhimurium* Hi (serum K267) as well as with sera raised against whole-cell lysate of *T. phagedenis* (serum K779) only weak cross-reactivity could be seen. The results are summarized in Table 2. In contrast,

serum K297, specific for recombinant *B. burgdorferi* flagellin, contained antibodies that recognized the 33 and 37 kDa flagellins of *T. phagedenis* and the flagellins of *E. coli* and *B. subtilis* (Fig. 4). Using the tryptic digest as antigen, only antisera raised against *Borrelia* species recognized the 14 kDa fragment (Table 2); the 4 kDa peptide seen in Coomassie-stained SDS-polyacrylamide gels was not recognized. No cross-reaction with the flagellins of *T. phagedenis*, *B. subtilis* and *E. coli* was observed using serum K270, specific for the 14 kDa fragment. Antibodies in this serum reacted with the flagellins of *B. hermsii*, *B. parkeri* and *B. turicatae* (Fig. 4).

The results of ELISA using intact recombinant flagellin and purified 14 kDa fragment as antigens corroborate data obtained by immunoblotting (Table 2). The cross-reactions predominantly seen between recom-

Fig. 6. Purification of the 14 kDa fragment by gel filtration (Superdex 75 HR 10/30). Fractions were lyophilized and separated on a 16.5% SDS-polyacrylamide gel, stained with Coomassie G-250. Lanes: M, molecular mass markers; A-D, various concentrations of the tryptic digest; 7-17, fractions. Fractions 10 and 11 contain the 14 kDa fragment.

binant flagellin and sera raised against the flagella of *B. subtilis* (serum K306) and *E. coli* (serum K215) were eliminated when using the 14 kDa fragment as antigen. Using intact *B. burgdorferi* flagella (Flagellum ELISA-test kit, Dakopatts) as antigen, marked cross-reactions were observed with sera raised against the flagella of *B. subtilis*, *E. coli* (sera K306 and K215) and with sera raised against whole-cell lysates of *B. hermsii*, *B. parkeri* and *B. turicatae* (sera K567, K4, K1).

Further analysis of the 14 kDa fragment by N-terminal sequencing revealed that the peptide is part of the variable region (amino acids 131-266) of the flagellin (Fig. 5). The first amino acid is valine at position 175. Amino acid analysis of the purified 14 kDa fragment pointed to a possible end at amino acid position 252. To verify this, the peptide was digested with endoproteinase Asp-N. N-terminal sequence analysis of Asp-N peptides (purified by reversed phase chromatography) corroborated the result of amino acid analysis. By reversed phase chromatography of tryptic peptides of recombinant flagellin and subsequent N-terminal sequence analysis of some peptides, we were able to identify the hexapeptide that immediately follows the 14 kDa fragment (amino acids 253-258). The 14 kDa fragment (apparent molecular mass in a 16.5% SDS-polyacrylamide gel) consists of 78 amino acids (amino acids 175-252) which corresponds to a theoretical molecular mass of 8.58 kDa. The peptide is rather hydrophobic, particularly in the region of amino acids 182-200, according to Kyte & Doolittle (1982). Gel filtration revealed that the peptide eluted in a peak that is in accordance with a molecular mass of 28 kDa (Fig. 6, fractions 10 and 11).

Discussion

Preparation and characterization of the 14 kDa fragment

Tryptic digestion of purified *B. burgdorferi* 41 kDa flagellin, expressed in *E. coli*, resulted in a peptide with an apparent molecular mass of 14 kDa. Further characterization by N-terminal sequencing and amino acid analysis revealed that the peptide constitutes a rather hydrophobic part of the variable region of the flagellin. The theoretical molecular mass of the amino acid chain is 8.58 kDa in contrast to the apparent molecular mass of 14 kDa in a 16.5% SDS-polyacrylamide gel. Slow migration of hydrophobic proteins in SDS-PAGE is not unusual. The result may also depend on the gel system chosen. Schagger & von Jagow (1987) observed such differences when separating protein in SDS-polyacrylamide gels which differed in their degree of cross-linking. Purification of the 14 kDa fragment by gel filtration revealed that the peptide eluted from the column in a fraction that corresponds to a molecular mass of 28 kDa; the molecules may be aggregating due to their hydrophobicity. The 14 kDa fragment is soluble in aqueous buffers, in contrast to recombinant flagellin that is soluble only in high concentrations of urea (6-8 M) or in detergents.

Cross-reaction of recombinant flagellin role of conserved regions and variable parts

The *B. burgdorferi* flagellin that consists of 336 amino acids shows semi-conserved and conserved regions at the

N- and C-terminal ends, while the middle part of the protein represents a variable region. *B. burgdorferi*-specific epitopes recognized by rabbit-hyperimmune-sera are clustered within a region flanked by amino acids 180–260. Cross-reactive epitopes recognized by sera raised against the flagella of *B. subtilis* and *T. phagedenis* are located in conserved regions of the N- and C-termini. The amino acid sequence of the *B. burgdorferi* flagellin reveals remarkable similarities to that of *B. hermsii* (95%), *T. pallidum* B2 (60%), *S. typhimurium* (51%), *E. coli* (51%), *Serr. marcescens* (50%) and *Ba. subtilis* (49%) (Gassmann *et al.*, 1991).

Marked cross-reactions were observed in immunoblotting and ELISA between recombinant flagellin and antisera raised against the flagella of *B. subtilis* and *E. coli* as well as with antisera raised against whole-cell lysates of *B. hermsii*, *B. parkeri* and *B. turicatae*, the etiological agents of North American tick-borne relapsing fever. The latter are of minor importance in Europe (Barbour & Hayes, 1986). Only weak cross-reactions were seen using sera raised against the flagella of *P. mirabilis*, *S. typhimurium* H1.2. and sera raised against whole-cell lysates of *T. phagedenis*. This latter result is unexpected because overall sequence similarity of the *B. burgdorferi* flagellin and the *T. pallidum* B2 flagellin is high at 60%; furthermore, class B flagellar proteins of *T. pallidum* and *T. phagedenis* share 67–95% homology of their N-termini (Norris *et al.*, 1988). Possibly our sera, raised against whole-cell lysates of *T. phagedenis*, contained only less cross-reactive antibodies. The result may be different using sera raised against the flagella of *T. pallidum* or *T. phagedenis*. Conversely a serum specific for *B. burgdorferi* recombinant flagellin revealed a strong cross-reaction with the flagellins of *T. phagedenis* in immunoblotting (Fig. 4).

Among the hyperimmune-sera available, only antisera to the genus *Borrelia* were reactive using the 14 kDa fragment as an antigen in ELISA and immunoblotting. A serum raised against the 14 kDa fragment showed no cross-reaction with the flagellins of *T. phagedenis*, *Ba. subtilis* and *E. coli* in immunoblotting (Fig. 4). Epitopes in the semi-conserved and conserved regions are responsible for the observed cross-reactions. These regions are possibly involved in flagellin export and organelle assembly, whereas variable parts seem to be less important for the structure of the flagella and may therefore represent variable antigenic determinants (Fedorov & Efimov, 1990; Kuwajima, 1988; Namba *et al.*, 1989). Due to the conserved regions, cross-reactions in routine serology may be traced back to contact with a multitude of motile bacteria.

Hansen *et al.* (1988) using intact *B. burgdorferi* flagella as an antigen in ELISA could improve the specificity of *Borrelia* serology; while Coleman & Benach (1987) found

flagellin eluted from SDS-polyacrylamide gels not to be superior to the whole-cell sonicate as the antigen in ELISA. Wilske *et al.* (1986) and Zöller *et al.* (1991) frequently observed antibodies, in immunoblots, against the 41 kDa flagellin in sera of patients without clinical symptoms of borreliosis. These data are in line with the idea that, using flagellin monomers, epitopes in the conserved regions are accessible for antibodies while in the intact flagella most parts of the conserved regions seem to be masked and the epitopes of the central region are exposed. However, even in intact flagella some cross-reactive epitopes may be recognized by antibodies as our results using intact flagella as antigen in ELISA show (Table 2). The antisera raised against flagella of *Ba. subtilis* and *E. coli* revealed a marked reaction. The cross-reactions we observed with intact flagella and recombinant *B. burgdorferi* flagellin mentioned above could be avoided by offering the 14 kDa fragment as an antigen in immunoblotting and ELISA.

Recently, Collins *et al.* (1991) reported that antibodies in patient sera were exclusively directed against epitopes at the N-terminus by using truncated recombinant fusion proteins of the *B. burgdorferi* flagellin as antigens in immunoblotting; a fragment containing the variable part was not recognized. Gassmann (1991) also obtained disappointing results using a Trp-E fusion protein representing the entire variable part of the *B. burgdorferi* flagellin. Only 35% of the patient sera tested showed a positive reaction in immunoblotting. These findings cannot be explained by the lack of antibodies specific for epitopes of the variable part of the flagellin, as our data with the 14 kDa fragment convincingly show with rabbit sera. Sera of rabbits immunized with antigen containing *Borrelia* flagellin, as well as sera of patients with Lyme borreliosis, recognize epitopes in the central region of the flagellin. When technical problems can be excluded (for example too little antigen offered) changes in conformation or post-translational modifications of the fusion proteins expressed in *E. coli* have to be considered. The conformation of a protein plays an important role in the interaction of antibodies and epitopes, as shown by the reaction of patient sera in a PIN-ELISA, using synthetic linear octapeptides as antigens. Only two of twelve Lyme borreliosis patients had antibodies that recognized linear epitopes (Gassmann, 1991).

The tryptic 14 kDa fragment seems to be a suitable antigen for determining specific antibodies in Lyme borreliosis, especially in the early course of the disease. It is not burdened by false positive results and it represents a part of the *B. burgdorferi* flagellin which carries a multitude of *Borrelia*-specific epitopes (Gassmann *et al.*, 1991). It is soluble in water, easy to handle and may be a convenient antigen for the detection of class-specific antibodies.

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References

- BARBOUR, A. G. & HAYES, S. F. (1986). Biology of *Borrelia* species. *Microbiological Reviews* **50**, 381–400.
- BARBOUR, A. G., BURGDORFER, W., HAYES, S. F., PETER, O. & AESCHLIMANN, A. (1983). Isolation of a cultivable spirochete from *Ixodes ricinus* ticks in Switzerland. *Current Microbiology* **8**, 123–126.
- COLEMAN, J. L. & BENACH, J. L. (1987). Isolation of antigenic components from the Lyme disease spirochete: their role in early diagnosis. *Journal of Infectious Diseases* **155**, 756–765.
- COLLINS, C. & PELTZ, G. (1991). Immunoreactive epitopes on an expressed recombinant flagellar protein of *Borrelia burgdorferi*. *Infection and Immunity* **59**, 514–520.
- FEDOROV, O. V. & EFIMOV, A. V. (1990). Flagellin as an object for supramolecular engineering. *Protein Engineering* **3**, 411–413.
- GASSMANN, G. S. (1991). *Analyse des Flagellins von Borrelia burgdorferi und antigenetische Charakterisierung des Genprodukts*. PhD thesis, Universität Tübingen, Tübingen, FRG.
- GASSMANN, G. S., JACOBS, E., DEUTZMANN, R. & GÖBEL, U. E. (1991). Analysis of the *fla* gene of *Borrelia burgdorferi* GeHo and antigenic characterization of its gene product. *Journal of Bacteriology* **173**, 1452–1459.
- HANSEN, K., HINDERSSON, P. & PEDERSON, N. S. (1988). Measurement of antibodies to the *Borrelia burgdorferi* flagellum improves serodiagnosis in Lyme disease. *Journal of Clinical Microbiology* **26**, 338–346.
- HANSEN, K., PII, K. & LEBECH, A. (1991). Improved immunoglobulin M serodiagnosis in Lyme borreliosis by using a μ -capture enzyme-linked immunosorbent assay with biotinylated *Borrelia burgdorferi* flagella. *Journal of Clinical Microbiology* **29**, 166–173.
- HILL, R. L. (1965). Hydrolysis of proteins. *Advances in Protein Chemistry* **20**, 37–107.
- KYTE, J. & DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.
- KUWAJIMA, G. (1988). Construction of a minimum-size functional flagellin of *Escherichia coli*. *Journal of Bacteriology* **170**, 3305–3309.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- NAMBA, K., YAMASHITA, J. & VONDERVISZT, F. (1989). Structure of the core and central channel of bacterial flagella. *Nature, London* **342**, 648–654.
- NORRIS, S. J., CHARON, N. W., COOK, R. C., FUENTES, M. D. & LIMBERGER, R. J. (1988). Antigenic relatedness and N-terminal sequence homology define two classes of periplasmic flagellar proteins of *Treponema pallidum* subsp. *pallidum* and *Treponema phagedenis*. *Journal of Bacteriology* **170**, 4072–4082.
- SCHÄGGER, H. & VON JAGOW, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166**, 368–379.
- STEERE, A. C., GRODZICKI, R. L., KORNBLATT, A. N., CRAFT, J. E., BARBOUR, A. G., BURGDORFER, W., SCHMID, G. P., JOHNSON, E. & MALAVISTA, S. E. (1983). The spirochetal etiology of Lyme disease. *New England Journal of Medicine* **308**, 733–740.
- STEERE, A. C. (1989). Lyme disease. *New England Journal of Medicine* **321**, 586–596.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- WILKE, B., PREAC-MURSIC, V., SCHIERZ, G. & VON BUSCH, K. (1986). Immunochemical and immunological analysis of european *Borrelia burgdorferi* strains. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene A* **263**, 92–102.
- ZÖLLER, L., BURKHARD, S. & SCHÄFER, H. (1991). Validity of western immunoblot band patterns in the serodiagnosis of Lyme borreliosis. *Journal of Clinical Microbiology* **29**, 174–182.