IMMUNOLOGICAL RESPONSE TO INFECTION

Borreliacidal activity of early Lyme disease sera against complement-resistant *Borrelia afzelii* FEM1 wild-type and an OspC-lacking FEM1 variant

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Sera obtained from 14 Lyme borreliosis patients at early stages (stages I and II) of the disease were examined for their borreliacidal properties against *Borrelia afzelii* isolate FEM1 by use of a growth inhibition assay. Five of 14 immune sera exhibited borreliacidal activity against isolate FEM1. Heat-inactivated immune sera failed to kill the spirochaetes. Immunoblotting experiments with outer-membrane preparations showed that OspC and 11 additional proteins of 14.0, 16.0, 17.7, 19.3, 21.7, 27.5, 32.7, 40.7, 48.9, 51.3 and 53.6 kDa were recognised by borreliacidal immune sera. To analyse the borreliacidal properties of anti-OspC antibodies, two sera (EM4 and EM5), which beside antibodies against a 51.3-kDa protein contained exclusively anti-OspC antibodies, were further investigated by comparative analysis with a FEM1 wild-type and a FEM1 variant lacking OspC in a growth inhibition assay. Only FEM1 wild-type and not variant FEM1OspC(–) was killed by immune sera EM4 and EM5. Complement-dependent killing of FEM1 wild-type was mediated by formation of the terminal complement complex that was found to be attached directly to the outer membrane as confirmed by immuno-electron microscopy. No complement deposition was observed on the surface of variant FEM1OspC(–) after incubation with immune sera EM4 and EM5, thereby suggesting that only anti-OspC antibodies in these two immune sera were responsible for borreliacidal activity. These results provide direct evidence that anti-OspC antibodies, once developed during the immune response, are of critical importance for efficient killing of borreliae in the early phase of infection.

Introduction

Borrelia burgdorferi is regarded as the most common tick-borne pathogen of the northern hemisphere. Following a tick bite, besides an erythema migrans (EM), the haematogenous dissemination of *B. burgdorferi* to other organs such as the joints, heart and central nervous system may lead to the human multisystemic disorder currently known as Lyme disease (LD) [1].

During the natural course of infection the humoral

immune response results in the production of antibodies that, in most cases, eliminate the pathogen and thereafter can temporally protect against re-infection. Protection can also be provided either by active immunisation of animals with whole spirochaetes [2, 3] or by vaccination with recombinant outer-surface proteins, especially OspA [4, 5], OspB [2], OspC [6, 7] and DbpA [8]. Recent investigations revealed that sera from patients at various stages of LD often display borreliacidal activity and these antibodies may play an important role in the host defence mechanism against borreliae [9–12]. There is evidence that antibodies against OspC are of critical importance for this borreliacidal activity [13]. However, antigens other than OspC may also be relevant for the induction of borreliacidal activity [9]. The aim of the present study was to characterise more precisely those antibodies that

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are relevant for the borreliacidal activity of early LD sera. This problem was addressed by the use of purified outer-membrane preparations of a *B. afzelii* isolate to characterise the antigen specificity of early borreliacidal LD sera. Subsequently, a *B. afzelii* wild-type isolate and a variant of this isolate lacking OspC, were tested against these pre-defined sera.

Materials and methods

B. afzelii isolates and culture conditions

B. afzelii isolate FEM1 (<15 passages, uncloned) was originally isolated from a skin biopsy of a patient with erythema migrans and proved to be pathogenic in C3H mice after cultivation *in vitro*. A variant of isolate FEM1 which did not express OspC was selected by long-term cultivation at 23°C. The culture was then shifted to 33°C and grown for a further two passages at 33°C before use.

Spirochaetes were grown in 10 ml of modified Barbour-Stoenner-Kelly (BSK) medium [14] at 33°C for 5–6 days up to a cell density of *c.* 10^7 /ml, dispensed into 1.8-ml screw-capped tubes, and stored at –70°C until used.

Non-immune human serum (NHS)

Twenty sera from healthy blood donors with no previous history of a spirochaetal infection were tested for the presence of antibodies against *B. burgdorferi* by an ELISA (Dade Behring, Marburg, Germany) and a recombinant immunoblot (Mikrogen, Martinsried, Germany). Only sera that gave negative results in all assays were used to form a NHS serum pool. Processing of all blood samples included clotting for 30 min at room temperature, centrifugation at 3000 *g* for 10 min and storage of serum in divided volumes at –20°C. Repeated freeze-thaw cycles were avoided to prevent loss of complement activity.

Immune sera

For the present study, 14 immune sera from seropositive patients suffering from early LD (stages I and II) as diagnosed by experienced clinicians were examined. Individual sera were obtained from six untreated patients presenting with EM and from eight neuroborreliosis (NB) patients with intrathecal synthesis of *B. burgdorferi*-specific antibodies.

Total complement activity (CH_{50}) of tested sera

All sera (NHS and immune sera) contained normal complement activity as determined by a haemolytic microtitration assay [9].

In-vitro borreliacidal growth inhibition assay

The growth inhibition assay was performed as described previously [9]. Briefly, a frozen 1.8-ml sample of spirochaete suspension was thawed and inoculated into 10 ml of fresh BSK medium. The cultures were then incubated at 33°C for 5–6 days up to a cell density of *c.* 10^7 /ml. After incubation, the number of spirochaetes was determined by dark-field microscopy with a Kova counting chamber (Hycor Biomedical, Irvine, CA, USA). BSK medium (50 μ l) containing phenol red 240 μ g/ml, BSK medium (50 μ l) containing 1.25×10^7 cells and immune serum 100 μ l were added to sterile, uncoated 96-well microtitration plates (Costar, Cambridge, MA, USA). Control wells were charged with 100 μ l of either heat-inactivated immune sera, NHS or heat-inactivated NHS (hi-NHS). Sera were heat inactivated by incubation at 56°C for 30 min. A growth control with BSK medium instead of serum was included in all assays. After gentle agitation, the plates were incubated for 3 h at 37°C and then for a cultivation period of 10 days at 33°C. Borreliacidal activity of immune sera was determined with an ELISA reader (PowerWave 200, Bio-Tek Instruments, Winooski, VT, USA) by measuring the decrease of indicator colour shift of the BSK medium at 562/630 nm. Growth inhibition of the spirochaetes and the presence of borreliacidal antibodies were indicated by no or a minor decrease of absorbance values in comparison with the absorbance values of the control (NHS) after incubation for 10 days.

Detection of deposited complement components on the surfaces of borreliae by indirect immunofluorescence

Deposition of complement components C3, C6 and C9 (neoantigen) on borrelial cells after incubation with immune sera was investigated by an indirect immunofluorescence assay (IFA) as described previously [15]. Borrelial cells (1.25×10^7) were incubated in veronal-buffered saline (VBS; supplemented with 1 mM Mg^{2+} , 0.15 mM Ca^{2+} , gelatin 0.1%, pH 7.3–7.4) 200 μ l containing immune serum 50% at 37°C with gentle agitation. After 30 min, half of the suspension was transferred to a new tube and the remaining 100 μ l of suspension were incubated for another 150 min at 37°C. Immediately after incubation, 10- μ l samples of both suspensions were mounted on to adhesion slides to assess the extent of blebbing and the motility of the spirochaetes by dark-field microscopy. The remaining spirochaetes were washed twice with cold Mg^{2+} - Ca^{2+} -free phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) 1% and transferred to adhesion slides, air-dried and fixed with methanol 100%. Deposition of complement components C3, C6 and C9 was identified by incubation with a goat anti-human C3 antibody (Sigma-Aldrich, Deisenhofen, Germany), a biotin-conjugated goat anti-human C6 antibody [16] or a biotin-conjugated mouse anti-human C9 (neoanti-

gen) antibody (WU13-15) as described previously [17]. For determination of fluorescent borreliae, 10 visual fields were counted (c. 300–500 cells) by immunofluorescence microscopy (Olympus CX40) at a magnification of $\times 1000$.

Isolation of borrelial outer-membrane fractions

The outer membrane of FEM1 wild-type (passage 7) was isolated as described by Radolf *et al.* [18] with minor modifications. Cultures (500 ml) were grown at 33°C in modified BSK medium to the late-log phase (determined by enumeration of spirochaetes by dark-field microscopy) and the spirochaetes were then harvested by centrifugation at 13 000 *g* for 30 min in a Beckman 19 rotor. The resulting pellets were washed gently once with OM buffer (OMB) consisting of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 150 mM NaCl and 1 mM MgCl₂ (pH 7.4), and centrifuged a second time at 13 000 *g* for 30 min in a Beckman 50 Ti rotor. After resuspension in a final volume of 12 ml of ice-cold OMB containing sucrose 20% w/v and 0.2 mM phenylmethylsulphonyl fluoride, the suspensions were incubated on a rocking platform for 2 h at 4°C with gentle agitation. Samples (3 ml) were layered on to 33-ml linear sucrose gradients containing sucrose 20–60% in OMB and centrifuged for 26 h at 112 000 *g* with a Beckman SW 28 rotor. After centrifugation, 1-ml fractions were removed from the top of the tubes, and those fractions containing outer-membrane material (upper band) and inner-membrane material (lower band) were pooled. After dilution in OMB (1:2), membranes were collected by centrifugation in a Beckman 50 Ti rotor (190 000 *g* for 3 h), washed in ice-cold OMB and harvested a second time. The final membrane pellet was suspended in 100 μ l of OMB containing 0.2 M phenylmethylsulphonyl fluoride and stored at -80°C .

Gel electrophoresis and immunoblotting of isolated outer-membrane fractions

Proteins were separated by Tricine-SDS-PAGE through 4% stacking and 10% separating gels as described previously [9]. Mol. wt standards were purchased from Sigma-Aldrich. For immunoblot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), which were then blocked with non-fat dried milk 5% – 50 mM Tris-HCl (pH 7.4)–200 mM NaCl-Tween 20 0.1% at 4°C for 12 h. The nitrocellulose strips were incubated at room temperature for 4 h with immune sera diluted 1 in 200 for IgG immunoblots or with monoclonal antibodies (MAbs) in dilution buffer (Mikrogen). After four washes with 50 mM Tris-HCl (pH 7.5) – 150 mM NaCl-Tween 20 0.2% (TBST), strips were incubated for 90 min with a 1 in 1000 or 1 in 500 dilution of peroxidase-conjugated rabbit anti-human IgG or peroxidase-conjugated rabbit anti-mouse IgG antibodies (Dako, Glostrup, Denmark). The strips were washed four times with TBST and then

developed with diaminobenzidine and H₂O₂. Molecular masses of all reactive proteins on each strip were determined by an HP Scanjet 6100C scanner and a Wincam gel styler software version 2.2, Cybertech, Berlin, Germany.

Detection of deposited C9 on the surface of borreliae by immuno-electron microscopy

Cultures of FEM1 wild-type and FEM1OspC(–) variant growing at mid-log phase were pelleted by centrifugation at 5000 *g* for 30 min and resuspended in VBS 200 μ l. Borreliae (1×10^8 cells) were then incubated in 100 μ l of VBS containing NHS 50% or immune serum 50% for 30 min at 37°C with gentle agitation. Following a second wash with PBS, cells were resuspended in 600 μ l of VBS. Cells were fixed by adding an equal volume of glutaraldehyde 4% in 0.2 M sodium cacodylate buffer (pH 7.4) followed by incubation at room temperature for 1 h. Fixed pellets were washed three times with PBS and incubated for 1 h at 37°C with a monoclonal anti-human C9 antibody (WU13-15) or with PBS containing BSA 1%. After three washes with PBS, cells were incubated for 1 h at 37°C with a goat anti-mouse IgG-10-nm diameter gold conjugate (AuroProbe, Amersham Pharmacia Biotech, Freiburg, Germany) and diluted 1 in 20 in PBS/BSA 1%. After three further 10-min washes with PBS, cell pellets were fixed with glutaraldehyde 2.5% in 0.1 M cacodylate buffer (pH 7.4).

For whole-mount electron microscopy, pellets of gold-labelled cells were suspended in distilled water and adsorbed to carbon films according to the procedure described by Valentine *et al.* [19]. Cells were then observed as whole mounts by transmission electron microscopy. For thin-section preparation, gold-labelled glutaraldehyde-fixed cells (see above) were prepared and additionally fixed in OsO₄, as described by Traub *et al.* [20]. The samples were embedded in Spurr's resin [21] and thin sections were contrasted with aqueous uranyl acetate 2% and lead citrate [22]. All specimens were examined with a model CEM 902 A microscope (Zeiss, Oberkochen, Germany).

Reverse transcription PCR (RT-PCR) for the detection of flagellin, OspA and OspC mRNA

Cells growing at mid-log phase were harvested by centrifugation and the total RNA was isolated with the RNeasy[®] Total RNA system obtained from Qiagen (Hilden, Germany). First-strand cDNA was transcribed from total RNA (100 ng/reaction, 50 μ l total reaction volume) with random nonamers with the Enhanced Avian RT-PCR Kit according to the manufacturer's instructions (Sigma-Aldrich). Reaction mixture (1 μ l) was amplified by PCR with oligonucleotide primers for *flagellin*, *ospA* and *ospC* at a final concentration of 100 nM each in the presence of 200 μ M dNTPs. PCR was performed for 30 cycles under the following

conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 70°C for 1 min for the *flagellin* PCR; denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 70°C for 1 min for the *ospA* PCR; denaturation at 94°C for 1 min, annealing at 45°C for 1 min and extension at 70°C for 1 min for the *ospC* PCR. 5' and -3' primers for PCR were as follows: *flagellin* (352-bp fragment), 5'-AACACACCAGCATCGCTTTCAGGGTCT-3' and 5'-TATAGATTCAAGTCTATTTTGGAAAGCACCTA-3'; *ospA* (311-bp fragment), 5'-GGGAATAGGTCTAATATAGCC-3' and 5'-CTAGTGTTTTGCCATCTTCTTTGA-3'; *ospC* (680-bp fragment), 5'-TGGACTATTAATTCATAAAAAGGAGG-3' and 5'-TCTTATTAAGTTTTTTTGGACTTTCTGC-3'.

MAbs against borreliacidal proteins

A panel of MAbs was used for the identification of borreliacidal antigens: L100 1G22 against p83/100, L41 1C11 against p41, L32 1F11 against OspA, L30 1B10 against p30 and L22 1F8 against OspC [23]. An additional MAb LA3 against HSP70 [24] was kindly provided by M.D. Kramer.

Results

Borreliacidal effect of early Lyme disease (LD) sera against isolate FEM1 as determined by growth inhibition assay and detection of deposited complement components

In this study, sera obtained from 14 LD patients at early stages (stages I and II) of the disease were examined for their borreliacidal activity against *B. afzelii* isolate FEM1 by use of a growth inhibition assay and by immunofluorescence-based detection of deposited complement components on the cell surface. The growth inhibitory effect against isolate FEM1 was assessed in parallel for all the selected immune sera by the determination of borreliacidal growth from the indicator colour shift in the assay over an incubation period of 10 days. Growth of isolate FEM1 was inhibited by five sera (EM4, EM5, NB3, NB4 and NB6), whereas nine sera did not inhibit growth (Table 1). To confirm whether or not growth inhibition was complement-mediated, the experiments were repeated with heat inactivation of all test sera before running the assay under identical conditions. After heat inactivation, none of the sera displayed a borreliacidal effect in the growth inhibition assay, indicating that growth inhibition was induced solely by an antibody-dependent but complement-mediated mechanism leading to bacteriolysis.

The study investigated whether or not the deposition of complement components C3, C6 and C9 on the surface of isolate FEM1 after incubation with all selected immune sera was in accordance with the results obtained in the growth inhibition assay. To determine

Table 1. Correlation of growth inhibitory activity of immune sera with percentage of deposited complement components C3, C6 and C9

Immune serum	Growth inhibitory activity*	Cells positive for deposited complement component (%)		
		C3	C6	C9
EM 1	—	<20	<20	<20
EM 2	—	<20	<20	<20
EM 3	—	<20	<20	<20
EM 4	+	88	79	98
EM 5	+	80	77	ND
EM 6	—	<20	<20	<20
NB 1	—	<20	<20	<20
NB 2	—	<20	<20	<20
NB 3	+	87	90	73
NB 4	+	46	45	28
NB 5	—	<20	<20	<20
NB 6	+	39	31	24
NB 7	—	<20	<20	<20
NB 8	—	<20	<20	<20

*Growth inhibitory activity of immune sera was determined by the growth inhibition assay after cultivation for 10 days: —, no borreliacidal activity; +, borreliacidal activity; percentage of fluorescent cells was the mean determined by counting 10 visual fields in duplicate experiments; ND, not determined.

the percentage of non-specific complement binding, isolate FEM1 was incubated with NHS. The baseline percentages of complement-positive cells were as follows: 13(SD6)% for C3, 20(SD6)% for C6 and 15(SD3)% for C9, respectively. No increase of fluorescent cells over the baseline could be demonstrated after incubation of borreliacidal with those immune sera that lacked growth inhibitory activity (Table 1). On the other hand, an increase of fluorescent borreliacidal was obvious with all inhibiting immune sera. Strong labelling (close to 100%) was observed with immune sera EM4, EM5 and NB3, whereas two other immune sera (NB4 and NB6) produced a lower percentage of fluorescent cells. In the latter two sera, the correct percentage of complement-positive cells was difficult to assess because of the large number of fragmented cells, indicating an overwhelming borreliacidal effect by these sera on viable spirochaetes. In summary, these findings correlated well with the growth characteristics of borreliacidal after exposure to these sera in the growth inhibition assay.

Immunoreactivity of borreliacidal LD sera with B. afzelii outer-membrane proteins

To identify relevant outer-membrane proteins (OMPs) which may serve as predominant targets for borreliacidal antibodies present in those LD sera that inhibited growth, outer-membrane preparations of isolate FEM1 were processed as an antigen source for immunoblotting. Initially, the purity of the membrane fractions derived from a whole cell lysate, i.e., protoplasmatic cylinder and inner-membrane fraction, as well as the outer-membrane fraction were analysed by SDS-PAGE in combination with specific MAbs to recognise

possible cross-contamination of proteins. Flagellin and the cytosolic protein HSP70 were regarded as representative markers for non-surface-exposed proteins; OspA and OspC were used as markers for the outer-membrane constituents. As shown in Fig. 1, the outer-membrane fraction showed little or no cross-contamination with the 41-kDa flagellar protein or with the internal marker protein HSP70. On the contrary, OspA (not shown) and OspC were detected in both the inner- and the outer-membrane fractions.

By use of immunoblots derived from outer-membrane preparations, a total of 11 immunoreactive proteins of 14.0, 16.0, 17.7, 19.3, 21.7, 27.5, 32.7, 40.7, 48.9, 51.3 and 53.6 kDa, in addition to OspC, were detected after incubation with the borreliacidal immune sera (Fig. 2). Strong immunoreactivity against OspC was observed in three of five immune sera (EM4, EM5, NB6), whereas immune serum NB3 showed only weak immunoreactivity against OspC. In contrast to these findings, anti-OspC antibodies were found in only one of nine sera lacking borreliacidal activity as demonstrated by immunoblotting with whole-cell lysates (data not shown). A broad pattern of bands (14.0, 16.0, 17.7, 19.3, 21.7, 27.5, 48.9, 51.3, 53.6 kDa) including OspC was observed in immunoblots with immune serum NB6. More than three immunoreactive bands were found by applying immune serum NB3, whereas immune sera EM4, EM5 and NB4 reacted with only two proteins. Immune serum NB4 recognised proteins of 32.7 and 40.7 kDa; immune sera EM4 and EM5 showed strong immunoreactivity against OspC and weak immunoreactivity against a protein of 51.3 kDa.

Because of the very restricted antibody response, immune sera EM4 and EM5 were selected to analyse whether or not anti-OspC antibodies predominantly affect the growth of borreliae. It was possible to address this problem directly inasmuch as both a wild-type OspC-expressing FEM1 and a FEM1 variant

Fig. 1. Western blot analysis of protein profiles of outer- and inner-membrane (OM, IM) fraction and whole-cell (WC) lysate from low passage *B. afzelii* FEM1 wild-type. Proteins (15 µg/lane) were subjected to Tricine-SDS-PAGE (acrylamide 10%) and blots of the proteins were reacted exposed to MAbs (α) against HSP70 (LA3), flagellin (L41 1C11) or OspC (L22 1F8).

Fig. 2. Immunoblot reactivity of IgG antibodies to OMPs of *B. afzelii* FEM1 in immune sera displaying borreliacidal activity. Outer-membrane fractions (15 µg) were subjected to 10% Tricine-SDS-PAGE, transferred to nitrocellulose and treated with immune sera. Lane 1, immune serum EM4; 2, EM5; 3, NB3; 4, NB4; 5, NB6. The calculated molecular masses of reactive bands are listed on the right and the antigenically reactive OspC is denoted by an arrow.

lacking surface-exposed OspC were available for additional studies.

Growth characteristics of FEM1 variants differing with regard to OspC expression

To analyse further those immune sera containing borreliacidal antibodies directed against OspC, a variant of the initially OspC-expressing isolate FEM1 was generated, which did not express appreciable amounts of that outer-surface protein (see *Materials*

and methods). This was achieved by incubation of spirochaetes at 23°C in modified BSK medium over several passages. As indicated by SDS-PAGE, spirochaetes produced high levels of OspC at 33°C but not at 23°C (Fig. 3a). In contrast to OspC expression, which clearly proved to be temperature-dependent, neither the amount of OspA nor flagellin expression of isolate FEM1 were significantly affected when spirochaetes were cultivated at either 23°C or 33°C (Fig. 3a). To confirm these SDS-PAGE observations further, RT-PCR was performed with primers directed against targets of the OspC, OspA and the flagellin gene. While no significant differences could be observed concerning the amount of OspA and flagellin expression at 23°C compared to that at 33°C, once again OspC was clearly down-regulated at 23°C as calculated by quantitative densitometric analysis of the amplified DNA products (Fig. 3b). Furthermore, the OspC-lacking variant of FEM1 (FEM1OspC(-)) did not re-express OspC after either continuous cultivation at 23°C or after the application of temperature shifts to 33°C and 37°C.

To assess the surface exposure of OspC on borrelial cells, immunofluorescence microscopy was performed with a MAb directed against OspC. The OspC-expressing FEM1 wild-type appeared brightly stained after incubation with anti-OspC antibodies, whereas the FEM1OspC(-) variant obviously lacked OspC (Fig. 4). Two non-surface-exposed antigens, the periplasmatic flagellin and the cytosolic protein HSP70, were analysed by applying identical test conditions for both variants. A weak fluorescence as a result of methanol fixation was seen after incubation with anti-flagellin, whereas no staining of spirochaetes could be observed after incubation with anti-HSP70 antibodies.

In summary, the results of these experiments demonstrate that only the FEM1 wild-type and not the FEM1OspC(-) variant expressed considerable amounts of OspC on the cell surface. Therefore, both variants proved useful for further analysis of the specific killing capacity of anti-OspC antibodies found in the pre-defined EM sera.

Characterisation of early LD sera with regard to their borreliacidal activity against FEM1 variants

The borreliacidal effect of immune sera containing anti-OspC antibodies against both the FEM1 wild-type and the FEM1OspC(-) variant was also investigated by monitoring borrelial growth in the growth inhibition assay over a cultivation period of 10 days at 33°C. Changes in OspC expression of both variants which may have occurred during the study were controlled by SDS-PAGE analysis, but no changes of OspC expression could be detected when processed borreliae were compared to the initially inoculated FEM1 variants during the assay or after the conclusion of the test.

Borreliacidal activity of the immune sera EM4 and EM5 selected for antibody analysis was observed only against the FEM1 wild-type, whereas growth of the FEM1OspC(-) variant was not affected by either immune serum throughout the entire incubation period (Fig. 5). Growth inhibition of immune serum EM4 on isolate FEM1 was clearly stronger than that exhibited by serum EM5. To underscore the importance of anti-OspC antibodies for inhibition of borrelial growth, no growth inhibition was seen after FEM1 variants were exposed to heat-inactivated immune sera and incubated with NHS.

Localisation of deposited C9 on borrelial surface and correlation with the borreliacidal activity of early LD sera

To localise the lytic complement complex, C9 deposition on the surface of the FEM1 wild-type and FEM1OspC(-) variant was investigated by immunoelectron microscopy after incubation with immune serum EM4. The immunoreactivity of borreliae was analysed after exposure to immune serum EM4 or to NHS with a MAb directed against C9 (neo-epitope) and a gold-labelled secondary antibody. By means of the whole-mount technique, high labelling of gold particles was found predominantly on amorphous slime material and on the outer surface of the FEM1 wild-type when cells were incubated with immune serum EM4 (Fig. 6A). In contrast, only weak labelling of the FEM1OspC(-) variant was observed under identical test conditions as demonstrated in Fig. 6B. In an additional attempt, thin-section immuno-electron microscopy was performed to examine the localisation of C9 deposited on the outer membrane of both variants. As shown in Fig. 6C, C9 was attached directly to the outer membrane of FEM1 wild-type, but no gold particles were observed on the outer membrane of the FEM1OspC(-) variant (Fig. 6D). Furthermore, FEM1 wild-type cells were mostly aggregated and formed cell clumps with dense immunogold-labelling distributed over the entire cell surface. In experiments with NHS, both FEM1 wild-type and FEM1OspC(-) variant displayed only a few randomly distributed gold particles on the cell surface, thus indicating that these variants were not affected by serum complement alone (data not shown).

Discussion

The results obtained in the present study illustrate that the immune response in patients suffering from early LD already contains borreliacidal antibodies. Moreover, the growth inhibition experiments showed that the borreliacidal activity of immune sera clearly requires complement insofar as sera no longer exhibited borreliacidal activity after heat inactivation. Immunofluorescence tests on immune sera revealed that only cells exposed to the five borreliacidal immune sera

Fig. 3. SDS-PAGE and RT-PCR from FEM1 wild-type and FEM1OspC(-) variant. **(a)** Whole-cell lysate (15 μ g/lane) was loaded on a 10% Tricine-SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, variant FEM1OspC(-) shifted from 33°C to 23°C; 2, low passage FEM1 grown at 33°C. Molecular mass markers (in kDa) are shown on the left and the position of flagellin, OspA and OspC are indicated on the right. **(b)** RT-PCR analysis of *flagellin*, *ospA* and *ospC* expression by FEM1 and FEM1OspC(-) spirochaetes cultivated at different temperatures. mRNA isolated from cultured spirochaetes was used to produce first-strand cDNA by use of random nonamers as described in *Materials and methods*. The cDNAs were amplified with specific sets of primers for *flagellin*, *ospA* and *ospC*. One μ l from each amplification reaction was subjected to agarose gel electrophoresis and stained with ethidium bromide. Mol. wt markers are shown on the left.

Fig. 4. Phenotype characterisation of FEM1 wild-type and FEM1OspC(–) variant with different MABs. After methanol fixation, slides were incubated initially with 1 in 10 dilutions of MABs (α) against OspC (L22 1F8), flagellin, (L41 1C11) and HSP70 (LA3) and then with a 1 in 1000 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin. Magnification $\times 100$.

were positive for complement deposition. Accordingly, exposure to borreliacidal immune sera EM4, EM5 and NB3 led to a dramatic increase of fluorescence in up to 98% of cells within 30 min (Table 1); in contrast, fluorescence did not exceed 20% of the cells when immune sera without borreliacidal activity were tested.

Controversy remains concerning the role of the complement system in the host defence directed against *B. burgdorferi*. Existing evidence suggests that killing of *B. burgdorferi* *in vitro* after binding of specific antibodies to the cell surface is brought about by at least two mechanisms. The first mechanism is comple-

ment-mediated but antibody-dependent so as to facilitate the interaction of the borrelial membrane with the terminal complement complex (TCC). Nevertheless, some authors propose a second, hitherto unknown mechanism that causes complement-independent killing of the spirochaetes in the presence of MABs directed against OspA [11], OspB [25, 26], BmpA [27] and DbpA [8]. However, by application of patients' sera or sera from laboratory animals infected with the pathogen, we [9] and others [10, 28–31] could demonstrate that the efficient killing of *B. burgdorferi* *in vitro* is caused by the first mechanism. This correlates with the findings of the current investigation, which underscore

Fig. 5. Growth kinetics of FEM1 wild-type and FEM1OspC(-) variant incubated with EM LD sera. Spirochaetes were incubated in 50% native immune sera EM4 and EM5 over a cultivation period of 10 days at 33°C. Colour changes were monitored by measurements of the absorbance at 562/630 nm. OspC-expressing isolate FEM1 wild-type incubated with IS EM4 (●), variant FEM1OspC(-) incubated with IS EM4 (○), OspC-expressing isolate FEM1 wild-type incubated with IS EM5 (■), variant FEM1OspC(-) incubated with IS EM5 (□).

the prominent role of the complement system in killing the spirochaetes.

The detection of borreliacidal antibodies in immune sera from patients suffering from LD clearly depends on the isolate applied in the test [9, 32]. The serum-resistant *B. afzelii* isolate FEM1 wild-type proved to be a suitable monitor for the detection of borreliacidal antibodies [9]. When this isolate was employed, borreliacidal antibodies were detected in five (36%) of 14 immune sera investigated from patients with early LD. In comparison to these observations, when *B. burgdorferi* isolate 297 and *B. afzelii* isolate EB1 were used, borreliacidal antibodies were detected in only 8% and 17%, respectively, of early LD sera [9]. Similar results were obtained by other investigators who found borreliacidal anti-*B. burgdorferi* 297 antibodies in only 12.5–15% of early LD sera [32, 33].

The current study attempted to identify surface-exposed proteins that may serve as predominant targets for borreliacidal antibodies found in LD sera with borreliacidal activity. Therefore, outer membranes of FEM1 wild-type were purified and used for immunoblotting experiments. The results demonstrated that borreliacidal immune sera EM4, EM5, NB3, NB4 and NB6 predominantly reacted with OspC, whereas two of five immune sera (NB3 and NB6) recognised more than three proteins, which made it difficult to specify the targets of borreliacidal antibodies in these sera. Because of the very restricted antibody response directed against only two proteins, immune sera EM4, EM5 and NB4 were chosen for the identification of borreliacidal epitopes. Whereas immune serum NB4 recognised two OMPs of 32.7 and 40.7 kDa, immune sera EM4 and EM5 showed a strong reactivity against OspC and an additional weak reactivity directed against

Fig. 6. Detection of bound C9 on cells of FEM1 wild-type and FEM1OspC(–) variant by immuno-electron microscopy. After incubation with either 50% NHS or immune sera EM4 for 30 min at 37°C, cells were treated with MAb WU13-15 raised against the neo-epitope of C9 and a gold-conjugated goat anti-mouse immunoglobulin as described in *Materials and methods*. Whole-mount immuno-electron microscopy of (A) serum-treated FEM1 wild-type and (B) serum-treated FEM1OspC(–) variant. Thin-section micrographs of FEM1 wild-type (C) and FEM1OspC(–) variant (D) after incubation with immune sera EM4. The gold particles (dark dots) indicate the distribution of C9 on the surface of borreliac cells. These figures are representative of the results obtained from *c.* 25 fields examined. S, slime material; Sph, spheroplast. Bar markers = 0.2 μ m in all micrographs.

an 51.3-kDa protein. Interestingly, anti-OspC antibodies were found in four of five borreliacidal immune sera, but in only one of nine sera lacking borreliacidal activity. These observations provide direct evidence that the borreliacidal activity in these immune sera is primarily mediated by anti-OspC antibodies. To confirm this hypothesis and to further investigate whether the killing by anti-OspC antibodies is complement-dependent or not, the present study focused on the investigation of antibody–pathogen interactions by using immune sera EM4 and EM5 in combination with a previously generated OspC-deficient FEM1 variant. Furthermore, other proteins besides OspC may also represent possible targets for borreliacidal antibodies as demonstrated by the findings for immune serum NB4. To date, additional information regarding these two proteins of 32.7 and 40.7 kDa is unavailable. It remains a matter of speculation whether antibodies that react with either a single or both proteins may exhibit borreliacidal activity.

In simulating the differential gene expression of OspC in ticks and in the mammalian host, several studies pointed out the temperature dependence of OspC expression, thereby showing that OspC is down-regulated at lower temperatures (23°C) and up-regulated at higher temperatures (37°C) [34, 35]. Based on these findings, the present study generated an OspC-deficient FEM1 variant (FEM1OspC(–)) by application of temperature shifts, to analyse whether anti-OspC antibodies in patient sera display borreliacidal activity. With immune sera EM4 and EM5, which in addition to antibodies directed against a 51.3-kDa protein contained only anti-OspC antibodies, the FEM1 wild-type but not the FEM1OspC(–) variant was killed rapidly in the growth inhibition assay. These findings provide direct evidence that antibodies against OspC but not against the 51.3-kDa protein were responsible for the killing of the FEM1 wild-type. Furthermore, these experiments provide additional information about the specificity of borreliacidal antibodies by comparing the growth kinetics of FEM1 in the presence of immune sera EM4 and EM5. Borreliacidal activity of immune serum EM4 was clearly higher than that of EM5. This observation can be explained by a yet inadequate amount or specificity of anti-OspC antibodies in EM5, or both.

The present study also aimed to specify the localisation of deposited C9 on FEM1 wild-type when compared with FEM1OspC(–) variant after exposure to immune serum EM4. While immunogold-labelling of FEM1 wild-type appeared to be distributed arbitrarily over the entire cell surface or concentrated on condensed slime material, cells of FEM1OspC(–) variant were not labelled at all. Thin-section immuno-electron microscopy demonstrated that C9 was deposited directly on the outer membrane of FEM1 wild-type. Thus, the serum resistance of this isolate can be overcome by formation of specific borreliacidal antibodies followed by integration of the TCC.

During the natural course of the infection, borreliae are mostly eliminated as a result of the first borreliacidal antibody response. However, spirochaetes are able to spread in the human host despite the presence of these specific borreliacidal antibodies. Accordingly, it is tempting to speculate that (i) dissemination of spirochaetes occurs in the very early phase of infection, or (ii) spirochaetes become sequestered in immunologically privileged sites before high concentrations of specific anti-OspC borreliacidal antibodies are developed, or (iii) spirochaetes down-regulate OspC by up-regulation of other Osps, including OspA, during this early dissemination [13]. Thus, anti-OspC borreliacidal antibodies may fail to kill spirochaetes when OspC is not surface-exposed or when the attachment of anti-OspC antibodies is hindered by the increased production of other Osps. Moreover, the host immune response may no longer be appropriate after an early change of the antigen pattern of the infecting organism. This is in accordance with the findings of Padilla *et al.* [36], who have shown that the borreliacidal antibody response waned rapidly after primary and secondary vaccination with OspA in human subjects and hamsters, and that the recognition of the protective epitopes is difficult for the host immune system [37].

In summary, the present study demonstrated that *B. afzelii* isolate FEM1 wild-type represents a suitable tool for the identification and characterisation of borreliacidal antibodies in sera from LD patients. Furthermore, the findings indicate that anti-OspC borreliacidal antibodies can already have developed in high concentrations shortly after an infection with *B. burgdorferi* and thus are of critical importance for effective killing of the pathogen in the course of the immune response. Studies are currently underway to identify additional antigens and their specific epitopes that may be involved in the induction of borreliacidal antibodies during the infection.

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