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Epitope Mapping of the Outer Surface Protein A (OspA) of the Spirochete *Borrelia burgdorferi* Using a Panel of Monoclonal Antibodies and Lanthanide Competition Fluoroimmunoassay

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Abstract—A panel of fourteen different monoclonal antibodies was used for detection and analysis of antigenic determinants located on the outer surface protein A (OspA) of the spirochete *Borrelia burgdorferi*, which is a causative agent of tick-borne borreliosis (Lyme disease). Two main and several minor partially overlapping antigenic determinants have been found on the surface of the OspA protein of *Borrelia burgdorferi* sensu stricto (strain 297) by lanthanide competition fluoroimmunoassay. One of the main antigenic determinants is located in the N- and the other in the C-half of the OspA molecule. The involvement of the OspA protein in intact *Borrelia burgdorferi* sensu stricto (four bacterial strains have been analyzed: 297, B31, FR90-594, and CA90-742) is associated with retention of the above-mentioned two major antigenic determinants, but unlike the case of the isolated OspA they are partially overlapping with each other and with other antigenic determinants. The protein of the spirochete *Borrelia afzelii* (two bacterial strains have been analyzed: Ip-21 and Pko) contains only one antigenic determinant, which is the same as the main determinant of the OspA protein of *Borrelia burgdorferi* sensu stricto located in the N-half of the OspA molecule.

Key words: Lyme disease, tick-borne borreliosis, spirochete *Borrelia burgdorferi*, outer surface protein A (OspA), monoclonal antibodies, competition analysis, lanthanide fluoroimmunoassay

The tick-borne borrelioses (Lyme disease) are a group of diseases with different etiology caused by different species of *Borrelia* spirochetes that are transferred from animals to humans by ticks (*Ixodoidea*) [1]. Humans are infected through a bite of a borrelia-infected tick that is usually accompanied by inflammation at the site of the bite (erythema migrans). At present, Lyme disease is the most widespread tick-transferred disease in virtually all continents of the world. In the former Soviet Union Lyme disease is recorded nearly everywhere: from the Baltic Sea coast to the Pacific ocean [2]. In the Russian Federation Lyme disease is transferred by the taiga tick *Ixodes persulcatus* and by the forest tick *Ixodes ricinus*. In European Russia 10-60% of ticks (depending on the collection area) are infected with a spirochete of the *Borrelia* genus [3].

The totality of *Borrelia burgdorferi* species (a complex of genospecies called *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.)) has been recently subdivided into ten separate species, and only three of them, such as *Borrelia burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *Borrelia garinii* (*B. garinii*), and *Borrelia afzelii* (*B. afzelii*) are pathogenic for humans, although are different in their organotropism. Thus, *B. burgdorferi* s.s. mainly strikes the locomotor system (arthritis), *B. garinii* causes nervous pathology (radiculoneuritis, meningitis, encephalitis, etc.), and *B. afzelii* promotes development of skin diseases (disseminated erythema migrans, acrodermatitis chronica atrophicans, focal scleroderma, etc.) [4]. Bacteria isolates found in the USA are mainly of the *B. burgdorferi* s.s. species. In contrast, in Europe and in the Eastern Asian countries in addition to *B. burgdorferi* s.s. two other species, *B. garinii* and *B. afzelii*, are found [5], and main-

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ly these two species are also found in the Russian Federation [6-8].

The structure of the *B. burgdorferi* spirochete includes two main outer surface proteins: OspA (M_r = 31 kD) and OspB (M_r = 34 kD) [9]. These proteins are encoded by the same linear, completely sequenced plasmid [10] of 49,000 nucleic bases in length.

OspA is the predominant protein in amount. It is a species-specific, highly variable, and highly immunogenic spirochetal protein involved in the structure of virtually all species pathogenic for humans [11]. It plays the main role in the immune response of the body in Lyme disease. Polyclonal and monoclonal antibodies specific to OspA were shown to prevent Lyme disease in mice [12] and immunization with this protein protected mice against tick-borne borreliosis [13]. This was the reason for development of an anti-borreliosis vaccine based on OspA. At present, a vaccine for humans that is produced by genetic engineering with the OspA expressed in *E. coli* as the immunogen is undergoing trials [14-16].

The antigenic diagnosis of tick-borne borreliosis with immunochemical approaches is also based on detection of the OspA in biological (ticks) and clinical (blood and synovial fluid of patients) specimens. To increase the specificity of the test-systems, monoclonal antibodies (mAbs) are usually used [17-19]. And to increase the sensitivity of diagnostic test systems, it is recommended to use pairs of immunoglobulins specific to different epitopes of the antigen to be detected [20]. Obviously, for both vaccine development and construction of diagnostic test systems, it is extremely important to have information about the number and location of antigenic determinants on the surface of the protein molecule.

It is now established that OspA is a lipoprotein with a protein chain of 273 amino acid residues. The fatty acid residue is covalently bound only to the cysteine-17 residue in the N-terminal of the polypeptide chain [21] and seems to be responsible for anchoring OspA in the bacterial membrane. OspA has been completely sequenced for a significant number of isolates (more than 50) of *B. burgdorferi* s.l. [22]. The N-terminal of the OspA molecule is shown to be very strain-conservative, whereas all amino acid replacements in different bacterial strains are located in the C-terminal region [23]. Based on differences in the primary structure, several structural (and antigenic) types of OspA have been determined [24]: type 1 is found in intact *Borrelia burgdorferi* sensu stricto and type 2 is found in intact *B. afzelii*, whereas five different OspA types (types 3-7) are found in the intact structure of different strains of *B. garinii* spirochete.

X-Ray crystallographic analysis at 1.9 Å resolution [25] of the recombinant delipidated OspA from *B. burgdorferi* s.s. (the B31 strain) crystallized in complex with the Fab-fragment of mAb 184.1 [26] has revealed that OspA mainly contains β-chains (21 per molecule) with only one α-helical region which is located in the C-terminal region. The mAb 184.1 used in this work [26] interacts with an antigenic determinant located in the N-terminal of the OspA molecule in the region of amino acids 17-119. This region of the polypeptide chain was shielded and inaccessible for mAb 184.1 on the OspA inintact bacteria. The authors suggested [26] that other antigenic determinants should exist which according to a subsequent structural computerized analysis [27] should be located in the regions of lysine-46, asparagine-82, and lysine-231, i.e., be distributed along the length of the molecule.

The structure and functions of the OspA from *B. burgdorferi* have been studied with several panels of mAbs, the majority of which were not strictly mapped but allowed detection of epitopes located in the variable C-terminal of the protein molecule. MAbs H5332, H3TS [28], CIII.78 [29], and LA-2 [12] were specific to antigenic determinants located in the large variable region (amino acids 133-273) of the polypeptide chain. MAb 105.5 [23] interacted with the region of amino acids 214-217. These antibodies suppressed the spirochete growth *in vitro* and displayed protective properties in mice. MAbs 9B3D [30], B3G11 [13], and 336 [31], which also were specific to the C-terminal half of OspA, suppressed the adsorption of *B. burgdorferi* on HUVE cells [30] and displayed a bactericidal effect and a complement-dependent killer activity [13]. Only two of the previously used mAbs, 8C4BC [29] and 184.1 [23, 26], were specific to the strain-conservative N-terminal of the OspA polypeptide chain: the first antibody interacted with amino acids from the region of 1-133 and the other interacted with amino acids located in the region of 17-119, as mentioned.

A relatively large panel of five mAbs was used for epitope mapping of the OspA of *B. burgdorferi* s.s. by Yang et al. [32]. Four antigenic determinants were detected on the surface of OspA isolated from the bacteria with trypsin and the detergent Triton X-114.

However, a still more complete panel of mAbs (14 clones) to OspA of *B. burgdorferi* s.s. (strain 297) has been prepared by Dr. D. Bucher (New York Medical College, USA). In the studies of Dr. A. Cozzolino [33] (a coworker of Dr. D. Bucher) the mAbs of this panel were characterized by epitope specificity and combined into seven groups depending on their reactivity to American and European borrelia strains. Using immunoblotting and OspA fragments prepared by genetic engineering and expression in the *E. coli* system, Dr. Cozzolino has mapped the OspA regions interacting with mAbs of this panel: five mAbs interacted with the C-terminal third of the polypeptide chain (amino acids 201-273), two mAbs interacted with the N-terminal third (amino acids 16-105), and the remaining seven mAbs interacted with the central third of the OspA (amino acids 108-201).

MAbs to the surface OspA of *B. burgdorferi* were also used earlier for typing isolated bacterial strains. A pronounced antigenic variability of these proteins from

American and European borrelia strains was found by Barbour and Schtrumpf with three mAbs specific for the OspA and OspB proteins [28] and also by Wilske et al. [24] with a panel of eight different antibodies specific to OspA.

The purpose of our studies was to develop a competition approach [34] based on the competition between Eu^{3+}-labeled and unlabeled mAbs for binding sites on the antigenic determinant of the OspA, both isolated and in the structure of various species of *B. burgdorferi*. For this purpose, we used a set of fourteen above-mentioned mAbs produced in the laboratory of Dr. Bucher (New York Medical College, NY). The competition level was quantitatively assessed by lanthanide fluoroimmunoassay (LFIA) [35].

MATERIALS AND METHODS

Antigens. The following bacterial antigens were used: intact spirochete *B. burgdorferi* s.s. (strains 297, B31, CA90-742, FR90-564), intact spirochete *B. afzelii* (strains Ip-21 and Pko), the surface protein OspA isolated from *B. burgdorferi* s.s. (strain 297). All above-mentioned bacteria (except strains CA90-742, FR90-564, and Pko) were grown in BSK-H medium and purified by a single centrifugation cycle in the laboratory of Dr. I. Schwartz (Department of Biochemistry, New York Medical College, Valhalla, USA). Bacteria of CA90-742, FR90-564, and Pko strains were kindly presented by Dr. Edward J. Orff, Department of Biochemistry, Microbiology, and Molecular Genetics, University of Rhode Island, USA).

A protein antigen OspA was isolated from *B. burgdorferi* s.s. (strain 297) by column chromatography as follows. The spirochetes were disrupted by addition of 10% SDS and by sonication. The lysate was placed onto a column (1.5 × 90 cm) containing BioGel A-5m (BioRad, USA), and chromatography was performed with 0.02 M Tris-HCl buffer (pH 7.4) supplemented with 0.1% SDS and 0.05% sodium azide. The resulting chromatographic fractions were analyzed by SDS-PAGE. The fractions containing OspA were combined and concentrated with an Amicon concentrator (Holland) using a PM10 membrane. The concentrated preparation was dialyzed against distilled water for four days to remove SDS. The final OspA preparation was a purified A-protein of the surface membrane of the spirochete with an admixture of another bacterial protein, OspB (by data of SDS-PAGE, the OspA/OspB ratio was 2 : 1). The protein concentration in both spirochete and OspA was determined by the Lowry method [36].

Monoclonal antibodies. A panel of mAbs specific to the surface protein OspA from *B. burgdorferi* s.s. (American strain 297) was used. To produce hybridomas, BALB/c mice were immunized with the OspA preparation (2 × 25 μg per mouse), and hybridomas (based on SP2/O cells) were produced using polyethylene glycol 1500 by the method of Kohler and Milstein with modifications [37, 38]. The hybridomas were cloned and monitored by enzyme-linked immunosorbent assay for production of anti-OspA immunoglobulins. Then the hybridomas were amplified in culture medium, and ascites were produced by injecting BALB/c mice with 500,000 hybridoma cells per mouse.

The mAbs were used either as ascites or as purified immunoglobulins (IgG) prepared by affinity chromatography of ascitic fluid on a column with immobilized protein A [39].

Some important characteristics of the fourteen mAbs used in the present work are given in Table 1. The specificity of the antibodies was checked by immunoblotting (data not presented). All mAbs were specific only to OspA, except the [L12-3C9(2C7)] clone (C7 as the abbreviated name), which reacted with both OspA and OspB.

Preparation of Eu^{3+} conjugates. Conjugates of antibodies with europium chelate were prepared by the method of Paik et al. [40] with modifications as described in [41]. The immunoglobulins were labeled with Eu^{3+} using diethylene triaminopentacetic acid (DTPA) dianhydride as the chelator. The covalent binding of DTPA dianhydride to IgG was performed for 1 h at room temperature in 0.1 M carbonate-bicarbonate buffer (pH 8.1). All subsequent reactions were performed on an ice bath. To the reaction mixture, 1% $EuCl_3$ in 0.02 M citric acid was added. After incubation for 15 min, excess Eu^{3+} was bound by addition of 0.02 M DTPA. The conjugates were purified from the unbound label on a column with Sephadex G-50 (medium, Pharmacia, Sweden) in 0.05 M Tris-HCl buffer (pH 7.75) + 0.15 M NaCl + 0.05 M NaN_3. The conjugates were characterized by protein concentration and by the molar ratio Eu/IgG (Table 1) that was determined by lanthanide fluoroimmunoassay. The mAbs labeled with europium were designated as D8-Eu, E6-Eu, etc.

Competition analysis by LFIA. The competition analysis was done using a direct adsorption of the antigen on polystyrene. Polystyrene plates from the Institute of Biological Engineering (Moscow) and from Labsystems (Finland) were used. The antigen (OspA or intact bacteria) in 0.05 M bicarbonate buffer (pH 9.6) was placed into the wells of the plate at the concentration of 1 μg per well and incubated at 4°C for 18 h. After a triple washing with a washing buffer (5 mM Tris-HCl (pH 7.75) + 0.15 M NaCl + 0.05% Tween 20 + 0.01% NaN_3), the wells were supplemented with 250 μl of an blocking buffer (50 mM Tris-HCl + 1% BSA + 0.01% NaN_3) and incubated at 37°C for 1 h. Then the antigen-containing wells were supplemented with 50 μl of europium-labeled mAbs diluted 1 : 100 with a reaction buffer (0.05 M Tris-HCl (pH 7.75) + 0.15 M NaCl + 20 mM DTPA + 0.5%

Table 1. Characteristics of monoclonal antibodies and their Eu^{3+} conjugates used in the present study

Full name of monoclonal antibody	Abbreviated name of mAb	Isotype of mAb	Molar ratio Eu/IgG	Initial concentration of IgG, mg/ml
L12-9B4(2D8)	D8	IgG2b	7.2	0.25
L14-7A6(1C3)	C3	IgG1	6.6	0.2
L14-3E10(2F8)	F8	IgG1	5.2	0.25
L14-2G10($2A7_1$)	$A7_1$	IgG2b	5.2	0.25
L14-4B5($2A7_2$)	$A7_2$	IgG2a	22.2	0.2
L9-E1D6	D6	IgG1	—	—
L9-E2E6	E6	IgG1	3.3	0.1
L11-1F8(1F11)	F11	IgG2a	—	—
L12-3C9(2C7)	C7	IgG2b	8.7	0.25
L12-5H2(1C6)	C6	IgG2a	5.2	0.25
L12-4D5(2F10)	F10	IgG2a	—	—
L12-8A6(2G6)	G6	IgG2b	21.6	0.25
L12-6H2(2F6)	F6	IgG1	—	—
L10-2C9(4A10)	A10	IgG3	3.4	0.1

BSA + 0.01% Tween 20 + 0.05% NaN_3). The wells of the polystyrene plate were concurrently supplemented with 50 µl of ascites containing unlabeled mAbs and diluted 1 : 40 with the same buffer. After incubation for 1 h at room temperature with shaking on a shaker and after a triple washing with the washing buffer, the wells were supplemented with an enhancement solution (0.2 M phthalate-acetate buffer (pH 3.5) + 0.02 mM 2-naphthoyl trifluoroacetone + 0.05 mM trioctylphosphinoxide + 0.1% Triton X-100) produced in the Institute of Biological Engineering, and the amount of the labeled antibody adsorbed on the antigen was determined by time-resolved pulse spectrofluorometry. The luminescent signal in the wells was recorded either with an Arcus-1234 spectrofluorimeter (Wallac, Finland) or with a similar IFI-01 device (Institute of Biological Engineering). Normal mouse ascites diluted 1 : 40 with the reaction buffer was used as the control. The competition of two antibodies for the binding site on the antigen was determined as the competition percent (C) by the formula:

$$C = (F_{contr} - F_{exp})/F_{contr} \cdot 100\%,$$

where F_{exp} is the fluorescence of the well after competition had been performed and F_{contr} is the fluorescence of the control well supplemented with the antibody-free buffer instead of the unlabeled competing antibody.

To determine the mAbs adsorption on bacterial antigens, the experiment was performed as follows. The antigens were adsorbed onto the wells of the polystyrene plates as described above and after the inhibition of non-specific binding sites with the inhibiting buffer the wells were supplemented with europium-labeled mAbs (diluted 1 : 100 with the reaction buffer). After washing, the antibody adsorption level was determined by fluorescence of the wells. It was assumed that mAbs were not adsorbed onto the bacterial antigen if the fluorescence of the corresponding well was not higher than the background of 10^3 pulses/sec.

RESULTS AND DISCUSSION

Competition analysis is a suitable approach for epitope mapping of proteins [34]. The main prerequisite for its being used is a complete structural identity of the labeled and unlabeled mAbs competing for the binding sites on the antigen. From this standpoint, lanthanide labels are more advantageous than labels used in other approaches (radioimmunoassay, fluoroimmunoassay) because their damaging effect on the native structure of antibodies is minimal due to the small size of the molecules, a mild labeling procedure, and also due to the absence of antibody damage during storage [35, 42].

During the development of the competition version of LFIA for the epitope mapping of OspA, first the optimal conditions for its execution were determined. Therefore, experiments were performed on immunoglobulin displacement from the binding sites on the antigen during the interaction of competing and non-competing antibodies. In the plate wells 50 µl of Eu^{3+}-labeled antibodies of the E6 clone were incubated with the same

amount of unlabeled antibodies of the E6 or D8 clones as ascitic fluids diluted 200-51,200-fold. The results are presented in Fig. 1.

Figure 1 shows that the competition occurred only during the interaction of the labeled and unlabeled antibodies of the same clone (E6). In this case, the unlabeled immunoglobulins even at great dilutions of the ascites (1 : 200-1 : 400) virtually completely displaced the labeled antibodies from the antigenic determinant. In the absence of competition (europium-labeled E6 antibody and unlabeled D8 antibody) the labeled antibody was not displaced from the binding sites on the antigen at any concentration of the unlabeled antibodies used in our experiments.

It has been already mentioned that after the initial steps of LFIA, competition analysis was performed with addition into the wells of the plate of 50 μl (or 50-125 ng, Table 1) of the europium-labeled antibodies along with 50 μl of the unlabeled antibodies in 40-fold diluted ascites fluid. Considering that the ascites fluid contained mAbs in the concentration of 1-10 mg/ml [43], in the competition experiments each well was supplemented with 1.25-12.5 μg unlabeled immunoglobulins, i.e., the amount of the unlabeled mAbs was 10-250-fold higher than the amount of the labeled antibodies.

Tables 2-5 present results of the competition analysis of OspA isolated from *B. burgdorferi* s.s. (strain 297) and also of this protein in the intact spirochete of various genospecies and strains (in the Tables 2-5 the cases of absent, partial, or complete competition of the labeled and unlabeled antibodies for the binding sites on the antigen are shown with different marking).

Table 2. Results of competition analysis of OspA isolated from *B. burgdorferi* s.s., strain 297 (competition percent, %)

Labeled antibodies → Unlabeled antibodies ↓	D8-Eu	D6-Eu	E6-Eu	C7-Eu	C6-Eu	G6-Eu	A10-Eu
D8	**99.1**	13.1	10.7	2.0	0.1	**97.3**	—
C3	**98.5**	1.8	4.0	—*	**41.9**	—	—
F8	**98.1**	8.0	14.5	—	**54.3**	—	—
$A7_1$	**98.0**	10.6	2.7	—	**54.0**	—	—
$A7_2$	**98.0**	—9.7	3.4	—	**61.0**	—	—
D6	0.8	**96.3**	**99.5**	—	**42.5**	—	—
E6	1.3	**94.0**	**97.8**	—1.3	**53.6**	7.0	—
F11	3.2	**89.1**	**92.4**	—	**29.8**	—	—
F6	**63.4**	**87.0**	**89.9**	—	**33.4**	—	—
C7	7.6	12.1	6.8	**99.4**	8.5	7.6	—
C6	7.0	**45.2**	**52.6**	5.5	**94.1**	13.2	—
F10	**40.0**	5.4	—4.3	8.7	11.4	**31.2**	—
G6	**96.6**	7.3	4.7	—1.2	**35.2**	98.1	—
A10	—0.6	—	2.1	8.7	2.4	4.1	—
Polyclonal IgG to OspA	**91.9**	**94.6**	**93.3**	—	**82.4**	—	—
Control of adsorption of mAbs**	2.069	—	0.249	0.238	0.185	0.060	0.001

Note: Here and in Tables 3-5 the competition level is shown with the shading: the dark shading shows the competition level of the unlabeled and labeled mAb higher than 85%; the gray shading shows the competition level of the unlabeled and labeled mAb from 20 to 85%; the absence of shading shows the competition level of the unlabeled and labeled mAb lower than 20%.

* Measurements were not performed.

** Here and in Tables 3-5 the level of the antibody adsorption is expressed as the fluorescence intensity ($\times 10^{-6}$ pulse/sec) of the control well (without the competition).

Table 3. Results of competition analysis of OspA in intact *B. burgdorferi* s.s., strain 297 (competition percent, %)

Labeled antibodies → Unlabeled antibodies ↓	D8-Eu	C3-Eu	F8-Eu	$A7_1$-Eu	$A7_2$-Eu	E6-Eu	C7-Eu	C6-Eu	G6-Eu	A10-Eu
D8	**99.5**	**99.5**	**99.6**	**99.5**	**98.9**	**55.3**	**25.5**	**54.9**	**81.1**	**49.5**
C3	**99.4**	**99.7**	**99.7**	**99.5**	**99.4**	**60.5**	—*	—	**83.2**	—
F8	**99.4**	**99.6**	**99.7**	**99.6**	**99.2**	**63.3**	—	—	**79.4**	—
$A7_1$	**99.6**	**99.6**	**99.7**	**99.8**	**99.7**	**70.1**	—	—	**72.0**	—
$A7_2$	**99.7**	**99.8**	**99.7**	**99.8**	**99.8**	**69.6**	—	—	**80.8**	—
D6	**44.4**	**51.2**	**45.6**	**55.1**	**33.4**	**98.8**	—	—	**57.5**	—
E6	**59.9**	**64.3**	**67.6**	**66.7**	**41.0**	**99.9**	**61.0**	**82.7**	**56.8**	**99.6**
F11	**71.5**	**90.9**	**94.2**	**86.0**	**90.6**	**99.8**	—	—	**74.4**	—
F6	**64.4**	**91.8**	**95.7**	**88.3**	**90.7**	**99.8**	—	—	**67.9**	—
C7	**28.1**	**36.8**	**39.8**	4.2	12.2	10.9	**89.7**	19.1	**42.3**	–2.5
C6	**46.1**	**47.2**	**74.9**	**66.5**	**70.5**	**54.3**	–2.6	**97.0**	**70.5**	–0.1
F10	17.3	**24.6**	**22.9**	**20.8**	1.3	1.8	–1.3	–3.7	**42.8**	–5.1
G6	**55.1**	**50.8**	**53.7**	**57.2**	**37.5**	1.7	–2.2	**46.7**	**92.3**	8.1
A10	**40.1**	**54.6**	**61.3**	**50.6**	**24.1**	**85.3**	**44.4**	**42.2**	**35.5**	**99.0**
Control of adsorption of mAbs	0.754	0.686	0.420	0.813	1.129	1.392	0.08	0.365	0.016	0.414

* Measurements were not performed.

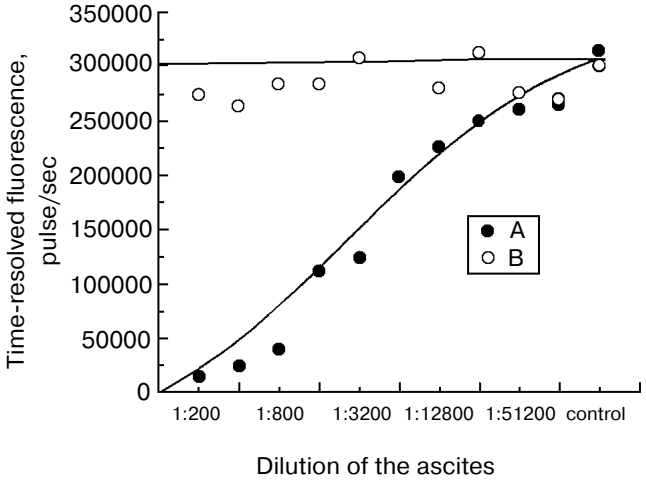

Fig. 1. Amount of labeled mAb E6-Eu adsorbed on the antigen (OspA) depending on the amount of the unlabeled antibody added: A) the competing variant (labeled E6 was supplemented with ascites containing unlabeled E6); B) the non-competing variant (labeled E6 was supplemented with ascites containing the unlabeled D8). Control: the well containing the antigen and the labeled mAb was supplemented with buffer without the unlabeled mAbs.

Table 2 presents the results of the competition analysis of the isolated OspA. Note the findings as follows. First, five mAbs (D8, C3, F8, $A7_1$, and $A7_2$) virtually completely competed with the europium-labeled homologous antibody (D8) and displayed poor or no competition with the labeled antibodies of other groups. We suggested that these antibodies should interact with the same antigenic determinant (designated as ADI) on the surface of the isolated OspA. Four other mAbs (D6, E6, F11, and F6) had the same property: they displayed a 100% competition with the homologous europium-labeled antibodies (D6 and E6) and poor or no competition with the antibodies that were not in this group. We suggested that these antibodies should interact with another antigenic determinant designated as ADII. Note that the antigenic determinants ADI and ADII are not overlapping in the case of the OspA isolated from the spirochete, i.e., the antibodies interacting with one determinant absolutely failed to compete with the antibodies interacting with another determinant.

For the remaining five mAbs of the panel the picture is more complicated. These antibodies seem to interact

Table 4. Results of the competition analysis of OspA in intact *B. burgdorferi* s.s., strain B31 (competition percent, %)

Labeled antibodies → Unlabeled antibodies ↓	D8-Eu	C3-Eu	F8-Eu	$A7_1$-Eu	$A7_2$-Eu	E6-Eu	C7-Eu	C6-Eu	G6-Eu	A10-Eu
D8	**99.3**	**98.8**	**98.8**	**99.1**	**99.1**	**25.1**	**75.0**	**81.1**	**92.1**	**66.0**
C3	**99.0**	**99.4**	**99.5**	**98.5**	**98.6**	**25.2**	—*	—	**91.2**	—
F8	**99.2**	**99.3**	**99.7**	**99.5**	**99.2**	**33.2**	—	—	**92.5**	—
$A7_1$	**99.6**	**99.7**	**99.8**	**99.7**	**99.7**	**29.0**	—	—	**90.6**	—
$A7_2$	**99.0**	**99.5**	**99.2**	**99.5**	**99.5**	**36.5**	—	—	**91.5**	—
D6	**70.3**	**49.4**	**47.5**	**50.3**	**56.2**	**96.6**	—	—	**39.3**	—
E6	**66.2**	**36.8**	**26.9**	**40.0**	**39.3**	**99.9**	**84.8**	**87.0**	**50.5**	**93.5**
F11	**74.3**	**79.5**	**87.4**	**82.7**	**88.5**	**99.3**	—	—	**60.2**	—
F6	**82.7**	**87.1**	**94.0**	**89.1**	**92.1**	**99.6**	—	—	**77.2**	—
C7	**35.0**	**39.6**	**45.9**	**45.4**	**49.4**	**25.5**	**98.6**	**36.2**	**39.6**	**39.8**
C6	**55.2**	**72.0**	**72.5**	**73.4**	**81.1**	**56.6**	**42.3**	**99.1**	**74.9**	**38.6**
F10	6.5	**49.0**	**47.4**	**40.9**	**46.0**	–0.4	1.7	**2.2**	18.3	1.8
G6	**28.2**	**33.6**	**24.8**	**44.3**	**46.2**	0.6	9.1	**54.7**	**96.9**	**30.8**
A10	**53.6**	**50.0**	**56.2**	**56.0**	**60.3**	**82.5**	**77.1**	**56.4**	42.9	**91.8**
Control of adsorption of mAbs	5.787	2.687	1.358	4.369	6.681	1.916	0.534	0.870	0.073	0.022

* Measurements were not performed.

with OspA molecular regions other than those described above and develop antigenic determinants designated as ADIII (the mAb C7), ADIV (C6), ADV (F10), and ADVI (G6). Virtually all these determinants are partially overlapped with other determinants, in particular, with ADI and ADII. This overlapping is associated with an incomplete competition of the labeled and unlabeled antibodies for the binding site. Thus, the mAb C7 interacts with the non-overlapping determinant ADIII, and there is no competition with antibodies of the ADI and ADII groups. On the other hand, the mAb C6 failed to compete with the europium-labeled antibodies D8 and C7 (i.e., the antigenic determinant ADIV was not overlapped with the antigenic determinants ADI and ADIII) but competed with the labeled antibodies D6, E6, and G6 (i.e., the antigenic determinant ADIV was partially overlapped with the antigenic determinants ADII and ADVI). Other minor antigenic determinants were similarly analyzed.

The mAb A10 was not adsorbed on OspA (last line of Table 2) and, naturally, competed with neither of the labeled mAbs. Therefore, it was believed that OspA isolated from the spirochete had no antigenic determinant capable of adsorbing mAb A10.

A similar antigenic mapping has been performed for intact borrelias of two genospecies: *B. burgdorferi* s.s. (strains 297, B31, FR90-564, and CA90-742) and *B. afzelii* (strains Ip-21 and Pko).

Results of this analysis of American strains 297 and B31 are presented in Tables 3 and 4. For the strains FR90-564 and CA90-742 the results were similar (data not presented). As in the case of the isolated OspA, the determinant analysis of *B. burgdorferi* s.s. revealed the same major (ADI and ADII) and several minor antigenic determinants. The main difference was, first, the partial overlapping of two major determinants ADI and ADII and, second, the presence of an additional minor antigenic determinant (ADVII) with which the mAb AD10 interacted.

The results of competition analysis of the strains of *B. afzelii* genospecies were quite different. First, only mAbs of the antigenic site ADI (D8, C3, F8, $A7_1$, and $A7_2$) interacted with intact borrelia. The antibodies interacting with the antigenic determinant ADII in isolated OspA and in OspA in intact *B. burgdorferi* s.s. were not

Table 5. Results of competition analysis of OspA in intact *B. afzelii*, strain Ip-21 (competition percent, %)

Labeled antibodies → Unlabeled antibodies ↓	D8-Eu	C3-Eu	F8-Eu	$A7_1$-Eu	$A7_2$-Eu	E6-Eu	C7-Eu	C6-Eu	G6-Eu	A10-Eu
D8	**99.7**	**99.6**	**99.1**	**99.8**	**99.7**	—*	—	—	**60.3**	—
C3	**99.1**	**99.5**	**98.9**	**99.7**	**99.3**	—	—	—	**45.1**	—
F8	**96.4**	**98.7**	**98.8**	**98.3**	**97.6**	—	—	—	**41.8**	—
$A7_1$	**99.4**	**99.5**	**98.8**	**99.6**	**99.6**	—	—	—	**40.8**	—
$A7_2$	**99.7**	**99.4**	**99.1**	**99.6**	**99.8**	—	—	—	**33.5**	—
D6	7.2	5.2	2.4	–4.7	2.8	—	—	—	2.5	—
E6	5.7	6.1	5.3	2.4	0.6	—	—	—	9.2	—
F11	2.9	–3.6	5.2	0.9	1.7	—	—	—	4.3	—
F6	5.0	19.9	4.8	0.2	11.5	—	—	—	5.7	—
C7	17.4	18.7	8.9	12.2	6.1	—	—	—	2.4	—
C6	4.6	14.5	5.7	15.9	9.3	—	—	—	0.8	—
F10	4.9	**22.8**	**33.0**	7.6	6.8	—	—	—	7.0	—
G6	**57.2**	**67.0**	**81.2**	**71.0**	**65.8**	—	—	—	**91.0**	—
A10	–0.2	2.8	–1.6	10.2	4.4	—	—	—	4.0	—
Control of adsorption of mAbs	1.000	0.594	0.140	0.952	1.436	0.001	0.001	0.001	0.009	0.001

* Measurements were not performed.

adsorbed onto *B. afzelii* (Table 5, last line). Therefore, it was concluded that the OspA of *B. afzelii* lacked the antigenic site ADII. Second, only one mAb G6 of five mAbs of the minor sites interacted with intact *B. afzelii*. And this minor antigenic determinant was partially overlapped with the major determinant ADI. The above-presented data on epitope mapping of all antigens studied in this work are summarized in Table 6 and shown in Fig. 2.

Very similar results have been recently obtained by Dr. A. Cozzolino [33], who is one of the authors of the present article. To detect and characterize antigenic determinants of OspA isolated from strain 297 spirochete, he used all fourteen mAbs which were used by us. For epitope mapping, Dr. Cozzolino used immunoblotting and genetically engineered constructions containing various fragments of OspA. The mAbs D6, E6, F6, and F11 were shown [33] to be of the same epitope group and to interact with the C-terminal third of the OspA (amino acids 201-273). Our findings (Table 6) suggest that these mAbs should produce the epitope group of the ADII antigenic determinant. It is known from the literature that the C-terminal part of OspA is highly variable [22-24]; thus, it is not surprising that this epitope was not detected in the OspA of *B. afzelii* (Fig. 2).

By the data of Cozzolino [33], the remaining ten mAbs interact with the N-terminal and with the central parts (amino acids 16-105 and 108-168, respectively) of the OspA and are adsorbed either onto one or onto the other part of this molecule. These regions of the OspA molecule seem to be brought close together and are involved in the formation of both one of the main antigenic determinants (ADI) and the minor epitopes from ADIII to ADVII. Because the N-terminal and the central part of the OspA molecule are conservative and similar in various genospecies of *B. burgdorferi* s.l. bacteria, it is clear why we have found the antigenic determinant ADI in both *B. burgdorferi* s.s. and in *B. afzelii*.

And the following circumstance should also be discussed. In some rare cases there is no symmetry in the overlapping of antigenic determinants. Thus, in intact borrelia the antigenic determinant ADIII is partially overlapped with the antigenic determinant ADII (competition percent $C = 61\%$, Table 3), but there is no reciprocal effect: the antigenic determinant ADII is not over-

Table 6. Summary table of location and features of antigenic determinants of *B. burgdorferi* s.l.

Object	Antigenic determinant	Presence	Features
Isolated OspA (*B. burgdorferi* s.s., strain 297)	I*	+	Partially overlapped with ADV and ADVI
	II	+	Partially overlapped with ADVI
	III	+	Not overlapped with other AD
	IV	+	Partially overlapped with ADI, ADII, and ADVI
	V	+	Partially overlapped with ADI and ADVI
	VI	+	Partially overlapped with ADI and ADV
	VII	—**	
OspA in intact *B. burgdorferi* s.s. (strains 297, B31, FR90-564, CA90-742)	I	+	Partially overlapped with all AD
	II	+	Partially overlapped with ADI, ADIV, ADVII
	III	+	Partially overlapped with ADI, ADII, ADVII
	IV	+	Partially overlapped with ADI, ADII, ADVI, ADVII
	V	+	Partially overlapped with ADI, ADVI
	VI	+	Partially overlapped with ADI, ADII, ADIII, ADIV, ADV, ADVII
	VII	+	Partially overlapped with all AD
OspA in intact *B. afzelii* (strains Ip-21, Pko)	I	+	Partially overlapped with ADVI
	II	—	
	III	—	
	IV	—	
	V	—	
	VI	+	Partially overlapped with ADI
	VII	—	

* Antigenic determinant I (ADI) is the adsorption region of mAb D8, C3, F8, $A7_1$, $A7_2$; ADII is the adsorption region of mAb D6, E6, F11, F6; ADIII is the adsorption region of mAb C7; ADIV is the adsorption region of mAb C6; ADV is the adsorption region of mAb F10; ADVI is the adsorption region of mAb G6; ADVII is the adsorption region of mAb A10.

** Antigenic determinant is absent.

lapped with the antigenic determinant ADIII (C = 10.9%). Similarly, ADVI is overlapped with the ADIII (C = 42.3%) but ADIII is not overlapped with the ADVI (C = 2.2%). Such asymmetry in the interaction of antigenic determinants is well known from the literature [34] and can be explained as follows. First, the molecular region considered as an antigenic determinant can include not five or six amino acid residues with which an individual mAb is interacting but be a significantly more extended region. mAbs of the same epitope group can interact with different areas of this region and sterically overlap or not overlap the adjacent determinant. Second, the interaction of a mAb with an antigenic determinant is known to influence the space structure of the protein molecule–antigen. This influence can result in a mediated structural overlapping of antigenic determinants when the mAb interaction with one of the antigenic determinants results in changes in the structure and reactivity of another. These two effects are sufficient to explain the lack of symmetry in the interaction of antigenic determinants on the OspA molecule.

Briefly summarized, the main findings of the present work are as follows.

First, based on the epitope mapping with a panel of fourteen mAbs and by lanthanide fluoroimmunoassay, a hypothetical model has been designed of the antigenic structure of the surface protein OspA of *B. burgdorferi* s.l. American strains of *B. burgdorferi* s.s. (four strains have been analyzed) are shown to contain two main (conservative and variable) antigenic sites located in the N- or C-

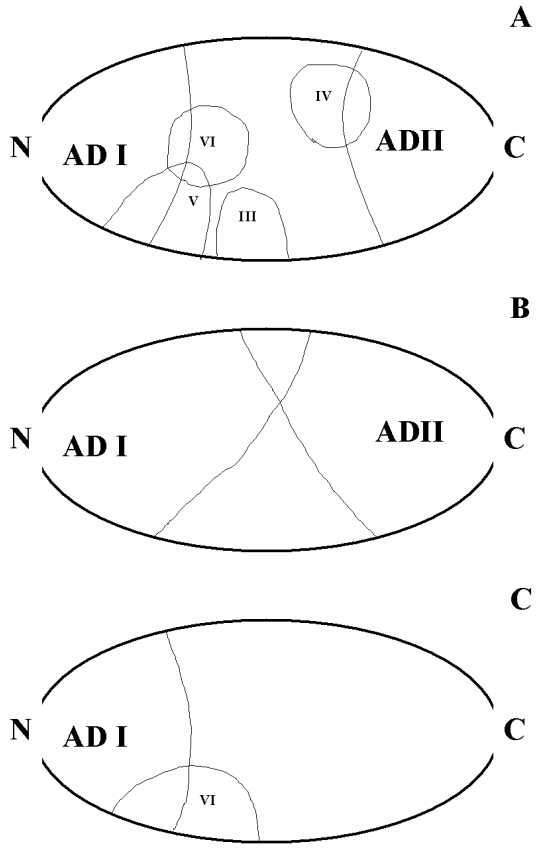

Fig. 2. Epitope mapping of OspA: A) isolated; B) in the intact *Borrelia burgdorferi* sensu stricto (for convenience, the scheme shows the location only of two main antigenic determinants ADI and ADII); C) in intact *Borrelia afzelii*.

parts, respectively, of the protein molecule. With the same panel of mAbs, in European strains of *B. afzelii* (two strains have been analyzed) only a conservative binding site in the N-half of the OspA has been detected.

Second, differences are found in the mAbs interaction with the antigenic determinants of the OspA isolated from bacteria and in the native structure. In the second case, the major antigenic determinants not overlapping in the isolated protein were partially overlapped to one another and with other minor determinants. This finding is consistent with the literature data that the native conformation of OspA is required to provide a full realization of its properties [25, 27].

Finally, a new approach is developed for the epitope mapping of the OspA of spirochete *B. burgdorferi* s.l. based on the use of lanthanide fluoroimmunoassay and of the mAb panel. It has been already noted that the LFIA advantage is minimal damage of europium chelate-based labels on the native structure of antibodies during both label incorporation and conjugate storage, and this is extremely important for successful competition immunoassay.

We think that the results of the present work can be used along several lines.

1. For construction of a detailed model of the antigenic structure of the OspA of *B. burgdorferi* based on the epitope mapping by competition LFIA and using the maximally complete panels of mAbs (including those prepared to Russian borrelia variants) and a significantly enlarged group of strains analyzed, first of all, with the addition of *B. garinii* isolates characterized by the greatest variability.

2. For elaboration of new highly sensitive test systems with a desired specificity. Such test systems, especially based on LFIA, can be used for detection of *B. burgdorferi* in laboratory and field specimens, e.g., for the quantitation of the causative agent in culture, for seroepidemiological monitoring of natural loci of Lyme disease, and also for the differentiation of natural isolates of bacteria. For these purposes, it is reasonable to elaborate group-specific test systems based on mAb interaction with the conserved region of the ADI of the OspA combined with mAbs specific to the variable site of the ADII to provide the strain differentiation. There is already an experience in the elaboration of such test systems based on the knowledge about the number and location of antigenic determinants in the structure of the surface protein OspA of *B. burgdorferi* s.s. [18, 19, 44].

3. For elaboration of new anti-borreliosis vaccines based on artificial polypeptide antigens (or on a set of antigens), providing that antibodies to these antigens should protect against the agents of Lyme disease circulating in a certain geographical region.

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