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Identification of Two Epitopes on the Outer Surface Protein A of the Lyme Disease Spirochete *Borrelia burgdorferi*

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ABSTRACT

A murine IgM monoclonal antibody (MA-2C6) with κ-light chains directed against an antigenic determinant of outer surface protein A (OspA) of the Lyme disease spirochete, *Borrelia burgdorferi*, is produced. This antibody could bind specifically to OspA antigen of several isolates of *B. burgdorferi*, but not to the non-Lyme disease bacteria such as *T. pallidum* and *B. hermsii*. Antibody MA-2C6 was purified by ion-exchange chromatography and used for purification of OspA antigen from *Borrelia burgdorferi* cell lysate. This antibody together with an IgG1 monoclonal antibody specific for OspA, that was previously characterized, were used to test whether these antibodies recognize different epitopes on OspA antigen of *Borrelia burgdorferi*. For this test, ELISA double antibody binding was used. Two antibodies were added to the antigen either separately or simultaneously, and the amount of bound antibody was quantitatively measured by the use of rabbit anti-mouse IgG conjugated with alkaline phosphatase. Additivity of the bound enzymatic activity was observed when the monoclonal antibodies bind to distinct epitopes. With this test, two distinct epitopes were recognized on the OspA molecule. This antibody can be used not only for the purification and subtyping of OspA, but also for neutralization and immunotherapy. *Iran. Biomed. J. 4: 7-12, 2000*

Keywords: OspA, Anti-OspA, Epitopes, Lyme disease, *Borrelia burgdorferi*

INTRODUCTION

Lyme disease (Lyme borreliosis) is a tick-born disease caused by *Borrelia burgdorferi*, a gram-negative spirochete [1, 2]. This disease is a complex series of clinical disorders manifested in several stages consisting of an inflammatory disorder of skin, heart, nervous system and joints [3]. Human Lyme borreliosis has been reported from more than 20 countries in Europe, Asia, Australia and North America [3] and more than 99,000 cases have been reported to the US Centers for Disease Control and Prevention from 1982-1996. Although patients with Lyme borreliosis develop specific antibodies (IgG and IgM) to B. burgdorferi antigens a few weeks after infection [4, 5] and generate specific T and B cell responses, their immune reactions do not convey total protection [2].

Since culture and direct visualization of spirochetes are often negative in Lyme disease, diagnosis serologic test procedures are used to detect the B. burgdorferi antibodies in the serum. These procedures include ELISA [6], indirect immunofluorescent and Western immunoblot [7]. These techniques are not 100% reliable and false reactions may occur both in healthy subjects and in patients with a variety of diseases especially syphilis [3, 8]. Therefore, the availability of specific monoclonal antibodies against B. burgdorferi antigens could eliminate the cross-reactivity with other bacteria. As a result, specific monoclonal antibody, if used together with other techniques, would be an alternative tool for a rapid and accurate diagnostic test for the early detection of Lyme disease [9, 10]. Monoclonal antibodies have been used for detection of intact spirochetes or for the presence of B. burgdorferi antigens (shed or degraded) in urine [9, 10], blood samples [11] or the tissues of patients. Also, the antibodies specific for OspA and OspC were used to identify the presence of parasite in the tick [12]. Recently, it has been shown that antibody against outer surface protein A (OspA) of B. burgdorferi could protect the immunodeficient mice against B. burgdorferi

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infection and development of the disease [13, 14]. Therefore, this protein was used for vaccine development [15].

Also, epitope mapping is a useful technique that helps us to predict suitable monoclonal antibodies to develop an assay which is both specific and highly sensitive for OspA subtype [16] and for neutralization assay for anti-*Borrelia* activities to define the immunodominant epitopes. Previously, a murine IgG1 monoclonal antibody specific for the OspA antigen of B. Burgdorferi has been reported [17]. In this report, a murine IgM monoclonal antibody specific for OspA antigen is prepared and used together with the IgG1 antibody for identification of OspA epitopes.

MATERIALS AND METHODS

Source of recombinant OspA (rec-OspA). Recombinant OspA (rec-OspA) was a gift from Dr. John Donn (Dept. of Biology, Brookhaven National Laboratory, Upton, New York, USA [18].

Immunization protocol and cell fusion. Female BALB/c mice were immunized intraperitoneally (i.p.) with 25 µg rec-OspA in Freund's complete adjuvant followed by a boost in Freund incomplete adjuvant. Animals that showed positive results by ELISA at serum dilution of 1:200,000 received a final i.p. injection of the same amount of antigen in saline, followed by fusion 3 days later. Spleen cells from immunized mice (about 10^8) were fused with 2 × 10^7 mouse P3-X63-Ag8.653 myeloma cells (ATTC, USA) at a ratio of 5:1, using 50% polyethylene glycol (PEG 1500, Boehringer Mannheim) [19]. The fusion mixture was suspended in HAT medium and aliquot of 200 µl (2.5×10^5 cells) were suspended into 96-well plates. After 2 weeks, supernatants of growing hybridomas were screened for antibody production by ELISA.

ELISA screening assay. An indirect ELISA was used to detect anti-OspA antibody in culture supernatant. Briefly, microtiter plates were coated with 50 µl of 10 µg/ml of rec-OspA or sonicatedspirochetal antigen at 50 µg/ml for 1 h at room temperature (RT). The wells were washed with washing buffer (PBS, 0.1% BSA and 0.05% Tween 20, pH 7.2) and blocked with 250 µl of 1% BSA (Sigma Chemical Company, St. Louis, USA). Fifty microliters of hybridoma supernatant was added to the wells and incubated for 1 h at RT. After washing, alkaline phosphatase-conjugated anti-mouse polyclonal antibody (Sigma, USA) was added and incubated for 1 h at RT. After addition of substrate solution, p-nitrophenyl phosphate disodium (Sigma, USA), positive wells were detected by ELISA reader at 410 nm. The positive clones were immediately subcloned by limiting dilution and were expanded and cryopreserved or grown as ascites into the pristane-primed mice. The isotype of the isolated immunoglobulin was determined by ELISA using Mouse-Type Sub-Isotyping Kit (Bio-Rad, Richmond, CA).

Purification of OspA by monoclonal antibody. Monoclonal antibody was purified from ascites fluid or cell culture supernatant by ion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia, Piscataway, NJ) using 0.1- 1.0 M NaCl in 0.1 M Tris buffer [17]. Eluted antibody was then applied to S-200 Sephacryl column for further purification. Protein concentration was determined by Pierce Protein Assay Reagent (Pierce, Rockford, IL). The purified antibody (2 mg) was coupled to 1 ml Reacti-Gel (6×) activated agarose beads according to the Pierce Chemical Company. Sonicated B. burgdorferi was centrifuged and the clear supernatant was added to the column and the OspA was eluted with 0.1 M Glycine-HCl (pH 2.5). Purity of OspA was determined by gel electrophoresis.

Gel electrophoresis and Western immunoblotting. SDS-PAGE was performed on 12.5% slab gel according to Laemmli's method [20]. Gels were stained with Coomassie Blue. Proteins separated by SDS-PAGE were transferred to 0.45 µm pore size nitrocellulose sheets (Schleicher & Schuell, Keen, NH) by semi-dry blotting technique [21]. Subsequently, the nitrocellulose sheet was blocked and then incubated with hybridoma culture supernatant overnight at 4ْC as described previously [17]. After washing, second antibody (anti-mouse polyvalent alkaline phosphatase-conjugated antibody) was added and the immune complex was detected by a color reaction using BCIP/NBT substrate (Sigma).

ELISA additive assay. The additivity test was performed according to the procedure of Friguet *et al.* [22]. This procedure is the same as ELISA described above. Briefly, a fixed amount of antigen

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was coated to microtiter plate (1 m g/ml) in order to achieve antigen saturation with each monoclonal antibody (50 m l). To quantitate the experimental results of the additivity test, the additivity index was determined by the following formula [22].

$$\text{Additivity Index} = \left(\frac{2A_{1+2}}{A_1 + A_2} - 1\right) \times 100$$

Where A_1, A_2 and A_{1+2} are the absorption reached in ELISA with the first antibody alone, the second antibody alone and the two antibodies together. If the two antibodies bind independently at distinct sites, A_{1+2} should be the sum of A_1 and A_2, and index will be equal to 100%.

RESULTS AND DISCUSSION

Identification and purification of monoclonal antibody. Hybridoma culture supernatants were screened by ELISA for specific antibody production against OspA, using rec-OspA and whole cell sonicates of B. burgdorferi coated plates. Culture supernatants showing reactivity with rec-OspA were cloned by limited dilution and subcloned twice. One clone, designated MA-2C6, was stable more than a year and retained strong reactivity to OspA. This antibody was determined by ELISA to be Ig Misotype with kappa light chains. Figure 1 shows the reactivity of MA-2C6 culture supernatant to different concentration of antigen coated to the 96-well plate. The minimum amount of OspA antigen recognized by MA-2C6 culture supernatant in ELISA was approximately 15 ng for rec-OspA and about 25 ng for B. burgdorferi cell lysate. No reactivity was observed with lysates of *B. hermsii* and *T. pallidum*. Monoclonal antibody from culture supernatant and from ascites fluid was purified by ion-exchange chromatography and gel filtration columns and was coupled to the activated agarose. Figure 2 shows the purity of the eluted OspA from affinity column after applying B. burgdorferi cell lysate to the column.

Specificity of MA-2C6. Current serological tests for Lyme disease diagnosis usually give false negative and more commonly, false positive results both in healthy subjects and in patients with a variety of other diseases [3, 8]. These diseases include: syphilis, rocky mountain spotted fever, autoimmune diseases, and neurologic disorders [3, 7].

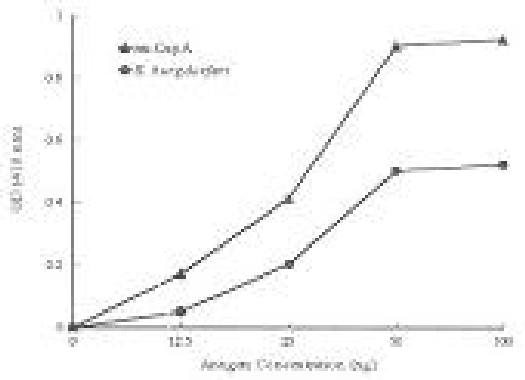


Fig. 1. ELISA titration curves of MA-2C6 monoclonal antibody against different concentration of antigen preparations. Different concentration of rec-OspA and *B. burgdorferi*, *T. pallidum*, *B. hermsii* cell lysates were prepared and coated on the microtiter plates.

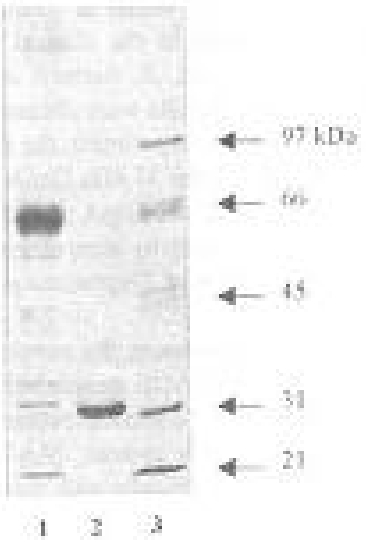


Fig. 2. SDS-PAGE analysis of the purified OspA from affinity column. Lane 1, *B. burgdorferi* cell lysate; lane 2, affinity purified OspA, and lane 3, protein markers.

To determine the antigenic specificity of the MA-2C6 monoclonal antibody toward OspA antigen of *B. burgdorferi*, its reactivity was investigated using several isolates of *B. burgdorferi* from USA and Europe and some non-Lyme disease bacteria. These bacteria were sonicated and the proteins were used for assay by ELISA and immunoblot. Table 1 shows reactivity of MA-2C6 antibody with several strains of *B. burgdorferi* except with Swedish (SW), German (G1) and Russian (IP3) isolates. No cross reactivity was observed with any proteins of

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Table 1. Reactivity of MA-2C6 antibody to different strains of *B. burgdorferi*by ELISA and Western blot. For ELISA 2.5 m g and for blot 10 m g of lysate were used.

***B. burgdorferi* strains**	**ELISA (OD 410)***	**Western blot**
B31 (New York, USA)	0.352	+
FR-2 (Germany)	0.293	+
CA 287 (California, USA)	0.308	+
126B (New York, USA)	0.293	+
297 (New York, USA)	0.361	+
SW (Sweden)	0.014	-
IP3 (Russia)	0.013	-
G1 (Germany)	0.012	-
Control bacteria:		
E. coli	0.013	-
S. aureus	0.011	-
T. pallidum	0.012	-
B. hermsii	0.009	-

*The data shown are the mean of three experiments.



Fig. 3. Western blot analysis of monoclonal antibody MA-2C6 against 31 kDa OspA protein of *B. burgdorferi.* Bacteria were sonicated and approximately 10 µg of each cell lysate was separated by SDS-PAGE and immunoblotted onto the nitrocellulose paper. Lane 1, *B. burgdorferi* 297 (New York isolate) cell lysate, Lane 2, affinity purified OspA from B1 (New York isolate); lane 3, non-Lyme disease bacterium *T. pallidum,* and lane 4 rec-OspA.

Treponema pallidum, which is responsible for the false positive results in the clinical diagnostic of Lyme disease, *E. coli*, *B. hermsii* and *S. aureus* lysates. The same results were obtained by Western blot analysis. In this experiment, the antibody could react specifically to the 31 kDa OspA protein of the B. burgdorferi lysate, rec-OspA and affinity purified OspA. No such reactivity was observed with non-Lyme disease proteins of *Treponema pallidum* (Fig. 3).

In the above experiment, the samples were boiled for 10 min in SDS-PAGE sample buffer containing SDS and 10% 2-mercaptoethanol. Despite denaturation of OspA protein, MA-2C6 antibody still could react specifically with the 31 kDa OspA protein suggesting that the antibody is directed against a linear epitope of OspA and not a conformational epitope.

ELISA additivity test. This test is based on an estimation of the number of antigenic sites simultaneously available to a pair of antibodies on the antigen [22]. For this test, the antigen must be saturated with each antibody tested. For this reason, the saturation curves of the antigen by each antibody were determined by ELISA. Table 2 shows the epitope specificity of the two monoclonal antibodies analyzed by adding to each well sufficient amount to saturate the coated antigen. The OD obtained for each antibody indicating that 2 monoclonal antibodies bind simultaneously to different epitopes of OspA. The additivity index of the results obtained in Table 2 can be written as 94.4% that

Table 2. ELISA additive assay for identification of epitopes .OspA antigen (1m g/ml) was coated to 96- well plate. Monoclonal antibodies were added (100 m l) either separately or simultaneously in order to saturate the antigen. Rabbit anti-mouse was used as second antibody. The data are the mean of three experiments.

Antigen	**First antibody 50 «-ª:H:RM:«-ª**	**OD (410 nm)**	**Theoretical sums**
OspA	MA-2G9 + buffer	0.50	-
OspA	MA-2G9 + MA-2G9	0.51	-
OspA	MA-2C6 + buffer	0.92	-
OspA	MA-2C6 + MA-2C6	0.93	-
OspA	MA-2G9 + MA-2C9	1.39	1.43
None	MA-2G9 + MA-2C9	0.02	-

clearly shows the binding of MA-2G9 and MA-2C6 are additive.

The data presented here show that monoclonal antibody MA-2C6 can be useful in a variety of applications. It could be used for purification of the OspA from B. burgdorferi lysate or for immunoprecipitation. It could be valuable for diagnostic purposes for developing a sensitive immunoassay for the early detection of B. burgdorferi antigen in urine [9, 10] or in serum [11] using ELISA [6] and Western blotting [7]. In the

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mouse model, it has been shown that anti-OspA antibody could protect the animal against B. burgdorferi infection and development of the disease [13, 14]. The anti-OspA of the immune hosts enters ticks during blood feeding and destroys spirochetes before transmission to the host [15]. It seems that OspA mediates spirochete attachment to the tick gut by binding to an *Ixodes scapularis* [15]. Recently, the world's first Lyme disease vaccine was made available to doctors [23]. The US Food and Drug Administration (FDA) in December 1998 approved SmithKline Beecham's Lymerix for vaccination of people ages 15 to 70. This vaccine offers the first immune protection and creates antibodies that recognize OspA of the Lyme bacterium [23]. Almost 74% of vaccinated humans with OspA generate growth-inhibiting antibody against B. burgdorferi [24]. This antibody may predict protection of vaccines against infection. Out of three forms of OspA vaccine [25], it was shown the lapidated OspA has the best potential for induction of a protective effect in human by activating monocytes to induce cytokines (IL-1β, IL-6, IL-10 and TNF).

Since OspA molecule is immunogenic, the MA-2C6 monoclonal antibody may be useful for identification and isolation of immunologic peptides representing non-variable part of the OspA molecule that could be important for vaccine development.

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