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Immunology

Monoclonal antibodies specific for the outer surface protein A (OspA) of *Borrelia burgdorferi* prevent Lyme borreliosis in severe combined immunodeficiency (*scid*) mice

(spirochete/humoral response/immunogenic epitope/protection)

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ABSTRACT **We have recently shown that viable *Borrelia burgdorferi* organisms induce a chronic infection associated with arthritis and carditis in severe combined immunodeficiency (*scid*) mice but not in immunocompetent mice. The disease is similar to that found in patients suffering from Lyme disease. We now show that *B. burgdorferi*-specific immune mouse sera as well as a monoclonal antibody to the spirochetal outer surface antigen A (31 kDa) but not monoclonal antibodies specific for the 41-kDa antigenic component of the periplasmic flagella are able to prevent (or mitigate) the development of the disease in *scid* mice when passively transferred at the time of the bacterial inoculation. The identification of a *B. burgdorferi*-associated protective antigen suggests that the corresponding spirochetal protein should be tested as a vaccine against Lyme disease.**

Lyme borreliosis is a tick-borne disease caused by the spirochete *Borrelia burgdorferi*. In humans Lyme disease is a multisystem illness with dermatologic, rheumatic, cardiac, and neurologic manifestations. Although patients with Lyme borreliosis develop specific antibodies and T-cell responses to *B. burgdorferi*, their immune reactions do not seem to convey protection (1). Laboratory models for Lyme arthritis have been described in rats (2) and hamsters (3, 4) and in severe combined immunodeficiency (*scid*) mice (5). All three species developed arthritic lesions similar to those found in patients with Lyme disease (2–5). Moreover, *B. burgdorferi*-infected *scid* mice showed additional clinical symptoms, such as inflammatory lesions of heart tissue (5). The facts that most normal mice develop only subclinical infections in response to *B. burgdorferi* and that hamsters could be passively immunized against infective doses of spirochetes with immune rabbit serum (6) prompted us to search for protective antibodies against the clinical consequences of *B. burgdorferi* infection in *scid* mice.

MATERIALS AND METHODS

Mice and Inoculation with *B. burgdorferi*. Adult mice of strains C.B-17 *scid* (*scid*), C.B-17, and C57BL/6 were bred under specific-pathogen-free conditions at the Max-Planck-Institut für Immunbiologie (Freiburg, F.R.G.). Female animals between 6 and 8 weeks of age were used in this study. Mice were inoculated with 1×10^8 *B. burgdorferi* ZS7 organisms s.c. in the tail.

Bacteria: Isolation and Identification. The low-passage (two *in vitro* passages) tick isolate *B. burgdorferi* ZS7 and the high-passage variant of the strain B31 (ATCC 35210) were used. The methods for reisolation of spirochetes from blood of infected *scid* mice as well as for detection of spirochetes in blood have been described (5).

Immune Sera, Monoclonal Antibodies (mAbs), Treatment of Mice, and Serological Tests. The anti-B31 immune serum (IS) was taken from C57BL/6 mice 91 days after s.c. inoculation with 1×10^8 *B. burgdorferi* B31 organisms, the anti-ZS7 IS was taken from C57BL/6 mice 68 days after subcutaneous inoculation with 1×10^8 *B. burgdorferi* ZS7 organisms; both sera contained specific antibodies (60 μg/ml), as measured in an ELISA system as described (5). The normal mouse serum (NMS) was taken from uninfected C57BL/6 mice. mAbs LA-2 (IgG2b) and LA-5 (IgG2a), which are specific for the outer surface protein A (OspA), and mAbs LA-10 (IgG2a) and LA-21 (IgG1), which are specific for the flagellin (Table 1), were obtained as described (7), purified, and stored at −20°C at 1 mg/ml.

At the time of inoculation and subsequently at 4-day intervals, the indicated mAbs, IS, NMS, or isotonic phosphate-buffered saline (PBS) were passively transferred i.p. to *scid* mice by the following protocol: day 0 and day 3, 100 μl; day 7 and day 10, 200 μl; day 13 and day 17, 300 μl.

Throughout the observation period, blood samples were taken from individual reconstituted *scid* mice and the *B. burgdorferi*-specific antibodies were measured in a solid-phase ELISA system on soluble antigens of strain B31 and strain ZS7 as described (5). Values of titers are expressed as the mean from three mice.

Western Blot Analysis. Whole cells of *B. burgdorferi* strains B31 or ZS7 (4×10^8 cells) were lysed in SDS buffer, separated by SDS/PAGE on 12% polyacrylamide gels and transferred to nitrocellulose. After blocking with 0.1% bovine serum albumin, the membrane was incubated with the respective mAbs (1:10,000 dilution), IS or NMS (1:50 dilution), or sera from previously reconstituted *scid* mice (1:50 dilution), as described (7).

Pathology and Histopathology. Mice were inspected daily for the appearance of clinical signs of arthritis in tibiotarsal joints. At 21 days after infection, *scid* mice were sacrificed and tissue sections from indicated organs were evaluated for histopathological alterations as described (5).

Macrophages and Bioluminescence Assay. Bone marrow-derived macrophages from (BALB/c × C57BL/6)F_1 mice were generated with modifications as described (8). Briefly, bone marrow cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 15% (vol/vol) supernatant from L929S cells in Teflon bags for 11 days. Approximately 2×10^6 *B. burgdorferi* strain ZS7 spirochetes freshly

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Abbreviations: mAb, monoclonal antibody; IS, immune serum; NMS, normal mouse serum.

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Table 1. Antigen specificities and isotypes of antibody preparations used for passive transfer

Antiserum or mAb	Specificity for *B. burgdorferi* structures of strains B31 and ZS7					Isotype
	20 kDa	OspA (31 kDa)	OspB (34 kDa)	41 kDa	60/65/70 kDa	
C57BL/6 anti-B31	+	+	+	+	+	ND
C57BL/6 anti-ZS7	+	+	+	+	+	ND
mAb LA-2		+				IgG2b
mAb LA-5		+*				IgG2a
mAb LA-10				+		IgG2a
mAb LA-21				+		IgG1

+, Specificity observed; ND, not determined.
*mAb LA-5 only recognizes OspA of strain B31 not OspA of ZS7.

isolated from cultured medium were washed twice in PBS and incubated for 1 hr at 37°C with the respective mAbs, IS, or NMS at a 1:10 dilution or with PBS; spirochetes were then washed three times in PBS and added to 2×10^5 adherent bone marrow cells in DMEM (400 μl) without phenol red supplemented with 10 μl of lucigenin (final concentration, 68.4 μg/ml; Boehringer Mannheim); tubes were centrifuged for 3 min at 5000 × *g* and 4°C and bioluminescence was measured for 30 min at 37°C in a Biolumat (Berthold, Wildbad, F.R.G.). Values represent the integrals of light emission over a 30-min period, measured as cpm and expressed as the mean ± standard deviation of three measurements. Background bioluminescence ($4 \pm 1.7 \times 10^3$ cpm) was the mean + 2 standard deviations from six measurements using spirochetes preincubated with PBS.

RESULTS AND DISCUSSION

scid mice inoculated s.c. in the tail with 1×10^8 viable *B. burgdorferi* organisms of the low-passage virulent strain ZS7 showed clinical signs of arthritis in their tibiotarsal joints around day 7 after inoculation and developed a persistent spirochetosis associated with a progressive disease involving mainly the joints and the heart, in the absence of any detectable specific immune response (5). By day 21, inflammatory reactions were found in the synovium and periarticular tissue as well as in the heart with infiltrations consisting of mononuclear cells including monocytes and granulocytes but no lymphocytes (Table 2 and refs. 5 and 23). Destructive bone erosions appear at multiple sites of the hind limb joints (unpublished results).

To study the influence of *B. burgdorferi*-specific antibodies on the development of the disease in *scid* mice, heterogeneous antibody and mAb preparations were used for passive-transfer experiments. The specificities of the individual antibody preparations as revealed by Western blot analyses on antigen preparations of strains ZS7 and B31 as well as the isotypes of mAbs are documented in Table 1 and Fig. 1*A*. mAbs LA-2 and LA-5 recognize OspA (9) and are of the isotypes IgG2b and IgG2a, respectively. The fact that mAb LA-5 only reacts with OspA of strain B31 but not with that of strain ZS7 indicates antigenic variations between different isolates of *B. burgdorferi* (7, 10). mAbs LA-10 and LA-21 are specific for the flagella-associated 41-kDa periplasmic protein (11). Immunofluorescence studies showed that both OspA epitopes recognized by mAbs LA-2 and LA-5 are exposed on living *B. burgdorferi* cells whereas the flagella-associated epitope(s) seen by mAbs LA-10 and LA-21 are, if at all, only marginally expressed (data not shown). As expected, both C57BL/6 IS (anti-ZS7 and anti-B31) were polyspecific and recognized, in addition to OspA and the 41-kDa protein, polypeptides with molecular masses of approximately 20 kDa (pC) (7, 10), 34 kDa (outer surface protein B, termed OspB; ref. 9), 55 kDa, 60 kDa (7), and 80 kDa, respectively. C57BL/6 NMS did not contain any *B. burgdorferi*-specific antibodies.

The first dose of each antibody preparation was passively transferred to *scid* mice i.p. at the time of the bacterial inoculation (1×10^8 spirochetes s.c. in the tail). Further injections of increasing amounts of antibody preparations were given at half-weekly intervals for 3 weeks to achieve increasing antibody titers in the host. The results show that roughly similar concentrations (1–5 μg of antibodies per ml) of the injected polyclonal IS or mAbs, except for anti-B31 IS (0.5 μg of antibodies per ml), could be maintained in the sera of treated *scid* mice throughout the observation period (Fig. 1*C*). In additional experiments even higher titers of polyclonal antibodies or mAbs (up to 25 μg/ml) were found in the sera of reconstituted *scid* mice, which were comparable to those found in the IS used for reconstitution (≈60 μg/ml). Western blot analyses of individual sera revealed that the specificity of the antibody recovered from the sera of treated *scid* mice (Fig. 1*B*) was identical to those of the injected mAb or polyclonal IS preparation (Fig. 1*A*).

The *scid* mice treated with anti-ZS7 IS, anti-B31 IS, or mAb LA-2 did not develop apparent clinical symptoms of

Table 2. Effect of *B. burgdorferi*-specific IS and mAbs on the evolution of experimental Lyme borreliosis in *scid* mice

Treatment	Mice, no.	Clinical arthritis	Histopathology		Detection of *B. burgdorferi*	
			Periarthritis/ arthritis	Carditis	Immuno-fluorescence	Cultivation
PBS	8	+	+	+	+	+
NMS	3	+	+	+	+	+
Anti-B31 IS	3	−	−	∓	±	+
Anti-ZS7 IS	2	−	−	∓	−	−
mAb LA-2 (OspA)	6	−	∓	∓	−	−
mAb LA-5 (OspA)*	3	+	+	+	+	+
mAb LA-10 (41 kDa)	3	+	+	+	±	+
mAb LA-21 (41 kDa)	3	+	+	±	+	+

Degree of histopathological alteration is indicated as follows: +, severe; ±, moderate; ∓, subclinical; −, none. Detection of *B. burgdorferi*: +, yes; ±, not detectable in every mouse; −, not detectable.
*mAb LA-5 recognizes only the 31-kDa protein of strain B31 but not that of strain ZS7.

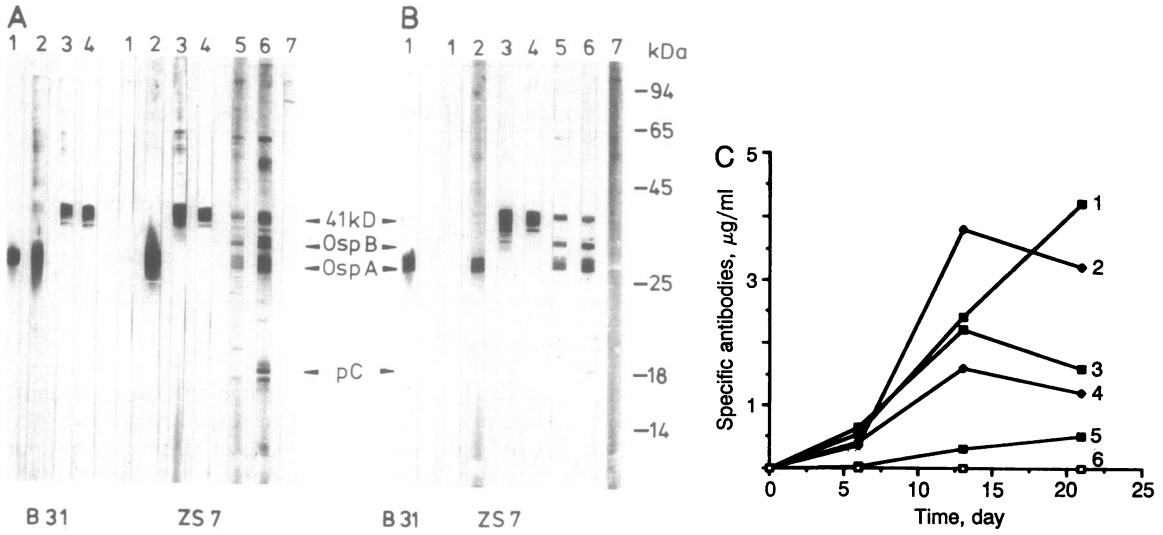

FIG. 1. Western blot analysis of *B. burgdorferi*-specific antibodies. (*A*) mAbs, IS, and NMS used for reconstitution of *scid* mice, tested on strains B31 and ZS7 of *B. burgdorferi*. (*B*) Sera from individual *scid* mice reconstituted with the respective mAbs, IS, or NMS, tested on strain ZS7 (strain used for infection of mice) and on strain B31. The following mAbs or sera were used: mAb LA-5 (lane 1), mAb LA-2 (lane 2), mAb LA-10 (lane 3), mAb LA-21 (lane 4), anti-B31 IS (lane 5), anti-ZS7 IS (lane 6) and NMS (lane 7). (*C*) Amount (μg/ml) of *B. burgdorferi*-specific antibody(ies) in reconstituted *scid* mice during the observation period (one representative experiment). Curves: 1, mAb LA-10; 2, mAb LA-21; 3, mAb LA-2; 4, mAb LA-5; 5, anti-B31; 6, PBS.

arthritis (i.e., no reddening and swelling of tibiotarsal joints was apparent during 21 days of observation). The fact that another OspA-specific mAb (LA-26.1) of the isotype IgG1 was also able to mitigate clinical symptoms of arthritis indicates that both immunoglobulin subclasses IgG1 and IgG2b may participate in the protection against arthritis in inoculated *scid* mice. In contrast, *scid* mice that had received PBS, NMS, mAb LA-5, mAb LA-10, or mAb LA-21 showed clinical signs of arthritis similar to those observed in untreated *scid* mice (Table 2); the severity of arthritis in those latter animals increased with time after inoculation and did not resolve during the entire observation period. No spirochetes could be isolated from *scid* mice injected with either anti-ZS7 IS or mAb LA-2. In contrast, they were readily detected either by immunofluorescence or by cultivation in blood samples of *scid* mice that had received PBS, NMS, or mAbs LA-5, LA-10, or LA-21 (Table 2).

These results suggest that antibodies to OspA but not those to the flagella-associated periplasmic protein protect against *B. burgdorferi* infection in *scid* mice. Although LA-5 is also specific for OspA, this mAb was not expected to be protective as it did not recognize OspA on strain ZS7 used for infection. Note that spirochetes were also found in the blood of *scid* mice treated with anti-B31 IS; however, these mice did not show any signs of clinical arthritis. Hence, at this concentration, antibodies against strain B31 are able to cross-protect at least partially against the consequences of the infection with *B. burgdorferi* strain ZS7 but do not seem to eradicate effectively with spirochetes from the host. Histological evaluation of the joints and the heart of mice in the individual groups revealed that *scid* mice treated with mAb LA-2 (Fig. 2 *b* and *d*) or with anti-ZS7 IS or anti-B31 IS (data not shown) did not develop the arthritic and cardiac lesions that are found in *B. burgdorferi*-inoculated control *scid* mice treated with PBS or NMS (Table 2). However, some of these mice (≈10%) presented evidence of subclinical inflammations in the joints and the heart. In contrast, *scid* mice that had received mAb LA-5 (Table 2 and Fig. 2 *a* and *c*), LA-10, or LA-21 (data not shown) developed destructive inflammatory lesions in the joints (Fig. 2*a*) and the heart (Fig. 2*c*).

Our findings that heterogeneous antibodies or mAbs specific for OspA at 0.5–5 μg/ml are able to prevent Lyme disease in *scid* mice confirm and extend previous studies of Johnson *et al.* (6, 12) and Schmitz *et al.* (13). They showed that exposure of hamsters to small amounts (0.0125 ml) of immune rabbit serum (6) or to highly diluted (1:20) immune hamster serum (13) conferred protection against spirochetemia or prevented induction of arthritis, respectively. Optimal protection in *scid* mice was only achieved when anti-ZS7 IS or mAb LA-2 were given at the same time with but not after the spirochetal inoculation (data not shown), which is also in line with the study of Johnson *et al.* (6). These data emphasize the necessity for an early antibody response to structures of the outer surface of *B. burgdorferi* to achieve optimal elimination of the spirochetes.

Our results suggest that OspA expresses an epitope(s) important for the induction of protective antibodies. In fact, it was found in preliminary experiments that purified native OspA induces specific antibodies in immunocompetent mice that, upon passive transfer, protect *scid* from spirochetal infections (unpublished results). In addition, we have now prepared antisera against recombinant OspA proteins previously isolated from expression libraries of strains B31 (9) and ZS7 (14). It was found in preliminary experiments that passively transferred anti-OspA IS (Z57) conveys protection against *B. burgdorferi* infection in *scid* mice (unpublished results).

At present, the mechanism(s) by which specific antibodies inhibit spirochetosis and the development of disease in *scid* mice is not known. The effective elimination of spirochetes by an IgG2b antibody (mAb LA-2) that is both able to fix complement (15) and to bind to Fc receptors (16) suggests at least two immune-clearance mechanisms that may either act in concert or independently: (*i*) spirochetes may be killed by antibody-dependent complement-mediated lysis, as shown (17); (*ii*) the organisms may be eliminated by Fc receptor-mediated phagocytosis, as proposed (16). The second possibility is supported by the finding that spirochetes preincubated *in vitro* with mAb LA-2, anti-ZS7 IS, or anti-B31 IS, but not with mAbs LA-5, LA-10, or LA-21 were potent activators of bone marrow derived macrophages, as revealed by the induction of oxygen burst (Fig. 3). The preliminary finding that mAb LA-26.1 of the isotype IgG1, which cannot fix complement (15), was also able to prevent, at least in part,

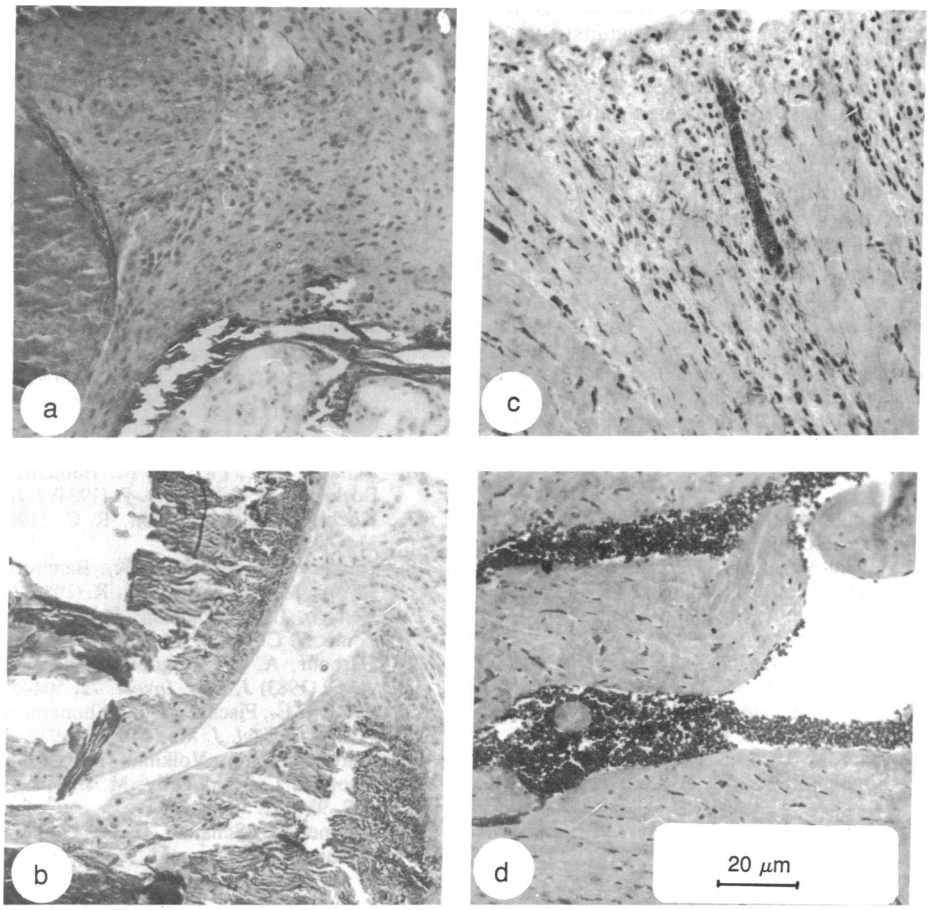

FIG. 2. Histopathology in *scid* mice simultaneously inoculated s.c. with *B. burgdorferi* and i.p. with mAb LA-5 (*a* and *c*) or mAb LA-2 (*b* and *d*). (*a*) Tibiotarsal joint of a *scid* mouse treated with LA-5. (*b*) Tibiotarsal joint of a *scid* mouse treated with the mAb LA-2. (*c*) Heart of a *scid* mouse treated with mAb LA-5. (*d*) Heart of a *scid* mouse treated with mAb LA-2.

arthritis in inoculated *scid* mice indicates that spirochetes may be eliminated independently of the classical complement pathway. However, these data do not exclude other effector mechanisms such as by factors that may be induced in mononuclear leukocytes during infection of *scid* mice (18).

In view of the present data and the fact that patients with Lyme disease produced considerable amounts of *B. burgdorferi*-specific antibodies, the question arises why the humoral response is protective in experimentally inoculated mice (5, 19) but not in patients with Lyme disease (20, 21). This may be due to qualitative differences between the antibodies generated by both species. Previous reports have shown that the first antibodies generated in patients with Lyme disease are restricted primarily to the 41-kDa flagella-associated antigen whereas antibodies to OspA and/or OspB are generated only in later stages of the illness (21, 22). Among the mAbs so far tested, only antibodies to OspA but not those to the 41-kDa polypeptide are effective in the elimination of spirochetes. The delayed appearance of antibodies to OspA in patients may allow time for the *B. burgdorferi* organisms to evade into immuno-privileged sites and to induce pathological and/or immunopathological reactions. This may also explain the persistence of spirochetes in untreated patients during all stages of the disease. In contrast, in the experimental infection of immunocompetent mice the first antibodies to be detected in the serum are those reacting with OspA and OspB of *B. burgdorferi* (19). The early appearance of such antibodies may, therefore, be a key factor in the prevention of a severe chronic disease in this species. Although the reason for the different kinetics in the generation of protective antibodies in mouse and man is not known, it is possible that they are due to the different ways of inoculation (i.e., experimental vs. natural infection) or to differences in the processing of bacterial antigens in both species.

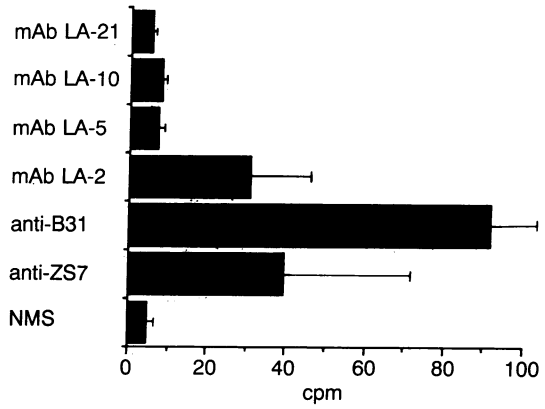

FIG. 3. Effect of preincubation of *B. burgdorferi* organisms of strain ZS7 with the respective mAbs, IS, or NMS on the induction of oxygen burst (and phagocytic activity) of bone marrow-derived macrophages. Cells were incubated with spirochetes pretreated with mAbs LA-2, LA-5, LA-10, or LA-21, anti-B31 IS, anti-ZS7 IS, or NMS and were tested for oxygen burst by bioluminescence. Spirochetes preincubated with PBS alone served as control. Data are cpm $\times 10^3$.

Our knowledge of the fate of invading spirochetes in the host, as well as on the processes leading to disease are only fragmentary at present. It is, therefore, premature to assume

that immunization with an outer surface structure of *B. burgdorferi* may generate complete protection against Lyme borreliosis. Additional studies are also needed to define whether the immune sera against OspA of one strain of *B. burgdorferi* are effective against other strains of the same species of Borrelia. However, the data presented here emphasize the potential role of antibodies against surface structures of *B. burgdorferi* in the control of Lyme disease. The definition of a protective epitope identified by mAb LA-2 may now allow the further testing of this antigen for its suitability as vaccine against Lyme disease.

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