Interactions of OspA Monoclonal Antibody C3.78 with *Borrelia burgdorferi* within Ticks

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The *Borrelia burgdorferi* outer surface protein A (OspA) vaccine induces antibodies that prevent transmission from the tick to the host. Here we describe studies with an OspA monoclonal antibody (C3.78) to understand the mechanism by which antibodies entering the tick block *Borrelia* transmission. Host complement in the tick's blood meal did not contribute to protection because the antibody was equally effective whether infected ticks fed on normal or complement-deficient mice. Antibody-mediated cross-linking of bacteria or cross-linking of OspA molecules was not required for protection because C3.78 Fab′ fragments were as effective as whole antibody molecules. At low C3.78 concentrations, transmission was blocked despite the presence of many live spirochetes within the tick, confirming that clearance of *Borrelia* organisms was not required to block transmission. We propose that OspA antibody binding to the surface of spirochetes blocks transmission by a mechanism that does not require bacterial killing.

Borrelia burgdorferi, the agent of Lyme disease, is the most common vector-borne disease in Europe and North America (12, 17). A recombinant protein vaccine, based on outer surface protein A (OspA) of the spirochete, has been shown to be an arthropod-specific transmission-blocking vaccine (3, 6, 7). When a *Borrelia*-infected tick feeds on a vaccinated host, OspA antibodies enter the gut of feeding ticks, bind to spirochetes, and prevent transmission. When infected ticks feed on hyperimmunized mice with high concentrations of circulating OspA antibody, the bacteria are killed within the tick and transmission is aborted (7, 13). When infected ticks feed on mice with low concentrations of circulating OspA antibody, live spirochetes persist within the feeding tick gut, yet spirochetes are not observed in the salivary gland and transmission to the host is blocked (4).

The mechanism by which transmission is blocked despite the presence of live bacteria within the feeding tick is not known. Here we report on studies done with OspA monoclonal antibody C3.78 to better understand the mechanism by which transmission is blocked at low antibody concentrations.

MATERIALS AND METHODS

Mice and ticks. Female C3H HenJ mice, 4 to 6 weeks of age, were obtained from the National Institutes of Health (Bethesda, Md.). The ticks used in this study were raised by placing larval *Ixodes scapularis* on mice infected with *B. burgdorferi* strain B31 (from Shelter Island, N.Y.). Transgenic C57BL/6 mice deficient in the C3 component of the complement system (kindly provided by M. C. Carroll, Center for Animal Resources and Comparative Medicine, Harvard Medical School, Boston, Mass.) and wild-type C57BL/6 mice were raised and used for the experiments (18). The ticks were kept in a humid chamber at 21°C and allowed to molt. After the molt, infection prevalence was assessed for the nymphs. Individual nymphs were homogenized in phosphate-buffered saline and spotted onto slides. The homogenates were acetone fixed and blocked in 5% fetal bovine serum–phosphate-buffered saline at room temperature for 1 h. Twenty-five microliters of goat anti-*B. burgdorferi*-fluorescein isothiocyanate (1:

200) was applied to each spot and incubated at room temperature for 1.5 h. The slides were washed, and Anti-Fade (Molecular Probes, Eugene, Oreg.) mounting medium was applied. Eighteen of 20 nymphs assessed were positive for *B. burgdorferi*.

Antibodies. Monoclonal anti-OspA antibody C3.78 (immunoglobulin G3) (kindly provided by Fred Kantor, Yale University) was purified from hybridoma supernatants produced in a hollow-fiber bioreactor unit. To purify the antibody, 10 ml of supernatant was passed over a 1-ml agarose-protein A column (Sigma, St Louis, Mo.) and washed twice with 20 ml of phosphate-buffered saline, pH 7.4. The monoclonal antibody was eluted in 1 ml of 0.1 M glycine, pH 3.0. Fractions containing antibodies were combined and dialyzed overnight in 1 liter of phosphate-buffered saline, pH 7.4. The purification yielded 3 ml of C3.78 at 8.4 mg/ml. The control immunoglobulin G3 (Southern Biotech, Birmingham, Ala.) was dialyzed in 1 liter of phosphate-buffered saline to remove sodium azide prior to use.

Fab′ fragments were prepared by digesting C3.78 with immobilized papain (Pierce, Rockford, I;ll.). Five milligrams of C3.78 was dialyzed into a 20 mM sodium phosphate–10 mM EDTA buffer, pH 7.0. Prior to digestion, cysteine-HCl was added to bring the concentration to 20 mM. The antibody solution was mixed with immobilized papain (prepared as per the manufacturer's directions) and incubated overnight at 37°C in a shaking water bath. Papain beads were separated by centrifugation, and the undigested antibody was removed by incubating with immobilized protein A for 1 h at 37°C in a shaking water bath. Digestion and removal of undigested antibodies were confirmed by using sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and visualizing the proteins by Coomassie blue staining.

Immunization. Mice were passively immunized intraperitoneally with phosphate-buffered saline, C3.78, C3.78 Fab′, or immunoglobulin G3 control antibodies. Antibodies were diluted in sterile phosphate-buffered saline to a final volume of inoculum of 100 μl/mouse.

Assays for determining total specific immunoglobulin G. Sera from passively immunized mice was tested for anti-OspA antibodies in 96-well enzyme-linked immunosorbent assay plates (Griener, Lake Murray, Fla.). Each well on the plate was treated with 10 ng of OspA–glutathione *S*-transferase fusion protein (OspA-GST) in 100 μl of carbonate binding buffer (pH 9.2) overnight at 4°C to coat the well with OspA. Plates were washed with Tris-buffered saline–0.2% Tween 20 (TBST) and blocked with 0.25% bovine serum albumin in Tris-buffered saline for 30 min. at 37°C. Sera from immunized mice were applied to enzyme-linked immunosorbent assay plates in multiple dilutions in blocking buffer and incubated at 37°C for 90 min. The plates were washed three times in TBST and incubated with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (1:1,000) for 60 min at 37°C. The plates were washed three times with TBST and developed with paranitrophenyl phosphate (Sigma, St. Louis, Mo.) before measuring absorbance at 405 nm.

Estimating bacterial load in ticks. Ticks were homogenized, spotted onto silylated slides, acetone fixed, and blocked with 5% fetal bovine serum–phos-

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TABLE 1. Titration of OspA-specific C3.78 necessary to protect mice[a]

C3.78 level		No. infected/total	
Immunization (μg)	Circulating (μg/ml)	Mice	Ticks
0	0	4/4	4/4
0.315	>0.5	4/4	4/4
3.15	0.55	4/4	4/4
31.5	5.3	1/4	4/4

[a] Groups of four mice were passively immunized with different amounts of C3.78 and challenged with four *B. burgdorferi*-infected *I. scapularis* ticks. The circulating concentration of C3.78 was determined by ELISA. At 3 weeks postrepletion, mice and ticks were assessed for infection by culture and indirect immunofluorescence, respectively.

phate-buffered saline at room temperature for 1 h. Twenty-five microliters of goat anti-*B. burgdorferi*-fluorescein isothiocyanate (KPL, Gaithersburg, Md.) (1:200) was applied to each spot and incubated at room temperature for 1.5 h. Slides were washed, and Anti-Fade (Molecular Probes, Eugene, Oreg.) mounting medium was applied. The bacterial load in ticks was compared by fluorescence microscopy as previously described (3).

Evaluation of mice for *B. burgdorferi* infections. Three weeks after tick detachment, mice were sacrificed, and serum, spleen, and bladder samples were obtained. Samples were placed in BSK-II medium, incubated at 35°C, and checked weekly for evidence of spirochetes by dark-field microscopy. The serum was probed for production of anti-*B. burgdorferi* antibodies by Western blot as previously done (6).

Estimating the number of *B. burgdorferi* in tick samples by quantitative PCR. Tick samples collected at 60 h of feeding and 1 week postrepletion were homogenized in 50 μl of phosphate-buffered saline, and DNA was purified with DNeasy tissue kits with the manufacturer's instructions for insect tissue (Qiagen, Valencia, Calif.). DNA was also purified with the same method from 3.0×10^7 cultured *B. burgdorferi*. The DNA from the cultured bacteria was used to set up standard curves for bacterial loads and the copy number of each gene in the mRNA analysis. The primers for *flaB* (FlaB-458F, TGCAGCCTGCAAAAATTAACA, and FlaB-559R, TCTTGGACTTTAAGAGTTCATGTTGG) amplified a 101-bp fragment. The purified DNA from each tick was used in duplicate reactions. SYBR Green was used as the detector, and the number of spirochetes per tick was determined by setting up a standard curve of the C_t (point of inflection) as read by the ABI Prism 7000 system.

RESULTS

C3.78 is a monoclonal antibody that binds to the C-terminal region of *B. burgdorferi* OspA (15). Passively administered monoclonal antibody C3.78 protects mice from tick-borne spirochetes (2, 7). We began by defining the minimum concentration of C3.78 required to protect mice from tick-transmitted *Borrelia burgdorferi* (Table 1). The majority of mice (three of

four) injected with 31.5 μg of antibody were protected, unlike mice receiving 3.1 μg or less, which were susceptible to tick-borne infection. Based on these results, we conclude that 31.5 μg of C3.78 antibody administered intraperitoneally protected mice from infection, whereas 10 times less antibody was not protective.

Role of host complement in protection of mice passively immunized with C3.78 antibody. Culture-grown *B. burgdorferi* is effectively killed by specific antibody in the presence of complement (9). Experiments were carried out with complement-deficient mice receiving low concentrations of C3.78 monoclonal antibody to determine if host complement played a role in protection. Wild-type and complement-deficient (C3-deficient) C57BL/6 mice were passively immunized with 62.5 μg of C3.78, which gave the mice circulating anti-OspA titers of approximately 10 μg/ml. Mice of both genotypes were challenged with five *B. burgdorferi*-infected nymphal ticks per mouse. The OspA antibody did not eliminate spirochetes from feeding nymphs, as similar levels of infection were observed in individual ticks irrespective of antibody treatment or mouse genotype (Table 2). However, both wild-type and C3-deficient mice receiving the C3.78 antibody were protected from infection, indicating that active complement and eradication of bacteria within ticks were not required for the protective effects of the C3.78 monoclonal antibody (Table 2).

C3.78 Fab′ fragments protect mice from *B. burgdorferi* infection. Monoclonal antibody C3.78 may block *Borrelia burgdorferi* transmission by extensive surface cross-linking of OspA proteins, cross-linking of spirochetes to one another, or by the large antibody molecules' masking a bacterial protein required for transmission. To further elucidate the mechanism of protection, experiments were done to determine if monovalent Fab′ fragments of C3.78 were capable of providing protection from infection in the tick-mouse model. C3H mice were passively immunized with whole C3.78 (300 μg), C3.78 Fab′ (200 μg), or an immunoglobulin G3 isotype control (300 μg). These concentrations result in equivalent numbers of antigen-binding domains in all the groups.

The mice were challenged 1 day after being passively immunized by placing eight infected *I. scapularis* nymphs on each mouse. One to two ticks were removed from each mouse 60 h into the blood meal and analyzed for infection by direct immunofluorescence. Ticks removed from all three groups were infected with *Borrelia burgdorferi* (Table 3). However, when the

TABLE 2. Ability of C3.78 to prevent transmission of tick-borne *B. burgdorferi* in the absence of host complement[a]

Passive immunization	Wild-type mice			Complement-deficient mice		
	No. of infected ticks/total	Relative bacterial load (no. of bacteria per tick) ± SE (n = 8)	No. of infected mice/total	No. of infected ticks/total	Relative bacterial load (no. of bacteria per tick) ± SE (n = 6)	No. infected mice total
PBS	8/8	95 ± 52	4/4	8/8	71 ± 38	4/4
C3.78 (62.5 μg)	8/8	126 ± 24	0/4	6/6	72 ± 37	0/4

[a] Groups of four normal or complement-deficient mice were immunized with either PBS or C3.78 (62.5 μg) and challenged with four infected *I. scapularis* nymphs per mouse. From each mouse, two of the nymphs were removed at 60 h and tested for spirochetes, and the remaining nymphs were allowed to feed to repletion. The results in this table are from one of two independent experiments. Ticks were assessed for infection by indirect immunofluorescence microscopy after 60 h of feeding. The bacterial load in each tick was estimated by counting the number of spirochetes in five microscope fields viewed with the 40× objective. The mean number of spirochetes per infected tick was not significantly different between C3.78 antibody-treated and contol groups or between normal and C3-deficient mice (Student's *t* test, *P* > 0.05; *n* = 6 to 8). Mice were assessed for infection by Western blot 3 weeks after tick feeding.

TABLE 3. C3.78 Fab fragments prevent transmission of tick-borne *B. burgdorferi*[a]

Immunization	Tick infection				No. of mice infected/total
	Fluorescence assay		Quantitative PCR		
	No. of infected ticks/total	Relative bacterial load (no. of bacteria per tick) $\pm$ SE ($n = 6$)	60 h of feeding (mean no. of bacteria per tick $\pm$ SE; $n = 4$)	7 days postrepletion (mean no. of bacteria per tick $\pm$ SE; $n = 4$)	
Whole C3.78 antibody (300 μg)	8/8	11.25 ± 3.25	ND	$2,076 \pm 1,216$	0/4
C3.78 Fab (200 μg)	7/8	20.38 ± 6.15	$42,529 \pm 5,897$	$1,720 \pm 1,443$	0/6
IgG3 control (300 μg)	6/6	33.17 ± 13.19	$32,136 \pm 4,173$	$4,086 \pm 2,199$	6/6

[a] Groups of mice were immunized with whole (300 μg) or Fab fragments (200 μg) of C3.78 or an immunoglobulin G3 isotype control (300 μg) and challenged with eight infected *I. scapularis* nymphs. At 60 h into the blood meal, one or two nymphs were removed from each mouse and tested for *Borrelia* infection. The remaining nymphs were allowed to feed to repletion. The results in this table are from one of two independent experiments. Ticks were assessed for infection by indirect immunofluorescence microscopy after 60 h of feeding. The bacterial load in each tick was estimated by counting the number of spirochetes in four microscope fields viewed with the 40× objective. The mean number of spirochetes per infected tick was not significantly different between the C3.78-treated and control groups (Student's *t* test, $P > 0.05$; $n = 6$). Quantitative PCR was used to determine the number of copies of *B. burgdorferi flaB* within feeding (60 h of feeding) and replete (7 days after feeding) ticks. The C3.78 antibody-treated and control groups were not significantly different from one another (Student's *t* test, $P > 0.05$, $n = 4$). Western blot and organ culture were used to determine mouse infection 3 weeks posttick repletion. ND, not determined.

mice were analyzed for infection, all mice immunized with whole or Fab′ fragments of C3.78 were protected, unlike mice immunized with the control antibody, which were infected (Table 3). Thus, simple binding of a C3.78 Fab′ fragment to the bacterial surface was sufficient to block transmission.

Do the levels of monoclonal antibody C3.78 required for protection reduce the number of bacteria within feeding ticks? From the preceding experiments it was clear that at low concentrations of C3.78 Fab′ fragments, *B. burgdorferi* transmission was blocked from ticks even though many bacteria were present within the gut of the ticks. It is plausible that the Fab′ fragments suppress growth and block transmission by preventing the bacteria from growing to a critical density required for transmission.

To explore the role of bacterial cell density, we used two approaches to estimate bacterial load within ticks. In the first approach, we removed ticks at 60 h into the blood meal and estimated the relative number of bacteria by direct immunofluorescence microscopy. The mean number of bacteria per tick was not significantly different between groups (Student's *t* test, $P > 0.05$) (Tables 2 and 3). In the second approach, quantitative PCR was used to estimate the bacterial burden in ticks feeding on control and C3.78-treated mice. Total DNA was purified from the ticks at 60 h of feeding and 1 week postrepletion. Quantitative PCR was performed with the *flaB* chromosomal gene as the target. The number of spirochetes per tick was determined by setting up a standard curve from DNA purified from known numbers of cultured *B. burgdorferi*. Similar numbers of bacteria were found in all the groups, indicating that differences in spirochete density within the tick gut were not the determining factor in protecting mice receiving a low concentration of whole C3.78 or its Fab′ fragments (Table 3).

DISCUSSION

Antibodies against OspA interfere with the transmission of *B. burgdorferi* from the vector to the host (3, 7, 8). Previously, working with hyperimmunized mice that had high levels of OspA antibody, we demonstrated that host complement was not required for spirochete clearance within ticks and for blocking transmission (13). In the current study we performed

experiments with low concentrations of OspA antibody because the synergistic effects of host complement may only be apparent at limiting antibody concentrations. Even though monoclonal antibody C3.78 has the ability to fix complement and kill spirochetes in vitro (10), we found no evidence for host complement interacting with low concentrations of C3.78 antibody entering nymphal ticks and blocking transmission.

Ticks produce anticomplement proteins that may inactivate complement at the site of tick feeding and in the blood entering the tick (13, 14, 16). Thus, in the case of both normal and C3-deficient mice, functional complement is unlikely to have been present in the blood entering the tick gut, and this may explain the similar results obtained with normal and C3-deficient mice. Our conclusion that host complement is unlikely to be functional in the tick vector also implies that complement-mediated bacterial killing is not a good assay for evaluating vaccines that target antigens expressed by or within ticks.

The ability of antibody fragments to kill *B. burgdorferi* has been shown previously (5, 11). Escudero and colleagues demonstrated that the Fab′ fragments that bound to the spirochete caused membrane blebbing, which they hypothesized was caused by destabilization of the bacterial membrane (1, 5). Recently Anderton and colleagues used microarrays to demonstrate that sublethal concentrations of an OspB monoclonal antibody induced changes in the expression of 21 genes, most of which were plasmid-encoded genes of unknown function (1). They also observed that the *blyA* and *blyB* genes were upregulated in response to antibody. These proteins have been characterized as prophage-encoded holins with the ability to insert into membranes and form pores. Anderton and colleagues propose that increased levels of functional holin proteins may damage bacterial membranes and account for the bactericidal effects of the monoclonal antibody.

When *Borrelia*-infected nymphal ticks feed on mice with high concentrations of OspA antibody, spirochetes are killed and cleared from the infected nymphs (4, 7, 13). In the current study, when mice were passively immunized to achieve a low concentration of circulating C3.78 with both immunofluorescence and quantitative PCR assays, we did not observe a significant difference in the number of bacteria in ticks feeding on immunized and control mice (Tables 2 and 3). We conclude that OspA antibody binding to spirochetes within ticks blocks

transmission by mechanisms utilizing bacterial killing and mechanisms independent of bacterial killing and that the relative contribution of these different mechanisms depends on the concentration of antibody entering the tick gut. In the current study, the mere binding of an Fab′ fragment to OspA was sufficient to block transmission.

We currently do not understand how anti-OspA Fab′ fragments block transmission from the tick to the host. Previous work has demonstrated that low concentrations of OspA antibody specifically block salivary gland infections without clearing infections in the tick gut (4). Thus, the mechanism by which Fab′ fragments blocks transmission most likely involves a block in the migration of spirochetes from the tick gut to the salivary glands. *B. burgdorferi* within feeding nymphal ticks changes the expression of many genes in preparation for transmission and adaptation to the new vertebrate host. Our current working hypothesis is that anti-OspA Fab′ fragments interfere with changes in protein expression required for tick transmission, and studies are under way to test this hypothesis.

REFERENCES

1. **Anderton, J. M., R. Tokarz, C. D. Thill, C. J. Kuhlow, C. S. Brooks, D. R. Akins, L. I. Katona, and J. L. Benach.** 2004. Whole-genome DNA array analysis of the response of *Borrelia burgdorferi* to a bactericidal monoclonal antibody. Infect. Immun. **72:**2035–2044.
2. **Bockenstedt, L. K., S. Barthold, K. Deponte, N. Marcantonio, and F. S. Kantor.** 1993. *Borrelia burgdorferi* infection and immunity in mice deficient in the fifth component of complement. Infect. Immun. **61:**2104–2107.
3. **de Silva, A. M., S. R. Telford, 3rd, L. R. Brunet, S. W. Barthold, and E. Fikrig.** 1996. Borrelia burgdorferi OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. **183:**271–275.
4. **de Silva, A. M., N. S. Zeidner, Y. Zhang, M. C. Dolan, J. Piesman, and E. Fikrig.** 1999. Influence of outer surface protein A antibody on *Borrelia burgdorferi* within feeding ticks. Infect. Immun. **67:**30–35.
5. **Escudero, R., M. L. Halluska, P. B. Backenson, J. L. Coleman, and J. L. Benach.** 1997. Characterization of the physiological requirements for the bactericidal effects of a monoclonal antibody to OspB of *Borrelia burgdorferi* by confocal microscopy. Infect. Immun. **65:**1908–1915.
6. **Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. Science **250:**553–556.
7. **Fikrig, E., S. R. Telford, 3rd, S. W. Barthold, F. S. Kantor, A. Spielman, and R. A. Flavell.** 1992. Elimination of Borrelia burgdorferi from vector ticks feeding on OspA-immunized mice. Proc. Natl. Acad. Sci. USA **89:**5418–5421.
8. **Gern, L., O. Rais, C. Capiau, P. Hauser, Y. Lobet, E. Simoen, P. Voet, and J. Petre.** 1994. Immunization of mice by recombinant OspA preparations and protection against Borrelia burgdorferi infection induced by Ixodes ricinus tick bites. Immunol. Lett. **39:**249–258.
9. **Kochi, S. K., and R. C. Johnson.** 1988. Role of immunoglobulin G in killing of *Borrelia burgdorferi* by the classical complement pathway. Infect. Immun. **56:**314–321.
10. **Kochi, S. K., R. C. Johnson, and A. P. Dalmasso.** 1991. Complement-mediated killing of the Lyme disease spirochete Borrelia burgdorferi. Role of antibody in formation of an effective membrane attack complex. J. Immunol. **146:**3964–3970.
11. **Kochi, S. K., R. C. Johnson, and A. P. Dalmasso.** 1993. Facilitation of complement-dependent killing of the Lyme disease spirochete, *Borrelia burgdorferi*, by specific immunoglobulin G Fab antibody fragments. Infect. Immun. **61:**2532–2536.
12. **Nadelman, R. B., and G. P. Wormser.** 1998. Lyme borreliosis. Lancet **352:**557–565.
13. **Rathinavelu, S., A. Broadwater, and A. M. de Silva.** 2003. Does host complement kill *Borrelia burgdorferi* within ticks? Infect. Immun. **71:**822–829.
14. **Ribeiro, J. M.** 1987. Ixodes dammini: salivary anti-complement activity. Exp. Parasitol. **64:**347–353.
15. **Sears, J. E., E. Fikrig, T. Y. Nakagawa, K. Deponte, N. Marcantonio, F. S. Kantor, and R. A. Flavell.** 1991. Molecular mapping of Osp-A mediated immunity against Borrelia burgdorferi, the agent of Lyme disease. J. Immunol. **147:**1995–2000.
16. **Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro.** 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, Ixodes scapularis. J. Biol. Chem. **275:**18717–18723.
17. **Wang, G., A. P. van Dam, I. Schwartz, and J. Dankert.** 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin. Microbiol. Rev. **12:**633–653.
18. **Wessels, M. R., P. Butko, M. Ma, H. B. Warren, A. L. Lage, and M. C. Carroll.** 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proc. Natl. Acad. Sci. USA **92:**11490–11494.