

NOTES

Decorin-Binding Protein A (DbpA) of *Borrelia burgdorferi* Is Not Protective When Immunized Mice Are Challenged via Tick Infestation and Correlates with the Lack of DbpA Expression by *B. burgdorferi* in Ticks

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Received 23 March 2000/Returned for modification 2 May 2000/Accepted 13 May 2000

Previous studies showed that decorin-binding protein A (DbpA) of *Borrelia burgdorferi* was a protective immunogen in the murine model of Lyme borreliosis when mice were challenged (needle inoculated) intradermally with *in vitro*-cultivated spirochetes. In the present study, DbpA-immunized C3H/HeJ mice were not protected from infection when infested with *Ixodes scapularis* nymphs harboring virulent *B. burgdorferi* 297. This lack of protection correlated with the failure to detect DbpA on *B. burgdorferi* in ticks, suggesting that DbpA is not available as a target for bactericidal antibodies in serum when *B. burgdorferi*-infected ticks take their blood meal from an immunized host. The failure of DbpA immunization to protect tick-challenged mice contradicts the results of earlier needle inoculation vaccination experiments and suggests that DbpA may not be suitable as a Lyme disease vaccine.

The new outer surface protein A (OspA) Lyme disease vaccine, recently approved for human use by a panel of the United States Food and Drug Administration, represents a significant medical advance (21, 22). However, there are a number of unresolved issues concerning this first-generation monovalent vaccine (23), including (i) uncertainty about its efficacy in children under the age of 15 (a group with a particularly high risk of contracting Lyme disease), (ii) heterogeneity among (and even absence of) *ospA* genes within United States isolates of *B. burgdorferi* (4, 17), and (iii) the fact that protective levels of anti-OspA antibodies in humans can wane after vaccination (18). Of equal potential importance is the fact that OspA expression by *B. burgdorferi* is sharply downregulated as the tick vector takes its blood meal and the spirochetes migrate from the midgut to the salivary glands of the tick (9, 20). OspA vaccination thus is efficacious via the killing of *B. burgdorferi* in tick midguts by OspA antibodies present in the blood meal (9, 11). One way of potentially enhancing the efficacy of the current OspA Lyme disease vaccine would be to expand the number of vaccinogens to include one or more also expressed during the mammalian phase of infection; such a multivalent vaccine would provide immune targets during both phases of the zoonotic life cycle of *B. burgdorferi*.

To this end, at least four studies demonstrated that decorin-binding protein A (DbpA), which is expressed by *B. burgdorferi* during the mammalian phase of infection, was protective in the murine model of Lyme borreliosis when immunized mice were

challenged (needle inoculated) intradermally or subcutaneously with borreliae cultivated *in vitro* (5, 10, 14, 15). As such, DbpA has emerged as a leading new candidate vaccinogen for Lyme disease. In view of the importance of DbpA as a potential human Lyme disease vaccinogen, we sought to extend our previous vaccination studies (14) to confirm that mice immunized with DbpA also were protected from infection when infested with *Ixodes scapularis* nymphs harboring *B. burgdorferi* 297, a challenge condition which mimics the natural mode of *B. burgdorferi* transmission.

Preparation of recombinant antigens (DbpA, OspA, and glutathione *S*-transferase [GST]) and immunization of C3H/HeJ mice were described previously (14). All mice achieved serum antibody titers against each respective immunogen of greater than 1:10,000, as determined by testing 10-fold serial dilutions of sera via immunoblotting (14) (data not shown). Infection of *I. scapularis* nymphs with *B. burgdorferi* 297 and infestation of mice for challenge were performed essentially as described previously (27); each mouse was infested with 10

TABLE 1. Immunization of C3H/HeJ mice with recombinant proteins and subsequent challenge by infestation with *I. scapularis* nymphs harboring *B. burgdorferi* 297

Expt.	Vaccinogen	No. of mice challenged	No. of mice protected
1	GST	4	0
	OspA	5	5
	DbpA	3	1
2	GST	5	0
	OspA	5	5
	DbpA	9	0

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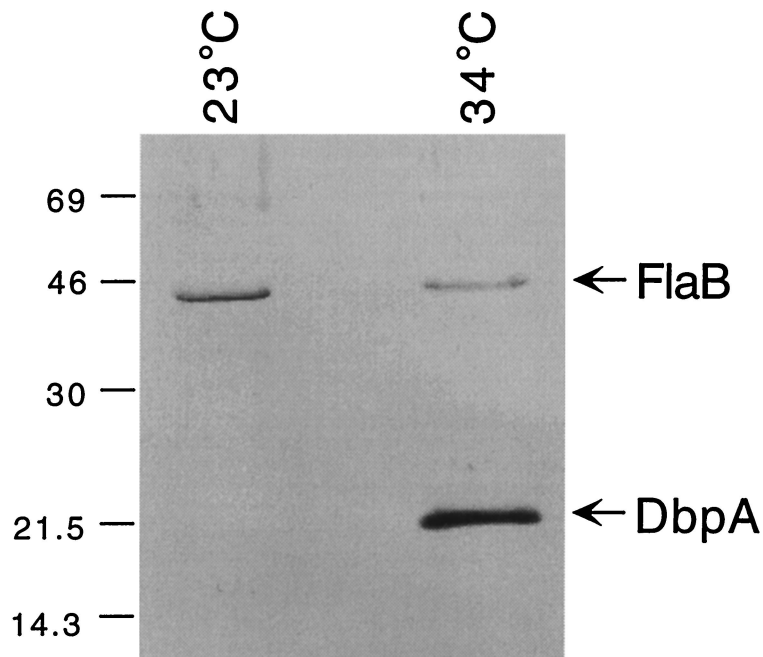


FIG. 1. SDS-PAGE and immunoblot analysis of DbpA expression in *B. burgdorferi* 297 cultivated at either 23°C (2×10^7 spirochetes) or 34°C (10^7 spirochetes). Polyclonal rat anti-DbpA antiserum and monoclonal antibody 8H3-33, directed against FlaB, were used as the respective antibody probes. Numbers at the left denote protein apparent molecular weights (in thousands).

infected nymphs which fed for 4 days. *B. burgdorferi* infections of mice were confirmed by culture of heart, urinary bladder, and punch biopsy specimens from ear pinna in BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) (14). Two separate vaccination experiments, using different lots of antigens prepared on different days, were performed. As predicted, the combined results of the two separate vaccination experiments showed that immunization with OspA protected 100% of mice (Table 1). In contrast, immunization with DbpA had virtually no protective effect (8.3%) (Table 1). GST, used as a negative control, also did not confer protection (Table 1).

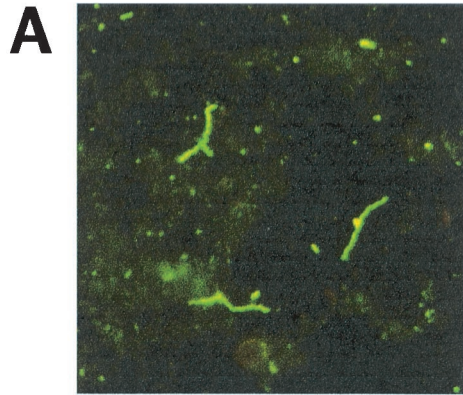
In view of the previous reports of immunoprotection conferred by DbpA (5, 10, 14, 15), the failure of DbpA to protect immunized mice challenged via tick infestation was surprising. Therefore, additional experiments were conducted in an attempt to explain these unanticipated results. In this regard, temperature regulation appears to be important for a number of *B. burgdorferi* lipoproteins, such as OspC, OspE, OspF, Mlp8, Mlp9, and Mlp10 (1, 24, 27). Of note, Cassatt et al. (5) reported that *B. burgdorferi* B31 sharply downregulates its expression of DbpA when cultivated at 23°C, a temperature corresponding more closely with that of the tick's natural habitat when it is not feeding. To examine whether DbpA expression also is temperature regulated in *B. burgdorferi* 297, low-passage-number spirochetes were first cultivated at 34°C to exponential phase in BSK-H medium; an aliquot of the culture was stored at -70°C for future use. The remainder was diluted to a final concentration of 10^3 spirochetes per ml in fresh

BSK-H medium and incubated at 23°C for about 14 days, or until the density of the culture reached about 10^7 spirochetes per ml. Culture volumes containing 10^7 34°C-cultivated *B. burgdorferi* or 2×10^7 23°C-cultivated *B. burgdorferi* were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses. In these analyses, anti-FlaB monoclonal antibody 8H3-33, which has the same specificity as 1H6-33 (1), was used to monitor the different protein levels within the two gel lanes. The rat polyclonal antiserum utilized, which was directed specifically against DbpA, was described previously (14). Spirochetes cultivated at 34°C expressed large amounts of DbpA, whereas DbpA was undetectable in even twice the number of organisms cultivated at 23°C (Fig. 1).

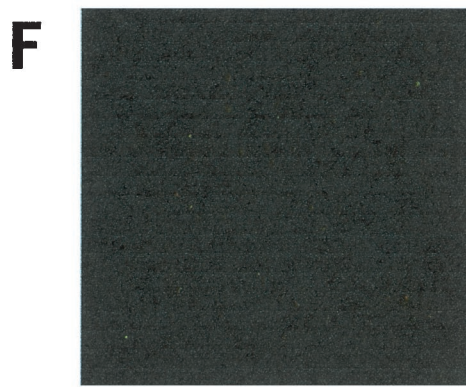
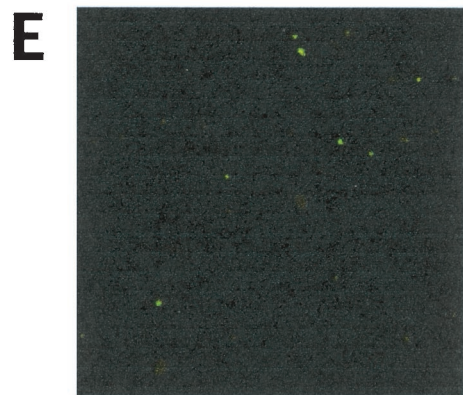
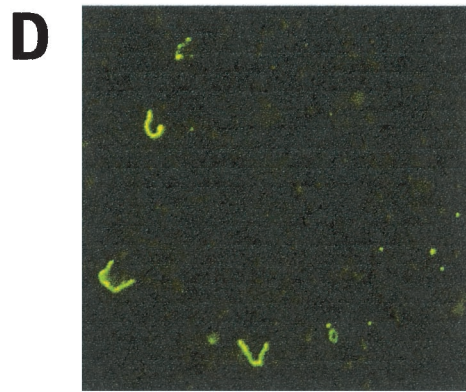
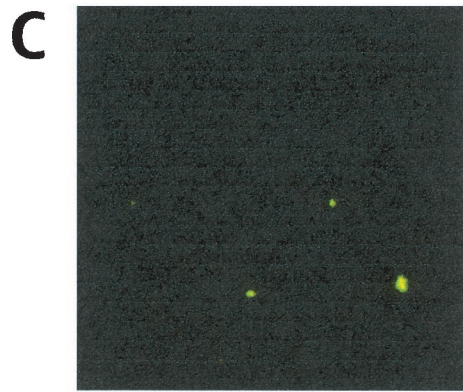
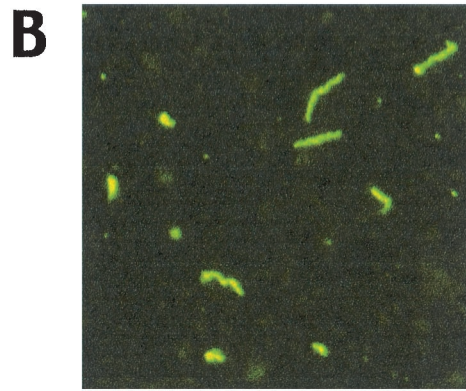
The results shown in Fig. 1, in conjunction with those of Cassatt et al. (5), imply that temperature is one important regulator of DbpA expression in *B. burgdorferi*. Given this, we hypothesized that DbpA may not be expressed by *B. burgdorferi* in tick midguts, at least those of flat ticks. To examine this possibility, indirect immunofluorescence assays (IFAs) were performed (1) on spirochetes harvested from the midguts of both flat- and engorged (4-day) nymphs. Spirochetes were readily detected in both flat- and fed-tick midgut smears with a rat polyclonal antiserum to *B. burgdorferi* 297, although, as expected, spirochetes were less numerous in flat ticks (Fig. 2A and B). OspC expression was virtually undetectable in borreliae from flat ticks (Fig. 2C), whereas OspC was upregulated in fed ticks (Fig. 2D), as predicted from previous studies of OspC

FIG. 2. (A to F) IFAs of borreliae harvested from the midguts of either flat or 4-day-fed *I. scapularis* nymphs harboring *B. burgdorferi* 297. Nymph midgut contents were expressed onto microscope slides, dried, fixed with acetone, and then probed with rat antiserum directed against either whole-cell lysates of *B. burgdorferi* 297 (A and B), OspC (C and D), or DbpA (E to G). (G) Smear of *B. burgdorferi* 297 cultivated in vitro at 34°C and probed with anti-DbpA antiserum. The secondary antibody probe was fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin G. Spirochetes were observed under a 40 \times objective; data were recorded via a charge-coupled device camera mounted on an Olympus dark-field and fluorescence microscope. Panels shown are representative of at least 30 microscope fields examined in each of two separate experiments.

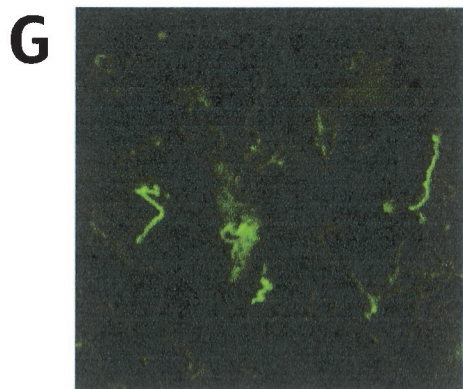
Flat Ticks



Fed Ticks



In Vitro



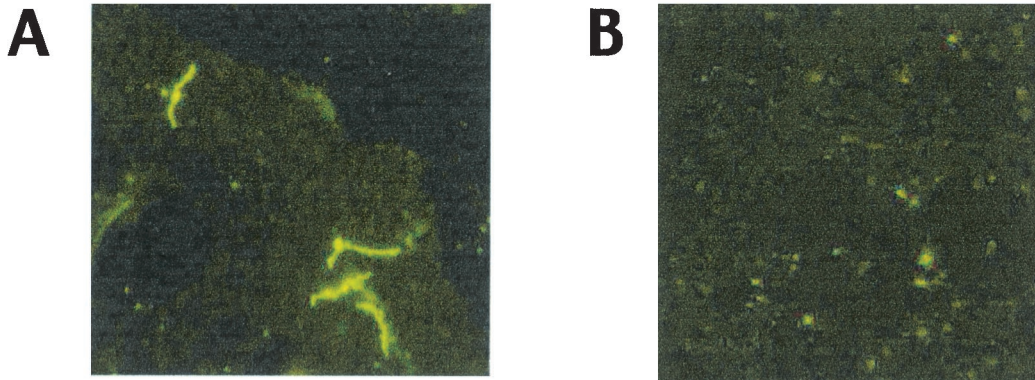


FIG. 3. IFAs of borreliae harvested from the salivary glands of *B. burgdorferi*-infected *I. scapularis* nymphs. Nymphs were allowed to feed on normal C3H/HeJ mice for 48 to 72 h, at which time the ticks were removed. Tick salivary glands were then dissected out (with caution to exclude midgut contents) and homogenized (by repeated gentle pipeting) in 10- μ l aliquots of phosphate-buffered saline. The samples were then divided into two equal portions on microscope slides, dried, fixed with acetone, and probed with rat antiserum directed against either a whole-cell lysate of *B. burgdorferi* 297 (A) or DbpA (B). All other procedures were as described for Fig. 2. The panels shown are representative of at least 30 microscope fields examined in each of five separate salivary gland smears probed with each antiserum.

expression (1, 20). Consistent with the immunoblotting data (Fig. 1), DbpA was readily detected by IFA among borreliae cultivated in vitro at 34°C (Fig. 2G). In contrast, DbpA was not detectable in borreliae from either flat (Fig. 2E) or 4-day-fed (Fig. 2F) ticks. Moreover, although borreliae were readily detectable by IFA (using antiserum to *B. burgdorferi* 297) within the salivary glands of ticks fed for 48 to 72 h (Fig. 3A), the time of maximal spirochete load (8), no borreliae expressing DbpA were found in the same sample of tick salivary glands (Fig. 3B). These results imply that the expression of DbpA by *B. burg-*

dorferi does not occur to any appreciable degree within either flat or fed ticks. That DbpA is not expressed by *B. burgdorferi* in ticks but is expressed on the surfaces of in vitro-cultivated spirochetes (5, 10, 12, 14, 15) suggests that such expression of DbpA, in essence, may be an artifact of culturing *B. burgdorferi* under the artificial growth conditions provided by BSK medium.

The lack of detectable DbpA in *B. burgdorferi* within either flat or fed ticks also suggests that DbpA is not available as a *B. burgdorferi* target for bactericidal antibody in tick midguts dur-

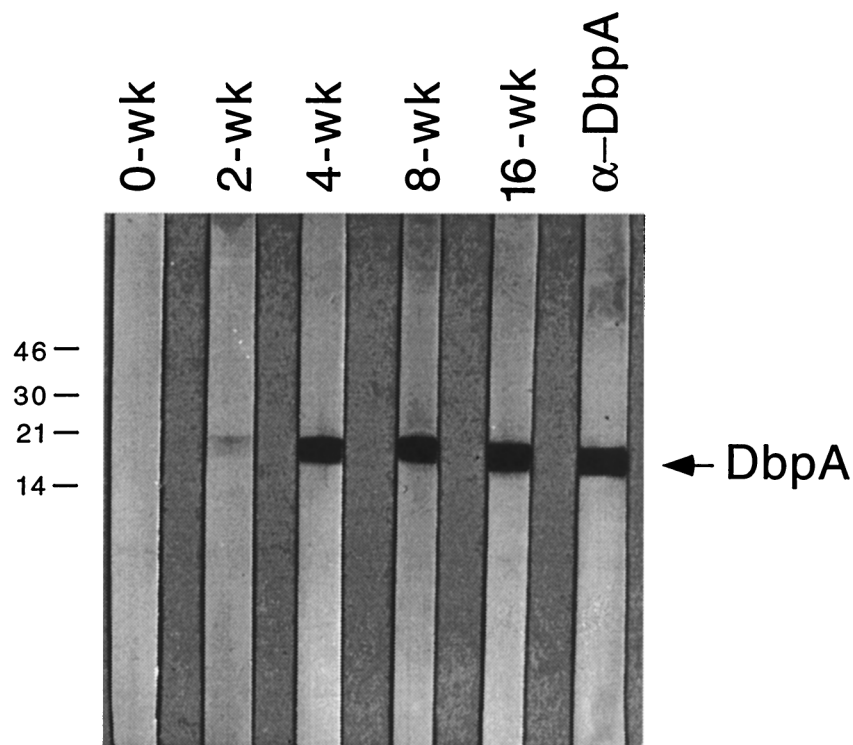


FIG. 4. Appearance of antibodies against DbpA during the course of tick-transmitted *B. burgdorferi* infection of C3H/HeJ mice. Groups of 10 mice were infested with *I. scapularis* nymphs harboring *B. burgdorferi* 297 (27); at various intervals postinfestation (denoted in weeks [wk] at the tops of the immunoblot strips), blood from mice within each group was collected and sera were then obtained and pooled. Recombinant DbpA was subjected to SDS-PAGE as previously described (14). Nitrocellulose strips were immunoblotted with 1:500 dilutions of the respective pooled sera. The lane at the right is a strip probed with rat antiserum against DbpA (α -DbpA). Numbers at the left denote protein apparent molecular weights (in thousands).

ing tick feeding, consistent with our finding that DbpA-immunized mice were not protected when challenged via tick infestation (Table 1). However, we also found that antibodies reactive with recombinant DbpA were detectable in the serum of tick-challenged mice as early as 2 weeks after tick infestation (Fig. 4), comparable to what was reported previously after low-dose needle inoculation of mice with in vitro-cultivated *B. burgdorferi* (14), suggesting that DbpA expression is upregulated relatively soon after *B. burgdorferi* is first introduced into mammalian tissues.

If DbpA is expressed relatively early in the course of mammalian infection by *B. burgdorferi*, it is reasonable to assume that antibody elicited by immunization with DbpA still should be bactericidal to *B. burgdorferi* just after tick transmission; thus, the data of Table 1 raise an interesting paradox. One plausible explanation for the inability of DbpA antibodies to kill *B. burgdorferi* transmitted by tick bite (Table 1) is that although DbpA is expressed sometime after *B. burgdorferi* enters mammalian tissue, this lipoprotein is not sufficiently shuttled to the surface of *B. burgdorferi*, where it can serve as an immune target. Another possibility is that changes in the expression of other outer surface lipoproteins during mammalian infection by *B. burgdorferi* somehow limit antibody access to DbpA, as has been proposed for P66 (3). Interestingly, in passive immunization experiments in which rabbit anti-DbpA antiserum was administered to mice at various times after challenge with in vitro-cultivated *B. burgdorferi* B31, Hanson et al. (15) showed that needle inoculation of mice with *B. burgdorferi* could be aborted only up to 4 days postinfection, suggesting that either DbpA expression or its membrane topology is altered relatively quickly so that the protein becomes refractory to bactericidal antibody. Nonetheless, normal antigen processing and presentation of subsurface DbpA, on the other hand, would still account for the appearance of antibodies early in the course of infection (Fig. 4); the subsurface localization of other *B. burgdorferi* lipoproteins, even some known to have surface topologies under certain conditions, has been well documented (6, 7, 16, 26). That DbpA is expressed but may not be surface exposed during the mammalian phase of infection by *B. burgdorferi* is consistent with the fact that DbpA antibodies do not clear an endogenous, chronic *B. burgdorferi* infection. This notion is inconsistent, however, with the findings of Cassatt et al. (5), who found that *B. burgdorferi* B31 within the plasma of spirochetemic mice were killed when exposed to DbpA antisera in vitro, suggesting that DbpA is surface exposed in *B. burgdorferi* during the mammalian spirochetemic phase. The reasons for these opposing results are unclear, but it is known that in vitro killing assays with spirochetes are prone to misinterpretation due to technical limitations. Finally, the notion that DbpA is not expressed on the surface of *B. burgdorferi* during mammalian infection calls into question whether DbpA really does serve as a surface ligand for the adherence of *B. burgdorferi* to tissue matrix proteins (e.g., decorin), as has been proposed elsewhere (2, 12, 13).

Both OspC (20) and DbpA (5) (Fig. 1) of *B. burgdorferi* are upregulated by elevated temperatures under in vitro growth conditions. However, it is noteworthy that whereas OspC expression is induced by the process of tick feeding, presumably due to both temperature and blood effects (20), this same phenomenon was not observed for DbpA. It is thus tempting to speculate that some other environmental factor(s) may be involved in the suppression of DbpA expression during tick feeding. In this regard, preliminary studies from our laboratory suggest that changes in the pH of tick midgut contents during feeding favor the upregulation of OspC but suppress DbpA expression (X. Yang, M. S. Goldberg, T. G. Popova, G. B.

Schoeler, S. K. Wikel, and M. V. Norgard, unpublished data). This does not preclude the possibility that other stimuli are involved in the induction of DbpA as *B. burgdorferi* enters mammalian tissue. The pattern of DbpA expression thus may represent a model system for determining how factors of mammalian tissue (other than blood) modulate (upregulate) differential antigen expression in *B. burgdorferi*.

There is much that is not yet understood about the factors that influence the regulation, expression, and membrane topology of DbpA, but it seems that there is something particularly enigmatic about the manner in which DbpA is expressed and shuttled to its membrane location(s). Regardless of the precise mechanism(s) involved in the control of DbpA expression, the combined findings of this study suggest that DbpA may not be suitable as a human Lyme disease vaccinogen. They also sound a cautionary note with regard to attempting to extrapolate the results of mouse vaccination experiments using needle inoculation as the challenge route for *B. burgdorferi* to what normally occurs during natural tick transmission. This notion seems to be consistent with a growing body of evidence that needle inoculation is not tantamount to the tick delivery of arthropod-borne *B. burgdorferi* (19, 25). Although needle inoculation of mice remains a mainstay of Lyme disease vaccine research, results of such experiments should not be taken as definitive evidence for immunoprotection. Implicit in this tenet is that tick challenges of immunized mice should become the "gold standard" prior to performance of human clinical trials with a candidate Lyme disease vaccinogen.

We thank Taissia Popova for excellent technical assistance and Anette Huebner for critical reading of the manuscript.

We gratefully acknowledge funding for this work provided by grants AI-45538 and AI-29735 from the Lyme disease program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Editor: J. T. Barbieri