Resistance to Tick-Borne Spirochete Challenge Induced by *Borrelia burgdorferi* Strains That Differ in Expression of Outer Surface Proteins†

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Hamsters were immunized with thimerosal-killed *Borrelia burgdorferi* 297 or a mutant of 297 (M297) that lacks the 49-kb linear plasmid and expression of outer surface proteins A and B (OspA and OspB). *Ixodes scapularis* nymphs infected with either the *B. burgdorferi* sensu stricto strain 297 or JMNT, similar in OspA and OspB but differing in OspC expression, were used to evaluate protection. In a homologous challenge, 24 hamsters were vaccinated, 8 each with 297 or M297 and 8 sham (adjuvant)-vaccinated controls. Hamsters vaccinated with either bacterin were completely protected against a natural tick bite or subcutaneous (s.c.) inoculation of 297. *Borreliae* were effectively eliminated from 80 to 90% of the 297-infected ticks that fed on four hamsters immunized with the 297 bacterin. Cultures of spirochetes isolated from the ticks that remained infected were infectious and induced joint inflammation in naive hamsters. There was no reduction of strain 297 spirochetes in ticks that fed on four hamsters immunized with M297, but the hamsters were protected. Results with the M297 bacterin indicate that proteins other than OspA or OspB can protect hamsters against a tick challenge without eliminating *B. burgdorferi* in the tick. In a heterologous challenge, 36 hamsters were vaccinated, 12 with each bacterin and 12 controls. None of the hamsters immunized with either bacterin were protected from a challenge involving JMNT-infected ticks, while two of four were protected against an s.c. challenge. Hamsters challenged s.c. with strain 297 spirochetes were protected. There was partial elimination of JMNT spirochetes in ticks that fed on the group of four hamsters immunized with the 297 bacterin, and infection rates were reduced by 50 to 60%. JMNT spirochetes reisolated from the ticks that fed on 297-vaccinated hamsters also remained infectious for hamsters. In the JMNT-infected ticks that fed on four M297-immunized hamsters, there was no decline in the proportion of infected ticks. Destruction of spirochetes in ticks that fed on the hamsters vaccinated with the 297 bacterin suggests that antibodies to OspA and OspB may have been responsible, since the mutant did not induce this activity.

Lyme disease is an infection of increasing importance in human and veterinary medicine (7, 8). The causative organism, *Borrelia burgdorferi* (6, 22), is transmitted by ticks in the *Ixodes ricinus*-*Ixodes persulcatus* complex (23), and in North America the main vector is *Ixodes scapularis* (30). Because of problems associated with tick control, chemotherapy, and diagnosis, the development of a vaccine is viewed as a desirable goal for the prevention of Lyme borreliosis (2). Johnson et al. (21) first reported that hamsters were protected against infection with *B. burgdorferi* when immunized with lyophilized spirochetes. However, bacterins have been implicated in the induction of destructive arthritis in challenged hamsters (26), and antigenic similarities between spirochetes and components of the nervous system have raised additional concerns (13). More recently, then, vaccine development has focused on immunoprotective outer surface proteins (Osp proteins) of *B. burgdorferi* that would be suitable for a subunit vaccine. These include OspA and -B (10, 14, 16, 20, 39), OspC (15), and OspE and -F (29). Among those, lipoproteins OspA and OspB and OspC confer protective immunity to challenge via needle as well as

infected-tick bites (4, 10, 14, 15, 16, 20, 39). Destruction of the spirochetes was observed within ticks that fed on animals vaccinated with recombinant OspA and OspB (10, 39) and OspE and OspF (29) but not OspC (15). This has led to the proposal that vaccines based on OspA dually protect by killing spirochetes both in the tick and in the mammal (10). However, protection of vaccinates from needle-inoculated spirochetes is likely due to the fact that *B. burgdorferi* cultured in vitro expresses OspA on its surface, unlike spirochetes naturally transmitted by ticks, which express OspC (35). This is supported by the discovery that mammalian-host-adapted spirochetes, i.e., those that have infected a naive host successfully, are infectious when transplanted within infected skin into a host immunized with OspA (4). Conversely, in unfed ticks, spirochetes express OspA (12, 35) and are thus vulnerable to anti-OspA antibodies (10, 11, 14, 16, 20, 39). It is only later during tick feeding that *B. burgdorferi* switches to OspC expression (35). Such switching in Osp protein expression possibly provides *B. burgdorferi* with a mechanism for escaping from antibody-mediated killing. Therefore, and also because *B. burgdorferi* shows considerable antigenic diversity, immunity to a single antigen may be incomplete (20). Specific Osp mutants may assist in identifying antigens that are differentially expressed in either the mammal or the vector tick and point to immunogens that will better protect vaccinates from heterogeneous strains of spirochetes. A noninfectious mutant of strain 297 (hereafter referred to as M297) that lacks the entire 49-kb plasmid and which does not

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express OspA or OspB or two additional lipoproteins of 7.5 and 20 kDa has been isolated (19). Hamsters experimentally immunized with lyophilized and thimerosal-killed M297 organisms were protected from needle challenge with the virulent, wild-type 297 strain. M297 induced antibodies to OspC and a 39-kDa protein (P39), indicating their possible role in eliciting immune protection from *B. burgdorferi*.

In this study, we sought further evidence for the role of spirochetal Osp proteins, particularly OspA and OspB, in protecting hamsters challenged via the bite of infected *I. scapularis* nymphs. Our analysis was aided by the use of bacterins prepared from 297 and M297. We examined the ability of these vaccines to immunize hamsters against tick challenge by 297 (homologous challenge) or another *B. burgdorferi* sensu stricto isolate, JMNT (heterologous challenge). In addition, we investigated the persistence and infectivity of spirochetes in the ticks that fed on vaccinated hamsters.

MATERIALS AND METHODS

Spirochetes. Two *B. burgdorferi* sensu stricto isolates were used: 297 cultured from human spinal fluid in Connecticut (38) and JMNT isolated from an *I. scapularis* tick collected in Minnesota (24). Both isolates were maintained by periodic passage through *I. scapularis* and hamsters. An avirulent and noninfectious mutant of 297 (M297) which lacks the plasmid that encodes OspA and OspB was also used (19). Borreliae were cultured in Barbour-Stoenner-Kelly (BSK) medium at 34°C as described elsewhere (1). The two isolates belong to the same genospecies and ribotyping group (18), but they differ in their plasmid profiles (28). The two isolates have similar *ospAB* operons, as detected by using probes for the *OspAB* locus and Southern analysis (28). Furthermore, JMNT and 297 react similarly with monoclonal antibodies for OspA and OspB (19, 28). However, unlike 297, JMNT is not recognized by the OspC monoclonal antibody L221F8 but is recognized by a polyclonal anti-OspC serum against OspC of *B. burgdorferi* Sh2-82 (19, 27a, 35).

Vaccine preparation and vaccinations. Vaccines were prepared as described elsewhere (19). Briefly, 297 and M297 grown in BSK medium were washed and suspended in phosphate-buffered saline (PBS), lyophilized, and stored at -20°C. Lyophilized preparations (60% protein) were killed by suspension in sterile saline solution containing thimerosal and mixed with adjuvant, aluminum hydroxide (alum). Hamsters (golden Syrian, *Mesocricetus auratus*) were inoculated subcutaneously (s.c.) with 0.2 ml of vaccine suspensions on days 0 and 14. Each vaccine dose contained 25 µg of lyophilized spirochete protein and 0.2% adjuvant. Control hamsters were inoculated s.c. with adjuvant only.

Hamsters. Male hamsters, approximately 2 months old when vaccinated, were purchased from Harlan Sprague Dawley, Inc. (Madison, Wis.). The hamsters were housed four per cage and provided food and water ad libitum. They were killed with carbon dioxide gas and exsanguinated by cardiocentesis.

Ticks. *I. scapularis* nymphs used in this study were from a laboratory colony originally established from adult females collected from hunter-killed white-tailed deer in Minnesota and Wisconsin. The ticks were free of *B. burgdorferi* as determined by PCR (33) using unfed tick tissues disrupted in sample buffer as described elsewhere (17) and by ear biopsy culture of host animals (36). The ticks were maintained in desiccator jars humidified with a supersaturated Na₂SO₄ solution and a photoperiod of 16 h light and 8 h dark. The rearing temperatures were 22°C during light and 18°C during dark periods. Larvae and nymphs were fed on hamsters, and adults were fed on Dutch belted rabbits. Ticks of the fourth and fifth laboratory generations were used in these studies.

Infection of ticks. Two groups of ticks were used in this study, one infected with *B. burgdorferi* 297 and the other infected with *B. burgdorferi* JMNT.

(i) **297-infected ticks.** We infested healthy hamsters with infected nymphs (1 month postmolt; 20 nymphs per hamster) whose larvae had acquired spirochetes by engorging on hamsters inoculated intraperitoneally 4 weeks earlier with 10⁸ strain 297 borreliae. Hamsters determined to be infected by ear biopsy culture (36) were infested with larvae 1 month after infected nymphs had completed engorgement. Following completion of the molt, nymphs were dissected in Hanks' balanced salt solution, and wet mounts of gut tissues were examined by phase-contrast microscopy at a magnification of ×600. A total of 60% (6 of 10) of the nymphs were infected with spirochetes. This cohort, i.e., nymphs whose larvae had acquired spirochetes from hamsters infected via tick bite, was subsequently used for homologous challenge of vaccinated hamsters.

(ii) **JMNT-infected ticks.** Hamsters, infected 4.5 weeks earlier by the bite of JMNT-infected nymphs, were infested with larvae to generate equivalent infected nymphs (i.e., nymphs whose larvae had fed on hamsters infected via tick bite) for the heterologous challenge experiment. Hamsters had bilateral tarsal joint swellings during tick feeding. When examined by phase-contrast microscopy (see above), 90% (27 of 30) of the ticks were found to be infected.

To confirm the transmission potential of nymphal cohorts to be used for challenge trials, four healthy hamsters were each infested with 25 nymphs (1

month postmolt) harboring either 297 or JMNT spirochetes. All hamsters became infected as determined by ear biopsy cultures.

Challenge procedures. Two separate challenges were done, one involving 297-infected ticks and the other involving JMNT-infected ticks. Hamsters were divided into six groups in the 297 trial (see Table 1) and nine groups in the JMNT trial (see Table 2), with four hamsters per group. The groups consisted of hamsters vaccinated with the 297 or the M297 vaccine and sham-vaccinated (adjuvant-only) controls. The groups of hamsters were challenged by infestation with nymphal ticks infected with one of the two challenge strains or by s.c. inoculation with one of the two challenge strains.

(i) **Tick challenge.** Vaccinated and control hamsters were infested with infected ticks 2 weeks after the second vaccination. The hamsters were housed separately in cages placed in pans filled with water, and each was infested with 40 nymphs. On days 4 to 6, engorged nymphs were collected from the water, counted, surface disinfected with a 0.1% solution of sodium hypochlorite, and stored in humidified desiccators. Ticks from each hamster were stored separately in sterile vials. Only ticks that had engorged and dropped off the hamsters were considered replete and evaluated for infection.

(ii) **Needle challenge.** Vaccinated and control hamsters were needle challenged 2 weeks after the second vaccination by s.c. inoculation of 10⁶ spirochetes (JMNT or 297, as appropriate) at the nape of the neck. The JMNT spirochetes were in the second in vitro passage and the 297 spirochetes were in the sixth in vitro passage after isolation from hamsters.

Determination of infection by culture in BSK medium. (i) **Hamsters.** Ear biopsies were cultured 3 weeks after challenge via needle inoculation of spirochetes or via infected-tick bite (36). In addition, 1 week later, hamsters were killed with carbon dioxide gas, and urinary bladder, heart, and spleen tissues were removed for cultivation in BSK medium (19).

(ii) **Ticks.** Tick tissues were processed for cultivation in BSK medium at two times after the ticks fed on vaccinated or control hamsters: just before molting to adults (1 month postrepletion) and 1 month after molting to adults. Prior to dissection, the ticks were surface disinfected by immersion (5 min each time) in solutions of sodium hypochlorite (1% sodium hypochlorite in water), benzalkonium chloride (0.5% in water), and ethanol (70% in water). A drop of Tween 80 was mixed into each solution to facilitate surface wetting of the tick. The surface-disinfected ticks were rinsed three times in sterile water and placed in a drop of BSK medium. Internal tissues were extirpated and disrupted by pipetting, and the tissues from each tick were transferred to a 4-ml culture tube containing BSK medium. All cultures, whether from tick or hamster tissues, were incubated at 34°C and sampled weekly for the presence of spirochetes by phase-contrast or dark-field microscopy.

Fluorescent-antibody (FA) determination of infection in ticks. Ticks were examined for borreliae by direct immunofluorescence as outlined elsewhere (3). Antisera were raised in hamsters inoculated intraperitoneally with live, washed, cultured borreliae of strains 297 and CT-1 (37). Equal portions of anti-297 (homologous titer of 1:1,024) and anti-CT-1 (homologous titer of 1:512) antisera were mixed and diluted 1:8 in PBS. Smears prepared from the extirpated midgut diverticula of ticks were fixed with anhydrous methanol, stored frozen (-20°C), and desiccated until use. After thawing, fixed smears were treated with fluorescein isothiocyanate-labeled antisera and examined by epifluorescence microscopy.

Infectivity of spirochetes recovered from ticks fed on vaccinated hamsters. Spirochetes (strain 297 or JMNT) isolated from replete nymphs and adults that had fed as nymphs on hamsters vaccinated with 297 or M297 were used to needle inoculate hamsters. Selected isolates of spirochetes from 21 separate ticks were tested for infectivity by inoculating naive hamsters s.c. with spirochetes (10⁶ borreliae per hamster).

Statistical analysis. Data on the extent of tick feeding and the presence of spirochetes were obtained for ticks that had fed on individual hamsters. Datum sets for each vaccine treatment were pooled, and the means and standard deviations were calculated. The significance of differences in results between ticks feeding on control hamsters (adjuvant only) and experimental groups (vaccinates) was determined by the chi-square test comparing two proportions.

RESULTS

Protection of vaccinated hamsters. Two separate experiments were done to evaluate the protective immunity in hamsters vaccinated with 297 or the OspAB-negative M297 bacterin. Vaccinated hamsters were challenged naturally with infected ticks or artificially by s.c. inoculation of cultured spirochetes. In one experiment, a homologous challenge was done with the virulent strain of 297 used to produce the 297 bacterin employed in this study. In a heterologous challenge experiment, we used the *B. burgdorferi* strain JMNT, which is being characterized in our laboratory. To assess if the vaccinated hamsters became infected, their tissues were cultured in BSK medium 3 to 4 weeks after the challenge.

TABLE 1. Protection of hamsters vaccinated with killed wild-type or mutant (M297) *B. burgdorferi* from infection following the bite of ticks infected with 297 or s.c. inoculation of 297

Vaccine ^a	Challenge	No. of culture-positive hamsters/total ^b
Adjuvant	Tick ^c s.c. ^d	4/4 4/4
297	Tick s.c.	0/4 0/4
M297	Tick s.c.	0/4 0/4

^a Total vaccine dose, 50 µg (dry weight). Adjuvant, aluminum hydroxide (alum).

^b Hamsters were sacrificed 4 weeks after challenge and assayed for infection by culturing tissues in BSK medium.

^c Hamsters were challenged 14 days after the last vaccination with an infestation of 40 nymphs infected with strain 297.

^d Hamsters were challenged 14 days after the last vaccination with an s.c. inoculation of 10⁶ 297 spirochetes.

The results of the homologous challenge are given in Table 1. *B. burgdorferi* was isolated from only the four sham-vaccinated control hamsters that were challenged with infected ticks and the four controls that were challenged by s.c. inoculation of 297. Thus, all vaccinates, regardless of the vaccine strain (297 or M297) or type of challenge (tick or s.c.), were protected.

The results of the heterologous challenge are given in Table 2. In contrast, all hamsters challenged with JMNT-infected ticks, controls and vaccinates alike, were culture positive for spirochetes. Two of the four hamsters in the 297 vaccine group were protected when challenged by s.c. inoculation with JMNT, but none of the hamsters in the M297 vaccine group were protected, similar to control animals. As in the homologous challenge study, the four control hamsters inoculated s.c. with 297 became infected but those vaccinated with either 297 or M297 were protected.

In neither study did we observe joint swelling or lameness in

TABLE 2. Challenge of vaccinated hamsters with *B. burgdorferi* JMNT-infected ticks or by s.c. inoculation of JMNT or 297

Vaccine ^a	Challenge	Strain	No. of culture-positive hamsters/total ^b
Adjuvant	Tick ^c	JMNT	4/4
	s.c. ^d	JMNT	4/4
	s.c.	297	4/4
297	Tick	JMNT	4/4
	s.c.	JMNT	2/4
	s.c.	297	0/4
M297	Tick	JMNT	4/4
	s.c.	JMNT	4/4
	s.c.	297	0/4

^a Total vaccine dose, 50 µg (dry weight) administered in two doses. Adjuvant, aluminum hydroxide (alum).

^b Hamsters were sacrificed 3 weeks after challenge and assayed for infection by culturing tissues in BSK medium.

^c Hamsters were challenged 14 days after the last vaccination with an infestation of 40 nymphs infected with JMNT spirochetes.

^d Hamsters were challenged 14 days after the last vaccination with an s.c. inoculation of 10⁶ spirochetes.

TABLE 3. Detection of spirochetes in challenge ticks that fed to repletion

Strain	Bacterin	No. of ticks positive by microscopy/no. examined ^a (%)	No. of ticks positive by culture/no examined ^a (%)	
			Nymphs ^b	Adults ^c
297	297	1/19 (5)	3/20 (15)	3/35 (9)
	M297	11/20 (55)	11/20 (55)	11/20 (55)
Control	12/20 (60)	14/20 (70)	14/20 (70)	
JMNT	297	14/20 (70)	7/19 (37)	10/21 (48)
	M297	12/20 (60)	15/19 (79)	16/23 (70)
	Control	14/20 (70)	14/16 (88)	13/15 (87)

^a Seven days after repletion, wet mounts of nymphal midguts in saline were examined by phase-contrast microscopy (magnification, ×600).

^b One month after engorgement and just before the molt to adults, tick tissues were cultured in BSK medium.

^c Adult tick tissues were cultured in BSK medium 1 month after the molt.

either the fore- or the hindlegs in any of the bacterin-vaccinated hamsters.

Intensity of tick challenge. In order to quantify the tick bite challenge, each hamster was housed separately and the nymphs that engorged on it were analyzed. The numbers of ticks that fed on each hamster were similar in the homologous and the heterologous challenges. In the group of ticks infected with strain 297 (homologous challenge), an average of 57% of the ticks engorged to repletion (mean ± standard deviation, 22.6 ± 4.9 ticks per hamster; range, 12 to 30 ticks per hamster) to give an average of 13.6 ± 1.5 infected ticks per hamster (from the infection rate of 60% multiplied by the average number of ticks recovered from each hamster). A slightly higher proportion (64%) of the ticks feeding on the four hamsters vaccinated with the M297 vaccine engorged successfully ($P < 0.05$). In the group of JMNT-infected ticks (heterologous challenge), an average of 53% (mean ± standard deviation, 21.3 ± 3.8 ticks per hamster; range, 14 to 26 ticks per hamster) of the ticks fed to repletion to give an average dose of 19.1 ± 3.0 infected ticks per hamster (from the infection rate of 90% multiplied by the average number of ticks recovered from each hamster). Again, a slightly higher proportion (61%) of ticks fed to repletion ($P < 0.05$) on the hamsters vaccinated with M297.

Spirochetes in engorged nymphs. We determined the proportion of ticks that remained infected after nymphs had engorged on the vaccinated hamsters. Our objective was to examine if these ticks would be cleared of *B. burgdorferi* as reported for ticks fed on mice vaccinated with recombinant OspA and -B (10, 11, 14, 39) and OspE and -F (29). In both studies, the guts from five ticks from each hamster were dissected and examined by phase-contrast microscopy for spirochetes.

The proportion of 297-infected ticks that remained infected after engorging on the 297 bacterin-vaccinated hamsters was 8% (5%/60%) of that for nymphs that had fed on sham-vaccinated control hamsters; the few spirochetes seen were distorted and had membrane blebs (Table 3). The 55% decline in the infection rate (from 60 to 5%) was significant ($\chi^2 = 13.14$, 1 df, $P < 0.001$). 297 spirochetes in ticks that had fed on control hamsters or hamsters vaccinated with M297 appeared motile and normal. These spirochetes showed the regular wave form and spiral shape, and there was no significant difference ($P > 0.05$) in the infection rates of these two groups of ticks.

In contrast, for ticks infected with JMNT, there were no significant differences ($P \geq 0.05$) in the percentages of infected ticks between the three groups (Table 3). Ticks that had fed on

the control (adjuvant-only) and M297-vaccinated hamsters harbored JMNT spirochetes that appeared normal, motile, and intertwined. However, JMNT spirochetes in ticks fed on 297-vaccinated hamsters were fewer, and most were nonmotile and misshapen. Spirochetes in the guts of some of the adults that emerged from these nymphs were examined by use of FA (data not shown). The proportion of adults infected with JMNT after having fed as nymphs on 297-vaccinated hamsters was 57% (12 FA-positive ticks of 21 ticks examined) and significantly lower ($\chi^2 = 6.17$, 1 df, $P < 0.025$) than that of ticks from control hamsters (94%, 15 of 16 ticks FA positive). Most (97%, 11 of 12) of the JMNT-infected adults that had fed on hamsters vaccinated with 297 had spirochetes that fluoresced weakly. The proportion of JMNT-infected ticks from hamsters vaccinated with M297 was similar to that of controls (15 of 16 [94%] from the controls were FA positive, and 20 of 21 from the M297 vaccinates were FA positive), but for 30% (6 of 20) of these adults the spirochetes fluoresced weakly.

Culture of spirochetes from ticks before and after molting.

The impact of borreliacidal antibodies on *B. burgdorferi* survival within the ticks was examined by inoculating BSK medium with tick tissues before and after the nymph-adult molt. Ticks from which we isolated spirochetes in culture were considered infected, and those that did not yield positive cultures were considered uninfected.

In the study using strain 297-infected nymphs, the elimination of spirochetes was most notable in ticks that had fed on hamsters vaccinated with the 297 bacterin. Only 3 of the 20 (15%) 297-infected nymphs feeding on hamsters vaccinated with the 297 bacterin yielded spirochete cultures (Table 3). This 55% decline (70 to 15%) was significantly different ($\chi^2 = 12.38$, 1 df, $P < 0.001$). After the molt to adults, only 3 of the 35 ticks were culture positive, significantly different from the control ($\chi^2 = 22.49$, 1 df, $P < 0.001$). There was no significant difference in the proportion of 297-infected ticks in this group before and after the adult molt ($\chi^2 = 0.66$, 1 df, $P > 0.05$). There was no clearance of strain 297 spirochetes in ticks that fed on M297-vaccinated hamsters. The proportion of 297-infected ticks remaining infected after having fed on control or M297-vaccinated hamsters was 55 to 70% (Table 3) and did not differ significantly from the controls or from one another, before or after the molt ($P > 0.05$).

We also observed a reduction in the proportion of JMNT-infected ticks remaining infected after feeding on hamsters vaccinated with the 297 bacterin (Table 3). The proportion of JMNT-infected ticks was significantly lower than that of the controls, both before and after the molt (pre-molt: $\chi^2 = 9.29$, 1 df, $P < 0.005$; post-molt: $\chi^2 = 5.78$, 1 df, $P < 0.025$). The pre- and post-molt results did not differ significantly ($\chi^2 = 0.47$, 1 df, $P > 0.05$) from one another. Most ticks fed on control and M297-vaccinated hamsters remained infected (Table 3). There was no significant difference ($P > 0.05$) between the proportions of ticks infected in the two groups, but cultures for ticks that fed on control hamsters had higher spirochete densities (data not shown) than those from the ticks that had fed on the M297-vaccinated hamsters. Ten-day-old cultures of samples from the ticks that fed on the 297-vaccinated hamsters also contained fewer spirochetes than those for ticks fed on control hamsters or animals vaccinated with M297 (data not shown).

Infectivity of spirochetes recovered from ticks having fed on vaccinated hamsters. We examined whether spirochetes isolated from the ticks that had fed on vaccinated hamsters remained infective. Naive hamsters were inoculated s.c. with reisolated spirochetes from the primary isolate or in the first passage. In the homologous challenge trial, all six isolates from the strain 297-infected ticks (three from nymphs and three

from adults) that had fed on 297-vaccinated hamsters were infective as determined by ear biopsy cultures. A total of 12 hamsters were inoculated s.c., and all gave positive ear biopsy cultures 4 weeks after the inoculation. Beginning 3 to 5 weeks postinoculation, six of the hamsters showed swelling of the tibiotarsal joints accompanied by lameness that lasted for 3 to 4 days. We also tested 3 of the 22 reisolates obtained from the ticks that had fed on the M297-infected hamsters; all 3 were infective. Finally, from the second trial, six of the JMNT reisolates were tested, three from ticks that had fed on 297-vaccinated hamsters and three from the M297-vaccinated hamsters. All six reisolates were infective, and one isolate induced swelling in the digital and metacarpal joints in the hamster.

DISCUSSION

We infested vaccinated hamsters with large numbers of ticks to provide a stringent test of immunoprotection and recover sufficient numbers of ticks for postrepletion analyses. In addition, the s.c. route and challenge dose were selected to simulate natural infection and ensure consistent (i.e., 100%) infection of the hamsters with *B. burgdorferi* (32). The initial number of ticks used per hamster, i.e., 40, was similar to the maximum number of nymphs observed on wild-caught white-footed mice, i.e., 35 (5). Furthermore, animals in nature are subject to continuous tick challenge throughout the season, and the cumulative tick load per season per animal is likely higher than that seen at the moment of capture. The proportion of ticks that fed to repletion in our study, 53 to 57%, was similar to that reported by others (10). On the basis of the proportion of ticks that yielded spirochete-positive cultures (approximately 70% of 297-infected ticks versus 80% of JMNT-infected ticks) after engorgement on sham-vaccinated hamsters (Table 3), we consider the challenge doses delivered by the nymphal cohorts infected with the two *B. burgdorferi* strains (297 and JMNT) to be similar. Culture isolation is more sensitive in detecting infected ticks than microscopic examination (16), and these data probably better represent the actual infected-tick challenge. Our results demonstrating solid immunity in M297-vaccinated hamsters against homologous challenge, together with severe reduction of the spirochete load in vector ticks, further support our contention that we did not overchallenge vaccinates. The lack of protection against the JMNT-infected-tick challenge was not due to a failure in vaccine administration, as the control hamsters inoculated s.c. with 297 were protected.

Although our results obtained with the two groups of infected nymphs were quite different, they correspond to results obtained by others. The B31 spirochete load was reduced, but not cleared, when ticks fed on mice vaccinated with lipitated OspA derived from another isolate of *B. burgdorferi* sensu stricto (16). We observed essentially the same in JMNT-infected ticks fed on 297-vaccinated hamsters. However, under homologous challenge conditions, i.e., when the vaccine and challenge strains are identical or vaccines are derived from the same geographical region as the challenge strains, protection of vaccinates is complete (10, 11, 14, 39). Our results with a bacterin-type vaccine, instead of recombinant OspA, have confirmed these data. Nevertheless, spirochetes still survive in a small number of ticks (10, 14, 39) and, as shown in the present study, are able to pass through the nymphal molt and retain infectivity for the mammal. Our morphologic observations indicate that spirochetal elimination in ticks that fed on hamsters vaccinated with the 297 bacterin involved membrane disruption and lysis for both 297 and JMNT. Similar observations have been reported for spirochetes exposed in vitro to poly-

clonal and monoclonal antibodies which caused agglutination, formation of surface blebs, damage to the outer envelope, and lysis (9, 34). In both groups of ticks (i.e., ticks infected with strain 297 and ticks infected with JMNT), results obtained by using postmolt adult tissues to inoculate BSK cultures were similar to those obtained with premolt tissues (Table 3). Thus, the molting process did not appear to enhance clearance of spirochetes previously exposed to borreliacidal antibodies. On the basis of results obtained *in vitro* (9, 33, 34), it is assumed that spirochetes that survive the onslaught of bactericidal antibodies in the tick gut would be debilitated and avirulent because of selection of escape mutants with altered (i.e., truncated or recombinant) Osp proteins (11). However, others (20) demonstrated that ticks feeding on OspA-vaccinated hamsters transmitted spirochetes which were subsequently isolated from ear biopsies. Moreover, these reisolates expressed OspA profiles indistinguishable from those of the challenge strain but varied in expression of proteins in the 19- to 23-kDa region. Our results further indicate that, instead of encouraging the emergence of noninfectious variants, antibody-mediated selection appears to favor retention of *B. burgdorferi* infectivity.

Experimental vaccines based on whole killed spirochetes have, in one study, been associated with induction of destructive arthritis in hamsters (26). Hamsters vaccinated with a bacterin prepared from the *B. burgdorferi* sensu stricto strain C-1-11 (27) suffered severe arthritis of the hind paws upon needle inoculation of homologous or heterologous strains of spirochetes. We did not observe induction of joint swelling and lameness when vaccinated hamsters were challenged by needle inoculation or by exposure to high numbers of ticks infected with either 297 or JMNT. However, spirochetes isolated from ticks that had fed on these vaccinated hamsters induced joint swelling and lameness in naive hamsters. The potential of JMNT spirochetes to induce joint swelling and lameness in naive hamsters has been described previously (24, 28). At present, it is not clear whether these contrasting results are a reflection of the particular isolate (C-1-11), the site of challenge (the hind paws), the mode of challenge (needle versus tick), or differences in hamster strains used.

Animals vaccinated with cultured *B. burgdorferi* mount a strong immune response to OspA and OspB and are protected from infection by needle-inoculated spirochetes (21). Recombinant OspA and, to a lesser degree, OspB, have been shown to confer essentially the same degree of immunity against tick-borne borreliae, indicating that OspA and -B may be the main mediators of immune protection (8, 10, 11, 14, 39). The efficacy of anti-OspA antibodies appears to reside in their ability to kill spirochetes contained within the tick's midgut (10). Nevertheless, results obtained from the use of other surface proteins, such as OspE and OspF (25, 29), or mutants that are specifically deficient in OspA and -B (19) have provided evidence that additional borreliacidal antigens are involved. We have shown here that M297, which lacks OspA and OspB, completely protected hamsters against tick-borne 297 challenge with minimal effect on the spirochetes in the tick. However, M297 has lost the entire 49-kb plasmid and differs considerably from strain 297. It also lacks two additional lipoproteins of 20 and 7.5 kDa but still expresses OspC and P39 (19).

There appear to be at least two distinct mechanisms whereby vaccinated animals can be protected from infection with *B. burgdorferi*, one mediated by destruction of borreliae within the vector (10, 29, 39) and another mediated by destruction in the mammal, and possibly by blocking acquisition of mammalian infectivity within the tick. A recent, elegant study on the temporally differential expression of OspA and OspC by *B. burgdorferi* demonstrated that in ticks, these organisms express

mainly OspA until after the beginning of the blood meal, when a switch to OspC is induced (35). This event coincides with the timing of reacquisition of infectivity by spirochetes for the vertebrate (31). However, thermal induction alone is not sufficient to induce OspC expression or the reacquisition of infectivity, and it appears that the ingestion of blood is required as well (31, 35). Once in the mammal, OspC is found on the spirochetes' surface, but not OspA (4), which may also explain why naturally infected animals mount no, or only a very minor, response to OspA (32). This discovery suggested that the protection afforded by vaccination with M297 might be due to OspC. Indeed, immunizing mice with recombinant OspC, but not P39, had the same effect of protecting the mice without apparent harm to the spirochetes within ticks used for challenge (15). In light of the fact that the timing for reacquisition of infectivity of Lyme disease spirochetes for the mammal (31) coincides with a switch from OspA to OspC expression (35), we speculate that this event (i.e., reacquisition of infectivity) may be blocked by anti-OspC antibodies induced by the M297 vaccine. This hypothesis is supported by the observation that spirochete viability was not affected but infection of the mammal did not result.

In conclusion, we have demonstrated that a bacterin-type vaccine mixed with an adjuvant approved for use in humans, i.e., alum, is highly effective in protecting hamsters against homologous, but not heterologous, tick-borne spirochete challenge, as has been shown for animals immunized with recombinant OspA or -B (8, 10, 11, 14, 16, 39). The 297 bacterin-induced antibodies caused destruction of both 297 and JMNT spirochetes within vector ticks in a manner similar to that demonstrated for recombinant OspA (10, 11, 14, 16, 39), but induction of swollen joints and lameness was not observed when the hamsters were challenged by the bite of infected ticks or by s.c. inoculation of *B. burgdorferi*. Spirochetes reisolated from the ticks that remained infected were infectious for naive hamsters and induced joint inflammation. We further show that an OspAB-negative mutant is as effective as the wild-type strain in protecting vaccinates against a homologous challenge, but not a heterologous challenge, without affecting viability of the borreliae resident in vector ticks.

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