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Elimination of *Borrelia burgdorferi* from vector ticks feeding on OspA-immunized mice

(Lyme disease/*Ixodes dammini*/spirochete/vaccine development)

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ABSTRACT **Although recombinant outer surface protein A (OspA) of *Borrelia burgdorferi* protects mice against injected Lyme disease spirochetes, the mode of protection has not yet been explored. Indeed, the efficacy of vaccine-induced immunity against a realistic vector-mediated challenge remains unexplored. Accordingly, we determined whether this immunogen protects mice against spirochetes delivered by nymphal *Ixodes dammini* ticks. Following challenge by tick bite, no spirochetes could be cultured from immunized mice, and no characteristic histopathology was found. The spirochete was not detected in ticks that fed on immunized animals and was present in virtually all ticks that fed on nonimmunized mice. We conclude that OspA-immunized mice are protected from spirochetal infection, at least in part, because the spirochete is destroyed in the infecting tick.**

Various model systems have been developed to provide a basis for developing vaccines against *Borrelia burgdorferi* and to probe the pathophysiology of this infection (1–4). Passive immunization of hamsters by means of polyclonal antiserum against syringe challenge of cultured spirochetes suggests that protection may be effective (5). Although the major surface antigen on the spirochete, OspA, is not immunodominant in naturally infected hosts, polyclonal and monoclonal antibodies to OspA similarly protect immune-intact C3H/HeJ mice (6), as well as the immune-deficient scid (7, 8). Indeed, we previously showed that mice actively immunized with a recombinant OspA were protected from infection and disease when challenged with an intradermal injection of three virulent *B. burgdorferi* isolates (6).

The effectiveness of immunization against the natural mode of transmission of this tick-borne infection, however, was not reported. Tick-mediated transmission may differ from syringe transmission in several crucial ways. Anti-inflammatory properties of the saliva of ticks may enhance pathogen transmission (9, 10) as occurs in the case of phlebotomine sandfly saliva and infection by *Leishmania braziliensis* (11). In addition, vector-borne pathogens may differ from those propagated *in vitro* in terms of immunogenicity as well as other transmission-related properties. A realistic challenge of a vector-borne agent of disease seems essential in evaluating a vaccine. We now demonstrate that active immunization with recombinant OspA protects mice against tick-borne spirochetal infection by destroying *B. burgdorferi* in ticks feeding on vaccinated mice.

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MATERIALS AND METHODS

Mice. Three-week-old, random sex, virus-antibody-free C3H/HeJ (C3H) mice were obtained from The Jackson Laboratory. They were shipped in filter-equipped crates and housed in micro-isolator cages. Food and water were provided *ad libitum*. Mice were killed with carbon dioxide gas. Outbred CD-1 mice were obtained from Charles River Breeding Laboratories.

B. burgdorferi. Low *in vitro* passage isolates of *B. burgdorferi* N40, with previously proven infectivity and pathogenicity in C3H mice, were utilized (12). The spirochetes were grown to logarithmic phase in modified Barbour–Stoenner–Kelly (BSK II) medium and counted in a hemocytometer under darkfield microscopy.

Recombinant OspA Fusion Protein. Recombinant OspA was expressed and purified as a fusion protein with glutathione transferase (GT) (6). In brief, the gene for OspA from N40 was ligated into plasmid pGEX-2T (Pharmacia) in frame with the GT gene. The recombinant plasmid was used to transform *Escherichia coli* strain DH5α. Production of the recombinant fusion protein was induced with isopropyl β-D-thiogalactopyranoside, and the protein was purified from the cell extract by affinity chromatography on a glutathione-Sepharose 4B column (Pharmacia) (6).

Infection of Ticks with *B. burgdorferi.* *Ixodes dammini* ticks were from a laboratory colony (maintained at the Harvard School of Public Health) derived from an Ipswich, Massachusetts, population, and had been determined to be free of inherited spirochetal infection. Outbred CD-1 mice were infected by means of intradermal inoculation of 10^3 low-passage N40 spirochetes 3 weeks prior to serving as hosts. Ticks were infected with *B. burgdorferi* by allowing larvae to feed to repletion on these mice. Upon repletion, engorged larvae were collected, pooled in groups of 100–200, and permitted to molt to the nymphal stage at 21°C and 95% relative humidity. Prevalence of infection in each pool of ticks was determined 3 weeks after molting, by examining a sample of 10 ticks with an immunofluorescence procedure. Only those pools in which spirochetal prevalence exceeded 70% were used for the challenge experiments.

Vaccination and Challenge of C3H Mice. Four-week-old mice were actively immunized with 10 μg of recombinant OspA fusion protein in complete Freund's adjuvant and given booster injections with the same amount of protein in incomplete Freund's adjuvant on days 14, 28, and 42. Control mice were actively immunized with GT in an identical manner. Fourteen days after the last boost, three or eight nymphs were placed on each mouse. All engorging ticks were permitted to feed to repletion and naturally detach over water.

Abbreviation: GT, glutathione transferase.

Ticks were collected from the water and stored at room temperature until their examination for the presence of spirochetes, 4 or 10 days later.

Upon sacrifice, animal joints and hearts were formalin-fixed, paraffin-embedded, sectioned, and examined microscopically for evidence of inflammation. Both tibiotarsal joints were examined. A mouse was considered to have arthritis if at least one joint showed evidence of periorbital edema and synovial infiltration with neutrophils and lymphocytes. Arthritis was blindly graded on a scale from 0 to 3: grade 0 represents the lack of inflammation, grades 1 and 2 indicate mild inflammation, and grade 3 signifies severe inflammation. Animals with grade 0 were considered free of disease. Carditis was characterized by aortitis, myocarditis, or atrial and ventricular pericarditis. Blood and spleen from experimental animals were collected aseptically, homogenized in BSK II medium (spleen), and cultured in BSK II medium. Cultures were incubated for 2 weeks and examined by darkfield microscopy as described (6). Twenty high-power fields were scanned per culture. Positive cultures had between 1 and 100 spirochetes, and typically contained 15 organisms, while negative cultures had no organisms. Mice were considered infected if at least one culture was positive and/or evidence of disease was present on histologic examination.

Immunofluorescence. Four or 10 days after detachment from the mice, engorged ticks were examined for spirochetes by a procedure designed to determine whether host antibody obscured our ability to detect infection. The protocol is a modification of published procedures (13). Individual ticks were homogenized in 100 μl of phosphate-buffered saline in a 1.5-ml microcentrifuge tube, and aliquots of 10 μl were spotted on each of three slides. Slides were allowed to air-dry, fixed in cold acetone for 10 min, and stained with fluorescein-labeled polyclonal rabbit antibody to *B. burgdorferi*, or with monoclonal antibodies H5332 (against OspA) and H9724 (against the 41-kDa flagellin) in an indirect immunofluorescence procedure. Tick homogenates were visualized by both direct and indirect immunofluorescence.

Random samples of the fresh tick homogenates were examined by darkfield microscopy. In addition, tick lysates were cultured (100 μl of tick triturate in 7 ml of BSK II medium at 32°C) for 4 weeks, to allow spirochetes to grow to the stationary phase, and examined by darkfield microscopy for the presence of spirochetes. Twenty high-power fields were examined per slide. A negative slide had no spirochetes. A positive slide had one or more spirochetes, and in virtually all cases more than four spirochetes, per high-power field.

RESULTS AND DISCUSSION

Mice immunized with the OspA fusion protein or GT (control) were used in the tick challenge experiments. Fourteen days after the last booster injection, three or eight ticks were placed on each mouse. To determine whether mice became infected by tick-transmitted spirochetes, samples of blood, spleen, and skin were cultured in BSK II medium at 2 weeks after exposure to infected ticks, and evidence of characteristic carditis and arthritis was sought in histopathological specimens. To determine whether ticks retained spirochetal infection after feeding on immunized mice, all engorged ticks were examined for the presence of spirochetes by immunofluorescence.

The prevalence of spirochetal infection in mice that had been immunized with OspA was compared with that in mice immunized with only the carrier protein (GT). Evidence of spirochetal infection was noted in all GT-exposed mice and virtually absent in mice exposed to the OspA protein (Table 1). Less than half as many GT-immunized mice became infected, however, when three infected ticks were used in the spirochetal challenge than when eight were used. We concluded that OspA-immunized mice were effectively protected against tick-borne spirochetal infection.

Table 1. Protection of mice immunized with recombinant protein against *B. burgdorferi* spirochetes transmitted by ticks

Immunogen	No. of challenge ticks	No. of mice examined	% mice		
			Spirochetes	Arthritis	Carditis
GT	3	21	38	19	38
	8	5	40	100	100
OspA	3	25	0	0	4
	8	5	0	0	0

Effect of recombinant GT carrier protein was compared with that of a similar preparation fused to OspA spirochetal antigen.

To determine whether ticks retained spirochetal infection after feeding on immunized mice, engorged challenge ticks were examined for evidence of infection. Of 230 ticks initially placed on the mice, 43% were recovered. The remainder were apparently eaten by their hosts as indicated by the frequent finding of fragments of ticks in the cages. Although more than 70% of these challenge ticks retained infection after feeding on GT-immunized mice, few of those feeding upon the OspA-immunized mice retained infection. Prevalence of infection was even less in ticks examined 10 days after feeding on OspA-immunized ticks than in those examined after 4 days (χ^2 test, $P < 0.001$; Table 2).

Morphologically intact spirochetes appeared within ticks that had fed on GT-immunized hosts but not within those that had fed on OspA-immunized mice (Fig. 1), regardless of whether monoclonal or polyclonal antibody was used to visualize these agents. Darkfield microscopy further confirmed that spirochetes were not detectable within ticks feeding on OspA-immunized mice but were present within the ticks feeding on GT-immunized mice. Furthermore, cultures from 6 of 10 ticks (examined 10 days after feeding) that had fed on GT-immunized mice were positive for spirochetes, whereas only 1 of 8 cultures from ticks that had fed on OspA-immunized mice were positive (χ^2, $P < 0.001$). Experiments designed to determine whether such ticks remained free of infection after molting to adults confirmed our immunofluorescence results: of 6 adults derived from nymphs feeding on OspA-immunized mice, none were determined by immunofluorescence to be infected, whereas 4 of 6 adults derived from ticks feeding on GT-immunized mice were infected (Fisher exact test, $P < 0.05$). We conclude that spirochetes are destroyed in ticks feeding on OspA-immunized animals and that this effect progresses following detachment of the ticks from the mice.

Although tick saliva theoretically has transmission-enhancing activities by local immunomodulation, spirochetes delivered via ticks are unable to evade the protective immune response in OspA-immunized mice. Adoptive transfer experiments suggest that antibody mediates protection within the

Table 2. Presence of *B. burgdorferi* in the guts of *I. dammini* ticks that had fed on mice immunized with recombinant OspA fusion protein

Immunogen	4 days		10 days	
	No. of ticks	% infected	No. of ticks	% infected
GT	20	80	40	72
OspA	14	14	25	0

Effect of recombinant GT carrier protein was compared with that of a similar preparation fused to OspA spirochetal antigen. The time at which engorged ticks naturally detached from the mice was considered day 1. The ticks were stored at room temperature and examined 4 or 10 days later, by direct and indirect immunofluorescence.

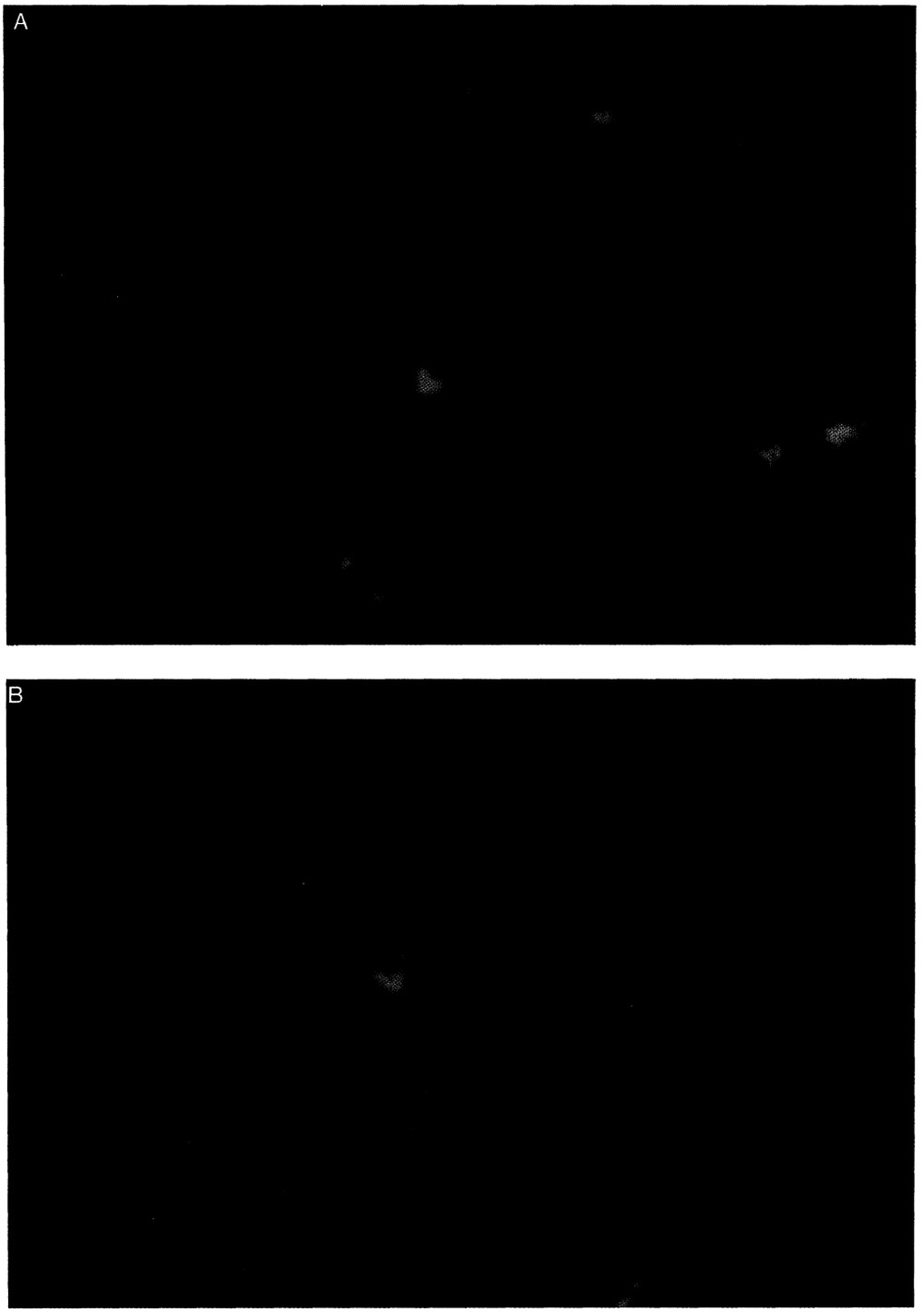

FIG. 1. Immunofluorescence of the guts of nymphal *I. dammini* ticks, infected with *B. burgdorferi* spirochetes, after feeding on a mouse immunized with recombinant OspA fusion protein (*A*) or on a mouse exposed only to the GT (control) carrier protein (*B*). No intact spirochetes were seen in ticks that had fed on OspA-immunized mice, and the photos are representative of the microscope fields seen from ticks that fed to repletion upon control or OspA-immunized mice. (×480.)

vertebrate host (6–8). It may be, however, that protection by anti-spirochetal antibody can be conferred by events that occur prior to the entry of the pathogen into the feeding site within the skin of the host. Lyme disease spirochetes undergo a precise developmental cycle in their vector tick (14). Of particular importance is the localization of these organisms within the gut of the nonfed ticks (15). Transmission is effected during the tick's attachment to the host when *B. burgdorferi* become activated, penetrate the gut wall, and migrate into the hemocoel and the closely apposed salivary glands (16). Because a minimum period of 24–48 hr of attachment is required before an infectious inoculum of spirochetes is delivered (17), a period of replication within the gut of the tick may precede dissemination into the hemocoel. Thus, these pathogens appear to be particularly vulnerable to destruction by antibody-mediated mechanisms within the gut prior to dissemination, because ticks concentrate the products of their blood meal. In other ixodid ticks, up to 20 ng of

intact IgG per microliter has been detected within the hemolymph of replete ticks (18–20), suggesting that even greater concentrations may be found within the gut itself.

The suppression of pathogens within their vector by host antibodies has been described for plasmodia (21), trypanosomes (22), and rickettsiae (23). This study, however, shows successful destruction of a pathogen within a vector feeding on a vaccinated host. It may thus be that a vaccine against infection by the agent of Lyme disease may be uniquely effective because of a dual mode of action: (*i*) destruction of the agent within the vector prior to transmission and (*ii*) antibody-mediated protection within the vertebrate host.

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