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Antibody Response of the Mouse Reservoir of *Borrelia burgdorferi* in Nature†

LAURA ROSA BRUNET, CATERINA SELLITTO, ANDREW SPIELMAN, AND SAM R. TELFORD III*

Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts 02115

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To determine whether the white-footed mouse reservoir host (*Peromyscus leucopus*) of the agent of Lyme disease (*Borrelia burgdorferi*) naturally mounts an immune response against the full range of antigens expressed by this zoonotic pathogen, we analyzed the pattern of immunoreactivity of these rodents at sites in which the intensity of transmission differs. Although the incidence of seroconversion within the reservoir population relates proportionally to the density of subadult deer ticks (*Ixodes dammini*), seroprevalence appears constant. About a fifth as many juvenile mice recognize spirochete antigens as do adult mice. Virtually all reservoir mice in nature recognize the p20, p35.5, p39, and p58 antigens, regardless of the intensity of transmission. Seropositive mice retain reactivity to a wide range of spirochetal antigens. Few mice recognize flagellin, OspB, and OspC. Although a third of serum samples include reactivity to a 31-kDa band, this reaction is irregular and may represent an uncharacterized antigen that comigrates with OspA. Mice captured where transmission is intense recognize the same spectrum of antigens as do mice captured where vector ticks are scarce.

The spirochetal agent of Lyme disease (*Borrelia burgdorferi*) is maintained in the northeastern United States mainly in white-footed mice (*Peromyscus leucopus*) (30). These rodents are readily infected by nymphal deer ticks (*Ixodes dammini*) and subsequently transmit spirochetes to larval ticks that feed on them. Even where transmission is so intense that virtually all mature mice would repeatedly have been exposed to infected ticks, only about a quarter recognize spirochete antigens, and their humoral response appears to be restrained (17). In the laboratory, syringe-inoculated mice and naturally infected mice, however, do recognize spirochetal antigens, rapidly mounting a prominent humoral response against antigens ranging in size from 60 to 16 kDa (20, 24, 28). Field observations suggesting that the antispirochetal immune response of white-footed mice is restrained thus stand in contrast to laboratory observations suggesting that these mice are immune competent and mount a vigorous humoral response.

The restrained immune response of white-footed mice against spirochetal infection may reflect the complex relationship that develops naturally between populations of pathogens and their reservoir hosts. Exposure to an infectious agent during early fetal development, for example, may alter subsequent immunological responsiveness (37). The offspring of filaria-infected mothers more readily develop patent infection than do those of noninfected mothers (22, 31), and the hypersensitivity granulomatous responses of the offspring of schistosome-infected mice are less intense than of those of noninfected mice (16). Other immunity-modifying factors operating in nature include the host age at time of infection (6) and the potentially immunosuppressive effects of coinfecting pathogens such as *Babesia microti* (18). The effects of tick salivary product may also modulate the mouse-spirochete relationship (35).

It may be that the immune response of the natural reservoir of the agent of Lyme disease varies with the force of transmission. To test this hypothesis, we compared seroprevalence and pattern of immunoreactivity against spirochetal antigens in serum samples from white-footed mice captured repeatedly at sites in which the force of transmission differed.

MATERIALS AND METHODS

Study sites and specimen collection. Great Island and Nantucket Island were selected for study because they differed in the force of transmission of the agent of Lyme disease. Great Island is a 240-ha tombolo located off of Cape Cod on the southern Massachusetts coastline. The reduction of its deer population has decreased Lyme disease transmission during the past decade (39). Nantucket Island, a 12,000-ha island situated 30 km southeast of Great Island, served as a comparison site. Here, the deer population has remained abundant, and Lyme disease is intensely zoonotic. Both sites are similar in appearance (32, 38).

White-footed mice were trapped as part of a capture-mark-release study from May through October during two consecutive years, 1992 and 1993. Mice were trapped at monthly intervals for 2 nights on permanently situated 0.4-ha grids (four on Great Island and two on Nantucket Island), each containing 49 Longworth box traps. Blood samples were collected from the retroorbital sinus of each mouse. Age, weight, and tick infestation were recorded for all mice, which were individually ear tagged and released at the site of capture. Mice with a mass of <18 g were considered to be juveniles (15, 31a).

Preparation of antigen for immunoblots. Strain GI-2 (passage 2) of *B. burgdorferi* (26), originally isolated from a rabbit fed upon by ticks collected on Great Island, was used as the antigen for immunoblots. The high-passage B31 strain (ATCC 35210) was used as a reference. Spirochetes were grown to a density of $\sim 3 \times 10^8$ cells per ml in BSK-H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum. Cells were harvested by centrifugation, washed three times in phosphate-buffered saline (pH 7.2) containing 5 mM $MgCl_2$, and resuspended in sodium dodecyl sulfate (SDS)-sample buffer (62.5 mM Tris-Cl [pH 6.8], 2% SDS, 50 mM dithiothreitol, 10% glycerol, 0.004% bromophenol blue). After being boiled for 5 min, the aliquoted antigen was stored at −20°C. Aliquots containing approximately 1.5×10^7 spirochetes were loaded on SDS-polyacrylamide (10%) gels (8.4 cm wide and 0.75 mm thick) and electrophoresed according to the method of Laemmli (14). Separated proteins were then electrophoretically transferred to nitrocellulose membrane (MSI, Westboro, Mass.) as previously described (36). Proteins on blots were visualized with Ponceau S staining (Sigma). The molecular masses of major spirochetal protein bands were calculated by comparing their electrophoretic mobilities with those of molecular

* Corresponding author. Phone: (617) 432-4079. Fax: (617) 738-4914. Electronic mail address: stelford@hsph.harvard.edu.

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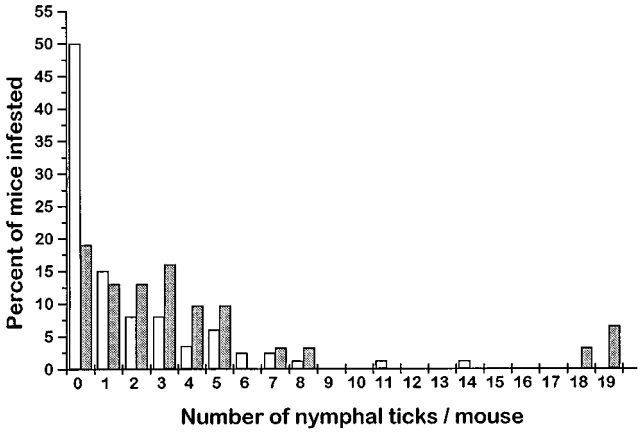

FIG. 1. Proportion of mice infested with nymphal deer ticks on Great Island (open bars) and Nantucket Island (shaded bars).

mass standards (Pharmacia, Piscataway, N.J.). After the positions of major known antigen bands were marked, the blots were destained and processed for immunostaining.

Immunostaining of blots. Mouse sera collected in the field were separated from whole blood and stored at −20°C. Negative and positive control sera were always included in the immunoblot assays as follows. (i) The sera of laboratory-reared white-footed mice either never exposed to Lyme disease spirochetes or exposed to the bites of clean vector ticks from our laboratory colony were used as negative controls. (ii) The sera of laboratory-reared white-footed mice either experimentally infected by tick bite or immunized with recombinant outer surface protein A (OspA) fusion protein (8) were used as positive controls. (iii) Monoclonal antibodies (MAbs) H9724 (2) and H604 (3) against flagellin, H5332 (5) and H3TS (3) against OspA, H6831 (4) and B10 (9) against OspB, and 1B3B6C6 against OspC (courtesy of S. Padula, University of Connecticut) and a MAb against p39 (25) were used to identify the positions of the respective antigens. For immunostaining, blots were blocked in Tris-buffered saline (pH 7.2), containing 0.05% Tween 20 (TBST) and 5% low-fat milk and then mounted inside a Miniblot apparatus (Immunetics Inc., Cambridge, Mass.). Fifty microliters of diluted mouse serum (1:50 in TBST) were used to probe the blots. After a brief wash in TBST, the specific immunoreactivity of mouse sera was detected with an alkaline phosphatase-conjugated goat anti-*Peromyscus* immunoglobulin G (H + L chains) serum (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:1,000 in TBST containing 1% low-fat milk. Immunoreactive bands were visualized with bromochloro-indolylphosphate substrate and nitroblue tetrazolium chromophor (Amresco, Solon, Ohio). The immunoblots were developed until the positive control reached a standardized color intensity at which no background staining was seen in the negative control. A serum reactive with at least three of the specific spirochetal antigens (p18-20, p29, p35.5, p37, p39, p41, p45, p58, p66, p88, and p93-100; 31-kDa OspA; 34-kDa OspB; and 23-kDa OspC [modified from reference 7]) was considered positive.

Statistical analysis. Nymphal tick infestation differences between the two study sites were evaluated by Mann-Whitney test. Differences in the frequency of reactivity against particular antigens between the two sites were determined by the chi-square test with Yates' correction. When sample sizes were small ($n < 10$), the Fisher exact test was used. A P value of 0.05 or less was considered significant.

RESULTS

Force of transmission. In a preliminary series of observations, we identified sites in which the intensity of transmission of the Lyme disease spirochete differed. Thus, the density of feeding ticks infesting mice in four trapping sites on Great Island was compared with that in two sites on Nantucket Island (Fig. 1). The median nymphal burden per mouse during May through June in two consecutive transmission seasons differed between Great Island and Nantucket Island. On Great Island, a median of 0.5 nymphs infested each mouse ($n = 84$), compared with 3 nymphs per mouse ($n = 31$) on Nantucket Island (Mann-Whitney test; $P<0.001$) (Fig. 1). During May through June, a mean of 1.7 (standard deviation, 2.6) and a maximum of 14 nymphs parasitized mice on Great Island in contrast to 5.16 (standard deviation, 7.6) and 35 nymphs, respectively, on Nantucket Island. Mice captured on Nantucket Island, therefore, provided samples representing intense transmission, and those on Great Island represented low-level transmission.

Prevalence of immunoreactivity. To select a spirochete isolate for use in our immunoblot assays, we compared the electrophoretic protein patterns and the reactivity to eight MAbs of a local isolate (GI2) with that of a standard reference isolate (B31). The overall electrophoretic protein pattern of the GI2 isolate was similar to that of the B31 strain (Fig. 2A). In the low-molecular-mass region of the GI2 isolate, an ~22-kDa protein migrated slightly faster than in the B31 strain. This band, visible in Ponceau S-stained blots (Fig. 2A), was immunologically nonreactive and failed to bind a MAb against OspC (1B3B6C6). The MAb instead identified OspC as a 23-kDa protein in GI2 (Fig. 2B, GI2, lane 8). Our B31 antigen did not react with the OspC MAb (Fig. 2B, B31 lane 8). Both strains were probed with MAbs to localize the positions of known spirochetal antigens (Fig. 2B). An anti-OspB MAb (H6831) failed to react with the GI2 isolate (Fig. 2B, GI2 lane 5), and the position of this antigen was established by means of the B10 MAb (Fig. 2B, GI2, lane 7). In order to avoid the possibility of changes in the antigenicity of spirochetal proteins as a function of prolonged in vitro cultivation of ATCC B31 (23), we selected the low-passage GI2 isolate for subsequent analyses.

We then determined whether the prevalence of antispirochetal seropositivity in white-footed mice on Nantucket Island, where transmission is intense, differs from that on Great Island, where transmission is more diffuse. The age and sex distributions of both populations of mice proved to be similar (Table 1). We considered a serum sample with reactivity to at least three specific spirochetal antigens as positive (see Materials and Methods). Our criterion of seropositivity was confirmed by separate observations of the development of the humoral response of white-footed mice experimentally infected with Lyme disease spirochetes by tick bite (Fig. 3 and unpublished observations). Laboratory-reared white-footed mice exposed to the bite of infected vector ticks develop reactivity to a small number of spirochetal antigens, which we consider to be specific for *B. burgdorferi*, during the initial 10 days of infection and then proceed to develop reactivity against the full range of antigens in the following weeks. On both Great Island and Nantucket Island, seroprevalence in adults was five times as great as that in juvenile mice (Fig. 4A). The prevalence of reactivity in adult mice from each site was similar, as was the prevalence in the two juvenile mice populations. Thus, the prevalence of seroreactivity of mice against the agent of Lyme disease may not correlate with the force of transmission.

Incidence of immunoreactivity. The incidences of seroreactivity against the agent of Lyme disease between these two sites were then compared. All mice that had been captured were marked and released, and 83 mice (including 54 on Great Island and 29 on Nantucket Island) were recaptured during the following months. The serum samples of 32 mice failed to react against spirochetal antigens when those mice were first captured (20 on Great Island and 12 on Nantucket Island). When recaptured the following month, one in three of the mice had seroconverted on Great Island and two in three had seroconverted on Nantucket Island (Fig. 4B and 5A). Although seroprevalence is similar at our study sites, incidence of infection is greater where tick density is greater.

Spectrum of immunoreactivity. To determine whether intensity of transmission affects the nature of the humoral response of mice, we compared the pattern of immunoreactivity of mice trapped on Great Island with that of mice trapped on

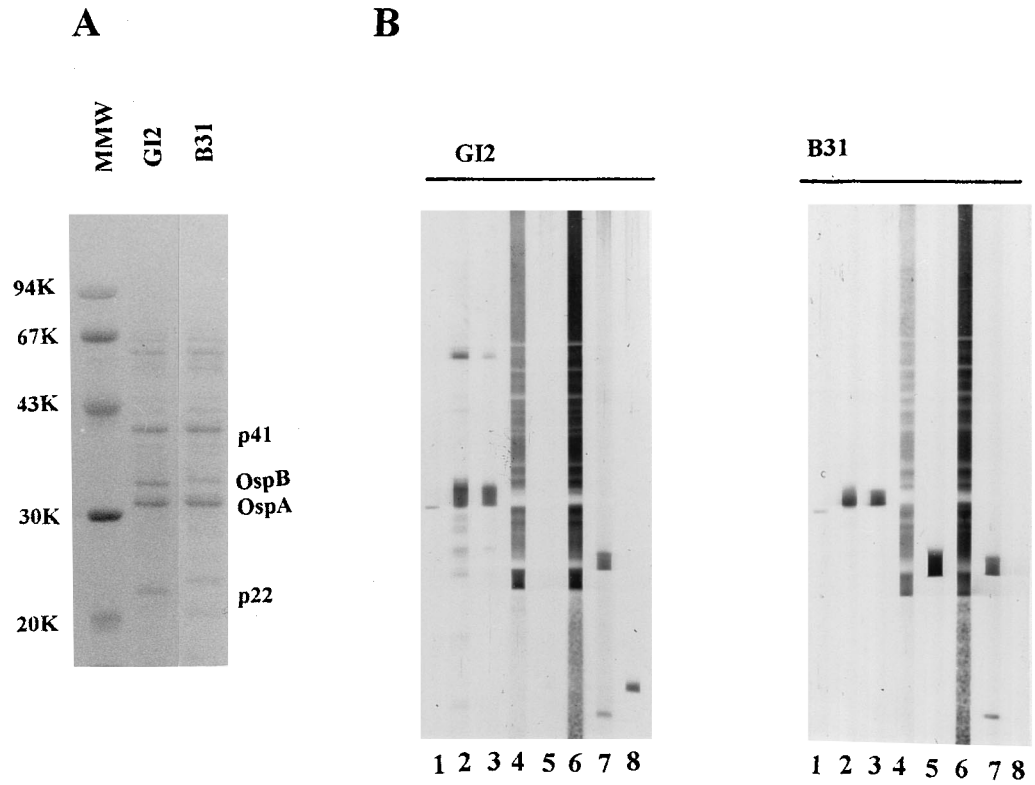

FIG. 2. Electrophoretic pattern of spirochetal antigens and their immunoreactivity with specific MAbs. (A) Ponceau S staining of *B. burgdorferi* isolates GI2 and B31. Standard molecular weight markers (MMW) are shown on the left. (B) Reactivity of GI2 and B31 isolates with the following MAbs (by lane): 1, anti-p39; 2 and 3, anti-flagellin H604 and H9724, respectively; 4 and 6, anti-OspA H5332 and H3TS, respectively; 5 and 7, anti-OspB H6831 and B10, respectively; and 8, anti-OspC 1B3B6C6.

Nantucket Island. A total of 224 serum samples from Great Island mice and 154 serum samples from Nantucket mice were probed on GI2 isolate blots (Fig. 5 and 6). In addition, the pattern of immunoreactivity of these sera was compared with that of sera of chronically infected mice bitten by five laboratory spirochete-infected ticks and the reactivity of MAbs (see Fig. 3 for representative samples). We identified one serum sample, from an experimentally infected mouse, able to discriminate as many as 17 bands spanning the entire length of the blot between 94 and 18 kDa (Fig. 5C and 6F), and we included it as positive control in all of our blots. Many of the field-derived sera were reactive to the p20, p29, p31, p35.5, p37, p39, p45, p58, p66, p88, and p93-100 antigens (Fig. 5D and 6G). These antigens, along with OspA (31 kDa), OspB (34 kDa), and OspC (23 kDa), were selected for the analysis that follows.

TABLE 1. Age and sex of white-footed mice sampled on Great Island and Nantucket Island during two successive transmission seasons from May through October of 1992 and 1993

Mouse age or sex	Data for sampling sites on:			
	Great Island		Nantucket Island	
	n	%	*n*	%
Juvenile	47	36.7	27	25.7
Adult[a]	88	68.7	80	76.2
Female	58	45.3	35	33.3
Various[b]	4	3.1	8	7.6
Total	128		105	

[a] Of these, 11 mice on Great Island and 10 on Nantucket Island had previously been captured as juveniles.
[b] Various, age and sex not determined.

To determine whether reactivity against each of these antigens may change over time, we compared the serum samples of the 83 mice that were captured repeatedly. Forty of these mice (30 from Great Island and 10 from Nantucket Island) had antibodies against at least four antigens at the time of the first capture (Fig. 5B 6A through E). Their pattern of immunoreactivity remained constant throughout the sampling period, and four mice (three from Great Island and one from Nantucket Island) retained reactivity against the same antigens over the winter months (Fig. 5B). The serum samples of only 11 mice (4 from Great Island and 7 from Nantucket Island) reacted with fewer than 4 of the 14 selected antigens when they were first captured. These antigens included the p35.5 (70%), p39 (100%), and p58 (20%) antigens. When these mice were subsequently recaptured, their serum samples reacted with at least 6 of the 14 antigens. These observations indicate that mice recognize p35.5, p39, and p58 soon after they are infected and that chronically infected mice retain reactivity against a wide spectrum of antigens.

We determined whether the frequency of immunoreactive bands on blots probed with Great Island reactive mouse serum samples ($n = 148$) may differ from that probed with Nantucket Island mouse serum samples ($n = 104$). Of the 14 selected antigens, 10 were present as frequently in sera from Nantucket Island mice as in those from Great Island mice (Fig. 7). Only the smallest ($P < 0.001$) and particularly the largest ($P < 0.001$) antigens appeared to differ in frequency of recognition.

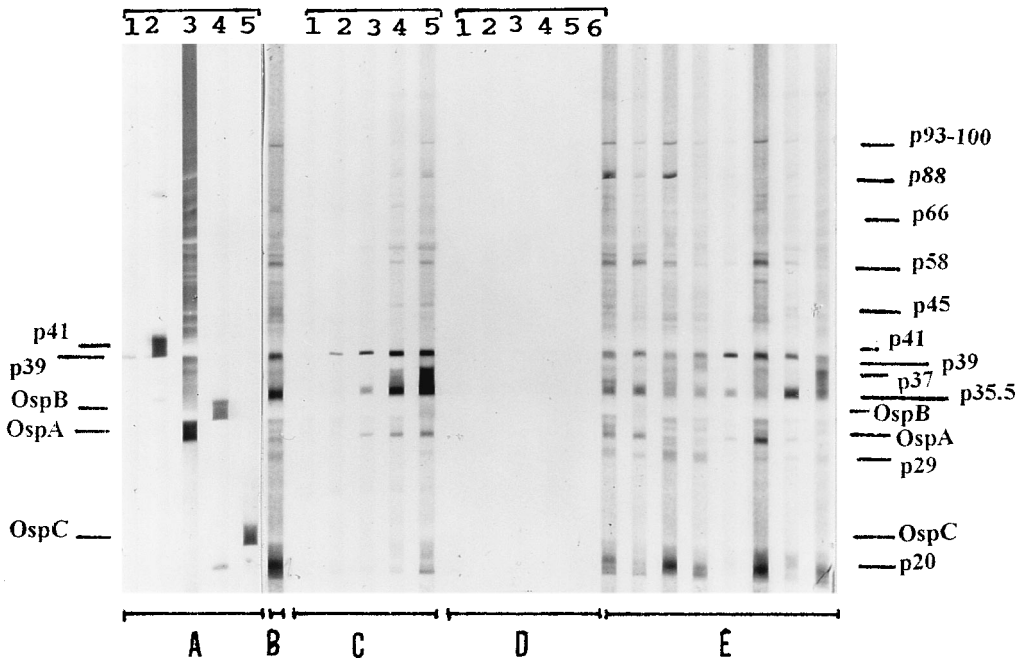

FIG. 3. Immunoblot analysis of representative serum samples collected from laboratory-reared white-footed mice and from wild mice captured at the two study sites. (A) Reactivity of G12 with MAbs anti-p39 (lane 1), anti-flagellin H604 (lane 2), anti-OspA H5332 (lane 3), anti-OspB B10 (lane 4), and anti-OspC 1B3B6C6 (lane 5). (B) Positive control serum sample from a laboratory-reared white-footed mouse infected by tick bite. (C) Serum panel from a laboratory-reared white-footed mouse bled on days 0 (lane 1), 5 (lane 2), 10 (lane 3), 38 (lane 4), and 65 (lane 5) postexposure to an infected tick bite. (D) Serum samples from six laboratory-reared white-footed mice free of *B. burgdorferi* infection and exposed to laboratory-reared uninfected vector ticks. (E) Representative serum samples collected from mice captured on Great Island and Nantucket Island.

Reactivity against four borrelial antigens, p20, p35.5, p39, and p58, was observed in more than 75% of the serum samples tested. We also observed strong reactivity against p29, p66, and p93-100. We conclude that the immune systems of reservoir mice recognize the same set of antigens regardless of intensity of transmission.

DISCUSSION

Our finding that white-footed mice frequently mount an immune response in nature against Lyme disease spirochetes contrasts with the results of similar observations of mice from other zoonotic sites (17). The sera of more than half of our mice reacted by immunoblot with spirochetal antigens, in contrast to only a quarter of serum samples from mice from these other sites in an enzyme-linked immunosorbent assay (ELISA) screen. It had been suggested that the "immunological response in *P. leucopus* may be delayed" and that the "antibody titers for these rodents were relatively low" (17). The humoral response in our mice, however, develops rapidly, and those that seroconvert remain intensely immunoreactive for the duration of their lives. These contrasting findings cannot be explained by differences in transmission intensity, because transmission is weak in one of our sites. On Great Island, no human cases of infection were detected during the 7 years that preceded our study. It may be that the difference derives from an overrepresentation of juvenile mice in the sites that were previously studied or from a poor performance of the secondary antibody used in the ELISA screen (17). We conclude that the mouse reservoir host of the agent of Lyme disease reacts strongly against antigens naturally presented by this spirochete.

Our observations suggest that reservoir mice born to a spirochete-infected mother are capable of mounting an immune response against the agent of Lyme disease. If such mice were to become immunotolerant, seroreactivity would vary inversely with prevalence of infection. At least two-thirds of adult mice in our enzootic sites have been infected, and no more than a third of their progeny would recognize spirochete antigen as nonself if they were tolerized in utero. We have monitored the epizootiology of the agent of Lyme disease in both field sites over a span of at least 10 years and find that transmission intensity has remained relatively constant. The nearly universal seroreactivity of these mice in nature, therefore, argues that fetal exposure to antigen may play no role in the natural history of this infection.

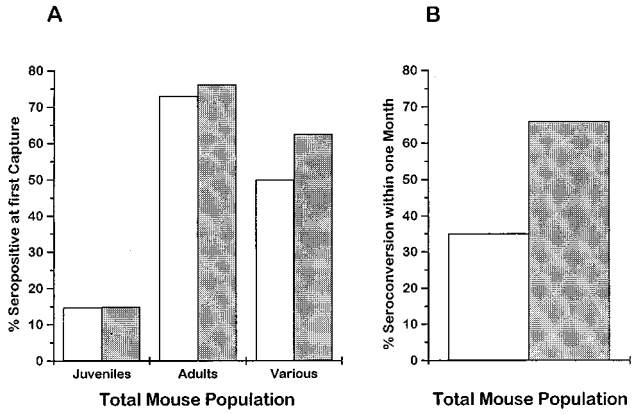

FIG. 4. Seroprevalence and incidence of spirochetal infection. (A) Proportion of mice on Great Island (open bars) and Nantucket Island (shaded bars) that at first capture were immunoreactive against spirochetal antigens. Various, age not determined. (B) Proportion of mice seroconverting within 1 month on Great Island (open bar) and Nantucket Island (shaded bar).

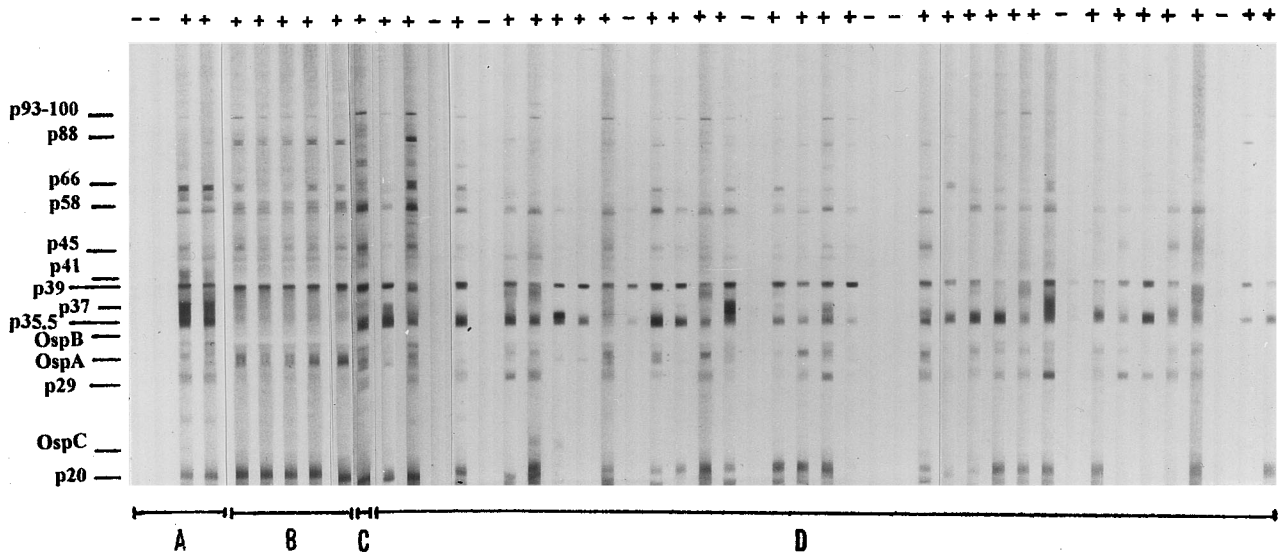

FIG. 5. Immunoblot analysis of representative serum samples collected from mice captured on Great Island. (A) Serum panel from a mouse which was seronegative in May and June (first two lanes), had seroconverted by August (third lane), and remained positive through October of the same year (fourth lane). (B) Serum panel from a mouse which was seropositive from May through October of the same year (first four lanes) and remained immunoreactive over the winter until May of the next year (fifth lane). (C) Positive control serum sample from a laboratory-reared white-footed mouse infected by tick bite. (D) Panel of representative positive (+) and negative (−) serum samples.

We considered the possibility that antigen recognition in mice on Nantucket Island may differ from that on Great Island because transmission is more intense there. In the course of infection, mice may recognize as many as 14 spirochetal antigens, including several (p20, p39, p35.5, and p58) that generally react with particular frequency. Only at the upper or lower ends of our blots do we detect differences in the frequency of antigen recognition. The biological basis for these differences remains unclear, but may be related to subtle differences in the antigenic profile of the spirochetes infecting the mice in our study sites or to differences in the major histocompatibility complex molecules expressed by the two reservoir populations (11).

Mice appear to recognize spirochete antigens in a particular sequence. Reactivity against the p35.5, p39, and p58 antigens develops before that against other spirochetal antigens. All 11 of the mice in our study whose serum samples reacted with these antigens when first captured recognized additional spirochete-associated antigens when they were recaptured. Reactivity to the p35.5 antigen is a dominant feature of the white-footed mouse immune response and merits further investigation. This antigen does not react with MAb preparations

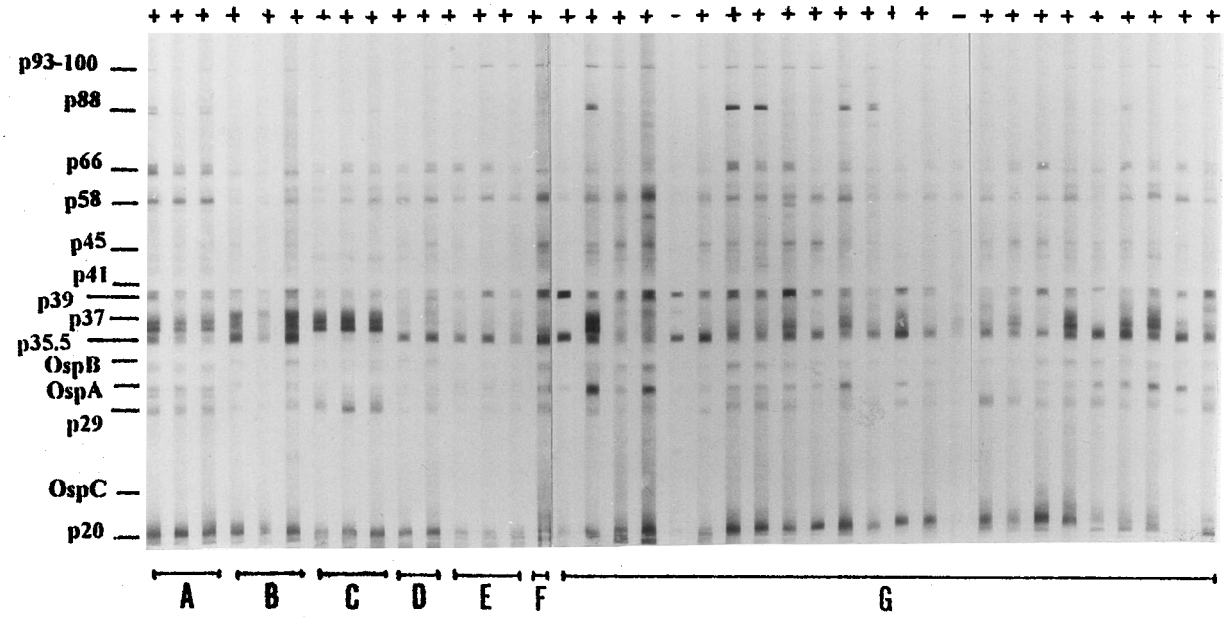

FIG. 6. Immunoblot analysis of representative serum samples collected from mice captured on Nantucket Island. (A, B, C, D, and E) Serum panels from mice captured during consecutive months. (F) Positive control serum sample as in Fig. 4C. (G) Panel of representative positive (+) and negative (−) serum samples.

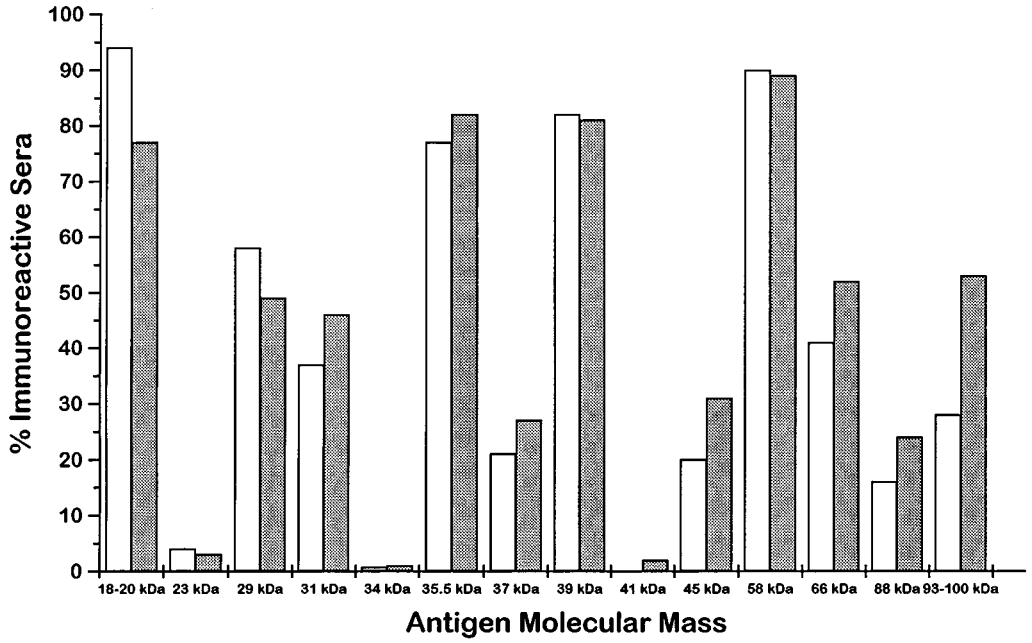

FIG. 7. Proportion of white-footed mouse sera from Great Island (open bars) and Nantucket Island (shaded bars) reactive with specific spirochetal antigens.

against OspB, and sera from chronically infected mice simultaneously recognize p35.5 as well as the p37 antigen. The persistent immunoreactivity against p39 in the white-footed mouse is consistent with previously reported experimental infections in these hosts (28), in mice (12), in hamsters (19, 21), and in guinea pigs (29). Reactivity against p58 has been previously described in recombinant inbred strains of mice (11). The prominent reactivity against the p35.5, p39, and p58 antigens may be derived from some unique feature of the Lyme disease spirochete in both of our study sites or from some peculiarity of the mouse-spirochete relationship in nature.

The mice in our study sites generally fail to react with flagellin, reflecting a disagreement in the literature. This antigen is said to be the first to be recognized in newly infected hosts, such as needle-inoculated mice or naturally infected humans (1, 27). Other research workers suggest, however, that anti-flagellin antibodies are only infrequently present in human sera and ascribe the problem to confounding effects such as exposure to nonpathogenic spirochetes and reactivity to p39 (28). We confirmed the presence of both p39 and flagellin on our blots by their reactivity with specific MAbs. We conclude that antibodies to p39 are ubiquitous in rodents in our study sites and may be indicators of active infection (28). As previously described for hamsters, anti-flagellin antibody may be present only transiently in the natural reservoir of spirochetal infection (19).

Mice in our study sites generally failed to react against OspB and OspC. We were surprised to find apparent reactivity against the OspA antigen in almost half of our mice because OspA and OspB are only infrequently and slowly recognized by naturally infected human as well as nonhuman hosts (2, 10, 11, 13, 19). Indeed, such antibodies would inhibit reservoir competence (33). OspA and OspB are almost invariably recognized, on the other hand, by parenterally infected hosts (19, 28). Reactivity to the 31-kDa band in naturally infected dogs and mice has been previously reported (11, 13). These reports concluded that the observed reactivity, described as a thin band situated near the leading edge of the band bound by anti-OspA MAbs is not against OspA. These, along with our own observations based on a recombinant OspA ELISA (unpublished observations), therefore, persuade us that the observed reactivity is directed against a protein that comigrates with OspA. The presence of this antigen may confound attempts to determine the natural frequency of recognition of OspA in reservoir mice, a study which might require the use of a number of different recombinant OspAs in immunoassays to avoid problems related to OspA epitope heterogeneity. The mice in our study sites, therefore, appear not to respond to the OspA, OspB, and OspC antigens, even though these antigens elicit strong immune responses in syringe-inoculated hosts (27).

Our study sites differed in the force with which the agent of Lyme disease is transmitted. On Great Island, the density of nymphal ticks has been drastically diminished by a deer reduction program, and no new human Lyme disease cases have been described since 1985. On Nantucket Island, however, ticks and deer remain abundant, and Lyme disease is intensely enzootic there. Regardless of the difference in nymphal tick density between the two sites, the prevalence of seropositivity in the reservoir population is similar. Perhaps, even when vector ticks are present in low density, Lyme disease spirochetes may efficiently be maintained. Alternatively, transmission of spirochetes may be spatially heterogeneous, even within a site with a size of approximately 200 ha such as Great Island (34). Although the prevalence of infection in the white-footed mouse reservoir population is similar at both sites, the probability of an uninfected mouse seroconverting within 1 month is nearly twice as great on Nantucket Island as on Great Island, reflecting the greater density of nymphal ticks at this site.

Our observations suggest a general absence of immunoreactivity of white-footed mice against spirochetal flagellin, OspA, OspB, and OspC. This may be related to the exceptional reservoir competence of these mice in nature. In contrast, we observe prominent reactivity against three less-well-characterized antigens (p35.5, p39, and p58). We conclude that the main enzootic reservoir mounts a rapid, intense, and long-

lasting humoral response to most spirochetal antigens. The absence of a response to the major borrelial outer surface proteins may represent coevolution to a seemingly benign interaction between the Lyme disease spirochete and its reservoir.

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