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Rapid Dissemination by the Agent of Lyme Disease in Hosts That Permit Fulminating Infection

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We determined whether the agent of Lyme disease (*Borrelia burgdorferi*) disseminates more rapidly following deposition in hosts that permit fulminating infection than in hosts in which infection is relatively benign. Thus, individual infected nymphal deer ticks (*Ixodes dammini*) were permitted to engorge on the ears of C3H mice, and the site of attachment was excised at intervals thereafter. Infection in each mouse was determined by serology and by examining previously noninfected ticks that had engorged on these mice. These results were compared with data obtained similarly by using the CD-1 strain of mice in which the agent is relatively nonpathogenic. When the site of inoculation was ablated within 2 days after the infected tick became replete, dissemination was aborted. Spirochetemia could not be demonstrated in any of these mice. We conclude that Lyme disease spirochetes disseminate from the feeding lesion of an infecting tick more rapidly in certain highly spirochete-susceptible mice than in others in which pathogenesis is less severe.

The etiologic agent of Lyme disease (*Borrelia burgdorferi*) remains localized for several days in the skin of a rodent host where the spirochetal inoculum had been deposited by an infecting deer tick (*Ixodes dammini*) (27). Although the mechanism for retention of these spirochetes close to the site of tick attachment has not been demonstrated, this finding is consistent with the facility with which these pathogens migrate in skin (14). The erythema migrans lesion in the skin of an infected person expands progressively after an initial period of delay (1, 6, 7, 29) of about 1 to 2 weeks.

The severity of the lesion produced by Lyme disease spirochetes differs according to the strain of mice used as hosts. In needle-inoculated CD-1 mice, an outbred strain, spirochetemia is only rarely demonstrable and pathogenesis is limited (16). Similar infection in inbred C3H mice, on the other hand, is fulminating and spirochetemia is said to be prominent between 3 and 30 days after inoculation (3, 4). Spirochetes are far more readily demonstrated in diverse tissues of these C3H mice than in those of the CD-1 strain. The courses of spirochetal dissemination, however, have not been compared.

It may be that Lyme disease spirochetes more promptly disseminate from their site of inoculation in hosts that permit fulminating infection than in others in which the agent develops less abundantly. To explore this possibility, we permitted spirochete-infected deer ticks to feed on spirochete-vulnerable mice and compared the span of time during which the spirochetes remain localized at the site of inoculation. In particular, we determined whether the site of spirochete inoculation in C3H mice must more promptly be ablated to abort infection than in CD-1 mice.

MATERIALS AND METHODS

Animals. C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine) which were 3 to 4 weeks old, were held at an ambient temperature of 22°C and exposed to 16 h of light per day. In one experiment, age-matched CD-1 mice (Charles River Laboratories, Wilmington, Mass.) were used.

Ticks. Ticks used in this study were the second generation progeny of adult deer ticks collected from the Crane Wildlife Reservation (Ipswich, Mass.). They were reared and maintained as described previously (27). Ticks were stored in mesh-covered plastic vials at 22°C, with 95% relative humidity and 16 h of light per day. These ticks were free of inherited spirochetal or babesial infection. To obtain spirochete-infected nymphs, these larvae were permitted to feed on infected CD-1 mice.

Spirochete strain. The spirochetal isolate of *B. burgdorferi* (JD1) used in these experiments was originally isolated from naturally infected deer ticks swept from vegetation in the site in which the tick colony originated (18). The isolate reacted with monoclonal antibody H5332 (2) and *B. burgdorferi*-specific DNA probes (26). These JD1 spirochetes have been maintained in a system of alternate passage between deer ticks and laboratory mice.

Experimental infection of mice. To ensure successful infection of mice, groups of infected nymphs derived from engorged larvae were used in these experiments only when >80% proved to be infected with Lyme disease spirochetes (as determined by either dark-field microscopy or direct immunofluorescent-antibody assay). One spirochete-infected nymphal deer tick was placed on the ear of each mouse, and tick-infested mice were caged individually over water, as described previously (27). Detached ticks were collected and examined for evidence of infection, as described previously (8). Thus, each experimental mouse was subjected to the bite of an individual tick; each tick was later examined to determine whether spirochetes were present in its gut.

Ablation of feeding sites. Mice were examined daily following attachment of the infecting tick, and the time of detachment was noted. The full depth of the ear at the site of feeding was excised at intervals thereafter with a 6-mm surgical skin punch, as described previously (27). Feeding sites were ablated at 0, 1, 2, 3, 5, 7, or 10 days after detachment.

Detection of infection in mice. To determine whether mice sustained disseminated spirochetal infection, we used a xenodiagnostic procedure. Thus, at least 50 laboratory-

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TABLE 1. Dissemination of Lyme disease spirochetes from the site of deposition in the skin of C3H mice[a]

Sample source and no. of days after tick repletion	Evidence of infection in mice		
	No. of mice tested	% of mice infectious to ticks	% of explants infected in cultures
Ablated site			
0	7	0	0
1	7	0	0
2	11	27	9
3	7	29	29
5	10	30	60
7	10	50	80
10	7	86	0
Intact site	7	100	

[a] Infectivity to ticks following ablation of the bitten site was compared with infectivity in cultures of the ablated tissue.

TABLE 2. Seroreactivity against the agent of Lyme disease in mice following the bite of an infected or noninfected tick

Source of bite and time after infection (week)	No. of mice tested	Reciprocal titer in infected mice[a]			
		IgM		IgG	
		Mean	SE	Mean	SE
Infected tick					
1	26	10.0	6.3	0	
2	26	50.0	26.8	100.0	34.6
4	26	100.0	34.6	1000.0	424.3
6	25	100.0	43.3	1000.0	353.6
8	24	50.0	28.0	1000.0	255.4
12	20	8.0	2.3	500.0	397.4
Noninfected tick[b]	30	0		0	

[a] Determined by indirect immunofluorescent-antibody assay.
[b] Mice were sampled 1 to 4 weeks after being bitten.

reared noninfected larval deer ticks were permitted to feed on each such mouse 4 weeks after it was bitten by infected nymphs. After xenodiagnosis, 10 engorged larvae and 5 to 10 derived nymphs were dissected and examined for spirochetes by direct immunofluorescent-antibody assay or by dark-field microscopy as described previously (27).

Isolation and identification of spirochetes. To culture spirochetes from the excised tissue of the ear of each mouse, tissues were incubated in microdilution plates containing BSK-II medium, as described previously (28). In one experiment (as specified), BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) was used. The identity of spirochetes isolated from excised tissues was verified by direct immunofluorescent-antibody assay, using fluorescein isothiocyanate-conjugated polyclonal anti-*B. burgdorferi* rabbit serum.

Serologic tests. To detect antibody against the Lyme disease spirochetes, sera collected from mice exposed to the bites of infected and noninfected ticks were serially diluted and tested by an indirect immunofluorescent-antibody assay as previously described (30). Reactivity of serum at a dilution of 1:100 served as a conservative criterion of infection (30). After xenodiagnosis was completed, those mice whose sera reacted in this test were assayed further at 6, 8, and 12 weeks after feeding.

RESULTS

To determine how rapidly Lyme disease spirochetes disseminate in C3H mice from their site of natural deposition, one infected (or noninfected) nymphal deer tick was allowed to feed to repletion on the ear of each mouse. We ablated the feeding site at various intervals after the infecting tick had detached, and two samples of each tissue taken from the ear of each mouse were cultured in duplicate in modified BSK-II medium. No infections became disseminated in mice when the site of feeding was ablated within 1 day after the infecting tick detached. Only a few mice (<30%) became infected when the feeding site was ablated after an additional 3 days, and the infection rate gradually increased to about 50% when the feeding site remained in place for 1 week (Table 1). In contrast, infections became disseminated in all but one mouse when the feeding site remained in place for 10 days, and all mice became infected when the feeding site remained intact. We conclude that spirochetes begin to disseminate from their site of inoculation in C3H mice at 2 days after they were deposited.

To determine whether spirochetes remain localized at their site of inoculation, tissues ablated in the above experiment were placed in culture medium. Spirochetes developed in only one of these samples that was ablated at 2 days after the infecting tick detached; however, the number of samples showing spirochete growth progressively increased to 80% of the specimens ablated at the end of the first week. No spirochetes could be cultured from tissues ablated at 10 days after detachment of the infecting tick. These observations indicate that spirochetes multiply at their point of inoculation and that their density subsequently diminishes.

We then assessed the humoral immune response of C3H/HeJ mice against Lyme disease spirochetes following ablation of the site of feeding of the infecting tick. Sera were sampled from the mice in the previously described experiment at intervals (1, 2, 4, 6, 8, and 12 weeks) after each infecting tick had detached. Immunoglobulin M (IgM) titers became elevated (1:100) at 4 to 6 weeks after infection and subsequently decreased and waned after 8 weeks. IgG titers became elevated (1:100) at 2 weeks after infection, increased markedly (1:1,000) at 4 to 8 weeks, and remained elevated through 3 months after infection (Table 2). In contrast, both IgM and IgG titers in sera from animals that were bitten by noninfected ticks never exceeded 1:10. The magnitude of the humoral immune response in these C3H mice appears to rise somewhat earlier than that in previously reported work with CD-1 mice.

Finally, we determined whether spirochete-infected C3H mice may be spirochetemic more frequently than are CD-1 mice. Thus, three infected nymphs was placed on each of eight mice of each strain. All animals were bled from the retroorbital sinus after 1 week and again at 2 weeks after the ticks had detached. About 20 μl of blood from each of these 16 samples was inoculated into 200 μl of BSK-H medium, and the cultures were examined weekly for 4 weeks thereafter. No spirochetes developed in any of these cultures (data not displayed). This experiment indicates that tick-transmitted Lyme disease spirochetes do not frequently invade the blood vascular system, even in highly susceptible hosts.

DISCUSSION

These observations confirm our previous finding that Lyme disease spirochetes remain where they were placed in

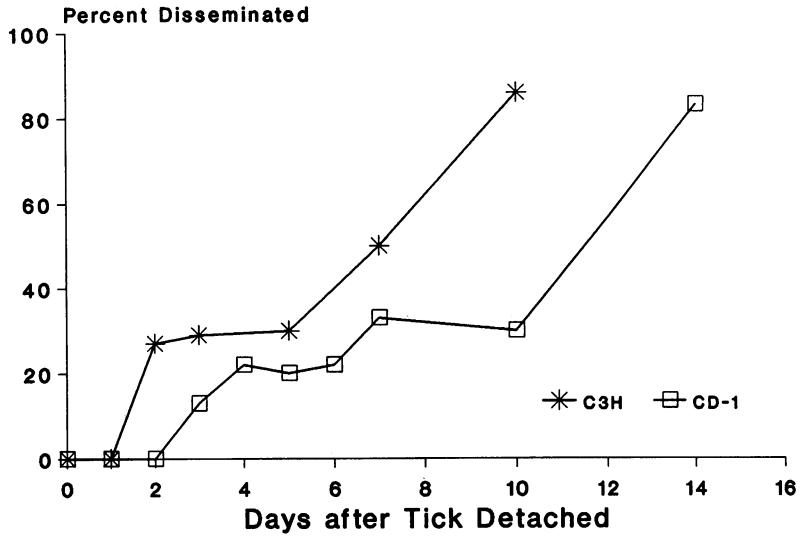

FIG. 1. Time of dissemination of Lyme disease spirochetes in C3H mice (Table 1) compared with that in CD-1 mice (27).

the skin of a mouse for some time after the infecting tick has finished feeding and has detached (27). This previously published study of outbred mice (27) was performed side-by-side with the study reported herein; the same batch of spirochete-infected ticks was used to infect mice in both studies, and both were performed by the same personnel as parts of the same study. Figure 1 displays data described in Table 1 in parallel with comparable data from the previous report (27). These sets of data differed at a P value of <0.01 (by analysis of variance, $F = 0.0296$). We concluded that Lyme disease spirochetes disseminate from the site of tick inoculation in certain spirochete-susceptible mice more rapidly than in mice that are more resistant to infection. The pathophysiological mechanism that regulates dissemination, however, remains unknown, and the route of dissemination from the skin site of inoculation has not been demonstrated.

It is interesting that the explanted site of spirochete inoculation tends to lose infectivity to cultures while remote tissues become increasingly infective to ticks. These observations confirm our earlier suggestion (27) that Lyme disease spirochetes rarely grow from explants when the sample is ablated soon after the infecting tick detaches or at many days later. Success in culturing spirochetes from a sample of tissue, presumably, depends largely on their abundance. Thus, we suggest that spirochetes initially proliferate where they are deposited by a vector tick, that they tend to remain there, and that they ultimately diminish in density as they disseminate away from this site. Culture-based methods for detecting spirochetes in skin may be less sensitive than in the case of a more fluid medium. It is curious that spirochetes ultimately seem to disappear from their initial site of inoculation in the skin of a mammalian host.

The course of Lyme disease in C3H mice differs markedly from that in CD-1 mice (3, 4). Highly susceptible C3H mice suffer a fulminating infection, and spirochetemia is said to be prominent even when the inoculum is inoculated intradermally by needle. The inflammatory cardiac and joint lesions in CD-1 mice, however, are more subtle, and spirochetemia is rarely demonstrable. We attempted to verify the observation that these C3H mice suffer massive spirochetemia but could not do so, even though our culture system detects as few as one to five cultured spirochetes (19). Perhaps spirochetemia is so sporadic that the analysis of blood generally would not be likely to detect this transient occurrence.

Intradermal inoculation by needle disrupts the architecture of the skin, places a million or so organisms in a massive bleb, and omits the profound pharmacological spectrum of effects (20–22) associated with tick bites. Vector ticks, on the other hand, inoculate far fewer spirochetes into the minute, immune-suppressed lesion that they create. Although Lyme disease spirochetes may occasionally be isolated from the blood of human patients (5), spirochetemia appears to be vanishingly sparse following natural infection. Tick-transmitted spirochetes seem to remain locally in the skin.

Humoral immunity in various needle-induced animal models of Lyme disease seems to protect against infection (10, 13, 23, 24). Vaccine-induced immunity acts, at least in part, within the vector (11). In spite of these observations, chronically infected mice retain infection, even in the presence of a prominent antibody response. One explanation for the ability of the spirochete to persist is based on the observation that the borreliacidal activity of protective antibody is strongest during the first 2 to 3 weeks of needle-induced infection and subsequently declines (24). Another explanation invokes antigenic variation in the protective epitopes of the spirochete as the adaptation that permits them to escape immune-mediated destruction (25). A third explanation for the ability of these spirochetes to survive in chronically infected hosts relies on their apparent ability to hide in immunologically privileged sites. Perhaps these spirochetes remain sequestered in particular tissues where they may not be exposed to the full force of the host's protective response (9, 12, 14).

It is interesting that ablation of the site of spirochete inoculation serves to prevent the development of a detectable humoral immune response to the spirochetes even when the tissue is removed several days after the infecting tick had detached (27). Spirochetes would then have been reproducing locally for some days (15). A protective immune response depends largely on exposure of surface epitopes that are recognized by the host's immune system. Because the entire resident spirochete population would be removed when the site of inoculation is excised, corresponding sero

logical evidence of infection would be absent. We suggest that anti-spirochete seroreactivity may become evident only after the spirochetal inoculum disseminates.

Spirochetes appear to disseminate from their site of inoculation at different rates, depending on the nature of the host. It is interesting that this process proceeds somewhat more rapidly in mice that suffer a fulminating infection (following needle infection) (3, 4) than in those in which infection proceeds more slowly. Differences in immune status of the hosts cannot be responsible for this difference because insufficient time would have elapsed for immunity to develop before the spirochetes begin to migrate. The difference does not seem to be related to the time of blood-borne dissemination because no spirochetemia is evident in either experimental host when infection is established via tick bite. Spirochetes are not released directly into the blood vascular system. Although some host property appears to influence the duration of retention (17) as well as the onset of dermal dissemination of Lyme disease spirochetes, the nature of this mechanism remains elusive.

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