BACTERIAL PATHOGENICITY AND CHARACTERISATION

Differential efficacy of passive immunisation against infection by Lyme disease spirochaetes transmitted by partially fed vector ticks

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The efficacy of passive immunisation against tick-transmitted Lyme disease spirochaetal infection was determined in relation to the duration of previous feeding of infected vector ticks. Thus, mice challenged with spirochaete-infected unfed or partially fed nymphal ticks were passively immunised with monoclonal and polyclonal antibodies against the Lyme disease spirochaete (*Borrelia burgdorferi*) at various intervals after tick attachment. Spirochaetal infection in challenged mice and engorged ticks was verified by xenodiagnosis and indirect immunofluorescent antibody assay, respectively. Although tick-transmitted spirochaetal infection could be aborted by anti-OspA antibodies and hyperimmune antiserum, nearly all immunised mice challenged with infected ticks that had previous 36-h attachment became infected. More than 72% of the nymphal ticks used in this challenge retained their *B. burgdorferi* infection after engorgement on mice immunised with anti-spirochaete antibodies, and their subsequent infectivity to mice remained effective. It is concluded that a higher efficiency of transmission by partially fed infected nymphs and a lower efficacy of passive immunisation against infection result from an effect of previous feeding of infected ticks that activates antigenic change and enables the spirochaetes to circumvent OspA-based humoral immunity.

Introduction

Passive immunisation appears to protect experimental animals from infection and disease caused by the aetiological agent of Lyme disease, *Borrelia burgdorferi* [1–6]. Passive administration of the antibodies produced against the major outer surface proteins (Osps) of *B. burgdorferi* may prevent spirochaetal infection and the subsequent development of disease [2, 4, 6]. Similarly, active immunisation with Osps of spirochaetes to elicit borreliacidal and protective antibodies also prevents subsequent persistent infection in hosts challenged by either bites of infected ticks or cultured spirochaetes [4, 7–14]. These observations demonstrate that humoral immunity protects hosts against infection when challenged by the agent of Lyme disease.

Efficiency of transmission of Lyme disease spirochaetes correlates directly with the duration of attachment of the infective ticks [15, 16]. Spirochaetal

infection can be transmitted efficiently by unfed (flat) infected nymphal ticks within 48 h of attachment [5, 16, 17]. However, the crucial period required for partially fed infected nymphs to transmit the agent of Lyme disease is less than that required by unfed infected nymphs [17]. Although passive transfer of immune serum or monoclonal antibodies (MAbs) is sufficient for protection of mice against tick-delivered spirochaetal infection [5, 6], the protective immunity afforded by passive immunisation is observed within a narrow time window before the transmission of spirochaetes [5]. In addition, antigenic variation of *B. burgdorferi* during tick feeding has been reported [18], and a temperature-related differential expression of Osps has also been demonstrated [19]. For these reasons, the efficacy of passive immunisation in preventing spirochaetal infection transmitted by partially fed infected nymphs needs to be further defined.

Spirochaetal infection may be transmitted more efficiently to the host by partially fed nymphal ticks and the duration of previous feeding of infected nymphal ticks is an alternative factor that may influence the efficacy of passive immunisation in protecting mice against *Borrelia* infection. To explore

this possibility, laboratory-reared infected nymphs were permitted to pre-feed on spirochaete-free mice for various periods after tick attachment and then allowed to re-attach on passively immunised mice until repletion, and the efficacy of protective immunity in mice against spirochaetal challenge by these partially fed infected nymphs was compared. In particular, the ability of anti-spirochaete antibodies to eliminate spirochaetes within engorged nymphs was defined, as well as their effect on subsequent infectivity for the host.

Materials and methods

Tick colony

The uninfected larval vector ticks used in this study were the third-generation progeny of adult *Ixodes dammini* (also known as *I. scapularis*) originally collected from the Crane Wildlife Reservation (Ipswich, MA, USA). This colony was introduced to our laboratory in November 1994 and was maintained free of inherited spirochaetal infection as described previously [20]. Spirochaete-infected nymphs were derived from the engorged larvae that had fed on spirochaete-infected mice. All ticks were stored in mesh-covered, plaster-bottomed plastic vials at 22°C, 95% relative humidity, and 16 h of light per day.

Experimental animals

Groups of 4–5-week-old spirochaete-free mice (C3H/HeNCrj) were purchased from the animal supply centre of National ChengKung University Medical Center (Tainan, Taiwan). All mice were ear tagged and caged by group according to the type of antibody to be infused. All experiments were conducted under the Guidelines for Animal Experiments of the National Science Council, Taiwan, ROC.

Spirochaete strain

The spirochaetal isolate (JD1) of *B. burgdorferi sensu stricto* used in the present study was originally derived from naturally infected vector ticks swept from vegetation at the endemic site of the Crane Wildlife Reservation as described previously [21]. This isolate reacted with MAb H5332 [22] and *B. burgdorferi*-specific DNA probes [23], and it is highly infectious to rodent hosts [24–26]. It had been introduced into this laboratory in November 1994 and was maintained in a system of alternate passage between vector ticks and laboratory mice, as described previously [20].

Tick attachment and feeding

To permit successful attachment, spirochaete-free mice (C3H/HeNCrj) were anaesthetised by injection of 0.2 ml pentobarbital solution (0.1%), shaved with an animal clipper and restrained individually in a small

wire cage, as described previously [17]. Infected nymphal vector ticks (35 for each mouse) were randomly placed on these mice and allowed to attach for specified periods. Because ticks may take from a few minutes to an hour to attach, the duration of attachment was measured beginning at 1 h after exposure to mice. Ticks that failed to attach within 1 h were removed and discarded. Attached nymphal ticks were removed at various intervals (24, 36 and 48 h) after attachment by gentle traction with fine forceps. Any tick that was injured during removal was discarded. These partially fed infected nymphs were subsequently allowed to re-attach on passively immunised mice until fully engorged. The engorged nymphs were collected and stored in separate mesh-covered vials for further investigations of the survival of spirochaetes within the engorged ticks and their subsequent spirochaetal infectivity for non-infected mice.

Antiserum and MAbs

Hyperimmune polyvalent rabbit serum was produced by injecting cultured JD1 strain spirochaetes into New Zealand White rabbits and the antiserum titre (>2048) was determined by the indirect immunofluorescent antibody test, as described previously [20]. Anti-OspA activity was dominantly detected in the hyperimmune serum. MAbs against the OspA (H5332) or OspB (H6831) of *B. burgdorferi* (courtesy of Dr A. G. Barbour, University of Texas, USA) were derived from the undiluted hybridoma supernates. For comparison, sera collected from non-infected rabbit or mouse were used as the control regimen.

Passive immunisation and challenge of mice

To determine the efficacy of passive immunisation against tick-delivered spirochaetal infection, six mice from each group were passively immunised with 0.5 ml of undiluted hyperimmune rabbit serum or MAbs against *B. burgdorferi* by intraperitoneal infusion at various intervals (1, 3 and 5 days) after attachment of unfed (flat) infected nymphal ticks. For comparison, mice challenged with flat infected nymphal ticks were infused with normal rabbit or mouse serum at 1 and 3 days after attachment. Similarly, mice challenged with partially fed infected nymphal ticks were also passively immunised with antiserum or MAbs against *B. burgdorferi* at intervals (1, 2 and 3 days) after re-attachment of partially fed infected nymphal ticks. In general, each mouse was challenged with 3–5 unfed or partially fed infected nymphal ticks until fully engorged.

Tick homogenates and infectivity assay

Homogenates of engorged nymphs that had previously fed for various intervals (24, 36 and 48 h) and subsequently re-attached on passively immunised mice

until repletion were prepared by grinding 5–7 engorged nymphs with phosphate-buffered saline (PBS) in a 1-ml Dounce tissue grinder (Wheaton Instruments, Millville, NJ, USA). Spirochaetal infectivity for mice was tested by inoculating mice with 0.3 ml of tick homogenate intradermally at multiple sites, as described previously [25] and the spirochaetal infection in inoculated mice was determined by xenodiagnosis 1 month later.

Detection of spirochaetes in engorged ticks

To determine the ability of passive immunisation to destroy spirochaetes within the engorged nymphs, manually removed infected nymphal ticks were allowed to re-attach and engorge on passively immunised mice until repletion. All engorged nymphs were dissected 2 weeks later and examined for evidence of spirochaetes by an indirect immunofluorescent antibody assay, as described previously [26]. Briefly, the midgut and other internal tick organs were dissected into a drop of PBS (pH 7.2) on ringed slides, squashed with a coverslip, and permitted to dry. Preparations were fixed in acetone for 10 min, treated with high titre polyvalent rabbit anti-spirochaete antiserum, and then stained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (F0382, Sigma). Slides were washed in PBS, air-dried, mounted with buffered glycerol, overlaid with a coverslip and examined for spirochaetes by epifluorescence microscopy.

Determination of infection in mice

A xenodiagnostic procedure was used to determine whether passively immunised mice became persistently infected after challenge with either unfed infected nymphs or partially fed infected nymphs. Briefly, challenged mice of each group were restrained individually in a small wire cage, and laboratory-reared

non-infected larval ticks (60–80 for each mouse) were placed randomly on these mice until repletion. At least 20 engorged larvae from each mouse were dissected 2 weeks later, and all ticks were examined for spirochaetal infection by an indirect immunofluorescent antibody (IFA) assay. Serological evidence of infection was also confirmed by seroconversion with an increase in IFA titre to *B. burgdorferi*.

Results

Abortion of tick-transmitted spirochaetal infection by passive immunisation

A preliminary experiment determined whether the tick-delivered spirochaetal infection could be aborted by passive immunisation with various antibodies produced against the Lyme disease spirochaetes. Thus, mice challenged with spirochaete-infected unfed nymphal ticks were immunised with hyperimmune rabbit antiserum or MAbs against the Osps (OspA or OspB) of *B. burgdorferi* at intervals of 1, 3 and 5 days after attachment of unfed infected ticks. For comparison, mice infused with normal rabbit or mouse serum were also challenged with unfed infected nymphs. After xenodiagnosis, no spirochaetal infection was detected in any mouse immunised with polyvalent antiserum or MAbs against OspA at 1 day after unfed infected nymphs had attached. Only one mouse immunised with MAbs against OspB was protected from infection by tick challenge (Table 1). Generally, mice became infected mainly when they were passively immunised with antiserum or MAbs 3 days after unfed infected ticks had attached. In contrast, all mice became infected when they were infused with normal rabbit or mouse serum at 1 day after the unfed infected ticks had attached. These results demonstrate that tick-transmitted spirochaetal infection can be aborted by

Table 1. Efficacy of passive immunisation of mice against Lyme disease infection after challenge by unfed infected ticks

Type of antibody infused*	Days tick attached until infused	Infection in mice	
		Number tested	Infected (%)
Anti-OspA H5332	1	6	0
	3	6	67
	5	6	100
Anti-OspB H6831	1	6	83
	3	6	100
	5	6	100
Polyvalent JD1	1	6	0
	3	6	67
	5	6	100
Rabbit sera	1	3	100
	3	3	100
Mouse sera	1	3	100
	3	3	100

*Undiluted hybridoma supernates (anti-OspA or anti-OspB), polyvalent rabbit anti-spirochaete (JD1) serum and normal rabbit or mouse serum were infused intraperitoneally in mice.

passive immunisation only when antibody is administered 24–48 h after unfed infected ticks had attached. This probably corresponds to a period prior to the deposition of Lyme disease spirochaetes by vector ticks.

Efficacy of passive immunisation against spirochaetal infection challenged by partially fed infected ticks

To determine the efficacy of passive immunisation in the protection of mice against spirochaetal infection transmitted by the partially fed infected nymphal ticks, spirochaete-infected nymphal ticks were removed from attached hosts at intervals of 24, 36 and 48 h after initial attachment and used to challenge the mice immunised with various anti-spirochaete antibodies at intervals of 1, 2 and 3 days after re-attachment of partially fed infected nymphs. Spirochaetal infection was detected in the majority (>67%) of mice immunised with hyperimmune antiserum or MAbs against OspA at 1 day after partially fed infected nymphs had reattached (Table 2). No mice were protected from spirochaetal infection when they were infused with MAbs against OspB. Regardless of the protective antibodies infused into mice, virtually all mice became infected when they were challenged with infected nymphal ticks that had previously fed on hosts >36 h after initial attachment. These results indicate

that spirochaetal infection is transmitted more efficiently by partially fed infected ticks and passive immunisation did not protect mice against spirochaetal infection from challenge by partially fed infected nymphal ticks.

Elimination of spirochaetes within infected ticks by passively infused anti-spirochaete antibodies

The ability of passively infused anti-spirochaete antibodies to eliminate spirochaetes resident in the infected nymphal ticks that had previously fed on hosts >24 h after initial attachment, and subsequently engorged on passively immunised mice until repletion was then assessed. Two weeks after repletion, spirochaetes were detected within the majority (>72%) of engorged ticks that had fed on mice infused with polyvalent antiserum or MAbs against *B. burgdorferi* (Table 3). In contrast, almost all ticks that had engorged on mice infused with normal serum remained infected. These observations indicate that spirochaetes resident in the partially fed infected ticks were not eliminated by passively infused antibodies.

Infectivity to hosts by spirochaetes that survive within ticks

The study determined whether spirochaetes survived within the engorged ticks that had fed on passively

Table 2. Efficacy of passive immunisation of mice against Lyme disease infection after challenge by partially fed infected ticks

Duration (h) of previous attachment of infected ticks	Type of antibody infused*	Days tick re-attachment until infused	Infection in mice	
			Number tested	Infected (%)
24	Anti-OspA	1	6	67
		2	6	83
		3	6	100
	Anti-OspB	1	6	100
		2	6	100
		3	6	100
	Polyvalent	1	6	67
		2	6	83
		3	6	100
36	Anti-OspA	1	6	83
		2	6	100
		3	6	100
	Anti-OspB	1	6	100
		2	6	100
		3	6	100
	Polyvalent	1	6	83
		2	6	100
		3	6	100
48	Anti-OspA	1	6	100
		2	6	100
		3	6	100
	Anti-OspB	1	6	100
		2	6	100
		3	6	100
	Polyvalent	1	6	100
		2	6	100
		3	6	100

*MAB to OspA (H5332) or OspB (H6831) and polyvalent rabbit antiserum (JD1) were infused in mice.

Table 3. Elimination of spirochaetes from the infected ticks that were detached while feeding and subsequently engorged on mice immunised with various anti-spirochaete antibodies

Type of antibody infused in mice	Detection of spirochaetes in engorged ticks*	
	Number examined	Infected (%)
Anti-OspA	42	83
Anti-OspB	38	89
Polyvalent	46	72
Control [†]	24	92

*Spirochaetal infection was examined 2 weeks after repletion of engorged ticks and verified by indirect immunofluorescence with polyvalent rabbit anti-spirochaete antisera as primary antibody.

[†]Normal rabbit or mouse serum was infused in the control groups.

immunised mice until repletion remained infective to the host. Thus, tick homogenates of 5–7 engorged infected nymphs that had fed on passively immunised mice were prepared 2 weeks after engorgement and then injected into non-infected mice by intradermal inoculation. After xenodiagnosis, only one mouse remained without infection when it was inoculated with homogenate of engorged ticks that had previously fed on the host for 24 h after attachment and subsequently engorged on mice immunised with polyvalent antiserum or MAbs against OspA of *B. burgdorferi* (Table 4). Virtually all mice became infected when they were inoculated with homogenates of engorged ticks that had previously fed on hosts >36 h after initial attachment. These results indicate that spirochaetal infectivity for the host was not affected by the passively infused anti-spirochaete antibodies.

Discussion

The effectiveness of humoral immunity against spirochaetal infection may depend on the mode of inoculation of spirochaetes and the timing of passive immunisation. Indeed, the effectiveness of passive immunisation against spirochaetal infection delivered by naturally infected ticks differs markedly from that induced by syringe inoculation, and the efficacy of passive immunisation against tick-delivered spirochaetes

tal infection has been attributed to the duration of attachment of unfed infected nymphal ticks [5, 6]. In the present study, spirochaetal infection delivered by unfed infected nymphs could be aborted by passively transferred anti-spirochaete antibody administered 24–48 h after attachment of unfed infected nymphs. Furthermore, the transmission of Lyme disease spirochaetes by an unfed infected nymphal tick occurs mainly at 48 h after initial attachment of unfed infected tick [5, 16, 17]. Thus, this study confirms that passive humoral immunity against tick-transmitted spirochaetal infection is only effective if applied within a defined time period (24–48 h) before the deposition of spirochaetes by an unfed infected nymphal tick.

The efficacy of passive immunisation against spirochaetal infection delivered by partially fed infected nymphal ticks has never been defined. The results of the present study indicate that spirochaetal infection can be efficiently transmitted to passively immunised mice when they were challenged with partially fed infected nymphal ticks that had previously fed on the host >24 h after initial attachment. These results imply that a partially fed infected nymphal tick transmits spirochaetes more rapidly than an unfed infected nymphal tick, and the span of spirochaetal transmission may be accelerated by the active dispersal of spirochaetes to tick salivary glands during the previous attachment of infected nymphal ticks [27]. Indeed, a previous study also demonstrated that partially fed infected nymphs become infectious to other hosts within only 1 day after re-attachment [17]. This is far more efficient than an unfed infected nymph. Thus, passive immunisation is not sufficient for protection of mice against spirochaetal infection challenged by partially fed infected nymphs, and the factors that modulate the effectiveness of humoral immunity against tick-transmitted spirochaetal infection remain to be defined.

The ability of passively transferred antibody to eliminate spirochaetes within the partially fed infected nymphs remains unclear. Although it had been proposed that the site of anti-spirochaete activity by

Table 4. Infectivity for mice of spirochaetes surviving within engorged ticks previously fed on a host for various periods and subsequently engorged on passively immunised mice until repletion

Type of antibody infused in mice	Duration (h) of previous feeding of engorged ticks	Infectivity to mice*	
		Number tested	Infected (%)
Anti-OspA	24	6	83
	36	6	100
	48	6	100
Anti-OspB	24	6	100
	36	6	100
	48	6	100
Polyvalent	24	6	83
	36	6	100
	48	6	100

*Spirochaetal infection was examined by xenodiagnosis 1 month after intradermal inoculation with 0.3 ml of tick homogenates of engorged nymphal ticks.

passively infused antibody was mainly within the guts of the attached ticks [5, 11], the present observations demonstrated that spirochaetes could be detected in the majority of the nymphal ticks that had been partially fed and subsequently engorged on passively immunised mice. Humoral immunity seemed to have no significant effect on the destruction of spirochaetes within those nymphal ticks that had previously fed on the host >24 h after initial attachment. Accordingly, it is suggested that the process of previous feeding of infected nymphal ticks may actually enhance the survival of spirochaetes resident in the gut of engorged infected nymphs.

It is interesting that spirochaetes within the partially fed nymphal ticks were not vulnerable to passively immunised MAbs against the Osps of *B. burgdorferi* when the challenged nymphs had previously fed on a host >24 h after initial attachment. Although OspA is an abundant protein on *B. burgdorferi* and vaccination based on OspA protects hosts against spirochaetal infection [7, 8, 11, 14], the expression of OspA within attached ticks may vary with the duration of feeding of the attached ticks. Indeed, previous studies demonstrated that Lyme disease spirochaetes are able to alter their outer surface during tick feeding [18], and the differential expression of OspA and OspC on *B. burgdorferi* occurs *c.* 24 h after tick attachment [6]. In addition, an OspC antibody-mediated immunity has proved to be effective in resolving established chronic Lyme disease [28] and in protection of mice against borrelia infection [29]. In the present study, spirochaetes within the partially fed nymphal ticks were not eliminated by passively infused anti-OspA MAbs and they remained infectious to other hosts. Thus, it is suggested that the previous feeding of infected nymphal ticks may actually activate the migration and antigenic change of spirochaetes within the partially fed infected nymphal ticks, and differential expression of OspC by spirochaetes may account for the circumvention of OspA-mediated humoral immunity.

Failure of vaccination against spirochaetal infection may result in exposure to partially fed infected ticks. Vector ticks feeding on infected hosts may detach prematurely because of grooming or host-derived anti-tick immunity [30, 31], and these partially fed ticks may already be infectious to hosts and avidly seek other hosts. Indeed, re-attachment by partially fed sub-adult ticks occurs commonly [17, 32–34]. Although the natural frequency of such partially fed infected ticks has not been determined, they may present a particularly great risk of transmission of spirochaetes.

In summary, these observations show that spirochaetal infection is transmitted more efficiently by partially fed infected nymphal ticks, and passively infused antibodies are not effective against spirochaetal infection from challenge by partially fed infected

nymphs. However, the mechanisms that regulate the anti-spirochaete activity within engorged ticks remain to be determined.

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