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GROWTH AND MIGRATION OF *BORRELIA BURGDORFERI* IN *IXODES* TICKS DURING BLOOD FEEDING

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Abstract. We have studied the growth of *Borrelia burgdorferi* in nymphal ticks (*Ixodes scapularis*) feeding on mice using confocal fluorescence microscopy to follow the distribution of spirochetes. In starved nymphs, the bacteria were only detected in the midgut and each nymph had a mean of 496 spirochetes. Upon attachment of nymphs to the host, the bacteria grew with a doubling time close to 4 hr and reached a mean of 7,848 spirochetes per nymph 15 hr after attachment. During this initial period (36 hr) of rapid growth, the bacteria appeared to be restricted to the gut, but after 48 hr, the spirochetes had disseminated to the salivary glands in the majority of nymphs examined. Thus, a critical event that allows the spirochetes to disseminate and infect the salivary glands takes place 36–48 hr after attachment. A maximum number of 166,575 spirochetes per nymph was noted 72 hr after attachment. Soon after completion of feeding and detachment from the host (96 hr), the mean number of spirochetes decreased to 95,410 per nymph and the spirochetes appeared to be cleared from organs other than the midgut. Thus, dissemination of spirochetes within the vector appears to be a transient phenomenon. These results provide strong evidence in favor of a salivary route of disease transmission while also demonstrating the utility of confocal microscopy to study vector-pathogen interactions in general.

Lyme disease is a vector-borne bacterial disorder that can progress to a severe multisystemic disease if left untreated. The infectious agent responsible for Lyme disease is the spirochete *Borrelia burgdorferi*, which is normally maintained in an enzootic cycle involving an *Ixodes* tick vector and a variety of vertebrate hosts.[1] Rodents often play an important role as reservoir hosts.[2] In the current study, we focus on the events that take place during transmission of the spirochete from nymphal ticks to rodents.

The route taken by the spirochete during transmission from the vector to the host has been an issue of some contention. In the majority of unfed nymphs, the spirochetes are found in the lumen of the midgut, often associated with the apical surface of the epithelium.[3] The overall preponderance of ticks with infections localized to the gut lent support to the hypothesis that disease transmission occurs not via saliva but by regurgitation during tick feeding.[4] Furthermore, Benach and others[5] failed to detect any spirochetes in the salivary glands of feeding adult female ticks. However, other studies support an alternate salivary route of transmission. Ribeiro and others[6] observed spirochetes in the hemolymph and saliva of adults and nymphs that were in the process of blood feeding. Zung and others[7] used electron microscopy to follow the route of transmission. In one group of unfed nymphs, the spirochetes were exclusively localized to the midgut lumen and in the same group after 48 hr of feeding spirochetes were present in the salivary acini as well as in the ducts. In the same study, a second group of infected nymphs was used to determine the route as well as time course of salivary gland invasion. Experiments on the time course of salivary gland invasion were inconclusive because these nymphs had disseminated bacterial infections prior to feeding.

In the present study, we have characterized the time course of salivary gland invasion by *B. burgdorferi* during nymphal tick feeding. We have used confocal fluorescence microscopy to examine the distribution of spirochetes in nymphal tissues. In contrast to conventional fluorescence microscopy, confocal microscopy is especially useful for surveying tissues for the presence of spirochetes because of its ability to optically section a given plane of an intact tissue. Thus, one can easily sample the interior as well as exterior of tissues without resorting to laborious embedding and sectioning methods. For an improved understanding of the vector-pathogen interactions during feeding, we have also performed a quantitative study on the growth of spirochetes within the vector. The implications of these morphologic and quantitative studies for understanding spirochete transmission from the vector to the host are discussed.

MATERIALS AND METHODS

Maintenance and infection of ticks. All ticks used in this study were derived from a colony of *I. scapularis* maintained at the Connecticut Agricultural Experiment Station (New Haven, CT). The spirochete strain used was derived from a cloned population of strain N40. Pathogen-free C3H/HeN (C3H) mice were obtained from the National Institutes of Health (Bethesda, MD) and infected by an intraperitoneal injection of 10^4 spirochetes. Four to eight weeks after infection, the mice were used in tick feeding studies. To infect ticks, approximately 70 uninfected larvae were placed on an infected mouse and allowed to feed to repletion. Larvae that were 10 days postrepletion were examined by direct fluorescence microscopy to estimate infection rates. Approximately 70% of the engorged larvae were infected. Larvae were kept in humid chambers at ambient temperature until they molted into nymphs.

Tick-mediated transmission of *B. burgdorferi* to C3H mice. Ten to fifteen infected nymphs were placed on an uninfected C3H mouse. Attached nymphs were carefully removed with forceps at the indicated times and immediately used in subsequent studies.

Preparation of nymphal organs for confocal microscopy. For each time point, 5–10 midguts and salivary glands were dissected out of nymphs in a drop of phosphate-buffered saline (PBS). The isolated organs were immobilized on silylated glass slides (PGC Scientific, Gaithersburg, MD) and fixed with 4% paraformaldehyde in PBS for 30 min at

room temperature or overnight at 4°C. After fixation, the organs were rinsed twice with PBS and sequentially incubated in PBS with 0.5% Triton-X 100 for 5 min and PBS with 5% fetal calf serum for 30 min at room temperature. Next, the organs were incubated for 1 hr at room temperature with a fluorescein isothiocyanate (FITC)–conjugated rabbit polyclonal antibody raised against the JD-1 strain of *B. burgdorferi* (kindly provided by Dr. Sam Telford, Harvard School of Public Health, Boston, MA) diluted 1:50 in PBS that also contained propidium iodide (10 gmg/ml). After the antibody incubation, the organs were washed three times with PBS and then completely dried before being mounted in glycerol for viewing. The whole organ, including the entire thickness, was examined for the presence of spirochetes.

The tissues were imaged on a confocal scanning laser microscope (MRC 600; Bio-Rad Laboratories, Microscience Division, Cambridge, MA) equipped with an argon/krypton laser. Filter blocks that permitted the viewing of the FITC signal alone (excitor filter at 488 nm, a dichroic reflector at 510 nm, and an emission barrier filter at 515 nm) or the simultaneous viewing of the FITC and propidium iodide signals on separate channels (dual band excitor filter at 488 nm and 568 nm, a dichroic reflector at 560 nm, and emission filters at 522 nm for the FITC and 585 nm for the propidium iodide) were used. The images were stored on an optical memory disk recorder (model TQ-3031F; Panasonic Industrial Company, Secaucus, NJ).

Counting the mean number of spirochetes per nymph. The mean number of spirochetes within a nymph were calculated using a procedure that was a modification of one published by Piesman and others.[8] At indicated times after attachment to the host, all the internal organs were dissected out of 5–10 nymphs and pooled in 0.5–1 ml of PBS. The tissues were ground using a 1-ml dounce homogenizer (Wheaton, Millville, NJ) to disrupt the tick cells and tissues while causing minimal damage to the much smaller spirochetes. From each homogenate, a total of four individual 5-μl drops were placed on silylated glass slides and allowed to air dry. The slides were dipped in acetone for 5 min prior to staining with an anti-*B. burgdorferi* FITC-conjugated rabbit polyclonal antibody (kindly provided by Dr. Sam Telford) to visualize the spirochetes. The spirochetes were counted using a conventional fluorescent microscope. The number of spirochetes within each 5-μl drop were counted and this number was used to calculate the mean number of spirochetes per tick.

RESULTS

Growth of *B. burgdorferi* during nymphal feeding. Previous investigators have noted that the number of spirochetes within a tick increase during tick feeding.[5] However, no attempt has been made to carefully quantitate this increase and to estimate growth rates for *B. burgdorferi* during nymphal feeding. To estimate the number of spirochetes in a nymph prior to feeding, all the internal organs were dissected out of starved infected nymphs (8–9 weeks postlarval-feeding) and prepared for counting spirochetes as described in the Materials and Methods. Prior to feeding, the mean number of spirochetes per tick was 496 (standard error = 408) (Figure 1).

To estimate the number of spirochetes during feeding, infected flat nymphs (unfed nymphs 8–9 weeks postlarval-feeding) were placed on an uninfected C3H mouse and removed at 15, 38, 50 and 72 hr after attachment (Figure 1). By 15 hr after attachment, the mean number of spirochetes per nymph had increased to 7,848 ± (standard error = 4,320) and this increase reached a maximum of 166,575 (standard error = 33,150) spirochetes per nymph after three days of attachment. By day 4, the nymphs had fed to repletion and fallen off the mouse. Following repletion, the mean number of spirochetes per tick decreased to 95,410 (standard error = 13,040). These data indicate that during the first 15 hr after attachment, the spirochetes multiply rapidly with a minimum doubling time close to 4 hr while by 50 hr postattachment, the minimum doubling time had increased to 17 hr.

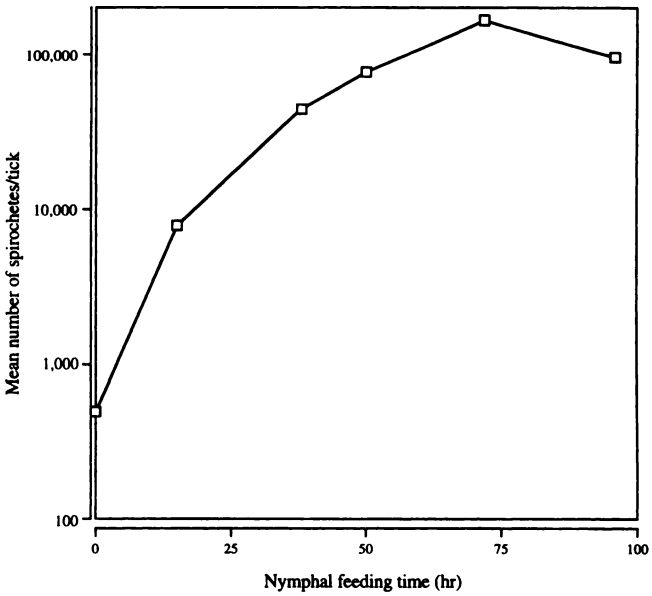

FIGURE 1. Growth of *Borrelia burgdorferi* during nymphal feeding. At 0 (five nymphs), 15 (five nymphs), 38 (eight nymphs), 50 (eight nymphs), 72 (four nymphs), and 96 (five nymphs) hr after attachment to C3H mice, nymphs were removed for estimating the mean number of spirochetes per tick. At the 96-hr timepoint, the nymphs had fed to repletion and fallen off the host. All the nymphs removed at a given time were pooled, homogenized, and prepared for counting as detailed in the Materials and Methods. Each homogenate was sampled and counted four times (n = 4).

Migration of *B. burgdorferi* within nymphs during feeding. After quantitating the steep increase in the number of spirochetes during nymphal feeding, we used confocal microscopy to examine the distribution of spirochetes during feeding. The entire gut and both salivary glands were isolated from infected nymphs that had fed for different times. The dissected organs were fixed with paraformaldehyde and incubated with an FITC-conjugated rabbit anti-*B. burgdorferi* serum and propidium iodide, a fluorescent dye that binds to nucleic acids. The propidium iodide was used to visualize the cellular organization of the tick organs. Both salivary glands and all the diverticulae in each nymph were examined for spirochetes. Occasionally, a salivary gland was lost during dissection or antibody staining and in these cases only the remaining organs were examined.

Flat nymphs. Midguts and salivary glands from eight infected flat nymphs were examined. Spirochetes were ob-

TABLE 1

Movement of *Borrelia burdgorferi* to nymphal salivary glands during feeding

Feeding time (hr)	No. of infected guts examined*	No. of infected salivary glands
0†	8	0/8
24	6	0/4
36	7	1/7
48	7	6/7
72	5	5/5
96‡	ND	0/6

* ND = not determined.
† Flat nymphs.
‡ Fully engorged and detached nymphs.

served in the midguts only (Table 1). Prior to attachment, the midguts were sparsely populated by spirochetes (Figure 2, 0 hr). In these images, it is not possible to distinguish whether the spirochetes are extracellular or intracellular because the propidium iodide stains only nuclei and the borders of the tick cells are not clearly demarcated. When different planes of the tissue were scanned for spirochetes, they were consistently found towards the middle of the organ, which is in agreement with the electron microscopy studies that have demonstrated the presence of spirochetes in the lumen of the midgut in flat nymphs.[7]

Twenty-four hours after nymphal attachment. The midgut was slightly enlarged, presumably because of the incoming fluid as well as morphologic changes in preparation for digesting the blood meal. By this time, the number of spirochetes in the midgut had increased (Figure 2, 24 hr). None of the salivary glands examined contained any spirochetes (Table 1).

Thirty-six to forty-eight hours after nymphal attachment. The nucleus as well as the cytoplasm of the midgut epithelial cells were stained by the propidium iodide (Figure 2, 48 hr), which probably reflected an increased amount of nucleic acid in this compartment as the cells prepare to digest the blood meal. The cytoplasmic staining allowed one to visualize the borders between cells as well as regions devoid of cells. During this time, there were a large number of spirochetes in the midgut. In the majority of cases, these spirochetes were present in concentrated arrays that were localized between cells (Figure 2, 48 hr). These intercellular arrays of spirochetes were observed as early as 36 hr after attachment (Figure 3).

By 36 hr after attachment, of the seven infected nymphs examined, one contained spirochetes in the salivary gland (Table 1). By 48 hr, six of seven infected nymphs had spirochetes in the salivary gland (Table 1). In the infected salivary glands, the majority of the acini did not have any spirochetes, and even in the infected acini, only a few spirochete were observed (Figure 4).

Seventy-two hours after nymphal attachment. The majority of the nymphs had not yet begun the rapid feeding phase, although they had more blood in the midgut than the nymphs removed at the previous timepoints. The spirochetes were present in large numbers, mainly concentrated in the regions between the epithelial cells of the midgut (Figure 2, 72 hr). All the salivary glands examined at this time contained spirochetes (Table 1). In comparison with the 48-hr timepoint, some of the acini of the salivary glands were greatly enlarged and these appeared to contain more spirochetes (Figure 5). However, even at this long time after attachment, many acini did not contain any spirochetes and the overall number of salivary spirochetes was at least 2–3 orders of magnitude lower than the number in the midgut.

Fully engorged nymphs. The majority of nymphs began the rapid feeding phase after 72 hr of attachment and fell off the mice on the fourth day. It was impossible to prepare midguts from these replete nymphs for confocal microscopy due to their greatly enlarged and fragile nature. Consequently, only the salivary glands were examined from these nymphs. Of the six salivary glands examined none contained any spirochetes (Table 1).

DISCUSSION

The current study has focused on the growth and migration of the Lyme spirochete, *B. burgdorferi*, during nymphal tick feeding. Our results with *B. burgdorferi* confirm that in starved nymphs, the bacteria were exclusively localized to the midgut lumen. Upon attachment and feeding, the spirochetes multiplied rapidly (doubling time of 4 hr) reaching a mean of 7,848 spirochetes per tick in the first 15 hr. This initial period of rapid growth appears to be restricted to the gut since spirochetes were not observed in salivary glands up to 24 hr after attachment. Even after 36 hr of attachment (a mean of 44,000 spirochetes per tick), only in one of seven infected nymphs did we observe spirochetes in the salivary gland. By 48 hr after attachment, the spirochetes had disseminated and infected the salivary glands in six of seven infected nymphs The maximum number of 166,575 spirochetes per tick was noted immediately preceding the rapid feeding phase (72 hr after attachment) and by this time all the infected nymphs examined had salivary gland infections. Upon completion of feeding and detachment from the host, the mean number of spirochetes decreased to 95,410 per tick and no spirochetes were observed in any of the salivary glands examined.

Nymphal ticks have to feed on mice for a minimum of 48 hr for efficient disease transmission.[9,10] This 48-hr lag for transmission correlates well with the present data on the localization of spirochetes to salivary glands. Shih and others[10] observed that after 36 hr of nymphal feeding, only 14% of mice became infected. Similarly, we observed only one of seven infected nymphs with salivary gland infections after 36 hr of feeding. In the same study, Shih and others[10] observed 100% infection of mice after 48 hr of feeding while in the present study, six of seven nymphs had salivary gland infections at this time. These data support the hypothesis that Lyme borreliosis is transmitted via tick saliva.

In a study with adult female ticks, Ribeiro and others[6] observed the sequential infection of hemolymph and saliva as the blood meal progressed, suggesting that adult females also transmit spirochetes via saliva. An interesting observation made by these investigators was that only a fraction of the adult females with midgut infections displayed spirochetes in saliva during feeding. Thus, salivary transmission by adults appears to be inefficient. This inefficiency may also explain why Benach and others[5] failed to detect any spirochetes in the salivary glands of feeding adult females. Instead of focusing on adults, in the present study we focused on the

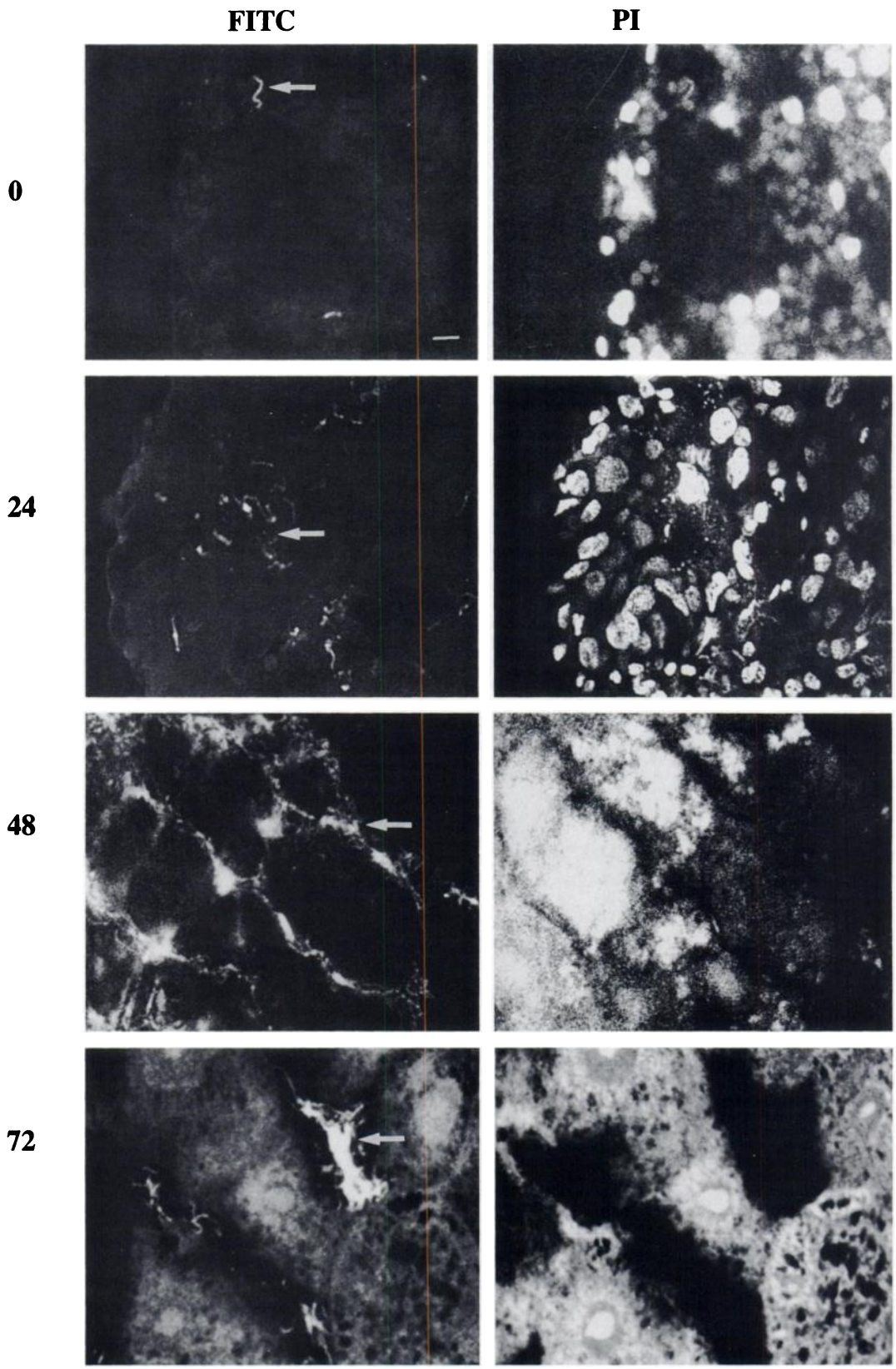

FIGURE 2. Confocal fluorescence microscopic images (43× objective) showing the distribution of spirochetes (arrows) in nymphal midguts after 0, 24, 48, and 72 hr of attachment to the host. The spirochetes were stained with a fluorescein isothiocyanate (FITC)–conjugated rabbit anti–*Borrelia burgdorferi* serum **(left panels)**, while the tick tissues were stained with propidium iodide (PI) **(right panels)**. Bar = 20 μm.

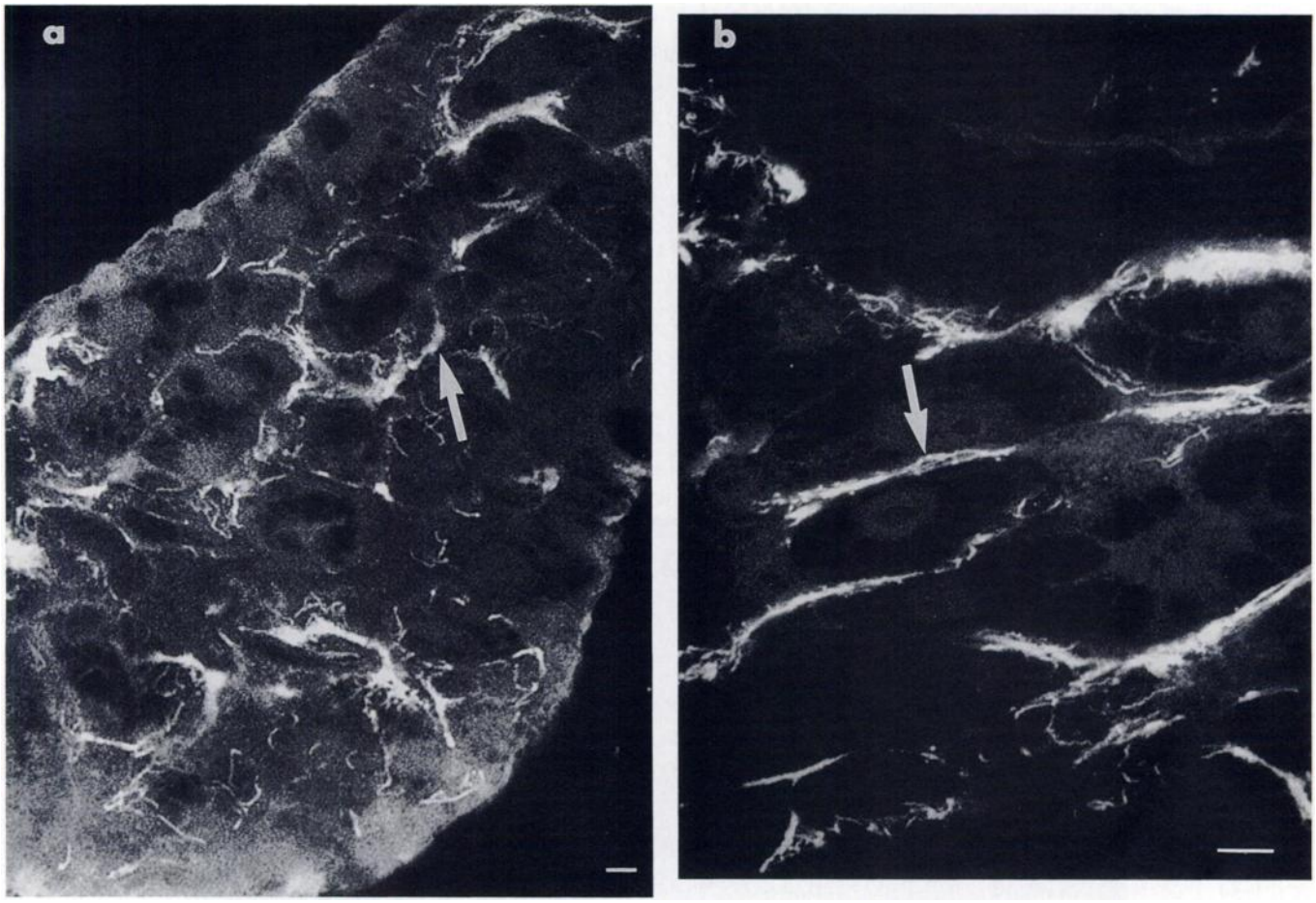

FIGURE 3. Confocal fluorescence microscopic images showing the distribution of spirochetes (**arrows**) in nymphal midguts (**a** = 43× objective and **b** = 63× objective) 36 hr after attachment to the host. The spirochetes were stained with a fluorescein isothiocyanate–conjugated rabbit anti–*Borrelia burgdorferi* serum. Bars = 20 μm.

nymphal stage because of its importance in disease transmission. In contrast to adult females, spirochetes appear to invade the salivary glands of nymphs efficiently since 100% of the nymphs had infected salivary glands 72 hr after attachment. The fact that nymphs transmit the disease very efficiently was directly demonstrated in a recent study that tested the ability of a single infected nymph to transmit the disease to a mouse.[11] In this study, all nine mice that were exposed to single nymphs developed infections.

An alternate explanation for the failure to detect efficient infection of salivary glands in past studies may be related to the sensitivity of detection methods used. In the present study with the aid of confocal microscopy, small numbers of spirochetes in salivary glands were detected and detection methods used in the past may not have identified such low level infections.

Our data also point to a critical event occurring during the period 36–48 hr postattachment that allows a few of the gut restricted spirochetes to invade the hemocoel and move to the salivary glands. The exact mechanism by which the spirochetes penetrate the midgut epithelium is unknown. In a study using adult female ticks, Benach and others[5] found no evidence for an intracellular route of invasion. In an electron microscopy study, Zung and others[7] observed that during nymphal feeding spirochetes were present intracellularly in vacuoles as well as in the spaces between cells. The confocal images used in this study clearly show that 36–48 hr after attachment, the majority of spirochetes in the midgut are localized to the spaces between the midgut epithelial cells. In preparation for digesting the blood meal, the tick midgut epithelium undergoes many changes that include the replacement of epithelial cells. During these changes, the integrity of the epithelium may be temporarily compromised, allowing a few spirochetes to invade the hemocoel. Alternatively, the spirochetes may penetrate the epithelium by an active mechanism that remains to be elucidated.

Burgdorfer and others[4] have questioned the role of salivary transmission because few ticks collected in nature show systemic infections. They argue that if saliva is the major route of transmission, most ticks should have systemic infections, especially after feeding to repletion. In the current study, we have found that during the time of disease transmission (48–72-hr postattachment), all the infected nymphs examined had disseminated infections. However, following repletion, the mean number of spirochetes decreased from 166,575 to 95,410 per tick and no spirochetes were observed in the salivary glands examined. These results indicate that systemic infections during feeding may

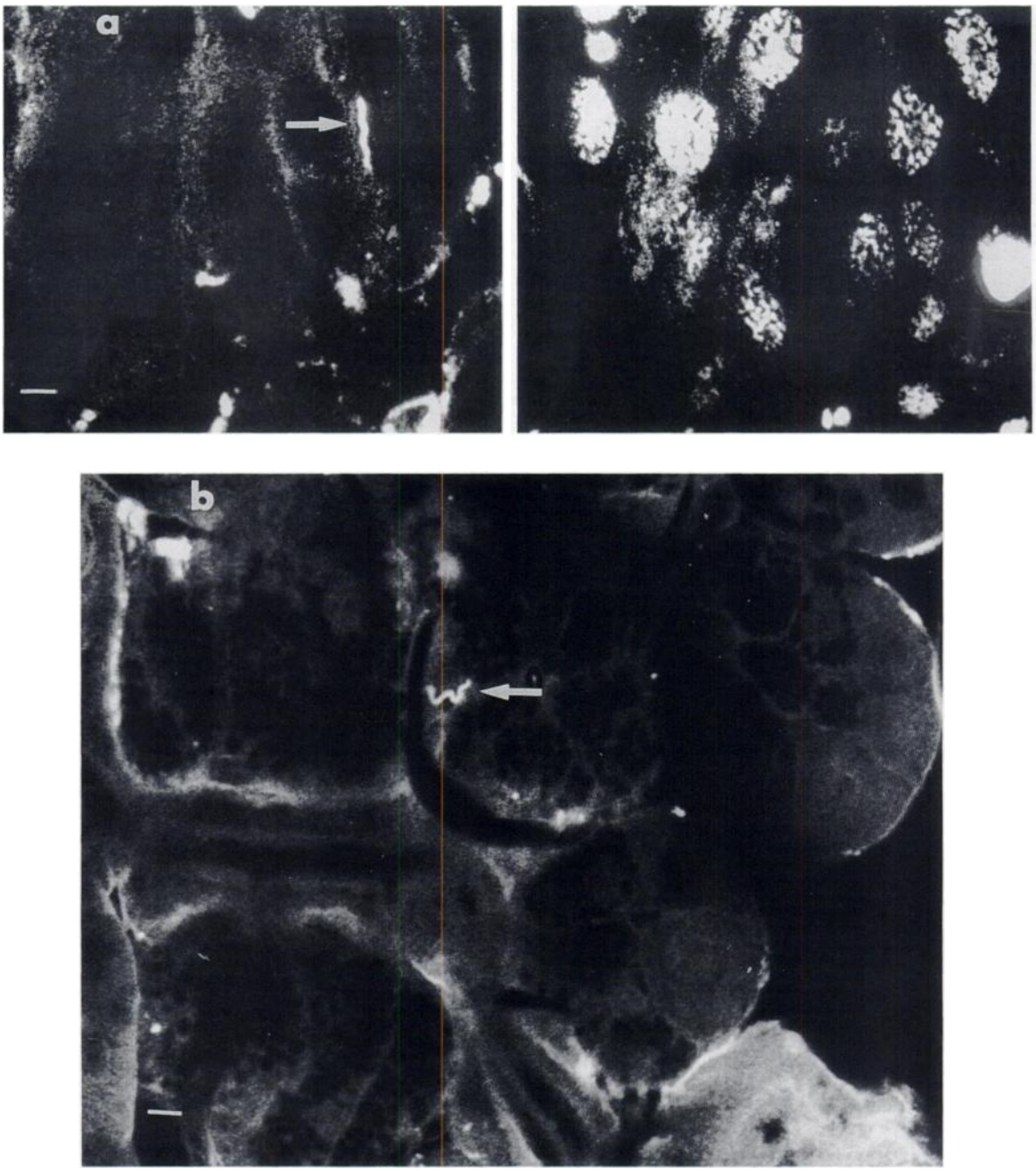

FIGURE 4. Confocal fluorescence microscopic images (63× objective) showing the distribution of spirochetes **(arrows)** in nymphal salivary glands after 48 hr of attachment to the host. **a,** spirochetes were stained with a fluorescein isothiocyanate (FITC)–conjugated rabbit anti–*Borrelia burgdorferi* serum **(left)** while the tick tissues were stained with propidium iodide **(right)**. **b,** staining with only the FITC-conjugated rabbit anti-*B. burgdorferi* serum. Bars = 12.5 μm.

be a transient phenomenon. During the rapid feeding phase or after feeding to repletion, the spirochetes may be cleared from organs other than midgut. The low percentage of ticks found with systemic infections may be due to a failure of this clearance process. The reasons for the low rate of permanent systemic infections warrant further study because these ticks may transmit the spirochetes after short periods of feeding.

The present study has quantitated for the first time the increase in the number of spirochetes during nymphal feeding. The results indicate that in the first 15 hr after attachment, the spirochetes multiply rapidly (doubling time of 4 hr) and that by 72 hr after attachment, they reach a maximum of 166,575 spirochetes per tick. The numbers after 48 hr of attachment do not reveal the absolute increase in the number of spirochetes since some spirochetes would have moved from the nymph to the host. It is also important to note that these numbers were obtained by pooling 5–10 nymphs at each timepoint and do not reveal the variation that may exist between individual nymphs. When individual nymphs were examined in the confocal microscopy studies, the majority appeared to have greater numbers of spirochetes

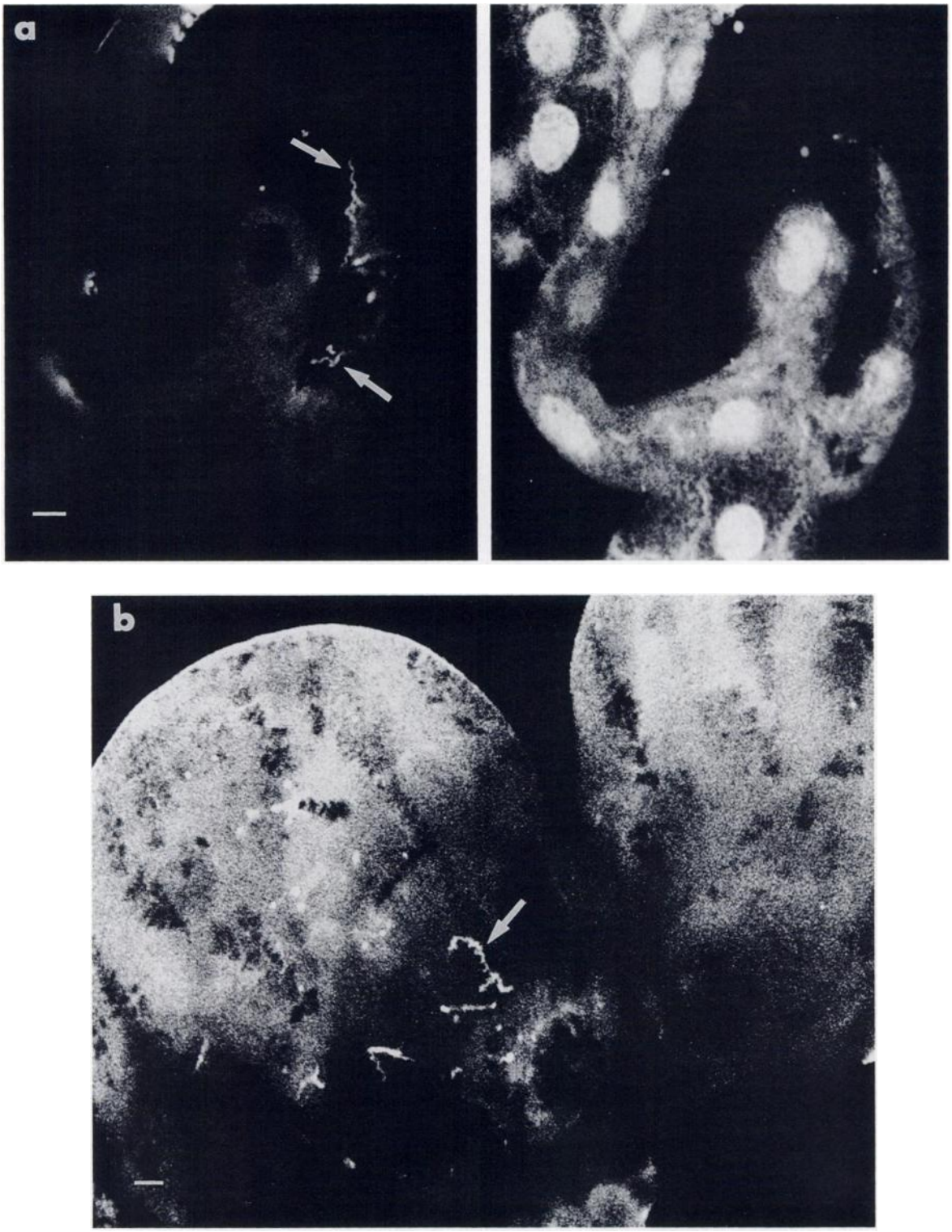

FIGURE 5. Confocal fluorescence microscopic images (63× objective) showing the distribution of spirochetes **(arrows)** in nymphal salivary glands after 72 hr of attachment to the host. **a,** spirochetes were stained with a fluorescein isothiocyanate (FITC)–conjugated rabbit anti–*Borrelia burgdorferi* serum **(left)** while the tick tissues were stained with propidium iodide **(right)**. **b,** staining with only the FITC-conjugated rabbit anti-*B. burgdorferi* serum. Bars = 12.5 μm.

as feeding progressed. Occasionally, nymphs that deviated from this pattern were also observed.

Piesman and others[8] examined the growth kinetics of spirochetes in *I. scapularis* during different stages of the tick life cycle. However, these investigators did not examine changes in spirochete numbers during feeding. Instead, they focused on the changes in numbers after feeding and after molting. In close agreement with our findings, they found a

mean of < 300 spirochetes in starved nymphs. By 10 days after feeding to repletion, the mean number of spirochetes per nymph was approximately 5,000. By the 75th day postrepletion, the mean number of spirochetes increased to a maximum of 60,000 spirochetes per nymph. The data that we have obtained in this study indicate that during actual nymphal feeding, the number of spirochetes (166,575 per nymph at 72 hr after attachment) is higher than at any other stage in the life cycle of the tick. Our results, together with those of Piesman and others,[8] indicate that soon after repletion, the mean number of spirochetes decreases steeply and then gradually increases over a 2–3-month period.[8]

Recently Burkot and others[11] used an enzyme-linked immunosorbent assay to quantitate the amount of OspA, a major spirochetal outer membrane protein, in ticks at different stages of their life cycle. These investigators found that flat nymphs had a mean of 10,530 spirochete equivalents (the amount of OspA in a spirochete grown in culture was defined as one spirochete equivalent) of OspA per nymph. Our results, together with those of Piesman and others,[8] indicate that a flat nymph has a mean of less than 500 spirochetes. These apparently contradictory results may be due to differences in *B. burgdorferi* strains used in these different studies. Alternatively, the results may indicate that OspA is expressed at a very high level in the few spirochetes present in a starved nymph. In fact, we have previously reported that spirochetes in infected nymphs are destroyed when nymphs feeds on an OspA-immunized mouse.[12] Burkot and others[11] also observed that during nymphal feeding, the mean OspA spirochete equivalents increased from 10,530 to a maximum of 49,820 per nymph after 72 hr of attachment. Thus, the total amount of OspA increases 4.5-fold during the blood meal. Our data indicate that the actual number of spirochetes increase from a mean of 496 to 166,575 (300-fold) per nymph over the same time period. These observations are consistent with a model in which the amount of OspA on the surface of spirochetes is drastically reduced during spirochete transmission. In fact, antibodies to OspA are not detected early in the host immune response to the spirochete.[13] If OspA is expressed by spirochetes to a high level during early stages of nymphal feeding, then in a OspA-vaccinated animal antibodies may bind to spirochetes in the tick midgut, preventing events that lead to spirochete growth and salivary gland invasion. Experiments are currently underway to directly examine the levels of OspA on spirochetes during disease transmission and to relate changes in OspA expression in the vector to antispirochetal immunity.

The present study has characterized the growth and migration of the Lyme disease spirochete in nymphal ticks. The data indicate that soon after attachment of the tick to the host, the bacteria multiply rapidly, reaching a mean of more than 150,000 spirochetes after three days of attachment. With the aid of confocal microscopy, we have found that the majority of spirochetes are present between the epithelial cells of the nymphal midgut. A critical event that allows the spirochetes to disseminate and infect the salivary glands takes place 36–48 hr after attachment to the host. The invasion of salivary glands is a highly efficient process since all the infected nymphs examined had spirochetes in the salivary gland after three days of attachment to the host. These results provide strong evidence in favor of a salivary route of disease transmission while also demonstrating the utility of confocal microscopy to study arthropod vector-pathogen interactions in general.

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