A Review of Development of Colonies of *Anaplasma marginale* in the Gut of *Dermacentor andersoni*

Katherine M. Kocan, *Ph.D.*
S. A. Ewing, *D.V.M., Ph.D.*
Department of Veterinary Pathology (Kocan)
Department of Veterinary Parasitology, Microbiology and Public Health (Ewing)
College of Veterinary Medicine
Oklahoma State University
Stillwater, Oklahoma 74078

Introduction

Anaplasmosis is a major disease of cattle in the United States and is one of several tick-borne maladies that severly hamper cattle production world-wide.[1] The causative agent, *Anaplasma marginale,* is currently classified in the Family Anaplasmataceae, Order Rickettsiales according to the 8th Edition of Bergey's Manual,[2] but little is known about the developmental cycle of the organism in either the invertebrate (tick) or vertebrate (cattle) hosts. Previous to studies in our laboratory, the only form of *A. marginale* that had been identified and described was the erythrocytic inclusion body found in cattle with anaplasmosis.[3 15] Although *A. marginale* had been reported by earlier workers to be transmitted by ticks,[16 18] it was not clearly established whether the organisms observed in previous studies were those ingested as part of the blood meal or were stages that developed within tick cells.[19 20]

The studies from our laboratory reviewed herein were undertaken to demonstrate *A. marginale* in *Dermacentor andersoni* and to describe its development from infection of nymphal ticks through transmission of the organism to cattle by subsequently molted adults. In these studies, *A. marginale* has been found to occur within membrane-bound inclusions that we have termed colonies. The colonies were first described in midgut epithelial cells of adult *D. andersoni* that were infected as nymphs.[21] Since publication of that finding, colonies have also been found in replete nymphal ticks. The colonies at various stages of tick development were found, by light microscopy, to have different morphologic characteristics and to contain several types of organisms—representing part of a developmental sequence of the organism in the tick. This review of the development of *A. marginale* in ticks will be presented chronologically beginning from infection of nymphs (while feeding on a calf with anaplasmosis) and continuing through the time ticks molt to the adult stage.

Development of *Anaplasma marginale* in *Dermacentor andersoni* feeding nymphs

Occurrence of colonies:—Nymphal ticks become infected soon after beginning to feed on a calf with anaplasmosis. Nymphs that fed for as little as 24 hours on an infected calf and were then transferred to an uninfected calf to complete feeding were able to transmit the disease as adults.[22] When tissues from nymphs collected on each of the 6 days of feeding were examined by light and electron microscopy, colonies of *A. marginale* were not observed.[23] Furthermore, no stages of *A. marginale* were seen with electron microscopy either within the midgut lumen or within epithelial cells of feeding nymphs. Gut contents of feeding nymphs were difficult to evaluate with electron microscopy because the midgut becomes filled with blood, obscuring parasite morphology. Once concentrated by the tick, the blood meal is very electron-dense. As a consequence, the concentrated hemoglobin masks parasite structures in the lumen of the tick's gut to an even greater extent than it does in intact, infected bovine erythrocytes.[15]

Replete nymphs—Colonies of *A. marginale* were observed in midgut epithelial cells of replete nymphs throughout development to the adult stage (approximately 20-30 days).[23] Two distinct morphologic types of colonies were observed and categorized by light microscopy as nymphal types 1 and 2. Colonies that were morphologically indistinct, with characteristics in common with both types, were termed transitional nymphal (T_sN) colonies. Nymphal type 1 (N_y1) colonies were first observed at five days post-repletion, and nymphal type 2 (N_y2) colonies were observed at 20 days after feeding. The first evidence of colony formation (5 days post-repletion) appeared more or less concurrently with the rapid formation of midgut epithelial cells and digestion of the blood meal. At completion of

feeding, the nymphal tick midgut was essentially a tubular structure filled with concentrated blood; the gut was composed of only a very thin layer of epithelial cells. In contrast, at 5 days post repletion the midgut lumen was not evident and the gut was filled with epithelial cells that had taken up most of the blood-meal. Therefore, the formation of colonies appeared to have been concurrent with rapid digestion of the blood meal by the tick and attendant cellular proliferation.

When representatives of the 3 nymphal colony types were examined by electron microscopy, organisms of different morphologic types were observed within the colonies. N_y1 colonies contained small particles and large reticulated forms that were round and some of which appeared to be dividing by binary fission. N_y2 colonies also contained reticulated organisms but they were rod-like in shape, and there was no morphologic evidence of binary fission. Organisms within N_y2 colonies appeared to be surrounded by two double-layered membranes. Electron-dense forms, commonly observed in adult ticks, were not seen in any of the 3 colony types (N_y1, N_y2 or T_sN) found in nymphal ticks.

Occurrence of Inclusion Appendages—Anaplasmal organisms and the associated inclusion appendages similar to those observed in bovine erythrocytes could be seen in replete nymphs.[24] At 5 days post-repletion, much of the hemoglobin has been removed from the lumen by the digestive action of tick gut cells, thus unmasking the parasite. Initial bodies similar to those generally found in bovine erythrocytes were observed in the lumen, but most of them appeared to be degenerating; they were not observed within gut cells. On the other hand, the morphology of inclusion appendages was quite similar to that described for inclusion appendages associated with the marginal bodies in bovine erythrocytes. Some appendages were free in the lumen of the midgut and occurred either alone or with clusters of small vesicular particles. Frequently inclusion appendages were observed attached to the luminal surface of midgut cells by a blunt, electron-dense attachment complex. The appendage attachment complex appeared to be extracellular and the pointed end of the appendage extended into the gut lumen. Small particles were often observed immediately across the host cell membrane from where the appendages were attached; the small particles appeared to be generated from the appendage itself and to have passed through the membrane of the midgut cells. The possible role of the inclusion appendage and of small particles in the infection of tick midgut cells was suggested; further research is needed to substantiate this hypothesis.[24]

Development of *Anaplasma marginale* in Adult *Dermacentor andersoni* that were Infected as Nymphs

Initial studies in our laboratory demonstrated that adult ticks, infected as nymphs, readily transmitted *A. marginale* to susceptible cattle.[25] Furthermore, homogenates of tick gut tissue from infected adults caused infection when inoculated intravenously into susceptible calves.[26] Adult ticks were found to be infective when they had fed as nymphs on either carrier calves with no apparent *A. marginale* parasitemia or on calves with acute anaplasmosis.[26] However, nymphs that fed on calves with higher parasitemias induced anaplasmosis more rapidly when they fed as adults on susceptible calves.

Colonies of *A. marginale* were found in midgut epithelial cells of adult *D. andersoni* experimentally infected as nymphs from groups of ticks that had proved to be infected by animal transmission studies.[27] No colonies were observed in associated control ticks, and the identity of the colonial organisms was confirmed as *A. marginale* with fluorescein- and ferritin-labeled antibodies[28,29] and with peroxidase-antiperoxidase-technique.[30]

Initial studies with the light microscope revealed colonies that varied in staining characteristics; the colonies were found to contain very pleomorphic organisms.[27] After examination of hundreds of colonies, it was confirmed that the staining characteristics of the colonies, as discerned with the light microscope, varied considerably and that the colonies could be categorized into 5 morphologic types.[31] Types 1 and 2 colonies contained densely staining masses of organisms, Type 3 colonies contained clumps of organisms and Types 4 and 5 contained distinctly separate organisms along with large amorphous particles. The mean diameter of the various colony types ranged from 5.64 μm for Type 1 to 10.49 μm for Type 5 colonies. It was hypothesized that the small type 1 colonies develop into the larger Types 4 and 5 colonies and that the 5 colony types represent a developmental sequence of *A. marginale* in midgut epithelial cells of adult *D. andersoni*.[31]

The structure of *A. marginale* appeared to vary within the 5 colony types and further suggested the occurrence of a developmental sequence of the organism in gut cells.[31] Representatives of each colony type were selected by light microscopy and were sectioned for examination by electron microscopy. The ultrastructural features of individual *A. marginale* organisms within colonies varied and included: (1) small electron-dense forms, (2) larger reticulated forms, (3) pleomorphic reticulated forms, and (4) small particles. Types 1 and 2 colonies contained small, electron-dense forms. Type 3 colonies contained electron-dense forms, early reticulated forms, and small particles that were often outside of the parasite's limiting membrane. Type 4 colonies contained many reticulated forms that often had small particles within cell membranes. Type 5 colonies contained fewer well-formed reticulated forms than did type 4 and, in some cases, larger masses of pleomorphic reticulated forms. The various morphologic forms of *A. marginale* within colonies were unique in our experience. They were more similar to stages described in the developmental cycle of *Chlamydia* than to any other organism with which we are familiar. During the course of a chlamydial life cycle, the organism changes from small electron-dense elementary bodies that can adsorb to and infect cells to larger reticulate

bodies that divide by binary fission and subsequently reorganize into elementary bodies.[32] [33] Although the morphologic evidence found in our study of *A. marginale* suggests a developmental sequence leading from electron-dense form to reticulated form, it is not known whether the reticulated forms reorganize into electron-dense forms as occurs in *Chlamydia.* What similarities, if any, exist between the forms of *A. marginale* we have described and the chlamydiae is not known at present.

Colonies of *A. marginale in Rhipicephalus simus*—Similar colonies of *A. marginale* were described in *Rhipicephalus simus,* a common tick from Republic of South Africa, confirming a pattern of development of the organism in a second species of tick.[34] As with studies of *D. andersoni,* 5 morphologic types of colonies were present in midgut epithelial cells of adult ticks that were infected as nymphs. The colonies were also studied by electron microscopy and found to contain organisms similar to those described in *D. andersoni* in North America.

Colony Density Studies—The density of *A. marginale* colonies (No. of colonies per .001 mm^2 of tick gut tissue examined) in adult *D. andersoni* infected as nymphs was compared in ticks infected on calves with low and high parasitemias; these values, in turn, were compared with those from unfed infected *D. andersoni* that had been exposed to elevated temperature (37° C) for various periods. Histologic and animal inoculation studies confirmed that infection levels of *A. marginale* were significantly higher in ticks infected as nymphs on calves with higher *A. marginale* parasitemias.[35] Incubation of adults also affected colony density. Densities were greatest in adults that had been incubated at 37° C for 2.5 days.[36] Corresponding tick transmission studies confirmed that ticks with the greater colony density also had greater potential to produce infection; calves inoculated with gut homogenates from *D. andersoni* that were incubated 2.5 days at 37° C (and had high colony density) developed anaplasmosis in a shorter time than did calves exposed to material from ticks a.) not incubated b.) incubated for 1.5 days or c.) 7 days.[36] The incubation studies also demonstrated that near to 100% of ticks that attached to calves with anaplasmosis became infected and that colonies of *A. marginale* developed almost uniformly in them.

Further Development of *A. marginale* in *D. andersoni*

Although a developmental sequence of *A. marginale* has been described in midgut epithelial cells, growth of the organism in the tick's gut does not imply that this is a likely location from which to transfer to the vertebrate host. Gut regurgitation has been suggested as the mode of transmission of *Cowdria ruminantium*[37] by ixodid ticks. However, this mechanism of transmission has not been demonstrated conclusively. In ixodid ticks, salivary glands are the main route by which fluid, electrolytes and disease-causing agents pass to the vertebrate host.[38] [44] For this reason, further experiments were designed to examine other possible sites of growth of *A. marginale.*

Hemolymph of infected ticks was studied because hemocytes are migratory and are likely cells to transport the infection to other tissues. Hemolymph from adult *D. andersoni* that were infected as nymphs was studied for the presence of *A. marginale* by two methods, viz., animal transmission and fluorescent antibody studies. The work was done with unfed ticks that were incubated and with feeding (for 6 days) adults. In both types of ticks, hemolymph was found to be another site of growth of *A. marginale,* but the development was more pronounced in ticks that had fed for 6 days.[44] Hemolymph from incubated ticks caused anaplasmosis in 2 of 4 trials, and hemolymph from feeding ticks caused anaplasmosis in 4 of 4 trials. Moderately fluorescing bodies were observed in some hemocytes from incubated ticks, whereas hemocytes from feeding ticks contained numerous clusters of more brightly fluorescing bodies. Fluorescing bodies were not observed in hemocytes from uninfected control ticks. Presence of *A. marginale* in hemocytes is an interesting observation but these host cells are not a likely mechanism for transfer of the parasite from the invertebrate to the vertebrate host. Hemocytes apparently circulate freely within ticks and could be important in transferring *A. marginale* within them. However, they have not been described to exit from ticks except when the acarines are injured.

Basic to understanding the mechanism of transmission of *A. marginale* by ticks is to determine specifically when, during feeding, *A. marginale* is passed from ticks to susceptible cattle. Transmission studies were conducted in which adult ticks were allowed to feed on calves for 1 to 9 days. Anaplasmosis developed only in calves on which ticks fed for at least 6 days. Therefore, we assume that the *A. marginale* organism could not be transmitted to calves until some further development or maturation occurred following attachment of adult ticks to the host. The long feeding time required for transmission to be effected suggests a progressive development of *A. marginale*; it also points to salivary glands as a site of development and transmission. Further study is required to prove this conjecture.

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