Cyclic Rickettsemia during Persistent *Anaplasma marginale* Infection of Cattle

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Submicroscopic levels of *Anaplasma marginale* rickettsemia in persistently infected cattle were determined by using nucleic acid hybridization. Within individuals, the rickettsemia levels steadily increased from less than 10⁴ infected erythrocytes per ml to a peak of more than 10⁹ infected erythrocytes per ml and then rapidly declined. This logarithmic variation parallels the variation of the rickettsemia level seen in acute infection and suggests that cyclic emergence of antigenic variants is a mechanism of rickettsial persistence.

Recovery from acute infection with organisms of the order *Rickettsiales* frequently results in persistent infection of the host (5, 16, 17). *Anaplasma marginale*, an obligate intracellular rickettsia found in cattle, infects 10 to 90% of the erythrocytes (>10⁸ infected erythrocytes per ml) during acute infection (15). Cattle that survive acute disease develop a chronic carrier state characterized by persistent low-level infection that cannot be detected microscopically (15, 18). Carrier cattle, although protected against clinical disease, maintain low-level *A. marginale* infection and serve as a reservoir for arthropod-borne transmission of *A. marginale* to susceptible cattle (15, 18). As with other rickettsial diseases, the basis of *A. marginale* persistence in the face of a competent immune response is unknown (12, 13).

Recently, we demonstrated that the levels of rickettsemia vary markedly in persistently infected animals sampled at approximately bimonthly intervals (from <10⁴ to >10⁸ infected erythrocytes per ml) (4). This variation could reflect cyclical multiplication of *A. marginale*, an event consistent with the emergence of antigenic variants not previously recognized by the immune system of the host. To determine if cyclical rickettsemia occurs in the persistently infected host and to compare the cycle with that of acute infection, we used nucleic acid hybridization to quantitate the development of rickettsemia during acute and persistent *A. marginale* infections.

Six Holstein steers were infected with the Florida isolate of *A. marginale* in 1984 and developed acute anaplasmosis with microscopically detectable rickettsemia. All cattle developed high antibody titers against *A. marginale* surface proteins and resolved the acute infection (13). Microscopic examination of blood smears for *A. marginale* was uniformly negative after 6 months postinoculation. By using nucleic acid hybridization and inoculation of blood into susceptible calves (4), the cattle were shown to be persistently infected. Blood was collected from each steer three times per week for 6 weeks during 1989, and the rickettsemia was quantitated by hybridization with an RNA probe. For comparison with acutely infected cattle, an additional 5 seronegative Holstein steers were inoculated intravenously with either 10⁸ or 10⁹ erythrocytes infected with the Florida isolate of *A. marginale*. Blood was collected daily from each steer during acute infection for quantitation of rickettsemia by using hybridization. All five cattle developed acute anaplasmosis with microscopically detectable infected erythrocytes and anemia.

 Determination of rickettsemia with an *A. marginale*-specific RNA probe was done as previously described (4, 6). Briefly, blood was washed in phosphate-buffered saline to remove plasma anduffy coat leukocytes. Samples were adjusted to 5 × 10⁹ erythrocytes per ml in phosphate-buffered saline and then lysed and pelleted to remove hemoglobin. *A. marginale* DNA was purified from the pellet by using sodium dodecyl sulfate, protease K, and phenol-chloroform extractions as previously described (4). DNA was denatured in NaOH (0.4 N final concentration) and transferred to nylon membranes via a dot blot manifold. Nylon membranes were incubated in a prehybridization solution for 6 h at 2°C prior to addition of the probe. A [³²P]CTP-labeled transcript was prepared by using the T7 promoter from pGEM-3 DNA, which contained a 2.0-kilobase fragment of the AmFl05L gene from *A. marginale* (1, 4). Membranes were hybridized overnight at 42°C and then washed five times with increasing stringency in various

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Infected erythrocytes/ml.

Days following initial detection by hybridization

FIG. 2. (A) Development of rickettsemia in five steers with acute \textit{A. marginale} (Florida isolate) infection: B497 (×), B498 (○), B501 (---), B504 (■), B513 (■). (B) Development of rickettsemia in four steers with persistent \textit{A. marginale} (Florida isolate) infection.

Solutions of SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) and sodium dodecyl sulfate (4). Membranes were air-dried and binding was detected by autoradiography. Rickettsemia levels were determined by comparing the autoradiographic hybridization signal obtained with known quantities of infected erythrocytes (10^2 to 10^7) and with a negative control of 8×10^6 uninfected erythrocytes on the same filter. Under these hybridization conditions, the RNA probe was capable of detecting a minimum of 10^4 infected erythrocytes per ml. The probe was specific for \textit{A. marginale} DNA, and there was no detectable binding to bovine leukocyte DNA (6).

Acute infection with \textit{A. marginale} results in a steady increase in rickettsemia followed by a decline (Fig. 1). The rickettsemia levels of all five cattle with acute \textit{A. marginale} infections followed a similar course, with a peak level of rickettsemia of more than 10^6 infected erythrocytes per ml developing by 14 to 18 days postdetection (Fig. 2A).

A cycle of a steady rise and decline in rickettsemia levels was seen in four of the six persistently infected cattle during the 6-week study. Rickettsemia was always less than the lower limits reliably detected by microscopic examination of blood smears. Animal 5 had an ascending level of rickettsemia when the initial sample was obtained, and therefore we did not observe the full cycle of rickettsemia. Animal 6 did not develop a detectable rickettsemia during the 6-week period. The other persistently infected cattle developed a similar rickettsemia, with a peak of more than 10^6 infected erythrocytes per ml developing by 7 to 16 days postdetection (Fig. 2B).
Cyclical multiplication of A. marginale during persistent infection in an immunocompetent host has not been previously reported and may have widespread implications for other persistent rickettsial infections. The rise and fall of rickettsiemia during persistent infection parallel the development and decline of rickettsiemia during acute infection (Fig. 1). This temporal similarity between acute and persistent infections suggests that the same host mechanism, a primary immune response, may control both cycles of rickettsiemia. On the basis of these findings, we hypothesize that A. marginale antigenic variants arise during intracellular parasitism and allow a new cycle of invasion and multiplication. This mechanism would account for the persistence of an obligate intraerythrocytic parasite in the face of a competent immune response. An alternate explanation for our observation is that waning of immunity allows the initial antigenic type to reemerge. However, the maintenance of high antibody titers, T-lymphocyte responsiveness, and resistance to homologous strain challenge in persistently infected cattle indicates that a significant waning of immunity does not occur (2, 3, 8, 13). In addition, the development of an anamnestic immune response would be expected to control the rickettsiemia in a much shorter period than was observed.

The ability of rickettsial pathogens, including A. marginale, to generate antigenic variants has been clearly established by the identification of antigenically distinct strains (7, 9–11, 14). Variation among strains of Rickettsia tsutsugamushi, which causes an acute, nonpersistent infection, appears to be relatively stable (7, 10). In contrast, within persistent rickettsiemia infections such as bovine anaplasmosis, stable antigenic variants may be accompanied by the more rapid variation necessary for persistence (9). The ability of A. marginale to rapidly generate variants may provide a model system for understanding antigenic variation and its role in persistent rickettsial infections.

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