

# Quantitative Evaluation of Ehrlichial Burden in Horses after Experimental Transmission of Human Granulocytic Ehrlichia Agent by Intravenous Inoculation with Infected Leukocytes and by Infected Ticks

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**This paper describes the kinetics of the human granulocytic ehrlichiosis agent in the blood of horses experimentally infected by intravenous inoculation with infected leukocytes and by infected ticks as evaluated by using a real-time quantitative PCR assay. The data obtained indicated differences in the period of incubation, duration of rickettsemia, and initial and maximal ehrlichial loads between the two routes of infection.**

Human granulocytic ehrlichiosis (HGE) is a newly recognized tick-borne disease in North America and in Europe caused by a rickettsial agent of the genus *Ehrlichia* (1, 5, 15). These small (0.5 to 1.5  $\mu\text{m}$ ), gram-negative, pleomorphic, and obligate intracellular bacteria show a tropism for granulocytic cells in the mammalian host and are transmitted by *Ixodes* spp. ticks (18). The agent of HGE belongs to the *Ehrlichia phagocytophila* genogroup, a cluster of phylogenetically and antigenically very similar agents. The *E. phagocytophila* group includes, besides the HGE agent, *E. phagocytophila*, the cause of tick-borne fever in goats, sheep, and cattle in Europe, and *Ehrlichia equi*, the cause of both equine and canine ehrlichiosis in the United States (7). The agent of HGE is also known to produce a febrile disease in horses similar to that caused by *E. equi* (2, 13, 14). The horse model has been shown to be highly reproducible for the study of the HGE agent. In this model, horses have mostly been infected by the intravenous route as well as with infected ticks. Although both routes are able to infect horses, to our knowledge, the course of infection and, especially, the kinetics of ehrlichial load in peripheral blood have never been compared. The purpose of this study was to compare both infection routes in horses experimentally infected with the agent of HGE.

## MATERIALS AND METHODS

**Horses.** The experimental infections were partially performed in earlier investigations (4, 13, 17). The clinically normal and ectoparasite-free horses were housed in a vector-proof facility at the Equine Research Laboratory, University of California, Davis, and were *E. equi* seronegative (titer,  $<1:10$ ) at the start of the experiment (12). In brief, infection was established by intravenous inoculation of blood stabilates ( $6.5 \times 10^6$  infected leukocytes) from an HGE agent strain Webster-infected horse (three horses) and 9 ml of whole blood ( $5.5 \times 10^6$  infected leukocytes) from a human patient infected with the HGE agent strain BDS (one horse), as well as through 20 to 30 infected adult female *Ixodes* ticks (70% infection rate) with the HGE agent strain Webster (four horses). The horses were monitored daily for clinical signs of illness. Blood samples were obtained daily for routine hematological and serological analyses. The procedures for inoculation and care of the horses were approved by the Animal Care

and Use Administrative Committee at the University of California, Davis. The animal holding facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care.

**Quantitative real-time assay.** Genomic DNA (gDNA) obtained daily from peripheral blood leukocytes (PBLs) was extracted by a standard method (13) and examined for the presence of rickettsiae of the *E. phagocytophila* genogroup with real-time TaqMan PCR as described elsewhere (16). In brief, the TaqMan PCR identified a 106-bp section of the 16S rRNA gene by use of a specific fluorogenic probe and two primers. This technique is specific for members of the *E. phagocytophila* group and has a detection limit of 10 copies of the target gene. After AmpliTaq Gold activation for 10 min at 95°C, the amplification conditions were 45 cycles of 15 s at 95°C and 60 s at 60°C. Amplification, data acquisition, and data analysis were carried out with an ABI 7700 Prism Sequence Detector (Perkin-Elmer, Applied Biosystems, Foster City, Calif.). The number of *Ehrlichia* equivalents per microgram of leukocyte DNA was determined by adjusting the TaqMan PCR results to the volume of the aliquot and the gDNA concentration.

## RESULTS

All of the infected animals developed typical clinical and hematological manifestations of equine granulocytic ehrlichiosis, including fever, lethargy, anorexia, petechiation, distal limb edema, leukopenia, thrombocytopenia, and the presence of morulae in circulating neutrophils. The hematological parameters were reported previously (4, 13, 17). The leukocyte count, differential leukocyte count, and number of infected leukocytes showed a similar pattern for all horses during the observation period. The incubation period was significantly longer in the tick-infected horses (mean, 10.5 days) than in the intravenously-infected horses (mean, 5.5 days; unpaired *t* test;  $P < 0.05$ ). The intravenously-infected horses seroconverted earlier (mean, 10.5 days) than the tick-infected horses (mean, 15.2 days; unpaired *t* test;  $P < 0.05$ ). The convalescent-phase serum obtained 30 days after inoculation showed a similar titer range in both infection routes, with geometric means of 320 and 280 for the intravenous and tick routes, respectively (unpaired *t* test;  $P > 0.05$ ).

Differences were found in the ehrlichial load in horses infected by the two different infection routes. Horses infected by the intravenous route showed a mean of *Ehrlichia* equivalents starting at  $1.1 \times 10^6$  equivalents per  $\mu\text{g}$  of leukocyte gDNA, followed by a rapid increase (Table 1). A plateau was reached after 4 days of PCR detection, with the highest load of  $1.8 \times 10^9$  equivalents per  $\mu\text{g}$  of leukocyte gDNA observed by day 6

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TABLE 1. Changes in ehrlichial load in horses following inoculation with the agent of HGE by the intravenous and tick routes<sup>a</sup>

Day of PCR detection	Ehrlichial load (equivalents/ $\mu$ g) in infection group <sup>a</sup>	
	Intravenous	Tick
1	$1.1 \times 10^6 \pm 1.5 \times 10^5$	$2.0 \times 10^4 \pm 3.0 \times 10^3$
2	$8.4 \times 10^6 \pm 1.5 \times 10^5$	$2.4 \times 10^5 \pm 1.9 \times 10^4$
3	$8.2 \times 10^7 \pm 2.0 \times 10^5$	$2.4 \times 10^6 \pm 7.5 \times 10^5$
4	$4.6 \times 10^8 \pm 1.6 \times 10^6$	$3.2 \times 10^7 \pm 5.0 \times 10^5$
5	$4.0 \times 10^8 \pm 4.5 \times 10^6$	$2.7 \times 10^8 \pm 2.7 \times 10^6$
6	$1.8 \times 10^9 \pm 1.4 \times 10^7$	$1.0 \times 10^9 \pm 4.0 \times 10^7$
7	$2.9 \times 10^8 \pm 3.5 \times 10^6$	$1.3 \times 10^{10} \pm 1.1 \times 10^{8b}$
8	$4.4 \times 10^8 \pm 2.6 \times 10^6$	$6.7 \times 10^9 \pm 5.4 \times 10^{7b}$
9	$3.3 \times 10^8 \pm 2.3 \times 10^6$	$3.1 \times 10^9 \pm 2.8 \times 10^{7b}$
10	$5.4 \times 10^8 \pm 3.4 \times 10^6$	$3.6 \times 10^8 \pm 2.9 \times 10^6$
11	$1.0 \times 10^8 \pm 4.0 \times 10^6$	$4.8 \times 10^8 \pm 3.0 \times 10^6$
12	$6.5 \times 10^7 \pm 7.4 \times 10^5$	$7.2 \times 10^8 \pm 1.2 \times 10^6$
13	$1.0 \times 10^8 \pm 1.1 \times 10^6$	$1.2 \times 10^8 \pm 8.1 \times 10^6$
14	$2.5 \times 10^7 \pm 2.7 \times 10^5$	$3.2 \times 10^7 \pm 2.3 \times 10^5$
15	$2.1 \times 10^6 \pm 2.0 \times 10^4$	$1.1 \times 10^7 \pm 1.0 \times 10^5$
16	0	$2.9 \times 10^6 \pm 2.0 \times 10^4$
17	0	$3.5 \times 10^4 \pm 7.0 \times 10^3$
18	0	0

<sup>a</sup> All values are means  $\pm$  standard deviations.

<sup>b</sup>  $P \leq 0.05$ .

of rickettsemia, followed by a slow decrease. The initial load was lower in horses infected by the tick route, with  $2 \times 10^4$  equivalents per  $\mu$ g of leukocyte gDNA. After a rapid increase, the highest load of  $1.3 \times 10^{10}$  equivalents per  $\mu$ g of leukocyte gDNA was observed by day 7 of rickettsemia, followed by a slow decrease. The mean ehrlichial load from day 7 to day 9 of rickettsemia was significantly higher in the tick group (unpaired  $t$  test;  $P < 0.05$ ). The detection period of a fluorescent signal was significantly longer for the tick route (mean, 16.75 days) than for the intravenous route (mean, 13.75 days; unpaired  $t$  test;  $P < 0.05$ ).

## DISCUSSION

Most HGE studies carried out with horses used needle inoculation of infected leukocytes. This route of inoculation and the ehrlichial source do not accurately reflect natural infection with the agent of HGE. To investigate whether tick transmission influences the course of granulocytic ehrlichiosis in the horse model, we compared the ehrlichial loads in susceptible horses following transmission of the agent of HGE by intravenous inoculation and tick bite.

Using *E. canis* as antigen, Gaunt et al. (8) have shown that the route of administration and the inoculation size can influence the course of ehrlichial infection, with thrombocytopenia and seroconversion occurring later by subcutaneous than by intravenous inoculation. Our results demonstrated similar disease severities in both infection routes; however, a significantly longer incubation time was observed with the tick route. The longer prodromal period probably resulted from the initial ehrlichia replication period in the vector and the smaller amount of infectious agent transferred during the tick bite. Recent studies (10, 11) have demonstrated that the onset of feeding stimulated replication of the HGE agent within nymphal ticks and that nymphal ticks, infected as larvae, transmitted infection to mice between 30 and 49 h. Although the tick-borne dose cannot accurately be determined with the HGE agent, infections with tick-borne agents, like *Borrelia burgdorferi*, showed that the number of bacteria transmitted

during a tick's blood meal is rather low (9, 19). Furthermore, the reported 55-times-lower initial rickettsial load in the PBLs from tick-infected horses suggested a low-dose inoculation. We realize that the measured ehrlichial load is based upon the amount of DNA, which does not necessarily reflect the infectivity of the tested material. The antibody response did not show marked differences between the two infection routes. The longer seroconversion time observed after tick infection seems to be more the result of the infection route and the number of bacteria initially transmitted.

This investigation showed that the courses of the ehrlichial load of the intravenous and tick routes were similar. However, differences in the initial and the maximal loads and in the duration of rickettsemia have been noticed. In a previous report, we showed that the number of equivalents is dependent on the leukocyte count, the percentage of infected leukocytes, and the differential leukocyte count (16). Since the patterns of hematological findings among all horses were similar, we assume that the quantitative differences were not due to differences in the leukocyte kinetics. While the differences in initial load are most probably the result of different infectious doses, the finding that tick-infected horses showed a higher load and a longer rickettsemia than those given intravenous inoculation is surprising. This discrepancy may be related to the site of infection, the immune reaction of the host, the quantity of ehrlichiae transferred, or changes of the HGE agent within the vector as it is transmitted into a mammalian host. Tick infection studies with *B. burgdorferi* in mice have shown that (i) in natural infection, spirochetemia may be greater and more naïve ticks would become infected when feeding on a recipient that was itself infected by tick bites; (ii) the transmission route influences the host immune response; (iii) the host immune system may be affected by the tick itself, which has been shown to secrete immunomodulatory constituents during feeding; and (iv) phenotypic changes in the spirochetes occur in the tick or mouse environment (3, 6, 9, 19). At present, detailed information on these points is lacking with regard to the agent of HGE.

In conclusion, we found similar disease severities in both infection routes. The longer incubation period and seroconversion time observed with the tick route probably reflect the slower kinetics of the HGE agent and the host-specific immune response. Whether the higher ehrlichial load observed with tick transmission affects the pathogenesis of granulocytic ehrlichiosis or is a biological mechanism allowing more efficient infection of ticks needs to be addressed in future studies.

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## REFERENCES

- Bakken, J. S., J. S. Dumler, S.-M. Chen, M. R. Eckman, L. L. Van Etta, and D. H. Walker. 1994. Human granulocytic ehrlichiosis in the upper midwest United States. A new species emerging? *JAMA* 272:212-218.
- Barlough, J. E., J. E. Madigan, E. DeRock, J. S. Dumler, and J. S. Bakken. 1995. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytotropic ehrlichia (HGE agent). *J. Clin. Microbiol.* 33: 3333-3334.
- Barthold, S. W. 1991. Infectivity of *Borrelia burgdorferi* relative to route of inoculation and genotype in laboratory mice. *J. Infect. Dis.* 163:419-420.
- Chae, J.-S., N. Pusterla, R. B. Kimsey, E. DeRock, M. T. Blanchard, J. S. Dumler, and J. E. Madigan. Experimental transmission of human granulocytic ehrlichia agent in horses by experimentally infected ticks (*Ixodes scapularis*). Submitted for publication.
- Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker. 1994. Identifi-

- cation of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. *J. Clin. Microbiol.* **32**:589–595.
6. **De Souza, M. S., A. L. Smith, D. S. Beck, L. J. Kim, G. M. Hansen, Jr., and S. W. Barthold.** 1993. Variant responses of mice to *Borrelia burgdorferi* depending on the site of intradermal inoculation. *Infect. Immun.* **61**:4493–4497.
  7. **Fritz, C. L., and C. A. Glaser.** 1998. Ehrlichiosis. *Infect. Dis. Clin. N. Am.* **12**:123–136.
  8. **Gaunt, S. D., R. E. Corstvet, C. M. Berry, and B. Brennan.** 1996. Isolation of *Ehrlichia canis* from dogs following subcutaneous inoculation. *J. Clin. Microbiol.* **34**:1429–1432.
  9. **Gern, L., U. E. Schaible, and M. Simon.** 1993. Mode of inoculation of the Lyme disease agent *Borrelia burgdorferi* influences infection and immune responses in inbred strains of mice. *J. Infect. Dis.* **167**:971–975.
  10. **Hodzic, E., D. Fish, C. M. Maretzki, A. M. De Silva, S. Feng, and S. W. Barthold.** 1998. Acquisition and transmission of the agent of human granulocytic ehrlichiosis by *Ixodes scapularis* ticks. *J. Clin. Microbiol.* **36**:3574–3578.
  11. **Katavolos, P., P. M. Armstrong, J. E. Dawson, and S. R. Telford.** 1998. Duration of tick attachment required for transmission of granulocytic ehrlichiosis. *J. Infect. Dis.* **177**:1422–1425.
  12. **Madigan, J. E., S. Hietala, S. Chalmers, and E. DeRock.** 1990. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. *J. Am. Vet. Med. Assoc.* **196**:1962–1964.
  13. **Madigan, J. E., P. J. Richter, R. B. Kimsey, J. E. Barlough, J. S. Bakken, and J. S. Dumler.** 1995. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. *J. Infect. Dis.* **172**:1141–1144.
  14. **Madigan, J. E., J. E. Barlough, J. S. Dumler, N. S. Schankman, and E. DeRock.** 1996. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic ehrlichia. *J. Clin. Microbiol.* **34**:434–435.
  15. **Petrovec, M., S. L. Furlan, T. A. Zupanc, F. Strle, P. Brouqui, V. Roux, and J. S. Dumler.** 1997. Human disease in Europe caused by a granulocytic *Ehrlichia* species. *J. Clin. Microbiol.* **35**:1556–1559.
  16. **Pusterla, N., J. B. Huder, C. M. Leutenegger, U. Braun, J. E. Madigan, and H. Lutz.** 1999. Quantitative real-time PCR for detection of members of the *Ehrlichia phagocytophila* genogroup in host animals and *Ixodes ricinus* ticks. *J. Clin. Microbiol.* **37**:1329–1331.
  17. **Reubel, G. H., R. B. Kimsey, J. E. Barlough, and J. E. Madigan.** 1998. Experimental transmission of *Ehrlichia equi* to horses through naturally infected ticks (*Ixodes pacificus*) from Northern California. *J. Clin. Microbiol.* **36**:2131–2134.
  18. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. *Clin. Microbiol. Rev.* **4**:286–308.
  19. **Schaible, U. E., L. Gern, R. Wallich, M. D. Kramer, M. Prester, and M. M. Simon.** 1993. Distinct patterns of protective antibodies are generated against *Borrelia burgdorferi* in mice experimentally inoculated with high and low doses of antigen. *Immunol. Lett.* **36**:219–226.