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Experimental Inoculation with Human Granulocytic Ehrlichia Agent Derived from High- and Low-Passage Cell Culture in Horses

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We report the successful infection throughout intravenous inoculation with low and high passage of in vitro-grown human granulocytic ehrlichiosis (HGE) agent in horses. Differences in disease severity but not in incubation time, hematological changes, PCR detection, ehrlichial load, seroconversion time, and titer range were noted between horses infected with a low and a high passage of in vitro-grown HGE agent.

Granulocytic ehrlichiae are tick-transmitted obligate intracellular bacteria which invade white blood cells and are known to cause a febrile disease in animals but were not known as likely human pathogens until recently. In 1994, an emerging tick-borne febrile illness in humans, called human granulocytic ehrlichiosis (HGE), was first described in North America and later in Europe (7, 17). The clinical and laboratory manifestations of HGE occur after an *Ixodes* spp. tick bite and include fever, headache, myalgias, malaise, chills, thrombocytopenia, anemia, leukopenia, elevations in serum hepatic transaminase activities, and the presence of morulae in circulating neutrophils (2, 8). The agent of HGE is also known to produce a febrile disease in the horse similar to that caused by *Ehrlichia equi*, which has been suggested as an appropriate animal model to study the pathogenesis of HGE. In this model, horses have mostly been infected by the intravenous route by using fresh blood from human patients or blood stabilates from an HGE agent-infected horse (3, 6, 14, 15). Recently, the HGE agent has been successfully cultured in a line of human promyelocytic leukemia cells (9). However, to the knowledge of the authors, cell culture-derived ehrlichiae have never been used for experimental infection in horses. The use of in vitro-cultivated HGE agents could allow the investigation of clinical and laboratory manifestations related to standardized type and size of inoculum. This study was undertaken to determine the susceptibility of horses to HGE agent grown in cell culture. A second objective was to evaluate the effect of in vitro passages on the virulence of the organisms in vivo.

Ehrlichial strain. The Webster strain of HGE was originally isolated from a human patient in northwest Wisconsin (1) and cultivated into an HL60 promyelocyte cell line according to Goodman et al. (9). In brief, 100 μl of human EDTA-anticoagulated blood was inoculated into 5 ml of an HL60 cell line in RPMI 1640 supplemented with 2 mM L-glutamine and 1% fetal bovine serum at a final concentration of 2×10^5 cells/ml. The cultures were maintained in a 5% CO_2 incubation chamber at 37°C and examined every 3 days by Romanowsky staining (LeukoStat; Fisher Scientific, Pittsburgh, Pa.) of cytocentrifuged cells for the presence of characteristic morulae. Once infection was established, the ehrlichiae were propagated by subsequent passage into uninfected HL60 cells for ≤6 (low passage) or ≥20 passages (high passage) in order to use the cells for inoculation.

Horses. Four healthy 3- to 16-year-old geldings (two Thoroughbreds, one Warmbred, one Hanoverian) and a 3- and a 17-year-old mare (one quarter horses, one standardbred) were assigned to a low-passage (LP) or a high-passage (HP) group each containing three horses (two geldings, one mare). The horses were housed in a vector-proof facility at the Equine Research Laboratory, University of California, Davis, and were *E. equi* seronegative at the start of the experiment. Horses of the LP and the HP groups were inoculated intravenously with 1×10^6 infected HL60 cells (not more than 1.2×10^6 total cells) passaged ≤6 and ≥20 times, respectively. An additional 7-year-old Thoroughbred gelding was challenged intravenously with 1×10^6 uninfected HL60 cells and served as an uninfected control. The animals underwent a thorough clinical examination before experimental infection and thereafter for 30 days. General attitude and behavior, appetite, rectal temperature, heart and respiratory rates, intestinal motility, and fecal consistency were assessed twice daily. 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.

Hematological, serological, and PCR examinations. Blood was collected from all horses into 10-ml evacuated glass tubes with and without anticoagulant (Vacutainer; Becton Dickinson, Franklin Lakes, N.J.) beginning on the day of experimental infection (day 0) and daily thereafter for 30 days. The leukocyte, erythrocyte, and platelet counts were determined, and blood smears were examined for characteristic inclusion bodies. HGE agent antibodies were detected by an indirect immunofluorescent-antibody assay using *E. equi*, a surrogate marker of the agent of HGE, essentially as described elsewhere, and the cutoff titer for a positive serological response was set at ≥10 (13, 14). DNA obtained from peripheral blood leukocytes of the horses was extracted by a standard method (4) and examined for the presence of HGE agent genomic DNA by nested PCR (4) and a quantitative real-time PCR assay (TaqMan PCR) in order to quantify the ehrlichial load

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TABLE 1. Laboratory findings in horses experimentally infected with high and low passages of in vitro-cultured HGE agent

Parameter (unit)	Low-passage group			High-passage group		
	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6
Leukocytes ($10^3/\mu l$)[a]	6.8–10.0[b]	8.1–12.3	5.3–9.9	7.1–12.7	4.8–7.7	5.3–6.9
Erythrocytes ($10^6/\mu l$)	6.9–9.6	7.9–9.4	5.4–9.1	7.1–9.4	7.3–8.7	6.6–10.2
Platelets ($10^3/\mu l$)	34–119	72–230	35–127	47–230	9–111	51–129
Ehrlichia detection by light microscopy (days PI)	10–14	9–12	9	6–14	8–14	8
Ehrlichia detection by nested PCR (days PI)	7–19	7–14	6–16	4–15	7–15	5–15
Time of seroconversion (days PI)	14	11	11	10	11	14
Antibody titer at day 30 PI	80	160	1,280	160	640	640

[a] Reference values: leukocytes, 5.4×10^3 to $14.3 \times 10^3/\mu l$; erythrocytes, 6.8×10^6 to $12.9 \times 10^6/\mu l$; platelets, 100×10^3 to $350 \times 10^3/\mu l$.
[b] Ranges of leukocyte, erythrocyte, and platelet counts are expressed as minimal and maximal values observed during 30 days postinoculation (PI).

(19). 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 genomic DNA concentration.

Results. The first clinical indication of infection was an abrupt rise in temperature in the horses of the LP and the HP groups following an incubation period of 5 to 7 days. The incubation time varied from 6 to 7 days (mean, 6.7 days) for the LP group and from 5 to 7 days (mean, 6.0 days; unpaired *t* test, $P > 0.05$) for the HP group. The temperature reached its maximum on the second day of fever, with peaks ranging from 40.1 to 40.8°C (mean, 40.5°C) for the LP group and from 39.7 to 40.0°C (mean, 39.9°C; $P = 0.03$) for the HP group. The febrile period lasted 5 days (mean, 5.0 days) for the LP group and 1 to 2 days (mean, 1.7 days; $P = 0.004$) for the HP group. During the febrile period, the horses of the LP group showed complete anorexia and severe depression (horses 1, 2, and 3), distal limb edema (horses 2 and 3), and reluctance to move (horse 2). In the HP group, horse 4 showed mild depression, horse 5 had distal limb edema, and horse 6 had no other clinical signs apart from fever. According to the duration and degree of the clinical signs, the disease was judged as more severe in the horses of the LP group. The inoculation of uninfected HL60 cells in the control horse caused no abnormal findings.

In the horses infected with the HGE agent, transient hematological changes occurred with the onset of clinical signs and were characterized by decreased leukocyte, erythrocyte, and platelet counts and inclusion bodies in the cytoplasm of neutrophils. Thrombocytopenia was observed in all horses, leukopenia was observed in three horses (horses 3, 5, and 6), and erythrocytopenia was observed in two horses (horses 3 and 6) (Table 1). The initial appearance of cytoplasmic inclusion bodies correlated closely with the onset of fever. The inclusion bodies were present for 1 to 5 days (mean, 3.3 days) in the LP group and for 1 to 9 days (mean, 5.7 days; $P > 0.05$) in the HP group. The maximum percentage of neutrophils containing inclusion bodies was similar in both groups and varied from 1 to 3%.

Ehrlichia DNA was detected by the nested PCR on blood buffy-coat cells on days 6 and 7 postinoculation for the horses of the LP group and on days 4, 5, and 7 for the horses of the HP group. Ehrlichia DNA was detected in buffy-coat cells for 8 to 13 days (mean of the LP group, 10.7 days; mean of the HP group, 10.7 days; $P > 0.05$). The detection of a positive signal by TaqMan PCR was identical to that of the nested PCR for each horse. Over the observation period, the ehrlichial loads of the two groups showed a similar course. In the LP and the HP groups, the means of *Ehrlichia* equivalents started at 3.1×10^6 and 2.1×10^6 equivalents per μg of leukocyte DNA, respectively, and increased thereafter. A plateau was observed after 5 and 6 days for both groups, followed by a decrease in ehrlichial load. The highest mean of *Ehrlichia* equivalents reached 6.2×10^8 and 3.8×10^8 equivalents per μg of leukocyte DNA in the LP and the HP groups, respectively ($P > 0.05$).

The serum antibody titer rose to ≥ 10 on days 11 to 14 postinoculation (mean, day 12.0 postinoculation) for the LP group and on days 10 to 14 (mean, day 11.7 postinoculation; $P > 0.05$) for the HP group. The convalescent-phase serum obtained 30 days after inoculation showed a similar titer range in both groups, with geometric means of 507 and 480 for the LP and the HP groups, respectively.

Discussion. The horse has been suggested as a model to study HGE because the disease is reproducible and characterized by clinical and laboratory findings and pathology similar to those observed in humans (H. Lepidi, J. E. Bunnell, M. Martin, J. E. Madigan, S. Stuen, and J. S. Dumler, submitted for publication). In this model, horses have mostly been infected by the intravenous route with fresh blood from human patients or blood stabilates from an HGE agent-infected horse (3, 6, 14). The disease severity of the reported studies varied over a wide range. While horses infected with blood from a human patient from Westchester County, New York, showed only fever for 3 to 4 days (6), horses infected with HGE agent originated in Wisconsin (BDS strain) fell severely ill (3, 14). The mentioned variation in disease severity could be related to the agent pathogenicity, type and size of the inoculum, or individual variation in the host response. The purpose of our study was to determine the susceptibility of horses to HGE agent grown in cell culture in order to optimize the experimental infection protocol and to enhance the reproducibility of this animal model. Further, horses were infected with low and high passages of cultured HGE agent in order to study the effect of in vitro passages.

Disease caused by *E. equi*, thought to be the typical granulocytic ehrlichial agent in horses, is characterized by high fever with a mean febrile peak of 40.8°C and duration of 5.5 days. These animals also developed depression and anorexia varying in degree and duration, subcutaneous edema, icterus, petechiation, reluctance to move, ataxia, leukopenia, erythrocytopenia, thrombocytopenia, and inclusion bodies detectable during 10 days and infecting up to 73% of the neutrophils (10). *E. equi* and the strain of HGE used in our study seem to be similarly virulent in horses. Peaks and durations of fever, depression, and anorexia are comparable among horses infected with *E. equi* in blood stabilates and with the low-passaged HGE agent; however, other characteristic clinical signs, like edema, icterus, petechiation, ataxia, and reluctance to move, are more often observed in *E. equi* experimentally infected horses. Thrombocytopenia was the only typical hematological sign found in all

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horses in our study. Leukopenia and erythrocytopenia were less frequent in our study than in horses infected with *E. equi* (10). The maximum percentage of infected neutrophils was surprisingly lower in HGE agent-infected horses (3%) than in *E. equi*-infected horses (73%) (10). The hematological discrepancies may be related to the ehrlichial strains, the quantity of ehrlichiae experimentally transferred, the immune reaction of the host, or changes of the HGE agent during the in vitro cultivation. Differences in the virulence of *E. phagocytophila* strains in cattle and sheep have been shown in previous studies (18, 21).

Loss of virulence after several in vivo or in vitro passages has been shown for a variety of rickettsial agents, such as *Anaplasma marginale* (20) or *Cowdria ruminantium* (12). The low- and high-passage cell culture-derived HGE agents were both able to induce a febrile disease in the infected horses; however, differences in the degree and occurrence of clinical signs were found. The HP-derived agent induced a mild infection, based on a lower degree and rate of clinical signs, but at the same time was able to produce laboratory findings similar to those of the LP group. The comparable results of blood cell counts, ehrlichial detection, ehrlichial load, seroconversion time, and antibody range between the two groups suggest a loss of virulence but not of infectivity of the HP-derived HGE agent. The loss of virulence occurring after several in vitro passages may be related to the outgrowth of low-virulence clonal populations, to metabolic changes as the agent adapts to in vitro growth, to differential expression of cell surface proteins and antigenic variations, or to altered gene expression due to some unidentified regulatory factors, as have been shown for other bacterial agents (5, 11, 16). At present, detailed information on these points is lacking for the agent of HGE.

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