Sequential Analysis of Anaplasma phagocytophilum msp2 Transcription in Murine and Equine Models of Human Granulocytic Anaplasmosis

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Anaplasma phagocytophilum causes human granulocytic anaplasmosis by inducing immunopathologic responses. Its immunodominant Msp2 protein is encoded by a family of >100 paralogs. Msp2 (msp2) expression modulates in the absence of immune pressure, and prolonged in vitro passage modulates in vivo virulence. Because programmed MSP2 expression occurs in Anaplasma marginale, we hypothesized a similar event in A. phagocytophilum in vivo, with specific Msp2 expression triggering immunopathologic injury or clinical manifestations of disease. We examined msp2 transcripts in 11 B6 mice and 6 horses inoculated with low- or high-passage A. phagocytophilum Webster strain. Blood was sequentially obtained through 3 weeks postinfection for msp2 reverse transcription-PCR. Horses were additionally assessed for clinical manifestations, seroconversion, complete blood count, blood chemistry, and cytokine gene transcription. In both species, there was no consistent emergence of msp2 transcripts, and all 22 msp2 variants were detected in both passage groups. Clinical severity was much higher for high-passage-infected than for low-passage-infected horses, preceded by higher levels of blood gamma interferon transcription on day 7. Antibody was first detected on day 7, and all horses seroconverted by day 22, with a trend toward lower antibody titers in low-passage-infected animals. Leukocyte and platelet counts were similar between experimental groups except on day 13, when low-passage-infected animals had more profound thrombocytopenia. These findings corroborate studies with mice, where msp2 diversity did not explain differences in hepatic histopathology, but differ from the paradigm of low-passage A. phagocytophilum causing more significant clinical illness. Alteration in transcription of msp2 has no bearing on clinical disease in horses, suggesting the existence of a separate proinflammatory component differentially expressed with changing in vitro passage.

Human granulocytic anaplasmosis (HGA) is a tick-borne disease caused by Anaplasma phagocytophilum. A. phagocyto-
philum strains from different geographic regions vary in their antigenic content (1, 11). Antigenic variability results chiefly from differential expression of immunodominant surface proteins encoded by a multigene family (msp2, p44) characterized by conserved sequences flanking a hypervariable region (HVR) which is predicted to contain B-cell and T-cell epitopes (8, 24). The related MSP2 antigenic variation of Anaplasma marginale leads to persistent, relapsing infection in ruminants, a feature not present in mammals or in humans who develop febrile infection with A. phagocytophilum. For A. phagocyto-
philum, Msp2 may have a role as an adhesin to bind to mammalian cells (19).

A. phagocytophilum msp2 transcription and expression change with increasing lengths of in vitro propagation (24). Horses experimentally infected by A. phagocytophilum passed in vitro develop clinical manifestations either typical of natural virulent disease when infected with low-passage bacteria or with significantly diminished clinical signs and laboratory features when infected with high-passage bacteria (20). In contrast, the murine model of HGA is imperfect since infection does not cause clinical signs in mice, yet the development of histopathologic lesions, such as inflammatory liver lesions that mimic those in infected humans and horses, indicates that this model is an important tool for investigating A. phagocyto-
philum pathogenesis (4, 7, 14). Moreover, histopathologic lesions in mice are directly linked to the production of gamma interferon (IFN-γ) and not to bacterial load (15), and Msp2 is the major immunological target of the host immune response (1, 13). In this study comparing two different animal models, we investigated whether there is a specific pattern of programmed Msp2 expression and whether that pattern differs between high- and low-passage infection in the mouse and horse models of HGA by analyzing transcript diversity and kinetics. In addition, whether programmed Msp2 expression correlates with clinical disease in the equine model of HGA was examined.

MATERIALS AND METHODS

Cultivation of A. phagocytophilum in vitro. A. phagocytophilum was maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine until approximately 90% of the cells contained morulae. Cultures were coordinated so that low-passage (passage 7 to 8) and high-passage (passage...
17 to 22) materials were available for inoculation. On the day of inoculation, infected and uninfected HL-60 cells were centrifuged (200 × g, 10 min) to concentrate the cells, and then the cell pellet was resuspended in serum-free RPMI 1640 medium.

**TABLE 1. PCR primer and TaqMan probe sequences for determination of msp2 HVRs by real-time RT-PCR**

| msp2 variant | Primer sequences (5’→3’) | Product length (bp) | Probe sequence (5’→3’) | Accession no. |
|--------------|--------------------------|---------------------|------------------------|---------------|
| RWMS11       | TCCACTATCGATGGGAAGGTTTG  | 84                  | TGACAAAGAAAGGAAAACGATACTCATGCG | AF443403     |
|              | CTTTATCTGCGATATCTGCTGCTGCTGCTG | 106        | CACTAGTAACTATGGAGCGCTGCTGCTGCTGCTG | AF443412     |
| WSM4         | TTCCGTATATGGGAAAGGTTTG  | 107                  | CTGCGGATAATTTTGGGAAAGTATGCG | AF443407     |
| WSM6         | CCGACTATGGGAAAGGTTTG   | 86                   | CGGGAAGGCTGTCAGTAGTAGCGCA | AF443411     |
| WSM16        | CTACGATGGGAAAGGTTTG    | 95                   | CGATACGCGAAAAAGATCATATTGCGA | AF443402     |
| WSM19        | CTTTATCTGCGATATCTGCTGCTGCTGCTGCTG | 95        | TGGACATAGTACACCAACGACGTAACTGCG | AF443404     |
| WSM5         | GCCTGTCGATGGGAAAGGTTTG | 104                  | AGCGGAAACAAAGATATGCTAATGACCTCAGAAGAG | AF443409     |
| WSM10        | ATTCTGACGTGGGAAAGGTTTG  | 94                   | AGCACGAATACGCAGAAATATGCTAGAGTAAACG | AF443400     |
| WSM25        | CGTCTTATCTGCATATACTCCATG | 135        | CGTGAGTACAGCGCAGGGAAAGGAG | AF443397     |
| WSM2         | GCGATCAGTGGGAAAGGTTTG  | 71                   | ACTGCTGACATCTGCAGCTAGCGCC | AF443405     |
| WSM45        | CACTTCTATGCGATGGGAAAGGTTTG | 84        | AGGAGGGAACAGATGGGGAAGTCAAGT | AF443408     |
| WSM15        | CCTACCGATGGGAAAGGTTTG  | 121                  | AGATAAATGCGTCTACTGCCGACTACCG | AF443398     |
| WSM3         | TTCTCTATCTCATTAGTGAAGAGGTTTG | 86        | TCTGCGGAAAGGAGGAAGGCGATAAACG | AF443406     |
| WSM52        | ATCCCGGTTATGGAAGAGGTTTG  | 87                   | CGGGAACGCGACCGCGGAGTAATATA | AF443410     |
| WSM1         | ATCCCTAATATGCGATGGGAAAGGTTTG | 94        | CACACGAGTGCCGATAGACTCCGTTCTG | AF443399     |
| WSM17        | TCCCGGTATTTGATAGAAGAGGTTTG | 85        | ACAGAAGCCCAAAAGTCTGGAGGAAAGTATGTA | AF443396     |
| WSM13        | TCCCGGATGTGGAAGAGGTTTG  | 92                   | CGACCCCAGACCGACGTTGGAACATAT | AF443413     |
| RW17         | ATCCCGGATGTGGAAGAGGTTTG  | 85                   | TGACGAAAGAAATCAGTGGGAGCAA | AF412831     |
| RW1          | ATCCCGGATGTGGAAGAGGTTTG  | 102                  | CGGAAAGAGCACTGAGGGTCTAGATGATGCG | AY253530     |
| RW23         | CCGGTTATGGAAGAGGTTTG    | 84                   | ACAGAAGCCCAAAAGTCTGGAGGAAAGTATGTA | AF135262     |
| RW20         | CCGGATGATGGGAAGAGGTTTTG | 125                  | CAATTTATATGCGGGTTATGCTGAGAGGACG | AY064524     |
cytophylum Webster strain-infected HL-60 cells. Three horses were infected with low-passage inoculum (<10 in vitro passages) and three with high-passage inoculum (20 to 26 in vitro passages). Blood was obtained from the horses on days 0, 2, 5, 7, 9, 13, 14, 15, 16, 20, and 22 by jugular vein venipuncture. Physical examination, complete blood count and serum chemistry, and body temperature were measured at these intervals to characterize clinical responses to infection. Clinical parameters that were examined and scored included ataxia, ranging from mild lack of coordination to recumbency (grade 1 to 5); lethargy, ranging from slightly quiet and less responsive to obtunded (grade 1 to 4); limb swelling, ranging from slight congestion above the fetlock to edema above and below the carpus and hock (grade 1 to 4); reluctance to move, ranging from slow but walk with leading to no walking even with leading (grade 1 to 4); and presence of petechiae (number observed on oral mucosa).

**Mice.** Eleven C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in a barrier facility and received food and water ad libitum. Baseline blood samples were taken from each mouse on day 0. Mice were later in each group) were then inoculated intraperitoneally with $1 \times 10^6$ HL-60 cells highly infected with low-passage (passage 5) or high-passage (passage 26) *A. phagocytophilum*. Three mice served as controls and received $1 \times 10^6$ uninfected HL-60 cells. Survival blood sampling was then performed on days 2, 7, 10, 14, and 21 by facial vein puncture, except for on day 21, when samples were acquired by terminal cardiac puncture. Mice were evaluated for clinical signs such as ruffled fur, reduced activity, failure to eat or drink, a hunched posture, and lack of interaction with cage mates.

**Serology.** Antibody responses were detected in serum samples of horses as has been previously described (20). Briefly, *A. phagocytophilum* antibodies were detected by an indirect immunofluorescent-antibody assay using cultured *A. phagocytophilum* as the antigen, and the cutoff titer for a positive serological response was $\geq 25$. Antibody responses in mice were not measured since the small serial samples taken were used exclusively for RNA extraction, with little serum left for adequate serological measurements.

**RNA preparation and RT-PCR for determination of bacterial load and equine cytokine transcript quantitation.** Total cellular RNA was isolated using the QIAsym RNA blood minikit (Qiagen Inc., Valencia, CA) from whole blood of low- and high-passage *A. phagocytophilum*-infected and mock-infected (uninfected HL-60 cell-infected) mice on days 2, 7, 14, and 21 and postinfection and from horses on days 0, 2, 5, 7, 9, 13, 14, 15, 16, 20, and 22. Blood was either used immediately or placed in RNAlater per the manufacturer's recommendations (Ambion, Austin, TX). For horses, RNA was used directly in quantitative reverse transcriptase 5' nuclease assays for analysis of equine cytokine mRNA and for 22 *A. phagocytophilum* msp2 variants (8, 24), with the primers, probes, and GenBank accession numbers listed in Table 1. Transcripts for msp2 were quantitated using a standard curve determined from plasmds for each of the 22 variants examined. Equine cytokine transcripts analyzed included interleukin-6 (IL-6), IL-8, IL-10, IL-12B (IL-12 p40 gene), IL-18, IFN-g, CCL5 (RANTES gene), and SLC11A1 (nRAMP gene) (21, 22). For mice, reverse transcription-PCR (RT-PCR) was performed with Superscript one-step RT-PCR and Platinum Taq (Invitrogen) with 30 ng RNA according to the instructions of the manufacturer. Broad-range primers for amplification of all msp2 cDNAs were used as previously described (8, 24). The reactions were subjected to electrophoresis in 1% agarose gels to confirm the presence of a 550-bp amplicon as visualized by ethidium bromide staining.

**Cloning and sequencing of RT-PCR products from mice.** RT-PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI), followed by transformation into Escherichia coli DH5a, and then plated onto LB agar containing 50 mg/ml ampicillin. Plasmids were purified (Wizard SV 96 plasmid DNA purification system; Promega) and assed for insert size after EcoRI digestion, and when an insert of the appropriate size insert (~550 bp) was detected, 10 to 20 from each cloning reaction were sequenced to compare at least 10 msp2 transcripts from each mouse at each interval. All sequences were aligned with *A. phagocytophilum* Webster strain msp2 references (18, 24) using ClustalX (25). The numbers of clones that aligned with each reference msp2 or into a new clade were identified to document the diversity of msp2 transcription that emerged as infection progressed in vivo with reference to low- or high-passage bacteria.

**Statistical analysis.** Chi-square analysis was conducted to compare clinical data from low- and high-passage-infected horses and the expected distribution of clinical signs. All other statistical analyses comparing continuous data between both low- and high-passage groups were analyzed using Student’s *t* test, with significance set at a *P* value of $<0.05$.

**RESULTS**

**Equine studies.** *A. phagocytophilum* morulae were detected in equine neutrophils at between days 13 and 16 postinfection, a result which was similar for both low- and high-passage-infected groups. Body temperatures started to increase at day 6 after infection and peaked between days 7 and 11. At day 15 body temperatures returned to baseline values. There were no differences noted in fever between the two infected groups. Antibody was first detected on day 7 for most horses in the low- and high-passage groups, and all seroconverted by day 22, with antibody titers reaching between 400 and 600 (Table 2). There was also a trend (*P* = 0.1, two-sided Student’s *t* test) toward a lower antibody titer in low-passage-inoculated horses (geometric mean of 400, versus 1,600 in high-passage-inoculated horses). Accumulated clinical scores reached a peak of 5 for low-passage-inoculated horses, compared to 13 in high-passage-inoculated horses (Fig. 1). Clinical signs were most prominent between days 5 and 18, with high-passage-inoculated horses still exhibiting clinical signs through day 22 (Fig. 1). There was also a bimodal pattern exhibited by both groups, in which the first peak of clinical manifestations occurred around day 7 to 9 and the second peak occurred during days 13 to 16.
In contrast to our prior work, the more severe clinical manifestations \((P < 0.001, \chi^2 \text{ test})\) were observed in high-passage \(A.\ phagocytophilum\) infection. Both clinical manifestations and thrombocytopenia, noted to begin predominantly around day 9 (Fig. 2), were preceded by elevated blood IFN-\(\gamma\) transcript levels on day 7, which were significantly higher in high-passage-infected horses than in low-passage-infected horses \((P = 0.045, \text{Student’s}’ t \text{ test})\) (Fig. 2). The elevations in IFN-\(\gamma\) transcript levels on day 7 also corresponded to the initiation of fever in these animals (Fig. 2). In concert with previous observations (20), when results were normalized to starting platelet counts, low-passage-infected horses developed a more profound thrombocytopenia than high-passage-infected animals, but only on day 13 \((P < 0.05, \text{Student’s}’ t \text{ test})\). No significant differences were noted in the white blood cell (WBC) count change between low- and high-passage-infected horses throughout the entire experimental period. Ap, \(A.\ phagocytophilum\). Error bars indicate standard errors of the means.

Multiple HVR transcripts were detected in each infected horse at each time interval. Between days 7 and 16, when the majority of \(msp2\) transcripts were detected, the diversity was very broad (Fig. 4A and B). The total number of transcripts detected, as a reflection of bacterial load, was not different between low-passage- and high-passage-infected horses \((P > 0.05)\) (Fig. 5). The differences in clinical findings could not be attributed to bacterial load or \(msp2\) transcript diversity, which varied considerably among all six low- and high-passage-inoculated horses; however, when assessed by group, there appeared to be a relationship between increased bacterial numbers and the second clinical score peak observed in both groups.

**Analysis of \(msp2\) transcripts in mice.** From among the \(msp2\) HVR amplicons after reverse transcription, 67 plasmid clones were generated from the low-passage \(A.\ phagocytophilum\)-infected mice and 8 clones from the low-passage inoculum prior to infection; 55 plasmid clones were similarly derived from...
high-passage *A. phagocytophilum*-infected mice and 12 from the high-passage inoculum prior to infection (Fig. 6). Four unique transcripts emerged from the initial inocula, including two from each passage. After infection in mice, msp2 transcripts were detected on days 2 through 21. As shown in Fig. 6, the most frequent transcripts were WMSP1 (low passage), WMSP2 (low passage), WMSP16 (low and high passages), WMSP17 (low passage), and WMSP19 (high passage), each comprising more than 10% of the total number of transcript clones. In contrast, in horses WMSP1 (high passage), WMSP2 (low and high passages), WMSP10 (low passage), and WMSP52 (low and high passage) comprised more than 10% of the total copy number detected. No reproducible pattern of msp2 transcription was found among mice, among inocula, or over time. Among mice in both the low- and high-passage groups, a total of 29 unique msp2 transcripts were detected. Each mouse had multiple (one to eight) msp2 transcripts representing heterogeneous populations of *A. phagocytophilum*. Some variants dominated the population (>50%) in individual animals, although these variants did not emerge with any pattern and occurred at different times. In the inocula, only two variants were shared between low and high passage, and the transcript repertoire present in both inocula was not reproduced in mice over the 21-day study.

**DISCUSSION**

In both the mouse and equine models of HGA, *A. phagocytophilum* msp2 transcription displays a high degree of diversity that emerges within 2 days in mice and by day 7 in horses and lasts through 21 to 22 days. The rapidity of variant emergence and lack of consistent variant emergence between and within animals suggest that msp2 gene recombination is not driven by adaptive or innate immunity and is instead a random and inherent property of the organism. The expression of a large repertoire of variants of *A. phagocytophilum* msp2 was also noted to occur within ticks and among various strains of *A. phagocytophilum* (3, 12), and this diversity also occurs at different time points of infection (2) and between mammalian and tick host cells (26). The benefit of diverse variants is the generation of heterogeneous bacterial populations, which ensure survival of the bacteria in the bloodstreams of various mammalian hosts until they are acquired in the tick blood meal. Additional diversity in ticks suggests a role in that host as well.

*A. phagocytophilum* msp2 transcription displayed a high degree of diversity in both experimental groups of horses, and this diversity in transcription of msp2 had no bearing on clinical disease in horses. This observation suggests the existence of a separate proinflammatory component differentially expressed with changing in vitro passage (10). These findings corroborate studies in mice, in which msp2 diversity did not explain differences in T-cell responses or differences in hepatic histopathology, a disease correlate (9). This discrepancy between msp2 expression and clinical disease in *A. phagocytophilum* differs from the case for its relative *A. marginale*, in which its MSP2...
has properties, such as segmental gene conversion, that contribute to specific CD4+ T-cell epitopes resulting in strong IFN-γ responses, clinical disease, and clearing of infection by macrophage activation (6).

Although no model is ideal for HGA investigation, given the clinical and pathological similarities to human disease, the distribution and kinetics of infection, and knowledge of the effects of the transmission vector and route, the horse provides many desirable attributes not attained with other models. It is clear that hypotheses generated from other models greatly benefit by validation in the horse. For example, we previously examined and demonstrated that IFN-γ plays a critical role in induction of inflammatory histopathology (15, 16). Although we did not use a histopathological correlate in horses, we demonstrated that IFN-γ expression in infected horses parallels that observed in the murine model and potentially plays a role in clinical manifestations and perhaps hematologic abnormalities such as platelet number and function (5). In this study, increased IFN-γ expression on day 7 corresponded with fever and clinical disease severity and immediately preceded thrombocytopenia in high-passage-infected horses and to a lesser extent in low-passage-infected horses (Fig. 2), as previously observed in mice (15, 16); a similar phenotype of increased cytokine expression and clinical severity observed has also been observed in a canine model of HGA (unpublished data). Increased IL-8 expression has not been shown to correlate with disease severity in HGA, although IL-8 plays a role in A. phagocytophilum infection and propagation (23) and ultimately HGA pathogenesis.

In both the murine and equine models, we also clearly demonstrated that there is a lack of a specific and reproducible program for A. phagocytophilum msp2 expression in animals inoculated with both low- and high-passage bacteria. The unique feature of this comparative study is that each animal was followed from inoculation for 21 to 22 days, when bacteremia or clinical signs subsided. Over this interval, msp2 variant emergence was unique for each individual and each species studied. Although we previously demonstrated a lack of programmed msp2 expression in mice inoculated with low- and high-passage organisms (9), the prior study design allowed for evaluation of transcript diversity only at various intervals in individual cohorts of mice per interval. The advantage of the current approach is the ability to sequentially evaluate transcript emergence within individual animals over time.

The evidence presented in this paper refutes our original hypothesis and supports the concept that changing Msp2 expression has little or no impact on clinical disease outcome and that passage in vitro, associated with Msp2 expression changes, is not followed by a specific repertoire of msp2 transcripts during infection. This is in contrast to the case for A. marginale, among other Anaplasmataceae, which establishes persistent infections and likely relies on changing pfam01617 surface antigen 2 expression to generate antigenic diversity and immune evasion (17). We propose to continue studies to elucidate the antigenic triggers that influence proinflammatory responses and that ultimately influence clinical outcomes and histopathologic severity. Once these are better understood, the goal of developing appropriate treatment regimens for patients and animals with granulocytic anaplasmosis will be more easily addressed.

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