Dexamethasone-Induced Cytokine Changes Associated with Diminished Disease Severity in Horses Infected with

\textit{Anaplasma phagocytophilum}^\textsuperscript{\textcopyright}

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\textit{Anaplasma phagocytophilum} is the zoonotic cause of granulocytic anaplasmosis. We hypothesized that immune response, specifically gamma interferon (IFN-\gamma), plays a role in disease severity. To test this, horses were infected and IFNG expression was pharmacologically downregulated using corticosteroids. Eight horses were infected with \textit{A. phagocytophilum}; 4 received dexamethasone on days 4 to 8 of infection. Clinical signs, hematologic parameters, and transcription of cytokine/chemokine genes were compared among treated and untreated horses. Infection was quantitated by \textit{msp2} real-time PCR and microscopy. As anticipated, there was significantly greater leukopenia, thrombocytopenia, and anemia in infected versus uninfected horses. The \textit{A. phagocytophilum} load was higher for dexamethasone-treated horses. Dexamethasone reduced IFNG transcription by day 12 and IL-8 and IL-18 by days 7 to 9 and increased IL-4 on day 7. The ratio of IL-10 to IFNG was increased by dexamethasone on day 9. There were no hematologic differences between the infected horses. Dexamethasone suppression of proinflammatory response resulted in delayed infection-induced limb edema and decreased icterus, anorexia, and reluctance to move between days 6 and 9 and lower fever on day 7. These results underscore the utility of the equine model of granulocytic anaplasmosis and suggest that Th1 proinflammatory response plays a role in worsening disease severity and that disease severity can be decreased by modulating proinflammatory response. A role for Th1 response and macrophage activation in hematologic derangements elicited by \textit{A. phagocytophilum} is not supported by these data and remains unproven.

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reproducibly restores virulence (37). Thus, we inoculated one horse with $10^8 A. phagocytophilum$-infected HL-60 cells propagated in vitro by intravenous injection and observed for onset of fever, morulae in neutrophils, and $A. phagocytophilum$ DNA in peripheral blood. Upon detection of infection on day 13 post-inoculation, 120 ml of fresh acid citrate dextran (ACD)-anticoagulated peripheral blood was transfused into a second horse. Upon evidence of fever and PCR detection of $A. phagocytophilum$, this animal served as the source of inoculum for the remaining study horses.

**Experimental infection.** Eight horses were infected intravenously with 18 ml of whole ACD-anticoagulated peripheral blood from the second experimentally infected horse. Four of the 8 horses (group 1) received 40 mg of dexamethasone on days 4 to 8 after infection, and the remaining 4 (group 2) did not. The inoculum was calculated to contain approximately $10^6$ infected cells ($1.14 \times 10^6$ infected neutrophils), in accordance with prior experimental infections in horses (29, 31, 37). Clinical assessments were conducted on days 0, 2, 4, 5, 6, 7, 9, and 12, and limb edema was also evaluated on days 14, 16, and 18. Blood samples for $A. phagocytophilum$ quantitative bacteremia by real-time PCR and cytokine/chemokine gene transcription in peripheral blood cells were obtained on days 0, 4, 7, 9, and 12; routine hematologic examination was conducted as well on these days, including day 6. The study was terminated on day 18, when infection and clinical signs had resolved in all animals.

**Clinical horses.** Two additional horses were mock infected with 18 ml of whole equine blood from a healthy $A. phagocytophilum$ PCR-negative donor horse. Two mock-infected horses received 40 mg of dexamethasone on days 4 to 8 (group 3), and the remaining 2 (group 4) did not.

**Quantitative $A. phagocytophilum$ bacteremia.** In order to assess the overall bacteremia with $A. phagocytophilum$, two methods were employed. First, routine Wright-stained blood smears were assessed for at least 100 neutrophils to determine the proportion containing $A. phagocytophilum$ morulae, and this value was used to determine the absolute infected neutrophil count. Likewise, direct bacterial burden in blood was determined by quantitative PCR targeting the $5'$ conserved region of $A. phagocytophilum$ msp2, a gene with $>100$ paralogous copies in the $A. phagocytophilum$ HZ strain genome (30, 31, 37), with all results normalized to those for total-blood DNA. For this, total bacteremia was calculated based on a comparison of threshold cycle ($C_T$) values from a standard curve of DNA obtained from known bacterial quantities (37). The values were examined by comparing treated and untreated horses on the same days, and total bacteremia over the course of the infection was calculated by determining the area under the curve for each group. Differences at individual days postinfection were assessed using one-sided, two-sample, unequal-variance Student $t$ tests, with a $P$ value of $<0.05$ considered significant. For overall quantitative bacteremia, the area under the curve was calculated for each condition and compared using the $\chi^2$ test, with a $P$ value of $<0.05$ considered significant.

**Clinical and hematologic assessment.** Blood was obtained from horses on days 0, 4, 6, 7, 9, and 12 by jugular vein venipuncture. Complete blood counts were determined using an automated hematology analyzer (Advia 120; Bayer Corporation, Norwood, MA) to characterize laboratory features of infection (Veterinary Medical Teaching Hospital, UC Davis). Clinical measurements of severity included the following: lethargy, ranging from slightly quiet and less responsive than normalities. Infected horses developed marked thrombocytopenia on days 4 through 9 ($P = 0.029$); fever was worse on day 7 ($P < 0.001$). In addition, other clinical conditions were worse for untreated horses on days 6 through 9, including anorexia ($P \leq 0.029$), icterus ($P \leq 0.029$), and reluctance to move ($P = 0.029$); fever was worse on day 7 ($P < 0.001$; Fig. 2). Ataxia did not differ between the treatment groups.

**Hematologic parameters.** As anticipated from a comparison with equine reference intervals, all infected horses developed thrombocytopenia, leukopenia, and anemia by day 6 of infection; untreated horses did not develop any hematologic abnormalities. Infected horses developed marked thrombocytopenia on days 6 through 12 ($P < 0.001$) (mean, 58,770 $\mu l^{-1}$; reference interval, 100,000 to 225,000 $\mu l^{-1}$), anemia on days 4 through 7 ($P = 0.038$) (mean, 25%; reference interval, 30 to 46%), leukopenia on days 6 through 9 ($P = 0.009$) (mean, 4,490 $\mu l^{-1}$; reference interval, 5,000 to 11,600 $\mu l^{-1}$), and lymphopenia on days 4 through 9 ($P < 0.014$) (mean, 789 $\mu l^{-1}$; reference interval, 1,600 to 5,800 $\mu l^{-1}$). There were no significant differences between dexamethasone-treated and untreated horses, with the exception of suppressed peripheral blood eosinophil counts in dexamethasone-treated horses on day 9 only ($P = 0.018$). The nadirs of thrombocytopenia and leukopenia corresponded closely with times of peak bacteremia (Fig. 3).

**Cytokine gene transcription measurements.** Transcription of cytokine genes among peripheral blood cells was assessed by real-time reverse transcriptase qPCR on days 0, 4, 7, 9, and 12 of infection. Total cellular RNA was isolated using the QIAamp RNA blood minikit (Qiagen, Inc., Valencia, CA) from the whole peripheral blood of $A. phagocytophilum$- and mock-infected horses as described previously (15); $ex vivo$ antigen stimulation was not used. Equine cytokine transcripts analyzed included $IL-4$, $IL-8$, $IL-10$, $IL-12B$ (IL-12 $p40$ subunit gene), $IL-18$, $IFNG$, and $CCL5$ (RANTES) (32, 33). Quantification was performed using the $\Delta C_{T}$ method and reported as relative transcription or the $n$-fold difference relative to the cDNA calibrator (22). For comparative purposes, transcription was normalized to that observed on day 0 for each horse; for infected horses, transcription was further normalized to account for changes in uninfected controls. Differences in transcription were assessed using repeated-measure ANOVA with a Bonferroni correction, with a $P$ value of $<0.05$ considered significant.

**RESULTS**

**$A. phagocytophilum$ PCR and microscopic quantitation.** All horses inoculated with $A. phagocytophilum$ were confirmed infected by real-time qPCR on days 4 through 12, with peak bacteremia burden on days 7 and 9; infection was also confirmed by microscopic examination of peripheral blood smears. Although the absolute number of infected neutrophils was higher on days 7 through 12 for the dexamethasone-treated horses, significant differences in the $A. phagocytophilum$ load did not exist when individual days were compared. However, the bacterial burden over the entire course of infection was higher for dexamethasone-treated than for untreated horses ($P = 0.022$; Fig. 1). Peak bacteremia coincided with nadirs of both leukocyte and platelet counts and coincided with or preceded peak severity of limb edema, anorexia, icterus, and reluctance to move.

**Clinical findings.** All infected horses manifested signs of disease regardless of treatment. By day 4, signs, including moderate anorexia, lethargy, reluctance to move, and icterus, appeared. Limb edema and ataxia appeared by day 6. The most dramatic difference between treated and untreated infected horses was a delay in limb edema with dexamethasone, as evident with lower edema scores for treated horses on days 6 to 9, reversing on days 14 to 16 (Fig. 2) ($P < 0.001$). In addition, other clinical conditions were worse for untreated horses on days 6 through 9, including anorexia ($P \leq 0.029$), icterus ($P \leq 0.029$), and reluctance to move ($P = 0.029$); fever was worse on day 7 ($P < 0.001$; Fig. 2). Ataxia did not differ between the treatment groups.
cantly differ over time except for changes in CCL5 and IL-4 on day 9, immediately after termination of dexamethasone treatment, and for IL-10 and the ratio of IL-10 to IFNG on day 12.

(ii) A. phagocytophilum-infected horses. Because transcription levels in mock-infected animals varied and were not directly related to dexamethasone treatment, cytokine gene transcription in infected horses was normalized to that in mock-infected horses to more precisely identify dexamethasone effects on infection-related cytokine gene expression. Infection had several broad effects on cytokine gene transcription in peripheral blood (Fig. 4), including a generalized increase in IL-12B, IL-18, and IL-8 and reduction in IL-4 and CCL5. After infection, regardless of dexamethasone treatment, transcription was upregulated 2- to 27-fold for IL-12B and 2-fold for IL-8 and downregulated 2- to 4-fold for IL-4 (P < 0.008), 3-fold for CCL5 (P < 0.001), and 3-fold for IL-10 (P < 0.001).

FIG. 1. Anaplasma phagocytophilum peripheral blood load, as determined by quantitative PCR and normalized to total-blood DNA content in untreated and dexamethasone-treated horses. The dashed lines represent the mean values at each time point; the shaded areas delineate the standard error of the mean (SEM) for the means for each group. Although bacterial loads did not differ between the groups at individual days postinfection, the overall bacterial load (area under the curve) was significantly higher for dexamethasone-treated than for untreated A. phagocytophilum-infected horses (P = 0.022, χ² test).

FIG. 2. Clinical severity of infection in untreated horses was worse or accelerated compared with these features in dexamethasone-treated horses after experimental infection with A. phagocytophilum. The median value is shown by the central bar within each box; the 1st and 3rd quartiles are delineated by the boxes, and the error bars show the maximum and minimum values after ranking. Significant differences between the treatment groups are illustrated by P values above comparisons for each graph.
Dexamethasone treatment abrogated the initial 9.5-fold down-regulation of IL-4 transcription in infected horses on days 7 and 12 to 2.3-fold ($P = 0.038$); this was followed by suppressed IL-4 transcription at day 12 (6.0- versus 2.6-fold; $P = 0.032$), after dexamethasone treatment was terminated. Similarly, 2.9- to 3.5-fold increases in IL-8 transcription on days 7 to 9 were suppressed by dexamethasone ($P = 0.026$ and 0.050, respectively) (Fig. 4). Dexamethasone treatment resulted in increasingly higher transcription ratios of IL-10 to IFNG (IL-10 transcription excess) over the course of infection, although this was significant only on day 9 ($P = 0.029$). Although the ratio of IL-10 to IL-12B was higher in dexamethasone-treated horses at every interval, the differences were not statistically significant in this small group.

Taken as a group, the changes in proinflammatory cytokine gene (IFNG, IL-12B, and IL-18) transcription were not significantly altered by the 4-day course of dexamethasone, and anti-inflammatory cytokine gene (IL-4 and IL-10) transcription was significantly lower only in the absence of dexamethasone at day 7 (ANOVA). However, when the ratios of IL-10 transcriptional change to IFNG and IL-12B transcriptional change were considered together, higher ratios that correspond to a lower overall proinflammatory response on both days 7 and 9 ($P = 0.029$, ANOVA), most likely impacted by the dexamethasone treatment on days 4 to 8, were observed.

**DISCUSSION**

The mechanisms by which *A. phagocytophilum* causes tissue injury and disease are unclear. Only limited data demonstrate any link between pathogen load and disease severity, and recent evaluations of infection in mice and in humans clearly establish links between immune response and histopathology or disease manifestations and severity (5, 7, 8, 10, 26). The chief immunological marker is IFN-γ, which when depleted results in a marked increase in pathogen load but complete loss of tissue lesions in mice (1, 26). In contrast, infection in mice that lack IL-10, a cytokine that suppresses IFN-γ expression, results in markedly worsened tissue lesions but no alteration in pathogen load (26). These findings indicate that in mice, *A. phagocytophilum* triggers a polarized response that favors inflammatory tissue injury and activation of macrophage effector functions (5, 8, 10, 35, 36, 38). In humans with *A. phagocytophilum* infection, moderate or high serum IFN-γ and IL-10 levels are observed, and a high ratio of IL-10 to IFN-γ is associated with increased disease severity, a likely reflection of the extent to which pro- and anti-inflammatory signaling occurs (10). In addition, serum IL-12, ferritin, and triglyceride levels correlate with increased disease severity, further reflecting the downstream activation of macrophages by the proinflammatory response and the association of this feature with a disease phenotype.

Unfortunately, the murine model suffers from the lack of clinical disease signs. To circumvent this shortfall in an experimental setting, horses offer the advantage that they develop a disease very similar to that in humans and share underlying histopathology after *A. phagocytophilum* infection with both experimentally infected mice and naturally infected humans (4, 21, 24). Among several disadvantages of the horse model, the inability to experimentally manipulate discrete immunological responses is potentially problematic. Here, we took advantage of the anecdotal observation that treatment of horses with dexamethasone results in a phenotype similar to that observed with IFN-γ knockout mice, in which blood pathogen loads increase substantially and disease manifestations seem curtailed. Dexamethasone has broad immunological effects, but major targets of its action include transrepression of NF-κB and AP-1 transcriptional activation by ligated glucocorticoid receptors and regulation of STAT1-modulated IFNG transcription and IFN-γ expression and signaling (16, 19, 28, 34).

Here we confirm some observations with murine models of GA which show that disease severity is lessened with attenuated host inflammatory and immune responses to *A. phagocyto-philum* infection (26, 35, 38). Furthermore, these data extend the observations with murine models by documenting the association of worsened clinical disease manifestations, such as anorexia, limb edema, icterus, reluctance to move, and fever with unmodified inflammatory response despite an overall lower bacterial burden. A feature of this analysis shows that IFNG transcription in peripheral blood is only marginally altered by dexamethasone treatment after established infection and that the only time point at which significant differences in IFNG transcription are detected is after the key differences in clinical features are observed, raising questions about the association of these features. However, assessment of cytokine responses among circulating leukocytes is not an absolute surrogate of responses or their magnitude in mononuclear phagocyte organs or at sites of inflammation. Perhaps subsequent experiments could better assess this possibility with ex vivo stimulation of peripheral blood elements by *A. phagocytophi-lum*, a situation that would mimic contact of immune cells with the pathogen in tissues. Moreover, proinflammatory responses attributable to IFN-γ signaling are amplified after priming or

![Graph showing leukocyte and platelet count changes normalized to day 0 values](https://example.com/graph.png)

*FIG. 3. A. phagocytophilum* infection induced significant leukopenia and thrombocytopenia for which nadirs corresponded to peak bacteremia regardless of dexamethasone treatment. The bars (left axis) depict leukocyte or platelet count changes normalized to day 0 values; the line (right axis) shows the level of bacteremia determined by microscopic counts of morulae-containing neutrophils in peripheral blood of infected (Aph-infected) horses only.*
by feed-forward loops even when IFN-γ concentrations are low and are counterbalanced by the effects of anti-inflammatory signaling of IL-10 and similar cytokines (17). Thus, these data could be underestimations, may not be reflective of local immunological responses, or could indicate more important roles for IL-12, IL-8, or other cytokines in horses. In contrast, the relationship between cytokine profiles that conform to Th1 proinflammatory or anti-inflammatory/regulatory responses, as reflected by transcription ratios of IL-10 to IFNG and IL-10 to IL-12B (9), is associated with the temporal occurrence of histopathologic lesions or clinical signs, as previously shown with mice and with humans with increased disease severity (10, 26). Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are known to synergistically activate macrophages; however, the inability to detect significant quantities of these factors in humans and animal models of A. phagocytophilum infection argued against their further study (10, 12, 25). In sum, A. phagocytophilum infection toggles the host response to a proinflammatory, macrophage-activating environment associated with histopathologic injury and clinical disease. In contrast, partial treatment with dexamethasone interferes with events, perhaps transcriptional, interwoven into the proinflammatory host response and tip its bias toward that often observed with the regulation of inflammation, dominated by suppressed proinflammatory cytokines and the presence of anti-inflammatory IL-10 (9, 27).

Interestingly, modulation of cytokine and inflammatory/innate immune responses did not substantially impact any of the hematologic changes associated with A. phagocytophilum, including leukopenia, thrombocytopenia, or anemia. This finding seems to contradict our prior hypothesis that such hematologic alterations in HGA are the result of IFN-γ-mediated macr-
phae activation, at least to the degree that such was modified by dexamethasone treatment after infection. This hypothesis posited that the predominant mechanism of pancytopenia involves macrophage activation by IFN-γ, leading to nonspecific extramedullary destruction of platelets and leukocytes after excessive or unregulated nonspecific phagocytosis or induction of inflammatory mediators within mononuclear phagocyte organs (2). However, here it must be noted that despite the inability to discern a linkage between the hematologic alterations and suppressed cytokine production, the dexamethasone treatment regimen used was limited to 5 days and was started only after onset of detectable bacteremia at a time when initiation of cytokine cascades that determine macrophage activation likely already occurred. Thus, further study will be needed to accurately determine the relationship between macrophage activation after cytokine stimulation and the development of pancytopenia in *A. phagocytophilum* infections. In contrast, Klein et al. proposed that the induced production of myelosuppressing cytokines and chemokines detected *in vitro* with *A. phagocytophilum* could account for the typical hematologic changes (18). The lack of an association between the alterations in IL-8 or CCL5 transcription and leukopenia, anemia, or thrombocytopenia provides evidence to refute this hypothesis.

The ramifications of these findings are potentially important for management of the severe consequences of *A. phagocytophilum* infection, since control of bacterial load appears to be only one consideration for minimizing disease, especially among the 7% or more with severe infections (10, 11). Rather, adjunctive measures that diminish inflammatory cascade induction, especially those that lead to macrophage activation, should be considered when disease becomes more severe, as with multiorgan involvement or acute respiratory distress syndrome. Additionally, as consideration is given to vaccines for animals or humans, it is imperative that the constructs used be carefully evaluated to avoid the triggering of such inflammatory sequelae (9). To better understand how this could be accomplished, studies will be needed to determine which components of *A. phagocytophilum* infection trigger the response (7), which cells in the host are predominantly responsible (8), and why some but not all individuals develop more profound inflammatory or innate immune responses that accompany or lead to severe inflammatory disease manifestations (10). Given the involvement of macrophage activation and the TH1 cytokine cascade, much attention should be given to the stimulation of this process by cytokines, such as IFN-γ, by innate immune subsets, such as NK and NK T lymphocytes, and control of the process by cytolytic or regulatory lymphocytes whose function is also in part down-modulation of macrophage activation.

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