Clinical signs and hematologic, cytokine, and plasma nitric oxide alterations in response to *Strongylus vulgaris* infection in helminth-naïve ponies

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Abstract

The objective of this study was to determine the effect of infection with *Strongylus vulgaris* on serum cytokines and plasma nitric oxide (NO) concentrations in helminth-naïve ponies. Group 1 (*n* = 21) was given 500 *S. vulgaris* L3 larvae and group 2 (*n* = 7) received a saline control. Ponies were monitored daily for clinical signs, and blood was collected for complete blood cell counts and serum cytokines (TNF, IL-1, IL-6) quantification. Group 1 ponies were depressed, anorexic, and febrile for variable periods of time. Plasma NO was increased on day 21 in group 1 and on days 9 and 21 in group 2. Significant increases in total white blood cell counts, fibrinogen, and plasma protein concentrations in group 1 were found. Significant decreases in red blood cell counts and packed cell volume were also noted in group 1. There were no differences in serum cytokines across time in either group of ponies. Despite the lack of proinflammatory cytokine induction with the apparent inflammatory response to *S. vulgaris* there is evidence of a potential role of NO.

Résumé

Les objectifs de l'étude étaient de déterminer chez des poneys jamais exposés aux helminthes l'effet d'une infection par Strongylus vulgaris *sur les concentrations de cytokines sériques et d'oxyde nitreux (NO) plasmatique. Les animaux du groupe 1 (N = 21) ont reçu 500 larves de* S. vulgaris *au stade L3 et le groupe 2 (N = 7) de la saline. Les poneys ont été examinés quotidiennement pour noter les signes cliniques et du sang prélevé pour un comptage cellulaire sanguin complet et une quantification des cytokines sériques (TNF, IL-1 et IL-6). Les poneys du groupe 1 étaient abattus, anorexiques et fébriles pour des périodes de temps variables. Le NO plasmatique était augmenté au jour 21 pour les animaux du groupe 1 et aux jours 9 et 21 pour les animaux du groupe 2. Des augmentations significatives du compte total de leucocytes, des concentrations de fibrinogène et de protéines plasmatiques ont été notées pour les animaux du groupe 1. Des diminutions significatives des comptages d'érythrocytes et de l'hématocrite ont également été notées pour les animaux du groupe 1. Pour ce qui est des cytokines, aucune différence dans le temps n'a été notée chez les deux groupes d'animaux. Malgré l'absence d'induction de cytokines pro-inflammatoires, la présence d'une réponse inflammatoire apparente envers* S. vulgaris *suggère un rôle potentiel pour le NO.*

(Traduit par Docteur Serge Messier)

Introduction

Strongylus vulgaris, a large strongyle of equids, is still considered one of the most pathogenic equine helminthes (1–3). The larvae cause a marked inflammatory reaction, characterized by an influx of neutrophils, eosinophils, and macrophages, when penetrating the intestinal mucosa. Subsequent arteritis of the ileocecocolic and associated arteries also occurs during larval migration with histologic evidence of multifocal inflammation, necrosis, and fibrosis (4,5).

There is evidence that tumor necrosis factor (TNF) and interleukin (IL)-1 are key mediators involved in initiating an inflammatory cascade (6,7) and administration of TNF and IL-1 results in increased activity of IL-6 in horses (7). It is possible that increased serum activities of TNF and IL-1 could be involved in mediating the clinical signs associated with the acute phase inflammatory response (fever, anorexia, depression, lethargy, and dullness) that helminth-naïve ponies exposed to *S. vulgaris* demonstrate during the first 2 wk after infection (4,8), and that the activity of IL-6 may reflect the presence of TNF and IL-1 (7).

Recent evidence suggests that NO can either attenuate or exacerbate gastrointestinal tract inflammation depending upon the source, magnitude, timing, and duration of its production (9). Nitric oxide

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is synthesized from L-arginine via nitric oxide synthase (NOS) (10). Nitric oxide synthase exists in 3 main isoforms; the first 2 are neuronal and endothelial NOS (nNOS and eNOS, respectively), grouped as constitutive NOS (cNOS) because they are present under basal conditions and are similarly regulated. Constitutive NOS consists of basal release of small quantities of NO for short periods, mediating physiological roles of NO (10). The 3rd isoform is inducible NOS (iNOS); its presence is induced by numerous factors (ischemia, endotoxin, cytokines) when the otherwise quiescent gene has been expressed (10,11). The iNOS isoform leads to synthesis of large quantities of NO for extended periods (11).

Fibroblasts, neutrophils, macrophages, and numerous other cell types express iNOS when exposed to TNF, IL-1, and IL-6 (11,12). Activation of NO synthesis via iNOS may exacerbate tissue injury through interaction of NO with superoxide anions (xanthine oxidase-derived and neutrophil-derived), which rapidly react forming the potent oxidant peroxynitrite (13,14), which can in turn initiate lipid peroxidation of cell membranes (13,14).

Stronglylus vulgaris infection could potentially initiate the expression of iNOS, resulting in excessive synthesis of NO. Increased NO could contribute, through its reaction with superoxide anions to form peroxynitrite (13,14), to tissue injury and potentially some of the clinical signs associated with *S. vulgaris* infection.

On the other hand, NO has been suggested as an effector molecule in parasite killing (15–17). Nitric oxide is regarded as the major effector molecule of macrophage cytotoxicity against a variety of microbial targets including helminthes, leishmaniasis, schistosomiasis, and toxoplasmosis (15,18). However, in other murine models of protection against NO generated via iNOS was shown to be necessary for the host to control a chronic infection, but not an acute infection (19). There may be a similar mechanism in equine helminth protective mechanisms.

The purpose of the study reported here was to determine and quantify the presence of pro-inflammatory cytokines and plasma NO concentrations after helminth-naïve ponies have been infected with *S. vulgaris,* and to determine if there is a relationship between increased concentrations of serum cytokines and plasma NO concentrations.

Materials and methods

Animals

This study was approved by the Louisiana State University Agriculture Center Institutional Animal Care and Use Committee. Twenty-eight helminth-naïve ponies, ranging in age from 4 to 6 mo, were raised and maintained under parasite-free conditions (20). Prior to the birth of these ponies, their dams were administered an anti-helminthic (Ivermectin, 200 μg/kg; Merial, Duluth, Georgia, USA), washed, and then placed in box stalls after foaling. The mares and foals were maintained in stalls until the foals were weaned at 3 to 4 mo of age. During this time the animals were administered pyrantal pamoate (2.64 mg/kg per os) daily.

After weaning, the ponies continued to be housed in stalls on wood shavings and were not exposed to parasites. Fecal exams were performed to confirm a negative status to helminths. They were fed a complete pellet feed and given water ad libitum. Group 1 ponies ($n = 21$) were administered 500 *S. vulgaris* L3 larvae via nasogastric intubation on day 0. Group 2 ponies ($n = 7$) were not given *S. vulgaris* larvae, but were given an equivalent volume of saline. The *S. vulgaris* L3 utilized in this experiment were obtained from fecal cultures of ponies with monospecific infections of the parasite. Ponies reared under parasite free conditions were infected by surgical introduction of living adult parasites into the cecum (21). Feces from these animals were cultured at room temperature and the L3 were recovered using a Baereman apparatus and stored at 4°C in water until used as previously described (22). All ponies had blood collected into tubes containing ethelyenediamine-tetraacetic acid (EDTA), and heparin, and tubes lacking anticoagulant (clot tubes) on days 0, 3, 6, 9, 14, 21, and 45 for complete blood (cell) count (CBC), plasma NO concentrations, and quantification of serum cytokine activities, respectively.

Clinical findings

Quantitative findings included daily rectal temperatures and body weight at the beginning and end of the study; subjective findings included whether the ponies were anorexic or were showing signs of abdominal discomfort.

Hematology

Blood collected with EDTA was analyzed routinely for CBC. Total and differential leukocyte counts, red blood cell indices, total plasma protein concentrations, packed cell volume, and fibrinogen concentrations were determined.

Measurement of NO

Jugular venous blood samples were collected into heparinized tubes and were centrifuged at 1500 × *g* for 10 min, the plasma supernatant was then collected and stored at −70°C until analyzed for NO concentration by using a chemiluminescence method (23,24). Aliquots of plasma were thawed and deproteinized by adding 100 μL of trichloroacetic acid (10% solution) to 100 μL of the sample, then vortexed for 30 s and allowed to stand for 15 min; afterward, the sample was placed in a microcentrifuge tube and centrifuged at 14 000 × *g* for 5 min. The supernatant then was removed for analysis. Aliquots of plasma (3 μL) were added to a purge chamber of vanadium chloride (100°C) in 1 N HCl under a nitrogen atmosphere. Nitric oxide (bound or in the form of nitrate) was liberated from the samples into the gaseous headspace and relayed to the NO analyzer (Model 280 [NOA]; Sievers Instruments, Boulder, Colorado, USA), where it reacted with ozone to produce a chemiluminescent signal in the 650 to 800 nm range. The amount of light was proportional to the NO concentration, which was calculated from a standard curve of known nitrate concentrations. Each sample was analyzed in triplicate. Assay sensitivity of liquid samples was 1 μM.

Serum cytokine bioassays

Ten milliliters of blood was collected into tubes containing no anticoagulant. After the clot was formed, the samples were centrifuged at 1500 × *g* for 10 min and the serum was pipetted into 3 tubes, which were stored at −70°C until analyzed for cytokine activities (IL-6) and concentrations (TNF and IL-1) (23).

Cell culture and preparation of equine cytokine standard

All cell lines were cultured in RMPI 1640 medium supplemented with 10% fetal bovine serum, 2.2 mM L-glutamine, 2.2 g of $NaHCO_3$/L, 25 mM HEPES, 2.7×10^{-4} mg of 2-mercaptoethanol/L, 100 U of penicillin and 100 μg streptomycin/mL of media (23). The B13.29 clone B9 cells were additionally supplemented with 50 U of recombinant murine IL-6 (R&D Sytems, Minneapolis, Minnesota, USA) per mL. Equine cytokine standards were generated by culturing 3×10^6 equine peripheral blood mononuclear cells/mL with 5 μg of bacterial lipopolysaccharide/mL for 6 h at 39°C in 5% CO_2. Cultures were centrifuged, the supernatant was filtered and aliquots frozen at −20°C (25–29).

Because dose-response curves for recombinant human and murine cytokines were not parallel with those obtained using equine standards, recombinant cytokines were used as a reference standard to determine units for equine cytokine standards in each of the bioassays (23). Equine cytokine standards were diluted serially 5-fold in the tumor necrotizing factor (TNF) assay and 3-fold in the IL-1 and IL-6 assays. Recombinant cytokines were added to the plates at concentrations of 10.0, 1.0, and 0.1 U/mL. Values obtained for recombinant cytokines were then used as a reference to determine units of cytokine per milliliter in the equine standards. Sensitivity of each assay was determined by serially diluting the equine standards to extinction.

Assay for TNF

The cytotoxicity assay was performed using the mouse fibrosarcoma cell line WEHI 164 clone 13. First, 50 μL of a 1:50 dilution of test supernatant was pipetted in triplicate into 96-well flat-bottomed microtitration plates (96 Well Flat Bottomed Plates; Corning, New York, USA). Next, 2×10^4 WEHI 164 clone 13 cells were added to each well in 50 μL of complete medium containing 2 μg of actinomycin D/mL (resulting in a final concentration of 1 μg/mL). Plates were sealed and incubated for 20 h at 37°C in 5% CO_2. Ten microliters of a solution of the tetrazolium dye 3-(4,5 dimethylthiazole-2yl)-2,5 triphenyl tetrazolium bromide (MTT); 5 mg/mL of calcium and magnesium free (CMF) phosphate buffered saline solution (PBSS) were added to each well. After a 4-h incubation at 37°C, 100 μL of 0.04 N HCL in isopropanol was added to each well, and the contents pipetted vigorously to dissolve the purple formazan crystals. Optical density (OD) of the plates was read at 570 nm in a multiwell scanning spectrophotometer. Sensitivity of the TNF assay was 0.04 U/mL (25).

Assay for IL-1

Interleukin-1 cytotoxicity activity against human melanoma cell line A375.S2 was determined. Triplicate samples were diluted 1:50 in 100 μL of culture medium in 96-well flat-bottomed microtitration plates, 2×10^3 A375.S2 cells were then added to each well in 100 μL of complete medium. Plates were sealed and incubated at 37°C in 5% CO_2 for 96 h.

After removal of the supernatant fluid, plates were washed in CMF-PBSS (pH 7.4). Next, 50 μL of 100% methanol was added to each well and the plates were then incubated at room temperature (20 to 22°C) for 30 min. Once the cells were fixed to the plate, the methanol was poured off and the plates were allowed to dry at room temperature for 24 h. The remaining adherent cells were stained with 50 μL of 0.05% crystal violet (Sigma Chemical Company, St Louis, Missouri, USA) in 20% ethanol for 10 min. Plates were rinsed in cold tap water, and the incorporated stain eluted with 100 μL of 50% methanol. Cell survival was determined by reading the OD of the plates at 570 nm in a multiwell scanning spectrophotometer (Multiwell Scanning Spectrophotometer; Dynatech Labs, Chantilly, Virginia, USA) (28). Sensitivity of the IL-1 assay was 7.21 U/mL.

Assay for IL-6

Supernatant IL-6 activity was measured by use of the murine hybridoma IL-6-dependent cell line B13.29 clone B9. Briefly, test samples were diluted 1:50 in culture medium, and 100 μL of was placed in each well of a 96-well flat bottomed microtitration plate (96 Well Flat Bottomed Plates; Corning). Two thousand B9 cells in 100 μL were seeded into each well, the wells were then incubated for 72 h at 37°C in 5% CO_2. During the last 8 h of incubation, plates were pulsed with 1.0 μCi/well of (^{3}H) thymidine per well. Proliferation was determined by harvesting the cells and determining (3H) thymidine incorporation by using liquid scintillation (Betaplate liquid scintillation counter 1205 LKB; Wallac, Turku, Finland) (25–27,29). Sensitivity of the assay for IL-6 was 1.677 U/mL.

Statistical analysis

The continuous data for all CBC variables, clinical findings (number of days the ponies were pyrexic, rectal temperatures on days blood was drawn), and NO concentrations was evaluated for normality, using the Shapiro-Wilk statistic and was considered to follow a normal distribution with failure to reject the null hypothesis of normality at $P \leq 0.05$. Normal data were summarized and graphed as mean ± standard deviation (*s*).

For clinical findings, the duration (days) of pyrexia followed a Poisson distribution and was analyzed using a log linear model. A $P \leq 0.05$ was considered significant. PROC GENMOD (SAS, version 6.12; SAS Institute, Cary, North Carolina, USA) was used for the analysis.

The CBC and NO data were analyzed using a mixed effect linear model, accounting for the random variance of horse and the repeated measurements across time. A 2-sided hypothesis with $\alpha = 0.05$ was used to determine significance of the main effects. PROC MIXED (SAS, version 6.12; SAS Institute) was used for the analyses. Comparisons within each group to baseline (time t = 0) and comparison between groups at specific times were made, using adjusted least squares means maintaining an experiment-wise error of $\alpha = 0.05$. Thus, where a difference was noted, unless specified, the *P*-value was ≤ 0.05.

The serum cytokine data, although considered to be continuous data, were highly skewed. For evaluation of a difference between groups, Mantel-Haenszel methods were used on the rank data controlling for time. For evaluation of the difference over time, Friedman's test for repeated data, blocking by horse was used. An experiment-wise *P*-value ≤ 0.05 was maintained by reducing the level of significance for individual tests, using a Bonferroni adjustment for the number of comparisons being made. For consistency, the data was summarized as mean ± *s*. PROC FREQ, PROC

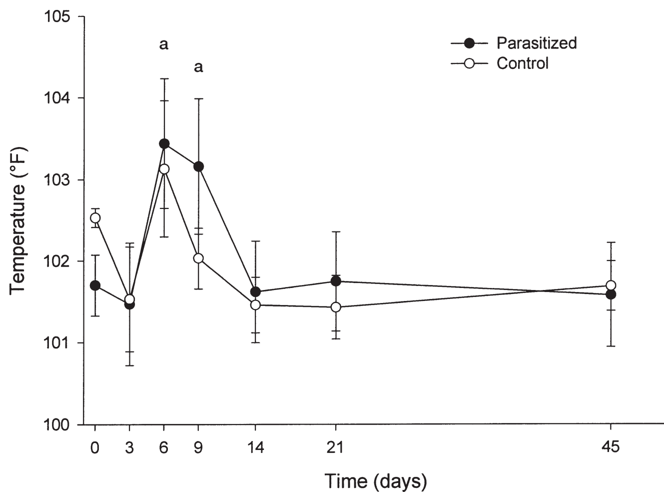

Figure 1. Mean (± *s*) rectal temperature in parasitized and control ponies. [a]Significant ($P < 0.05$) differences from baseline within the parasitized group.

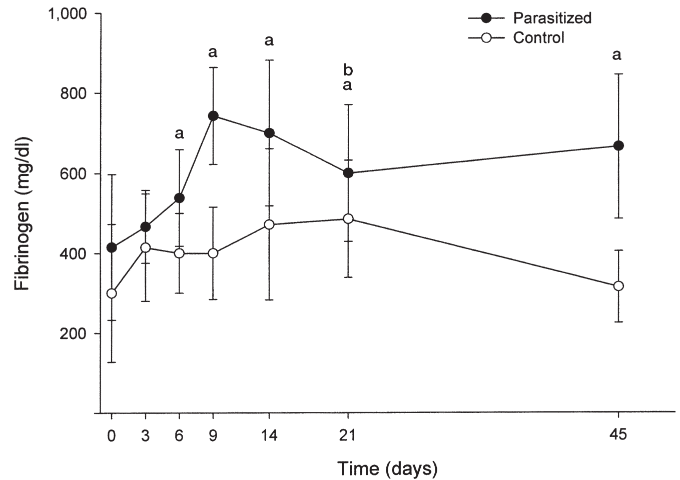

Figure 3 — Mean (± *s*) fibrinogen concentration in parasitized and control ponies. [a]Significant ($P < 0.05$) differences from baseline within the parasitized group. [a]Significant differences ($P < 0.05$) from baseline within the control group.

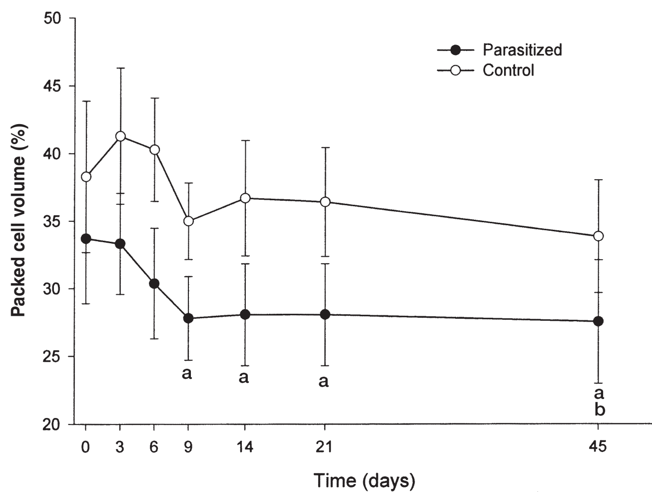

Figure 2 — Mean (± *s*) packed cell volume in parasitized and control ponies. [a]Significant differences ($P < 0.05$) from baseline within the parasitized group. [b]Significant differences ($P < 0.05$) from baseline within the control group.

NPAR1WAY, and PROC UNIVARIATE (SAS, version 6.12; SAS Institute) were used for the analysis.

Results

Clinical signs

Parasitized ponies exhibited significantly greater number of days of pyrexia compared with the control ponies. The parasitized ponies had less weight gain over the period, an increase of 18% in body weight compared with 31% for the control ponies.

Rectal temperatures

Parasitized ponies did not have significantly different rectal temperatures compared with control ponies. Within the parasitized group, there were significantly increased rectal temperatures on days 6 and 9, compared with baseline values, which then returned to baseline values on days 14 through 45. There were no significant changes from baseline values for the control ponies (Figure 1).

Hematological variables

Packed cell volume — Parasitized ponies had significantly lower PCV compared with the control ponies at all times. Within the parasitized group, there were significant decreases in the PCV on days 9, 14, 21, and 45 postinfection, compared with baseline value. There was a significant decrease in the PCV within the control ponies on day 45 compared with baseline value (Figure 2).

Total erythrocyte count — Parasitized ponies had significantly decreased erythrocyte counts, compared with the control ponies at all times. Within the parasitized group, there were significant decreases in the erythrocyte count on days 9, 14, 21, and 45 postinfection compared with baseline value. There was a significant decrease in the erythrocyte count within the control ponies on day 45 compared with the baseline value.

Total plasma protein concentration — Parasitized ponies had significantly decreased total protein concentrations compared with the control ponies at all times. Within the parasitized group, there was a significant increase above baseline values on days 14 and 45 postinfection. There were no differences from baseline values across time for the control ponies.

Fibrinogen concentration — Parasitized ponies had significantly greater fibrinogen concentrations compared with the control ponies at all times. Within the parasitized group, there were significant increases above baseline values on days 9, 14, 21, and 45 postinfection. There was a significant increase above baseline values on day 21 in the control group (Figure 3).

Total leukocyte counts — Parasitized ponies had significantly greater leukocyte counts, compared with control ponies at all times, except on the day of infection (day 0) and on day 3. Within the parasitized group, there was a significant decrease from baseline values on day 3 and then significant increases above baseline values on days 6,

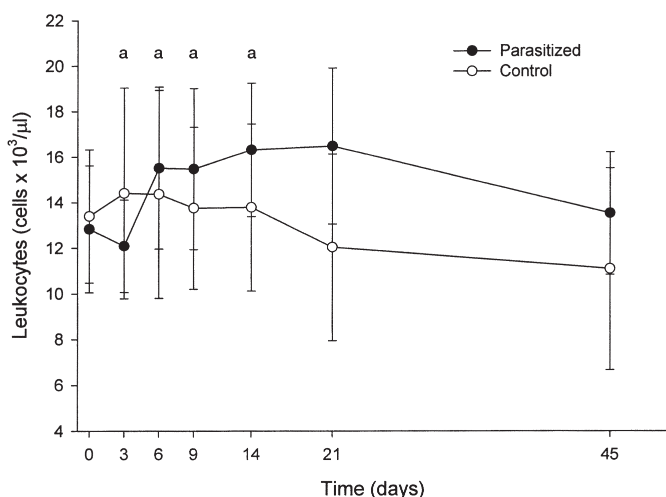

Figure 4 — Mean (± *s*) leukocyte counts in parasitized and control ponies. [a]Significant ($P < 0.05$) differences from baseline within the parasitized group.

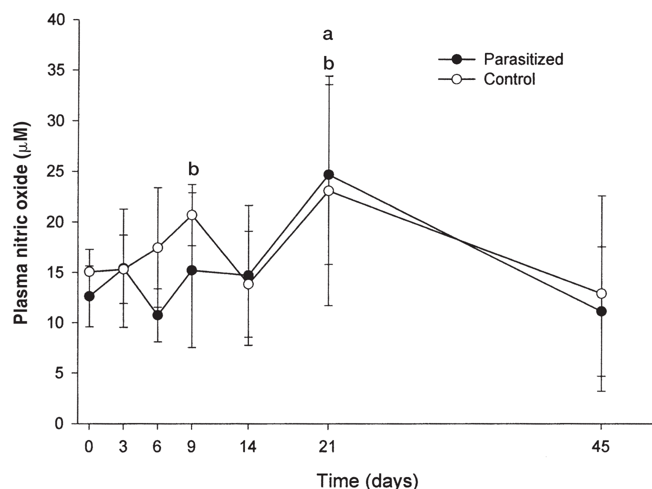

Figure 5 — Mean (± *s*) nitric oxide concentrations in parasitized and control ponies. [a]Significant ($P < 0.05$) differences from baseline within the parasitized group. [a]Significant differences ($P < 0.05$) from baseline within the control group.

9, and 14. There were no significant changes from baseline values for the control ponies (Figure 4).

Segmented neutrophil counts — Parasitized ponies did not have significantly different segmented neutrophil counts compared with control ponies at any time. Within the parasitized group, there was a significant increase above baseline values on day 6 and then returned to baseline values. There were significant decreases from baseline values on days 14, 21, and 45 in the control group.

Lymphocyte counts — Parasitized ponies did not have significantly different lymphocyte counts compared with control ponies at any time. Within the parasitized group, there was a significant decrease from baseline values on days 6 and 9. There were significant increases from baseline values on days 14, 21, and 45 in the control ponies.

Plasma NO concentrations — Parasitized ponies did not have significantly different plasma NO concentrations compared with control ponies at any time. Within the parasitized group, there was a significant increase above baseline values on day 21 (Figure 5).

Cytokine concentrations/activities

Tumor necrosis factor, Interleukin-1, Interleukin-6 — No significant differences were noted between the parasitized and control ponies for these serum cytokine concentrations at any time and there were no significant changes from baseline values within either group across time.

Discussion

The clinical signs exhibited by the parasitized ponies included depression, pyrexia, variable periods of anorexia, and decreased weight gain, and were consistent with characteristic findings subsequent to *S. vulgaris* infection (4,8). Hematologic variables evaluated also provided evidence of an acute inflammatory stimulus. Previous studies involving inoculation of *S. vulgaris* in helminth-naïve ponies had similar hematological changes (4,30,31). Despite the ponies developing clinical signs and hematologic changes associated with an acute phase response, no increases in serum cytokine activities or plasma NO concentrations were observed.

The illustration of a marked inflammatory response to a single 500 L3 innoculum is expected as the initial host response to *S. vulgaris* involves the influx of inflammatory cells into the bowel wall, especially neutrophils, eosinophils, and macrophages (4). These inflammatory cells are capable of generating oxygen free radicals (OFR); NO; leukotrienes; cytokines; and other toxic mediators that are involved in the killing of bacteria, parasites, and other invading microorganisms (32). Substantial evidence suggests that TNF-α initiates an inflammatory cascade resulting in the synthesis and release of additional mediators including interleukins (6) and mononuclear phagocytes in many tissues that are capable of synthesizing TNF (33). Neutrophils or endothelial cells may synthesize IL-1 and IL-6 in response to circulating TNF. Interleukin-1 acts on endothelial cells to induce factors that make up the acute phase response. Macrophages are also stimulated by IL-1, resulting in further synthesis of TNF, which is then capable of potentiating the overall inflammatory cascade. Normally, the effects of TNF are beneficial to the injured host, but prolonged or exaggerated release has been shown to cause injury (33).

However, analysis of serum TNF, IL-1, and IL-6 values did not reveal any significant increases, despite the clinical signs indicative of host tissue injury resulting from an exacerbated immunologic or inflammatory response that would be sufficient to expect the presence of these serum cytokines (33). In previous studies involving the administration of endotoxin in horses using the same bioassay techniques, TNF and IL-6 were shown to peak approximately 1 h after endotoxin administration and immediately decrease back to near baseline levels (TNF) or remain increased for 8 h before returning to baseline (IL-6). Interleukin-1 was not detectable after endotoxin administration (23). In this study, sampling occurred at 3 d intervals and the concentrations of TNF and IL-6 may have peaked and returned to baseline values between samplings, therefore, no increase in cytokines was observed. Alternatively the proinflammatory

cytokine response may not be as profound and detectable systemically in *S. vulgaris* infection compared with endotoxin administration.

The cytokine response to *S. vulgaris* may not result in expression of proinflammatory cytokines, despite the strong inflammatory response. A previous study comparing the humoral and cytokine responses of vaccinated and nonvaccinated ponies to *S. vulgaris* infection revealed that the cytokine response of helminth-naïve ponies was a type II response, with IL-5 and IL-4 being the predominant cytokines expressed (34). These ponies exhibited clinical signs similar to the ponies parasitized in our study. Helminth infections have been shown to be characteristically associated with an increased eosinophil response (35) and IL-5 is required for this response (36); however, the ponies in our study did not develop eosinophilia. It has been postulated that ponies vaccinated against *S. vulgaris* are able to generate a protective response within the intestinal submucosa and larval antigens are cleared via the portal vasculature (37). By blocking the parasites' progression into the intestinal arteriolar system, these ponies did not develop the verminous arterial lesions characteristic of *S. vulgaris* infection (37). However, if the primary defense was mediated within the intestinal wall, then larval killing would result in the local intestinal expression of proinflammatory mediators such as TNF, IL-1, and IL-6. The lack of a measurable increase in these proinflammatory cytokoines in the parasitized ponies in our study and prior studies may be due to the fact that clearance of these mediators is via the portal system, which is less susceptible to the degenerative effects of this cascade (34,37).

In our study, despite demonstrating a severe inflammatory response, the parasitized ponies did not have any significant increase in plasma NO concentration, even though expression of iNOS could be expected. In previous studies of small intestinal tract ischemic injury in the horse, there was immunohistochemical evidence of iNOS expression in leukocytes in the mucosa and submucosa of the small intestine, and plasma NO concentrations were increased in these horses (24). Evidence of severe inflammation of the small intestine was provided in these horses by histological evaluation of the bowel wall (24). Evidence of NO synthesis in horses has been provided in other systems, such as the endothelium of digital arteries (38) and synovial membrane when exposed to IL-1β; however, whether the NOS isoform expressed is iNOS has not been determined (39). Horses with summer pasture associated obstructive pulmonary disease were reported to have a trend toward increased plasma NO concentrations and evidence of significantly increased iNOS immunohistochemical staining in bronchoepithelial cells (40).

In a study where plasma NO was quantified after low-dose endotoxin infusion in horses, the plasma NO concentration did not increase, despite an increase in serum TNF and IL-6 concentrations (23). Possible explanations provided for the lack of an increase were the dose of endotoxin used, insufficiently high or prolonged cytokine expression, and the degradation of samples before analysis. However, the more plausible reason given was that the mechanism and time course of iNOS expression in horses is different from that observed in numerous other species.

There are several possible reasons as to why there was a lack of measurable increase in plasma NO concentration. The inflammatory response may not have been sufficient to initiate the synthesis of mediators, such as TNF and IL-1, capable of inducing iNOS and a subsequent increase in plasma NO concentrations. As NO is a labile, diffusible gas, measurement via the chemiluminescent method involves the reduction of either nitrite (NO_2^-) or nitrate (NO_3^-) from the plasma samples (41,42). Interference in NO measurement could occur during sample collection, processing and storage. Samples were collected, immediately centrifuged, stored in a closed container on ice, and frozen at −70°C within 1 h to minimize light exposure for prolonged periods, which has been reported to degrade nitrites resulting in lower NO concentrations (43). Nitrates were reduced and converted to NO in this study as nitrate is a more stable end metabolic product and its half-life in plasma has been documented in other species to be longer than nitrite (41,42,44). Nitrate is formed by NO reacting with hemoglobin and superoxide anion, whereas nitrite forms when NO reacts with oxygen alone. Therefore, the predominant end metabolite of NO was expected to be nitrate as hemoglobin and superoxide anions were likely to be available in abundance in our model.

Despite the apparent lack of proinflammatory cytokine synthesis in this study, there is still sufficient evidence that further work is warranted to deduce the effector molecules that provide protection against *S. vulgaris*. It is apparent that eosinophils play a role in protection against *S. vulgaris* (34,35,37) and that eosinophils have the potential to express iNOS, thus synthesizing, and releasing NO themselves (45). Eosinophils have also been shown in vitro to kill leishmania parasites when enhanced by IFN-γ; large quantities of nitrite were measured, and this accumulation could be blocked by NOS inhibitors, illustrating a role of NO in parasite killing (46). Additional studies to determine the role of NO and eosinophils in equine helminth protection are warranted.

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