The Mare Reproductive Loss Syndrome (MRLS) and the Eastern Tent Caterpillar: Immunological Testing of Aborting Mares

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ABSTRACT

Mare reproductive loss syndrome (MRLS) describes a condition of equine early-term and late-term fetal losses in which the etiology has not been fully characterized. Involvement of Eastern tent caterpillars (ETC) has been highly considered because oral administration of ETC rapidly induces abortion in pregnant mares. Pathologic lesions observed in placental and fetal tissues of horses with characteristics of MRLS were consistent with bacterial infection. In addition, a small percentage of the naturally affected MRLS horses developed metritis, pericarditis, and/or unilateral uveitis. Therefore, we hypothesized that the MRLS includes an immunosuppressive condition that may predispose the affected horses to infection with opportunistic organisms.

To test this hypothesis, we performed functional immunological testing in blood samples collected before and 7 days after oral administration of irradiated and non-irradiated ETC to pregnant mares involved in an experimental MRLS model. Peripheral blood samples were tested for lymphocyte subpopulation distribution, lymphocyte proliferation, phagocytosis and oxidative burst capacity, opsonic capacity of serum, and serum immunoglobulin isotype levels. Although the administration of non-irradiated and irradiated ETC via nasogastric intubation induced abortion promptly in

pregnant mares, no definitive evidence of an immunosuppressive condition could be observed in this study. Nevertheless, lymphocyte subpopulation distribution and proliferation between control and treated groups warrant further investigation given some trends observed. The evaluation of a potential immunodeficiency factor in MRLS may involve unknown environmental factors and could be more informative when performed under the natural conditions of the disease.

INTRODUCTION

In 2001, an outbreak of equine early-term fetal losses (ETFL, abortions between 40–60 days) and late-term fetal losses (LTFL, abortions of late-term pregnancy, stillborns, compromised foals) affected farms in the states of Kentucky, Ohio, Indiana, and Illinois.[1–4] Because of the epidemiological aspects of this outbreak, this phenomenon became known as the mare reproductive loss syndrome (MRLS). The etiology (e.g., bacterium, virus, toxin, other) of this condition has not been fully characterized to date; however, the involvement of Eastern tent caterpillars (ETC) in wild cherry and other fruit trees has been highly considered based on studies that used nasogastric administration of ETC to pregnant mares.[5–8]

Pathologic lesions observed in placental and fetal tissues of horses with characteristics of MRLS were consistent with bacterial infection. Isolated organisms included nonbeta hemolytic *Streptococcus* spp, alpha *Streptococcus*, *Actinobacillus* spp, and *Serratia*.[9] In addition, the observation that a small percentage of the MRLS-affected horses developed metritis, pericarditis, and/or a unique unilateral uveitis suggests that MRLS factor causes changes in the immune system.[10] Therefore, we hypothesized that the MRLS includes an immunosuppressive condition that may predispose the affected horses to infection with opportunistic organisms. The identification of a potential immunosuppressive condition in affected animals may provide useful information about the pathogenicity of the MRLS factor, and important guidelines for clinical monitoring and treatment.

Recurrent, severe infections caused by pathogenic or opportunistic organisms are typical clinical manifestation in immunodeficiencies. Humoral disorders may be associated with encapsulated organism infections (*Streptococcus* spp, *Staphylococcus* spp, *Mycoplasma* spp); cellular immune disorders may be associated with intracellular pathogens (viruses, fungi, protozoa, mycobacteria); and phagocytic dysfunctions may involve distinct organisms (*Staphylococcus* spp, *Pseudomonas* spp, *Serratia* spp, *Klebsiella* spp, *Aspergillus* spp, or *Candida* spp).[11–13]

Immunodeficiency disorders may be primary (genetically based) or secondary to a disease process (bacterial or viral infection), drug or irradiation therapy, toxins, metabolic disorders, or malnutrition. Immunodeficiency predisposes hosts to infectious disease and should be suspected in recurrent or persistent infections, failure to respond to antimicrobials, and infection with normally nonpathogenic organisms.[14] In immunodeficiency cases, the innate and/or adaptive immune systems may be affected at the humoral and/or cellular level.

Phagocytes play an important role in maintaining the innate host defense against bacterial infections via chemotaxis, adherence, phagocytosis, and intracellular killing. The phagocytosis process is markedly impaired in the absence or in the presence of low concentrations of serum opsonin (complement C3 and antibodies); opsonization capacity is critical in phagocytosis and killing of organisms.[15,16] The lymphocyte subpopulation distribution may be affected in immunodeficiency conditions due to a direct effect (cell death) or an indirect effect (impairment of cell maturation and activity) of the insulting agent. In these cases, lymphocyte subpopulation distribution (percentage of CD4+ T cells, CD8+ T cells, B cells) or cell activity (cell proliferation, cytokine synthesis or antibody production) may be impaired.

Using blood samples from horses involved in an ETC-induced MRLS study model, we measured the phagocytic capacity of peripheral blood phagocytes of control and treated animals, before and after treatment with the ETC.[6] In addition, opsonic capacity was tested in the sera of these animals using the phagocytosis and oxidative burst assay, and by measuring serum immunoglobulin isotypes. The contribution of complement and immunoglobulin was separately measured by testing heat-inactivated and normal sera. The lymphocyte subpopulation distribution was tested by flow cytometric analysis using lymphocyte markers, and lymphocyte activity was tested using proliferation assays. For the latter, normal horse pooled serum or autologous serum was used in an attempt to identify the presence of a potential toxic effect in the serum of the horses in the study. The objective of our study was to use these assays to determine if there was a detectable immunologic change in horses administered irradiated or non-irradiated ETC.

MATERIALS AND METHODS

Horses and the MRLS Model

Eighteen healthy Draft and Standardbred mares with an average age of 10 years (range, 8–12 years) were obtained from a commercial breeder. The mares were diagnosed in the third trimester of pregnancy, identified by numbers, and randomly assigned to 3 separate groups (A, B, and C). Seven days prior to the study, the mares were placed in their experimental stalls to allow for acclimation. Mares were maintained in these stalls throughout the experiment and had no contact with other horses. The feeding regimen consisted of sweet feed and hay, with water ad libitum.

Group A mares (n = 6) received 100 g in 100 mL normal saline of freshly thawed, non-irradiated ETC homogenate by gastric gavage once a day until they aborted (abortions occurred from 32 to 120 hours after treatment). Group B mares (n = 6) received 100 g in 100 mL normal saline of freshly thawed, irradiated ETC homogenate by gastric gavage once a day for 10 days. Irradiation of the caterpillars was performed with 30 kGy according to standardization for destruction of bacteria and viruses.[6] Group C mares (n = 6) were administered 100 mL of normal saline by gastric gavage once a day for 10 days.

Clinical parameters including temperature, heart rate, respiratory rate, mucous membrane color, capillary refill time, appetite, udder distention, and vaginal discharge were monitored twice daily (AM and PM) during the 10 days when ETC were administered, and once daily (AM) thereafter for 10 additional days.

Blood samples were collected aseptically in blood culture tubes (Becton Dickinson, Cockeysville, MD) at 24-hour intervals from all the horses in Groups A, B, and C until the last abortion occurred in Group A (total of 4 samples per horse). The blood samples were cultured for aerobic organisms at the bacteriology division of Livestock Disease Diagnostic Center, University of Kentucky, Lexington, KY. Additional blood samples were collected via jugular venipuncture in vacuum tubes containing heparin or no additives (Becton Dickinson Vacutainer Systems, Rutherford, NJ) on Day 0 (before administration of ETC) and Day 7 of the experiment. The samples were shipped cooled, overnight to Cornell University, College of Veterinary Medicine, Ithaca, NY, for immunological testing. The samples were labeled with the corresponding numbers of the horses, and the key for the group distribution was revealed after all the immunological testing was performed. The experimental protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

Phagocytosis, Oxidative Burst Activity, and Opsonization Assays

To determine phagocytosis and oxidative burst activity (phagox), experimental horse peripheral blood phagocytes were individually isolated by layering whole blood onto

Table 1. Monoclonal antibodies against equine leukocyte antigens used in the flow cytometric analysis

Monoclonal Antibody	Molecular Target	Cell Type	References
ELAW II # 72	CD4	T cells	Lunn et al, 1998
ELAW I # 53	CD5	T cells	Kydd et al, 1994
ELAW I # 12	CD8	T cells	Kydd et al, 1994
ELAW II # 73	CD19-like	B cells	Lunn et al, 1998
B-ly4	CD21	B cells	Mayall et al, 2001
ELAW II # 15	IgA	B cells	Lunn et al, 1998
ELAW II # 7	IgGa	B cells	Lunn et al, 1998
ELAW II # 4	IgGb	B cells	Lunn et al, 1998
ELAW II # 108	IgGc	B cells	Lunn et al, 1998
ELAW II # 11	IgGT	B cells	Lunn et al, 1998
ELAW II # 23	IgM	B cells	Lunn et al, 1998
ELAW I # 45	LFA-1	leukocytes	Kydd et al, 1994
LAW II # 57	MHC class I	all cells	Lunn et al, 1998
ELAW II # 43	MHC class II	APCs	Lunn et al 1998
Negative control	Anti-canine parvovirus	not applicable	Parrish et al, 1982

ELAW I and II = First and Second International Workshop on Equine Leukocyte Antigens; CD = cluster of differentiation; LFA = leukocyte function-associated antigen; MHC = major histocompatibility complex; APC = antigen-presenting cells.

Histopaque-1077 (Sigma Diagnostics, St. Louis, MO), 2 parts blood to 1 part gradient, and washed in calcium- and magnesium-free Hanks balanced salt solution (HBSS; Gibco, Grand Island, NY). Cells were counted and placed on ice until use. Commercially inactivated *Staphylococcus aureus* (Sa) bacteria (Pansorbin; Calbiochem-Novabiochem Corp., La Jolla, CA) were labeled with propidium iodide (PI; Sigma Diagnostics, St. Louis, MO) as described in Flaminio et al.[17] Aliquots of labeled bacteria were opsonized with 40% of pooled normal horse serum or heat-inactivated (65˚C for 30 minutes to destroy complement) serum diluted in HBSS (40%) for 30 minutes in the dark at 37˚C, and subsequently washed in HBSS. A separate aliquot was not treated with serum (non-opsonized control). Peripheral blood leukocytes (2×10^6 cells in 1 mL HBSS) were incubated with either 10 µg/mL of opsonized PI-Sa or non-opsonized PI-Sa for 15 minutes at 37˚C. Dehydrorhodamine 123 (Molecular Probes, Eugene, OR) was added at a final concentration of 3.5 µg/mL in each tube, and further incubated for 15 minutes at 37˚C. For the opsonization assays, bacteria were individually opsonized with autologous serum or heat-inactivated autologous serum from the experimental horses, and phagocytes from a normal control horse were used to test the samples. Analysis was performed using 2-color flow cytometry after the addition of 10 µL Trypan blue (Sigma Diagnostics, St. Louis, MO). Fluorescence signals are displayed as a 2-color dot plot mean fluorescence determined by quadrant statistics.

Lymphocyte Phenotyping

Peripheral blood lymphocytes were isolated from each experimental horse heparinized blood sample using Ficoll gradient centrifugation.[18,19] Isolated cells were analyzed by flow cytometry for lymphocyte antigen markers from a panel of monoclonal antibodies characterized in the First and Second International Workshop on Equine Leukocyte Antigens and elsewhere (Table 1).[20–23] Lymphocytes (10^6/well) were treated with a 20-minute blocking step using 10% normal goat serum in FACS-Buffer (0.01% sodium azide and 0.1% bovine serum albumin in PBS) in a 96-well plate. The cells were washed and resuspended in 50 µL of each primary monoclonal antibody for a 45-minute incubation at 4˚C. The secondary stage used was FITC-conjugated F(ab') fragment goat anti-mouse Ig(H+L) antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 50 µL/well of a 1:200 dilution in the 10% normal goat serum blocking solution. The cells were washed with FACS-Buffer in between

steps. After staining, the cells were fixed with 1% paraformaldehyde in PBS. Samples were analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with a 488-µm argon laser using Cell Quest Analysis software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Leukocyte subpopulations were displayed in a dot plot and gated according to size based on forward light scatter (FSC), and according to granularity based on 90 degree side light scatter (SSC). A region was placed around lymphocytes and data were collected on ten thousand gated cells. Results indicate percent positive cells in the lymphocyte-gated area.

Lymphocyte Proliferation Assay

Isolated peripheral blood lymphocytes were tested in vitro for their proliferation response to mitogenic stimulation.[24] Lymphocytes (6,250, 12,500, and 25,000 cells/well) were cultured in triplicate in RPMI medium (Gibco, Grand Island, NY) with 5% normal horse serum or 5% autologous serum, in the presence or absence of mitogens: pokeweed mitogen (PWM, 2.5 µg/mL final concentration), phytohemagglutinin (PHA, 5 µg/mL), concanavalin A (ConA, 5 µg/mL), or lipopolysaccharide (LPS, 2.5 µg/mL; Sigma Diagnostics, St. Louis, MO). The plates were incubated for 3 days at 37˚C and 5% CO_2. The salt 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; Sigma Diagnostics, St. Louis, MO) was added to each well at 0.5 µg/mL final concentration. Plates were further incubated at 37˚C and 5% CO_2 for 2 hours to allow reduction of the tetrazolium agent by the metabolic active cells. Wells were washed 2 times to removed excess MTT without disturbing the cells. The solubilization of the formazan was achieved by resuspending the cells with 200 µL of isopropanol. Absorbance is proportional to the metabolic and proliferative rate of the cells. Results are expressed as optical density read at 570 nm for a relative proliferation response.

Serum Immunoglobulin Isotypes

For the detection of immunoglobulin isotypes on control and testing horses, ELISA plates were coated with goat anti horse-IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as capture antibodies for the serum immunoglobulins.[25] Serial dilutions (1:10^2 to 1:10^8) of horse serum samples were tested. Murine monoclonal antibodies against equine IgGa, IgGb, IgGc, IgGT, and IgM (Table 1) were used to determine immunoglobulin isotypes. Peroxidase-conjugated goat anti mouse-IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used to detect bound mouse monoclonal antibodies. Results are expressed in optical density read at 450 nm for a relative concentration of immunoglobulins.

Statistical Analysis

Commercially available software (SAS; SAS/STAT Online Doc: version 8, CD-ROM, 2000, SAS Institute Inc., Cary, NC) was used to perform distribution-free analysis. Within each of the 3 treatment groups, pre- and post-treatment samples for each assay were compared using a non-parametric, paired analysis (Wilcoxon Signed Rank Test, SAS, PROC Univariate). Post-treatment samples were compared between all 3 treatment groups using a non-parametric analysis for 3 or more independent samples (Kruskall-Wallis Test, SAS, PROC NPAR1WAY with Wilcoxon option). The alpha value was set at 0.05.

RESULTS

The 6 mares in Group A (non-irradiated ETC) each aborted at experimental hours 32, 32, 46, 48, 84, and 120, respectively. In Group B (irradiated ETC), 3 mares aborted at post-dosing hours 280, 326, and 567. None of the mares aborted in Group C (control). Body temperature, heart rate, respiratory rate, mucous membrane color, capillary refill time, and other clinical signs of all the horses were within normal limits throughout the entire experiment.

Table 2. Differences between pre- and post-treatment values within each group (*P*-value ≥0.03)*

	Lymphocyte Phenotyping	Immunoglobulin Isotypes (autologous serum)	Proliferation Assays	Phagox*	Opsonization
GROUP A (non-irradiated)	↓ IgM, MHC II	IgGc, IgGT	↑ PHA	none	none
GROUP B (irradiated)	↓ B cell, IgM, MHC II	none	↓ PWM, ConA	none	none
GROUP C (placebo)	↓ B cell, IgM, CD21, MHC II; ↑CD2, CD4, CD5	↓IgGa, IgM	↓ConA	none	none

*Phagox: phagocytosis and oxidative burst activity.
Arrows indicate the direction of difference between pre- and post-treatment samples.

Table 3. Differences among Groups A × B × C post-treatment samples*

	Lymphocyte Phenotyping	Immunoglobulin Isotypes	Proliferation Assays	Phagox	Opsonization
Groups A & B vs. C	↑ B cell: 0.006 ↑ IgM: 0.009 ↓ CD4: 0.005	none	↑ PHA: 0.03	none	none

**P*-value shown with data; arrows indicate the direction of differences of Groups A & B in comparison to Group C.

Aerobic culture of the blood collected aseptically from all horses showed no bacterial growth of relevant organisms in any of the groups, before or immediately following abortions. The blood from one mare in Group B cultured positive for *Pantoea agglomerans* on Day 2 of the experiment. Because *P. agglomerans* is a common environmental contaminant, this finding was most likely due to contamination during collection or processing of the blood sample. This same mare aborted on Day 12 of the experiment, and *Streptococcus* spp was isolated from her placenta and aborted fetus. The pathology results are reported elsewhere.[6]

Lymphocyte Phenotyping

This assay characterizes the distribution of lymphocyte subpopulations. Various T (CD2, CD4, CD5, CD8) and B (cz2.1, CD21, IgM) cell markers were used, in addition to markers common to both cell types (MHC class I, MHC class II, LFA-1). Table 2 depicts the statistical difference ($P \leq 0.03$) between pre- and post-treatment samples within each group. In general, there was a decrease in the percentage of B cells in the post-treatment samples of all 3 groups (Figure 1). In Group C, the percentage of CD4+ T cells increased in the post-treatment sample compared with the pre-treatment sample (Figure 1). Table 3 depicts the statistical difference when comparing the post-treatment samples of Groups A, B, and C. Groups A and B revealed lower percentage of B cells and a higher percentage of CD4+ T cells in comparison to Group C.

Lymphocyte Proliferation Assay

This assay tests the response of lymphocytes to stimulation with T (PHA, ConA, PWM) and B cell (PWM, LPS) mitogens. In the comparison between pre- and post-treatment samples within the groups, there was a decrease in the lymphocyte response to ConA in Groups B and C (Table 2). When comparing the post-treatment samples, there was an increase in lymphocyte response to PHA in Groups A and B compared with Group C (Table 3 and Figure 2).

Phagocytosis and Oxidative Burst Activity of Peripheral Blood Neutrophils and Monocytes

This assay characterizes the capacity of neutrophils and monocytes to phagocytose and to activate the intracellular killing mecha-

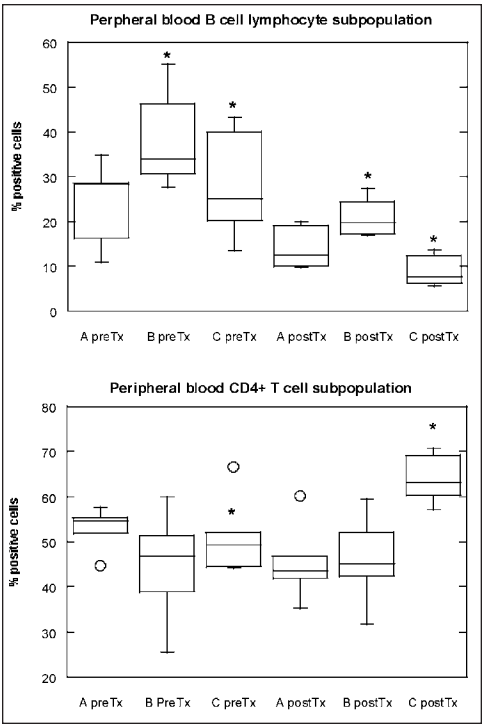

Figure 1. Box plot analysis of peripheral blood B cell and CD4+ T cell percentage distribution in pre- and post-treatment samples: Group A (non-irradiated ETC), Group B (irradiated ETC), and Group C (control). Among post-treatment sample (A × B × C) statistical analysis revealed $P = 0.006$ and $P = 0.005$ for B and CD4+ T cells, respectively. Within the box, the middle line indicates the median of the data; the upper and lower lines indicate the upper and lower quartile values; the bars indicate the lowest and the highest values; circles indicate outliers. $*P \leq 0.03$ between pre- and post-treatment samples within a group.

nisms. The results revealed no differences in the pre- and post-treatment samples within each group (Figure 3). In addition, there were no differences among the post-treatment samples of all groups.

Opsonization Capacity of Autologous Serum and Heat-Inactivated Autologous Serum

This assay tests for the opsonization ("antigen coating") capacity of the serum to facilitate phagocytosis. Opsonization is primarily accomplished by complement and antibodies present in the serum. The results revealed no differences in the pre- and post-

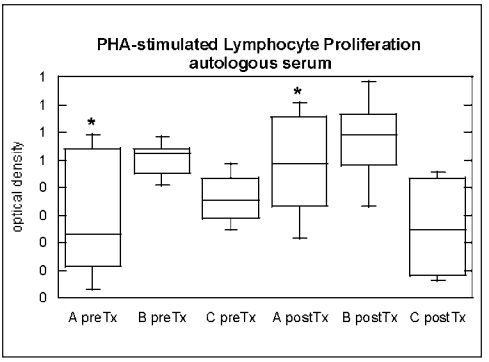

Figure 2. Box plot analysis of PHA-stimulated peripheral blood lymphocyte proliferation in pre- and post-treatment samples: Group A (non-irradiated ETC), Group B (irradiated ETC), and Group C (control). Post-treatment sample (A × B × C) statistical analysis revealed $P = 0.03$. $*P \leq 0.03$ between pre- and post-treatment samples within a group.

treatment samples within each group for both autologous serum and heat-inactivated autologous serum (Table 2 and Figure 3). In addition, there were no differences among the post-treatment samples of all groups for both autologous serum and heat-inactivated autologous serum (Table 3).

Serum Immunoglobulin Isotypes

This assay measured the relative concentration of serum IgGa, IgGb, IgGc, IgGT, and IgM isotypes. There were statistical differences in the pre- and post-treatment samples in Group A (IgGc and IgGT) and Group C (IgGa and IgM) (Table 2). There were no differences among the post-treatment samples of all groups for the different immunoglobulin isotypes (Table 3).

DISCUSSION

The administration of non-irradiated and irradiated ETC via nasogastric intubation induced abortion in pregnant mares. This rapid and reproducible model can be useful in the study of mechanisms of pregnancy losses related to MRLS. Nevertheless, peripheral blood samples from the horses undergoing MRLS type abortions did not show definitive signs of an immunosuppression condition. Although the time of sample collection was in the midst of the abortion

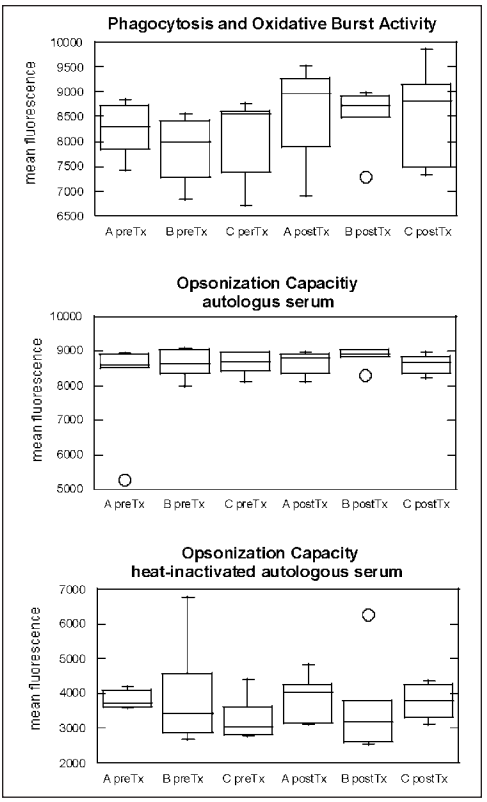

Figure 3. Box plot analysis of peripheral blood phagocytosis, oxidative burst activity, and opsonization capacity in pre- and post-treatment samples: Group A (non-irradiated ETC), Group B (irradiated ETC), and Group C (control). Post-treatment sample (A × B × C) statistical analysis revealed $P \leq 0.05$. $^{*}P < 0.05$ between pre- and post-treatment samples within a group.

events, it is not known if the potential immunodeficiency condition coincides chronologically with this parameter. In addition, the fact that only a small percentage of horses develop pericarditis, metritis, or unilateral uveitis associated with the natural occurrence of MRLS may suggest the involvement of unknown predisposing factors for the manifestation of a clinical immunodeficiency state.

Lymphocyte subpopulation distribution may be altered in immunodeficiency conditions, and this variation can be measured using immunological testing.[18,19,26,27] The peripheral blood lymphocyte phenotyping revealed a change in the lymphocyte subpopulation distribution in all groups between the pre- and post-treatment samples: a statistically significant decrease in the percentage of B cells was detected using 3 different B cell markers. In the control group, the drop in the percentage of B cells was accentuated and was followed by an increase (likely relative) in the CD4+ T cell population. Important inferences from these observations should be highlighted: a) the immune system of horses is dynamic and responds to environmental conditions (i.e., stress involved during adaptation period); and b) pre- and post-treatment evaluations within the same individual and population should be performed when testing for an effect on the immune system to consider these dynamic changes. Although there was a statistical difference between treatment and control groups in post-treatment samples, the drop in the B cell population within the control group should caution a precipitated conclusion that ETC promotes an immune system effect by altering the B cell and CD4+ T cell subpopulation distributions. Additional studies would be necessary to confirm this possibility.

Similarly to the lymphocyte phenotyping, there was a decrease in the response to ConA mitogen in the post-treatment samples of all groups, with statistical significance for the group treated with irradiated ETC and the control group. Isolated peripheral blood lymphocytes (T plus B cells) were stimulated with different mitogens. Concanavalin A is a mitogen that stimulates primarily T cells with some effect on B cells and monocytes. Pokeweed mitogen also has an effect on both T and B cells in horses. The decrease in the post-treatment B cell percentage may have contributed to the decrease in the proliferative response to these mitogens. In contrast, PHA stimulates T cells. Although there was a significant increase in the proliferative response of lymphocytes to PHA in the non-irradiated group, there was not a concomitant increase in the T cell population. T cells comprise the majority of the lymphocyte subpopula-

tion in physiological conditions, and T cells from normal horses often respond robustly to this mitogen.

Neutrophils and monocytes share common oxygen-independent (proteases, anti-bacterial peptides) and oxygen-dependent mechanisms of microbial killing termed the respiratory burst or oxidative burst activity.[28] The study of phagocytic function has been used in the assessment of chronic bacterial diseases in horses and other species under immunosuppressive conditions, including exercise-induced stress, septicemia, and infections caused by *Serratia* spp.[29–32] In addition, we have previously used the opsonization assay with autologous serum to test age-dependent capacity of foal serum to opsonize bacteria for phagocytosis and oxidative burst activity.[33] The phagocytosis and oxidative burst activity of pre- and post-treatment samples within each group were essentially the same. In addition, there were no differences among the post-treatment samples of all 3 groups. The opsonization capacity of the serum of the horses was also tested individually and no differences were detected. This finding was supported by the absence of changes in the relative concentration of serum immunoglobulin isotypes. Taken together, these results suggest that phagocytic function and serum opsonization capacity remained intact despite the ETC treatment.

Immunological testing of peripheral blood samples has been useful in the characterization of immunodeficiencies in isolated clinical cases. However, analysis of peripheral blood may not completely reflect the activity of secondary lymphoid tissues (i.e., lymph nodes, spleen), detect minor abnormalities in the immune system, and may require precise timing of sample collection due to dynamic characteristics of disease and immune response. Therefore, immune dysfunction may not be ruled out by normal blood parameters exclusively. The evaluation of a potential immunodeficiency condition in the MRLS could be more informative when performed under the natural conditions of the disease, in particular in horses that develop the systemic disease. Based on the results observed in this study, re-evaluation of peripheral blood lymphocyte subpopulation distribution and response to mitogens would be advised.

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