Evaluation of polymyxin B in an ex vivo model of endotoxemia in horses

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Objective— To evaluate effects of polymyxin B sulfate (PMB) on response of horses to endotoxin, using an ex vivo model.

Animals—8 healthy horses.

Procedure—In a crossover design, 3 doses of PMB (100, 1,000, and 10,000 U/kg of body weight) and physiologic saline solution (control) were evaluated. Prior to and for 24 hours after administration of PMB, blood samples were collected into heparinized tubes for use in 2 assays. For the endotoxin-induced tumor necrosis factor (TNF) assay, blood samples were incubated (37 C for 4 h) with 1 ng of *Escherichia coli* or *Salmonella* Typhimurium endotoxin/ml of blood. Plasma was harvested and assayed. For the residual endotoxin activity assay, plasma was collected into sterile endotoxin-free borosilicate tubes, diluted 1:10 with pyrogen-free water, and incubated for 10 minutes at 70 C. *Escherichia coli* endotoxin (0.1 or 1 ng/ml of plasma) was added to the thawed samples prior to performing the limulus ameobocyte lysate assay. Serum creatinine concentrations were monitored for 1 week.

Results—Compared with baseline values, PMB caused a significant dose- and time- dependent decrease in endotoxin-induced TNF activity. Compared with baseline values, residual endotoxin activity was significantly reduced after administration of 10,000 U of PMB/kg. Compared with baseline values, 1,000 and 5,000 U of PMB/kg should inhibit 75% of endotoxin-induced TNF activity for 3 and 12 hours, respectively. Serum creatinine concentrations remained within the reference range.

Conclusion and Clinical Relevance—Results of the study suggest that PMB is a safe, effective inhibitor of endotoxin-induced inflammation in healthy horses. (*Am J Vet Res* 2001; 62:72–76)

Several common diseases of horses such as septicemia in neonates, colic, peritonitis, and pleuropneumonia are complicated by endotoxemia.[1-3] Lipopolysaccharide (endotoxin) in the systemic circulation of horses with these diseases stimulates the synthesis of multiple endogenous mediators, which may lead to fatal sequelae such as disseminated intravascular coagulopathy and cardiovascular collapse. Specific treatments for horses with endotoxemia include administration of flunixin meglumine, antiendotoxin antibodies, and more recently, **polymyxin B (PMB)**.[3]

Benefits of flunixin meglumine in the treatment of horses with endotoxemia have been described.[4] However, this treatment is limited, because it affects only 1 branch of the endotoxin-induced inflammatory cascade (ie, prostanoid synthesis). Flunixin meglumine does not exert a direct effect on the lipopolysaccharide molecule or its ability to bind to inflammatory cells.

A second specific treatment option is administration of pooled, antibody-rich plasma or serum harvested from donor horses vaccinated with endotoxin from mutant *Salmonella* spp or *Escherichia coli*.[3] Because of the expense involved in preparing these antibodies, they can be costly. Additionally, acute hypersensitivity reactions are possible following administration of these antibodies.

Polymyxin B, an antibiotic with efficacy against gram-negative bacteria, has been investigated as a treatment for animals with endotoxemia. The ability of PMB to bind the lipid A component of an endotoxin molecule is widely known.[5-9] Polymyxin B neutralizes lipopolysaccharide and alters the release of mediators and the response of target cells.[7,8,10] However, concerns about nephrotoxicosis and neurotoxicosis have limited the use of PMB.[9,10] In a study to evaluate the efficacy of PMB at 3 doses (3,600, 18,000, and 36,000 U/kg of body weight, IV, q 6 h for 48 hours) in horses subjected to grain overload, investigators reported minimal benefit in prevention of laminitis and severe signs of neurotoxicosis.[10] In a more recent investigation in foals, scientists reported that 6,000 U of PMB/kg was more effective than antiendotoxin antibodies for the treatment of horses with endotoxemia.[11] Furthermore, the foals of that study did not have signs of neurotoxicosis or nephrotoxicosis. The objective of the study reported here was to investigate the pharmacodynamic properties of PMB in an ex vivo model of endotoxemia in adult horses to determine whether safe, effective doses of PMB can be identified.

Materials and Methods

Animals—Eight healthy light-breed horses that ranged between 334 and 544 kg were used in the study. Each horse was administered 3 doses of PMB. Between treatments, horses were maintained on pasture for a 3-week wash-out period. In preparation for collections, all horses were transported to our research facility, housed in box stalls, and fed coastal Bermuda hay. Water was provided ad libidum.

Treatments and sample collection—Using a crossover design, we evaluated 3 doses of PMB sulfate[a] in an ex vivo whole-blood model of endotoxemia. In random order, each horse received 1 of 4 treatments as a bolus injection (100, 1,000, and 10,000 units of PMB/kg of body weight and an

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equal volume [10 ml] of sterile saline [0.9% NaCl] solution). All blood samples were collected percutaneously from a jugular vein, using sterile techniques. Blood samples were placed into tubes containing heparin (10 U/ml of blood). Two blood samples were collected prior to administration of each treatment. Additional samples were collected 0.25, 0.5, 1, 2, 3, 6, 12, and 24 hours after administration of PMB or saline solution. The ability of PMB to neutralize endotoxin was investigated by 2 methods: quantification of ex vivo endotoxin-induced activity of **tumor necrosis factor (TNF)** and quantification of residual endotoxin activity after ex vivo addition of endotoxin to plasma samples.

Ex vivo endotoxin-induced activity of TNF—For each collection time, duplicate heparinized blood samples (4.5 ml in sterile 15-ml polypropylene conical screw top tubes) were treated with endotoxin (500 uµl of a solution containing 10 ng/ml) derived from *Salmonella* Typhimurium or *E coli* (O55:B5).[b] Samples were incubated on a rocker at 37 C for 4 hours. After incubation, 250 uµl of 100 m*M* EDTA/10 uµmol of meclofenamate was added to each tube, and the plasma was collected and stored at –70 C. Activity of TNF in plasma was determined by use of the WEHI cell bioassay.[12] Human recombinant TNF was used as the assay standard.[c] All plasma samples assayed were diluted 10-fold with RPMI 1640 media[d] prior to use in the assay. One unit of TNF activity per milliliter was defined as the dilution that produced death in 50% of the cells.

Quantification of residual endotoxin activity

Heat stability of PMB—Preliminary use of an assay was conducted to evaluate heat stability of PMB in diluted plasma samples. This was performed prior to the limulus ameobocyte lysate assay in the ex vivo portion of the study, because all samples for the limulus ameobocyte lysate assay must be heat inactivated prior to analysis. Blood samples from a healthy adult horse were collected into heparinized tubes and centrifuged, and the platelet-rich plasma was harvested. Saline solution or PMB was added to aliquots of the plasma to achieve final concentrations of 0, 0.5, 5, and 50 U PMB/ml of plasma. Plasma was diluted 1:10 with pyrogen-free water and heated for 10 minutes at 70 C. To test for heat stability of PMB, saline solution or PMB also was added to aliquots of plasma that had been diluted 1:10 and heat-treated. *Escherichia coli* O55:B5 endotoxin was added to each of the diluted, heated plasma samples to achieve a final concentration of 1 ng of endotoxin/ml. Endotoxin activity was determined, using the chromogenic limulus ameobocyte lysate microtiter plate assay.[e] Each sample was compared against blanks containing reagents and plasma appropriately diluted with reagent-grade pyrogen-free water.

Ex vivo study—For each collection time, blood samples were collected into sterile, pyrogen-free borosilicate tubes containing heparin. Samples were centrifuged and platelet-rich plasma harvested. Plasma samples were diluted 1:10 in sterile pyrogen-free water. Samples were incubated for 10 minutes at 70 C and subsequently frozen at –4 C. On days when samples were assayed, plasma samples were thawed, and *E coli* O55:B5 endotoxin (final concentrations of 0.1 and 1 ng/ml of plasma) was added to each sample. Endotoxin activity was determined, using the chromogenic limulus ameobocyte lysate microtiter plate assay.[13,14] Each sample was compared against blanks containing reagents and plasma appropriately diluted with reagent-grade pyrogen-free water.

Toxic effects—Blood samples were collected prior to and on days 0, 1, 3, and 7 after administration of PMB. Samples were analyzed for serum creatinine concentration to assess nephrotoxicosis. Horses also were monitored for evidence of abnormal neurologic signs.

Statistical analysis—Statistical analysis of the results was performed, using an ANOVA. The TNF data were log transformed prior to analysis. Comparisons of the log of TNF activity were made between the sources of endotoxin (*E coli and Salmonella* Typhimurium), doses of PMB and saline solution, collection times, and order of treatments. Similarly, residual endotoxin concentration was compared between doses of PMB, dose of endotoxin (0.1 or 1 ng/ml), and collection times. Means of interest were compared, using the Scheffe F test. Inhibition of *E coli*-induced TNF activity, as a percentage of the baseline value, was calculated for each dose of PMB. Linear regression analyses of percentage of inhibition of *E coli* endotoxin-induced TNF activity versus log of dose were calculated at 3, 6, and 12 hours after administration of PMB. Endotoxin data were analyzed, using an ANOVA. Means of interest were analyzed by use of paired *t*-tests. Values of $P < 0.05$ were considered to be significant.

Results

Ex vivo endotoxin-induced activity of TNF—Order of treatments did not significantly affect TNF activity. The TNF activity was not significantly different on the basis of source of endotoxin, although TNF activity induced when *E coli* was used as a source of endotoxin (**Fig 1**) was less than when *Salmonella* Typhimurium was used as a source of endotoxin (**Fig 2**). Compared with baseline values (ie, before administration of PMB or saline solution), administration of a bolus of saline solution did not significantly alter endotoxin-induced TNF activity over time (data not shown). Compared with baseline values, IV administration of a bolus of PMB significantly decreased ex vivo endotoxin-induced TNF activity. The effect of PMB was dependent on dose of PMB and time after administration of PMB. Compared with baseline values, 100 U of PMB/kg significantly inhibited *Salmonella* Typhimurium endotoxin- and *E coli* endotoxin-induced TNF activity for 1 and 3 hours, respectively. Compared with baseline values, 1,000 U of PMB/kg significantly inhibited *Salmonella*

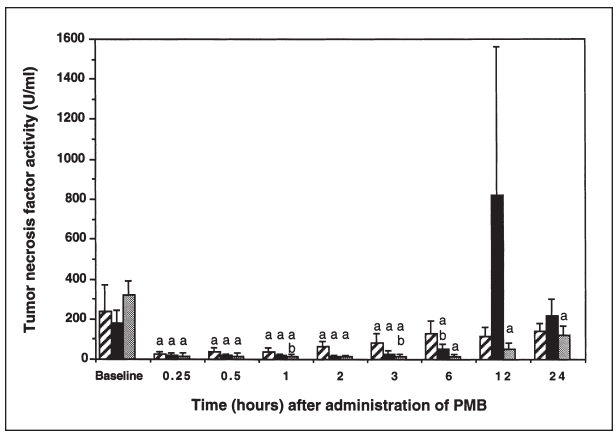

Figure 1—Activity of tumor necrosis factor (mean ± SE) in plasma samples determined after ex vivo addition of 1 ng of *Escherichia coli* endotoxin/ml of blood obtained from healthy horses that had been administered doses of polymyxin B (PMB), IV, at rates of 100 U of PMB/kg (striped bar), 1,000 U of PMB/kg (solid bar), and 10,000 U of PMB/kg (shaded bar). a = Significantly different ($P < 0.05$) from baseline value before administration of PMB. b = Significantly different($P < 0.05$) from values for 100 U of PMB/kg. c = Significantly different ($P < 0.05$) from values for 1,000 U of PMB/kg.

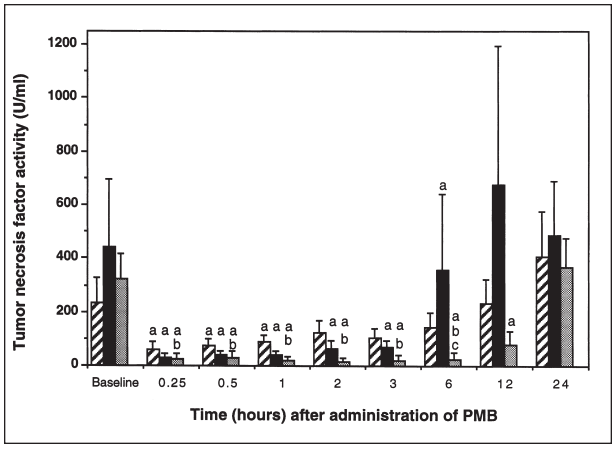

Figure 2—Activity of tumor necrosis factor (mean ± SE) in plasma samples determined after ex vivo addition of 1 ng of *Salmonella* Typhimurin endotoxin/ml of blood obtained from healthy horses that had been administered doses of PMB, IV, at rates of 100 U of PMB/kg (striped bar), 1,000 U of PMB/kg (solid bar), and 10,000 U of PMB/kg (shaded bar). *See* Figure 1 for key.

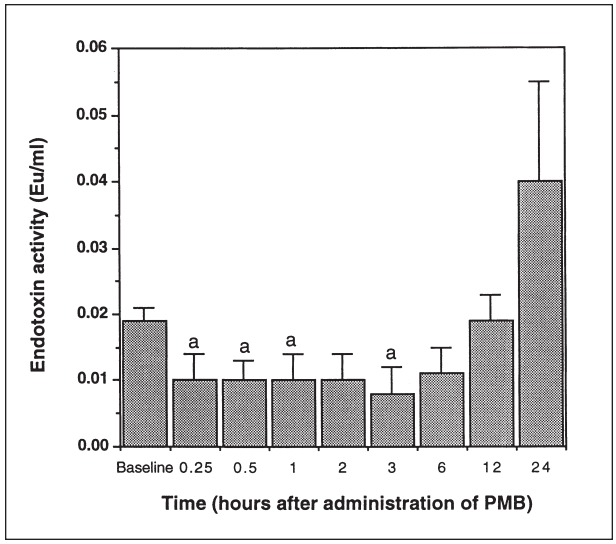

Figure 3—Endotoxin activity (mean ± SE) in plasma samples determined after ex vivo addition of 0.1 ng of *E. coli* endotoxin/ml of plasma obtained from healthy horses that had been administered doses of PMB at a rate of 10,000 U of PMB/kg of body weight, IV. a = Significantly different ($P < 0.05$) from baseline value before administration of PMB. Eu = Endotoxin units.

Typhimurium endotoxin- and *E coli* endotoxin-induced TNF activity for 6 hours. Compared with baseline values, 10,000 U of PMB/kg significantly inhibited *Salmonella* Typhimurium endotoxin- and *E. coli* endotoxin-induced TNF activity for 12 hours.

Linear regression analysis for samples obtained 3, 6, and 12 hours after administration of PMB indicated a strong association between dose of PMB and percentage inhibition of ex vivo endotoxin-induced TNF activity. At least 75% of the *E coli* endotoxin-induced TNF activity was inhibited for 3 and 12 hours after IV administration of approximately 1,100 and 5,200 U of PMB/kg, respectively.

Quantification of residual endotoxin activity

Heat stability of PMB—We did not detect a difference in residual endotoxin activity when values for plasma samples to which PMB was added prior to dilution and heat treatment were compared with values for plasma samples to which PMB was added after dilution and heat treatment (**Table 1**). Compared with plasma samples to which saline solution was added as a control vehicle, PMB in plasma reduced endotoxin activity in a dose-related manner.

Ex vivo study—Compared with baseline values, endotoxin activity for horses administered PMB at a rate of 10,000 U of PMB/kg significantly decreased over time for samples to which 0.1 ng of endotoxin/ml of plasma was added (**Fig 3**). Significant differences were not detected in endotoxin activity for horses administered PMB at a rate of 10,000 U of PBM/kg for samples to which 1 ng of endotoxin/ml of plasma was added or for horses administered PMB at a rate of 100 or 1,000 U of PMB/kg for samples to which 0.1 or 1 ng of endotoxin/ml of plasma was added ex vivo (data not shown).

Toxic effects—All measured serum creatinine values remained within the reference range. None of the horses in the study developed abnormal neurologic signs.

Discussion

Beneficial effects of PMB for the treatment of animals with endotoxemia have been documented in rats, dogs, goats, foals and humans.[8,9,11,15-17] In the study reported here, ex vivo endotoxin-induced TNF activity was decreased in a time- and dose-dependent manner after in vivo administration of a bolus of PMB to healthy horses. A similar dose-dependent response to PMB treatment was documented in human neutrophils with endotoxin-induced chemotaxis.[7] Such findings are attributable to the ability of PMB to bind to the

Table 1—Endotoxin activity (units/ml) in plasma samples containing polymyxin B (PMB) to evaluate heat stability of PMB and effectiveness in reducing endotoxin activity, as measured in the limulus ameobocyte lysate assay

	Concentration of PMB (Units of PMB/ml of plasma)			
Variable	0.0 (Saline)*	0.05	0.5	5.0
PMB added to plasma before dilution and heating	0.06	0.04	0.03	0.02
PMB added to plasma after dilution and heating	0.06	0.04	0.03	0.01

*Saline = 0.9% NaCl solution.

lipid A portion of the lipopolysaccharide molecule, thereby altering the 3-dimensional confirmation of the lipopolysaccharide molecule. This conformational change possibly inhibits the PMB-endotoxin molecule from binding to the receptor (CD 14) on monocytes, which inhibits release of inflammatory mediators such as TNF.[8,9] The binding of PMB to the outer membrane of target leukocytes can provide residual protection against endotoxin.[8,9] Because the ratio of binding is 1 molecule of PMB to 1 molecule of lipopolysaccharide, a dose-dependent response would be expected.[7] Therefore, the higher the concentration of endotoxin, the more PMB molecules that would be required for neutralization. Although an ex vivo model of endotoxemia was used in the study reported here, the concentration of endotoxin was approximately 5 times the mean plasma concentration of endotoxin reported in adult horses with naturally acquired acute gastrointestinal disease and foals with septicemia.[13,14] In the study reported here, depending on the dose of PMB administered, a single dose of PMB could effectively block a high concentration of endotoxin for several hours ex vivo. Given these facts, it would seem reasonable to assume that equivalent doses of PMB would provide similar benefit in vivo to horses with endotoxemia. Keeping in mind the limitations of the model used in this study, the ability of PMB to effectively block endotoxin in vivo, assuming endotoxin is in the blood prior to administration of PMB, remains to be determined.

In the study reported here, the ability of PMB to significantly reduce the ex vivo expression of endotoxin-induced TNF activity was clearly documented, supporting the use of this drug for treatment of horses with endotoxemia. However, the ex vivo residual endotoxin assay revealed the persistence of endotoxin activity in plasma samples unless PMB was administered at a rate of 10,000 U of PMB/kg. There were 3 fundamental differences between the endotoxin-induced TNF assay and the residual endotoxin activity assay: type of sample (blood vs plasma), heat inactivation, and dilution. Endotoxin was added ex vivo to blood samples for the TNF activity assay, whereas plasma samples were used in the residual endotoxin assay. Because PMB is lipophilic, the dramatic reduction in endotoxin-induced TNF activity may reflect the fact that most of the administered PMB was bound to the cells prior to the collection of blood and subsequent ex vivo exposure to endotoxin. Hence, when given in vivo, PMB would more effectively bind endotoxin in an assay that used a whole-blood assay, compared with an assay that used plasma. Furthermore, a 1:10 dilution and heat inactivation of plasma were required for the residual endotoxin activity protocol. Dilution of the plasma samples also could have sufficiently diluted the PMB concentration to less than that needed to effectively bind the added endotoxin. The PMB was heat stable in plasma samples. Clearly, it appears that PMB was stable with the heat treatment, and its effectiveness in reducing detectable endotoxin activity detected by the limulus ameobocyte lysate assay was dependent on the concentration of PMB in the plasma.

Adverse effects of neurotoxicosis and nephrotoxicosis caused by PMB in 1 study of horses[10] were not observed in our study. Although serum creatinine concentration is a crude test for subclinical nephrotoxicosis,[18] in the study reported here, serum creatinine concentrations were maintained within the reference range when a single bolus of PMB at a rate of up to 10,000 U of PMB/kg was administered IV to healthy horses. This finding is in agreement with the report that healthy foals also maintained serum creatinine concentrations within the reference range when administered a single bolus at a rate of 6,000 U of PMB/kg, IV.[11] In the study reported here, doses administered at rates as low as 500 U of PMB/kg inhibited 50% of the endotoxin-induced TNF activity for 12 hours, suggesting that even low doses of PMB could potentially provide some benefit during endotoxemia. Theoretically, these lower doses of PMB would be less likely to cause renal toxicosis, even in critically ill animals. However, PMB should be used cautiously in hypovolemic or azotemic animals, especially when other potentially nephrotoxic drugs are administered concurrently.

In horses, endotoxemia is a complication of many disease processes that affect neonates and adults. Because of its extensive clinical importance and influence on prognosis and morbidity, the search for an effective and economically acceptable treatment for animals with endotoxemia is of paramount concern. On the basis of results of this study, PMB has potential clinical use as a treatment for animals with endotoxemia. Doses at rates between 1,000 and 5,000 U/kg administered at 12-hour intervals could safely and effectively decrease the expression of endotoxin-induced TNF activity. Polymyxin B could theoretically neutralize all or most of the endotoxin, leaving a smaller quantity of endotoxin to stimulate a more controlled and appropriate immune response. Polymyxin B could offer an easier-to-use, more effective, and less expensive treatment alternative to other drugs that bind endotoxin, such as antiendotoxin antibodies. It must be mentioned that all of the horses in the study reported here were healthy. In ill horses, PMB should be used at the lowest possible therapeutic dose with careful monitoring of renal function during treatment.

[a]Polymyxin B, Bedford Laboratories, Bedford, Ohio.
[b]Endotoxin (*Salmonella* Typhimurium and *Escherichia coli*) List Biological Laboratories Inc, Campbell, Calif.
[c]Human Recombinant TNF Genzyme, Boston, Mass.
[d]RPMI 1640 media, Bio Whitaker, Walkersville, Md.
[e]QCL-1000 Chromogenic LAL, Bio Whitaker, Walkersville, Md.

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