Protection Against Clinical Endotoxemia in Horses by Using Plasma Containing Antibody to an Rc Mutant E. coli (J5)

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Thirty-two horses with clinicopathologic evidence of endotoxic shock were randomly selected for a double-blind trial of hyperimmune lipopolysaccharide (LPS) core antigen plasma. Horses were suffering from acute toxic enteritis (n=13), 360° volvulus of the large colon (n=9), proximal jejunitis/duodenitis (n=6), or strangulating obstruction of the small intestine (n=2). Plasma was harvested from suitable equine plasma donors (preimmune plasma) and horses were immunized with a whole-cell bacterin of an Rc mutant E. coli (J5). Plasma was again harvested from these horses when IgG ELISA titers recognizing LPS core antigen were ≥1:32,000. All horses included in the trial received either preimmune or hyperimmune plasma in addition to traditional therapy (fluids, antimicrobials, antiinflammatory agents, etc.) as dictated by the attending clinician. The mortality rate in the group of horses receiving hyperimmune plasma was 13%; in the control group receiving preimmune plasma, the mortality rate was 47% (P=.045). Horses receiving J5 hyperimmune plasma had a significantly improved clinical appearance 48 hours after plasma administration (P<.05) and a shorter period to recovery than control horses (P=.069).

Key words: endotoxin, gram-negative core antigen, passive immunotherapy, equine colic, plasma transfusion

INTRODUCTION

Gram-negative septicemia and endotoxic shock associated with equine diseases such as acute toxic enteritis and strangulating obstructions of the intestine are costly problems. A high mortality rate occurs despite vigorous antimicrobial therapy due to the inability to counteract the lethal effects of bacterial endotoxins once cascades of immunologic mediators are fully initiated [1–6]. The frequent sequelae to disease,

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including laminitis, venous thrombosis, or multiple organ failure, add to economic losses [6].

Endotoxin, also referred to as lipopolysaccharide (LPS), is a cell wall component of gram-negative bacteria and present in the intestinal contents of normal animals. Endotoxin is released by bacterial multiplication or bacteriolysis [2] and is absorbed into the circulation when the intestinal mucosal barrier is disrupted by intestinal obstruction, ischemia, or enteritis [3,7–9]. Gram-negative septicemia, secondary to inadequate colostral absorption or immunosuppression, offers another means for endotoxin to access circulation [6]. When a critical amount is absorbed or released, a shock state is produced, which includes tachycardia, reduced pulse pressure, prolonged capillary refill time, injected mucous membranes, cold extremities, and abdominal discomfort. Laboratory data observed with endotoxemia reveal numerous alterations, including hemoconcentration, leukopenia due to neutropenia and lymphopenia, hyperglycemia followed by hypoglycemia, elevated hepatic enzymes, elevated blood lactate, and coagulopathy suggestive of disseminated intravascular coagulation (DIC) [1–3,5]. Treatment for gram-negative sepsis and endotoxemia is costly in large animals and includes intense fluid administration, antimicrobials, corticosteroids, nonsteroidal antiinflammatory drugs, and other therapeutic agents [3,5,6].

A recent approach to therapy of endotoxic shock is the administration of plasma or serum products containing antibody directed toward endotoxins. Passive administration of antisera to a genetically stable rough mutant of *E. coli* 011:B4 (J5) [10,11] and of polyclonal antisera against LPS [12] has been shown to improve the survival rate of humans suffering from gram-negative bacteremia and endotoxemic shock. J5 is a mutant strain of *E. coli* which lacks specific oligosaccharide side chains, so the core polysaccharide, which is similar among gram-negative bacteria, is exposed. Antibody to these core polysaccharides may thus be cross protective, binding to many gram-negative organisms or endotoxins [13–15]. The host immune reaction to endotoxin is believed to be an important determinant of mortality in diseases such as acute toxic enteritis and strangulating obstructions of the large and small intestine [3,5,6]. Previous studies utilizing antiendotoxin antibody in horses with clinical evidence of endotoxemia reported decreased mortality and recovery periods [16,17]. However, some studies in horses failed to show protection of LPS core antigen antibody against endotoxin challenge [18] or neonatal septicemia [19]. While the protective effects of LPS antibody are still debated [18–26], strong evidence exists for their application in therapy for gram-negative disease. The purpose of this study was to perform a controlled clinical trial to determine if hyperimmune plasma containing antibody to gram-negative core antigens would reduce mortality in horses with clinical and laboratory evidence of gram-negative septicemia or endotoxia.

**MATERIALS AND METHODS**

**Preparation of Plasma**

Three healthy horses were selected as plasma donors based on blood typing and absence of alloantibody.¹ All donors were negative for A, C, and Q red blood cell

¹Serology Laboratory, University of California, Davis.
antigens, antierythrocyte antibodies, and equine infectious anemia. Horses were dewormed every 3 months and were vaccinated every 6 months against eastern and western encephalomyelitis, tetanus, and equine influenza A1 and A2. Donors were monitored for anemia by hematocrit determination prior to collection, and volume was replaced with lactated Ringer’s solution. Eight liters of blood was collected aseptically at approximately 30-day intervals. Sterile acid citrate dextrose (10%) was used as the anticoagulant and plasma was harvested after centrifugation (221 G for 40 minutes). All plasma was collected in 1 L plastic bags,² coded by number, and stored at −20°C. Once sufficient plasma was banked to serve as preimmune (control) plasma, the horses were immunized with a heat-killed *E. coli* J5 bacterin with incomplete Freund’s as adjuvant. The *E. coli* J5 bacterin was prepared as described by Ziegler et al. [10]. Horses were immunized intramuscularly with 3.5 ml of J5 (5 × 10⁹ CFU/ml) mixed with 1 ml of Freund’s incomplete adjuvant. Plasma was harvested when the anti-J5 IgG ELISA titer was ≥1:32,000. Horses were boosted every 30 days with bacterin to maintain elevated titers. J5 antibody titers were measured prior to all collections. The number of immunizations required to reach a titer of ≥1:32,000 was variable between donors, usually requiring at least four injections at 10-day intervals and two injections at monthly intervals. All preimmune plasma had an anti-J5 IgG titer of <1:500; the geometric mean IgG titer of hyperimmune plasma units was 1:41,499. All plasma batches (approx. 5 L plasma/harvest) were coded with a three-digit number picked at random and recorded by a research assistant. Horses were administered plasma from one code number. The plasma was stored in a freezer accessible to the large-animal clinicians. Horses were not preassigned to treatment groups; the selection was arbitrary based upon the coded plasma selected for the patient. Neither the clinicians or the investigators knew if the plasma was hyperimmune or control until the end of the trial. Equal amounts of hyperimmune and control plasma were harvested. The trial was terminated when the plasma stores were exhausted. No attempts were made to achieve equal division of hyperimmune and control plasma between disease groups.

**Serology**

An indirect enzyme-linked immunosorbent assay (ELISA) was used to determine anti-J5 IgG antibody activity [38]. In brief, 96-well flat-bottom microtiter plates³ were coated with heat-killed, washed whole-cell *E. coli* strain J5 (100 μl/well) that had been adjusted spectrophotometrically with 0.9% sterile saline to an optical density of 0.854 at 610 nm. Plates were incubated overnight at 37°C, washed, and sequentially incubated with 100 μl of diluted test plasma, conjugate (peroxidase-labelled antiequine IgG⁴), and substrate (40 mM ABTS, 0.05 M citrate buffer, 0.5 M H₂O₂). Stopping reagent (5 mM Na azide) was added and plates were read immediately with an automated plate reader.⁵ All samples were processed in triplicate with titers defined as the reciprocal of the highest dilution in which a

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²Transfer pack unit with coupler, Fenwal Laboratories, Deerfield, IL.

³Falcon Probind 3916, Becton Dickenson Labware, Oxnard, CA.

⁴Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD.

⁵Dynatech Laboratories, Alexandria, VA.
minimum of two of three identical wells exceeded 0.05 optical density after blanking on the arithmetic mean of eight control wells with no serum.

**Horses Chosen for Study**

All horses were clinical cases at the Veterinary Medical Teaching Hospital, University of California, Davis. All horses were hospitalized in the Large Animal Intensive Care Unit (ICU) or isolation facility with 24-hour supervision. Adult horses (>1 year age) were considered suitable for the clinical trial if there was owner consent and they met the following criteria: horses had to be severely ill with acute onset of severe diarrhea, proximal jejunitis/duodenitis, or strangulating obstruction of the intestine, and a high suspicion of gram-negative septicemia or endotoxemia. The diagnosis of proximal jejunitis/duodenitis was made either at exploratory laparotomy or based upon the clinical findings of abdominal pain, continuous gastric reflux for >24 hours, and results of rectal palpation. In addition, all horses had to display signs of acute toxemia or shock (altered mucous membrane color, increase in capillary refill time, tachycardia with weak pulse pressure, weakness, depression [i.e., lethargy, unresponsive to surroundings], cold extremities) to be included in the study. All nonsurviving horses had complete necropsies performed to confirm diagnoses. Each horse was administered either preimmune control plasma or hyperimmune plasma, intravenously, over 1–2 hours. Plasma was warmed to body temperature and administered through a blood filtration system. Horses weighing <250 kg received 1 L, while horses over 250 kg received 2 L. All plasma was coded by a research assistant, and neither the investigators or the attending clinicians knew whether J5 hyperimmune plasma or preimmune control plasma had been given. All plasma was stored at −20°C in a freezer accessible to hospital clinicians. Clinicians were asked to select the coded plasma and record the code number on the patient’s record. If additional plasma was needed to correct hypoproteinemia, normal plasma was administered from the hospital plasma bank. All horses received conventional therapy as dictated by attending clinicians. All horses received nonsteroidal antiinflammatory drugs, parenteral antimicrobials, and intravenous crystalloid supportive therapy. In addition, a reasonable commitment to support the patient following plasma administration was made. If euthanasia was performed, it was for humane reasons only (severe laminitis with uncontrollable pain or if death was imminent). If economic constraints were present, the horse was not included in the study.

**Laboratory Data**

Horses with acute toxic enteritis had at least three serial fecal cultures for *Salmonella* spp., *Campylobacter*, and *Clostridium perfringens* type A. Serology for *Ehrlichia risticii* was performed only on selected cases as the test was not available when the study was started. Laboratory data collected included complete blood count, platelet count, blood chemical analysis, blood gases, electrolytes, osmolality, and coagulation panel (PT, PTT, FDP). An attempt was made to collect and record these values daily for 3 days and at the time of recovery (discharge from the hospital) or death.

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*Y-type surgical blood-solution administration set, Travenol Laboratories, Inc., Deerfield, IL.*
garis, antimicrobial-resistant strains of E. coli, Pseudomonas aeruginosa, Enterobacter spp., Salmonella spp., and Serratia marcescens [13,15,21,29]. The mechanisms of protection provided by antibody to gram-negative antigens are likely multifactorial. Mechanisms include 1) targeting intact gram-negative bacteria or endotoxin for cellular effectors or opsonization and RE clearance [10,23] or 2) blocking of pharmacologically active lipid A epitopes [15]. Antibody to LPS has been shown to enhance bacterial clearance [22,30] and phagocytosis [22] and to decrease levels of detectable endotoxin [33]. Alteration in coagulation or reduction in the thrombogenic capacity of multiplying bacteria by antibody to core antigen has been suggested by experimental work in swine [23]. Protection against the hemodynamic sequelae following heterologous bacterial challenge was observed in dogs, suggesting an antitoxin effect of core antibody [35]. The immunoglobulin isotype (IgM or IgG) which provides protection is controversial [10,22,32,34–36]. The bulk of gram-negative core antibody in normal animals or patients recovering from gram-negative bacteremia is IgG [36,37]. Previous studies performed in our laboratory [23,39,40] have agreed with others [35] that IgG may be more strongly associated with protection than IgM. Until a broader understanding of the protective isotype and titer is known, specific recommendations for immunization schedules and dosages of plasma or serum cannot be made. From this study, it appears that 4.4 ml/kg of high-titer plasma (≥1:32,000 anti-15 IgG) improves clinical appearance and survival in adult horses with clinical evidence of endotoxemia.

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REFERENCES

Prognostic Value of Clinicopathologic Variables Obtained at Admission and Effect of Antiendotoxin Plasma on Survival in Septic and Critically Ill Foals

Simon F. Peek, Sue Semrad, Sheila M. McGuirk, Ace Riseberg, Jo Ann Slack, Fernando Marques, Dane Coombs, Lauren Lien, Nicholas Keuler, and Benjamin J. Darien

This prospective study compared survival rates of critically ill and septic foals receiving 1 of 2 different types of commercial equine plasma and analyzed admission variables as possible predictors of survival. Standardized clinical, hematoletic, biochemical, and hemostatic admission data were collected and foals received either conventional commercially available hyperimmune equine plasma or equine plasma specifically rich in antiendotoxin antibodies in a double-blinded, coded fashion. Sepsis was defined as true bacteremia or sepsis score >11. Overall survival rate to discharge was 72% (49/68). Foals that were nonbacteremic and demonstrated a sepsis score of ≤11 at admission had a 95% (18/19) survival rate. The survival rate to discharge for septic foals was 28/49 (57%), with truly bacteremic foals having a survival rate of 58% (14/24), whereas that for nonbacteremic, septic foals was 30% (14/22). Sensitivity and specificity for sepsis score >11 as a predictor of bacteremia were 74 and 52%, respectively. For the entire study population, a higher survival rate to discharge was documented for those foals receiving hyperimmune plasma rich in antiendotoxin antibodies (P = .012, odds ratio [OR] 6.763, 95% confidence interval [CI] 1.311, 34.903). Administration of plasma rich in antiendotoxin antibodies also was associated with greater survival in septic foals (P = .019, OR 6.267, 95% CI: 1.186, 33.109). Statistical analyses demonstrated that, among 53 clinical and clinicopathologic admission variables, high sepsis score (P < .001), low measured IgG concentration (P = .01), high fibrinogen concentration (P = .018), low segmented neutrophil count (P = .028), and low total red blood cell numbers (P = .048) were the most significant predictors of overall mortality.

Key words: Plasma; Sepsis; Systemic inflammatory response syndrome.

Neonatal sepsis is the most common cause of death in foals during the 1st week of life. A strict consensus definition for sepsis in horses has not been established, but the terms sepsis, septic, and septicecititis often are used in the literature interchangeably. In human medicine, sepsis, severe sepsis, systemic inflammatory response syndrome (SIRS), multiple-organ dysfunction (MODS), and septic shock are terms that have been developed to describe the sequentially worsening systemic pathology and impaired organ homeostasis that occurs as a consequence of an infectious process that causes the release of inflammatory mediators into the systemic circulation. Similar criteria have been used to describe sepsis in adult horses and foals. In the majority of cases, the inciting cause is a bacterial infection, but it can occur due to viral, fungal, or parasitic infections. In foals, bacterial sepsis, commonly associated with failure of passive transfer, and primary gastrointestinal and pulmonary conditions are the most likely causes of sepsis and septic shock. Although much of the available literature focuses on the role of gram-negative bacterial infections and endotoxin in the pathogenesis of sepsis, clinical signs of disease and multiple-organ dysfunction are seen with both gram-positive and gram-negative infections. Previous studies examining survival rates in critically ill foals admitted to referral hospitals in the United States have documented survival rates that vary widely. These studies have been predominantly retrospective in design and, in those instances in which prospective information was collected, it was done to examine the prognostic value of specific clinicopathologic variables rather than approaches to treatment. Several referral practices and university hospitals throughout the world devote considerable resources, time, and effort to the treatment of neonatal foals at considerable financial cost to owners, but there is very little data from controlled studies to validate or confirm many of the commonly used components of therapy for critically ill foals. The use of equine plasma of varying types in the treatment and prevention of sepsis in foals is commonplace, only 1 prospective study compared the efficacy of different plasma types in hospitalized foals. Consequently, we designed a double-blinded, prospective clinical study to examine the effect of 2 different plasma types in the treatment of neonatal sepsis in foals. One of the plasma types was a commercially available hyperimmune equine plasma product, and the other was a product obtained from mares that had been hyperimmunized with a core mutant gram-negative Escherichia coli isolate and which contained much higher concentrations of antilipopolysaccharide (anti-LPS) antibody compared with the...
conventional product. The hypothesis was that survival of critically ill and septic foals would be enhanced by the administration of plasma rich in anti-LPS antibodies. Furthermore, by obtaining a standardized clinicopathologic data set from each foal at admission, we were able to analyze 53 variables as possible predictors of survival for 96 hours after hospitalization as well as to discharge from the hospital.

**Materials and Methods**

**Animals**

Foals referred for treatment to the Veterinary Medical Teaching Hospital (VMTTH) at the University of Wisconsin during the 2002, 2003, and 2004 foaling seasons provided the case material for this study. All foals <7 days of age admitted to the neonatal intensive care unit (ICU) were eligible for the study.

**Sample Collection**

Each foal underwent a standardized series of clinicopathologic tests at admission, including CBC, serum sodium, potassium, chloride, calcium, total protein, phosphorus, magnesium, aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (AP), glucose, total CO₂, anion gap, creatine kinase (CK), creatinine, blood urea nitrogen (BUN), antithrombin III (ATIII), fibrinogen degradation products (FDP), 1 stage prothrombin time (OSPT), activated prothrombin time (APTT), endothoim membrane assay, and selenium concentration. An individual sepsis score was calculated for each individual according to a modified system of Brewer and Keterba. Passive transfer of humoral immunity was assessed at the time of admission by a foal-side semiquantitative assay and specifically quantified by radioimmunoassay of IgG using a simultaneously acquired serum sample. Both aerobic and anaerobic blood cultures were obtained at admission. In cases in which antimicrobials had been administered before referral, antibiotic filtration resin were used to maximize chances for a positive culture.

Each foal received 1 of 2 commercial equine hyperimmune plasma products immediately after admission tests were performed regardless of passive immunity status. Foals >25 kg in body weight received 2 L administered consecutively over the 1st 6-24 hours of therapy, whereas foals <25 kg received 1 L. Plasma bags were heparinized with 3,000 IU/L of heparin before administration. Plasma was stored frozen at 0°C and thawed in warm water (approximately 35°C) immediately before use. Plasma administration was performed in a double-blinded fashion under code, with the coding system preserved for the entire 3 years of the study.

Initial antimicrobial treatment was standardized as follows:

(a) If serum creatinine concentration was >5 mg/dL at admission: potassium penicillin at 22,000 IU/kg IV q6h, and amikacin at 20 mg/kg IV q24h.

(b) If serum creatinine concentration was >5 mg/dL or if the foal was azuric at admission: potassium penicillin at 22,000 IU/kg IV q6h and cefazolin at 20 mg/kg IV q8h.

Flunixin meglumine was also administered at 0.25 mg/kg IV q8h for the 1st 24 hours of hospitalization. All other treatments (eg, fluids, pressor agents, anticonvulsants) varied during hospitalization on a case-by-case basis.

Survival was described in 2 ways: 1st as survival to 96 hours from hospitalization and 2nd to discharge from the hospital.

**Statistical Analyses**

**Analysis of Sepsis Score.** For each foal in the study, the sepsis score on admission was determined as previously described. The sensitivity and specificity of a sepsis score >11 compared with a gold standard of positive blood culture was calculated.

**Survival Data and Comparisons between Plasma Types.** A total of 53 variables were examined for prognostic value between surviving and nonsurviving foals. The following clinical and clinicopathologic variables were examined using admission data from each foal: pulse rate, rectal temperature, respiratory rate, total white blood cell count, segmented neutrophil count, band neutrophil count, presence or absence of metamyelocytes, lymphocyte count, monocyte count, eosinophil count, basophil count, presence or absence of toxic changes in neutrophils, red blood cell count, hemoglobin concentration, hematocrit, platelet count, packed cell volume, presence or absence of abnormal erythrocyte morphology, anion gap, sodium, potassium, chloride, calcium, phosphorus, magnesium, carbon dioxide, glucose, BUN, creatinine, albumin, globulin, total bilirubin, total protein, fibrinogen, as well as serum enzyme activities for AP, CK, AST, and GGT. In addition, AT III, OSPF, APTT, FDP concentration, whole-blood selenium concentration, IgG quantitation (by radial immunodiffusion [RIDD]), and endotoxin concentration were also examined for prognostic value using admission samples. Additional variables included from admission data were volume of plasma administered, body weight, sepsis score, presence or absence of a positive blood culture, gram-positive or gram-negative organism, year of the study, and plasma type administered.

For each of the variables measured, the 68 study foals initially were tested to determine whether recipients of the 2 different plasma types were approximately identical in all measurable respects. Continuous and categorical variables were screened using t-tests and chi-squared tests, respectively. The treatment groups were found to be significantly different for 3 of the 53 variables screened (sodium higher by 3.33 mEq/L in the group receiving plasma rich in anti-LPS antibody, P < .049; temperature higher by 1.09°F in the group receiving conventional hyperimmune plasma, P = .033; and anion gap, higher by 2.98 mEq/L in the group receiving plasma rich in anti-LPS antibody, P = .083). Because of the existence of these possible confounding variables, the evaluation of differences in the 2 plasma treatment groups was corrected by fitting a logistic regression model on survival that included all 3 confounders in addition to the plasma effect. For comparison purposes, the uncorrected analyses were performed as well using Fisher's test. Survival rates to 96 hours of hospitalization and discharge from the hospital for foals receiving the 2 different plasma types were compared for the following groups:

(a) The entire study population (n = 68).

(b) Foals that were bacteremic at admission (n = 24).

(c) Foals that either were bacteremic or had sepsis scores >11 at admission (septic foals; n = 46).

(d) Foals that were not bacteremic and that had sepsis scores ≥11 at admission (n = 22).

**Analysis of Admission Variables as Potential Predictors of Survival.** This analysis was performed as a 2-step procedure:

(a) All 53 variables initially were screened by comparing the mean values for foals that survived versus foals that died. Each variable was examined independently using a t-test for continuous variables and a chi-squared test for categorical variables. The necessary P-value to progress to the next step was arbitrarily set at .4.

(b) The pool of variables with P-values <.4 from step (a) was analyzed by logistic regression in a forward-selection procedure. The 1st term added to the survival model was that which had the lowest associated P-value from step (a). The next term added was that which achieved the smallest P-value when added to the
Table 1. Survival data for foals receiving hyperimmune equine plasma rich in anti-lipopolysaccharide antibodies (plasma a) or conventional equine hyperimmune plasma (plasma b).

<table>
<thead>
<tr>
<th>Overall Survival</th>
<th>Lived</th>
<th>Died</th>
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<tr>
<td></td>
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<td>Plasma b</td>
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<th>Survival to 96 hours</th>
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<tr>
<td></td>
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<td>Septic foals</td>
<td>18</td>
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model already containing the previously added term. This process was continued until no additional resulted in a P-value <.05. P-values for addition to the survival model were calculated using the likelihood ratio test.

Results

Sixty-eight foals were enrolled in the study. Forty-six foals were defined as septicemic on the basis of positive blood culture or sepsis score >11 using clinicopathologic data and samples obtained at admission. There were 24 blood culture-positive foals, 14 of which had single gram-negative infections. There were 39 foals with sepsis score >11, 17 of which also had positive blood cultures. Seven foals with sepsis score of ≤11 had positive blood cultures. Sensitivity and specificity for sepsis score (>11) as a predictor of true bacteremia were 74 and 52%, respectively.

Data describing survival to 96 hours of hospitalization as well as to discharge from the hospital are summarized in Table 1. Of the 19 foals that did not survive to discharge, 8 died, 7 were euthanized primarily for financial concerns, and 4 were euthanized primarily based on a hopeless prognosis. Only 3 foals were alive at 96 hours of hospitalization that did not survive to discharge, all of which were euthanized due to financial concerns. Survival rates to 96 hours of hospitalization and discharge from the hospital did not differ significantly for each category of foal analyzed.

Statistical analyses demonstrated significant differences in survival for foals that received plasma that was rich in anti-LPS antibodies (Table 2). Twenty-seven of the 36 foals that received plasma that was rich in antiendotoxin antibodies survived (75% survival) to discharge, compared with 22/32 (68% survival) of the foals that received the other plasma type. For the entire study population, a significant difference

Table 2. Determination of differences in survival due to plasma type.

<table>
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<tr>
<th>Overall Survival</th>
<th>n</th>
<th>Deviance</th>
<th>df</th>
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<th>95% Upper</th>
<th>LR P-Value</th>
<th>Fisher's P-Value</th>
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<td>0.279</td>
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<td>1.000</td>
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<td>59.597</td>
<td>0.137</td>
<td>25.867</td>
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<td>.627</td>
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<th>df</th>
<th>OR a/b</th>
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<th>95% Upper</th>
<th>LR P-Value</th>
<th>Fisher's P-Value</th>
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* Odds ratio for probability of survival for foals receiving hyperimmune equine plasma rich in antilipopolysaccharide antibodies (plasma a) compared with foals receiving conventional equine hyperimmune plasma (plasma b).
* Likelihood ratio test P-value.
difference was seen in the survival to 96 hours ($P = .042$) as well as to discharge from the hospital ($P = .012$) for foals that received hyperimmune plasma that was rich in antiendotoxin antibodies compared with those that received the other plasma type. Statistical analyses demonstrated that these differences were independent of foal body weight and the volume of plasma administered. Administration of plasma rich in antiendotoxin antibodies also was associated with greater survival to discharge in the 46 septic foals ($P = .019$). No difference was seen in overall survival rate for those foals that were bacteremic at admission according to plasma type administered ($P = .320$). When considering only foals with gram-negative infection (n = 14), the survival rates were not statistically significantly different for the 2 different plasma types ($P = .055$).

For survival to discharge, statistical analyses demonstrated that of the 53 clinicopathologic admission variables examined, only 5 were retained in the final survival model. Specifically, sepsis score ($P < .001$) and fibrinogen concentration ($P = .018$) were negatively related to survival, whereas measured IgG concentration ($P = .01$), segmented neutrophil count ($P = .028$), and total red blood cell numbers ($P = .048$) were positively related to survival. When considering survival to 96 hours, the retained variables were sepsis score ($P < .001$) and fibrinogen concentration ($P = .007$), which again were negatively associated with survival, and segmented neutrophil count ($P = .055$) and blood glucose concentration ($P = .024$), which were positively associated with survival. The details of the model’s fit are presented in Table 3. The logistic regression equations for survival to discharge and survival to 96 hours of hospitalization are given below:

logit (survival to 96 hours) = 5.582 + (−0.346)sepsis score + (0.270)segmented neutrophil count + (−0.005)fibrinogen + 0.017(glucose).

**Discussion**

Our finding that the administration of plasma harvested from mares hyperimmunized with LPS was associated with greater survival in all foals admitted to our ICU as well as the septicemic foal subpopulation is a unique observation. It contrasts with findings in a prior study by Morris and Whitlock that also prospectively looked at anti-LPS antibody-rich plasma in comparison with lower anti-LPS titer plasma. That study was a multicenter study that did not demonstrate any significant difference in survival for critically ill neonates receiving either plasma type. We were able to demonstrate enhanced survival for the entire study population as well as for foals that we defined as septic by virtue of positive blood culture or sepsis score >11 at admission. Although statistical significance was identified both for overall short-term survival (96 hours of hospitalization, $P = .042$) and survival to discharge ($P = .012$) for those foals receiving hyperimmune plasma that was rich in anti-LPS antibody, the clinical relevance was less compelling, with 75% survival to discharge versus 68% for the 2 different plasma types. Statistical significance does not necessarily imply clinical relevance. From a statistical standpoint, it can be seen from the higher values for Fisher’s P-values in Table 2 that, if corrections had not been made in our data analysis for those factors that were not randomly distributed between foals receiving the 2 plasma types (sodium, temperature, and anion gap), we would not have identified a significant difference at all. There were no significant differences noted in survival between foals that were bacteremic and those that were not nor between those that had a gram-negative isolate obtained compared with a gram-positive isolate from samples obtained at admission. The overall difference in survival rate suggests that administration of plasma rich in anti-LPS conferred the greatest benefit on nonbacteremic foals. It is not clear from this study whether anti-LPS antibody-rich plasma may confer a therapeutic or survival benefit on foals with gram-negative sepsis.

Another important fact to consider is that, although 1 of our plasma types was obtained from donors that were hyperimmunized with the J5 vaccine product, it would be incorrect to assume that the other plasma type used in our study was devoid of anti-LPS antibody. Precise anti-LPS antibody titrations were not performed on every bag or batch of plasma used in this study. All of the donors for the anti-LPS antibody-rich plasma had titers of >1:12,000, but approximately 10% of the donors for the other plasma type also had titers this high. However, 50% of donors of the other plasma type had negative anti-LPS antibody titers at a dilution of 1:1,600. Therefore, our data actually may underestimate the potential beneficial effect of anti-LPS antibody in treating sick foals.

Another relevant difference between our study and that of Morris and Whitlock was the fact that all plasma was heparinized before administration to the

<table>
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<th>Table 3. Details of logistic survival model.</th>
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<tr>
<td><strong>Overall Survival</strong></td>
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<tr>
<td>Variable</td>
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<tr>
<td>Intercept</td>
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<td>Sepsis score</td>
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<td>SEG5</td>
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<td><strong>Survival to 96 hours</strong></td>
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<td>FIB</td>
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<td>Glucose</td>
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*LR, likelihood ratio test P-value; SEG5, segmented neutrophil count; RID, radial immunodiffusion; RBC, red blood cell; FIB, fibrinogen concentration.
foals in our study. Some hemostatic variables differ between newborn and 1-month-old foals\textsuperscript{19} as well as between newborn foals and adults,\textsuperscript{11,12} but clinically relevant disorders of hemostasis only become apparent during acquired conditions, such as septicemia.\textsuperscript{21,24} Hemostatic abnormalities in association with neonatal septicemia are well established in foals,\textsuperscript{5,13} but our findings are in accordance with those of Barton et al.,\textsuperscript{2} who observed that survival did not significantly correlate with conglobopathy. The heparin used in our study was of porcine origin\textsuperscript{8} and of mixed molecular weight and may have conferred a beneficial effect on the treatment of sepsis when combined with plasma by virtue of both its anticoagulant and anti-thrombotic properties.\textsuperscript{12} Heparinization of plasma alone, however, could not have been the only factor involved in enhancing survival rates compared with previous studies because both plasma types were treated with 2,000 I.U/L of sodium heparin, and statistically significant differences were seen between the plasma types administered. All foals in this study received flunixin meglumine in addition to heparinized plasma and antibiotics on the 1st day of hospitalization, even those for which the sepsis score was >11 on admission. Arguably, the use of this drug was not justifiable in all study foals, but in designing a prospective study, we sought to provide a standardized initial therapeutic regimen for comparative reasons and to ensure that the quality of care for the sickest client-owned foals was not compromised. Because of concerns regarding ulcerogenesis and renal toxicity associated with the use of nonsteroidal anti-inflammatory medications, especially in compromised neonates, other clinicians may reasonably elect not to use flunixin meglumine in all cases.

Of the 53 admission variables measured on every foal in this study, 4 were significantly associated with survival to discharge from the hospital. In terms of P-value, sepsis score was the most significant (P < .001), with higher sepsis score foals having a poorer chance of survival. No metabolic variables were retained as being significantly associated with survival to discharge from the hospital. This finding is in contrast with previous studies that have demonstrated high anion gap and low venous oxygen tension,\textsuperscript{2} or low glucose, low albumin, and low pH,\textsuperscript{4} to be associated with poorer survival rates for foals admitted to ICUs at different institutions. Interestingly, low blood-glucose concentration at admission was associated with poorer survival to 96 hours of discharge in our study but was not retained in the model examining survival to discharge. Other admission variables that were significantly associated with survival to discharge were IgG concentration (as measured by RID), fibrinogen concentration, total neutrophil count, and total red blood cell (RBC) count. Low IgG concentration, as a consequence of failure of passive transfer, potentially compounded by antibody consumption in the early stages of infection, was not a surprising risk factor for failure to survive in critically ill neonates.

Three studies comparable with ours examined factors potentially associated with survival in septicemic foals or foals admitted to referral hospitals for intensive care,\textsuperscript{2,3} and in 2 of those,\textsuperscript{3,4} low neutrophil count was documented as a variable that was significantly different between survivors and nonsurvivors. Although low neutrophil count was significantly associated with failure to survive in our study (P = .028) it was not as statistically significant as sepsis score, IgG concentration, and fibrinogen concentration. The finding that a high fibrinogen concentration was significantly associated with failure to survive (P = .018) is a novel finding, and one that suggests that foals with evidence of acute-phase inflammation, in conjunction with failure of passive transfer and neutrophil sequestration, are the poorest survival candidates. The admission variable with significance (P = .048) as a predictor of survival that potentially is the hardest to explain is the total RBC count. Although it demonstrated the lowest P-value in the final set of retained variables, its persistence in our analyses indicates that low RBC numbers are somehow related to survival. Whether this observation reflects anemia, low-grade hemolysis, or both granulocytic and erythropoietic precursor suppression is an area worthy of further study.

Our final survival models included both sepsis score and several of its components (ie, segmented neutrophil count, fibrinogen, and IgG for overall survival and segmented neutrophil count, fibrinogen, and glucose for survival to 96 hours). This finding is evidence that a component measure may provide information on survival that is not captured by that component's contribution to the sepsis score. This result may occur because of the inherent loss of information that occurs when a continuous predictor is categorized, as is the case for most of the other components of the sepsis score. It therefore seems reasonable and desirable to allow some predictors to be included twice, once as a component of the sepsis score and once as a separate predictor. When performing multiple logistic regression, correlation between the potential predictor variables (also known as multicollinearity) is always a major concern. Multicollinearity can lead to misleading or inaccurate estimates, standard errors, or P-values. In most cases, highly correlated predictors will not appear together in a model formed through a forward-selection procedure such as the one used in this study. This is because correlated predictors explain roughly the same portion of the response and generally will not both show as significantly predictive when included together, even if they may be predictive when included alone. Because forward selection implicitly protects against multicollinearity, correlations among all 53 potential predictors were not calculated. However, because it was known that sepsis score is composed of several of the other measured variables, it seemed prudent to explicitly check for correlations between sepsis score and those variables. Correlations were quite small for all such variables and thus should not have caused problems in the model fitting.

Although the precision of sepsis scoring with respect to accurately identifying truly septic foals has been the subject of recent debate,\textsuperscript{10} our data suggest sepsis scoring can be useful prognostically in a referral population. When defining the sensitivity and specificity of sepsis
score in our hospital, we used a gold standard of true bacteremia, which is more stringent and arguably too narrow by comparison with the definition originally used by Brewer and Koterba. Consequently, our calculated values for sensitivity and specificity are low (74% and 52% compared with 93% and 86%, respectively). Undoubtedly, there are many non-bacteremic foals that could reasonably be described as septic and there is variation in acceptance of sepsis score among institutions and individual clinicians. However, our findings suggest that the calculation of sepsis score using the system and criteria described by Brewer and Koterba gave the most prognostically useful index of survival for neonatal foals in our hospital.

The overall survival rate for foals admitted to our ICU was 72%, with the septicemic population and truly bacteremic foals having lower survival rates of 58 and 57%, respectively. The survival rate for all ICU admissions in our study was comparable with those documented by Hoffman et al (66%) and Furr et al (74%), but our survival rate for septicemic foals was higher than those previously published for comparably defined populations by Gayle et al (45%) and Morris and Whitlock (32.5%). Comparisons among referral hospitals are obviously of limited value when criteria for admission to neonatal intensive care units likely vary among institutions. In our hospital, not all foals <7 days of age are admitted to the ICU, several categories of foals (eg, angular limb deformities and other neonatal orthopedic problems in which foals can still nurse, mild enteric and respiratory disease cases) seen by the surgery and medicine services remain in the main hospital population. Although all foals that require IV fluid support and close monitoring are admitted to our ICU, the ultimate decision is clinician dependent and empirical, such that we may have different criteria compared with other studies and therefore a slightly different population base for comparative purposes. Our observation that approximately equal proportions of truly bacteremic and septicemic foals survived to discharge from the hospital was somewhat surprising; however, the fact that we performed blood cultures routinely, using antimicrobial retrieval systems for those foals that had been pretreated with antibiotics, may have increased the numbers of foals from which positive cultures were obtained. Survival rates for septicemic and bacteremic foals likely have increased over the last decade given improvements in critical care, therapeutics, and intensive nursing care available at university and private referral practices.

For ethical reasons, it is unlikely that a controlled, prospective study establishing whether plasma administration to critically ill foals confers a survival advantage over antibiotics and intensive supportive therapy alone will be conducted. However, we have been able to demonstrate that, within our referral population, the administration of anti-LPS-rich antibody plasma is associated with higher survival rates. Furthermore, the combination of failure of passive transfer, neutropenia, high fibrinogen concentration, and low RBC counts as measured at admission, warrants a graver prognosis.

Footnotes
*Polyneum, Veterinary Dynamics, Templeton, CA
Polyneum Plus antiendotoxin Ab, Veterinary Dynamics, Templeton, CA
Limulus Amebocyte Assay, BioWhittaker Co, Walkersville, MD
Snake IgG Test, Idexx Laboratories, Westbrook, ME
BBL Septi-Chek Columbia Broth, Becton Dickinson and Company, Sparks, MD
BBL Septi-Chek TSD With Resins, Becton Dickinson and Company, Sparks, MD
Heparin sodium injection USP, Elkins Sinn Inc, Cherry Hill, NJ
St Hygeline Biological Corporation, Woodland, CA
Personal communication, Dennis Brook, Veterinary Dynamics, Templeton, CA

Acknowledgment
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References
Endotoxemia in horses: Protection provided by antiserum to core lipopolysaccharide

Debra Deem Morris, DVM; Robert H. Whitlock, DVM, PhD; Lynette B. Corbeil, DVM, PhD

SUMMARY

An equine antiserum to core lipopolysaccharide was produced by inoculation of 6 horses with a boiled cell bacteria made from the J-5 mutant of Escherichia coli O111:B4. The antiserum immunoglobulin G titer to J-5 mutant E. coli, as determined by enzyme-linked immunosorbent assay, was 1:15,006. Pooled serum prepared before inoculation (preimmune serum) had a J-5 immunoglobulin G titer of 1:350.

The J-5 antiserum was tested for its protective efficacy in sublethal endotoxemia in 14 horses. Four horses served as nontreated controls and were given nothing before endotoxin challenge exposure (10 μg/kg of body weight, IV). Pooled preimmune serum (3 ml/kg, IV) was administered to 5 horses and J-5 antiserum (3 ml/kg, IV) was administered to 5 other horses 2 to 15 hours before endotoxin challenge exposure. During the 24 hours postendotoxin challenge exposure, endotoxemia was accompanied by significant (P < 0.05) time-related changes in temperature, heart rate, pulse character, respiratory rate and character, capillary refill time, mucous membrane color, fecal consistency, attitude, PCV, total plasma protein, WBC count, platelet count, plasma fibrinogen, prothrombin time, activated partial thromboplastin time, fibrinolytic degradation products, plasma glucose, and plasma lactate in all horses. There were no apparent treatment vs time interactions (P > 0.05). Two horses (1 control and 1 given J-5 antiserum) died suddenly from unknown causes immediately after endotoxin challenge exposure. Seemingly, equine antiserum to core lipopolysaccharide did not provide protection from the adverse effects of experimental endotoxemia produced by bolus IV infusion of 10 μg of endotoxin/kg.

Endotoxin, a lipopolysaccharide (LPS), cell wall component of gram-negative bacteria, has been implicated as a primary pathologic factor in equine gastrointestinal disorders and gram-negative bacterial infections. Clinical and laboratory abnormalities that occur during intestinal strangulation obstruction, colitis X, acute salmonellosis, or neonatal septicemia strongly resemble the syndrome of shock and coagulopathy produced by endotoxin administration to horses. The concentration of endotoxin within the equine large intestine increases rapidly when there are changes in gram-negative flora, such as occurs with an increase in dietary carbohydrate or intestinal strangulation. Luminal lactic acid accumulation, which also accompanies high carbohydrate intake, intestinal ischemia, and severe inflammation of the intestinal wall are thought to cause loss of mucosal integrity, allowing transmural movement of endotoxin into the peritoneal cavity and systemic circulation. Endotoxin has been demonstrated in the peritoneal fluid and peripheral blood of ponies coincident with the loss of villous epithelial cells after experimental small intestinal strangulation obstruction. Endotoxin-like substances have been isolated from the blood of horses with spontaneous gastrointestinal disease, and this finding was associated with a high mortality. Endotoxin also accompanied gram-negative bacteremia.

Most current research on equine endotoxemia is aimed at pharmacologic means to prevent endotoxin-induced cardiovascular derangements and formation of reactive metabolites. The use of flunixin meglumine, an inhibitor of prostaglandin synthesis, before endotoxin administration reduces vasodilation and enhances perfusion to vital organs but does not prevent general tissue damage and coagulopathy during endotoxemia. The pathophysiologic mechanisms of the numerous variable effects of endotoxin on thermoregulation, the cardiovascular system, coagulation mechanism, complement cascade, cellular constituents of the blood, and other body systems have not been elucidated completely, but probably involve more than one mechanism of action. Therefore, direct destruction or inactivation of endotoxin seems most appropriate in treatment of endotoxin-mediated diseases.

There has been extensive research to develop an antiserum against endotoxin that could be used as an adjunct to antibiotics in the treatment of gram-negative bacteremia in human beings. The 3 major structural components in endotoxin that are theoretic targets for protective antibody include: lipid A, the innermost region of the LPS molecule and the toxic moiety; core polysaccharide, composed of inner and outer parts linked to lipid A by 2-keto-deoxyoctonate; and the O polysaccharide, repeating short polysaccharide side chains located in the...
Antiserum to the J-5 mutant of Escherichia coli O111:B4, produced in rabbits or people, protected animals against adverse effects of endotoxins from many unrelated gram-negative organisms and improved survival from bacteremia caused by enteric gram-negative rods. Antiserum to rough mutants of Salmonella typhimurium protected mice against lethal challenge exposure with Klebsiella pneumoniae, whereas smooth Salmonella antigen was ineffective.

Antiserum production—Six healthy horses (5 mares and 1 gelding) between the ages of 7 and 14 years and weighing between 450 and 500 kg were used for antiserum production. The horses were fed a diet of timothy-alfalfa hay (0.45 to 0.90 kg/45 kg of body weight/day) and were given free access to grass pasture (5 horses/ha), water, and salt-mineral mix. Horses were dewormed, tested negative for equine infectious anemia antibodies, and inoculated with tetanus toxoid 1 month before the start of the experiment.

Samples of blood were collected from the jugular vein for determination of preinoculation serum titers against the J-5 mutant of E. coli O111:B4. Jugular venous blood (6 L) was collected from each horse into sterile, evacuated, 1-L bottles for preparation of preimmune serum. The blood was allowed to clot for 2 hours in a 37-C waterbath, the bottles were centrifuged, and sera were harvested and pooled into a sterile 20-L plastic carboy. After samples were taken for bacterial culture, the pooled serum was partitioned into sterile, evacuated 1-L and 0.5-L bottles, frozen at −20 C, and stored until later use in the endotoxin trials.

The 6 horses were inoculated on postinoculation days (PID) 1, 3, 5, 9, 12, and 17, with 2 ml of J-5 bovine cell bacterin. The bacterin was prepared from J-5 mutant E. coli O111:B4 bacterial cells in a stationary growth phase of an 18-hour Trypsinized broth culture. Bacterial cells were removed by centrifugation, washed 3 times in sterile 0.9% NaCl, and boiled for 2.5 hours. The concentration was adjusted spectrophotometrically, by the addition of 0.9% sterile saline solution, to approximately 5 x 10^8 bacteria/ml (233 light transmission at 510 nm), and phenol was added to a final concentration of 0.4%. The first dose of bacterin, mixed with an equal volume of Freund's incomplete adjuvant, was administered in 2 1x sites. Subsequent doses without adjuvant were administered subcutaneously in 2 different sites.

Because the serologic response to J-5 bacterin administration was not known, samples of blood were collected for the determination of titer to J-5 at PID 10, 17, 21, 38, 45, 59, 66, 73, 87, and 94. Jugular venous blood (6 L) was collected and handled as previously described, on PID 10, 38, 66, and 94, for preparation of postimmunization serum from these dates. It was planned.

<table>
<thead>
<tr>
<th>TABLE 1—Abnormally score grading scale for pulse character, respiratory character, fecal composition, and attitude of horses given endotoxin</th>
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<tbody>
<tr>
<td>Abnormality score</td>
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<td>1</td>
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<tr>
<th>TABLE 2—Serum immunoglobulin G titers to J-5 mutant E coli in horses, inoculated with J-5 bovine cell bacterin</th>
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<tr>
<td>Horse</td>
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Horses were inoculated on PID 1, 3, 5, 9, and 87 with 10^8 J-5 bovine cells. The 1st inoculation included Freund's incomplete adjuvant.

PID = postinoculation days.
TABLE 3—Mean values (± sd) for selected clinical data in horses after endotoxin administration

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
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<td>Values</td>
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<tr>
<td></td>
<td>Temperature (°C)</td>
<td>37.5 ± 0.3³</td>
<td>37.5 ± 0.4</td>
<td>37.9 ± 0.5</td>
<td>37.5 ± 0.6</td>
<td>38 ± 0.5⁴</td>
<td>38.2 ± 0.6³</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>41 ± 4</td>
<td>51 ± 13</td>
<td>50 ± 10</td>
<td>57 ± 12³</td>
<td>69 ± 12³</td>
<td>62 ± 15³</td>
<td>62 ± 15³</td>
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<tr>
<td>Respiratory rate (breaths/min)</td>
<td>17 ± 9³</td>
<td>31 ± 12³</td>
<td>28 ± 13³</td>
<td>25 ± 10³</td>
<td>28 ± 12³</td>
<td>30 ± 14³</td>
<td>21 ± 8³</td>
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<tr>
<td>Capillary refill time (s)</td>
<td>2²</td>
<td>3 ± 0.9²</td>
<td>3 ± 0.5²</td>
<td>3 ± 1.0³</td>
<td>4 ± 1.1³</td>
<td>4 ± 1.1³</td>
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<tr>
<td>Fecal composition*</td>
<td>0.5 ± 0.6</td>
<td>1.2 ± 0.6²</td>
<td>1.5 ± 0.5³</td>
<td>3.3 ± 1.1³</td>
<td>4.4 ± 0.8³</td>
<td>4.7 ± 0.7³</td>
<td>4.7 ± 0.7³</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>3.8 ± 1.1³</td>
<td>4.4 ± 0.8³</td>
<td>4.7 ± 0.7³</td>
<td>4.7 ± 0.7³</td>
<td>4.7 ± 0.7³</td>
<td>4.7 ± 0.7³</td>
<td>4.7 ± 0.7³</td>
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* Fecal composition abnormality score: 0 = normal; 1 = soft balls; 2 = cow-like consistency; 3 = fluid with fiber; 4 = fluid without fiber. 1 Attitude abnormality score: 0 = normal, bright; 1 = mild colic; 2 = mild depression; 3 = marked depression; 4 = ataxia; 5 = recumbency.

There were no significant treatment (group) effects (P > 0.05). Values without common superscripts are significantly (P < 0.05) different.

The horses were monitored from 2 hours before through post-inoculation hours (pH) 24. The following clinical values were recorded immediately after endotoxin administration and then every 15 minutes during the first 3 hours after endotoxin and at pH 4, 6, 8, 10, 12, and 24: temperature, heart rate and pulse character, respiratory rate and character, mucous membrane color and capillary refill time, fecal composition, and attitude. Pulse character, respiratory character, fecal composition, and attitude were assigned an abnormality score subjectively based on degree of deviation from normal.

Various blood was drawn for the following laboratory tests immediately before endotoxin administration (0 hour) and at pH 0.5, 1, 1.5, 2, 3, 6, 12, and 24: PCV, total plasma protein (TPP), WBC count and differential, platelet count, plasma fibrinogen, prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinolytic degradation products (FDP), plasma glucose, and plasma lactate. The PCV and TPP were measured by microhematocrit centrifugation and refractometry, respectively. The WBC and platelet counts were determined on a hemacytometer, with commercial dilution reagents, according to manufacturer’s recommendations. Plasma fibrinogen was determined by the microhematocrit heat-precipitation method. The PT and APTT were determined on a single-channel photo-optical clot-time device, using commercial thromboplastins. The FDP were determined by a latex agglutination reaction. Plasma glucose was measured by the hexokinase method and plasma lactate by an enzymatic pyruvate trapping reaction. Blood was drawn for determination of IgG titer to J-5 mutant E coli by ELISA, as previously described: before serum administration (groups II and III) and immediately before endotoxin administration (0 hour) and at pH 24.

Praunzig-Küster (P-K) test—A P-K test to detect type I (mast cell-mediated) hypersensitivity was performed on 2 clinically normal horses as follows: A serum sample from each of 2 horses inoculated 10 or 14 days previously with J-5 boiled cell bacterin was divided, and half was heated at 56°C for 120 minutes to inactivate IgE. Aliquots (0.1 ml) of heated and unheated sera were injected intradermally in the cervical region of 2 healthy horses. Twenty-four hours later, 0.1 ml of endotoxin (1 mg/ml) was injected intradermally at each site of serum injection. Presence and size of a wheal at each injection site were evaluated 30 minutes later. A positive reaction was characterized by wheal formation within 30 minutes. A positive reaction in the heated serum would indicate the presence of cephalosporin IgG to endotoxin, whereas positive in the unheated serum indicated the presence of IgE to endotoxin. Positive in both serum sites would indicate the presence of endotoxin-specific IgG and IgE.
in the serum, which had tagged tissue mast cells. Saline solution (0.9%) and histamine served as negative and positive controls, respectively. Endotoxin, injected intradermally in the group region, was another negative control.

Effects of treatment and time on clinical and laboratory values in horses after endotoxin administration were evaluated, using 3-way analysis of variance for repeated measurements. The Student's-Newman-Keuls' test was used to make comparison, where appropriate.

Results

Antiserum production—The serum J-5 IgG titer in the 6 horses used for antiserum production peaked at PID 10 to 30 (Table 2) and did not increase with subsequent doses of the bacterin. Four other horses inoculated with a larger antigenic mass (10^11 J-5 organisms) in Freund's incomplete adjuvant did not achieve higher titers. Because the geometric mean titer on PID 38 and 94 were similar and greater than those on PID 66, sera collected on PID 38 were selected for combination with an equal volume of that collected on PID 10 for preparation of the pooled postimmune serum later used in the endotoxin trials. The serum IgG titer against J-5 mutant E coli was 350 to 1,225 in the pooled preimmune serum and was 15,006 to 52,521 in the pooled postimmune serum.

Endotoxemia in horses—There were no significant treatment effects on clinical values with the exception of fecal composition, which was increasingly abnormal in groups I, III, and II (P < 0.05; Table 3). Because only fecal composition was statistically different among the groups, data are presented as means for all horses. There was a significant effect of time on all clinical values after endotoxin administration, including temperature, heart rate, pulse character, respiratory rate, character, capillary refill time, mucous membrane color, fecal composition, and activity (Table 3; P < 0.05). There were no apparent treatment group/time interactions (P > 0.05). The pulse was bounding immediately after endotoxin administration, then became weak and imperceptible, remaining abnormal throughout PID 24. An abnormal respiratory pattern, characterized by marked abdominal effort, nostril flaring, or obvious dyspnea, developed within 15 minutes of endotoxin administration, and the respiratory character remained abnormal during 24 hours after endotoxin. All horses developed congested, hyperemic mucous membranes within 15 minutes after endotoxin administration. During the time when capillary refill time was most prolonged (PID 6 to 12), some horses had cyanotic oral mucosa. Fecal composition after endotoxin administration ranged from soft fecal balls to fluid (without firm) and feces remained softer than normal in most horses through PID 24. Horses in all groups developed an abnormal attitude after endotoxin administration, characterized by mild colic, depression, and/or ataxia. Most horses became recumbent between PID 3 and 4, but had again risen by PID 6. Sweating, cool extremities, and salivation were shown by most horses during the first 3 hours after endotoxin. Five horses had an abnormal head motion, with the muzzle thrust forward and the poll directed laterally. Anal dilatation developed in 8 horses. Depression persisted through PID 24.

One horse in group I was euthanatized at PID 12 when showing signs of severe endotoxin shock. Other horses in the experiment were not so severely affected by endotoxemia. One horse each from groups I and III died unexpectedly within 5 minutes of endotoxin administration. Postmortem examination of these horses revealed splenic and/or hepatic congestion with edema and petechial hemorrhages of the respiratory and gastrointestinal tracts.

Similar to the clinical observations, there were no significant treatment effects on laboratory values (P > 0.05; Table 4). There was a significant effect of time on all laboratory values after endotoxin administration, including PCV, TTP, WBC, platelet count, plasma fibrinogen, PT, APTT, FDP, plasma glucose, and lactate (Table 3; P < 0.05).

There was a significant linear increase in total number of hand neutrophils after endotoxin (P < 0.05), which corresponded to the increase in total WBC count (data not shown). Although the TTP increased in each individual horse after endotoxin administration, the variability among horses resulted in a nonsignificant time effect on overall means. There were no interactions between treatment group and time (P > 0.05).

There was a significant interaction between treatment group and time on the mean titers (P < 0.05; Table 5). There were no group differences at any time and groups I and II had no difference over time (P > 0.05). There were significant time differences within group III: geometric mean titers were significantly greater after administration of postimmune serum and at PID 24 than before administration of postimmune serum (P < 0.05).

Four horses inoculated with J-5 boiled cell bacterin by the protocol used to produce antiserum were given a sublethal dose of endotoxin (10 μg/kg) on PID 10 or 14. Two horses died of anaphylaxis within 5 minutes after endotoxin administration, and 2 others had endotoxemia with clinical signs and laboratory abnormalities indistinguishable from horses in groups I to III. Three horses had a titer of 15,006 and the other had a titer of 1,225 to J-5 at the time of endotoxin administration (geometric mean = 8,021). Prausnitz-Kustner tests, using serum from the 2 inoculated horses that died suddenly after the administration of endotoxin, were negative.

<table>
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<tr>
<th>PH</th>
<th>2.5</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>24</th>
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<tbody>
<tr>
<td>38.3 ± 0.7a</td>
<td>38.3 ± 0.7a</td>
<td>38.4 ± 1.1a</td>
<td>39.9 ± 0.7a</td>
<td>39.6 ± 0.7a</td>
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</tr>
<tr>
<td>65 ± 14a</td>
<td>65 ± 14a</td>
<td>65 ± 14a</td>
<td>67 ± 10a</td>
<td>67 ± 14a</td>
<td>64 ± 10a</td>
<td>62 ± 22a</td>
<td>63 ± 10a</td>
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<tr>
<td>19 ± 7a</td>
<td>19 ± 4</td>
<td>19 ± 7</td>
<td>22 ± 14a</td>
<td>38 ± 22a</td>
<td>27 ± 9a</td>
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</tr>
<tr>
<td>4 ± 1</td>
<td>4 ± 1.8a</td>
<td>4 ± 1</td>
<td>6 ± 2.0a</td>
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<td>4 ± 1.8a</td>
<td>4 ± 1.5a</td>
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</tr>
<tr>
<td>23 ± 7.4a</td>
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<td>2.6 ± 0.9a</td>
<td>2.4 ± 1.0a</td>
<td>2.3 ± 1.0a</td>
<td>1.8 ± 1.0a</td>
<td>1.6 ± 0.9a</td>
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</tr>
<tr>
<td>4.4 ± 0.7a</td>
<td>4.3 ± 0.7a</td>
<td>4.3 ± 0.7a</td>
<td>3.8 ± 0.7a</td>
<td>3.8 ± 0.7a</td>
<td>3.2 ± 0.6a</td>
<td>3.2 ± 0.6a</td>
<td>3 ± 0.7a</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 5—Geometric mean serum IgG titers to J5 mutant E. coli in horses treated with preimmune or J5 postimmune serum before endotoxin administration.

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>n</th>
<th>Postserum and/or preendotoxin (0 hr)</th>
<th>Postendotoxin (24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nothing (I)</td>
<td>3</td>
<td>350 (2.54 ± 0.54)</td>
<td>350 (2.54 ± 0.54)</td>
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<tr>
<td>Preimmune serum (II)</td>
<td>4*</td>
<td>479 (2.54 ± 0.54)</td>
<td>350 (2.54 ± 0.54)</td>
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<tr>
<td>Postimmune serum (II)</td>
<td>4</td>
<td>130 (2.21 ± 0.54)</td>
<td>350 (2.54 ± 0.54)</td>
</tr>
<tr>
<td>Serum (III)</td>
<td>4</td>
<td>130 (2.54 ± 0.54)</td>
<td>350 (2.54 ± 0.54)</td>
</tr>
</tbody>
</table>

* One horse was excluded from calculations because of missing values. † Geometric means in group III, with superscripts, are significantly different from unmarked mean over time (P < 0.05).

Data in parentheses are the log mean ± SE.

n = No. of horses.

Discussion

Administration of equine antiserum to the rough mutant of E. coli O111:B4 (J-5) was not protective against equine endotoxemia produced by smooth E. coli O111:B4 endotoxin. Possible reasons for lack of protective efficacy of the equine J-5 antiserum include: failure of inoculated horses to produce sufficient cross-protective antibodies, ie, poor quality postimmune serum; insufficient dose of antiserum given to endotoxin challenge-exposed horses; failure of the J-5 boiled cell bacterin to elicit production of antibodies capable of protecting against heterologous endotoxemia.

The present inoculation protocol was similar to that described for production of protective J-5 antiserum in people, and inoculation of horses with a larger antigenic mass (1011 organisms) did not result in higher titers. All inoculated horses had a 12 to 150 times increase in titer (1.2 to 1.8 log) at PTD 10 to 38, which is greater than that in people. In trials indicating efficacy of J-5 antiserum, antibodies within the antiserum have been measured by bacterial cell agglutination and/or by passive hemagglutination, using RBC sensitized with J-5 LPS. Equine antibodies to J-5 were quantitated by an ELISA, designed to detect IgG. Rabbit titers of IgG for J-5 were similar in magnitude when determined by ELISA or hemagglutination, even though the latter measures predominantly IgM. Boiled bacterial cells were used as the antigen because there was not a pure source of J-5 LPS and to circumvent problems associated with adherence of rough endotoxin to the solid phase. Although work with laboratory animals indicates that titers to J-5 boiled cells correspond with those against J-5 LPS, it is possible that equine antibodies against nonprotective determinants or the wrong Ig class were being measured. Horses did attain a significantly higher J-5 titer after administration of the pooled postimmune serum.

Horses were given 1.5 L of preimmune or postimmune serum that corresponded to a dose of 3 to 4 ml/kg, the dose of human J-5 antiserum that was effective in reducing mortality in human beings with gram-negative sepsis. Rabbits have been protected from the adverse effects of endotoxin and gram-negative bacteremia by doses of J-5 antiserum ranging from 5 to 20 ml/kg. Neither the minimum J-5 titer nor the Ig class necessary for protection from the toxic effects of endotoxin or gram-negative sepsis has been established in the host or pro-
tective sera. Mortality from bacteremia was significantly less in human patients treated with J-5 antiserum with hemagglutination titers ≥ 128. The 7-S (IgG) and 19-S (IgM) fractions of apine J-5 antiserum protected rabbits against bilateral renal cortical necrosis, induced by 2 doses of heterologous LPS (Shwartzman reaction), therefore, pooled postimmune serum was made from sera collected at PJD 10 and 58, with the intent to ensure high titers of IgM and IgG against J-5. However, the IgG titer peaked in 5 of 6 inoculated horses on PID 10. This indicated that the horses were responding anamnestically to the organism and that the pooled postimmune serum may have been low in IgM. Natural antibody to core glycolipid has been found in serum from human beings, rabbits, dogs, and the predominant Ig class is IgG. The IgM level is elevated by 4-fold increase in IgG titer, as seen in horses inoculated with J-5 bacteria. Therefore, it seems that horses responded appropriately to inoculation and that the J-5 antiserum should have been qualitatively similar to that produced in rabbits and people.

One endotoxin challenge-exposed horse given preimmune serum had a base-line titer to J-5 of 1,225 before and after serum administration. Titters obtained in group II horses by administration of the postimmune serum were all 1,225. Because titers were determined retrospectively, this horse had a high base-line titer produced an unpredicted inequality among individuals in the groups and minimized differences between treatment groups. Nonetheless, mitigation of the deleterious effects of endotoxin was not observed in any of the horses, even those that were actively inoculated and had a titer of 15,006 when challenge exposed.

The cause of sudden death in 2 horses after being given endotoxin is perplexing. Results of the P-K tests, using serum from the inoculated horses that died, were negative. The last horse that died had not been given serum, had not been inoculated, and had a low titer to J-5 (100).

Although this horse and others may have had unmeasured cytotoxic IgE to LPS, it is equally likely that the endotoxin preparation contained an antigen to which some of the horses had been previously sensitized or that an unknown mechanism caused death. The administration of the same endotoxin solution to mice did not result in sudden death.

The protective antigen in J-5 has not been characterized, but there is evidence to indicate that it lies in the lipid A-2-keto-deoxyoctonate portion of the LPS molecule; protection to heterologous endotoxemia and gram-negative sepsis has been reported using antisera to the mutant of Salmonella minnesota, which lacks core polysaccharides. A common determinant group retained by the lipid-deficient (carbohydrate) and lipid-rich (toxic) fractions of the LPS molecule when they are chemically isolated may represent the protective moiety.

Protective antibodies induced by J-5 mutant E. coli may bind some portion of the LPS core and prevent access of lipid A to mediators of toxicity. Most naturally occurring antibodies induced by gram-negative infection belong to the IgM class; however, these are directed against the O side chains, function as opsonins and enhance complement-mediated bacteriolysis, and are not active against heterologous organisms. Most research has proven that O-specific antiserum affords better protection against bacterial infections than does antiserum to rough mutants, even though both antisera show a similar protective activity against lethal challenge exposure with LPS. Although the J-5 antiserum has protected against the toxicity of purified LPS, the equine J-5 antiserum, prepared in the present manner, did not protect horses from endotoxemia.

References