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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=15), 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 endotoxemia.

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

[1]Serology 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 (221G for 40 minutes). All plasma was collected in 1 L plastic bags,[2] coded by number, and stored at −20C. 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 (5×10^9 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 boostered 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 plates[3] 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 IgG[4]), and substrate (40 mM ABTS, 0.05 M citrate buffer, 0.5 M H_2O_2). 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

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

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

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

[5]Dynatech 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.[6] 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.

[6]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-J5 IgG) improves clinical appearance and survival in adult horses with clinical evidence of endotoxemia.

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