Effects on plasma endotoxin and eicosanoid concentrations and serum cytokine activities in horses competing in a 48-, 83-, or 159-km endurance ride under similar terrain and weather conditions

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Objective—To determine plasma endotoxin concentration in horses competing in a 48-, 83-, or 159-km endurance race and its importance with regard to physical, hematologic, or serum and plasma biochemical variables.

Animals—83 horses.

Procedure—Weight and rectal temperature measurements and blood samples were obtained before, during, and after exercise. Blood samples were analyzed for plasma endotoxin concentration; serum antiendotoxin antibody titers; thromboxane B_2 (TxB_2) and 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) concentrations; tumor necrosis factor alpha (TNF_α) and interleukin-6 (IL-6) activities; WBC, plasma protein, lactate, serum electrolyte, and calcium concentrations; PCV; and creatine kinase activity.

Results—Detection of plasma endotoxin increased during exercise for horses competing at all distances but occurred more frequently in the 48- and 83-km groups. Plasma lactate concentration was significantly greater when endotoxin was concurrently detected. Endotoxin in plasma was not significantly associated with success of race completion. Plasma TxB_2 and $PGF_{1\alpha}$ concentrations and serum IL-6 activity significantly increased with exercise. Horses that had an excellent fitness level (as perceived by their owners) had greater decreases in serum antiendotoxin antibody titers during exercise than did horses perceived as less fit. In horses with better finish times, TxB_2 and $PGF_{1\alpha}$ concentrations were significantly greater and TNF_α activity was significantly less than that of slower horses.

Conclusions and Clinical Relevance—Endotoxemia developed during endurance racing, but was significantly correlated with increased plasma lactate concentration and not with other variables indicative of endotoxemia. Plasma TxB_2 and $PGF_{1\alpha}$ concentrations and serum TNF_α activity may be associated with performance success. (*Am J Vet Res* 2003;64:754–761)

Received January 14, 2002.
Accepted December 12, 2002.
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Supported by the Section 1433 USDA Animal Health and Disease Research Fund, the American Horse Shows Association, and the University of Georgia Research Foundation.
The authors thank Dr. Khris Crowe for technical assistance and Dr. David Smith for statistical consultation.
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Endotoxin is a lipopolysaccharide component of the outer membrane of gram-negative bacteria that is released during the rapid phase of cell growth or bacteriolysis. Thus, horses with gram-negative infections, such as neonatal septicemia, pleuropneumonia, peritonitis, colitis, and metritis, may have endotoxin released into the bloodstream as bacteria multiply.[1] Free endotoxin in horses is mostly derived from the normal turnover of enteric gram-negative bacteria in the lumen of the gastrointestinal tract. In horses, this release of endotoxin is generally harmless. However, disorders that cause ischemia or inflammation of the gastrointestinal tract may disrupt the integrity of the intestinal mucosal and allow endotoxin to enter the circulation. Whether attributable to gram-negative bacterial infection or loss of the intestinal mucosal barrier, the net effects of endotoxemia are the same.

Although endotoxin has some direct toxic effects, most of its harmful properties are associated with endogenous mediators that are released from endotoxin-activated leukocytes, principally the mononuclear phagocyte.[1] The biologic actions of these endogenous mediators are responsible for the pathophysiologic events that occur during endotoxemia. The roles of several important endogenous mediators in horses, such as thromboxane A_2, prostacyclin, **tumor necrosis factor-alpha (TNF_α)**, **interleukin-6 (IL-6)**, thromboplastin, free radicals, and platelet activating factor during endotoxemia, have been well characterized.[2-7] The effects of uncontrolled mediator release include malaise, fever, hypovolemia, cardiovascular collapse, multiple organ failure, and possible death.

Strenuous exercise can also be accompanied by an acute inflammatory response, malaise, fever, and cardiovascular collapse, which are similar to the signs of endotoxemia or sepsis.[8,9] The pathophysiologic events of the exercise-associated inflammatory response include leukocytosis, release of endogenous mediators and acute phase proteins, biochemical changes indicative of hypoxemia or tissue damage, production of free radicals, immunosuppression, activation of complement, coagulation, and fibrinolysis.[8,10] Because of these similarities, it has been speculated that the inflammatory response to exercise is compounded by endotoxemia during exercise, which results from splanchnic hypoperfusion caused by the combined effects of adrenergic receptor activation, diversion of blood flow away from abdominal viscera to supply active muscles, and hyperthermia compounded by dehydration.[8,11,12]

Indeed, intestinal ischemia[1,13-15] and hyperthermia[16] are known to cause endotoxemia and increased permeability of the intestinal tract barrier in human athletes that are undergoing long-term strenuous exercise.[a] It seems reasonable, therefore, to hypothesize that a transient alteration of the intestinal tract barrier during strenuous exercise in horses could lead to translocation of endotoxin.

Development of endotoxemia accompanied by decreasing serum antiendotoxin antibodies during strenuous exercise has been documented in human triathletes[11] and athletes competing in a 90-km marathon,[12] as well as in horses that have sprinted distances of 1 to 2.8 km[17] and endotoxemia has been reported in horses during and after endurance racing[b] and after the cross-country phase of a 3-day event.[c] In a novel field investigation of a marathon race conducted in 1988,[12] endotoxemia was more frequently reported in competitors who collapsed during the race or complained of nausea, vomiting, or diarrhea after the race, compared with competitors who finished the race or did not have gastrointestinal tract disorders. Furthermore, horses with the lowest plasma concentrations of endotoxin at the end of sprinting had completed the course before those with high concentrations.[17] The purpose of the study reported here was to determine plasma endotoxin concentration in horses competing in a 48-, 83-, or 159-km endurance race and assess hematologic and serum or plasma biochemical markers of stress, inflammation, or endotoxemia. It was hypothesized that endotoxemia would develop during endurance competition in horses and that the severity of endotoxemia would depend on the duration or intensity of exercise and the level of physical fitness of the horses. It was further speculated that endotoxemia could be associated with induction of endogenous mediator production, signs of inflammation, and poor performance.

Materials and Methods

Horses—Eighty-three horses competing in a 48- (n = 25), 83- (33), or 159-km (25) endurance ride at the Biltmore Estate in Asheville, NC, were included in the study. These horses were included in a study[18] performed concurrently to investigate the effect of endurance racing on body weight, hematologic findings, and serum or plasma biochemical findings. Horses competed on the same day at approximately the same time, in similar weather conditions, and over similar terrain. Written consent was obtained from the owners and riders of the horses before inclusion in the study.

Procedures—The day before competition, owners of the horses completed a questionnaire, as described[18]; information collected regarding each horse included the number of endurance competitions in which the horse had participated during the preceding 12 months, number of hours in transit (trailer transport) to the competition site, number of years of endurance race experience, and owner's assessment of the horse's fitness (eg, poor, average, good, or excellent). This competition was not specifically sanctioned for drug use, and owners were not asked about recent administration of medications to the horses. However, the American Endurance Ride Conference Rule Book specifies that performance of horses in competition is not to be influenced by use of any drug; abidance to this rule was on an honor system. Data regarding completion or reason for disqualification or withdrawal from competition (lameness, metabolic [development of synchronous diaphragmatic flutter, rhabdomyolysis, or poor cardiac or respiratory recovery rate], or rider decision) were collected from the riders' cards. Place of finish (eg, first, second, or third place) and lap speed data were obtained from the event organizers.

Blood samples and measurements of body weight and rectal temperature were obtained from horses before (12 to 18 hours before ride), during, and immediately after completion of the ride; sampling and data collection were conducted at distances at which competition organizers required veterinary inspection of the horses. For horses competing in the 48-km ride, data were obtained at 22 and 48 km. For horses competing in the 83-km ride, data were obtained at 19, 46, and 83 km. For horses competing in the 159-km ride, data were obtained at 19, 51, 79, and 159 km. Because horses were examined at the same location after completing course laps, all data collection occurred at a single stationary site. Measurements of weight without tack were obtained with a portable calibrated scale with a digital readout.[d] Mercury thermometers were used to obtain rectal temperatures. After thorough cleansing of the skin in the region of the jugular vein with ethanol (70%) solution, blood samples were obtained by a single venipuncture with an 18-gauge needle attached to a 20-mL syringe for determination of WBC, **plasma protein (PP)**, lactate, endotoxin, **thromboxane B_2 (TxB_2)**, **6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$)** concentrations; serum TNF_α and IL-6 activities; PCV; serum antiendotoxin antibody titer; serum sodium, potassium, chloride, bicarbonate, and calcium concentrations; and serum creatine kinase activity. Immediately after venipuncture, the needle was removed from the syringe, and aliquots of blood were first transferred to pyrogen-free borosilicate glass tubes containing USP heparin (10 U/mL of blood) for determination of plasma endotoxin concentration. Blood was then transferred to tubes containing EDTA[e] for determination of WBC and PP concentrations and PCV. For determination of plasma lactate concentration, blood was transferred to tubes containing potassium oxalate and sodium fluoride.[e] Blood was transferred to chilled tubes containing 100mM EDTA and 10µM meclofenamate (1:20 with blood) for determination of TxB_2 and $PGF_{1\alpha}$ concentrations. For the remaining serum biochemical analyses, blood was transferred to glass tubes containing no anticoagulant. After transfer to tubes, blood samples were immediately placed into temperature-controlled (4 to 8°C) electric storage coolers. Packed cell volume and PP concentration were determined on site by use of a microcentrifuge and temperature-controlled refractometer. For determination of WBC concentration, blood samples were stored in the electric coolers for ≤ 48 hours until analyzed at the authors' research laboratory at the University of Georgia. For determination of endotoxin concentration, blood samples were centrifuged on site to yield platelet-rich plasma, which was aseptically transferred to pyrogen-free borosilicate tubes and frozen on site by use of dry ice in polystyrene storage coolers. For determination of all other tests, blood samples were centrifuged on site, and platelet-poor plasma or serum was transferred to 1.5-mL polypropylene microfuge tubes and frozen on site by use of dry ice in polystyrene storage coolers.

Hematologic and serum and plasma biochemical analyses—An electronic cell counter[f] was used to determine WBC concentration. Serum and plasma samples were transported on dry ice to the authors' research laboratory and stored at –70°C until analyzed. Plasma lactate, serum sodium, potassium, chloride, bicarbonate, and calcium concentrations and creatine

kinase activity were analyzed[g] with commercially available reagents and standards at the Veterinary Teaching Hospital Clinical Pathology laboratory at the University of Georgia.

Plasma endotoxin concentration—Plasma samples were transported on dry ice to the authors' research laboratory and stored at –20°C until analyzed. Plasma samples were thawed, diluted with pyrogen-free water (1:10), and heated for 10 minutes at 80°C. Endotoxin concentration in the diluted samples of plasma was determined by means of a chromogenic limulus amebocyte lysate assay[h] in a microplate assay, which was read on an automated 96-well plate reader.[i] All samples were blanked against matched subject plasma appropriately diluted with reagent pyrogen-free water. The range of standards used in the assay was 0.01 to 0.1 endotoxin U/mL. Blanked test samples that had an **optical density (OD)** greater than the OD of the 0.01 endotoxin U/mL standard were considered to be positive for endotoxin. Mean recent recovery, interassay coefficient of variation, and intraassay coefficient of variation for this assay in the authors' research laboratory are 100 ± 32, 9, and 7%, respectively.[19]

Serum antiendotoxin antibody titer—Serum samples were transported on dry ice to the authors' research laboratory and stored at –70°C until analyzed. Antibody titers against endotoxin were quantified by an ELISA. Ninety-six-well microtiter plates[i] were precoated for 30 minutes with 10 µg/mL of poly-L-lysine in tween-20 solution (0.05%; pH, 7.2) to a concentration of 100 µL/well. The plates were washed 3 times with PBS solution containing tween-20 (0.05%). Endotoxin from *Escherichia coli* O55:B5 and J-5 mutant *E coli* O111:B4[j] were used as solid-phase antigens; 100 µL of the antigens (8 µg/mL for each antigen) was added to each well and incubated for 16 hours at 37°C on a rocking platform. Plates were washed 3 times with PBS solution containing tween-20 (0.05%), and 200 µL of test sera diluted 1:100 was added to wells in triplicate and further serially diluted 1:2. Plates were incubated for 20 minutes at 37°C on a rocking platform and washed 3 times with PBS solution containing tween-20 (0.05%). To each well, 100 µL of peroxidase-labeled goat antihorse IgG[k] (1:1,000) was added, and plates were incubated for 20 minutes at 37°C on a rocking platform. Plates were washed 3 times with PBS solution (including soaking for 90 seconds with each wash) containing tween-20 (0.05%) before addition of hydrogen peroxide substrate containing 2'-azino-bis (3-ethylbenzthiazolinesulfonic acid).[k] Twenty minutes after addition of the substrate, OD values were read on an automated 96-well plate reader[i] with the test filter set at 405 nm and the reference filter set at 450 nm. The titer was considered to be the reciprocal of the highest serum dilution at which the mean OD value from the triplicate wells exceeded twice the OD values recorded for control wells that contained no serum. Negative and positive control sera were included in each plate and most consistently had titers of 400 and 3,200, respectively. Data were adjusted in reference to the positive control titer.

Plasma eicosanoid concentration—Plasma samples were transported on dry ice to the authors' research laboratory and stored at –70°C until analyzed. One milliliter of thawed plasma was extracted on C-18 columns[l] before determination of TxB_2 and $PGF_{1\alpha}$ concentrations (the stable metabolites of thromboxane A_2 and prostacyclin, respectively) by use of radioimmunoassay, as described[7]; commercially available tracer,[m] antisera,[n] and standards[n] were used. The percent recovery of the eicosanoids from extracted plasma samples was determined by the addition of known quantities of standard to plasma before extraction.

Serum cytokine activity—Serum samples were transported on dry ice to a laboratory and stored at –70°C until analyzed. For determination of cytokines, serum was diluted 1:10 with RPMI 1640[h] medium. For determination of IL-6, samples were heat-inactivated at 56°C for 30 minutes. Serum TNF_α and IL-6 activities were quantified in 96-well microtiter plate bioassays with WEHI 164 clone-13 murine fibrosarcoma cells and murine hybridoma cell line B13.29 clone B9, respectively, as described.[5,6] Assays of serum TNF_α and IL-6 activities were standardized by use of recombinant human and murine standards, respectively, and quantified on each assay plate. One unit of TNF_α activity was defined as the dilution of test sample that caused cell lysis by 50%. One unit of IL-6 activity was defined as the dilution of test sample required to induce cell proliferation by 50%.

Statistical analyses—Statistical analyses of the data were performed with commercial software[o] by members of the Statistics Department at the University of Georgia. Data were compared by ANOVA among distance groups at each collection point. To retain data from horses that did not complete the ride in the repeated measures analyses, analyses of data with distance were performed with the mixed procedure followed by the Tukey-Kramer test. Presence or absence of endotoxin in plasma was compared among collection points and distance groups by categorical repeated measures ANOVA and compared with answers to the owner questionnaire and all other variables by logistic regression analysis or χ^2 analysis. All data were compared with place of finish by the Spearman rank correlation test. Significance was set at $P < 0.05$ for all comparisons. Data are presented as mean ± SD.

Results

In each distance group, the distances in kilometers that horses had traveled at each data collection point were noted, and the mean speed (m/min) of the horses between data collections was calculated.[18] In general, horses in the 83- and 159-km distance groups had faster lap speeds than did horses in the 48-km group. Of the 25, 33, and 25 horses that began the 48-, 83-, and 159-km rides, respectively, 24, 25, and 18 horses, respectively, crossed the finish line. However, only 22, 24, and 13 of these horses from the 48-, 83-, and 159-km races, respectively, completed the rides successfully, as judged by veterinarians after evaluation of those horses in compliance with the American Endurance Ride Conference Veterinary Handbook guidelines. Compliance with data collection at the veterinary inspection site during and after completion of the race was variable, and all data were not available for all horses and riders at each collection point. The day of the rides was warm and sunny; morning temperature and relative humidity were 7.8°C and 61%, respectively, and increased to a maximum temperature and relative humidity of 22.8°C and 100%, respectively. There was no precipitation that day.

Body weights; rectal temperature; PCV; WBC, PP, serum electrolyte and calcium, and plasma lactate concentrations; and serum creatine kinase activity—Compared with preride values, body weights and serum sodium, potassium, chloride, bicarbonate, and calcium concentrations of horses competing in each distance group were significantly decreased at certain collection points during the ride,[18] with the exception of a significant increase in the serum sodium concentration in the 159-km group during the competition. Compared with preride values, rectal temperature;

WBC, plasma lactate, and PP concentrations; PCV; and creatine kinase activity in horses competing in each distance group significantly increased at certain collection points,[18] with the exception that rectal temperature did not significantly change in the 48-km group.

Plasma endotoxin concentrations—Mean regression coefficient for the standards included in each assay plate was 0.96. Compared with preride findings, the number of horses in all distance groups that had detectable levels of endotoxin in plasma was significantly greater at collection C; this significant difference from baseline was also detected at collections D and E in the 159-km group (**Table 1**). Approximately 50% of horses in the 48- and 83-km distance groups (13/23 and 12/22 horses, respectively) had detectable endotoxin in their plasma at collection C. The overall positive sample mean plasma concentration of endotoxin during exercise was 0.19 ± 0.22 endotoxin U/mL. There was no significant difference among distance groups at any collection point.

Serum antiendotoxin antibody titer—In horses in the 83-km group, serum antiendotoxin antibody titer measured before and during the ride was significantly greater than titers in horses in the 48- and 159-km groups (Table 1). Among distance groups, there was no significant effect of collection point (distance) on serum antiendotoxin antibody titer. Horses whose owners perceived them to have an excellent level of fitness had greater decreases in antiendotoxin antibody titers from collection time B to C than did horses whose owners perceived them to have a poor, average, or good level of fitness. There were no significant effects on antiendotoxin antibody titer associated with the horses' success of completion of the ride, place of finish, number of competitions undertaken in the preceding year, number of years of endurance race experience, lap speed, or hours in transit to the competition site.

Plasma eicosanoid concentrations—Mean percent recoveries after extraction for TxB_2 and $PGF_{1\alpha}$ were 76 and 77%, respectively. In our study, intra-assay coefficients of variation for TxB_2 and $PGF_{1\alpha}$ were 5 and 3.5%, respectively. The pattern of changes in TxB_2 concentration among distance groups was not consistent (**Table 2**). Compared with preride values, plasma TxB_2 concentration significantly increased at certain distances during the ride only for horses in the 48- or 159-km groups, with the 48-km group having the largest change (collection B). Plasma $PGF_{1\alpha}$ concentration significantly increased during the ride for horses in all distance groups, but was similar to preride values at the end of the ride for horses in the 83- and 159-km groups. At collection B, plasma $PGF_{1\alpha}$ concentrations were significantly greater in horses in the 83- and 159-km distance groups, compared with horses in the 48-km group. For horses in the 83- and 159-km distance groups, the difference in plasma TxB_2 concentrations at collection C and D was significantly greater in horses that were withdrawn from the ride for metabolic reasons (D minus C mean value: 106 ± 89 pg/mL), compared with horses that were pulled for lameness (D minus C mean value: 20 ± 41 pg/mL) and horses that completed the ride (D minus C mean value: –7 ± 58 pg/mL). At collection E in horses completing the 159-km course, plasma TxB_2 concentration was significantly greater in horses that had faster final lap speeds (final 19 km of the ride), compared with those that had slower final lap speeds. Among horses in the 159-km group, plasma TxB_2 concentration was significantly greater at collections C and E, and plasma $PGF_{1\alpha}$ concentration was significantly greater at collection E in horses with a better place of finish, compared with horses that placed higher at finish. There were no significant effects on plasma eicosanoid concentrations associated with the owners' perceived fitness of the horse, number of competitions undertaken in the preceding year, years of endurance race experience of the horse, or the hours in transit to the competition site.

Serum cytokine activity—Intra- and interassay coefficients of variation for the TNF_α assay were 8.6 and 27.9%, respectively. Intra- and interassay coefficients of variation for the IL-6 assay were 5.1 and

Table 1—Plasma endotoxin and antiendotoxin antibody titer for horses competing in a 48-, 83-, or 159-km endurance race

		Plasma endotoxin concentration (endotoxin U/mL)				
Distance group	Collection point	Mean ± SD of positive samples	Minimum positive sample value	Maximum positive sample value	No. of horses with detectable endotoxin	Mean ± SD Serum antiendotoxin antibody titer
48 km	A	0.20 ± 0.11	0.13	0.28	2/24	846 ± 531 (24)
	B	0.26 ± 0.11	0.19	0.34	2/21	753 ± 406 (23)
	C	0.07 ± 0.03	0.06	0.12	13/23*	1,038 ± 721 (24)
83 km	A	0.23			1/25	1,300 ± 650[a] (33)
	B	0.10 ± 0.11	0.03	0.24	3/23	1,178 ± 641[a] (24)
	C	0.18 ± 0.23	0.07	0.89	12/22*	1,413 ± 697[a] (22)
	D	0.56 ± 0.20	0.37	0.77	3/20	1,381 ± 788 (25)
159 km	A	0.06			1/25	832 ± 523[b] (25)
	B	0.33			1/20	739 ± 363[b] (21)
	C	0.11 ± 0.04	0.08	0.21	9/19*	1,146 ± 683[b] (19)
	D	0.21 ± 0.14	0.10	0.43	5/15*	853 ± 373[b] (16)
	E	0.35 ± 0.47	0.06	1.29	6/16*	711 ± 393 (16)

[a]Significantly ($P < 0.05$) different from 48-km group at same collection time. [b]Significantly ($P < 0.05$) different from 83-km group at same collection time. *Significantly ($P < 0.05$) different from within-group preride (A) value. The number in parentheses indicates the number of samples analyzed at that time.

Table 2—Plasma eicosanoid concentrations and serum cytokine activities (mean ± SD) for horses competing in a 48-, 83-, or 159-km endurance race

Distance group	Collection point	Thromboxane B_2 (pg/mL)	Prostaglandin $F_{1\alpha}$ (pg/mL)	Tumor necrosis factor activity (U/mL)	Interleukin-6 activity (U/mL)
48 km	A	48.7 ± 42.4 (24)	177.1 ± 44.0 (24)	0.63 ± 1.72 (24)	0.04 ± 0.21 (24)
	B	329.3 ± 365.1* (22)	222.4 ± 101.7 (22)	0.94 ± 0.81 (22)	0.36 ± 1.59 (23)
	C	136.0 ± 128.9 (22)	262.2 ± 164.5* (22)	2.01 ± 4.16 (23)	1.63 ± 2.88* (24)
83 km	A	136.9 ± 270.7 (33)	179.7 ± 37.8 (33)	0.58 ± 0.98 (33)	0.00 ± 0.00 (33)
	B	122.5 ± 141.9[a] (23)	337.7 ± 137.1[a]* (23)	1.08 ± 1.32 (25)	0.34 ± 1.66 (24)
	C	118.6 ± 63.5 (23)	269.9 ± 70.9* (23)	1.04 ± 2.40 (24)	0.00 ± 0.00[a] (22)
	D	114.4 ± 82.5 (24)	221.3 ± 77.1 (24)	1.00 ± 3.12 (25)	0.28 ± 1.40 (25)
159 km	A	52.3 ± 36.3 (23)	166.1 ± 52.4 (23)	1.00 ± 0.88 (25)	0.11 ± 0.11 (25)
	B	72.9 ± 31.9[a] (23)	389.1 ± 77.9*[a] (20)	0.80 ± 1.12 (21)	0.39 ± 1.07 (21)
	C	120.8 ± 116.3* (16)	279.4 ± 74.8* (16)	0.04 ± 0.07* (19)	0.15 ± 0.64[a] (19)
	D	150.9 ± 69.9* (15)	227.0 ± 63.3* (15)	0.35 ± 0.48 (18)	0.21 ± 0.86 (16)
	E	91.6 ± 42.8 (17)	185.0 ± 56.7 (17)	0.68 ± 0.98 (17)	0.11 ± 0.46 (17)

See Table 1 for key.

13.4%, respectively. Serum TNF_α activity did not significantly change during the ride for horses in the 48- and 83-km groups, but was significantly reduced at collection C, compared with preride values of horses in the 159-km group (Table 2). Compared with preride values, serum IL-6 activity significantly increased at collection C for horses in the 48-km group, and this increase was significantly greater than values obtained at the same collection for horses in the 83- and 159-km groups. Among all horses at collections C and D, serum IL-6 activity was significantly greater for horses with more years of endurance race experience, compared with horses with fewer years' experience. Among all horses at collection C, serum TNF_α activity was significantly greater in horses that had more years of endurance race experience, compared with horses that had few years' experience. For horses ridden a distance of 159 km, serum TNF_α activity was significantly greater at collection D in horses that had slower finish times, compared with horses that had faster finish times. There was no significant effect of serum IL-6 activity on place of finish. There were no significant effects on serum cytokine activity associated with success of completion of the ride, owners' perceived fitness of their horse, number of competitions undertaken in the preceding year, lap speed, or hours in transit to the competition site.

Data correlations with plasma endotoxin concentration—Among all competing horses at collection B, plasma lactate concentration was significantly ($P = 0.027$) greater in horses in which endotoxin was concurrently detected in the plasma (mean plasma lactate concentration, 2.13 ± 0.70 mmol/L), compared with the mean plasma lactate concentration in horses in which endotoxin was not detected (1.59 ± 0.54 mmol/L). Likewise, among all competing horses, the difference in plasma lactate concentration values between collections B and A was significantly ($P = 0.024$) greater in horses in which endotoxin was concurrently detected in the plasma at collection B (mean difference in plasma lactate concentration, 1.09 ± 0.67 mmol/L), compared with the values obtained horses in which endotoxin was not detected at collection B (mean difference in plasma lactate concentration, 0.59 ± 0.44 mmol/L). Among all competing horses at collection C, horses in which endotoxin was detected in the plasma had significantly ($P = 0.018$) lower mean rectal temperature (37.9 ± 0.3°C), compared with the temperature of horses in which endotoxin was not detected (38.2 ± 0.5°C). There were no other significant effects of the presence of endotoxin on the remaining hematologic and serum or plasma biochemical data. For all horses, there was no correlation of the presence of endotoxin in the plasma on completion of the ride with the reason for withdrawal, owners' perceived fitness of their horse, number of competitions undertaken in the preceding 12 months, number of years of endurance race experience of the horse, lap speed, number of hours in transit to the competition site, or place of finish.

Discussion

In our study, endotoxemia, defined as the presence of detectable endotoxin in plasma, developed in approximately 50% of horses during or on completion of endurance racing. This finding is consistent with that of another study[b] investigating the incidence of endotoxemia in horses in endurance competition by means of a whole blood assay. Although the hypothesis that endotoxemia develops in horses during endurance competition was correct, there was no evidence that the degree of endotoxemia was associated with the duration of endurance exercise or the horse's level of fitness (as perceived by the owner). It could be argued that some of the endotoxin detected in the plasma was spurious contamination. However, the most difficult venipuncture was that performed at the preride collection when horses were less distracted and not fatigued from exercise, but the incidence of positive preride samples was low; furthermore, significant increases in endotoxin concentration were detected during exercise, and it seems more likely, therefore, that increases in plasma endotoxin concentration were indeed from an endogenous source. Endotoxemia during exercise has been detected in humans and horses in other field investigations,[11,12,17,20,b,c] though information regarding its clinical significance is limited. The second hypothesis of our study was that exercise-induced endotox-

emia in horses was associated with induction of endogenous mediator production, detection of hematologic and serum or plasma biochemical markers of inflammation, and reduced performance. Our data indicated that only 1 of the variables evaluated in blood samples could be correlated with detection of endotoxin in the plasma; in horses in which endotoxin was detected in plasma, there was a mild but significant increase in plasma lactate concentration. In horses after the cross-country phase of a 3-day event,[c] increased plasma lactate concentrations were also significantly correlated with the presence of endotoxin in plasma. In those event horses, mean plasma lactate (14.9 mmol/L) and mean endotoxin (0.29 U/mL) concentrations were considerably greater than the mean values of those variables obtained in our endurance exercise study. Although plasma lactate concentration is not a direct measure of the degree of blood flow to or oxygenation of any specific tissue, it does provide some evidence of the intensity of exercise and extent of anaerobic glycolysis. The correlation of endotoxemia with increased plasma lactate concentrations lends credence to the hypothesis that endotoxemia can develop as the vigor of exercise increases or the effect of exercise on an individual intensifies.[8]

There was no association between detection of endotoxin in plasma at any collection point (ie, with distance) and completion of the race, reason for withdrawal, place of finish, total distance ridden, owners' perception of their horse's fitness level, or years of endurance race experience of the horse. These findings are consistent with those of studies[11,20,21] of human athletes in which the development of endotoxemia during exercise could not be correlated with success of performance. The overall low mean plasma endotoxin concentration and lack of correlation to hematologic and serum or plasma biochemical markers of endotoxemia or inflammation, fitness, or place of finish make it difficult to assess the clinical importance of exercise-induced endotoxemia and its relationship to the inflammatory response to exercise or performance under the conditions of endurance exercise in our study. However, because horses competing in a 3-day event had higher mean plasma lactate and endotoxin concentrations than values obtained in the endurance horses of the study reported here, further investigation of these variables during types of exercise that induce significant lactic acidosis in horses is warranted.

Although our data appear to refute the clinical importance of endurance exercise-induced endotoxemia, it should be noted that endotoxin rapidly binds to naturally occurring ligands after it enters the circulation. Ligand-bound endotoxin cannot be detected by the assay used in our study. Difficulty in detecting total (bound and unbound) endotoxin in the blood and its rapid clearance from the circulation may contribute to underestimation of its presence in blood or role during exercise. To overcome some of these assay limitations for detection of endotoxin in plasma, antiendotoxin antibody titers were measured to provide indirect evidence of endotoxemia. Decreases in serum antiendotoxin antibody titer during exercise have been identified in marathon runners, triathletes, and sprinting Thoroughbreds[11,12,17]; the decreases correlated with detection of endotoxin in plasma, intensity of exercise, and performance. It was further suggested in those studies[11,17] that conditioned athletes undergoing regular strenuous exercise had high antiendotoxin antibody titers from self-immunization during training-induced endotoxemia. In the study reported here, there was no correlation of serum antiendotoxin antibody titers with plasma endotoxin concentration, place of finish, or years of endurance race experience of the horse. These results are similar to those of a more recent study[20] in marathon runners in which there was no correlation of exercise-induced endotoxemia with decreases in antiendotoxin antibody titer. In that study, serum antiendotoxin antibody titers were highly variable and depended on the type and source of antigen used in the assay. In our study, only 2 solid-phase antigens (phenol-extracted *E coli* and core antigen) were used, which may have contributed to failure to optimally detect antibodies. Changes in total water balance associated with dehydration during exercise also may have contributed to the observed changes in antiendotoxin antibody titers. Nonetheless, horses in the 83-km group had significantly greater antiendotoxin antibody titers before and during the competition than did horses in the 48- or 159-km groups. In addition, at certain distances, decreases in serum antiendotoxin antibody titer in horses that were perceived by their owners as being well conditioned were significantly greater than titers in horses perceived by their owners as being less fit. The importance of these findings is unknown but may suggest that moderate exercise in well-conditioned horses results in both endotoxemia and self-immunization.

Exercise-induced increases in plasma TxB_2 and $PGF_{1\alpha}$ concentrations have been reported in horses and humans,[22-27] although the prostaglandin affected and the magnitude of change differs among studies and may be attributed to differences in the type and intensity of exercise and timing of blood sampling. Other studies[23,24,26,28] investigating the effect of blockade of eicosanoid synthesis on the physiologic response to exercise also present conflicting results. To the authors' knowledge, investigation of eicosanoid synthesis with concurrent measurement of plasma endotoxin concentrations during exercise has not been previously undertaken. In the study reported here, plasma TxB_2 and $PGF_{1\alpha}$ concentrations increased with exercise, but neither correlated with detection of endotoxin. Although endotoxin is a potent stimulus for eicosanoid synthesis, it does not appear to be the main cause of eicosanoid synthesis during this type of exercise. Other potential sources of thromboxane and prostacyclin during exercise are shear stress or catecholamine-activated platelets and endothelium.[22,29] Vessel wall damage, including that which occurs during venipuncture, may also trigger eicosanoid release. In a sedentary group of horses undergoing repeated venipuncture, Hinchcliff et al[23] found that plasma TxB_2 concentration increased significantly after 4 consecutive venipunctures performed in a period of 75 minutes. There was no effect of repeated venipuncture on plasma $PGF_{1\alpha}$ concentration. A sedentary population of horses was not evaluated in our study; thus, the effect of venipuncture on plasma TxB_2 concen-

tration cannot be clearly separated from the effect of exercise. Interestingly, plasma TxB_2 or $PGF_{1\alpha}$ concentrations were significantly greater in horses that placed well at the completion of the race, compared with horses that placed poorly. Although it seems clear that eicosanoids increase during exercise, the physiologic relevance of these findings remains uncertain. The correlation of increased plasma eicosanoid concentrations with improved performance cannot be ascertained from our data alone.

Exercise-induced changes in TNF_α and IL-6 activities in humans have been reported,[10,30-35] although the cytokine affected and the magnitude of change in plasma or urine cytokine concentration differ among studies; the changes are typically subtle. Of all the cytokines evaluated, IL-6 appears to consistently increase with exercise, and measurement of its activity provides the most reliable results.[32,33] To the authors' knowledge, serum cytokine activity has not been quantified in exercising horses. A significant increase in serum cytokine activity during exercise was detected only for IL-6 in the 48-km group of horses at the end of competition. There was no correlation between the detection of endotoxin in the plasma and serum cytokine activity. Because endotoxin is a potent inducer of TNF_α and IL-6 synthesis, it seems unlikely that endotoxemia during exercise is solely responsible for the observed increase in serum cytokine activity.

Direct correlations of cytokine synthesis with parameters of performance or excessive stress have not been clearly demonstrated in humans, although inhibition of cytokine synthesis is found most reliably after prolonged and excessive exercise.[32] In the horses of the study reported here, increases in plasma TNF_α and IL-6 activities were significantly associated with increased years of endurance race experience. In humans, the stress of exercise results in concentrations of glucocorticoids that suppress TNF_α production,[36] and perhaps the greater cytokine response in horses with more years of race experience is an indirect indication that these more experienced equine athletes were less stressed. Plasma TNF_α activity was significantly greater in horses that placed better at finish in the 159-km group, which may indicate that horses with greater TNF_α activity performed better.

Endotoxemia develops during endurance racing in horses. Our data indicated an association of endotoxin and mild increases in plasma lactic acid concentration, but there were no clear relationships between endotoxemia and performance, years of race experience of the horse, and hematologic and serum or plasma biochemical markers of endotoxemia or inflammation. However, because of the high incidence of endotoxemia during exercise, the potential risk that it could exacerbate the inflammatory response to exercise in horses should be considered, especially in horses that are heat-stressed, over-exerted, hypoxemic, or poorly conditioned.

[a]Moses F, Singh A, Smoak B, et al. Alterations in intestinal permeability during prolonged high intensity running (abstr). *Gastroenterology* 1991;100:A472.

[b]Barton M, Williamson L. Horses become endotoxemic during endurance racing (abstr). *J Vet Intern Med* 1995;9:225.

[c]Williamson L, Barton M. Endotoxemia in 3 day event horses (abstr), in *Proceedings*. Equine Exer Physiol Res Meet 1996;12.

[d]Allflex USA Inc, Dallas, Tex.

[e]Vacutainer Systems, Becton-Dickinson, Franklin Lakes, NJ.

[f]Baker Instrument Co, Allentown, Pa.

[g]Abbott Laboratories, Irving, Tex.

[h]QCL-1000 Chromogenic LAL, Biowhittaker Inc, Walkersville, Md.

[i]Immulon 1 96-well microtiter plates, Dynatech Laboratories, Chantilly, Va.

[j]List Biological Laboratories Inc, Campbell, Calif.

[k]Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.

[l]Bond Elut C-18 columns, Analytichem International, Harbon City, Calif.

[m]New England Nuclear, Boston, Mass.

[n]Advanced Magnetics Inc, Cambridge, Mass.

[o]SAS, SAS Institute Inc, Cary, NC.

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