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Systemic effects of Escherichia coli lipopolysaccharide-induced endotoxic shock in the horse

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SYSTEMIC EFFECTS OF ESCHERICHIA COLI LIPOPOLYSACCHARIDE INDUCED ENDOTOXIC SHOCK IN THE HORSE

Iowa State University PH.D. 1981

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Systemic effects of *Escherichia coli* lipopolysaccharide-induced endotoxic shock in the horse

by

Darryl Henry Patrick

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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GENERAL INTRODUCTION

Preamble

Gram-negative bacterial extracts have the ability to cause profound effects on both man and animals. The bacterial product or endotoxin exerts its pathogenic effect through the outer lipopolysaccharide portion of the cell wall. Experimental systemic exposure to bacterial endotoxin causes activation of most humoral and cellular defense mechanisms. The hyperactive systemic activation of these systems causes vascular alterations resulting in circulatory shock.

The role of bacterial endotoxin in the pathogenesis of natural disease syndromes has been demonstrated within the past decade. Systemic manifestations of bovine coliform mastitis such as neutropenia and temperature elevation are due to mammary gland absorption of endotoxin. In rabbits, typhilitis and acute diarrhea are associated with cecal growth of nonenterogenic Escherichia coli. Lesions are reproduced using intravenous inoculation of freeze-thaw (endotoxic) extracts.

In equine species, bacterial endotoxin absorption has been suggested in the pathogenesis of several syndromes. Carbohydrate overload in the horse results in quadrapedal laminitis. The cause is postulated to be enteric bacterial changes and endotoxin absorption. Colitis X (Exhaustive

shock syndrome) and one syndrome of Salmonellosis are characterized by acute circulatory collapse. The cause in both cases is thought to be endotoxic shock.

Experimental equine endotoxemia studies have emphasized hemodynamic, metabolic, and hematologic alterations. Changes observed include transient arterial hypertension, pulmonary hypotension, pyrexia, neutropenia with development of a neutrophilia, metabolic lactic acidosis, hypoglycemia, and irreversible shock. Necropsy findings have been limited. Lesions observed include widely scattered petechial and ecchymotic hemorrhages throughout the body, pulmonary congestion and edema, and adrenal cortical necrosis.

In animal species other than the horse, the dynamic changes observed either induce or are caused by vascular injury and hemostatic alterations manifested as disseminated intravascular coagulation and thrombosis. The location, severity, and development of these pathologic changes vary between species. The relationship of bacterial endotoxins as a cause of natural disease in the horse can only be conjectured until pathologic changes are characterized and their course of development examined. Similarities and differences between natural and experimental syndromes can then be more crytically examined.

OBJECTIVES

The objectives of this study were to 1) determine the clinical, hematological, and hemostatic manifestations of *E. coli* lipopolysaccharide-induced endotoxemia in the horse 2) characterize the lesions and 3) examine the potential role of endotoxemia in acute equine colic-shock syndromes.

DISSERTATION FORMAT

This dissertation is presented in alternate format and includes 2 manuscripts to be submitted to the American Journal of Veterinary Research. The manuscripts are presented in the format required by the American Journal of Veterinary Research except for footnote notation which conforms with the Graduate College Thesis Manual (Iowa State University, 1979). References cited are at the end of each manuscript and conform to journal format. The manuscripts are preceded by a general introduction that includes a preamble, objectives of the study, and critical review of the literature. General discussion and conclusions of the study follow the last manuscript. Additional literature references cited refer to citations in the general introduction and general discussion and are compiled in journal format consistent with that used in the manuscripts.

The Ph.D. candidate, Darryl H. Patrick, was the principal investigator for each of the studies and is the senior author for each of the manuscripts. Co-authors with direct but limited involvement in each study and specific assistance of others are indicated in the by-line and footnotes of each manuscript.

LITERATURE REVIEW

Bacterial Endotoxins

Historical aspects Initial studies of endotoxins began prior to the recognition of bacteria as an etiologic agent. In 1856, Panum (reviewed by Milner et al.)[1] studied the role of putrification and disease. He defined this response as 'putrid intoxication' and set out to isolate the toxic principle from feces, blood, and tissues. A toxic substance was found in the filtered and cleared biological samples. The extract could be resuspended in water but not alcohol and was heat stable. The aqueous suspension would cause a pyrogenic response at low doses and 0.12 gm would kill a dog.

Nearly 100 years ago, bacterial extracts were found to induce fever in both animals and man. The term 'bacterial pyrogens' was used to define these products from killed bacteria (reviewed by Westphal et al.)[2]. In 1892, Pfeiffer discovered that in certain bacterial strains, a toxin could be produced only upon death and lysis of bacterium[3]. He called this substance endotoxin because it is part of the living substance of bacteria and only liberated upon disintegration of the cell.

Continued research on bacterial endotoxins produced conflicting results because of different crude procedures of toxin extraction. Selective extraction procedures of the endotoxic substance provided the basis for specific biologic

and chemical property studies. Boivin and Mesrobeanu, in 1935,[4] using cold trichloroacetic acid were able to collect a potent endotoxin extract made up primarily of lipopolysaccharide but which contained proteins and free lipids. Westphal, Luderitz, and Bister[5] used a hot aqueous phenol solution to extract protein-free lipopolysaccharide. The extract still had potent endotoxic effects.

Numerous other extraction procedures have been developed. These include an EDTA extraction procedure by Levie et al.[6], diethylene glycol extraction by Morgan[7], and aqueous ether extraction by Ribi et al.[8] All these procedures yield bacterial lipopolysaccharides with varying degrees of purity.

Chemical composition Bacterial endotoxins are cell wall extracts of Gram-negative organisms. The cell wall consists of an outer and inner membrane.[9] The inner membrane (cytoplasmic membrane) has a trilaminar structure made up of globular proteins in fluid mosaic of phospholipids. The outer membrane consists of a rigid internal peptidoglycan layer; a middle layer of lipoproteins and phospholipids; and an outer lipopolysaccharide layer (LPS).[10,11]

Braude (in Winholz)[12] defines endotoxin as "lipopolysaccharide-protein complexes contained in the cell wall of Gram-negative bacteria". Purified lipopolysaccharide is the toxic component of a bacterial endotoxin.[13] The LPS molecule consists of lipid portion, termed Lipid A by Westphal and Luderitz[14], and a repeating chain of hexose subunits in a

polysaccharide portion.[10,11,15] Even and odd numbered length long chain fatty acids are ester and amide linked to the B-1-6-diglucosamine backbone of Lipid A.[11,15,16] Pyrophosphate groups make the lipid structure cationic.[16] The lipid part of LPS is structurally similar in Gram-negative species.[15,16] The polysaccharide portion has two regions;[11] a core polysaccharide,[17,18] and the O-antigen[19,20] polysaccharide. The core polysaccharide is distinct for groups of Gram-negative bacteria.[21]

The core structure consists of repeating units made up of hexoses, heptoxes, phosphorylethanolamine, and a unique deoxysugar, 2-keto-3-deoxyoculosonate (KDO).[17,18,22]

The O-antigen is an oligosaccharide of 3 or 4 different hexoses, and is specific for each type of Gram-negative bacteria.[19,20] The number of repeating units in the O-antigen oligosaccharide will vary in smooth or rough strains of bacteria.[21,23]

Westphal and co-workers theorized that the lipid fraction of LPS was responsible for the endotoxic effects.[14,24] In 1966, Luderitz and Westphal,[25] and others[11,24] discovered that a mutant strain of Salmonella minnesota (RE 595) retained endotoxic properties. RE 595 has only Lipid A because the organism lacks the linkage enzyme necessary to bind the lipid and polysaccharide portions together.[26] The water solubility and antigenic properties of LPS are associated with the polysaccharide component.[27]

Endotoxin is produced by death and lysis of Gram-negative bacteria. Under certain growth conditions, blebs of the cell wall can be liberated which make the media toxic without cell death.[28,29,30] Examples are Salmonella[28] or Pseudomonas[29] species which are grown in media with limited phosphate and protein-synthesis-inhibited Escherichia coli cultures.[30] Under these conditions, these bacteria produce a toxin similar to an exotoxin. Therefore, Gram-negative bacterial extracts classically defined as endotoxin would better be classified as lipopolysaccharide containing toxins.

Endotoxemia and Host Responses

Cellular reactions in endotoxemia Endotoxin changes many cell types[31,32,33,34] but its actions on cells of the myeloid and reticuloendothelial systems (RES) are responsible for endotoxic shock.[32,33]

Fixed macrophages of the liver are considered responsible for uptake and degradation of circulating endotoxins.[35,36] Nolan postulated that constant low grade exposure to endotoxin from the gastrointestinal tract stimulates cells of the reticuloendothelial system in the liver.[37] If other factors cause hepatic injury, the impairment of function allows endotoxin to enter peripheral circulation and induces systemic injury.[38,39,40]

Macrophages treated with endotoxin respond in two stages. First, there is an initial depression of phagocytosis and

foreign material degradation.[41] This is followed by a surge in cellular activity.[42] Anaerobic metabolism is stimulated with increased glucose utilization and lactic acid production.[42] Phagocytosis is enhanced.[42,43] There is an increase in lysosomal enzyme production with release of lysosomes into the surrounding media.[44] Numerous mediators are produced including colony stimulating factor, collagenase, and glucocorticoid antagonizing factor. Macrophage lysosomal changes and mediators produced are reviewed in depth by Allison et al., 1973[44] and Berry, 1978.[45]

Endothelial cells react to endotoxin in a dose related manner. Using fluorescent antibody labeling, sublethal doses of endotoxin are rapidly removed from circulation by capillary endothelium.[46,47] Higher endotoxin dosages cause endothelial swelling, disruption of tight junctions, and loss of vascular integrity.[48,49,50]

Endotoxin, in vivo, produces a rapid transient neutropenia followed by leukocytosis.[51,52] The transient leukopenia is caused by a shift of neutrophils from the circulating granulocyte pool to the marginating granulocyte pool and the sequestration in capillaries of visceral organs, primarily the lungs.[53,54] Both complement independent[55] and dependent[56] mechanisms cause vascular endothelial injury and greater adhesiveness of neutrophils.[57] Leukocyte sequestration enhances organ damage.[54] In the monkey, pulmonary injury and shock lung is observed.[54] within 15 minutes after

infusion, sequestration of granulocytes occurs with loss of specific granules. By 1 hour, marked degranulation and fragmentation of neutrophils is observed. Damage to vascular endothelium becomes pronounced.[54]

In vitro, neutrophils exposed to endotoxin have increased cellular activity similar to macrophages.[57] They have changes in basal metabolism including increased glycolysis,[58] a stimulated hexose-monophosphate shunt,[59] and marked decrease in ATPase activity.[60] Neutrophil phagocytosis and migration are altered.[61]

The most important mediator released by leukocytes is leukocytic endogenous mediator (LEM).[45,62] LEM acts as an endogenous pyrogen. Additional biological effects[45] include: lowered serum concentrations of iron and zinc, lysosomal release, and increased serum levels of alpha globulin.

Leukocytosis is due to endotoxin-induced myeloid stimulation mediated by colony stimulating factor (CSF).[45,62] This factor promotes the release of immature leukocytes from the bone marrow maturation pool.[63]

In recent years, effort has been put into the study of the immunologic effects of lipopolysaccharides. Lipopolysaccharide stimulates B-lymphocytes to dedifferentiate and increase mitoses.[64,65] The relationship of endotoxin to immunity and resistance is reviewed by Rudbach, 1976,[66] and Morrison and Ryan, 1979.[67] Lymphocytes do play a role in the acute response to endotoxin. Mergenhagen et al., 1976,[68]

demonstrated *in vitro* an amplification effect of B-lymphocytes on macrophages. B-lymphocytes enhanced the uptake of C^{14}-glucosamine in macrophages and produced a chemostatic factor for macrophages.[68] This factor was isolated in the supernatant similar to a T-lymphocyte lymphokine. T-lymphocyte depleted sheep had near normal myocardial contractibility and pO_2 compared to the endotoxin exposed controls.[69]

Platelets in most species respond to bacterial endotoxins like neutrophils. Five minutes to 1 hour after endotoxin infusion, there is a marked drop in circulating platelets.[70,71] Platelets become sequestered in thrombosed capillaries of lung, liver, and other visceral organs.[72,73] Platelet factor 3 (PF3)[74] and serotonin[75] are released from activated thrombocytes. The role of platelets in endotoxemia is controversial. **Rodriquez-Erdmann** was able to induce a generalized Shwartzman reaction by infusion of PF3 and endotoxin.[76] From et al. treated dogs with estradiol cypionate to deplete platelets.[77] Hemodynamic response, mortality, and necropsy findings were similar in thrombocyte depleted and control animals exposed to endotoxin.[77] Margaretten and McKay studied the development of a generalized Shwartzman reaction in thrombocyte depleted rabbits.[78] Platelet depleted animals did not develop glomerular thrombi and it was concluded that platelets were necessary.[78] Busulphan-induced neutropenic and thrombocytopenic animals did not develop intravascular coagulation when given

endotoxin.[79,80] In neutrophil transfused animals, thrombosis occurred.[79] Platelet replacement had no effect.[80]

Humoral mediators in endotoxemia The principal humoral mediators activated in endotoxic shock are the complement system (alternate and classical), coagulation scheme (extrinsic, intrinsic, and fibrinolytic), and kinin system. In addition, vasoactive amines and prostaglandins are also synthesized and released.

Endotoxin infusion stimulates both the coagulation and fibrinolytic systems.[81,82,83] This reaction is characterized by disseminated intravascular coagulation (DIC) in a generalized Shwartzman reaction and reviewed in several recent publications.[81,82] Both the intrinsic and extrinsic pathways of the coagulation schemes are activated.[79,84,85,86,87,88] Factor VII of the extrinsic cascade[79,84,85] and Factor XII of the intrinsic cascade[86,87,88] are activated by both indirect and direct mechanisms. Efforts to show direct activation of Hageman Factor (Factor XII) have been accomplished with two different methods. Morrison and Cochran were able *in vitro* to prove direct binding of Hageman Factor to endotoxin.[87] The complex could convert pre-Kallikrein to its active form and reduce clotting time of Factor XII-deficient plasma.[88] Kinins are detectable in serum of animals treated with low doses (3 ng/kg) of endotoxin.[89] Activation of the Hageman Factor is necessary for kinin synthesis.[86] Muller-Berghaus and others feel that indirect activation of Factor XII is the most

important route of coagulation activation.[90] Coagulation is initiated through complement fragment and via a mediator released from platelets, macrophages, endothelial cells, and neutrophils.[90]

Shen et al. were able to induce DIC in rabbits that were selectively depleted of Factor VII.[91] McGrath and Stewart presented morphological evidence of vascular damage exposing underlying basement membranes.[92] Factor VII is activated by tissue factors from damaged vessels.[79] The concept of extrinsic pathway activation aiding in amplification of DIC was shown in a study comparing the effects of endotoxin in hereditary deficient Factor VII dogs.[79] Vascular thrombosis was prominent in control animals but in Factor VII deficient animals thrombosis was markedly reduced.[79]

The role of DIC in the course of endotoxic shock is unclear. From et al. were unable to show any difference in hemodynamic or humoral responses to endotoxin in either normal or defibrinated dogs.[93] Bleyl postulated that thrombus formation is due to lack of adequate fibrinolysis and that consumptive coagulopathy is the result in endotoxemia.[83] In normal hosts, endotoxemia produces an activation of coagulation with formation of fibrin monomers which are rapidly degradated via the RES and fibrinolytic systems.[83] These fibrin split products have highly reduced coagulability, antithrombin activity, and antipolymeric activity.[83]

In conclusion, endotoxemia induces activation of both the intrinsic and extrinsic coagulation cascades. The development of vascular thrombosis is dependent on the inability of the fibrinolytic pathway to degrade monomeric fibrin units.

Activation of complement by endotoxin occurs via both the alternate and classical pathways.[94,95,96,97] The importance of complement in the outcome of endotoxic shock cannot be disputed. Spink and Vick found that in dogs, lethality was related to complement activation.[98] They could prevent death by transferring heat-treated plasma to dogs prior to endotoxin infusion.[98] McCabe, in a case study of bacteremia due to Gram-negative bacteria, was able to correlate the frequency of shock and fatality with the degree of lowering of C3 levels.[99]

Experimentally, anti-C3 antibody depleted C3 from the plasma of guinea pigs and prevented the development of hemorrhagic lesions in endotoxemia.[100] Immediate hypotensive changes are caused by C3 and terminal complement factors.[101] The role of complement factors in the development of either the generalized or localized Shwartzman reaction is disputed with contradictory experimental results.[102,103]

Complement activation of the classical pathway is associated with two distinct mechanisms. The first is via antibody-antigen activation of C1.[104] Kostka and Sterzl attempted to activate complement in serum from adult and precolostral (IgG-free) pigs.[104] Although both groups had similar complement titers, serum complement activation

occurred only in adult pigs' sera.[104] This suggested a role for antiendotoxin antibody. Muller-Eberhard et al. demonstrated endotoxin preparations could bind directly to C1.[105] Bacterial endotoxin, specifically the lipid A, could bind to C1 and activate the classical complement pathway in an antibody independent method.[106]

The terminal factors of complement are activated in C3 deficient or anti-C3 treated serum.[107-108] This occurs through the alternative, properidin pathway. The polysaccharide portion of LPS is necessary for initiation of the alternate pathway.[109]

Histamine is elevated in endotoxic shock.[110,111] With lethal injections of E. coli endotoxin, there is a rapid (as soon as 30 to 60 seconds) decrease in blood pressure which occurs at the same time as a rapid release of histamine.[111]

Kinins have been suggested and contested as the mediator of irreversible shock. Urbaschek and Urbaschek could not find any lowering of kininogen levels or increases in kinins.[110] Others were able to show a direct increase of bradykinin in plasma mixed with Salmonella abortus equi endotoxin.[89] Nies et al. demonstrated in rhesus monkeys after endotoxin administration, a decrease in kininogens.[112] Administration of trasylol, a proteinase inhibitor of kinin synthesis, has increased survival rate in endotoxemia.[113]

Prostaglandins with their potent vasoactive properties have been implicated in the development of endotoxic shock.[114]

Increased circulating levels of A, E, and F groups of prostaglandins develops.[115] Mlczoch et al. suggested that production of vasodilator prostaglandins is stimulated by the direct effects of endotoxin on pulmonary and systemic circulation, preventing hypoxic pulmonary vasoconstriction.[115] Weir et al. using prostaglandin synthesis inhibitors, were able to prevent both vasoconstriction and vasodilation in endotoxemia.[116]

Hemodynamic and vascular alterations Vascular and hemodynamic changes are the main manifestations of bacterial endotoxemia. For this reason, numerous experiments have been accomplished in an effort to understand these changes. Several reviews have been written in an effort to collate experimental results.[111,117,118,119]

Intravenous administration of bacterial endotoxin causes hypotension of such severity to be defined as shock.[117] Two classic animal models are endotoxic shock in the dog and monkey.[111,117]

The initial response to endotoxin in the dog is an early rapid fall in blood pressure.[120] Early systemic hypotension is caused by hepatic and splenic venous constriction,[121] decrease in venous return,[122] and drop in cardiac output.[123] Portal venous pressure rises sharply[121] while central venous pressure drops.[122] The small intestine becomes congested as a result of portal hypertension.[124] Visceral organ congestion is only transient and recovery of systemic pressure is because

of partial restoration of venous return.[117] The small intestine hemodynamics do not return to normal[125] and there continues to be fluid movement into the interstitium.[124] The pulmonary vascular response is primarily transient pulmonary hypertension and increased vascular resistance.[117,126] Both pulmonary venous and arterial resistance occur with a capillary bed congestion.[126] This response is also transient.[126] Renal blood flow also is diminished with decreased glomerular filtration rate.[127] This response is caused by adrenergic nervous system activation which causes decreased renal cortical blood flow.[128]

Following a period of partial recovery, the dog will become progressively worse with a second drop in blood pressure[120] and cardiac output.[129] This response is caused by decreased systemic vascular resistance, decreased cardiac output, and increased intravascular and extravascular pooling.[120,129] In the mesenteric blood vessels, arterial pressure exceeds mesenteric blood flow and this results in increased mesenteric vascular resistance with further intravascular-extravascular fluid shift.[130]

The monkey does not respond like the dog to bacterial endotoxins.[111] It has a gradual decrease in systemic arterial pressure and cardiac output.[131] Hepatosplenic pooling and portal hypertension are not characteristic features.[132] Mesenteric blood vasculature responds with vasodilation.[130,133] Increased arterial resistance and pressure do not develop.[111]

Extracellular fluid movement does not occur.[130] The progression to irreversible shock is caused by systemic vasodilation and vascular pooling with a pronounced decrease in vascular resistance.[134] This suggests a vasodilator mediator such as kinin. Nies et al. reported that kinin concentration was elevated and plasma kininogen was decreased in the early phase of endotoxemia.[112] Progressive systemic vasodilation results in visceral organ ischemia.[132] The small intestine therefore on post mortem examination is pale and ischemic as compared to the congested hemorrhagic intestine of the dog exposed to endotoxin.[130] Poor visceral organ perfusion results in metabolic acidosis with elevated blood lactate and blood pH.[111] Progressive metabolic changes and hypoxia result in irreversible shock and death.[111]

Endotoxemia in the horse The systemic effects of bacterial endotoxins and their potential role in equine disease were first suggested by Rooney et al. in the description of Colitis X.[135] In the first experimental study, Aerobacter aerogenes endotoxin was given intraperitoneally.[136] Clinical pathological response included hemoconcentration, leukopenia, and decreased blood glucose.[136] Post mortem findings were inconclusive with petechial hemorrhages on most visceral organs being the only significant finding.[136]

The first studies on the hemodynamic manifestations of endotoxemia were presented in the 1970s first in anesthetized

and then conscious ponies.[137,138,139] In anesthetized ponies given both rapid and slow intravenous infusion of endotoxin, there is a period of early systemic arterial hypotension with accompanied pulmonary and central venous hypertension.[137,138] This is followed by a gradual prolonged development of systemic hypotension and near normal pulmonary hemodynamics.[137,138] Hemoconcentration and leukopenia occurred.[137] In conscious ponies, similar hemodynamic changes were observed but hematologic changes were slightly different.[139] Leukopenia was followed by development of a neutrophilia.[139] Hypoglycemia and a febrile response were also observed.[139] In another experiment in anesthetized ponies, significant rises in blood lactate and pyruvate with increased arterial-venous oxygen difference indicated circulatory shock and systemic anaerobic metabolism.[140] Intravenous and intraperitoneal infusion of Escherichia coli organisms induced similar hematologic and hemodynamic changes, but pulmonary lesions predominated at post mortem examination.[141] The lesions included interstitial and alveolar pneumonia with pulmonary edema, acute inflammatory cell infiltration, and atalectasis.[141]

These experimental models produced clinical patterns similar to acute abdominal crisis in the horse.[142,143] The duration of experiments was shorter with more abrupt recovery or death than natural colic syndromes. Multiple intraperitoneal or sublethal endotoxin infusion does produce a more

gradual and sustained response more closely resembling clinical disease syndromes.[144] The experiments were limited to hematologic, hemodynamic, and metabolic alterations but failed to address the question of the source of bacterial endotoxin in clinical syndromes.

The alimentary tract is the principal site where numbers of organisms are large enough to produce endotoxin sufficient to cause shock syndromes.[145] Oral inoculation of endotoxin has failed to induce endotoxic shock.[146] Clinically, horses with intestinal strangulation or obstruction have shock signs with fluid and electrolyte loss.[147] Experimentally, one of four syndromes of Salmonellosis has clinical signs of shock, although Salmonella sp. organisms do not produce a bacteremia.[148] Preliminary studies in two horses indicated that carbohydrate overload induces elevation of blood lactate levels with development of circulatory changes manifested in laminitis.[149] This suggests a role for Gram-negative bacterial endotoxins in the development of systemic disease in the horse.

CLINICAL-PATHOLOGICAL MANIFESTATIONS OF ACUTE *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE-INDUCED ENDOTOXEMIA IN THE EQUINE

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the American Journal of Veterinary Research

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The authors thank Ms. J. Carter, Dr. S. Whipp, and Dr. D. Cassidy (United States Department of Agriculture) for laboratory facilities, equipment and technical assistance, and Dr. G. Booth and Mr. P. Christensen for assistance in statistical analysis.

SUMMARY

Sixteen ponies were given E. coli lipopolysaccharide intravenously (40 µg/kg in 1 liter of saline each) to evaluate clinical and hematological effects. Clinical responses included intermittent diarrhea, pyrexia, cyanosis, laminitis, and elevated heart and respiration rates. Initial neutropenia with subsequent neutrophilia, persistent lymphopenia, and prolonged thrombocytopenia were seen. Hemoconcentration and hyperproteinemia occurred within 15 minutes post infusion with subsequent development of hypoproteinemia. A gradual progressive consumptive coagulopathy with prolonged activated clotting time, prothrombin time, and activated partial thromboplastin time were observed. These studies suggest that equine endotoxic shock involves peripheral circulatory insufficiency with extraluminal fluid movement followed by development of disseminated intravascular coagulation.

INTRODUCTION

A systemic role has been reported for endotoxin in the pathogenesis of Gram-negative bacterial diseases such as bovine mastitis and porcine enteritis.[1,2] Experimentally, bacterial cell wall extracts in the form of purified lipopolysaccharide or crude endotoxin induce hematological, hemostatic, and hemodynamic alterations manifested as septic shock.[3,4]

In horses, carbohydrate overload allows for production of large quantities of Gram-negative endotoxin in the cecum and colon.[5] An enteric source of endotoxin has been postulated for the pathogenesis of alimentary laminitis and Colitis X (Exhaustive shock syndrome).[6,7]

Experimental equine endotoxemia produced by intravenous and intraperitoneal endotoxin treatment or infusions of Gram-negative bacteria have produced a syndrome of transient arterial hypotension, pulmonary hypertension, neutropenia with subsequent development of neutrophilia, pyrexia, hemoconcentration, and lymphopenia.[8,9,10] Unlike studies in other animal species, the potential role of coagulation aberrations and development of disseminated intravascular coagulation (DIC) during endotoxemia in the horse have not been examined.[11,12] The purpose of this study was to evaluate hemostatic changes after the systemic E. coli lipopolysaccharide infusion and the associated clinical and hematologic alterations.

MATERIALS AND METHODS

Eighteen clinically normal adult ponies were given anthelmentics upon arrival at the National Animal Disease Center and placed in stalls for a 2-week acclimation period. They were maintained on an alfalfa cube-grain ration and water. One day prior to treatment, the ponies were weighed.

Physical examinations and blood sampling were done on each pony 2, 1, and 0 days before treatment to establish individual pretreatment parameter baselines.

All ponies with the exception of #9 and #18 were given 40 µg/kg body weight Westphal purified 0111:B4 Escherichia coli lipopolysaccharide[a] in a liter of physiological saline intravenously via a jugular catheter. The infusion was completed in approximately 60 minutes. Pony #9 was used as a non-treatment control. Pony #18 was given 1 liter physiological saline in a 60 minute intravenous infusion as a treatment control. Ponies were unrestrained at all times except during initial intravenous infusion and blood sampling when light halter restraint was necessary. Water and feed were available free choice during the course of the experiment.

Clinical examinations included ascultation of the chest and abdomen and determination of heart rate, respiration rate,

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[a]Control No. 610243, Difco Laboratories, Detroit, MI.

and rectal temperature. Venous blood samples were collected with evacuated collection tubes.[b] After initiation of infusion, examinations and blood sampling were done at 15 minute intervals for the first 1½ hours, hourly from 2 to 8 hours, then every 2 hours until euthanasia or death. All animals were observed continuously through the course of treatment (Table 1).

White blood cell, erythrocyte, and platelet counts were done within 15 minutes after collection on an electronic cell counter (Celloscope®, Model 111-NB 3TX)[c] using whole blood collected in 7.2 mg EDTA-containing evacuated tubes. Hematocrit (HCT) and plasma protein determinations were done using routine laboratory procedures. Blood cell smears were prepared in duplicate and stained with Wright's Rapid[d] and May-Grunwald Giemsa stains.[13] Two differential counts were done on each blood smear.

Activated clotting time was determined by drawing 2 ml of whole blood into a 37C preheated evacuated glass tube containing 12 mg purified siliceous earth. After thorough mixing of blood and siliceous earth, the tube was returned to a 37C water bath. After 1 minute and at 5 second intervals, the

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[b]Becton-Dickinson, Rutherford, NJ.

[c]Particle Data Inc., Elmhurst, IL.

[d]MCB Manufacturing Chemists, Inc., Cincinnati, OH.

tube was examined for visible clot formation. The end point was determined to the nearest 5 seconds.

Activated partial thromboplastin time (APTT) and one stage prothrombin time (PT), Quick Test, assays were done using a fibrometer[e] with paired control and test plasmas.[14,15] Whole blood samples for the APTT and PT assays were collected in 3 ml evacuated tubes containing 11.4 mg sodium citrate. Citrated blood was immediately centrifuged after collection at 1500xG for 15 minutes. Plasma samples were then stored at 4C. Tests were completed within 2 hours after collection.

Quantitations of citrated plasma fibrinogen were carried out using a heat precipitation microhematocrit procedure described by Millar et al. (1971).[16]

Statistical analysis of the variables over time (minutes or hours) was performed utilizing a control chart procedure with a 95% confidence interval (CI) on the pretreatment mean.[17]

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[e]Bioquest, Cockersville, MD.

RESULTS

Clinical-Pathological Findings

Clinical observations Fifteen minutes after beginning infusion, heart rate (Figure 1) and respiratory rate (Figure 2) were markedly elevated. Oral mucous membranes were pale and cyanotic. Lethargy, characterized by head and lower lip droop, was observed. By 30 minutes, oral mucous membrane cyanosis and scleral congestion were clearly visible. Eight ponies passed soft feces. Salivation, sweating, and lacrimation were observed in the first 60 minutes. In all ponies, lethargy progressed to ataxia then prostration 1 to 2 hours after treatment began. Five ponies became comatose and died within 8 hours after prostration. The remaining eleven treatment ponies became more alert and were standing 5 to 10 hours post treatment. Oral mucous membrane color gradually changed from dark purple to brick red-coral 1 to 2 hours prior to attempts of each pony to stand. Abdominal ascultation revealed intermittent periods (approximately 2 hours) of hyperperistalsis followed by periods of quiescence with gas and fluid accumulation. Ponies that survived longer than 10 hours passed soft, unformed to watery feces every few hours. Quadripedal laminitis developed 8 to 12 hours post treatment. Fourteen to 18 hours post treatment, all surviving ponies became more alert: drinking water and urinating. Twenty-two to 24 hours post treatment, the clinical appearance changed

from an alert state to increasing ataxia. Dyspnea, muscle tremors, and unusual behavioral changes including head pressing and standing with muzzle in the waterer were observed. Four ponies became prostrate 26 to 30 hours post treatment and remained recumbent until euthanasia.

A febrile response was observed in all treated ponies (Figure 3). The maximum hyperthermic state occurred 8 to 12 hours post infusion initiation. Pyrexia was preceded by a transient (maximum 3 hours post treatment) period of hypothermia.

Hematologic findings Within 15 minutes of infusion of lipopolysaccharide, there was significant leukopenia ($P \leq 0.05$) (Figure 4), lymphopenia (Figure 5), and marked neutropenia (Figure 6). Neutrophils on smears (15 and 30 minutes) were hypersegmented with dense nuclear chromatin and pale vacuolated cytoplasm. Lymphocytes were small with a high nucleus to cytoplasm ratio.

Leukocytes began to increase 7 hours post infusion initiation (PI) and were significantly ($P \leq 0.05$) elevated over the baseline 14 hours PI. Non-segmented or band neutrophils (Figure 7) were not observed in the peripheral blood smears in the pretreatment blood sample or early phase of endotoxemia. Coinciding with the development of neutrophilia, non-segmented neutrophils, many with basophilic vacuolated cytoplasm, and occasional metamyelocytes, were observed in differential

smears. Both the leukocyte count and absolute neutrophil count remained elevated after 14 hours, but lymphopenia persisted during the entire course of treatment.

Erythrocyte count (Figure 8) and hematocrit (Figure 9) increased significantly ($P \leq 0.05$) to a maximum at 2 hours PI and remained elevated throughout the course of treatment. Plasma protein values (Figure 10) increased immediately, 15 minutes PI. After 2 hours, the concentration began to gradually decrease to below baseline, 12 to 26 hours PI, and returned to above normal, 32 to 40 hours PI.

Hemostatic findings Platelet counts (Figure 11) fell significantly ($P \leq 0.05$) within 15 minutes PI and persisted below the baseline normals throughout the entire experiment. Activated clotting time (Figure 12) gradually increased throughout the course of the experiment. Both intrinsic and common pathway coagulation assays (Figures 13 and 14), APTT and PT respectively, were significantly increased ($P \leq 0.05$) after 3 hours and gradually increased throughout the course of the experiment. Fibrinogen assays (Figure 15) presented a different picture with an initial elevation (maximum at 90 minutes PI, 160 mg/dl) then gradually declining to near normal values during the middle 1/3 of treatment (12 to 26 hours PI) and rising in the last third of the experiment to (28 to 38 hours PI).

DISCUSSION

Coagulopathies have been reported with Gram-negative bacteremia or experimental endotoxic shock in both animals and man.[4,18] These changes are caused by the initiation of the extrinsic and intrinsic coagulation cascades with the development of fibrin and platelet thrombi. This state is commonly referred to as disseminated intravascular coagulation (DIC).[19] DIC often produces a clinical picture of consumption of platelets and clotting factors. Rapid activation and sequestration of platelets is suggested by the marked thrombocytopenia within the first 15 minutes PI. The prolonged and gradual increase in PT and APTT times are indicative of a consumptive coagulopathy of factors in the extrinsic, intrinsic, and common pathways. The decreased number of platelets, prolonged ACT, PT, and APTT; all represent a clinical picture of DIC induced by E. coli lipopolysaccharide.

Previous studies have postulated that the increase in packed cell volume results from the initial effects of endotoxin such as splenic contraction and erythrocyte mobilization.[20,21] The concurrent increase in plasma protein and erythrocytes suggests that there is rapid intercompartmental fluid movement from vascular spaces into extravascular space. This hypothesis of rapid intercompartmental fluid movement is supported by endotoxin experiments in rhesus

monkeys in which pulmonary edema developed 15 minutes following infusion.[22] In the horse, the large intestine accounts for a large portion of the blood vascular bed, and the cecum and colon are the primary shock organs. Rapid fluid movement from the large intestine vascular bed into extravascular spaces could account for the observed hemoconcentration and hyperproteinemia. Further, the subsequent decrease in plasma protein can be attributed to a loss of capillary integrity with extraluminal movement of proteins into the extravascular space. The damaged capillary with exposure of the connective tissue would activate clotting and enhance DIC, as observed in this experiment. Near the termination of the experimental period, the increase in plasma protein levels is due to compensatory hepatic production. Endotoxemia is known to induce production of vascular proteins including fibrinogen and in this experiment, it is reflected in the terminal increase in fibrinogen.[23]

Neutropenia in the early phase of endotoxemia is due either to intravascular lysis of neutrophils or their sequestration in major visceral organs.[24] Subsequent development of neutrophilia is a result of a compensatory premature release of cells from the bone marrow maturation pool into the peripheral circulatory pool. This is exemplified by the pronounced regenerative left shift accompanying the neutrophilia

with the presence of toxic neutrophils and immautre myelogenous elements including metamyelocytes.

Elevation in body temperature was consistent with previous equine experiments, but hypothermia is not.[25] The hypothermic phase is seen in other species treated with Gram-negative bacterial extracts.[26] The mechanism of hypothermia that developed is now know, however, and in this experiment, the ponies were prostrate during this phase. It is probable that hypothermia developed because of the decreased peripheral vascular perfusion coupled with an environmental temperature lower than the animal's body temperature resulting in hypothermic imbalance and peripheral decrease in temperature.

Clinically, there was a distinct three phase hemodynamic response which is similar to the response in dogs.[3] The first phase characterized by cyanosis and scleral congestion; prostration was due to the abrupt development of systemic arterial hypotension. This was followed by a slower change in mucous membrane color and a more alert state which was associated with a return of near normal systemic and pulmonary pressures. The third phase was a gradual decrease in systemic pressure which terminated in irreversible shock.

The role of Gram-negative bacterial endotoxin in clinical syndromes in horses including Colitis X has been suggested.[27] Intravenous and intraperitoneal endotoxin infusions in horses have produced changes similar to the disease syndrome of

Colitis X including hemoconcentration, neutropenia, and lymphopenia. Salmonellosis in both field cases and experimental treatment can induce a syndrome of circulatory collapse and septic shock.[28,29] The similarity of Colitis X and Salmonellosis in horses has in the past caused some workers to suggest that they are the same condition with discrepancies associated with failure to isolate Salmonella sp.[30] Other Gram-negative organisms have been implicated as the causitive agent in Colitis X including E. coli and Klebsiella.[31] Common to all of these etiologies is the organism's lipopolysaccharide-containing cell wall.[32] These experimental findings of a consumptive coagulopathy were compatible with the post mortem finding in clinical cases of Colitis X of dark, non-clotted blood suggestive of a consumptive coagulopathy.[33] The commonality of low dose extravenous E. coli endotoxin treatment in animal models and the hematologic-hemodynamic pattern in Colitis X and the acute syndrome in Salmonellosis in the equine give credence to Gram-negative bacterial endotoxins as a principal contributor to the disease process. Determination of the specific source and mechanism of endotoxin activation of host mediation systems must be paramount in future experimental studies.

Table 1 Sex, weight, and duration of experimental endotoxemia in ponies. Duration is the length of endotoxemia prior to euthanasia or premature death. Pretreatment death is pretreatment control euthanasia.

Pony	Sex	Weight (Kg)	Duration (hrs)
1	M	210	26
2	M	168	32
3	M (C)	117.5	5 - death
4	M	137.3	40
5	M	126.7	8 - death
6	M	138.6	10 - death
7	F	103.6	36
8	M	102.0	32
9	M	128.5	Pretreatment Death
10	M	188.0	16
11	F	115.4	36
12	F	93.2	40
13	M (C)	128.5	10 - death
14	M	150	16
15	M (C)	117.6	8
16	M	138	8
17	M	119.5	7 - death
18	M	159.7	16

Fig 1 Mean heart rate of all ponies after beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

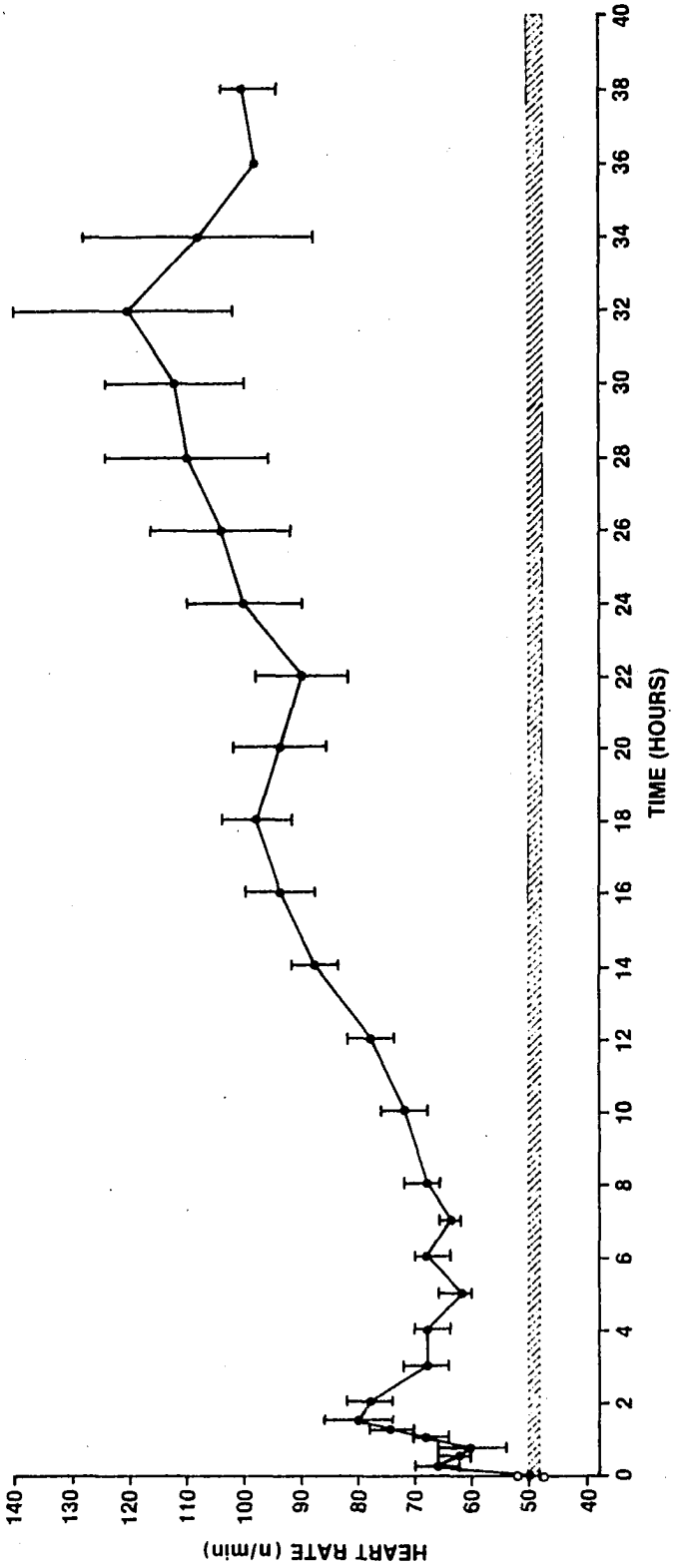

HEART RATE (n/min)
140
130
120
110
100
90
80
70
60
50
40
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 2 Mean respiratory rate of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

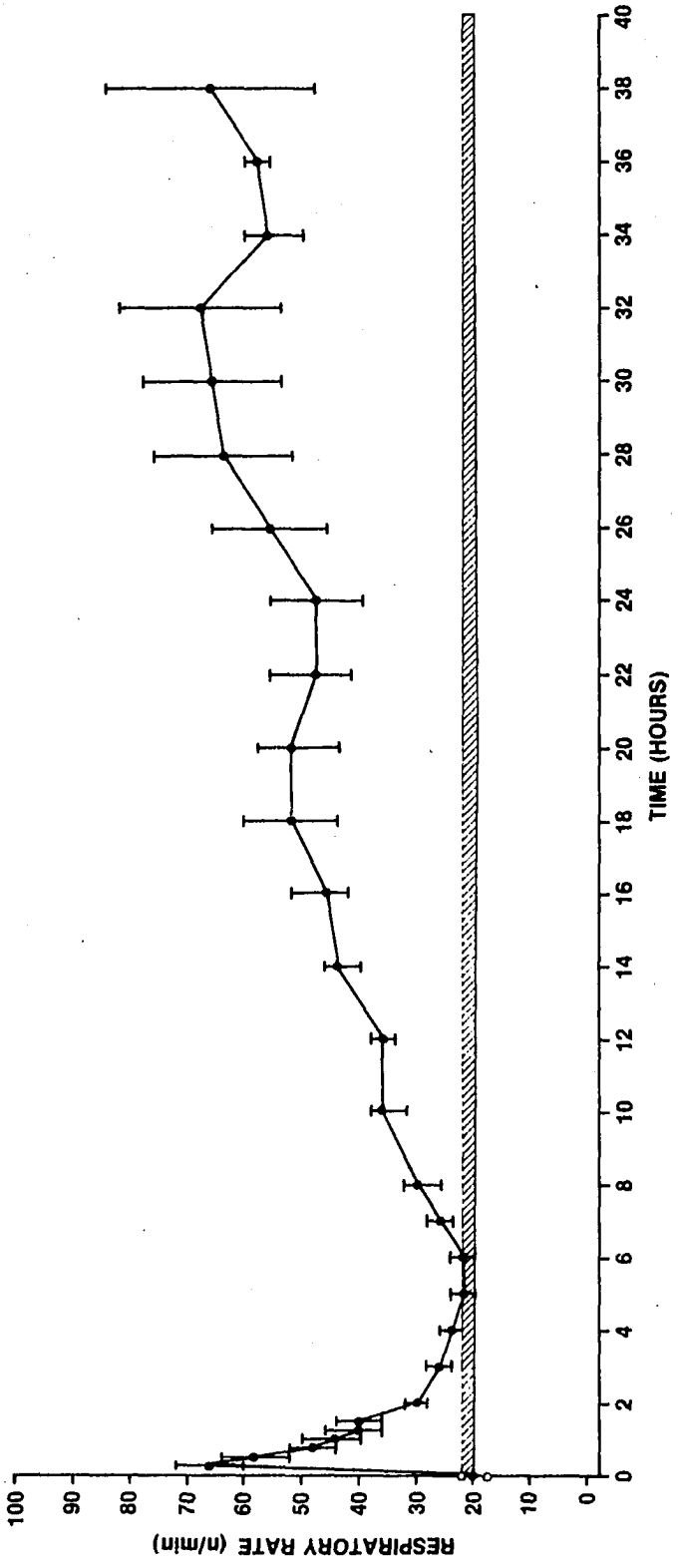

RESPIRATORY RATE (n/min)
100
90
80
70
60
50
40
30
20
10
0
TIME (HOURS)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

Fig 3 Temperature of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

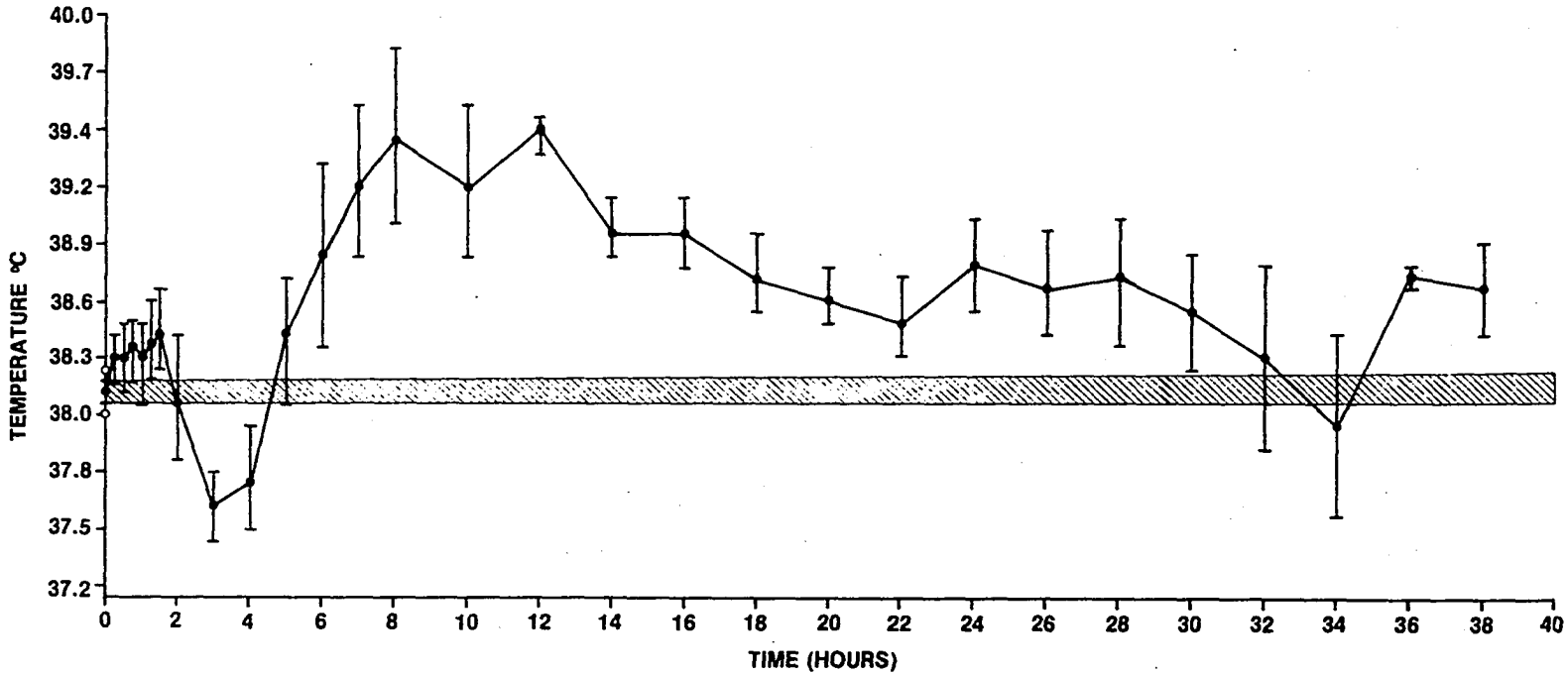

40.0
39.7
39.4
39.2
38.9
38.6
38.3
38.0
37.8
37.5
37.2
TEMPERATURE °C
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 4 Mean white blood cell counts of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

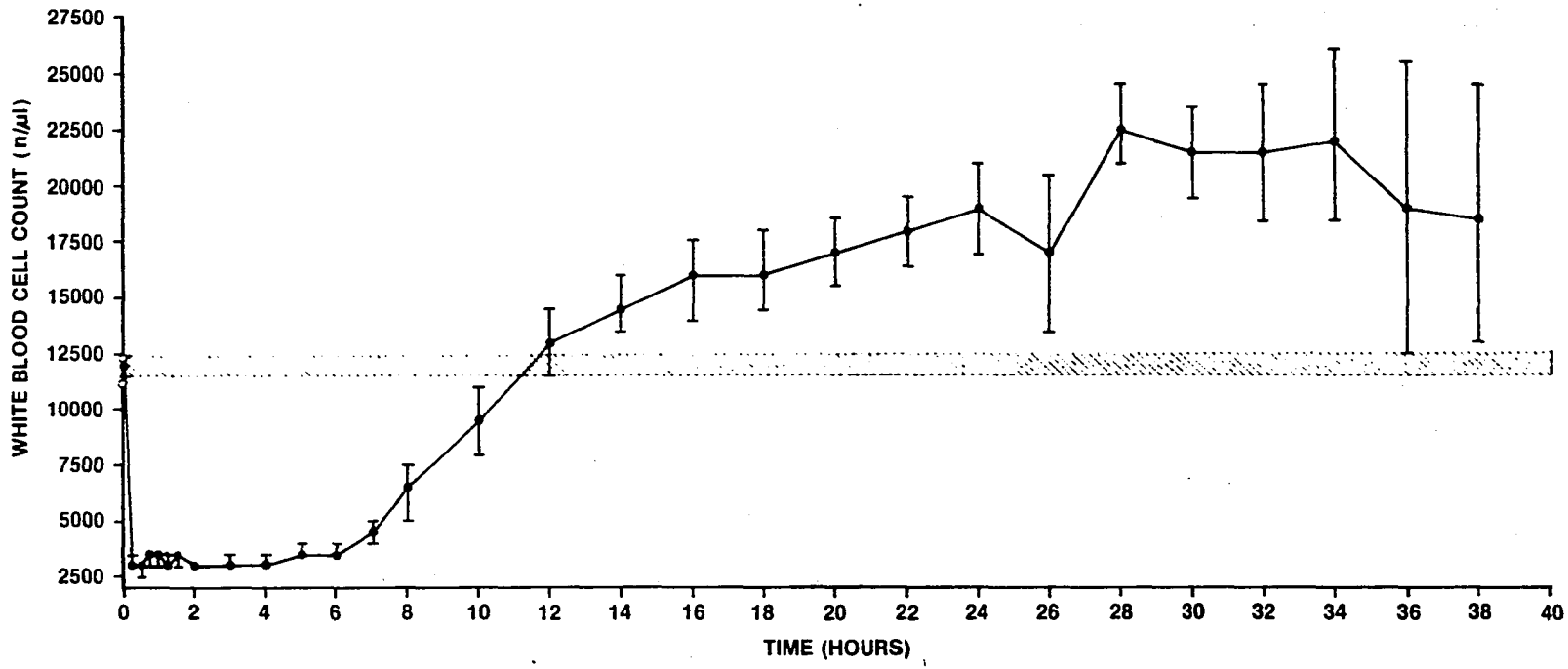

WHITE BLOOD CELL COUNT (n/µl)
27500
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 5 Mean lymphocyte count of all ponies beginning treatment at time zero. Shaded area represents pretreatment means $\pm$ 1 SEM. The vertical bars = treatment $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

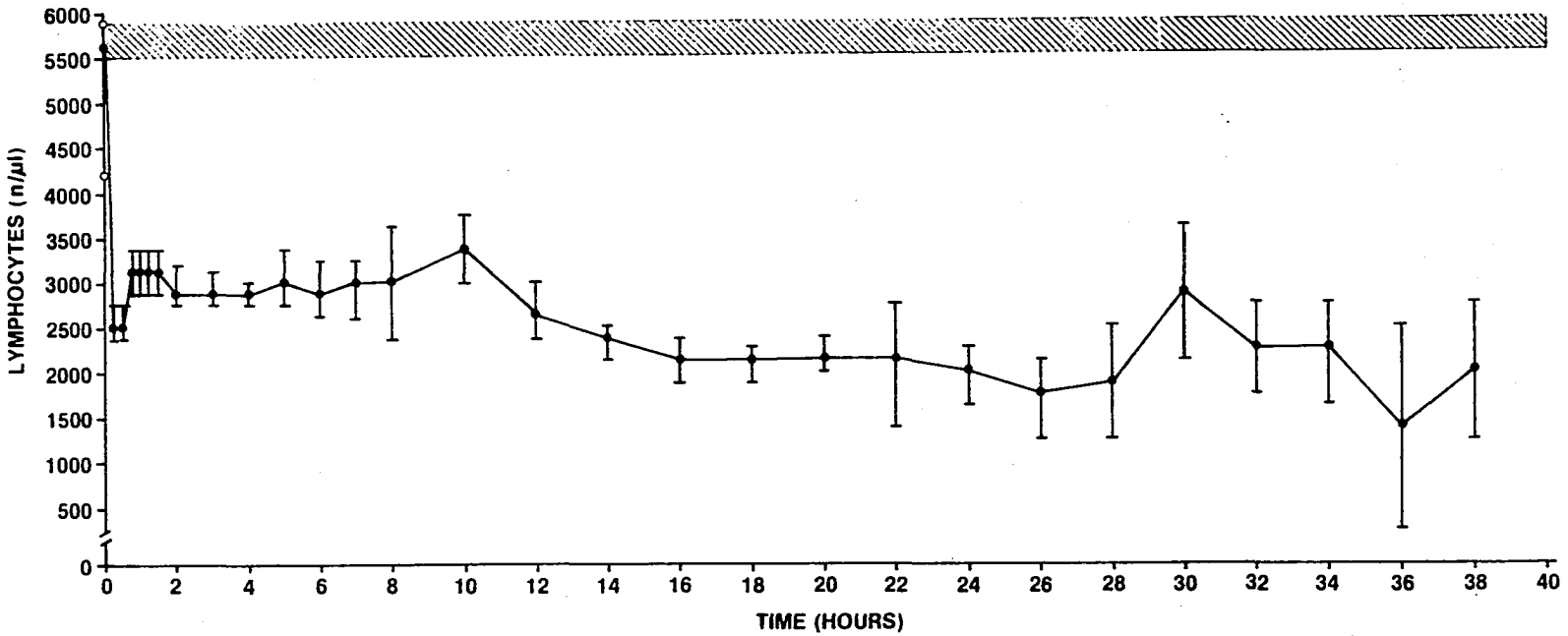

LYMPHOCYTES (n/µl)
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 6 Mean segmented neutrophil count of all ponies beginning treatment at time zero. Shaded area represents pretreatment means ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

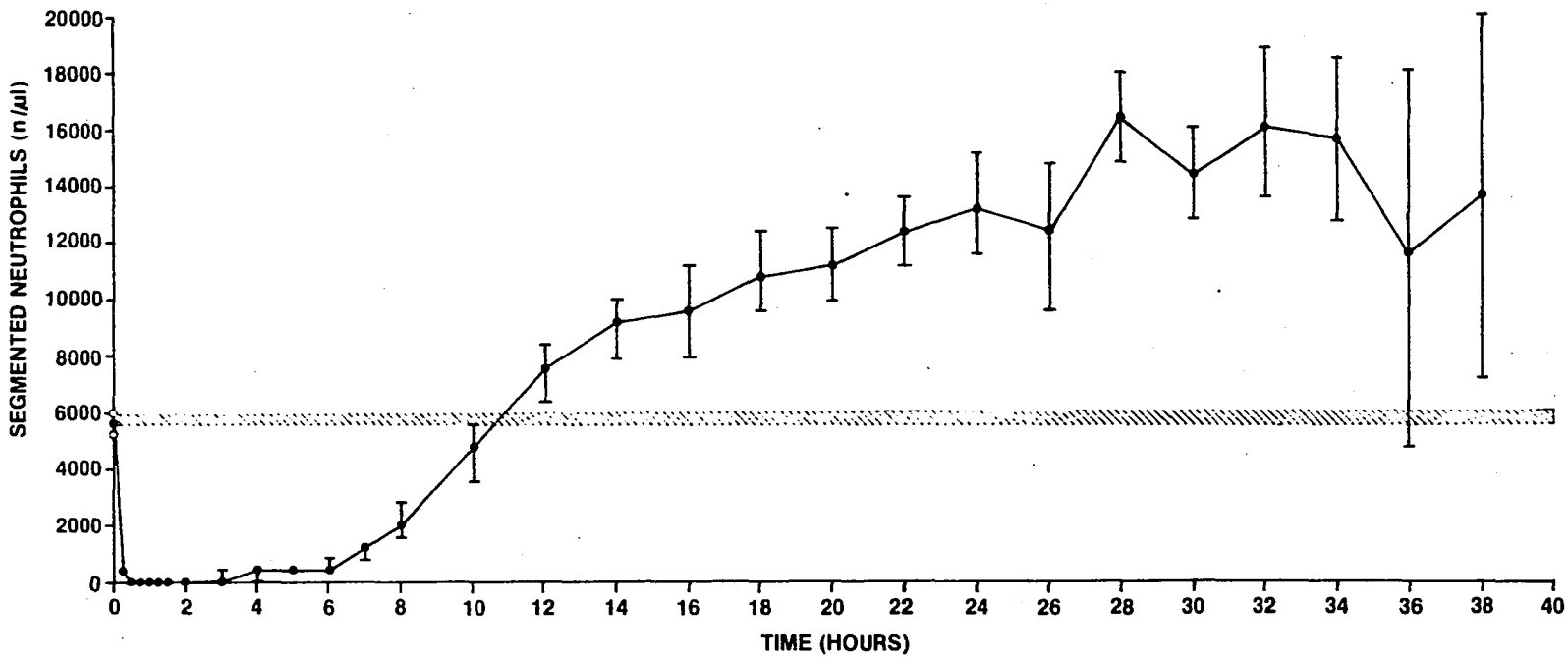

SEGMENTED NEUTROPHILS (n/µl)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 7 Mean band neutrophil count of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

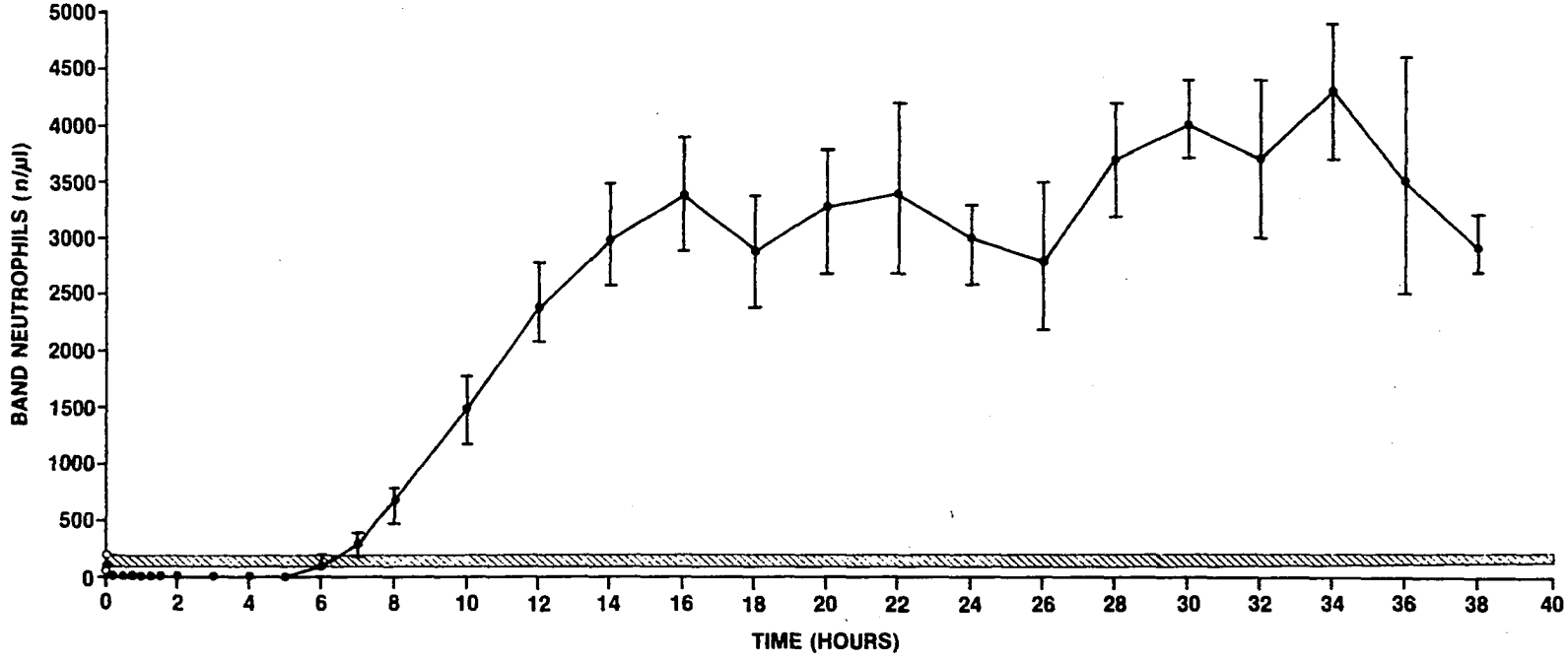

BAND NEUTROPHILS (n/µl)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 8 Mean erythrocyte counts of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

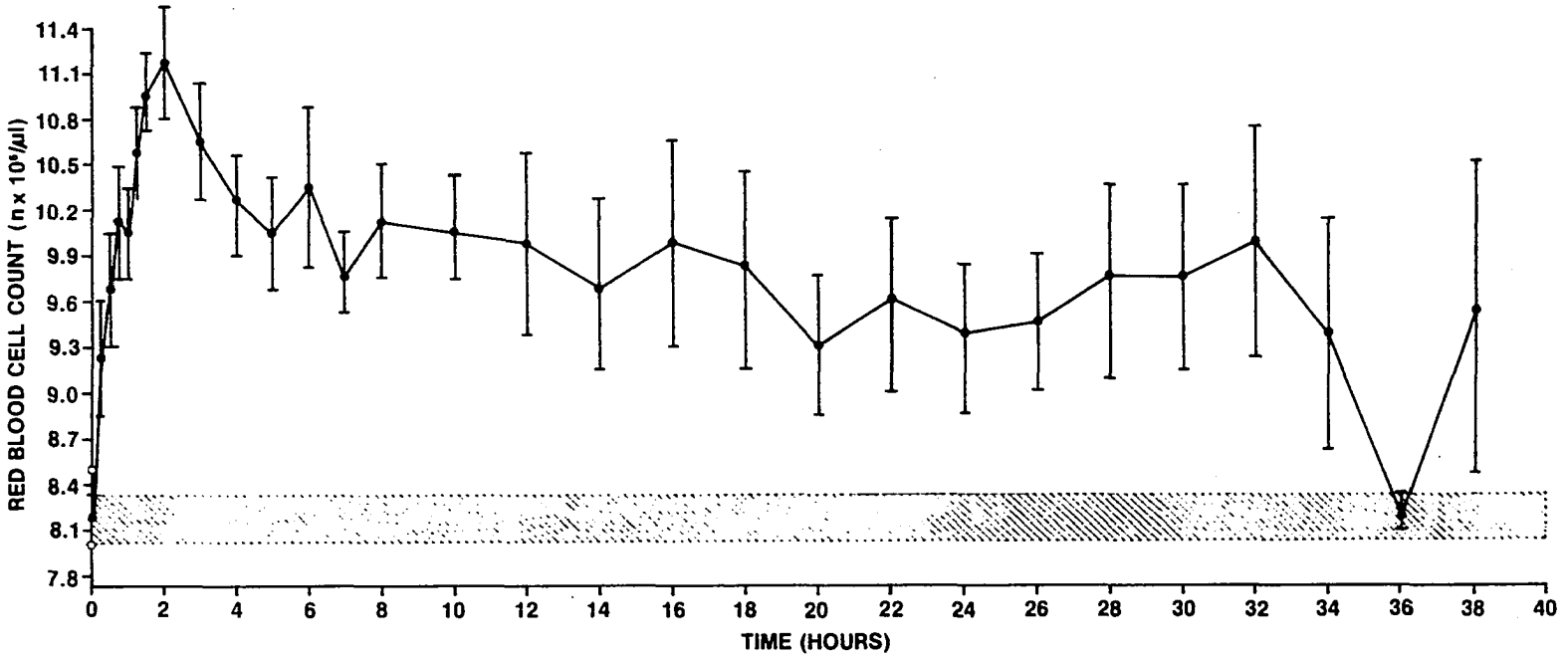

RED BLOOD CELL COUNT (n x 10⁶/µl)
11.4
11.1
10.8
10.5
10.2
9.9
9.6
9.3
9.0
8.7
8.4
8.1
7.8
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 9 Mean hematocrit of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

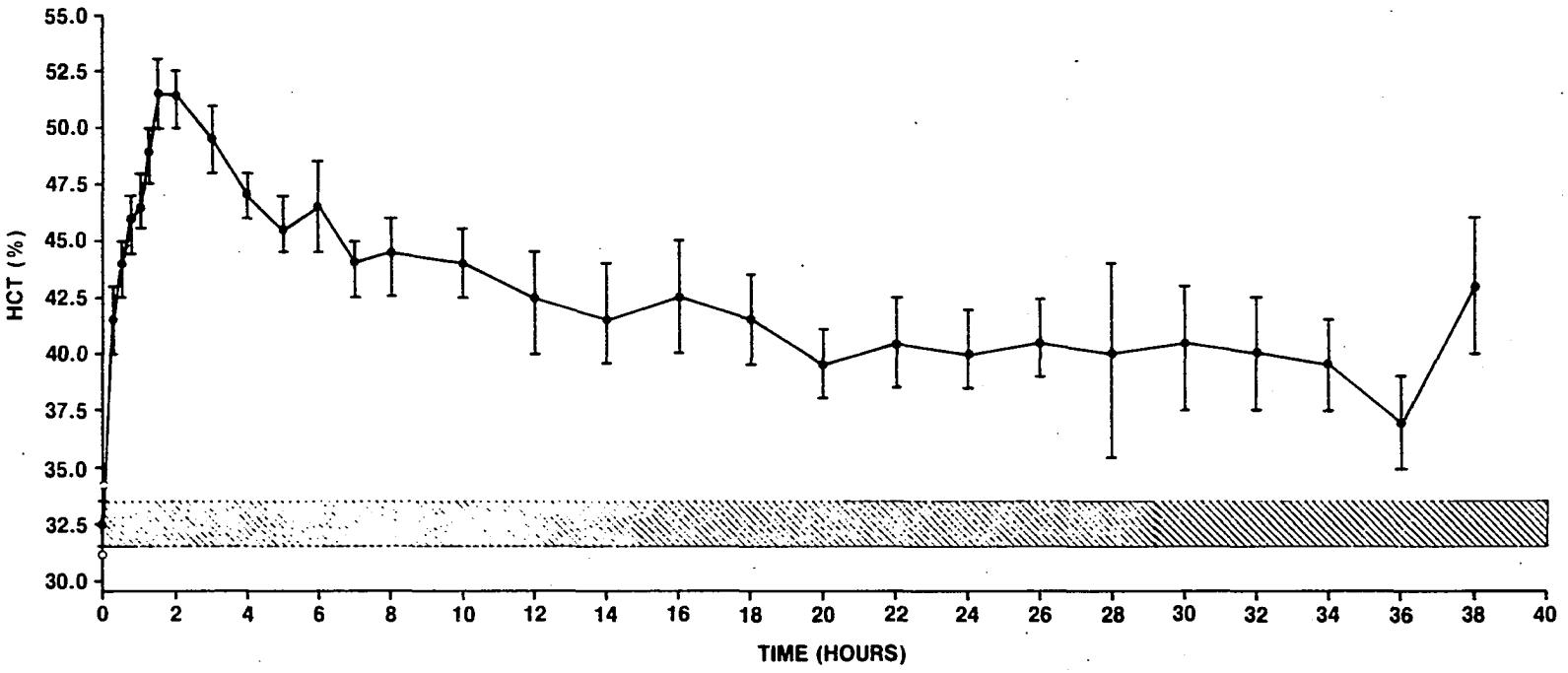

55.0
52.5
50.0
47.5
45.0
42.5
40.0
37.5
35.0
32.5
30.0
HCT (%)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 10 Mean plasma protein concentration of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

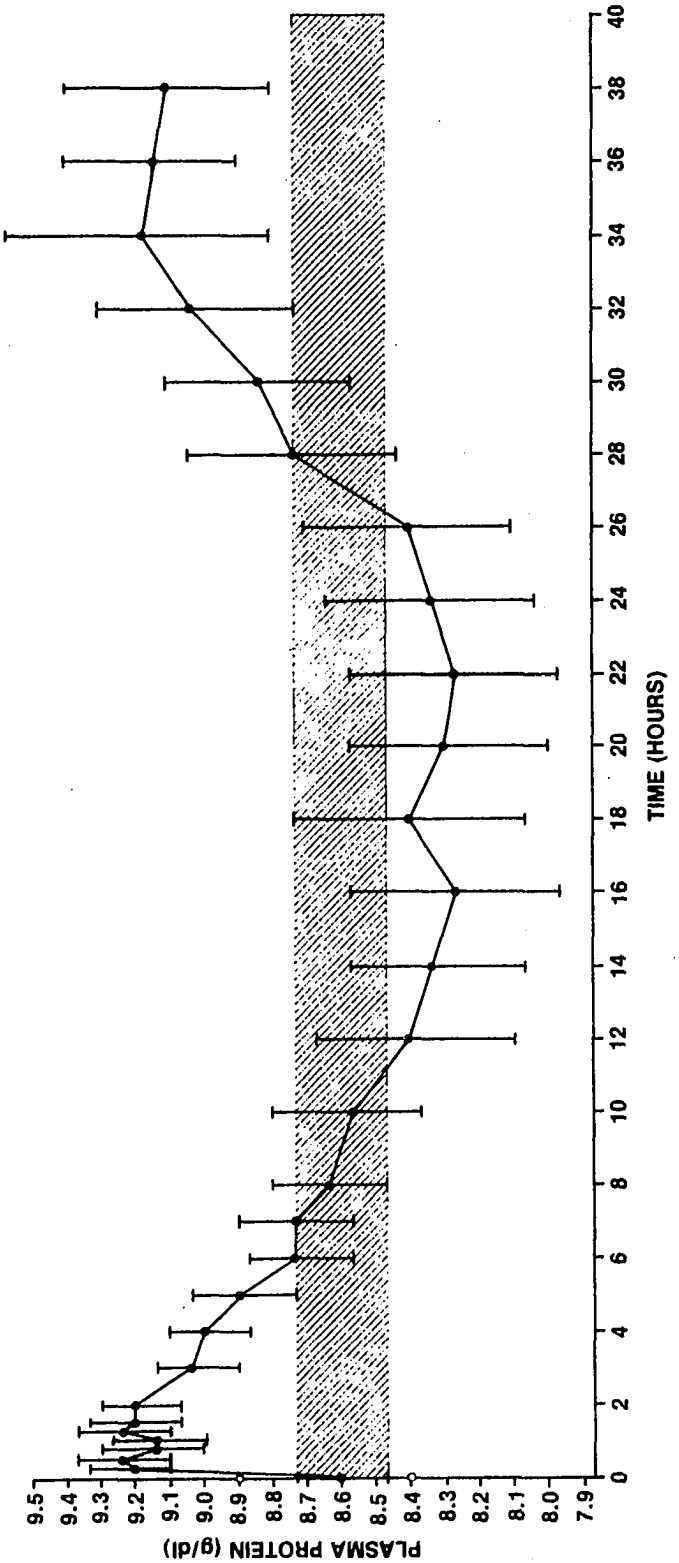

PLASMA PROTEIN (g/dl)
9.5
9.4
9.3
9.2
9.1
9.0
8.9
8.8
8.7
8.6
8.5
8.4
8.3
8.2
8.1
8.0
7.9
TIME (HOURS)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

Fig 11 Mean thrombocyte count of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

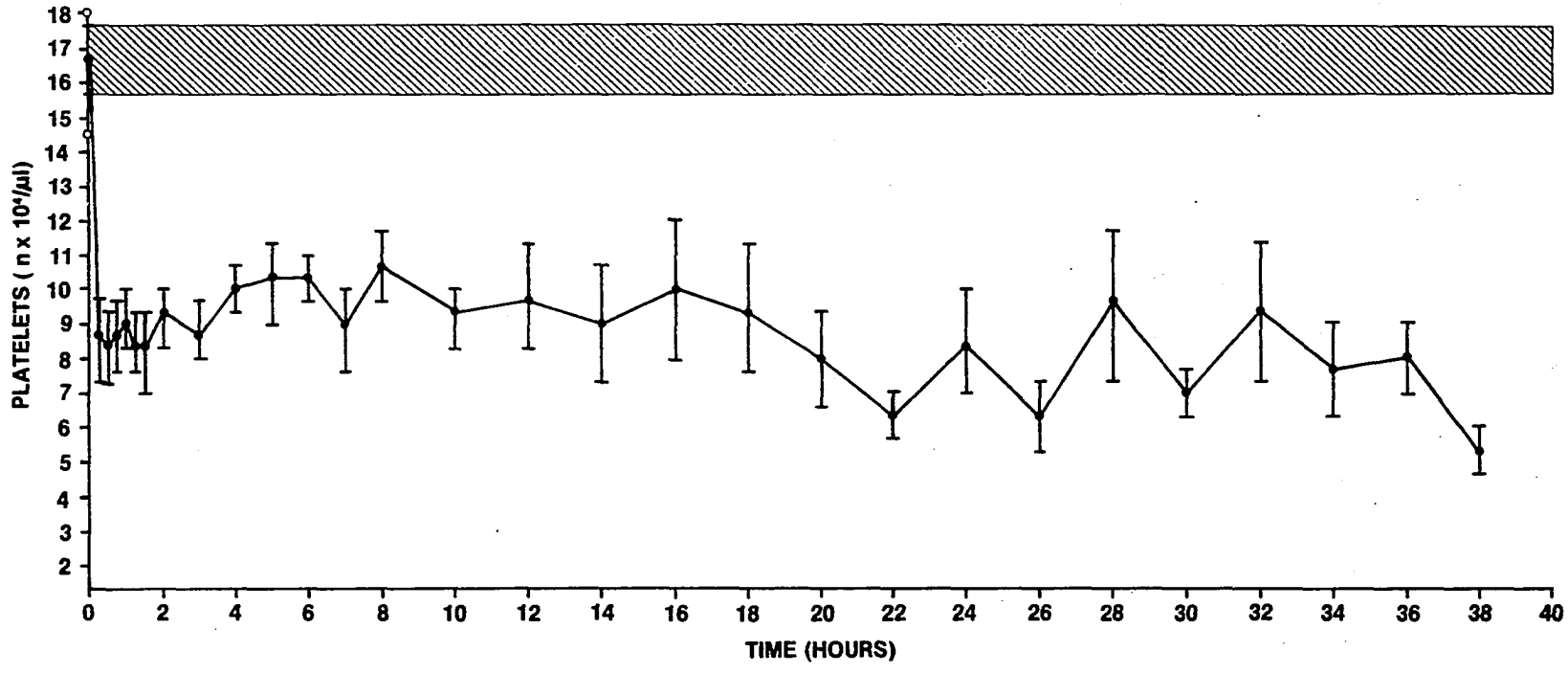

PLATELETS (n x 10⁴/µl)
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 12 Mean activated clotting time of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

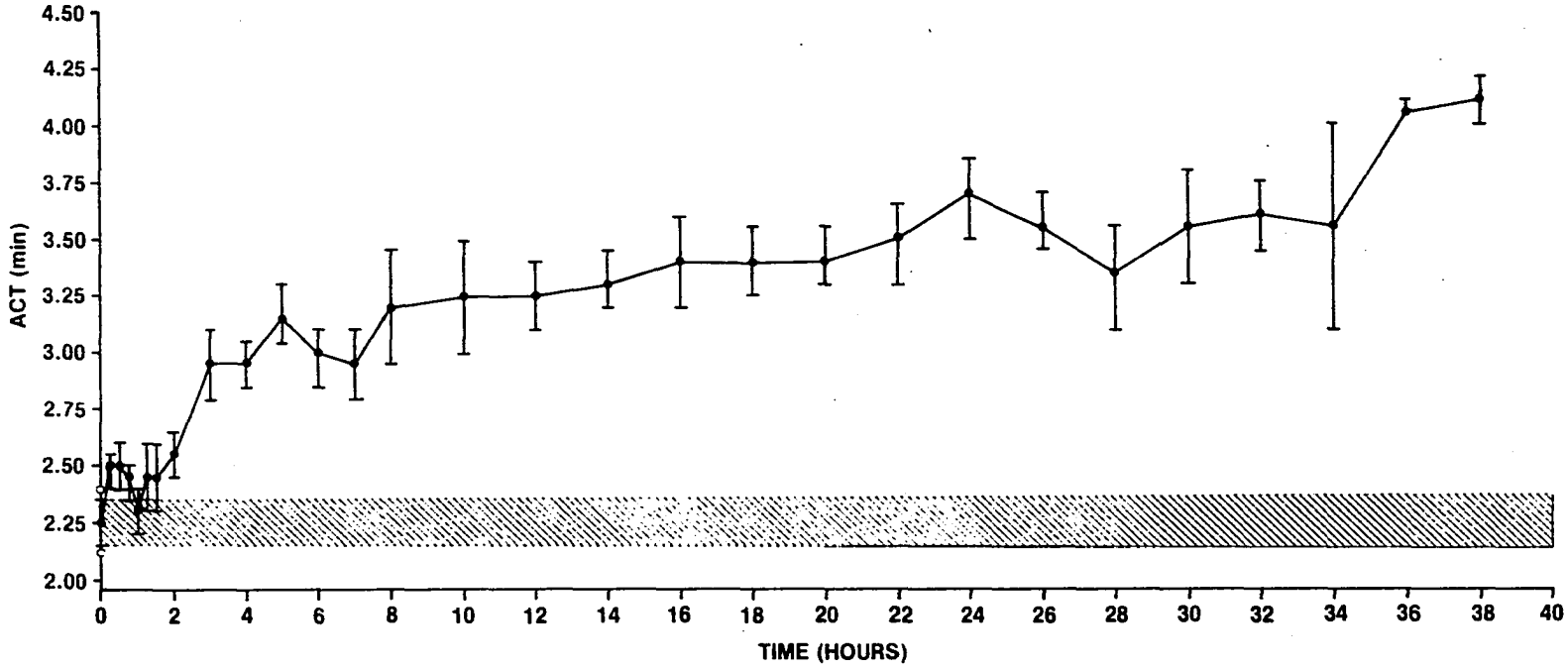

4.50
4.25
4.00
3.75
3.50
3.25
3.00
2.75
2.50
2.25
2.00
ACT (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 13 Mean activated partial thromboplastin time of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

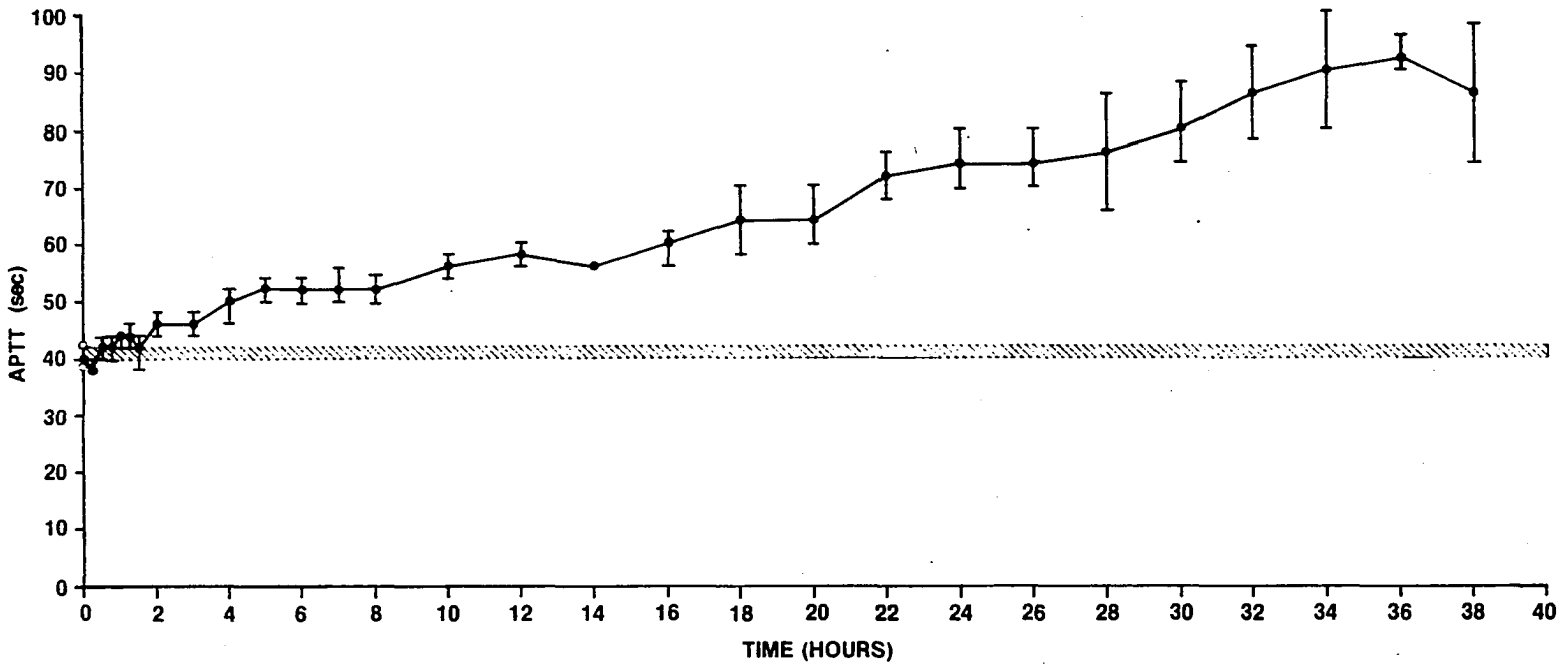

APTT (sec)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 14 Mean prothrombin time of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean $\pm$ 1 SEM. The vertical bars = treatment mean $\pm$ 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.

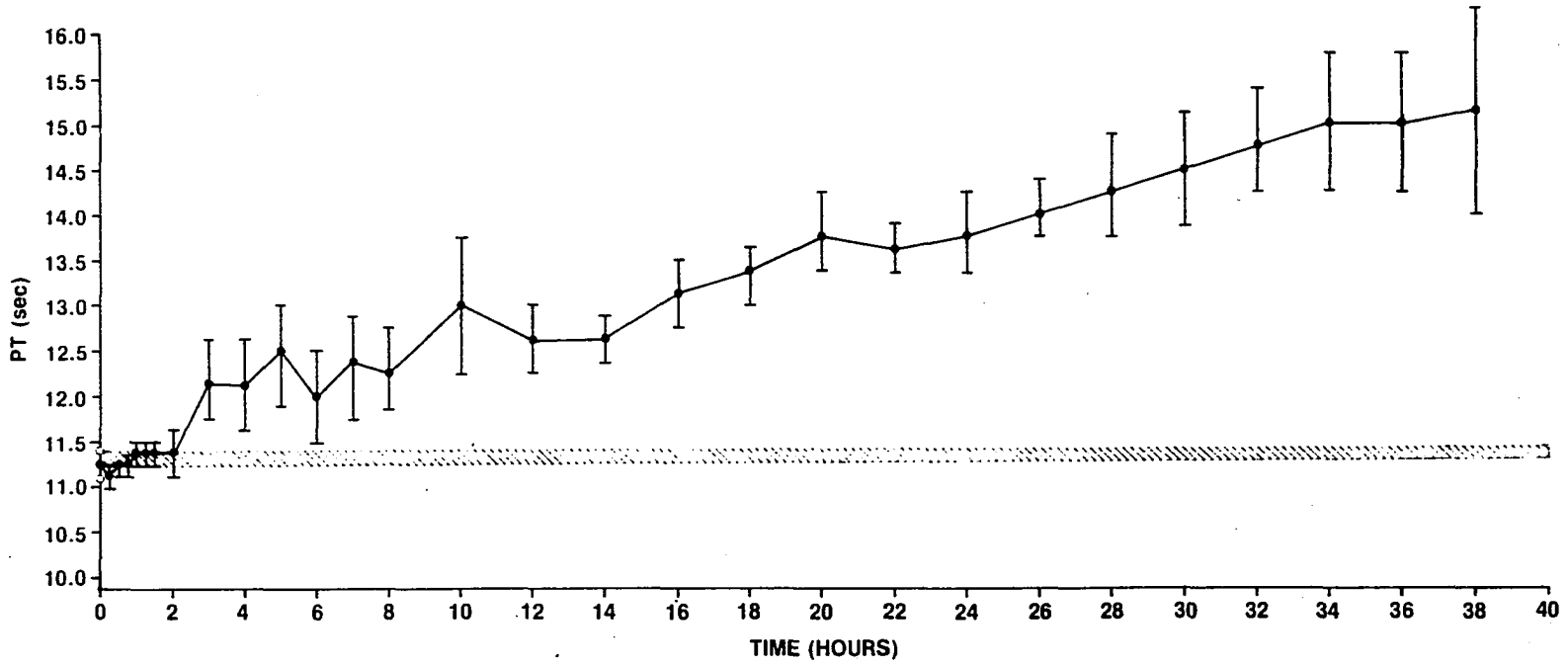
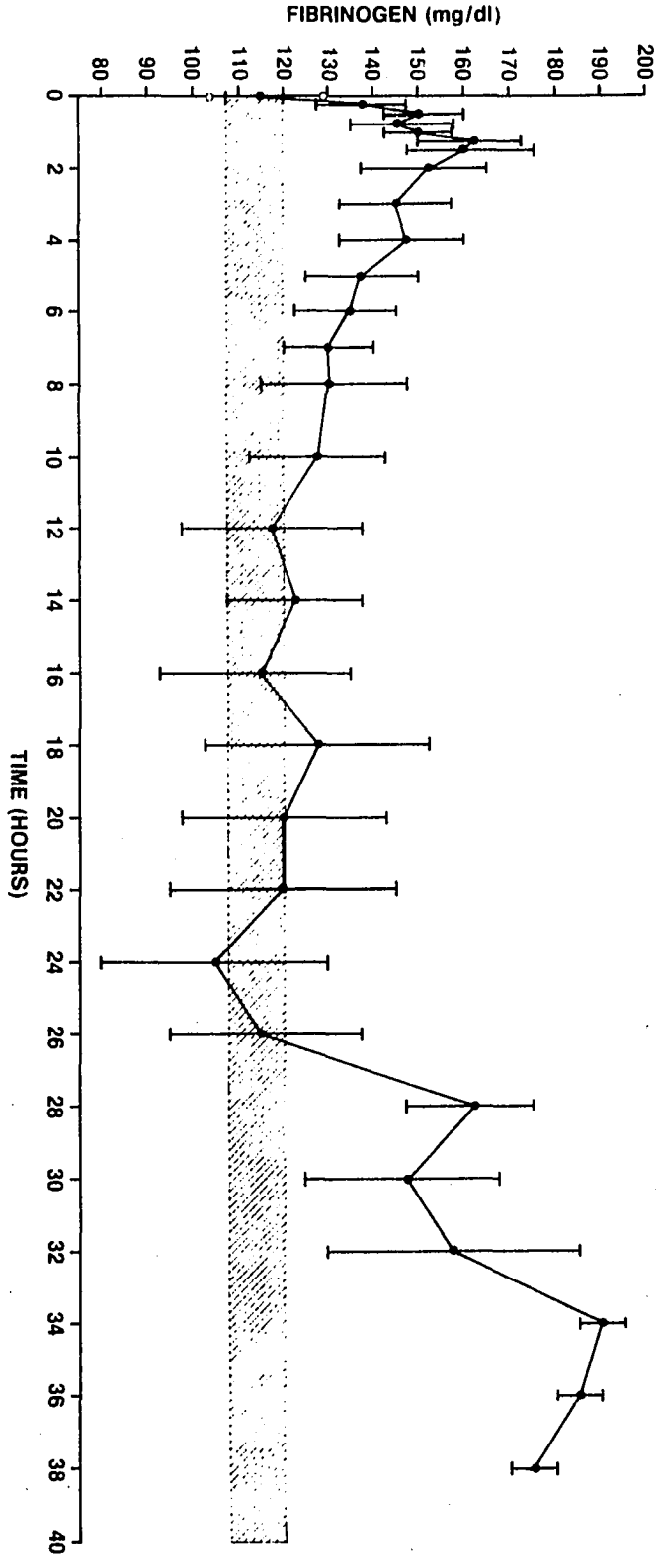

PT (sec)
16.0
15.5
15.0
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
10.5
10.0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
TIME (HOURS)

Fig 15 Mean plasma fibrinogen concentration of all ponies beginning treatment at time zero. Shaded area represents pretreatment mean ± 1 SEM. The vertical bars = treatment mean ± 1 SEM. Open circles on the vertical axis indicate the 95% confidence interval ($P \leq 0.05$) on the pretreatment mean.


FIBRINOGEN (mg/dl)
80
90
100
110
120
130
140
150
160
170
180
190
200
TIME (HOURS)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

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LESIONS SEEN IN *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE-INDUCED ENDOTOXIC SHOCK IN PONIES

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SUMMARY

Each of eleven ponies was given 40 µg/kg Escherichia coli lipopolysaccharide intravenously. Ponies were necropsied at 8, 16, 26, 32, 36, and 40 hours post infusion initiation (PI). The most severe lesions were in the cecum and ventral colon which were distended with fluid and gas. Large intestinal mucosal lesions included focal to diffuse congestion, hemorrhage, and ulceration. Hyperemia, edema of lamina propria, and erosion of mucosal epithelium were seen histologically. Lesions were the most severe at 36 and 40 hours PI. Vascular endothelial disruption of tight junctions with suffusion of vascular fluid, dissolution of basal laminae, and stromal edema were seen by 32 hours PI. Endothelial intercellular edema and organelle changes were observed by 8 hours PI. Adrenal cortical vascular congestion was most severe in capillary sinusoids of the zona glomerulosa and was observed by 8 hours PI. Hemorrhage obliterated adrenal cortical architecture 26 hours PI and necrosis was seen at 36 hours PI. Petechial and eccymotic hemorrhages were seen on most organ serosal surfaces at various times PI. Lesions caused by systemic endotoxemia are postulated to be caused by selective large intestinal vascular injury and extraluminal fluid flux. Necrosis of the adrenal cortex is the sequalae to vascular stasis.

INTRODUCTION

Experimental equine endotoxemia produces hemodynamic, hematologic, and metabolic alterations that are similar to acute shock syndromes of Salmonellosis and Colitis X (Exhaustive shock).[1,2,3,4] In general, the lesions in the natural disease conditions appear to be related to vascular damage. The lesions include petechial and ecchymotic hemorrhages on most organs; adrenal cortical hemorrhage and edema; hyperemia, hemorrhage, and necrosis of the large intestine (cecum and large colon) mucosa and submucosa.[5,6] Description of lesions in experimental equine endotoxemia has been brief with adrenal cortical hemorrhage and petechiae and ecchymoses scattered throughout the body being the most significant findings.[7]

In other animal models of endotoxemia, the lesions that develop are caused by vascular changes and disrupted hemostasis. Vascular alterations seen by light and electron microscopy are the result of loss of vascular integrity. Gerrity et al. studied intravenous endotoxemia in the dog and its effect on endothelium.[8] Within 5 minutes of endotoxin injection, an increase in circulating endothelial cells was observed in pulmonary circulation. In 1 hour, endothelial junctions were permeable to colloid dyes, confirming endothelial injury and membrane permeability to protein. In

other studies on mesenteric vessels of rabbits, endothelium became swollen and detached from basal laminae. Tight junctions were disrupted 3 to 24 hours after endotoxin treatment.[9]

Altered hemostasis and thrombosis are the other lesions observed. These lesions are caused by triggering of disseminated coagulation. Fibrin formation with leukocyte and platelet aggregation in capillary beds of visceral organs is the principal lesion. Vascular lesions and endothelial damage may be the cause or result of thrombosis with activation and release of mediators and lysosomal enzymes from platelets and leukocytes. The site and severity of lesions vary in the animal species studied with different organ systems being variably affected in endotoxic shock.[10,11]

In the horse, comparisons between natural conditions and experimental endotoxemia cannot be effectively compared until post mortem changes in the experimental model are evaluated. Therefore, the purposes of this study were to: (1) describe morphological changes in equine endotoxemia; (2) examine course of development of these lesions; and (3) consider mechanisms that may play a role in the pathogenesis of the lesions.

MATERIALS AND METHODS

Animal and Specimen Preparation

Animal experimental protocol Thirteen adult ponies (93 to 210 kg) were kept in box stalls and maintained on grain and alfalfa-cube rations. Animals were acclimated for two weeks prior to treatment.

Each of eleven ponies was given 40 µg/kg body weight Westphal purified O111:B4 Escherichia coli lipopolysaccharide[a] in a liter of physiological saline via a jugular catheter. Infusion was completed in approximately 60 minutes. Two ponies were used as experimental controls. One pony was used as a non-treatment control. The other was given 1 liter of physiological saline in a 60 minute intravenous infusion and monitored for 16 hours as a treatment control.

All ponies were killed and exsanguinated. Treatment ponies were randomly selected for death at a predetermined time with 2 ponies each at 8, 16, 32, 36, and 40 hours and 1 pony at 26 hours after beginning the endotoxin infusion. Water and feed were available free choice during the experiment. All animals were monitored continuously during the experiment.

Post mortem and histological preparations Tissues collected included: cecum; ventral, dorsal, and small colon;

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[a]Control No. 610243, Difco Laboratories, Detroit, MI.

cecal and mesenteric lymph nodes; kidney; adrenal glands; jejunum; ileum; duodenum; heart; liver; lung; gonad; spleen; and stomach. Additional specimens were collected based on observations of gross lesions. Large intestine samples were trimmed to allow rapid direct exposure of mucosal and serosal surfaces to fixative. After fixation, tissues were routinely processed for paraffin embedding. Five um paraffin sections were stained with hematoxylin and eosin (H&E) and Mallory's Phosphotungstic Acid Hematoxylin Method (PTAH) for fibrin.

Electron microscopy Tissues for transmission electron microscopic (TEM) examination were taken from the dorsal and ventral colon; cecum; and adrenal glands. Samples of organs were taken from predetermined areas and placed in cold 2.5% glutaraldehyde. All samples were rinsed twice in cacodylate buffer (pH 7.2) 2 and 12 hours after collection. Tissues were post fixed in 1% osmium tetraoxide, dehydrated in graded ethanol, and embedded in epoxy resin.[b] Thick sections (1-3 um) were mounted on glass slides and stained with toluidine blue. Light microscopic examination of thick sections was used in screening for thin sectioning areas. Ultrathin sections (approximately 600Å) were cut with a diamond knife

[b]Epon 812, Shell Chemical Co., Kansas City, MO.

on an ultramicrotome and stained with lead citrate and uranyl acetate. TEM examination was done on a Phillips 200[c] electron microscope.

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[c]Phillips Electronic Instruments, Inc., Mahwah, NJ.

RESULTS

Pathologic Alterations

Gross lesions The cecum and colon were distended in all treated ponies. In ponies killed 8 and 16 hours post infusion (PI), the cecum and ventral colon were filled with large amounts of gas and fluid. The amount of water in the luminal contents was less in the ventral colon and was near normal in the small colon. The mucosal surface of the cecum and colon had focal patchy areas of congestion several cm in size (see Figure 1). The serosal surface appeared normal. Distal portions of the large intestine were normal. Cecal and colonic lesions were more severe in ponies killed 32, 36, and 40 hours PI. The serosal surface of the cecum and colon was dark red-purple with petechial hemorrhages. The luminal fluid contents were red-brown and foul-smelling. The amount of fluid in the contents of the large intestines was graded but the fecal matter in small colon was soft and adhered to the mucosa. In the 26 and 32 hour PI ponies, large intestinal mucosal lesions consisted of varying degrees of congestion with petechial hemorrhages. In the 36 and 40 hour PI ponies, erosions of the mucosa approximately 5 mm to 3 cm were seen with hemorrhage, adherence of ingesta, and fibrinous tags (Figures 2,3). The cecal lesions were least severe at the apex. In the colon, lesions were less prominent distally. The cecal submucosa was thickened, wet, and gelatinous.

Mesenteric and cecal lymph nodes were variably congested and edematous. Mesenteric vessels were congested with dark red-purple blood. The amount of peritoneal fluid diminished with time. At 8 and 16 hours PI, peritoneal fluid was near normal in viscosity, color, and quantity. At 36 and 40 hours PI, abdominal organ serosal surfaces were sticky to dry.

Gross lesions were prominent in adrenal glands of all ponies. Patchy to diffuse adrenal cortical congestion was seen in adrenal glands from 8, 16, and 26 hours PI ponies (Figure 4). Adrenal glands were swollen (twice normal), edematous, and friable at 32, 36, and 40 hours PI. On cut surface, the adrenal cortex was dark red, thickened, bulging from the surface, compressing the medulla, and wet with red tinged fluid (Figure 5).

The livers of all ponies at the various necropsy times were swollen and severely congested. The spleen was normal size but the red pulp was soft and drained from the cut surface in all post mortem examinations. Serosal ecchymotic hemorrhages were present in later (26 hours PI) spleen samples. The stomach fundus was dark purple in all samples.

Other abdominal organs were variably involved. Petechial and ecchymotic hemorrhages were observed on the serosal surfaces of the small intestine. Similar subcapsular hemorrhages were seen on the kidney. The distribution and time of lesion development were variable.

The character of the blood in congested organs, especially in the liver, changed with time. In the 8 and 16 hour PI animals, blood was normal color and readily clotted. In 32, 36, and 40 hour PI samples, the blood was dark red-purple and failed to clot after exposure to air. Serous atrophy of perirenal fat was observed in 32, 36, and 40 hour PI ponies. Icterus was seen in 36 and 40 hour PI ponies.

The lungs and heart had less severe lesions. The serosal surface of the lung had petechial hemorrhages in all samples but distribution and prominence was variable. Subendocardial, subvalvular, and pericardial hemorrhages were seen in the heart of samples from ponies of all time periods. Severity of lesions varied between animals at different times of necropsy.

Microscopic lesions Tissue samples for histopathologic examination were representative of gross lesions. The most significant histopathologic changes were observed in the large intestine. Focal dilatation of small veins and capillaries of the submucosa and laminae propria was seen in the cecum and ventral colon specimens at 8 and 16 hours PI. Lymphoid follicular necrosis with accumulation of cellular debris was present in 8 hour PI samples (Figure 6). Scant amounts of eosinophilic globular material were seen in the laminae propria underlying the luminal mucosa of the cecum and dorsal colon, in one of the 8 hour and both the 16 hour PI samples (Figure 7). In the 26 hour PI sample of the ventral colon,

distended lymphatics and edema of the submucosa accompanied lymphoid necrosis and vascular congestion. Mucosal epithelial damage of the ventral colon was absent. In the 32, 36, and 40 hour PI samples, mucosal lesions were present. They were most prominent in the cecum and ventral colon specimens. There was capillary distension with blood in the laminae propria (Figures 8,9,10). In the luminal one half of the mucosa, the laminae propria was disrupted with the suffusion of eosinophilic proteinaceous fluid and edema (Figures 11,12). The mucosal epithelium was focally disrupted with underlying blood, cells, and debris escaping into the lumen (Figure 13). Mucosal erosions were also seen which had loss of epithelium and infiltrations of inflammatory cells. In the submucosa, there was venous dilatation, arterial constriction, and foci of hemorrhage. The lesions were most severe in ventral colon and cecal samples.

Cecal and mesenteric lymph nodes also had follicular lymphoid necrosis as early as 8 hours PI. In 16 hour PI samples, edema was also a feature with medullary sinusoids containing many swollen macrophages with highly granular eosinophilic cytoplasm. Splenic lymphoid follicles had central necrosis. The red pulp was diffusely filled with erythrocytes.

In 8, 16, and 26 hour PI samples, the adrenal glands had capillary sinusoids distended with blood. The severity

increased from the zona glomerulosa toward the medulla. Adrenal cortical cells were swollen and the cytoplasm was foamy in appearance (Figure 14). In adrenal gland specimens after 26 hours PI, focal to diffuse adrenal cortical hemorrhage obliterated the architecture. In one 36 hour PI specimen, focal necrosis was pronounced and characterized by aggregated acellular debris surrounded by hemorrhage and focally intense infiltrations of neutrophils.

Liver samples had diffuse portal and sinusoid distension with blood and hepatocellular swelling with biliary stasis. Other organs had similar vascular changes but varied between individual animals.

Electron microscopic alterations The earliest vascular lesions were observed at 8 hours PI. Endothelium of small vessels in the laminae propria of the large intestine mucosa was affected. Endothelial cells were swollen and protruded into the vascular lumen. Intercellular edema with separation of organelles, swollen mitochondria, and fragmented cytocavity network was prominent. Pinocytotic vesicles were few. At 26 hours PI, extravascular edema was prominent in the laminae propria with separation of stromal cells. The basal laminae of the vessels were indistinct with derangement of fibers. Thirty-two hours PI, endothelium was severely damaged and separated from the basal laminae (Figure 15). Numerous autophagosomes and vesicles were present. Plasma membranes were irregular and tight junctions disrupted. Basal laminae were

severely deranged and fragmented. Collagen fiber bundles were damaged with individual fibers fragmented. Pericytes were separated from basal laminae. Stromal cells were separated by edema and amorphous granular protein similar to that within vascular lumens. Erythrocytes were scattered in the laminae propria.

At 16 hour PI, the crypt basement membrane in the cecum was indistinct and fragmented. Epithelial cells had disruption of tight junctions at 32 hours PI (Figures 16,17). Edema separated epithelial cells and numerous plasma membrane finger-like pseudopodia were present. Crypt basement membrane was severely damaged with only scant fragments of fibers remaining.

Damage to adrenal gland cortical epithelium was observed 8 hours PI. Individual cells and nests of cells were swollen, and had fragmented endoplasmic reticulum and enlarged mitochondria. Secretory granules were few. Capillaries were severely distended with erythrocytes. At 26 hours PI, extravasated erythrocytes obliterated normal adrenal cortical cellular architecture. Cortical cells were necrotic and had condensed nuclear matrix, fragmented organelles, and damaged plasma membranes. There were numerous neutrophils in the adrenal cortex and lysosomal degranulation was prominent. Small aggregates of fibrin were observed both in capillaries and surrounding degenerate adrenal cortical cells. Thrombosis and occlusion of vessels was not observed.

DISCUSSION

The development of lesions in equine endotoxemia is caused by vascular damage and loss of fluid homeostasis. Lesions were most severe in the large intestine and suggest selective endothelial sensitivity to endotoxic induced injury. Variation in vascular lesion severity is also reported in other species. In the rat and monkey, Kupffer cells of the liver were damaged while endothelium of other organs was not.[12,13] In rabbits, endothelial damage caused by endotoxin was greater in the aortic arch and abdominal aorta than in the thoracic aorta.[14] Selective response of vascular endothelium to endotoxic injury may reflect special functional adaptations for various organs. In the horse, the functional relationship of fluid movement and homeostasis is most significant in the large intestine.[15] Fluid movement into the large colon and cecum from the plasma is a minimum of 10 L daily. The volume that must be recovered from the large intestine in a 24 hour period is approximately equal to the total extracellular fluid volume.[16] Selective endothelial function no doubt plays a role in such fluid flux in the large intestine. Any damage of these cells would result in severely disrupted fluid homeostasis and be reflected as circulatory shock.

Mucosal lesions in the large intestine are directly related to the effects of endotoxin and the vascular injury it

causes. With damage to the capillaries, protein and fluid escape the vessels into the extraluminal spaces. This is histologically evident in the luminal one half of the large intestinal mucosa. Mucosal epithelial barrier is disrupted and the osmotic gradient in large intestinal contents potentiates fluid movement into the gut lumen. Direct exposure to endotoxin may play a role in mucosal epithelial tight junction disruption. Walker and Poruaznik were able to demonstrate intestinal epithelial tight junctional leakage using freeze-fracture techniques in rats.[17] Damage to the intestinal mucosal barrier may permit added exposure of the host to doses of endotoxin from an enteric source. Lillehei and MacLean postulated that this 'intestinal factor' may be responsible for development of irreversible shock.[18] This experiment supports that concept. In a previous experiment, the second phase of circulatory collapse corresponded in time with the disruption of the mucosal epithelial tight junctions.[19]

Adrenal gland changes are similar to those of the Waterhouse-Friderichesen syndrome in man,[20] which is associated with fulminating infection and results in shock, adrenal hemorrhages, and high mortality. In cattle, these lesions can be induced by intravenous endotoxin infusion.[21] These changes most likely are caused by blood stasis, metabolic disturbance, and hypoxia. Similar lesions can be produced in rabbit adrenal gland following orthostatic collapse.[22]

Lesions produced in this experiment are similar to those observed in acute equine colic syndromes such as Colitis X.[3,4] The specific mechanism causing the shock syndrome is unknown although Rooney et al. suggested an 'exhaustion' shock initiated and promoted by stress factors.[23] An initiating stress could damage the enteric mucosal barrier or alter portal hepatic clearance of endotoxin, permitting systemic exposure to scant amounts of endotoxin. Walker et al. have been able to quantitate low dose levels of circulating endotoxin in rats following stress of gamma radiation or hyperbaric stress.[24,25] Intestinal epithelium was briefly disrupted, then rapidly returned to normal with no evidence of injury. Hepatic injury could also permit systemic exposure to endotoxin.[26]

Once small amounts of endotoxin enter systemic circulation and vascular damage occurs, the clinical picture would closely resemble experimental intravenous low dose lipopolysaccharide infusion (acute endotoxic shock). In rabbits, a similar syndrome to Colitis X and experimental equine endotoxemia has been reported.[27] The cause is a non-enterotoxigenic Escherichia coli in the cecum. This disease was experimentally reproduced using freeze-thaw endotoxic extracts. The similarity of the equine and rabbit large intestine, both in response to endotoxin and in structure and function, suggests that Colitis X in horses and E. coli

colitis in rabbits have the same pathogenesis. Specific mechanisms which alter the mucosal barrier in the horse are important in future experiments if the development of acute colic syndromes is to be understood.

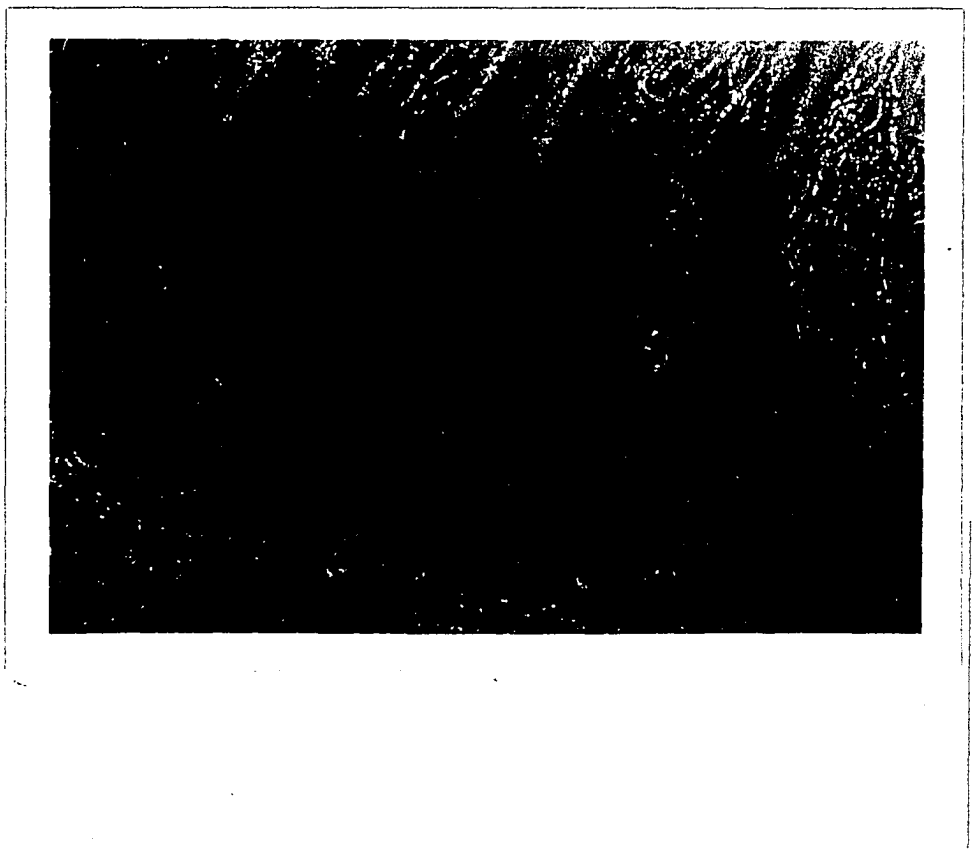
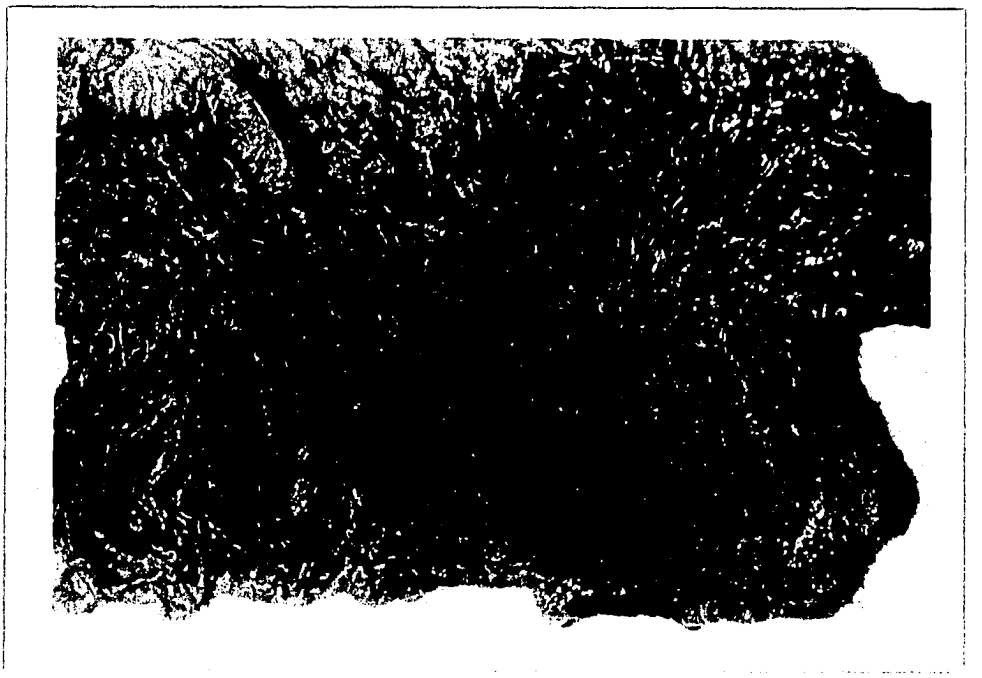


Fig 1 Focal area of intense mucosal congestion in the cecum of a pony intravenously infused with E. coli lipopolysaccharide and killed 16 hours post infusion.

Fig 2 Multifocal areas of mucosal congestion and hemorrhage in the cecum of a pony intravenously infused with E. coli lipopolysaccharide and killed 40 hours post infusion.

Fig 3 Multiple focal areas of congestion, hemorrhage, and ulceration of colonic mucosa of a pony intravenously infused with E. coli lipopolysaccharide and killed 40 hours post infusion. Note mucosal ulceration with adhesion of intestinal contents (arrow).



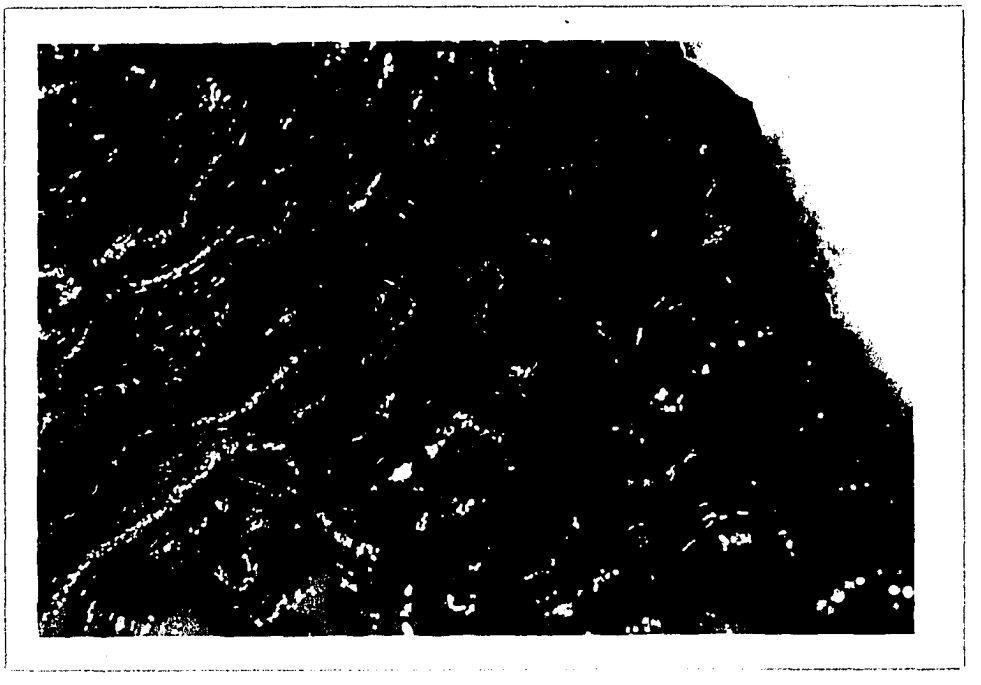


Fig 4 Patchy to diffuse cortical congestion in the adrenal gland of a pony intravenously infused with E. coli lipopolysaccharide and killed 26 hours post infusion.

Fig 5 Extensive cortical congestion and hemorrhage in adrenal glands of a pony intravenously infused with E. coli lipopolysaccharide and killed 36 hours post infusion. Note the thickened adrenal gland cortex bulging from cut surface with compression of adrenal gland medulla.

Fig 6 Necrosis and accumulation of cellular debris in a colonic lymphoid follicle of a pony intravenously infused with E. coli lipopolysaccharide and killed 8 hours post infusion. H & E, 340X.

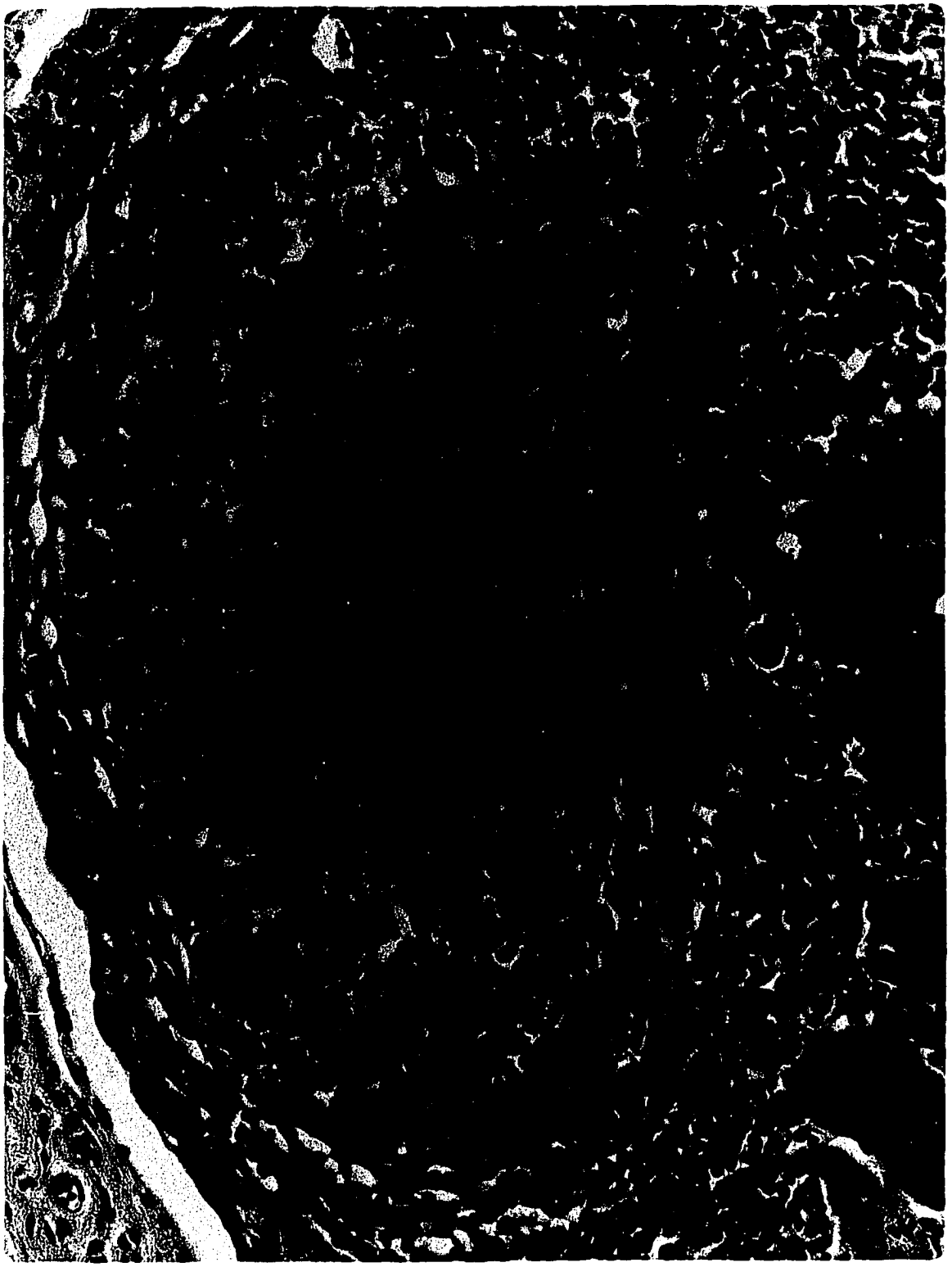


Fig 7 Cecal mucosa and laminae propria of a pony intravenously infused with E. coli lipopolysaccharide and killed 16 hours post infusion. Note capillary distension with blood and extravascular accumulation of scant amounts of globular material in the lamina propria. H & E 680X.

Fig 8 Cecal submucosa and mucosa of a pony intravenously infused with E. coli lipopolysaccharide and killed 32 hours post infusion. Note the submucosal venous and mucosal capillary distension with blood, loss of mucosal epithelium, and disruption of normal lamina propria architecture. H & E, 170X.

Fig 9 Cecal mucosa of a pony intravenously infused with E. coli lipopolysaccharide and killed 32 hours post infusion. Note the loss of mucosal epithelium and obliteration of the lamina propria architecture in the luminal one half of the mucosa. H & E, 340X.

Fig 10 Cecal mucosa of a pony intravenously infused with E. coli lipopolysaccharide and killed at 32 hours post infusion. The lamina propria architecture is obliterated by cellular debris, blood, and globular material. Mucosal epithelium is focally disrupted with luminal exposure of underlying stroma. H & E, 680X.

Fig 11 Cecal mucosa of a pony intravenously infused with E. coli lipopolysaccharide and killed 40 hours post infusion. There is suffusion of proteinaceous fluid in the lamina propria. A focus of ulceration is adjacent to intact mucosal epithelium. H & E, 170X.

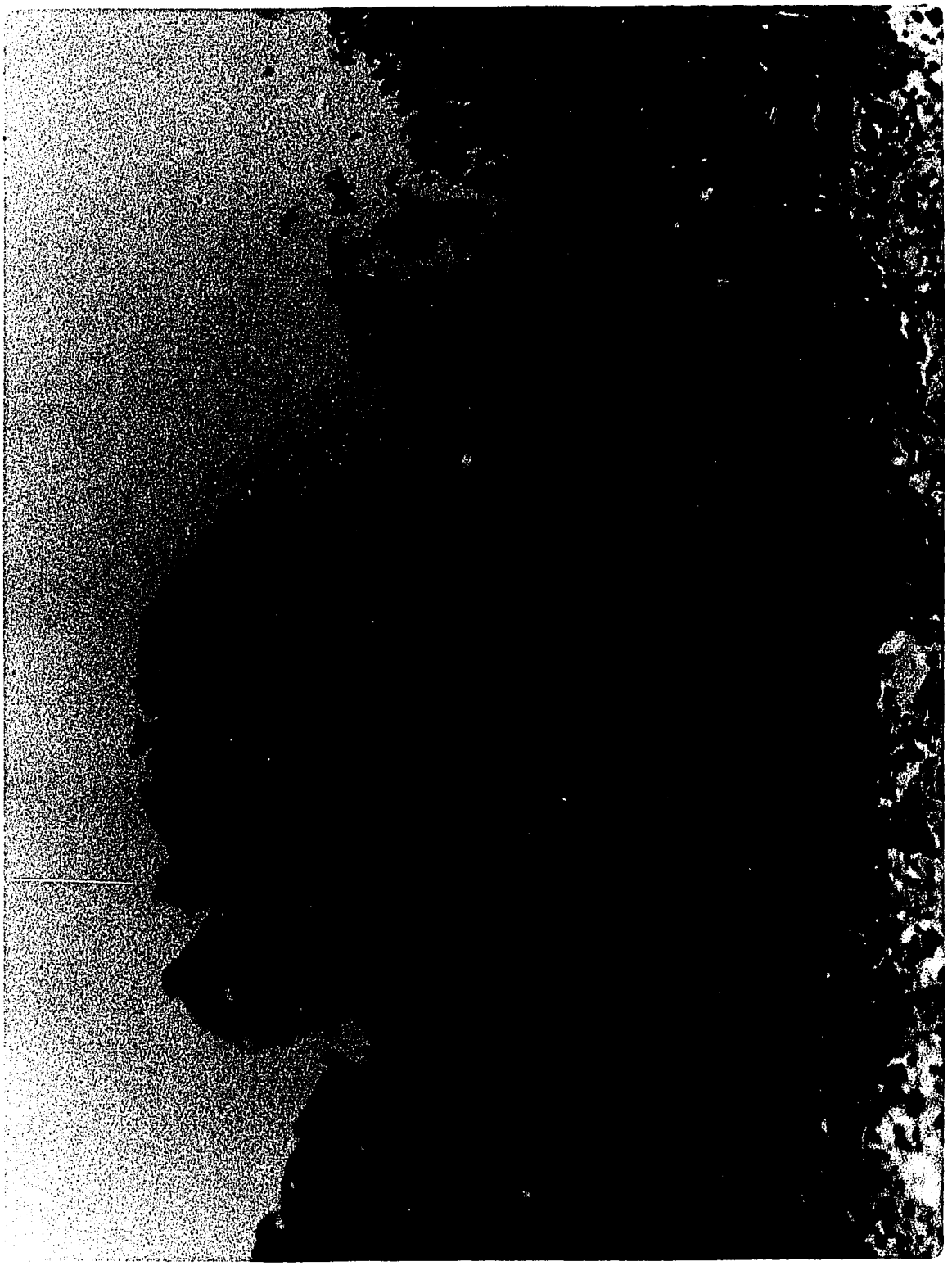


Fig 12 Cecal mucosa of a pony killed 40 hours after intravenous infusion of E. coli lipopolysaccharide. Note midmucosal lamina propria suffusion of protein and cellular debris. H & E, 340X.

Fig 13 Cecal mucosa of a pony intravenously infused with E. coli lipopolysaccharide and killed 40 hours post infusion. The mucosal epithelium is disrupted. Escaped cells, blood, and debris are present in the cecal lumen. H & E, 340X.

Fig 14 Focal hemorrhage and capillary distension with blood in the adrenal cortex of a pony intravenously infused with E. coli lipopolysaccharide and killed 26 hours post infusion.

Fig 15 Vessel within the lamina propria of the colon of a pony intravenously infused with E. coli lipopolysaccharide and killed at 32 hours post infusion. The endothelium (E) is separated from the pericyte (P). Amorphous granular material (Am) separates the vessel from stromal cells. Collagen fibers (C) are fragmented and disaggregated. 9030X.

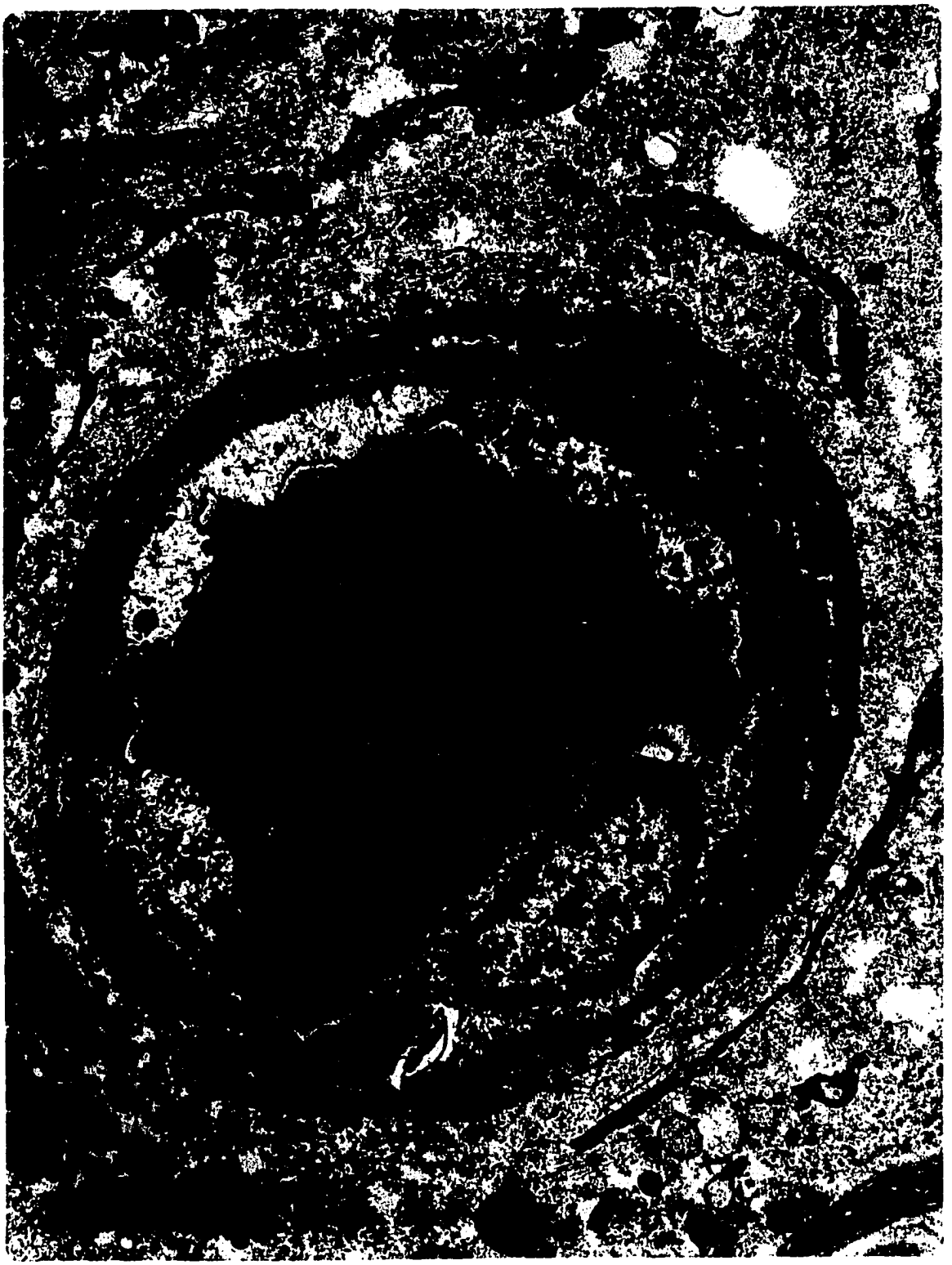


Fig 16 Colonic crypt epithelium and stromal cells of a pony intravenously infused with E. coli lipopolysaccharide and killed 32 hours post infusion. Epithelial cells (Ep) are separated from each other and the crypt basement membrane (Bm). 7056X.

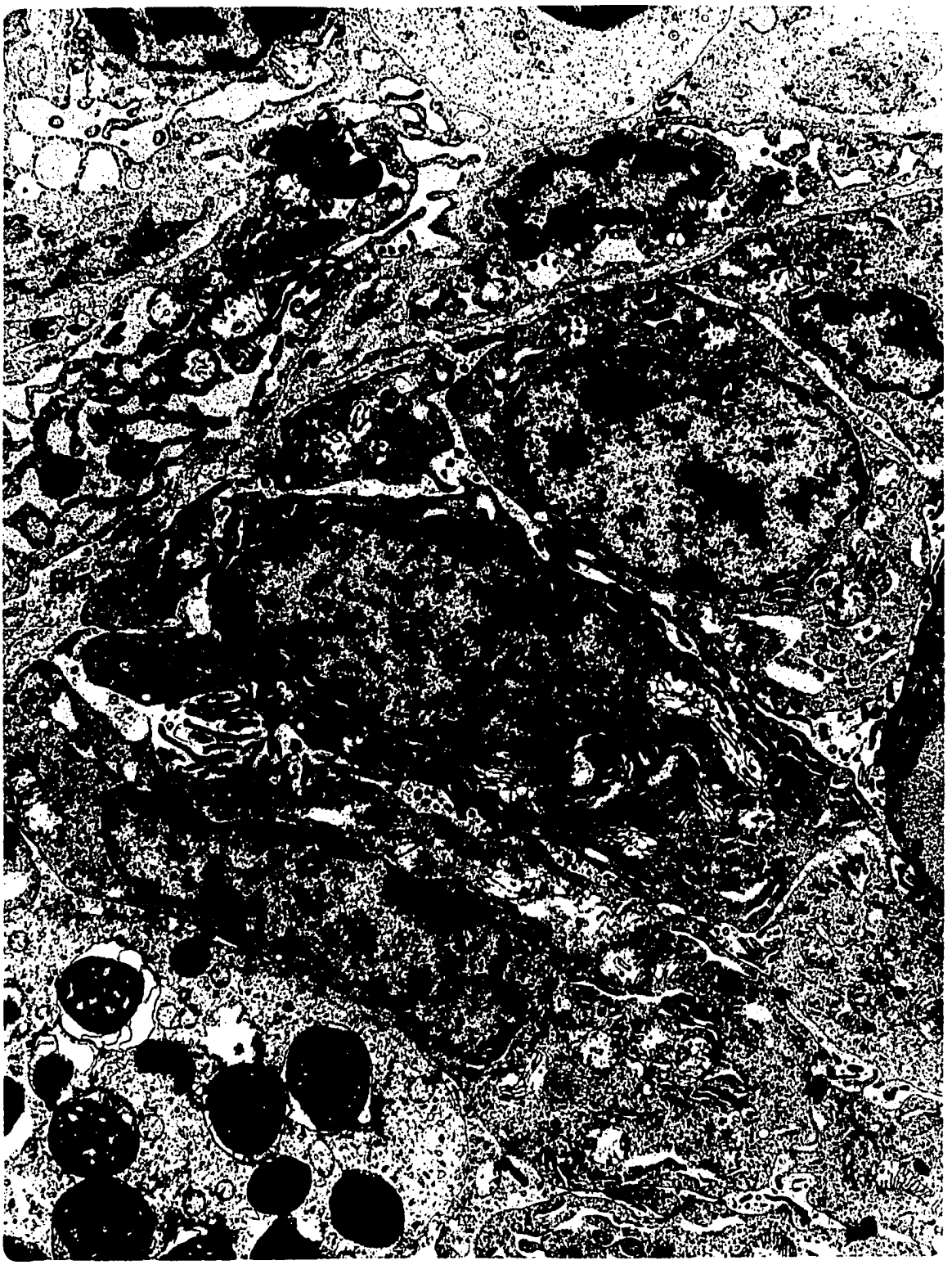
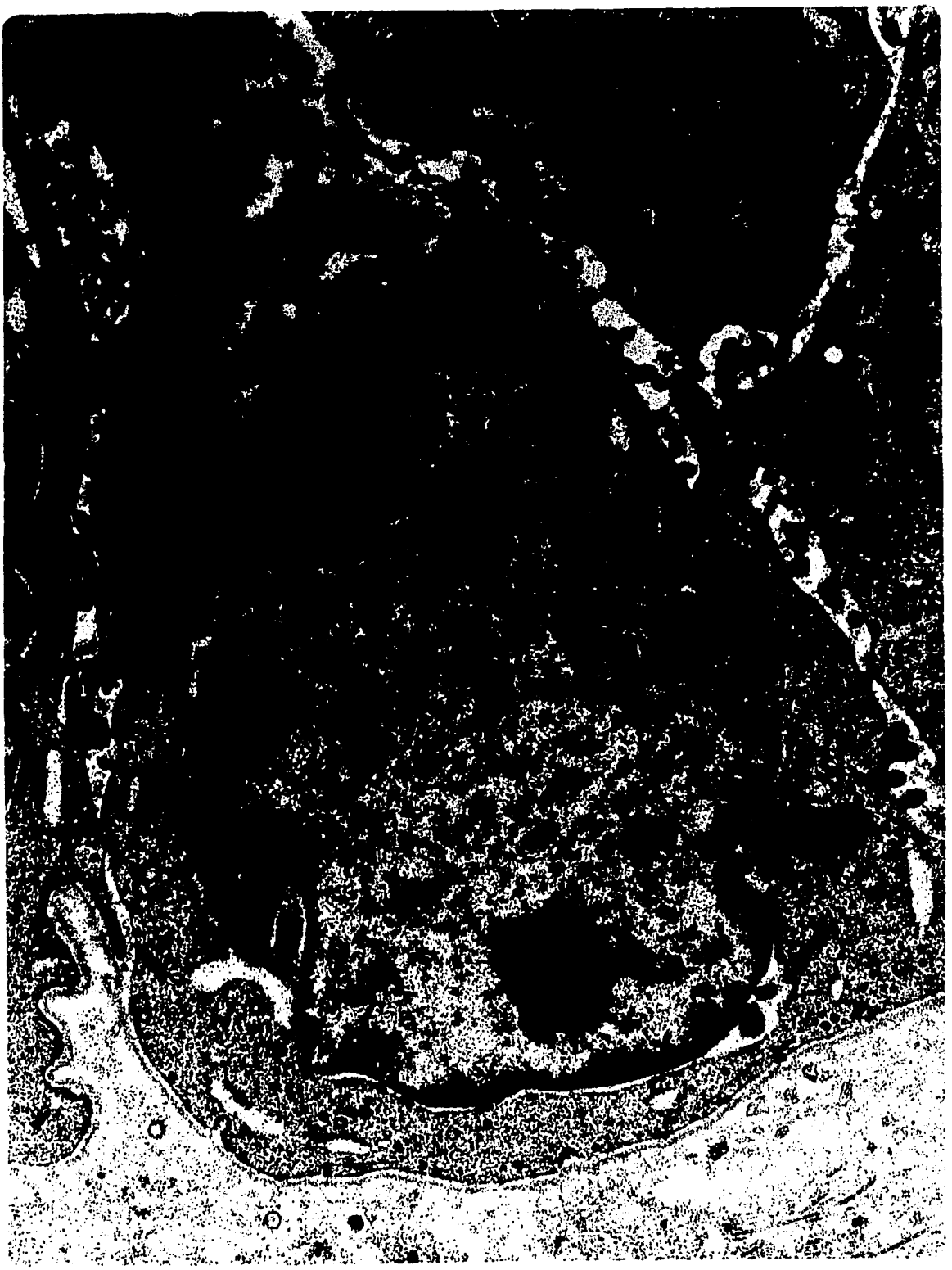


Fig 17 Colonic crypt epithelial cells of a pony intravenously infused with E. coli lipopolysaccharide and killed 32 hours post infusion. The tight junctions between epithelial cells are disrupted (arrows) and plasma membrane pseudopodia are present. 19,110X.



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GENERAL DISCUSSION AND CONCLUSIONS

This investigation provides the first description of both the clinical-pathological manifestations and lesions produced in Escherichia coli lipopolysaccharide-induced endotoxemia in ponies. This study is also a chronological characterization of the pathologic alterations and gives insight into the course of lesion development. Third, the similarity between experimental equine endotoxemia and natural acute colic syndromes supports the role of bacterial endotoxins in these conditions.

Lesions that developed in 40 μg/kg body weight infusion of E. coli lipopolysaccharide (LPS) were caused by vascular injury and intercompartmental fluid flux. The result was circulatory shock. Hemoconcentration and hyperproteinemia, 15 minutes after LPS infusion, suggested rapid extravascular fluid movement. This hypothesis could not be confirmed since post mortem examination was not performed before 8 hours post infusion (PI). At 8 hours PI, however, the cecum and ventral colon were filled with contents abnormally high in fluid. Twelve to 26 hours PI, hypoproteinemia developed even though animals were clinically dehydrated. These findings suggested development of vascular injury. This hypothesis was supported by histologic and ultrastructural observations. In the cecum and colon, capillaries were distended with blood. Edema and

proteinaceous suffusions were observed in the mucosal laminae propria of the large colon and cecum. Ultrastructurally, endothelial damage with disruption of tight junctions and proteinaceous material around vessels were seen.

The mechanisms that caused the vascular injury were not established in this study. Neutropenia and thrombocytopenia developed 15 minutes after infusion. Sequestration and activation of neutrophils and platelets with release of lysosomes and mediators could cause vascular injury. Light and electron microscopic examination of tissues 8 hours PI failed to locate the site of neutrophil sequestration, so this hypothesis cannot be substantiated.

Lesions of endotoxic shock were most severe in the cecum and ventral colon. Selective injury to vessels in the mucosa was the cause for lesion development. The interference in normal fluid resorption in the large intestine could play a role in the significant fluid imbalance observed in equine endotoxic shock.

Mucosal epithelial damage with erosion of the mucosa of the cecum and ventral colon was the most serious lesion observed. Ulceration in the 36 and 40 hours PI large intestines was also directly related to vascular injury. Fluid movement from intravascular to extravascular spaces in the laminae propria is enhanced by the high osmotic pressure of the large intestine luminal contents. Either because of direct injury

by the LPS or high extravascular hydrostatic pressure, mucosal epithelial tight junctions were disrupted. The loss of the mucosal barrier allows for uninterrupted movement of vascular fluids into the large intestine lumen.

Disruption of the mucosal barrier may have a second adverse effect, that is site for lethal exposure to an enteric source of bacterial endotoxins. The observation of a terminal circulatory collapse after apparent recovery from clinical signs corresponded in time with the lesion of disrupted mucosal epithelial tight junctions.

The clinical course of experimental endotoxemia and the lesions observed in the ponies were similar to syndromes of Colitis X and Salmonellosis. Clinically, the conditions were similar in the development of: 1) hemoconcentration; 2) neutropenia with subsequent neutrophilia; 3) consumptive coagulopathy; 4) cyanosis; 5) digestive disturbances; 6) pyrexia; and 7) irreversible shock. Lesions produced included large intestine luminal fluid accumulation; cecal and colonic mucosal hemorrhage, edema, and ulceration; adrenal cortical necrosis and hemorrhages; and visceral organ congestion with scattered serosal hemorrhages. Only speculation as to initiation of the natural disease syndromes can be given but a role for endotoxic shock is strongly supported by the findings of this study.

Considerations for further investigation include: 1) epidemiological and pathological examination of natural

acute shock cases with ultrastructural studies and systemic endotoxin quantitation; 2) examination of the role of large intestinal mucosal vascular bed in fluid secretion and resorption in normal horses; 3) the role of large intestine mucosal epithelium in fluid movement; and 4) mechanisms that may alter the large intestine mucosal barrier permitting endotoxin absorption.

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