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INTERACTION OF EQUINE NEUTROPHILS AND THE CONTAGIOUS EQUINE
METRITIS ORGANISM

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Interaction of equine neutrophils and
the contagious equine metritis organism

by

Timothy Allyn Bertram

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

Major: Veterinary Pathology

Approved:

Signature was redacted for privacy.

~~In~~ Charge of Major Work

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For the Graduate College

Iowa State University
Ames, Iowa

1983

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GENERAL INTRODUCTION

Contagious equine metritis (CEM) is a venereal disease of the horse which spread to several countries within the 1977 breeding season. This disease is caused by the contagious equine metritis organism (CEMO), a Gram-negative bacterium. It is manifested as a severe mucopurulent uterine discharge (105, 128). The neutrophilic efflux into the uterus can continue for weeks following initial infection (84). The predominant inflammatory cell, in the uterine lamina propria in early stages of the disease is the neutrophil (87, 96). As the disease progresses, the inflammatory cell population changes to include macrophages, lymphocytes, and plasma cells. The role that each of these cells has in protecting the horse from this bacterium has not been completely determined. Lesions are limited to the oviduct, uterus, cervix, and vagina. CEMO is found in the uterine exudate but has not been demonstrated in tissues deep to the basement membrane of the uterine mucosa.

Horses infected with CEMO have a detectable antibody titer within ten days after infection (20). The antibody titer is variable and has a relatively short duration but can coincide with remission of clinical signs (20). Investigations into the use of parenteral vaccination have not resulted in an ability to induce immunity to infection by CEMO (31, 103). Furthermore, vaccination, previous infection, or elevated antibody titers have not been demonstrated to prevent a "carrier state" in horses recovering from infection (20, 31, 103).

In vitro evaluations of the neutrophil responses to various bacteria (7, 58, 100, 109, 110) has lead to a better understanding of disease mechanisms and potential therapy (22). The importance of the neutrophil in ridding the body of infectious agents has been realized for some time (57, 75), while antibody and complement can work synergistically with the neutrophil in removing injurious bacteria (42). Some body fluids have been demonstrated to impair the neutrophil's ability to kill certain pathogenic bacteria (82). Impaired neutrophil functions, both acquired and congenital, have been demonstrated to be a significant factor in predisposing the body to bacterial infections (82).

The purposes of this research include i) evaluation of the effects of in vitro isolation on equine neutrophil morphology as well as develop a rapid procedure to obtain homogeneous populations of these cells, ii) determine the ability of equine neutrophils to ingest and kill CEMO in the presence of serum with high and low specific antibody titers, seminal plasma, and uterine flushings, and iii) evaluate neutrophil functions after exposure to CEMO or one of two lipopolysaccharides isolated from CEMO.

This dissertation is presented in alternate format including four manuscripts which have been submitted or accepted for publication in professional scientific journals. The first manuscript has been published in Veterinary Pathology, the second manuscript has been published in Infection and Immunity, the third manuscript has been accepted for publication in the American Journal of Veterinary Research, and the fourth manuscript has been submitted to the journal, Infection and

Immunity. The format used in this dissertation is similar to that of the journal, *Infection and Immunity*. A general introduction and literature review precedes the first manuscript and a general summary and discussion follows the final manuscript. Literature cited throughout this dissertation is listed in alphabetical order after the general summary and discussion. Throughout the manuscript, "CEMO" will be used as singular or plural to indicate contagious equine metritis organism(s).

Timothy A. Bertram was the principal investigator for each of these studies.

LITERATURE REVIEW

Historical background. In Great Britain during the 1977 equine breeding season, a unique equine venereal disease was recognized. In April, a large breeding farm in the Newmarket area had a high proportion of breeding mares return to estrus after a shortened diestrus period. Forty percent of these mares had a profuse vaginal discharge that appeared 48 hours after breeding. Further cases were reported in the Newmarket area during May and June. Crowhurst made the first English report of this venereal disease, pointing out that most mares presented with a copious amount of uterine discharge and that the disease was contagious (18). Because of the contagious nature and limitation to the horse, the disease was called contagious equine metritis (CEM). In the initial outbreaks, 29 farms in the Newmarket area were involved with 5 to 30% of the mares on given farms showing clinical signs. Identification of the causal agent was done by Platt et al. (88). In that same breeding season, reports of a similar disease and similar bacterial isolation came from Ireland (130), France (64, 86), West Germany (74), Australia (48), and Belgium (90).

In February 1978, a Kentucky mare bred to an imported French stallion had a mucopurulent vaginal discharge 8 days after breeding. In March 1978, two mares bred to two different French stallions had clinical evidence of CEM 10 days after being bred. By June 1978, 45 Kentucky mares and 5 stallions were culture positive for the causative agent of CEM and 13 other stallions were incriminated. The Kentucky outbreak involved both streptomycin-sensitive and streptomycin-resistant strains

(123). Another United States outbreak, involving only streptomycin-sensitive strains, occurred in Missouri in 1979 (28).

Clinical signs. Clinical signs are seen only in the female horse although both male and female can be infected (90). The most striking and consistent clinical sign of the mare is the premature return to estrus after a shortened diestrus period of 8 to 10 days (90). Mares have variable amounts of vaginal discharge ranging from copious amounts seen on the perineum and dripping from the labia to small amounts visible only with vaginoscopic examination. Some mares manifest only a reddened hyperemic cervix and vagina. Severely affected mares have an external vaginal discharge which covers the buttock and coats the tail hair (26, 90, 123).

The appearance of a uterine discharge is seen one to ten days following breeding (26, 90, 123), but may be as long as 80 days after breeding (97). In most cases the discharge lasts approximately two weeks (26). There is no evidence of stillbirth, abortion, elevated temperature, reluctance to move or anorexia. The absence of these clinical signs suggest that the causative bacterium of CEM does not induce a bacteremic/septicemic condition (26, 90). The stallion has no clinical signs at any time after infection (90).

Since clinical signs are variable and often absent, it is not possible to differentiate CEM from other genital infections in the horse based on clinical signs. These clinical signs also suggest dose of inoculum, virulence of the specific infecting bacterium, and

immune status of the host may play a role in the clinical appearance of CEM (90).

Experimental infections have reproduced clinical disease essentially identical to natural cases (84, 87, 105, 128). Experimentally, the mare is susceptible to infection by the causative agent of CEM at any period of the estrous cycle (84, 87, 128). Mares infected experimentally can have uterine exudate as early as 6 hours post uterine inoculation (1). Clinical disease cannot be induced experimentally if the inoculum is placed in the vagina, vestibule, or clittoral sinus (84, 105).

Pathology. Pathological changes in the horse are confined to the genital tract with the uterus and cervix most severely affected (87, 90). There is a mild vaginitis but the frequency and intensity varies among animals (84). Most of the early morphological studies were done with uterine biopsies and this has influenced results between various reports. Using biopsied tissue, several histological changes have been reported to be specific for CEM (84, 95). These changes included 1) a proliferation of luminal epithelial cells, 2) vacuolation at the base of luminal epithelial cells, 3) high numbers of neutrophils present in the luminal epithelium and stratum compactum, and 4) marked infiltration of plasma cells and macrophages into the stratum compactum and stratum spongiosum. Of these changes, vacuolation of epithelial cells and mononuclear cell infiltration were seen most consistently. Subsequent studies using tissues taken at necropsy demonstrated the characteristic vacuoles are not frequently observed and likely result from the biopsy technique (1).

Histological changes consisting of macrophage and neutrophil infiltration have been reported to last up to 205 days post inoculation (96). Culture of the causative bacterium was also possible throughout the 205-day sampling period (96).

The profuse uterine exudate reported in most infected mares is composed primarily of neutrophils throughout the course of the disease (87, 96). Neutrophil numbers decrease as the amount of exudate decreases, but this does not correlate with ability to isolate the bacterium (96, 143). Neutrophilic exudate may be profuse, yet it may not be possible to culture the bacterium from the reproductive tract. Some neutrophils within the exudate contain the bacterium in various stages of degeneration and high numbers of the bacterium may be seen extracellularly (143).

Ultrastructurally, uterine epithelial cells from an infected uterus have large round nuclei with extensive amounts of euchromatin suggesting epithelial hyperplasia (96). Further, vacuolation, myelin figures, and many lysosomes have been seen in the uterine epithelial cytoplasm (96). Apical microvilli were absent, cilia clumped, with fragmentation and blebbing of the luminal surface plasma membrane and cytoplasm (96). All changes support epithelial cell degeneration and an acute endometritis. Other ultrastructural findings included a unique but unidentified crystalline material 1 to 2 μm by 0.1 to 0.3 μm in size (96). These structures were located on the uterine luminal surface of mares with CEM (96). There are no reports of the causative agent being in tissue below the uterine epithelial basement membrane.

Causative bacterium. The causative agent of CEM is a newly recognized bacterium which has yet to be classified. In 1978, Taylor et al. proposed that this new agent be classified in the genus *Hemophilus* (127). This classification was not generally accepted and current literature is referring to this agent as CEM bacterium (104) or CEM organism (7, 26, 90).

Morphologically the organism has a wrinkled surface like most Gram-negative bacteria (122). CEMO is approximately 0.7 μm in width, ranges from 0.7 to 1.8 μm in length and can form filaments 5 to 6 μm in length (122, 126). CEMO has a 15 to 20 nm capsule but has no pili or flagella (126). The morphological appearance of the bacterium can be markedly influenced by the in vitro growth medium.

CEMO is Gram-negative, non-acid fast, and non-motile (127). The DNA base composition has been estimated from 36.1 to 39% GC (88, 112, 127). CEMO has large amounts of both 18 carbon unsaturated and 16 carbon saturated fatty acids with relatively lesser amounts of 16 and 18 carbon hydroxylated and 14 carbon saturated fatty acids (121).

Of the routine biochemical tests, CEMO is positive only for catalase, cytochrome oxidase, phosphatase, and the porphoryin tests (124, 127). CEMO is asaccharolytic in all media (124, 127), but does have detectable activity of various cellular enzymes (124, 127). CEMO requires neither the X factor (hemin) or the V factor (NAD), but growth is stimulated by the X factor (112, 124, 127). CEMO is best grown on chocolate blood agar at 37^o C with added moisture and in a 5 to 10% CO₂ atmosphere (124, 127).

Epidemiology. CEM occurred in more than five countries within three years (59, 123). The first cases were in France in 1975, Ireland in 1976, England and Australia in 1977, and the United States of America in 1978 (59). CEM has been found in several other European countries (59) and Japan (120) since the first reported outbreaks in Europe.

In England, 62% of the public breeding farms and 50% of the stallions in the Newmarket area were infected with CEMO (91). In this same region of England, 14% of all mares were infected with 37% of the mares at public breeding farms involved (91). Mares bred at the "nine-day heat" had a more severe clinical syndrome. Nearly all mares bred by an infected stallion became infected with CEMO. Contaminated instruments also played a role transferring CEMO from one horse to the next (91).

CEM was first observed in the United States of America on February 28, 1978 (123). Between the first report and March of 1978, 45 thoroughbred mares and five thoroughbred stallions were culture positive for the causative agent of CEM (123). Thirteen other stallions were incriminated but had been washed and treated with antibiotics before bacteriological examination. Stallions imported from France were incriminated with introducing CEM into the United States of America (23, 123). The spread of CEM appeared to be venereal between mares and stallions and by human contact of the external genitalia between stallion and stallion (123). The wet moist conditions of the breeding environment were also incriminated in improving stallion to stallion spread (123).

In April 1979, the second United States outbreak occurred in Missouri (28). This outbreak involved a Trakhener stallion and seven mares. All mares were culture positive for CEMO but the stallion was culture and serologically negative (28).

CEMO has been demonstrated in nonbreeding colts and foals (129). There have been 18 isolations of CEMO in young nonbreeding animals from Ireland, England, and Australia (129). Of these isolations, all CEMO have been streptomycin resistant. CEMO was isolated from one colt that was born in 1973 and had no breeding experience, raising the possibility CEM existed prior to 1976. In this report, only two of the 18 animals were female suggesting that the preputial sheath may facilitate infection during the birthing process (129).

CEMO has not been cultured from species other than the horse but antibodies to the agent have been detected in several other species. Approximately 600 cattle tested in the United Kingdom had titers between 1:10 to 1:160 to the CEMO with most of the titers ranging from 1:20 to 1:40 (17). The titers did not correlate with reproductive failure or clinical signs similar to those seen in CEMO infected horses (17).

A British study raised the possibility that CEMO infects humans (136). Healthy English men and women as well as those which had non-gonococcal urethritis were tested for serum agglutinins to CEMO (126). The percentage of people with titers greater than 1:20 was recorded as positive for anti-CEMO antibodies. From these findings, 2 to 5% of the healthy men and women had titers to CEMO. Twenty percent of randomly selected men and women seeking medical attention for genital disorders

and 40% of men with nongonococcal urethritis had CEMO titers (126). Serum agglutinins to CEMO have also been reported in the bovine population (17).

The significance of finding serum agglutinins to CEMO in cattle and humans is unclear. The authors speculated that these titers may be from undetermined organisms with one or more antigens in common with CEMO (17, 126). Isolation of CEMO was not successful in either the bovine or human cases (17, 126).

Neutrophils. In the bone marrow, granulocyte precursors constitute about 50% to 60% of all cells. The myeloblast constitutes approximately 2%, promyelocyte 5%, myelocyte 12%, metamyelocyte and band 22% and the mature neutrophil approximately 20% of the bone marrow granulocytes (57, 144). One myeloblast gives rise to two promyelocytes, four myelocytes, and eight metamyelocytes (24). The metamyelocytes mature with no further cell division (24).

Granule formation has been studied in the human and rabbit bone marrow (3, 4). Peroxidase (a component of the neutrophil primary granule) was detected in the promyelocyte stage. This reaction was observed throughout the endoplasmic reticulum, perinuclear cisternae, Golgi apparatus, and all granules of that stage (3). These granules were called azurophil or primary because of their staining affinity and time of development (3). In the myelocyte, peroxidase was present only in the cytoplasmic granules (primary). After this stage, many granules are peroxidase negative and were then classified as secondary or specific granules (3).

The primary granule contains peroxidase as well as other lysosomal enzymes such as acid phosphatase, and aryl sulfatase (3). Secondary granules contain many nonenzymatic proteins (37), but no lysosomal enzymes (3).

Primary granules are formed on the concave face of the Golgi apparatus and the secondary granules on the convex or outer face (75). The primary granules are reduced in number as the cell matures because cell division continues after primary granule formation ceases (75).

Equine neutrophils are morphologically unique compared to other mammalian neutrophils. Equine neutrophil primary granules are elongate, electron dense, and larger than secondary granules (106). The secondary granule is pleomorphic, being round, oval, or bilobbed, and less electron dense than primary granules (106). The nucleus of the equine neutrophil is hypersegmented and the nuclear membrane is denticulated (24).

Cytochemical properties of equine neutrophils have been previously evaluated (50, 51). Alkaline phosphatase and acid phosphatase activities are low or nonexistent in equine neutrophils (50, 51). Peroxidase and nonspecific esterase activities have been demonstrated (51). Glycogen content in the cytoplasm is low or nondetectable in most circulating equine neutrophils (51).

The functional significance of nuclear segmentation is not established. Metchnikoff felt this segmentation allowed for easier migration through the blood vessels (75). Since lymphocytes and monocytes also

emigrate through blood vessels, this hypothesis is unlikely. Further, the Pelger-Huet anomaly, where no nuclear segmentation occurs, does not alter neutrophil function or emigration ability (62).

Several different organelles are present in the equine neutrophil. The most prominent cytoplasmic organelles are the granules. There is considerable variation in granule size and shape between species (24, 106). Enzymatically there are two classes of granules. Both classes provide the bacteriostatic and bacteriocidal properties of the neutrophil (75).

An inactive genome is likely since nuclear chromatin is coarsely clumped and there is no morphological evidence of a nucleolus (57, 75). This would also suggest neutrophils cannot synthesize ribosomes and thus have minimal protein synthesis (75). The neutrophil has very little smooth or rough endoplasmic reticulum. Polyribosomes have been demonstrated, thus the cell may have some residual protein synthesizing capacity (75). Mitochondria are present in mature neutrophils but their numbers decrease as the cell matures (75). The amount of ATP provided by these remaining mitochondria is uncertain (75).

Glycogen is not readily observed ultrastructurally nor has it been demonstrated cytochemically in equine neutrophils (50). Glucose is the main source of energy for the neutrophil with the greatest source obtained from the extracellular tissue fluids (75). Oxygen has no effect on the glycolytic rate of neutrophils (no Pasteur effect), but glycolysis and O_2 consumption are greatly increased during phagocytosis (16). Neutrophils utilize the hexose monophosphate shunt to metabolize

glucose (75). One to two percent of glucose metabolism proceeds by this route in the resting neutrophil but this increases to 30% in cells actively phagocytizing bacteria (116).

Phagocytosis of microbial agents is dependent on the surface properties of the microbe and is an energy-dependent event (47). Antibody and complement are serum specific ligands which interact with the bacteria and neutrophil receptors to improve phagocytosis (42).

The human neutrophil has three unique complement fragment receptors on its surface. Some of these complement receptors may be lost during neutrophil maturation (99). Antibody receptors like the complement receptors are also present on the cell surface (75). Antibody or complement attachment, to their receptors triggers organization of cytoplasmic contractile filaments subjacent to the plasma membrane receptor site (41). These contractile elements include actin, myosin, actin binding protein, a protein that stimulates magnesium-dependent adenosine triphosphatase, and gelsolin, a calcium dependent actin regulatory protein (119, 147). Plasma membrane movement occurs as a result of coordination of these proteins and microtubules (118).

Neutrophil microtubules are composed of tubulin dimers and associated proteins (4, 57, 75). Tubulin consists of two unique protein subunits called α and β tubulin. The microtubule associated proteins may play a role in controlling tubulin polymerization (4).

Concentrations of tubulin dimers also participate in control of tubulin polymerization (80, 107). The critical concentration of tubulin dimers which causes polymerization can be lowered by nucleating centers

such as centrioles (80, 107). The paired centrioles can separate in a stimulated neutrophil resulting in two or more nucleating centers and thus more rapid microtubule assembly (107).

Cyclic nucleotides are also involved in control of tubulin polymerization (57, 75, 80, 107). Cyclic adenosine monophosphate (cAMP) and drugs which increase cAMP levels, in the cell, cause microtubule depolymerization (57, 80). Cyclic guanosine monophosphate (cGMP) and drugs which increase cGMP result in microtubule polymerization and stabilization of polymerized microtubules (80).

The various contractile elements have various sizes. Microtubules are 24 nm in diameter with a variable length (80). The rough edges seen ultrastructurally are a result of the microtubule associated proteins (MAPs) (80). Actin filaments are 6 nm in diameter and intermediate filaments are 10 nm in diameter (80).

Antibody and complement work in concert to promote phagocytosis of microbes (137). Antibody acts by promoting the fixation of the third component of complement to the bacterial surface, thus improving ingestion of the microbe by the neutrophil (47). In vitro, antibodies of the IgG isotype or complement may improve bacterial ingestion (47).

When bacteria are phagocytized by neutrophils, they are enclosed by a membrane-bounded vacuole that was originally located on the cell surface (47, 75, 106). Cytoplasmic granules fuse with the phagosome and the granular proteins are discharged into the phagosome (75, 106).

These proteins include lysozyme, which hydrolyzes a mucopeptide found in the cell wall of the bacteria; lactoferrin, which sequesters iron; and cationic proteins, which are highly basic and involved in bacteriocidal mechanisms (57, 75).

Myeloperoxidase is a bacteriocidal enzyme found in the primary granules of neutrophils (29). This enzyme works in concert with hydrogen peroxide and halide ions and is one of the most potent bacteriocidal mechanisms of the neutrophil (75). Generation of hydrogen peroxide requires molecular oxygen, NADPH oxidase, and NADPH from the hexose-monophosphate-pathway (29). Neutrophils obtain halide ions from their extracellular environment (75).

Reactive oxygens other than H_2O_2 include superoxide anion, singlet oxygen, and hydroxyl radicals (29). These ions are generated during NADPH oxidation (29). The exact role of these active oxygen radicles in bacteriocidal mechanisms is not currently established (75).

The neutrophil can kill bacteria by nonoxidative mechanisms that are independent of myeloperoxidase and nicotinamide adenine dinucleotide phosphate oxidase (57, 75). These mechanisms include low phagolysosome pH and high concentrations of lactic acid both of which are generated in the ingestion process (75); lysozyme from primary granules (57, 75); and lactoferrin and cationic proteins (75). The nonoxidative mechanisms must frequently function synergistically with complement and antibody to be bacteriocidal (75).

The consequences of phagocytosis are different for intracellular and extracellular pathogens. Different pathogenic microorganisms have

various antiphagocytic mechanisms (22, 47). For the intracellular bacteria, various mechanisms occur which alter the ability of the neutrophil to perform its killing mechanisms (22). These mechanisms may include inhibition of microtubules, microfilaments, and degranulation. Inhibition of key enzymes such as nicotinamide adenine dinucleotide phosphate oxidase, myeloperoxidase or other granule enzymes may also alter neutrophil killing abilities. Some pathogens have been reported to utilize neutrophil enzymes for metabolic substrates or are resistant to neutrophil enzymes and oxygen metabolites (22).

MORPHOMETRY OF EQUINE NEUTROPHILS ISOLATED
AT DIFFERENT TEMPERATURES

ABSTRACT

Equine neutrophils were evaluated ultrastructurally and by morphometric analysis. Homogenous populations of neutrophils were isolated from peripheral blood at 4° C and 22° C by centrifugation on two sequential Ficoll-Hypaque density gradients. Isolation procedures at both temperatures resulted in neutrophil degranulation but not cell swelling. Degranulation was more extensive in cells isolated at 22° C. Isolation temperature affected the neutrophil content of secondary granules more than primary granules. Granules similar to immature specific granules of human neutrophils were observed. Granules with a flocculent matrix were more frequent in cells processed at 22° C. These granules were considered to be involved in the degranulation process.

INTRODUCTION

Damage resulting from isolation procedures can adversely affect neutrophil function in vitro. Studies with human neutrophils after short periods of storage at different temperatures show reduction of intracellular bacterial killing and chemotaxis at 4° C (38, 60, 69, 70). With rabbit neutrophils, both of these functions are reduced at 22° C but maintained at 4° C storage (69). Killing of intracellular microorganisms occurs only after release of granule content into phagosomes. This function can be evaluated by structural and morphometric analysis of the neutrophil granules (55, 56, 85, 139, 142). Human neutrophil morphology studies indicate that there is a 15% decrease of intact cells within 48 hours after collection (61, 69).

Most leukocyte studies require homogeneous, viable, and normally functioning cells. Harsh isolation procedures may alter cellular function and lead to cell death. Structural or functional evaluation of degenerative cells or heterogeneous populations may lead to incorrect conclusions. Most leukocyte isolation procedures seldom consider isolation temperature and many result in heterogeneous polymorphonuclear leukocyte populations (11, 25, 32, 78, 125).

The purposes of this study are to evaluate the effect of isolation temperature on neutrophil morphology and to present an effective and rapid method of isolating a homogenous population of equine neutrophils.

MATERIALS AND METHODS

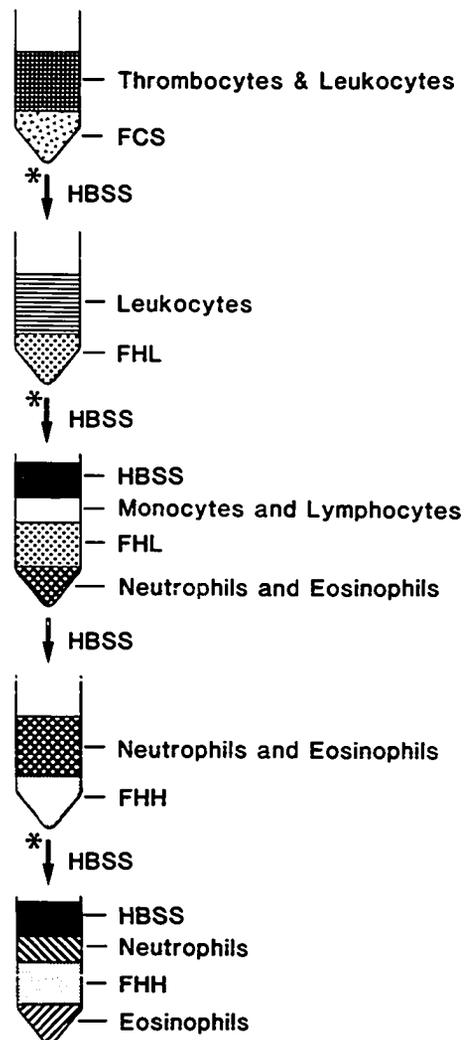
Animals. Three mature horses were included in this study, with samples being collected from each horse for the different processing temperatures and controls. The horses were clinically normal at the time of blood collection.

Blood collection. Whole blood was collected into 500 ml evacuated blood collection bottles by phlebotomy of the jugular vein. Each bottle initially contained 21.47 gm/100 ml dextrose, 1.32 gm/100 ml sodium citrate, 0.4 gm/100 ml citric acid, and 10,000 units of heparin. Two 500 ml samples were taken from each horse. One was chilled immediately in ice water (0-4° C) and the other was kept at room temperature (22° C).

Leukocyte separation. The following isolation procedures were completed at 22° C or 4° C, coinciding with collection temperature (Fig. 1). Samples were allowed to stand for 45 minutes to permit sedimentation of red blood cells. The supernatant was harvested and centrifuged at 1500g for five minutes. The pelleted cells were placed into a hypotonic solution of ammonium chloride in tris buffer, (pH 7.2) for five minutes to lyse erythrocytes, centrifuged at 1500g for five minutes, and suspended in calcium-magnesium free Hanks' balanced salt solution with 0.2% dextrose. The cells were layered carefully over an equal volume of fetal calf serum and centrifuged at 250g for 20 minutes to decrease thrombocyte numbers. The leukocytes were suspended in calcium-magnesium free Hanks' balanced salt solution with 0.2% dextrose and placed over an equal volume of Ficoll-Hypaque

Fig. 1. Leukocyte separation procedure, HBSS: Hanks' balanced salt solution, 0.2% dextrose, no calcium or magnesium, FCS: Fetal calf serum, FHL: Ficoll-Hypaque low (specific gravity 1.080), FHH: Ficoll-Hypaque high (specific gravity 1.125), *: Centrifugation, 20 minutes, 1,500g

- Blood sedimentation - 45 minutes
- Collection supernatant - Leukocytes & Thrombocytes & Erythrocytes
- Centrifugation - 5 minutes - 1500g
- Pellet suspension in NH_4Cl - Lysis Erythrocytes - 5 minutes
- Centrifugation - 5 minutes - 1500g
- Pellet suspension in HBSS - Leukocytes & Thrombocytes



LEUKOCYTE SEPARATION PROCEDURE

(Ficoll 400, Pharmacia Fine Chemicals; Hypaque Sodium, Winthrop Laboratories) density gradients with specific gravities of 1.010, 1.075, 1.080, 1.085, 1.090, and 1.095. After centrifugation at 250g for 20 minutes, the pellet was resuspended in calcium-magnesium free Hanks' balanced salt solution with 0.2% dextrose.

The granulocyte suspension was placed over an equal volume of Ficoll-Hypaque density gradients with specific gravities of 1.105, 1.110, 1.115, 1.120, 1.125, and 1.135, and then centrifuged at 250g for 20 minutes. The neutrophil layer was removed and washed with equine serum. Neutrophil viability was evaluated by light microscopy using trypan blue exclusion (108).

Control samples were collected and centrifuged immediately at 1500g for five minutes. These samples were considered to appear as normal neutrophils in the circulatory blood. Plasma was removed and 2.5% glutaraldehyde placed over the buffy coat surface for 45 minutes (138).

Electron microscopic examination. Samples for transmission electron microscope processing were postfixed in 1.0% OsO_4 for 30 minutes, dehydrated in graded alcohols, cleared in propylene oxide, infiltrated and embedded in epoxy-resin (Epon 812, Shell Chemical). Ultrathin sections were cut at 70-90 nm and stained with uranyl acetate and lead citrate.

Identification of primary granules was done as previously reported (68). Neutrophils were fixed in 2.5% glutaraldehyde, washed with

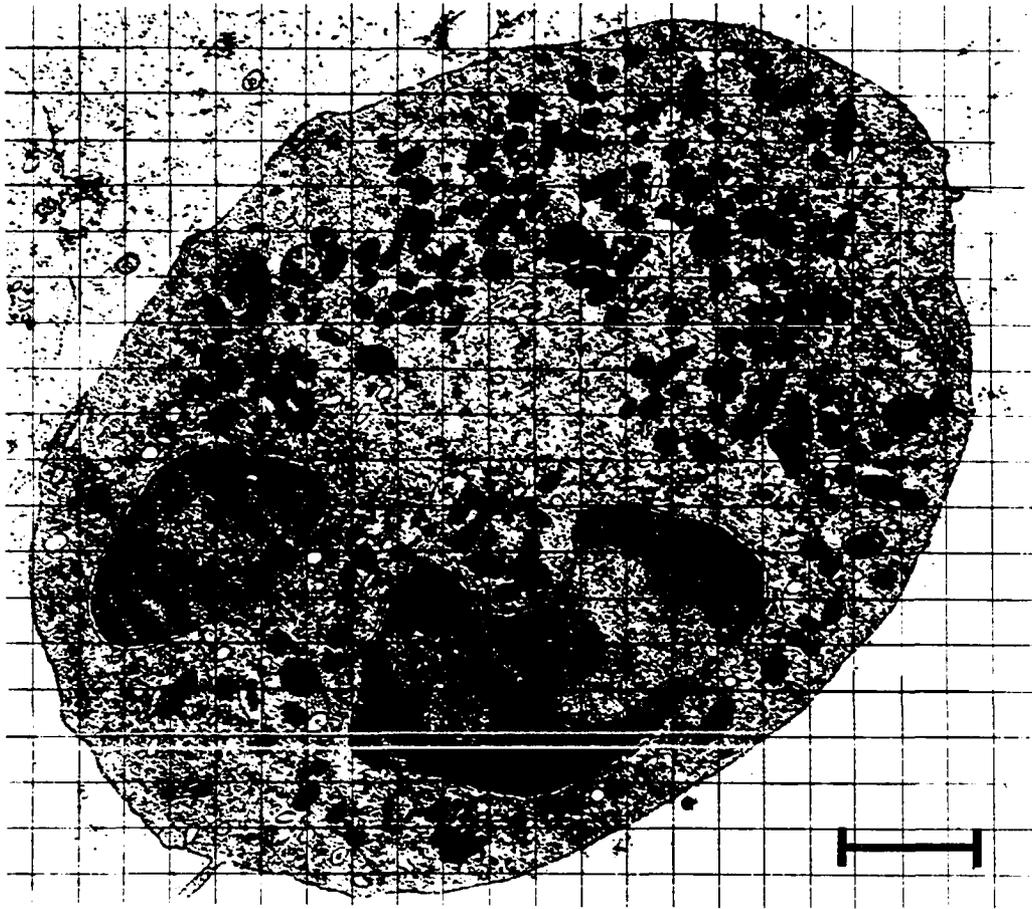
cacodylate buffer, and suspended in the peroxidase substrate of Graham and Karnovsky (0.1% H_2O_2 , 0.05% diaminobenzidine in 0.05M tris buffer at pH 7.6) (40).

Two tissue blocks were made from the buffy coat sample and the neutrophil layer from each horse at each temperature. One ultrathin section was cut from each block and six micrographs were taken from each section at a magnification of 13,000. Final magnification of each micrograph was 34,000.

Morphometry. Volumetric morphometry was done with a 25 cm x 25 cm lattice divided into 1-cm^2 squares. The lattice was placed over each photograph and the transection points on the individual organelles counted (Fig. 2).

Volumetric analysis of cell compartments was based on the Delesse principle (21). The volume of a cell organelle is directly proportional to the planimetric area of that organelle. By calling the grid unit area A_{g1} , each corner point of each grid square corresponds to a unit. Averaging this over the total grid gives, $A_A = (A_g) (P_A)$, where A_A is the transectional area of the compartment and P_A is the number of points within that compartment. The ratio of the corresponding organelle volume (V_V) will be: $V_{V1} : V_{V2} : V_{Vx} = A_{A1} : A_{A2} : A_{Ax}$. If the whole cell is used as the reference compartment (T), volume to volume ratios of each organelle will be $V_V/V_T = A_A/A_T$ (45, 78, 81, 125).

Fig. 2. Application of point counting test system to isolated neutrophil, Bar = 2 μm



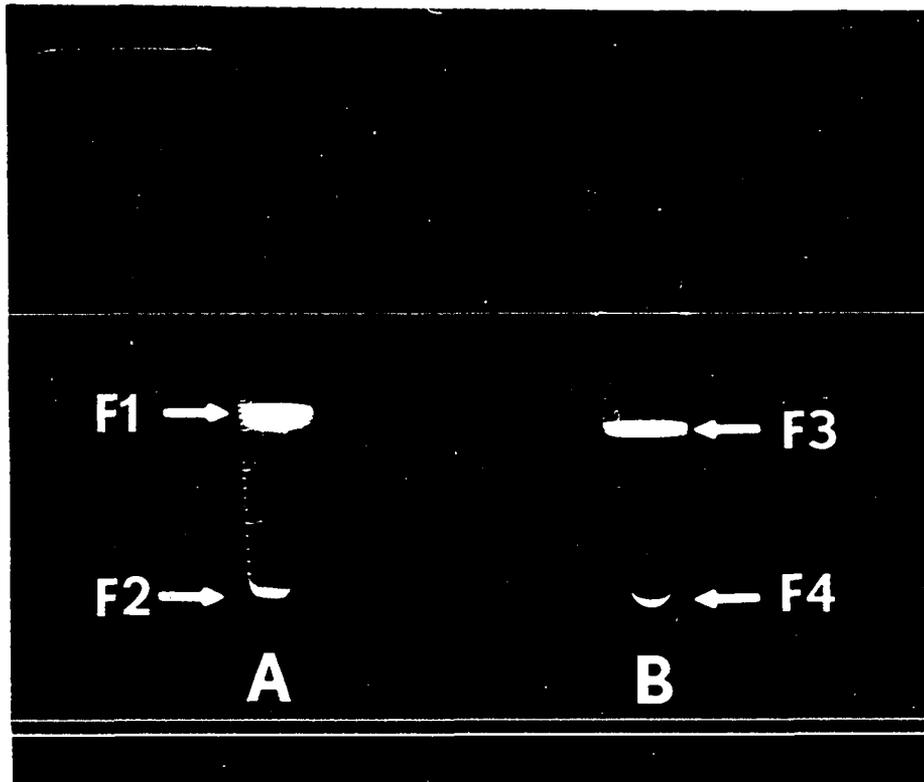
RESULTS

Granulocyte separation. Separation of monocytes and lymphocytes from granulocytes was achieved at a specific gravity of 1.080 (56 ml Hypaque, 21 ml of 10% Ficoll, and 23 ml distilled water:FHL). Granulocyte separation was optimal at a specific gravity of 1.125 (35 ml Hypaque, 38 ml of 14% Ficoll, and 27 ml distilled water:FHH).

Equine leukocytes separated into four distinct fractions using these two Ficoll-Hypaque density gradients (Fig. 3). Fraction 1 located at the HBSS-FHL interface, consisted of 97% monocytes and lymphocytes. Fraction 2 passed through FHL to the bottom of the centrifuge tube and was 99% granulocytes. In the normal horse, eosinophils can constitute 10% of the total leukocyte population (24). When granulocytes are concentrated, eosinophils can potentially represent 25% of the isolated cell population (94). Fraction 3, located at HBSS-FHH interface, was 99% neutrophils. Fraction 4 sedimented through FHH to the bottom of the centrifuge tube and was 95% eosinophils and 5% neutrophils. In tests for survival, trypan blue was excluded by 88% of the neutrophils processed at 4° C and by 90% at 22° C.

Ultrastructural examination. When evaluated by transmission electron microscopy, neutrophils were round to ovoid with multiple cytoplasmic projections. Nuclei were single or multiply lobulated. Euchromatin was centrally located and surrounded by the more electron dense heterochromatin. Peripheral cytoplasm had a coarse appearance and was free of organelles. Granules were the predominant organelle

Fig. 3. Equine leukocyte fractions on Ficoll-Hypaque. F1=monocyte and lymphocyte fraction; F2=granulocyte fraction (neutrophils and eosinophils); F3=neutrophils; F4=eosinophils. Tube A=Ficoll-Hypaque specific gravity 1.080; tube B=Ficoll-Hypaque specific gravity 1.125



in the central cytoplasmic region and varied in size, shape, and electron density. When observed, the Golgi complex was located centrally near the nucleus. Multiple clear vacuoles were distributed randomly throughout the central region of the cell. Lipid droplets, glycogen, and phagolysosomes were seen occasionally.

Four morphologically characteristic granule types were seen in neutrophils of all groups. Primary or azurophil granules were large and round to ovoid with a dense matrix (Fig. 4). These granules were 0.17 μm to 0.23 μm in width. Primary granules appeared electron dense in unstained cells when placed in peroxidase substrate of Graham and Karnovsky (Fig. 5).

Specific or secondary granules were observed as rod, dumbbell, and occasionally round structures. These granules were 0.13 μm to 0.67 μm in length and 0.04 μm to 0.13 μm in width. The matrix was less dense than the primary granules (Fig. 4).

A third granule type with a crystalline matrix arranged parallel to the longitudinal axis of the granule also was observed. The periodicity of the crystalline matrix was approximately 8 nm. The granule was ovoid, with a length ranging from 0.29 μm to 0.44 μm and the width ranging from 0.11 μm to 0.23 μm (Fig. 6).

Morphologically distinct from the above three types was a round to oval granule with a flocculent matrix. Granule dimensions were 0.26 μm to 0.58 μm in length and 0.14 μm to 0.26 μm wide. Many of these were similar in size and shape to the primary granule (Fig. 7).

Fig. 4. Granules. Primary (arrowhead) and secondary (arrow) granules. Peroxidase substrate, uranyl acetate, and lead citrate. Bar = 1 μ m

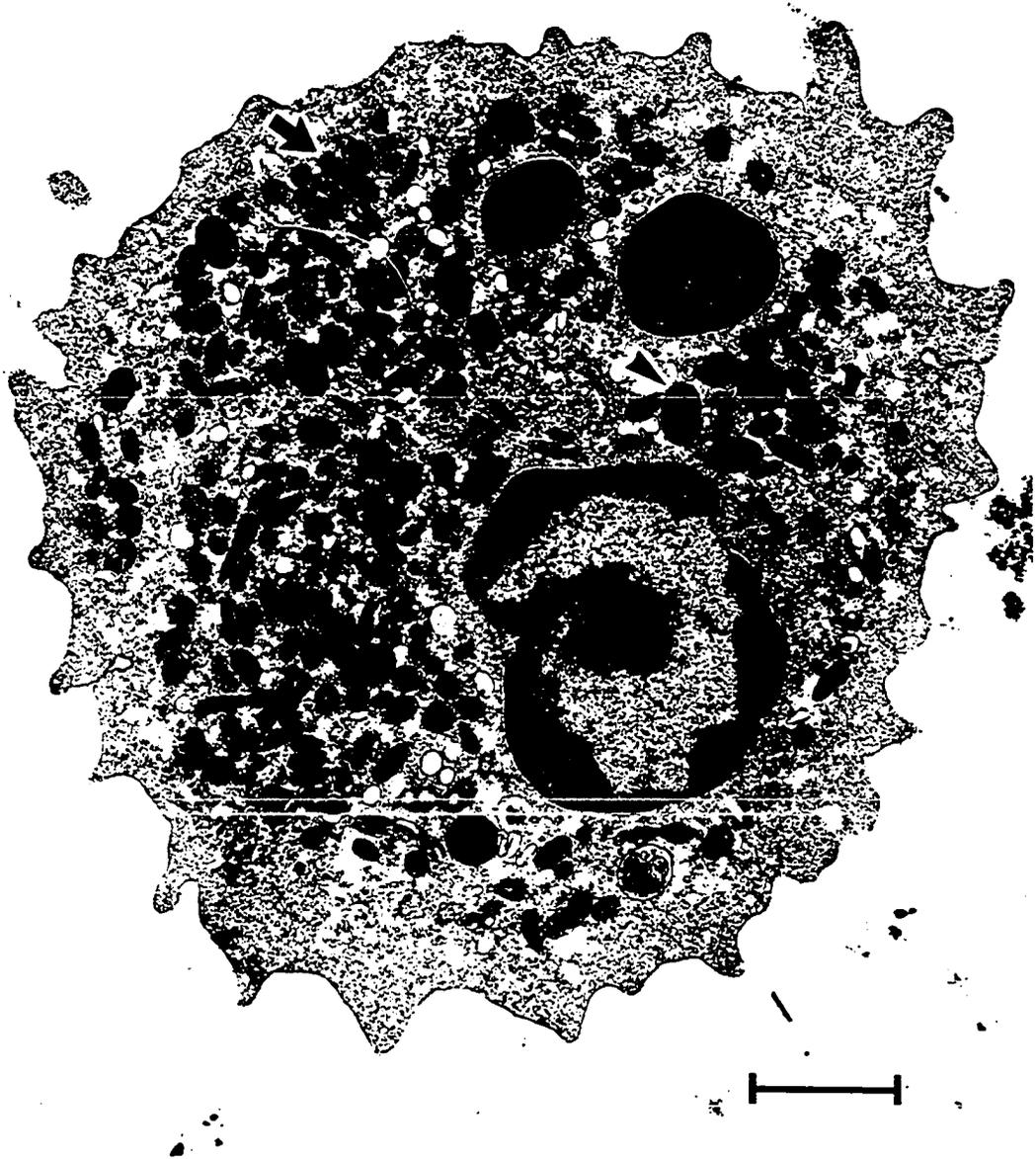


Fig. 5. Primary granules. Peroxidase substrate of Graham and Karnovsky. Unstained. Bar = 2 μ m

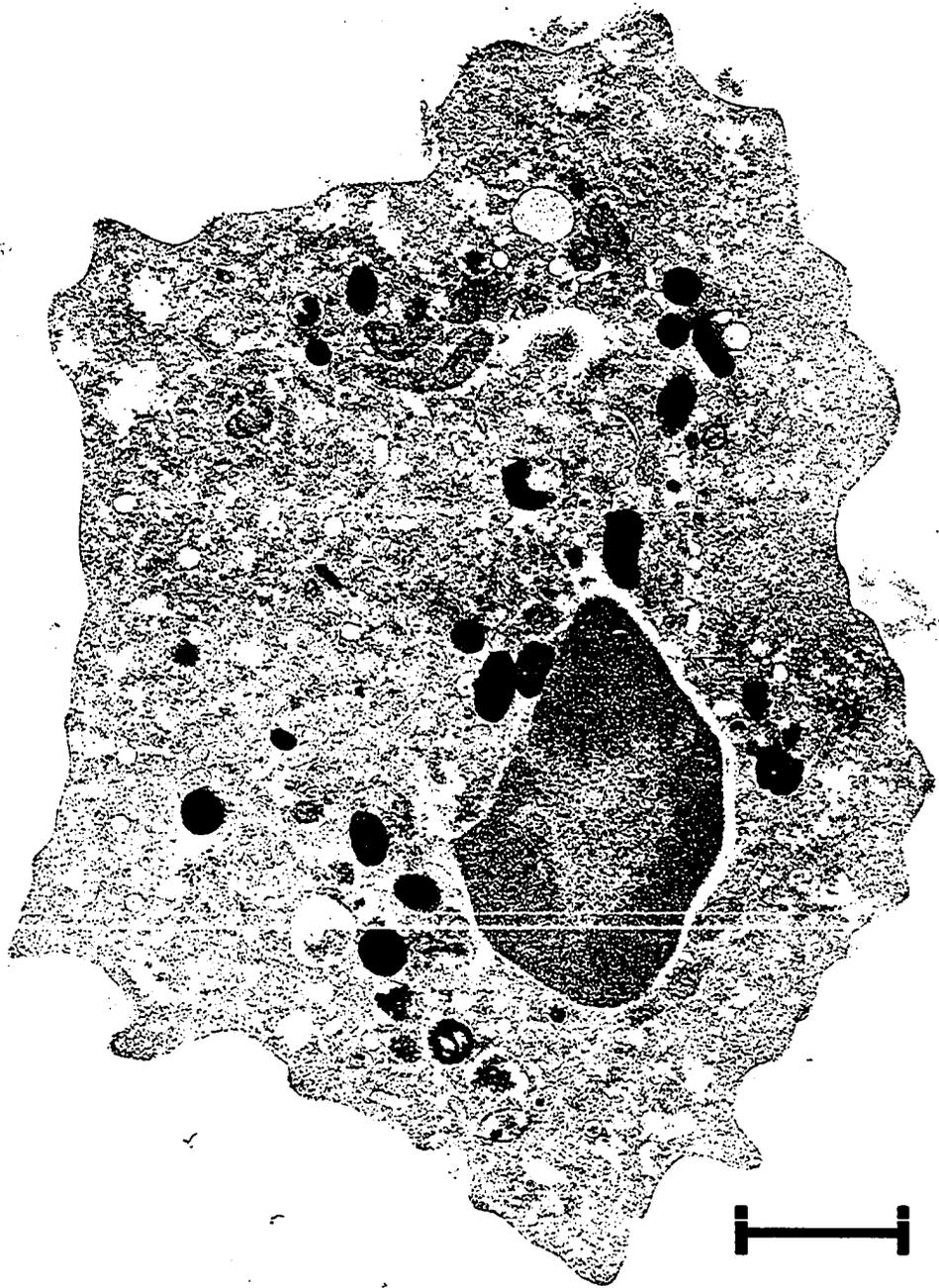


Fig. 6A. Granule with crystalline matrix. Rhomboid with polarity of striations. Uranyl acetate and lead citrate. Bar = 0.5 μm

Fig. 6B. Granule with crystalline matrix. Fusiform with striations occupying a central location. Uranyl acetate and lead citrate. Bar = 0.5 μm

Fig. 6C. Granule with crystalline matrix. Oval with striations throughout granule. Uranyl acetate and lead citrate. Bar = 0.5 μm

Fig. 6D. Granule with crystalline matrix. Round and oval with polar and central striations. Uranyl acetate and lead citrate. Bar = 0.5 μm

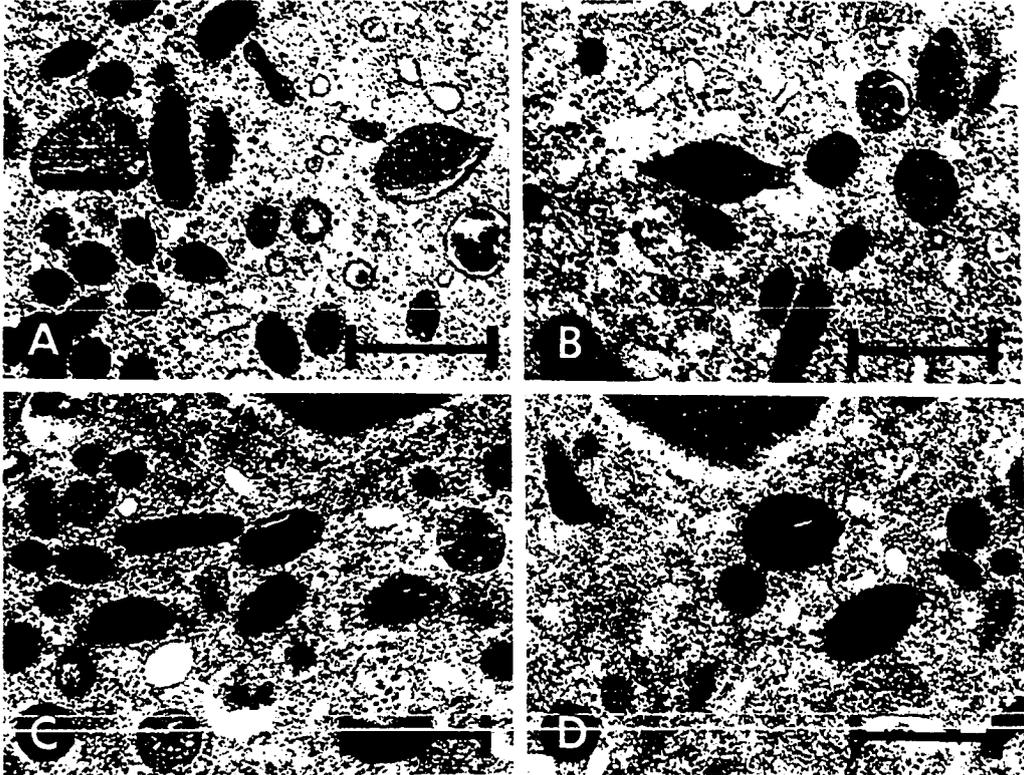


Fig. 7. Granules with flocculent matrix. Size and shape similar to primary granules. Uranyl acetate and lead citrate. Bar = 0.5 μm



Morphometric examination. Total transectional area of the four types of granules was $11.94 \pm 2.13 \mu\text{m}^2$ for the control neutrophils, $11.37 \pm 2.24 \mu\text{m}^2$ for 4°C , and $9.47 \pm 2.61 \mu\text{m}^2$ for 22°C processed cells. Control neutrophils had a mean secondary granule transectional area of $9.54 \pm 1.78 \mu\text{m}^2$ and a secondary granule volume ratio of 10.3%. Cells processed at 4°C had a mean secondary granule transectional area of $7.95 \pm 1.91 \mu\text{m}^2$ and a secondary granule volume ratio of 8.9%. Processing at 22°C resulted in secondary granule transectional area of $6.55 \pm 1.76 \mu\text{m}^2$ and a secondary granule volume ratio of 6.9%. Flocculent granule transectional area and volume ratio were greater in the treated than in the immediately fixed neutrophils. Primary granule transectional area and volume ratio were approximately the same in all groups (Table 1).

Cytocavitary network values were obtained by addition of perinuclear space, Golgi complex, vacuoles and endoplasmic reticulum transectional areas or volume ratios. The transectional area was $3.45 \mu\text{m}^2$ in the control cells and $3.00 \pm 0.72 \mu\text{m}^2$ and $3.04 \pm 0.73 \mu\text{m}^2$ in the 4°C and 22°C neutrophils. Volume ratio of the cytocavitary network was 3.7%, 3.3%, and 3.2% in the control, 4°C , and 22°C cells, respectively (Table 1).

Table 1. Morphometric data of equine neutrophils. Mean values, standard errors and volume ratios of 108 neutrophil photomicrographs from 3 horses

Organelle	Transectional Area (μm^2)			Volume Ratio (percent)		
	Control	4C	22C	Control	4C	22C
Nucleus	20.17 \pm 3.89	19.67 \pm 4.66	24.10 \pm 4.63	21.8	21.6	25.7
Perinuclear space	1.14 \pm 0.28	0.79 \pm 0.28	1.36 \pm 0.45	1.2	0.8	1.4
Perinuclear membrane	1.38 \pm 0.38	1.29 \pm 0.50	1.78 \pm 0.60	1.4	1.4	1.8
Endoplasmic reticulum	0.19 \pm 0.17	0.17 \pm 0.23	0.17 \pm 0.23	0.2	0.2	0.1
Golgi	0.31 \pm 0.35	0.23 \pm 0.26	0.05 \pm 0.09	0.3	0.2	0.1
Vacuole	1.81 \pm 0.58	1.81 \pm 0.49	1.46 \pm 0.61	1.9	2.0	1.5
Plasma membrane	1.32 \pm 0.36	1.36 \pm 0.42	1.51 \pm 0.49	1.4	1.5	1.6
Primary granule	1.18 \pm 0.63	1.44 \pm 0.56	1.27 \pm 0.66	1.2	1.6	1.3
Secondary granule	9.54 \pm 1.78	7.95 \pm 1.91	6.55 \pm 1.76	10.3	8.9	6.9
Flocculent granule	0.62 \pm 0.41	1.50 \pm 0.71	1.37 \pm 0.79	0.6	0.5	0.3
Crystalline granule	0.60 \pm 0.31	0.48 \pm 0.35	0.28 \pm 0.18	0.6	0.5	0.3
Phagolysosome	1.29 \pm 0.58	1.17 \pm 0.53	1.50 \pm 0.86	1.4	1.3	1.6
Mitochondria	1.12 \pm 0.88	0.77 \pm 0.40	0.78 \pm 0.43	1.2	0.8	0.8
Glycogen	0.17 \pm 0.14	0.18 \pm 0.13	0.45 \pm 0.35	0.1	0.2	0.4
Lipid	0 \pm 0.00	0.06 \pm 0.09	0.02 \pm 0.04	0	0.1	0.1
Cytoplasm	52.54 \pm 4.84	50.36 \pm 4.88	51.04 \pm 7.06	56.8	56.6	4.5

DISCUSSION

In the horse, isolation temperature is an important factor controlling neutrophil degranulation. Horse neutrophils isolated at 4° C have 3.5% less total granule transectional area than the controls; neutrophils isolated at 22° C have 21% less. Microtubule depolymerization may provide partial explanation for the relatively little degranulation found in the cells processed at 4° C. Microtubules are involved in neutrophil granule release and cold temperatures cause microtubule disassociation (46, 81). With more microtubules in the depolymerized state, granules cannot be extruded from the neutrophil. Cytosolic and granule enzyme release occurs in human leukocytes as a result of isolation procedures (78). With fewer granule enzymes, the ability to perform intracellular killing will be modified in cells isolated at 22° C. For in vitro neutrophil function in the horse to be evaluated with precision, there is a need for the cell to be biochemically intact, and this is accomplished best if the cells are isolated at 4° C.

The crystalline-granule type has not been reported previously in equine neutrophils. A similar granule type has been reported in humans (4, 138). It was demonstrated to be an immature secondary granule type and alleged to be a unique human characteristic. Since we found these granules in the control and treated neutrophils they probably were not induced by the separation procedure. This granule type in the horse may represent the immature secondary granule found in humans.

Granules with a flocculent matrix may be involved in the process of degranulation. This granule has been visualized previously adjacent to phagosomes in neutrophils containing exogenous material. The electron dense material of the granules was in phagocytic vacuoles at sites of granule attachment (14). These granules were seen most frequently in the treated neutrophils, thus suggesting that both treatments cause degranulation. Primary and secondary granules may appear as this flocculent form before being released from the cell. Flocculent granules may represent an intermediate form in the degranulation process. The large difference in total granule transectional area between the two isolation temperatures suggests that the flocculent granules are formed and released from the cell at different rates.

The cytocavitary network transectional area was not increased in the treated neutrophils, thus cell swelling was not present. Cytoplasmic vacuolation has been reported as the earliest sign of degeneration in human neutrophils stored at 4° C. Decreased granule stainability and degranulation are reported as occurring later (25). The presence of degranulation without cell swelling in the treated neutrophils suggests the first sign of modification in the equine neutrophil is degranulation. This discrepancy may be due to species variation or isolation technique differences.

Duration of isolation is important if viable cells are to be used in vitro. Some leukocyte separation procedures minimize isolation time but do not provide a homogeneous neutrophil population (32). Our

additional centrifugation step adds only 20 minutes to the isolation time and results in a higher percentage of neutrophils in the recovered cell population.

PHAGOCYTOSIS AND INTRACELLULAR KILLING OF THE CONTAGIOUS EQUINE
METRITIS ORGANISM BY EQUINE NEUTROPHILS IN SERUM

ABSTRACT

Equine neutrophils were combined with Haemophilus equigenitalis (CEMO) or Escherichia coli (E. coli) in low and high specific antibody titer serum to evaluate the neutrophils ability to phagocytize and kill these bacteria. More E. coli than CEMO were phagocytized at each time period. After 120 minutes in low antibody titer serum, 56.3% of the E. coli and 34.3% of the CEMO were phagocytized. Forty-five percent of CEMO and 74.9% of E. coli were phagocytized by 120 minutes when neutrophils were in high antibody titer serum. Greater than 75% of the ingested E. coli and 90% of the ingested CEMO were killed within 210 minutes of incubation. Fewer E. coli than CEMO were killed at any given time period. Ultrastructural examination showed CEMO to be degraded in the neutrophil. Degradation was the most extensive in neutrophils in high titer serum. It is suggested that CEMO is a pathogenic extracellular bacterium incapable of prolonged intracellular survival and that it is slower to be phagocytized than a nonpathogenic E. coli.

INTRODUCTION

Contagious equine metritis (CEM) was first described in Europe and subsequently in the U.S.A. (84, 123). The etiologic agent was originally called contagious equine metritis organism (CEMO) and tentatively classified as Haemophilus equigenitalis (127). This organism is an oxidase, catalase, and phosphatase positive, microaerophilic, Gram-negative coccobacillus. It is metabolically inactive with carbohydrates and is stimulated by, but does not require hematin and nicotinamide adenine nucleotides (123).

CEM is a venereal disease characterized by a purulent genital tract exudate in recently bred mares (84). Lesions are limited to the genital tract and are characterized by a moderate diffuse infiltration of neutrophils into the epithelium and lamina propria. The epithelial layer remains intact but is covered with a thick purulent exudate. Neutrophils containing CEMO can be demonstrated on the surface epithelium and in luminal exudate. CEMO, free or associated with neutrophils, is not found deep to the surface epithelium. The antibody response is of short duration and varies considerably between animals (20).

In vitro phagocytosis and killing methods have been used to evaluate virulence factors of pathogenic bacteria from man and animals (52, 133). We examined neutrophil-CEMO interactions using in vitro methods because (i) CEMO causes a surface infection (76), (ii) infection cannot be prevented by parenteral vaccination (31, 103), and (iii) CEMO is able to survive and produce disease despite an intense neutrophilic inflammatory

response. We assayed the ability of equine neutrophils to phagocytize and kill CEMO in the presence of (i) equine serum with high and low specific antibody titers and (ii) complement inactivated equine serum with high and low specific antibody titers. We also examined the ability of equine neutrophils to phagocytize and kill E. coli in the presence of equine serum with high and low specific antibody titer. From this work, it is suggested CEMO is not able to survive in equine neutrophils and is consistently slower to be phagocytized than E. coli.

MATERIALS AND METHODS

Serum. Equine sera for CEMO samples had tube agglutination titers of 1:1250 (HT) and 1:40 (LT) respectively and were used in all CEMO experiments. HT equine serum was produced by an initial subcutaneous inoculation of dead CEMO in Freund's complete adjuvant followed by two intramuscular injections of live CEMO in saline on days 40 and 68 and an intravenous inoculation of live CEMO on day 77. Serum was collected on day 84. The challenge CEMO used was the same as used for phagocytosis and killings assays. LT serum was collected at the time of neutrophil collection. Blood was collected into evacuated bottles followed by a 60 minute incubation at 37° C. Serum was then harvested, centrifuged, chilled to -60° C and stored at -60° C.

Inactivated-complement serum samples were heated in a water bath to 56° C for 30 minutes. LT and HT equine sera were used and heating was done on the day of each experiment. These samples were designated as HLT (heated low-titer serum) and HHT (heated high-titer serum).

HT equine serum for E. coli samples had tube agglutination titers of 1:1250. LT equine serum for E. coli samples had titers of 1:40. LT serum for E. coli samples was produced by using a previously reported serum absorption procedure (27). Briefly, the E. coli was heat killed and incubated with equine serum to remove E. coli-specific agglutinins.

Neutrophils. Neutrophils were harvested daily by a previously reported procedure (6). Briefly, whole blood was collected by jugular venipuncture from 3 clinically normal female horses. Red blood cells

were sedimented by gravity at 4° C and the residual red blood cells of the supernatant fluid were lysed with hypotonic ammonium chloride in tris buffer. Thrombocyte numbers were reduced by centrifugation through fetal calf serum. Neutrophils were separated from other leukocytes by centrifugation on two different Ficoll-Hypaque density gradients.

Bacteria. A streptomycin-sensitive strain of contagious equine metritis organism (78M3056) was used in all CEMO experiments. This culture was originally isolated in a Kentucky outbreak of CEM and was obtained from Dr. Billy Blackburn, Diagnostic Bacteriology Laboratory, National Veterinary Service Laboratory, Ames, Iowa. The CEMO used in all experiments had been passed 3 times in vitro since initial isolation. This isolate has been shown to be virulent after 30 in vitro passes and retains a capsule after 5 in vitro passes. A strain of E. coli (serotype O2:K-:H6) used as a nonpathogen control in previous studies (2) was utilized in all E. coli experiments. The stock cultures of bacteria were placed in glycerol and stored at -60° C. For each experiment CEMO was cultured in Brucella broth with 0.02% cysteine hydrochloride (Gibco, Grand Island, NE) at 37° C, with gentle agitation, for 3 days. E. coli was cultured at 22° C (to reduce pilus production) for 1 day in Brucella broth.

Broth cultures were centrifuged at 1500 x g for 25 minutes and resuspended in Hanks' balanced salt solution (HBSS) with 0.1% gelatin. Viable bacteria were counted and standardized by absorption spectrophotometry to 1×10^6 per ml. This standard concentration was confirmed

for each experiment by titration and plating CEMO on Eugon's Chocolate Blood Agar with 10% horse blood (ECBA) and E. coli on 5% bovine blood agar (BAP).

Phagocytosis assay. Phagocytosis assays were conducted as previously described (133). Briefly, 1×10^6 neutrophils in HBSS with 10% equine serum and 1×10^6 bacteria in HBSS with 0.1% gelatin were combined to give a final volume of 2 ml per tube, placed into a 37° C incubator, and rotated at 4 to 6 revolutions per minute. Tubes were collected at 0, 60, and 120 minutes and placed into an ice water bath for five minutes. Tube contents were then washed and centrifuged to separate extracellular bacteria and neutrophils. The neutrophil-free supernatant was collected, sonicated for 15 seconds to break up clumped bacteria, and plated. Sonication did not alter bacterial viability (unpublished data). Bacteria numbers were determined by counting colony forming units. To evaluate the bacteriocidal effect of serum, CEMO and E. coli samples were prepared as above but no neutrophils were added. The percent phagocytized bacteria was determined by:

$$\frac{\text{Bacterial numbers in serum alone} - \text{Bacterial numbers in neutrophil free supernatant fluids}}{\text{Bacterial numbers in serum alone}} \times 100$$

Intracellular killing assay. Killing assays were conducted as previously described (133). Briefly, 1×10^6 neutrophils in 10% equine serum and 1×10^6 bacteria in HBSS with 0.1% gelatin were combined and incubated for 60 minutes at 37° C with continuous rotation. Tube contents were washed to remove extracellular bacteria attached to

the test tube. The washing supernatant fluids were discarded and 5% equine serum was added to the cell pellet. Tubes were then placed into a 37° C incubator, rotated at 4 to 6 revolutions per minute, and collected 60, 90, 120, 180, and 210 minutes after the initial mixing of neutrophils and bacteria. Tube contents were sonicated to lyse neutrophils and break up clumped bacteria. Numbers of viable intracellular bacteria were determined by titrating the lysate and counting colony-forming units of bacteria. The number of phagocytized bacteria was determined as above and the percent killed bacteria was determined by:

$$\frac{\text{Number of phagocytized bacteria} - \text{Number of bacteria in lysate}}{\text{Number of phagocytized bacteria}} \times 100$$

Cell-associated bacteria. This test was conducted as previously described (133). Briefly, cell pellets from the phagocytosis experiments were placed on glass slides and stained with a Romanowsky-Giemsa stain. Two hundred to 220 neutrophils were randomly counted for associated bacteria.

Electron microscopy. Neutrophils and CEMO were combined as above, allowed to incubate for 120 minutes, fixed in 2.5% glutaraldehyde for 45 minutes, and then postfixed in 1.0% osmium tetroxide for 30 minutes. Samples were dehydrated in graded alcohols, cleared in propylene oxide, and infiltrated and embedded in epoxy-resin (Epon 812, Shell Chemical). Ultrathin sections were cut at 70 to 90 nm and stained with uranyl acetate and lead citrate. Fifty-five to 60 neutrophils with CEMO were

evaluated for lipid droplets, vacuoles, granule morphology, intracellular bacteria, and surface bacteria.

Statistical analysis. Student T test and linear regression of CEMO samples were done on the 0, 60, and 120 minute samples in the phagocytosis analysis and the 60 and 210 minute samples in the killing analysis.

RESULTS

Phagocytosis. The number of CEMO and E. coli in all samples with no neutrophils gradually increased with time (Fig. 1). In the presence of neutrophils the number of bacteria gradually decreased during the incubation period (Fig. 1). The largest decrease in CEMO was seen in the tubes with HT serum and neutrophils suggesting a role for antibody and complement in phagocytosis of CEMO by neutrophils. The greatest decrease of all samples was seen with HT serum, neutrophils, and E. coli (Fig. 1).

The percentages of CEMO phagocytized by neutrophils were significantly different ($p < 0.03$) between an average of the heated and unheated serum samples at the 120-minute sample period. Significant differences ($p < 0.04$) were found only at the 120-minute sample period in the HT and LT serum samples. E. coli in HT serum with neutrophils had the largest percentage of phagocytosis (Table 1). Table 1 figures are derived from data in Fig. 1.

Intracellular killing. Intracellular killing proceeded rapidly the first 60 minutes of incubation. Seventy-three to 92 percent of the ingested bacteria did not form colonies after 60 minutes. Fresh and heated immune sera samples had the largest decrease in viable intracellular CEMO. Intracellular killing in HLT and LT serum samples were not significantly different but both had higher numbers of viable intracellular CEMO than the HT serum samples. Significant differences ($p < 0.01$) in intracellular killing at the 60 and 210 minute sampling time

Fig. 1. Growth of CEMO and E. coli in serum (left graph). Growth of CEMO and E. coli in serum with neutrophils (right graph). Values are means of 3 replicates from 3 horses

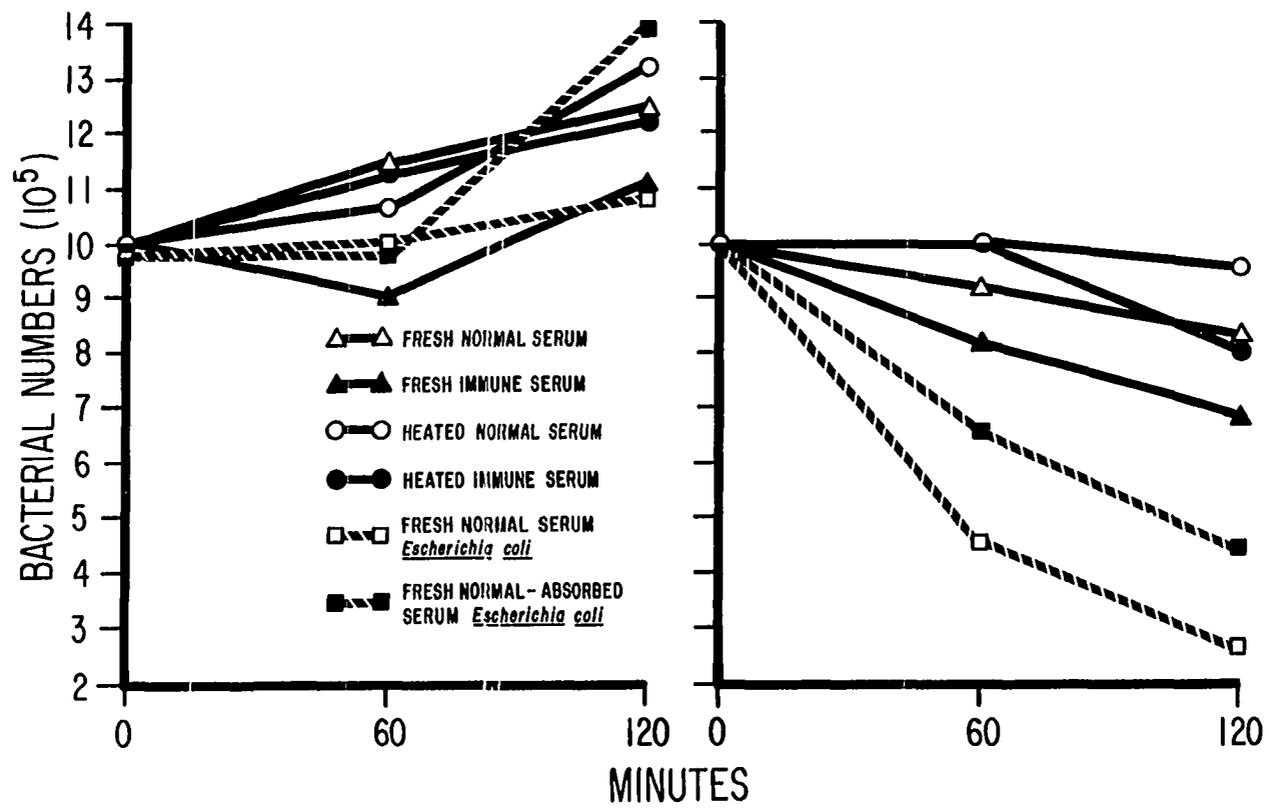


Table 1. Percentage of 1×10^6 bacteria phagocytized by 1×10^6 equine neutrophils^a

Bacteria	Serum	Percentage phagocytized bacteria		
		0 min	60 min	120 min
CEMO	High antibody titer	2.4	17.6	34.3
	Low antibody titer	0.5	13.3	45.3
	Heated low antibody titer	2.1	7.1	29.0
	Heated high antibody titer	0.3	12.7	33.9
<u>E. coli</u>	High antibody titer	1.0	50.0	74.9
	Low antibody titer	1.0	35.0	56.3

^aValues are derived as shown in text from Figure 1. CEMO samples are the average of 3 replicates from horses. E. coli samples are the average of 3 replicates from 2 horses.

were found between the HHT and HLT serum samples suggesting antibody has a role in intracellular killing of CEMO (Fig. 2). No significant difference was found between the HT and LT serum samples at any time suggesting complement has a role in the intracellular killing of CEMO (Fig. 2). The number of intracellular E. coli killed was not as high as the number of CEMO killed at any time (Fig. 2). The slopes from 0 to 210 minutes were not significantly different between any two CEMO plots.

Morphological examination. The percentage of neutrophils with associated bacteria paralleled the percentage of phagocytized bacteria for a given time period and serum group. The percentage of neutrophils with bacteria, as observed histologically, for each serum group is shown in Fig. 3. The number of neutrophils containing CEMO increased with incubation time. The number of CEMO per neutrophil was similar for all serum groups at a given time. The number of E. coli per neutrophil gradually increased with increasing incubation time. The number of bacteria per neutrophil is given in Table 2.

Ultrastructural examination. Ultrastructurally, phagolysosomes were observed to most frequently contain one bacterium. Thirty percent of the intracellular bacteria in LT serum samples and 83% in the HT serum samples had structural alterations. Structural alterations consisted of cytoplasmic flocculation, cell wall irregularities, and cell wall rupture (Fig. 4). Less than one percent of the neutrophils were associated with extracellular bacteria.

Clusters of secondary granules were observed around ingested bacteria. Separating the ingested bacteria and granules was a thin

Fig. 2. Percent viable intracellular CEMO and E. coli. Values are means of 3 replicates from 3 horses

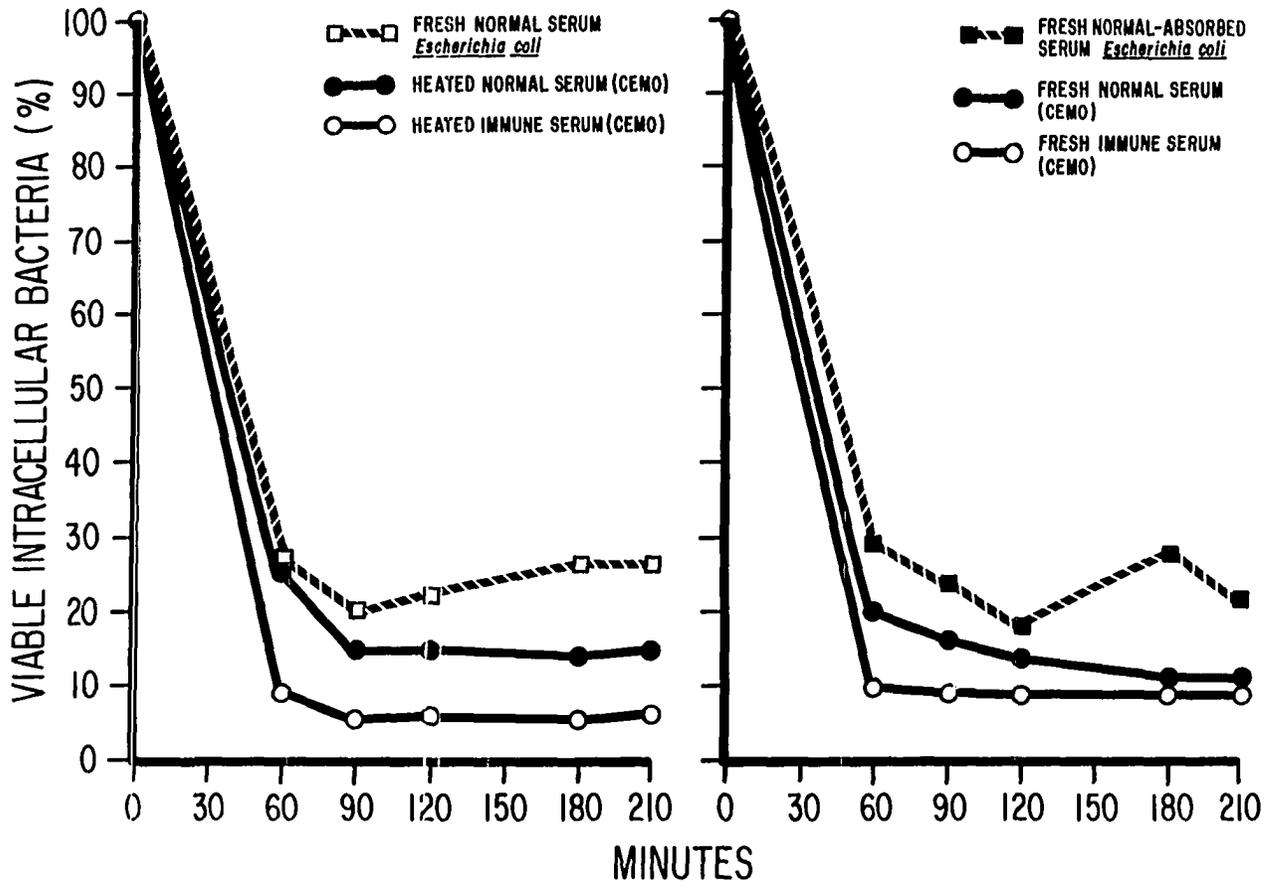


Fig. 3. Percent neutrophils with associated bacteria

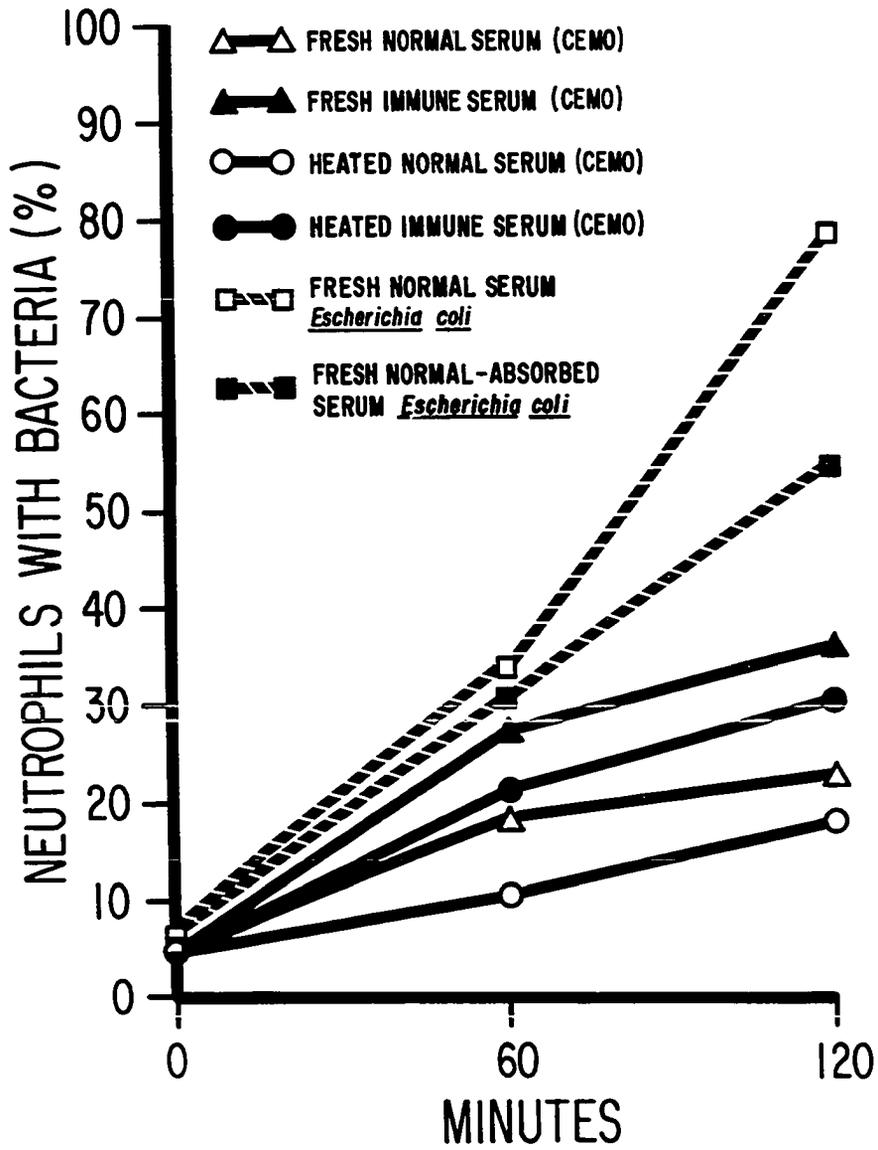
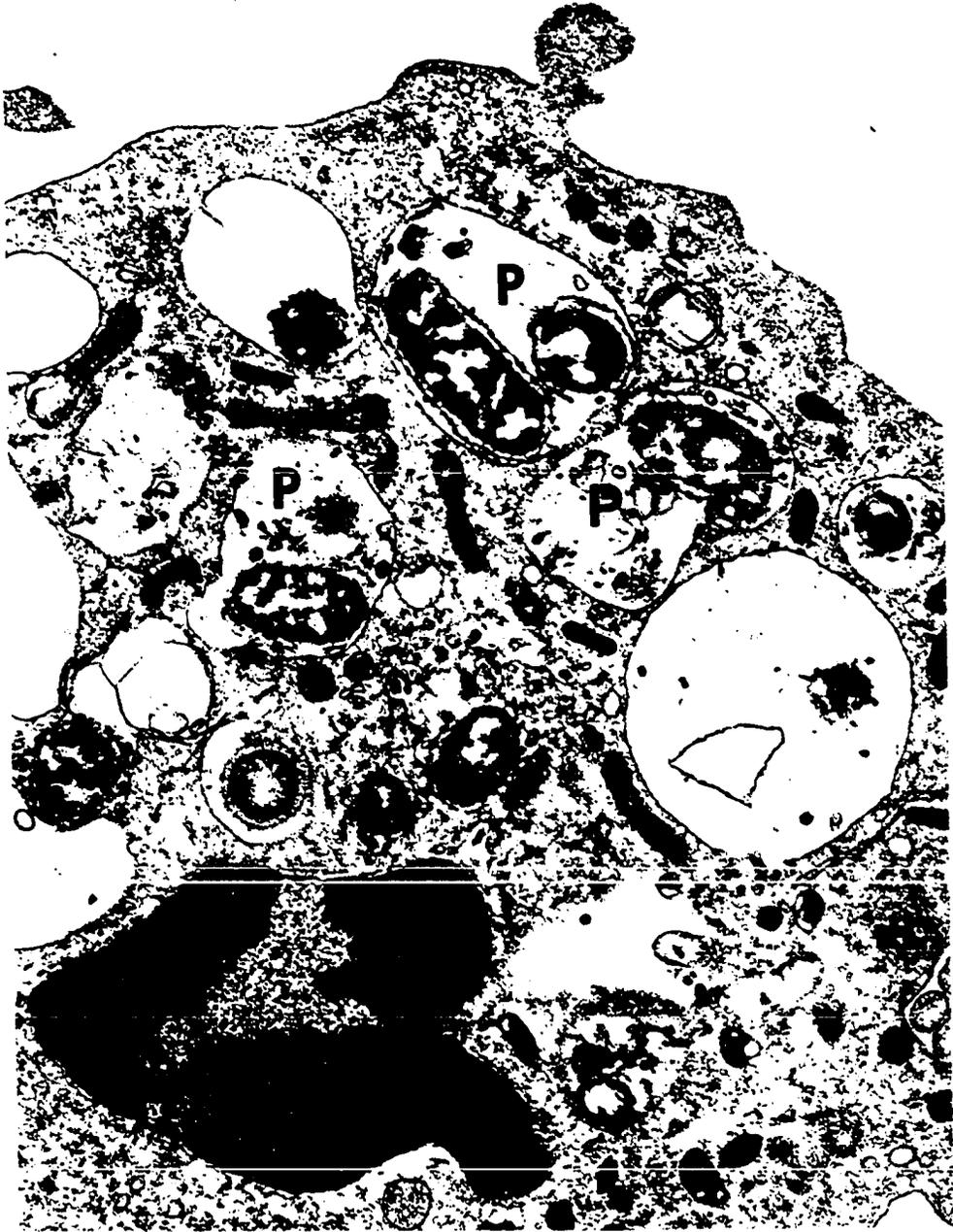


Table 2. Average number of bacteria phagocytized per horse neutrophil in various sera^a

Bacteria	Serum Treatment	Average number of bacteria per neutrophil (n=1200) ^a					
		LT serum			HT serum		
		0 min	60 min	120 min	0 min	60 min	120 min
CEMO	Unheated	1.0	1.6	1.6	1.0	1.3	1.6
	Heated	1.0	1.6	1.5	1.0	1.6	1.4
<u>E. coli</u>	Unheated	1.1	1.9	4.0	1.2	2.8	3.2

^aValues determined only for neutrophils which contained bacteria.

Fig. 4. Phagolysosome (P). Bacteria in various stages of breakdown. Bacterial wall irregularities, rupture, and dissolution, and flocculation of cytoplasm. Large and small vacuoles in neutrophil cytoplasm



zone of organelle free cytoplasm. Phagosomes located in agranular areas of cytoplasm were seen in 50% of the neutrophils with LT serum and 19% of neutrophils in HT serum samples (Fig. 5). Fewer granules were present in cells with multiple phagolysosomes and those remaining were mostly secondary granules and primary granules with a flocculated matrix.

A space was usually observed between the phagolysosome membrane and the bacterial cell. These phagolysosomes contained granular electron dense material and bacterial debris (Fig. 6).

Lipid droplets were observed near the phagocytized bacteria in 30% of the neutrophils. Lipid was most frequently observed in neutrophils which contained multiple bacterial laden phagolysosomes (Fig. 7).

Vacuoles were also observed near phagosomes as well as throughout the cytoplasm in 30% of the neutrophils in LT serum and 82% of the neutrophils in HT serum. Vacuoles were larger and more numerous in neutrophils which contained many bacteria.

Fig. 5. Polarity of neutrophil granules. CEMO (C) in agranular area of cytoplasm. Many secondary granules are present (S). Primary granules with flocculated contents (A). Lipid droplets (L) and vacuoles (V) throughout cytoplasm

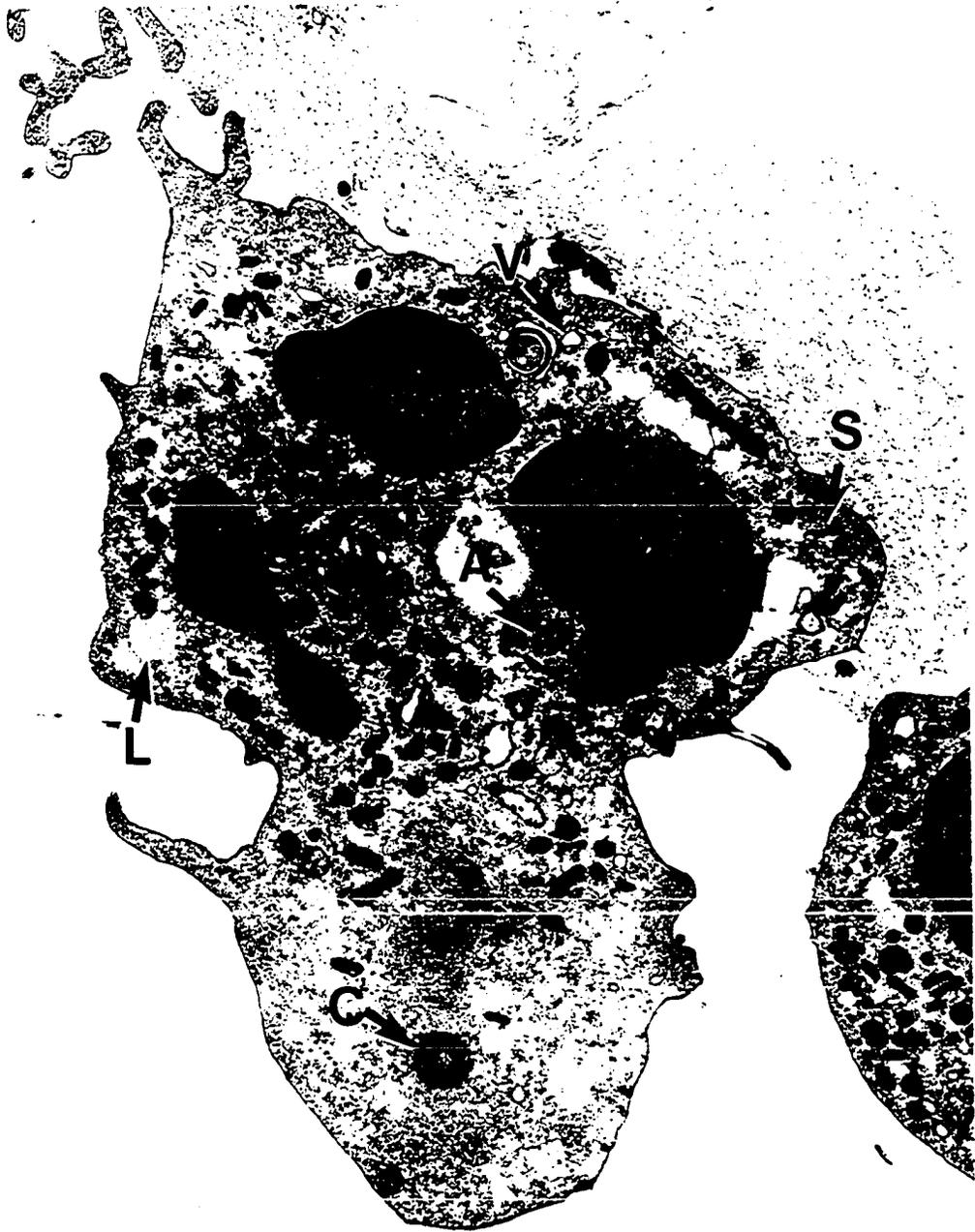


Fig. 6. Phagosome membrane closely apposed to dividing bacterium (P1). Phagosome membrane loosely apposed to ruptured bacterium (P2)

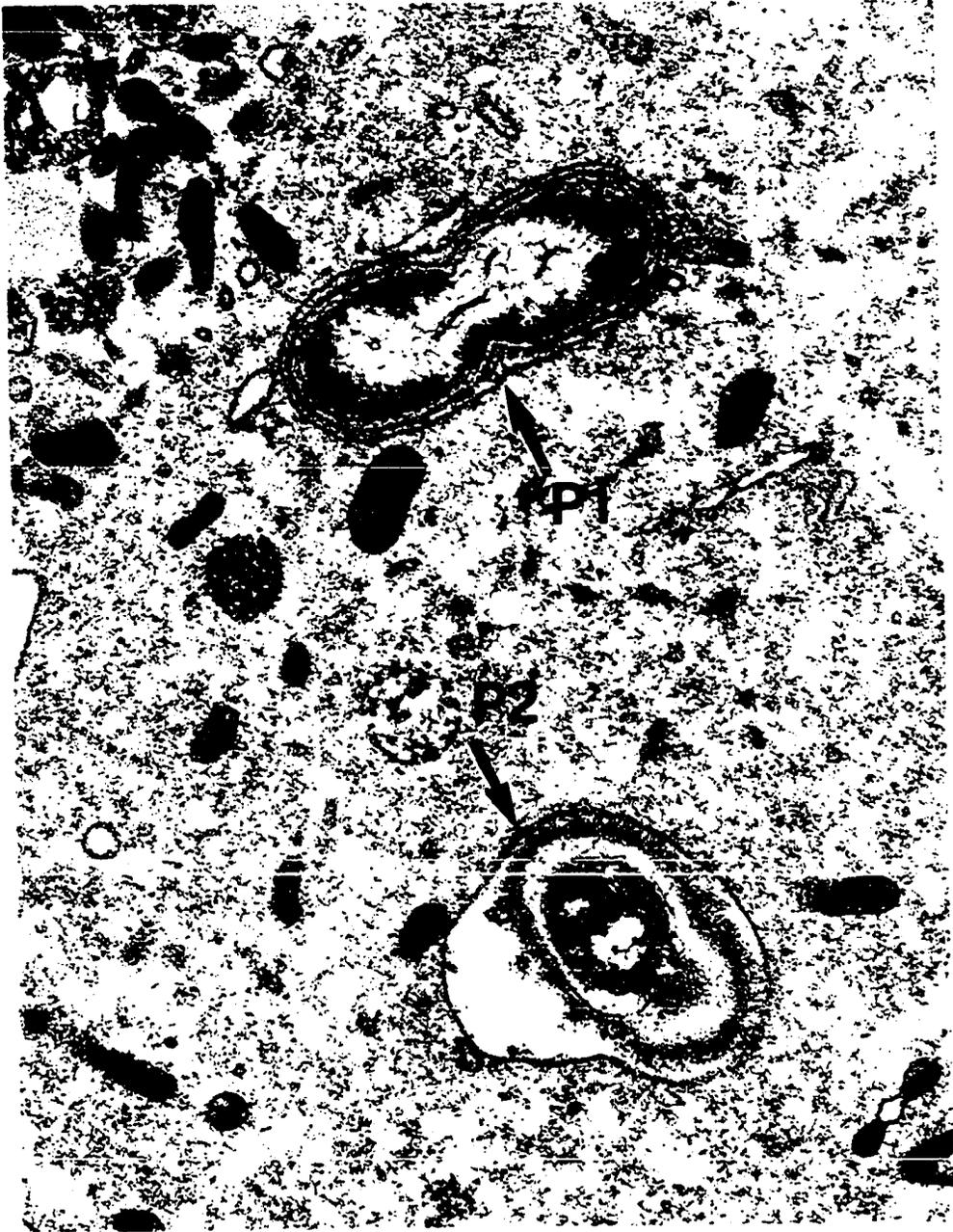
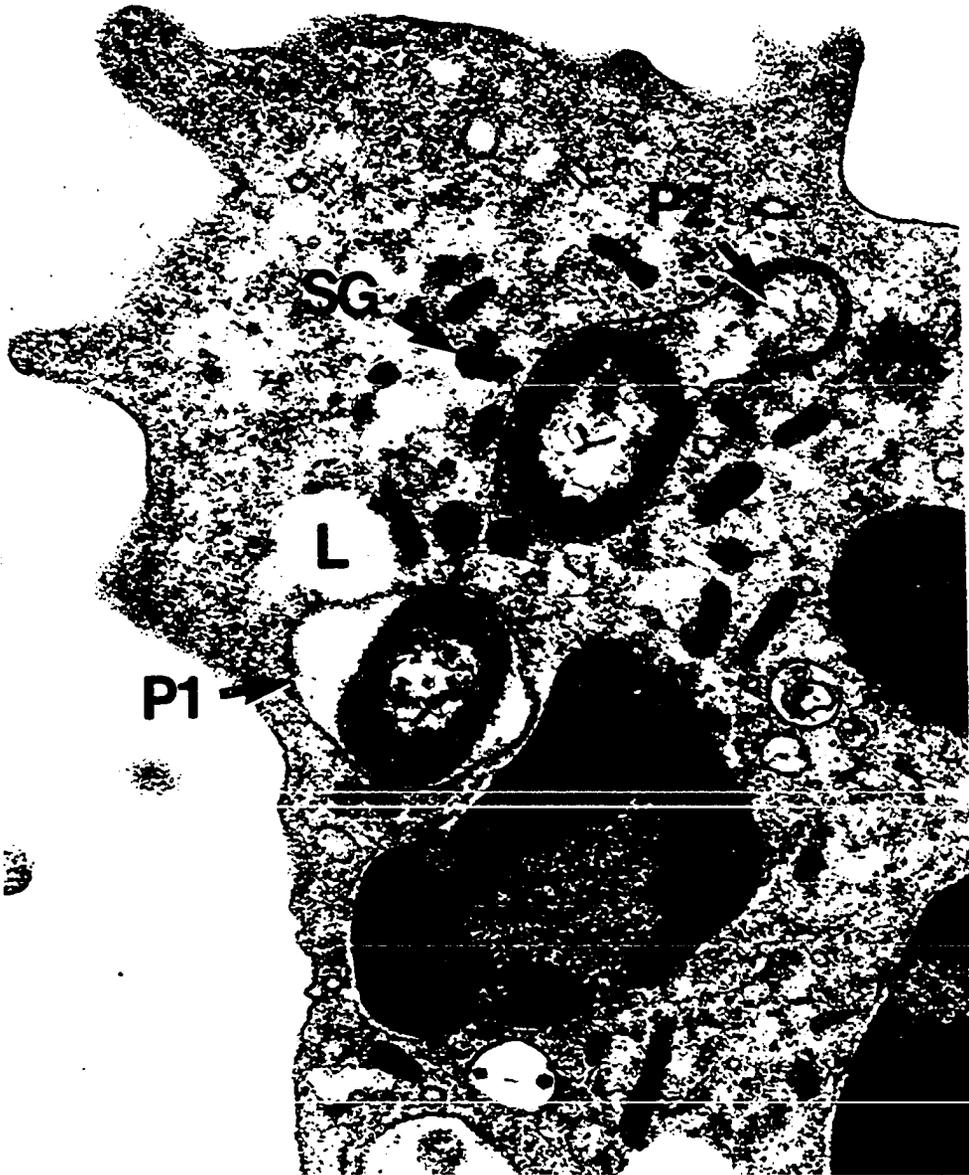


Fig. 7. Phagolysosome. Swollen phagolysosome containing bacterium (P1). Lipid droplets (L) adjacent to phagolysosome. Primary granule and contents within phagolysosome (P2). Secondary granules (SG) around phagolysosome containing bacterium



DISCUSSION

Our data suggest CEMO is consistently slower to be phagocytized than E. coli. Thirty-four percent of the CEMO and 56.3% of the E. coli were taken up after 120 minutes of incubation in LT serum. This slow uptake of CEMO may be the result of a CEMO virulence factor. There are several possible mechanisms by which CEMO could inhibit phagocytosis. Many pathogenic bacteria utilize capsular material to inhibit phagocytosis (22). The capsule reported for CEMO (122) may be involved in the inhibition of phagocytosis.

Although we noted statistically significant increase in neutrophil phagocytosis of CEMO between LT and HT serum, the absolute increase was small. A similar effect on phagocytosis seen with Neisseria gonorrhoeae was explained as a decrease in membrane fluidity of the phagocyte (110). Because fluidity of the membrane is essential for the neutrophil to engulf particulate material, increased attachment would not result in increased phagocytosis. Generally, increasing species specific antibody levels results in greatly increased attachment and subsequent phagocytosis of a bacterium (63). This was observed with samples of E. coli and neutrophils in LT and HT serum. If CEMO alters the neutrophil at the site of attachment, phagocytosis may be slowed even with increased amounts of opsonins. Other mechanisms that could inhibit engulfment include a decrease in acylation of phospholipids, chelation of divalent cations, or alteration of sulfhydryl groups on the neutrophil membrane (117).

Once ingested by the neutrophil CEMO does not inhibit continued phagocytosis. This is suggested by the presence of more than one bacterium in many neutrophils. This observation is compatible with our hypothesis that the effect of CEMO on phagocytosis is a localized phenomena at the site of engulfment and does not affect the entire neutrophil. The number of bacteria per neutrophil is less with CEMO than E. coli and is probably a result of phagocytosis inhibition seen with CEMO. Inhibition of continued phagocytosis has been reported with other pathogenic microorganisms (22).

CEMO is a pathogenic extracellular bacterium that does not survive intracellularly. Most CEMO were killed in the first 60 minutes of incubation. Of the ingested bacteria, more than 85% of the CEMO and 75% of the E. coli were killed by 210 minutes of incubation. In the phagolysosome, the pH is lower than 4 (75) which will result in rapid death of CEMO (104). Morphological evidence of bacterial digestion was not observed in all intracellular CEMO and some were observed to have division septae. These observations may be a result of recent ingestion or the possibility that CEMO was viable but not capable of sustained division and reproduction and thus not detected by our system. However, the clustering of primary and secondary granules around phagosomes, the presence of granule contents in phagosomes, and the breakdown of bacterial cells also indicate CEMO does not survive in neutrophils.

CEMO is resistant to the bactericidal effects of low serum antibody concentrations. We have demonstrated CEMO numbers increase despite the

presence of serum antibody and complement. There was a gradation of CEMO susceptibility since HT serum did not allow as much growth as HLT serum. Gram-negative bacteria responsible for localized infections are usually sensitive to the effects of serum whereas those causing bacteremia are resistant to the effects of serum (98, 135). Some studies have correlated serum resistance and virulence (75, 100). Since avirulent strains of CEMO have not been reported and CEMO does not cause a septicemic disease, these findings are of unknown significance.

Intracellular digestion of CEMO occurred with both LT and HT sera. Morphologically, alterations of bacterial cells were most extensive in the HT sera groups. Intracellular microorganisms may have been breaking up before ingestion but this is unlikely since bacterial numbers increased if neutrophils were not present. This observation may provide partial explanation for the difference in neutrophil killing ability in the HHT and HLT serum groups. Serum with elevated specific antibody titers is reported to enhance intracellular destruction of some chlamydia, treponemes (5), and mycobacteria (39, 65) but has no effect on intracellular digestion of certain protozoa (113, 146). The interpretation of these observations is difficult since each animal species has different abilities to digest various microbes (39, 113, 146).

Two factors may underlie the reported failure of parenteral vaccination to prevent CEM (31, 103). First, parenteral vaccination usually does not improve mucosal immunity (5, 10); since CEMO does not penetrate the uterine mucosa it would not be greatly influenced by

increased blood antibody titers. Second, this study suggests that even if circulating antibody did pass into the uterus, a drastic improvement of phagocytosis and killing would not be seen. This is supported by our observation of a limited increase of phagocytosis and intracellular killing with complement and elevated antibody levels.

PHAGOCYTOSIS AND INTRACELLULAR KILLING OF THE
CONTAGIOUS EQUINE METRITIS ORGANISM BY EQUINE NEUTROPHILS
IN GENITAL SECRETIONS

ABSTRACT

Equine neutrophils were combined with contagious equine metritis organism (CEMO) or Escherichia coli (E. coli) in vitro in the presence of seminal plasma, uterine flushings, or Hanks' balanced salt solution. Phagocytosis and intracellular killing were estimated by bacterial culture, and both light and electron microscopy. With lysed neutrophils the numbers of colony-forming units of CEMO and E. coli increased in seminal plasma and uterine flushings. CEMO numbers decreased in Hanks' balanced salt solution. CEMO numbers increased more in the presence of seminal plasma than the other media. When neutrophils were in the various media, 29 to 32 percent of the CEMO were phagocytized by 120 minutes. At all sampling times and all media types more E. coli than CEMO were observed to be associated with neutrophils. By 210 minutes of incubation in uterine flushings, seminal plasma, and Hanks' balanced salt solution 60 to 75 percent of the intracellular CEMO were killed. Eighty-five to 90 percent of the intracellular E. coli in the various media were killed by 210 minutes. We suggest that CEMO is an extracellular pathogenic bacteria and that immunoglobulins in seminal plasma and uterine flushings do not enhance CEMO phagocytosis or intracellular killing.

INTRODUCTION

Contagious equine metritis (CEM) is a venereal disease characterized by a purulent genital tract exudate in recently bred mares (85). It was first described in Europe and subsequently in the U.S.A. (85, 123). The etiologic agent was originally called Contagious Equine Metritis Organism (CEMO) and Hemophilus equigenitalis has been a proposed classification (127). This organism is an oxidase-, catalase-, and phosphatase-positive, microaerophilic, Gram-negative coccobacillus. Carbohydrates are not metabolized in vitro by CEMO and it is stimulated by, but does not require hematin and nicotinamide adenine nucleotides (127).

Lesions of CEM are limited to the genital tract and are characterized by a severe diffuse infiltration of neutrophils into the epithelium and lamina propria. As the disease progresses, the neutrophilic influx subsides and the lamina propria and submucosa are infiltrated with histiocytes and plasma cells (87). The serum antibody response in infected animals is of short duration and varies considerably between animals (20). Previous in vitro work has demonstrated that neutrophils in serum phagocytized CEMO more slowly than a nonpathogenic E. coli and that CEMO is not capable of prolonged intraneutrophil survival (7).

The neutrophils ability to phagocytize and kill bacteria has been evaluated in various physiological secretions (9, 35, 82, 109). The demonstration and role of immunoglobulins in these secretions has been evaluated for various diseases (43, 132). Immunoglobulin A is the

predominant immunoglobulin in most body secretions (54, 132). The poor opsonizing qualities of IgA have been demonstrated for both Gram-positive and Gram-negative bacteria (93, 150). In this study, we have assayed the ability of equine neutrophils to phagocytize and kill CEMO in the presence of equine seminal plasma, equine uterine flushings, and Hanks' balanced salt solution. We examined neutrophil-bacteria interaction in vitro by evaluating the ability of equine neutrophils to phagocytize and kill CEMO because (i) CEMO causes a mucosal surface infection and is limited to the genital tract (87, 96), (ii) the predominant inflammatory cell type early in this disease is the neutrophil (87), and (iii) semen has been reported to enhance the virulence of CEMO in horses (128). This work suggests that CEMO is an extracellular pathogen and that immunoglobulins in seminal plasma and uterine flushings do not enhance CEMO phagocytosis or intracellular killing.

MATERIALS AND METHODS

Seminal plasma. Semen was collected from two clinically normal and CEMO-free thoroughbred stallions on three different days. Spermatozoa were centrifuged from the semen, the seminal plasma filtered through a 0.45 micron filter (Millipore, Millipore Corporation), and stored at -60° C.

Uterine fluids. Uterine fluids were collected as previously described (149). Briefly, 100 ml of Hanks' balanced salt solution (HBSS) was infused into the uterus followed by uterine massage per rectum for 10 minutes. The fluid was collected, filtered through a 0.45 micron filter, and stored at -60° C.

Immunoglobulin evaluation. Uterine flushings and seminal plasma were concentrated by ultrafiltration using a 30,000 molecular weight filter (Millipore, Millipore Corporation). Seminal plasma was concentrated 3 fold and uterine flushings 10 fold. Cellulose-acetate electrophoresis (Model R101, Beckman Instruments) and immunoelectrophoresis (Universal electrophoresis cell, Searle Analytic Inc.) were used to detect the presence of immunoglobulins. Monospecific rabbit anti-equine IgA & IgG sera were obtained commercially (Antispecies antibody, Miles Lab).

Neutrophils. Neutrophils were harvested on the day of each experiment according to a previously reported procedure (6). Briefly, whole blood was collected by jugular venipuncture from three clinically normal female horses. Red blood cells were sedimented by gravity at

4° C and the residual red blood cells of the supernatant fluid were lysed with hypotonic ammonium chloride in tris buffer. Thrombocyte numbers were reduced by centrifugation through fetal calf serum. Neutrophils were separated from other leukocytes by centrifugation on two successive Ficoll-Hypaque density gradients.

Bacteria. A streptomycin-sensitive strain of Contagious Equine Metritis Organism (78M3056) originally isolated from an outbreak of CEM in Kentucky was obtained from Dr. Billy Blackburn, Diagnostic Bacteriology Laboratory, National Veterinary Services Laboratories, Ames, Iowa. This bacterium was passed three times on Eugon 10% chocolate horse blood agar (Eugon's Agar, Becton Dickinson, Co.) prior to use. This isolate has been shown to be virulent and retained a capsule after 5 in vitro passes (Bertram, unpublished data). A strain of E. coli (serotype 02:K-:H6) used as a nonpathogen control in previous studies (2) was utilized in all E. coli experiments. Stock cultures of bacteria were placed in glycerol and stored at -60° C. For each experiment CEMO was cultured in Brucella broth with 0.02% cysteine hydrochloride at 37° C, with gentle agitation, for 3 days. E. coli was cultured at 22° C for 1 day in Brucella broth (Brucella broth, Gibco).

Broth cultures were centrifuged at 1500 x g for 25 minutes and resuspended in HBSS with 0.1% gelatin. Viable bacteria were counted and standardized by absorption spectrophotometry to 1×10^6 bacteria per ml. This standard concentration was confirmed for each experiment

by titration and plating CEMO on Eugon's chocolate blood agar with 10% horse blood (ECBA) and E. coli on 5% bovine blood agar (BAP).

Phagocytosis assay. Phagocytosis assays were conducted as previously described (133). Briefly, 1×10^6 neutrophils in the various media and 1×10^6 bacteria were combined to give a final volume of 2 ml per tube. The neutrophil bacterial suspensions were placed into a 37° C incubator and rotated at 4 to 6 revolutions per minute. Tubes were collected at 0, 60, and 120 minutes and placed into an ice water bath for five minutes. Tube contents were then washed and centrifuged at 800 x g for five minutes to separate extracellular bacteria and neutrophils. The neutrophil-free supernatant fluid was collected, sonicated for 15 seconds to break up clumped bacteria and plated. Sonication did not alter bacterial viability (Bertram, Iowa State University, 1983). Bacteria numbers were determined by counting colony-forming units. To evaluate the bacteriocidal effect of the various media, CEMO and E. coli samples were prepared with lysed neutrophils and processed as above. The percent phagocytized bacteria was determined by:

$$\frac{\text{Bacterial numbers in supernatant fluid of tubes with lysed neutrophils} - \text{Bacterial numbers in supernatant fluid of tubes with intact neutrophils}}{\text{Bacterial numbers in supernatant of tubes with lysed neutrophils}} \times 100$$

Intracellular killing assay. Killing assays were conducted as previously described (133). Briefly, 1×10^6 neutrophils in the various media and 1×10^6 bacteria in HBSS with 0.1% gelatin were

combined and incubated for 60 minutes at 37° C with continuous rotation. Cold HBSS without calcium and magnesium was added to the tubes to stop phagocytosis. Tubes were centrifuged to separate cell free bacteria and bacteria associated with neutrophils. This procedure was repeated three times. The washing supernatant fluids were discarded and a solution of the corresponding media was added to the cell pellet. Tubes were then placed into a 37° C incubator, rotated at 4 to 6 revolutions per minute, and samples were collected 60, 90, 120, 180, and 210 minutes after the initial mixing of neutrophils and bacteria. Numbers of viable intracellular bacteria were determined by lysing neutrophils by sonication, titrating the lysate, and counting colony-forming units. The number of phagocytized bacteria was determined as above and the percent killed bacteria was determined by:

$$\frac{\text{Number of phagocytized bacteria} - \text{Bacterial numbers in lysate}}{\text{Number of phagocytized bacteria}} \times 100$$

Cell-associated bacteria. This test was conducted as previously described (133). Briefly, cell pellets from the phagocytosis experiments were placed on glass slides and stained with a Romanowsky-Giemsa stain. Two hundred to 220 neutrophils were randomly counted for associated bacteria.

Electron microscopy. Neutrophils and bacteria were combined as above and allowed to incubate for 120 minutes. Neutrophils were then fixed in 2.5% glutaraldehyde for 45 minutes and postfixed in 1.0% osmium tetroxide for 30 minutes. Samples were dehydrated in graded alcohols,

cleared in propylene oxide, and infiltrated and embedded in epoxy-resin (Epon 812, Shell Chemical). Ultrathin sections were cut at 70 to 90 nm and stained with uranyl acetate and lead citrate. Neutrophils were evaluated for intracellular and extracellular bacteria.

Statistical analysis. Student T tests were done on data from 0, 60, 120 minute samples in the phagocytosis analysis and from the 60 and 210 minute samples in the killing analysis.

RESULTS

Phagocytosis. In the presence of seminal plasma, uterine flushings, and lysed neutrophils CEMO numbers increased with time but CEMO numbers in HBSS samples with lysed neutrophils decreased with time (Fig. 1). E. coli numbers in all media with lysed neutrophils increased with time (Fig. 2). The number of CEMO in all samples with intact neutrophils decreased with time; the greatest decrease occurred with neutrophils in HBSS samples (Fig. 1). The number of E. coli in all samples with intact neutrophils increased with time (Fig. 2).

The percent phagocytized CEMO increased with time in all samples (Table 1). A significant difference ($p < 0.05$) between HBSS and the other media was demonstrated at 60 minutes only (Table 1). The average number of bacteria per neutrophil increased in all samples with time (Table 2).

Intracellular killing. Intracellular killing proceeded rapidly during the first 60 minutes of incubation in all CEMO samples. Fifty-five to 75% of all intracellular CEMO did not form colonies after 60 minutes of incubation. The percent killed was similar, between types of media, throughout the sampling times (Fig. 3). Significant differences ($p < 0.05$) between semen and uterine flushings were found in the 60 and 210 minute sample from one horse. No significant differences were found between any media at the 60 and 210 minute samples when all horses were combined.

The ingested E. coli that did not form colonies after 60 minutes of incubation varied from 30% (HBSS) to 70% (seminal plasma). The

Fig. 1. Number of CEMO in various media with lysed neutrophils (left graph). Number of CEMO in various media with intact neutrophils (right graph)

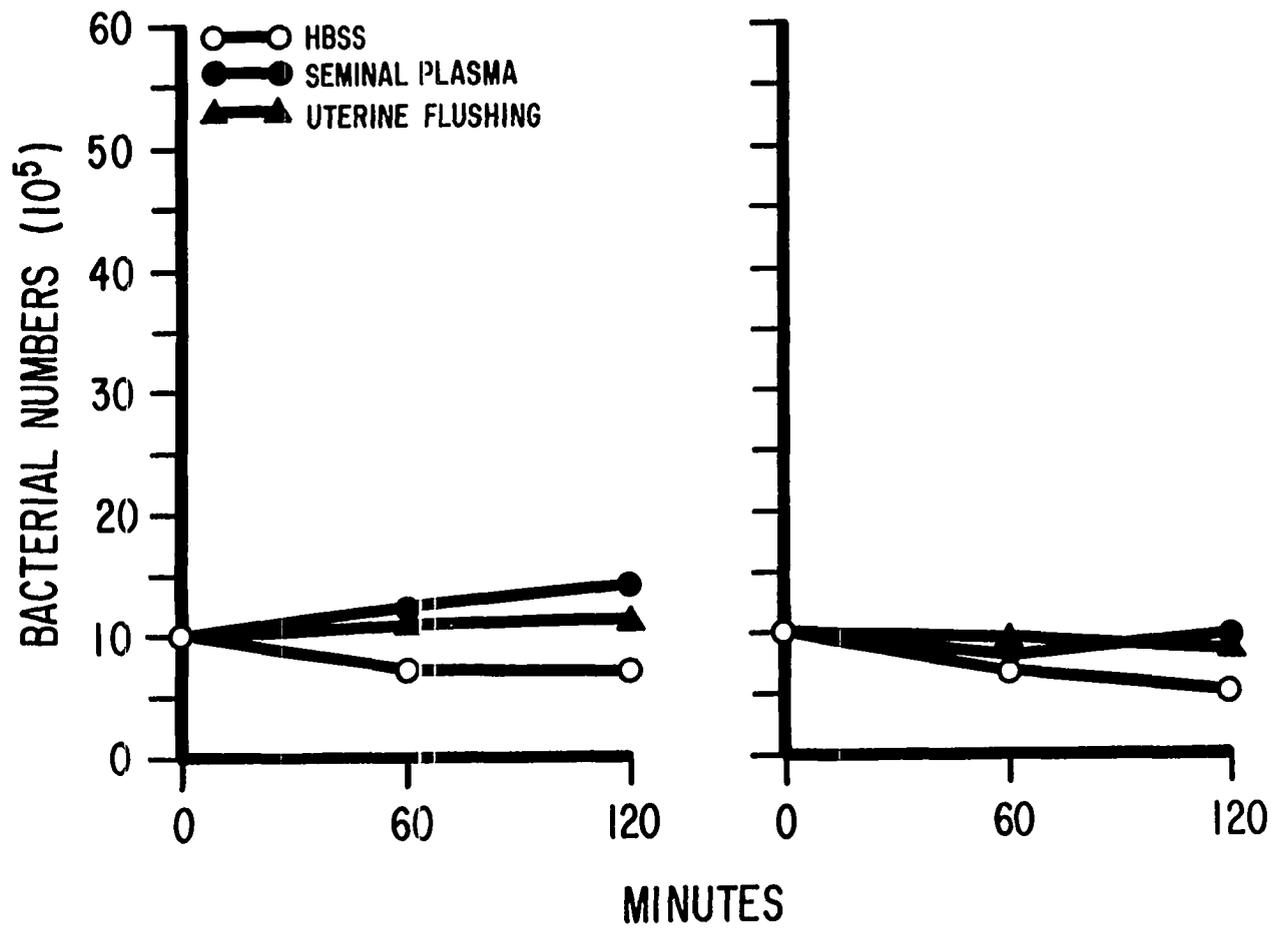


Fig. 2. Number of E. coli in various media with lysed neutrophils (left graph). Number of E. coli in various media with intact neutrophils (right graph)

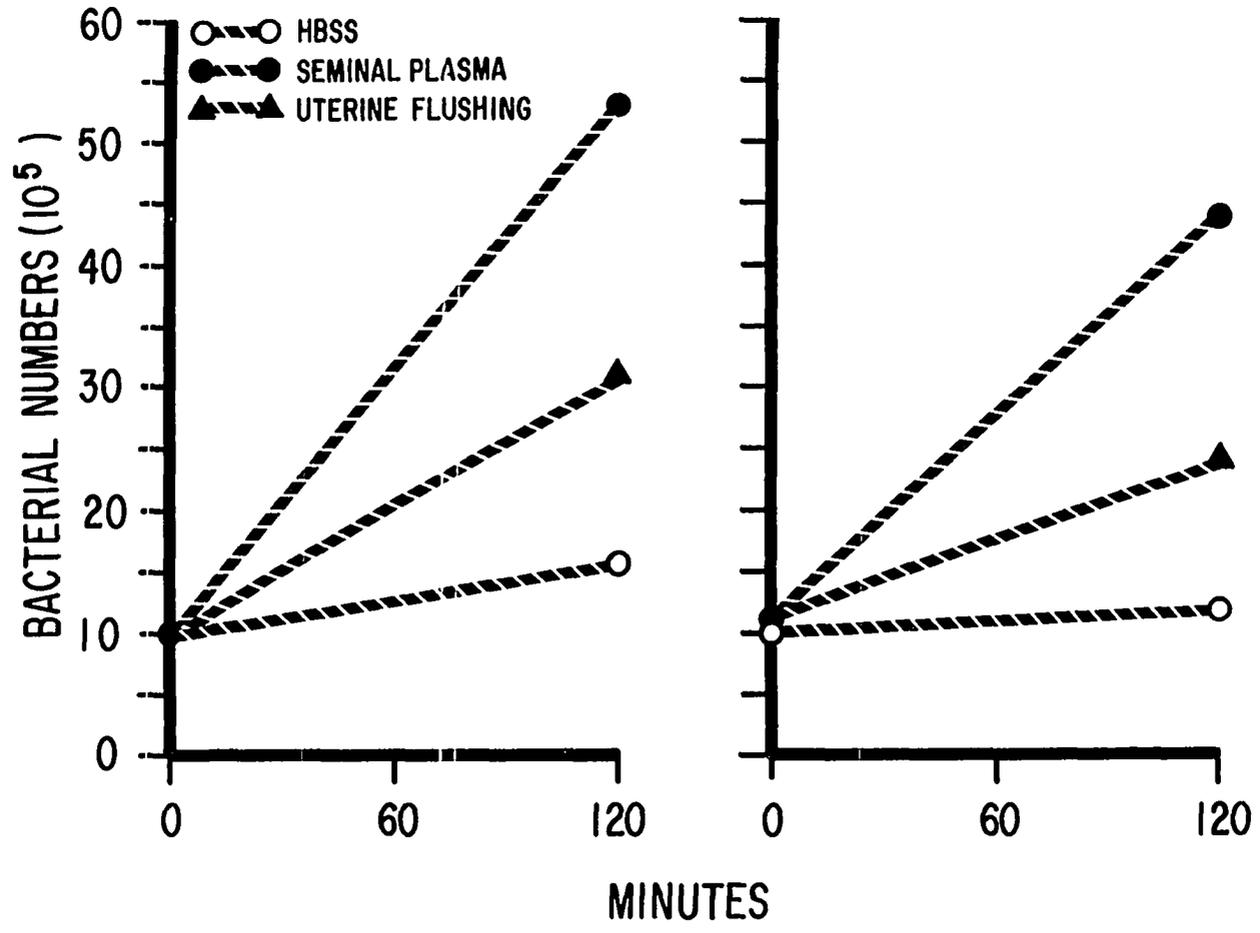


Table 1. Percentage of 1×10^6 CEMO phagocytized by 1×10^6 equine neutrophils^a

Medium	Percentage phagocytized bacteria		
	0 min	60 min	120 min
HBSS	2.0	7.0	29.0
uterine flushings	1.0	22.0	29.0
seminal plasma	0.0	28.0	32.0

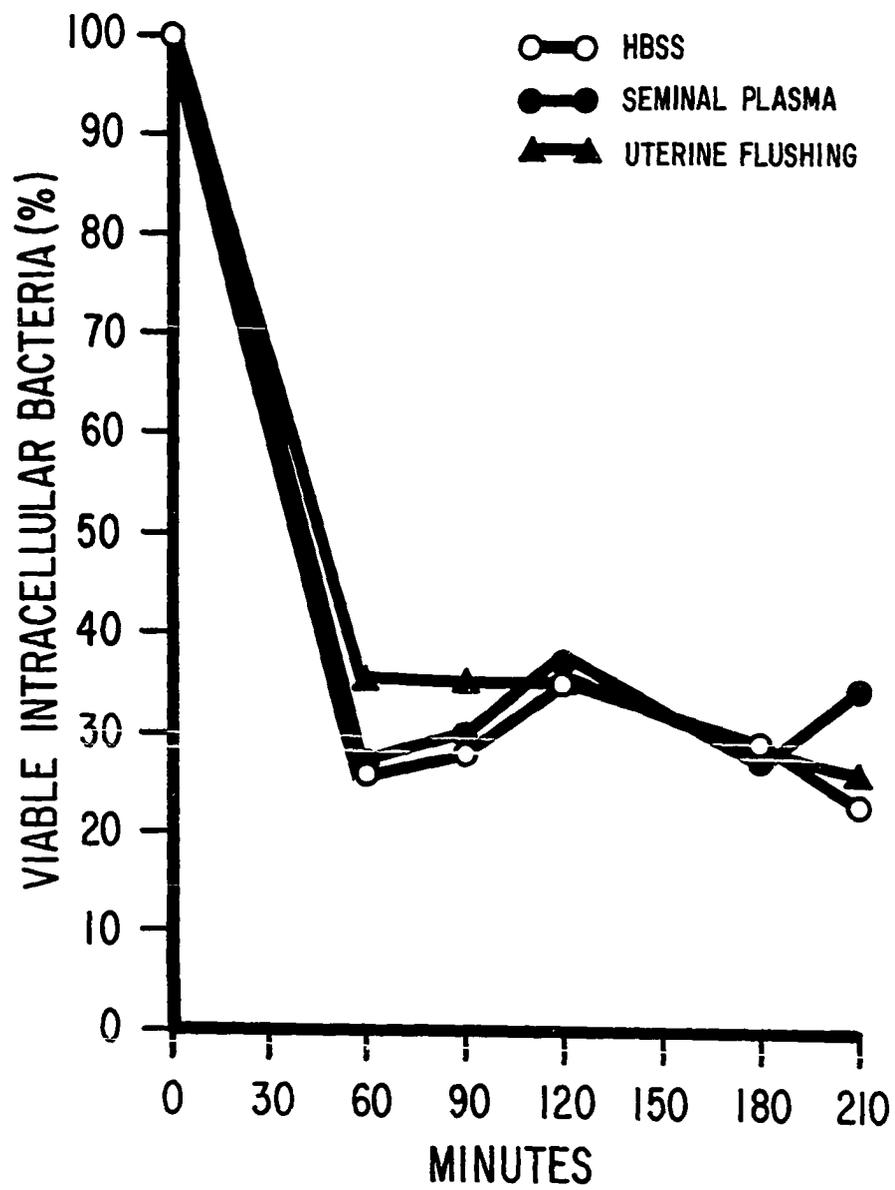
^aValues are derived as shown in text from Figure 1. CEMO samples are the average of 3 replicates from 3 horses. HBSS - Hanks' balanced salt solution.

Table 2. Average number of bacteria phagocytized per horse neutrophil in various media^a

Bacteria	Medium	Average number bacteria per neutrophil		
		0 min	60 min	120 min
CEMO	HBSS	1.0	1.3	1.4
	uterine flushings	1.0	1.3	1.4
	seminal plasma	1.0	1.3	1.3
<u>E. coli</u>	HBSS	1.1	1.2	1.9
	uterine flushings	1.4	1.5	1.6
	seminal plasma	1.4	1.5	2.6

^aValues determined only for neutrophils which contained bacteria. Means of 800 neutrophils from each horse. HBSS = Hanks' balanced salt solution.

Fig. 3. Percent viable intracellular CEMO



percent that did not form colonies after 210 minutes was similar for the various types of media (85-90%). Samples from seminal plasma consistently had fewer colony-forming units than did HBSS or uterine flushings. Until 210 minutes of incubation HBSS, samples had the greatest number of colony forming intracellular bacteria (Fig. 4).

Morphological examination. The percentage of neutrophils with visible associated CEMO paralleled the percentage of phagocytized CEMO for a given time and medium. The percentage of neutrophils with bacteria, as observed histologically, for each medium is shown in Fig. 5 and 6. The number of CEMO per neutrophil was similar for all medium groups at a given time. The percentage of neutrophils with intracellular bacteria and the average number of bacteria per neutrophil determined by electron microscopy corresponded directly with the light microscopy results. More E. coli than CEMO were observed intracellularly and the number of neutrophils with E. coli was greater than the number with CEMO. No extracellular bacteria were observed adherent to neutrophil membranes.

Electrophoresis and immunoelectrophoresis. Proteins migrating in the gamma globulin region were demonstrated in seminal plasma but not in the uterine flushings with electrophoresis. Immunoglobulins A and G were demonstrated in both semen and uterine flushings with immunoelectrophoresis (Fig. 7).

Fig. 4. Percent viable intracellular E. coli

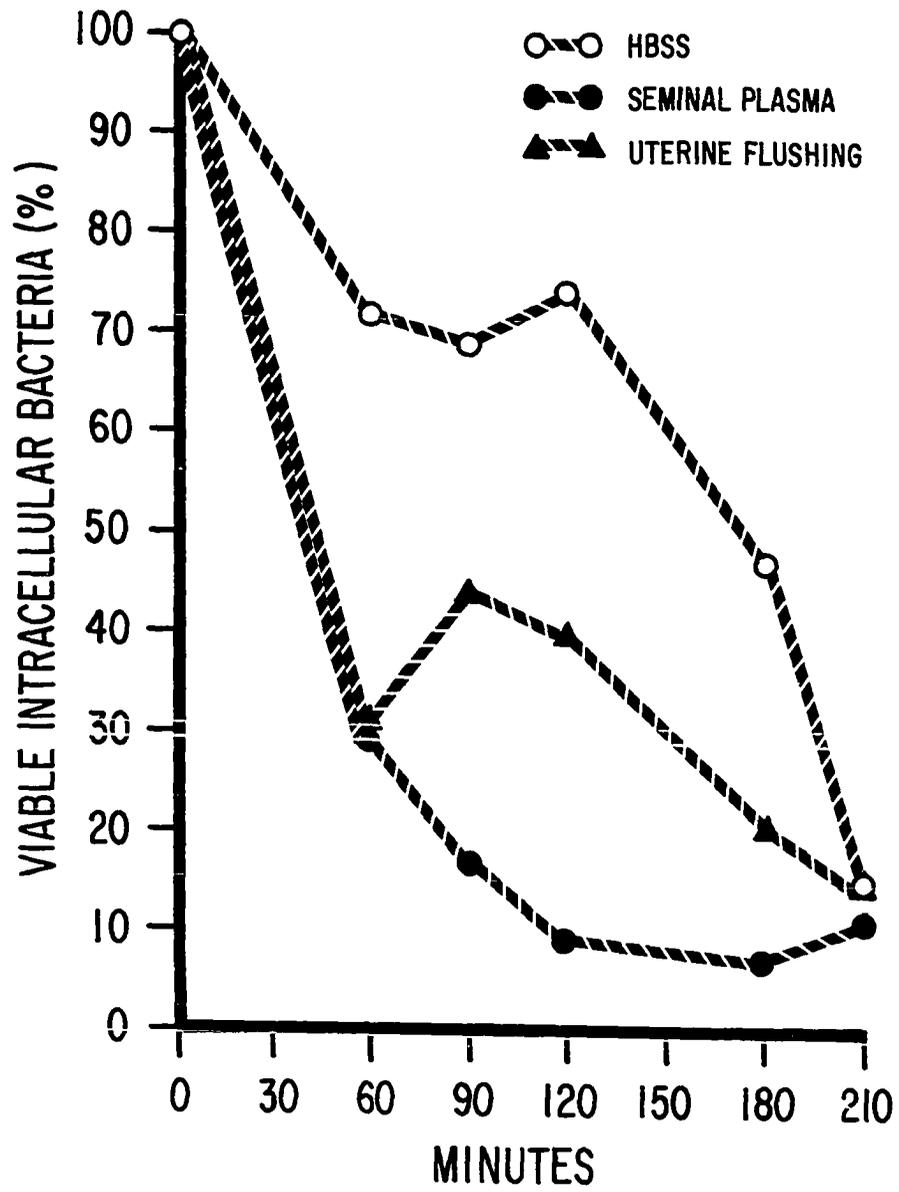


Fig. 5. Percentage of neutrophils with CEMO

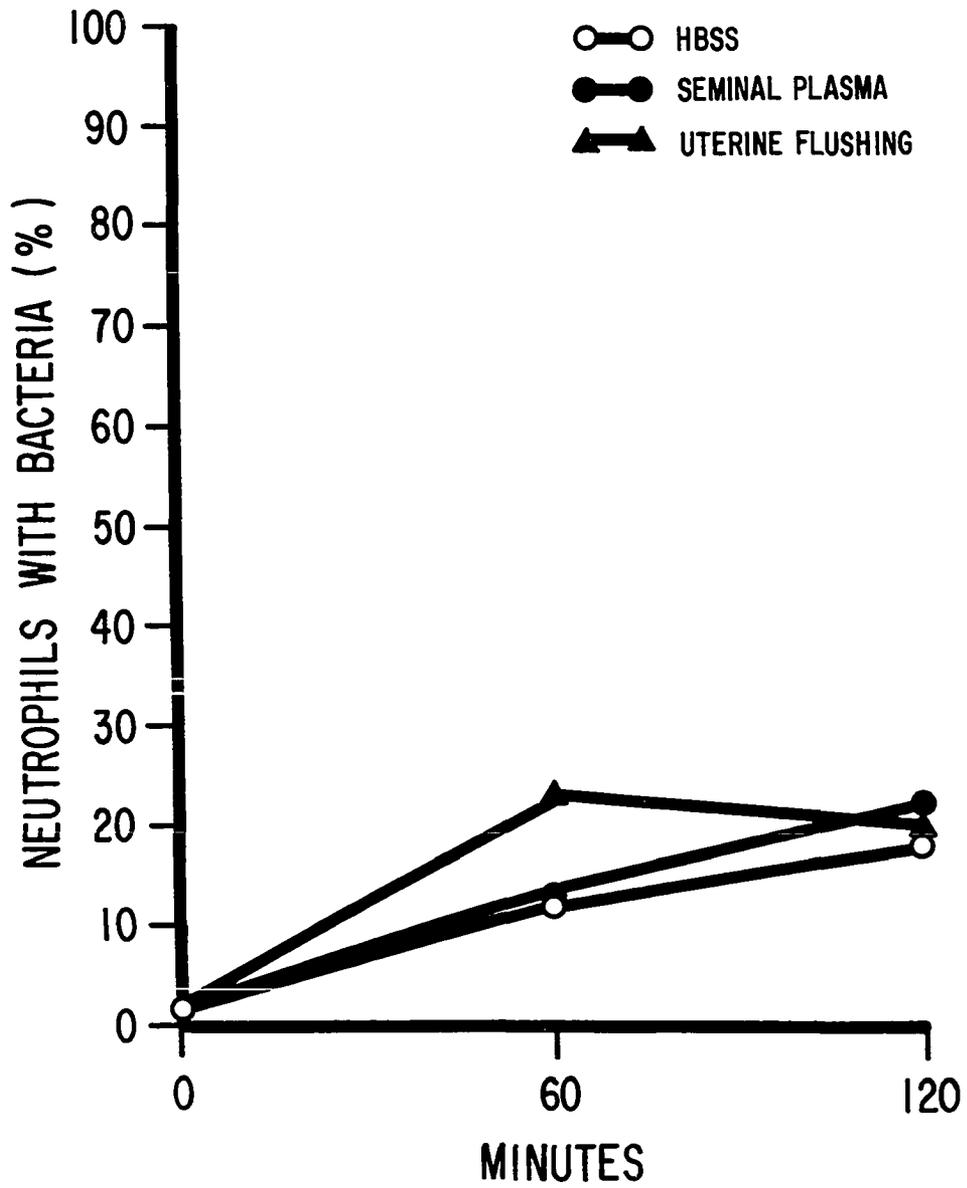


Fig. 6. Percentage of neutrophils with E. coli

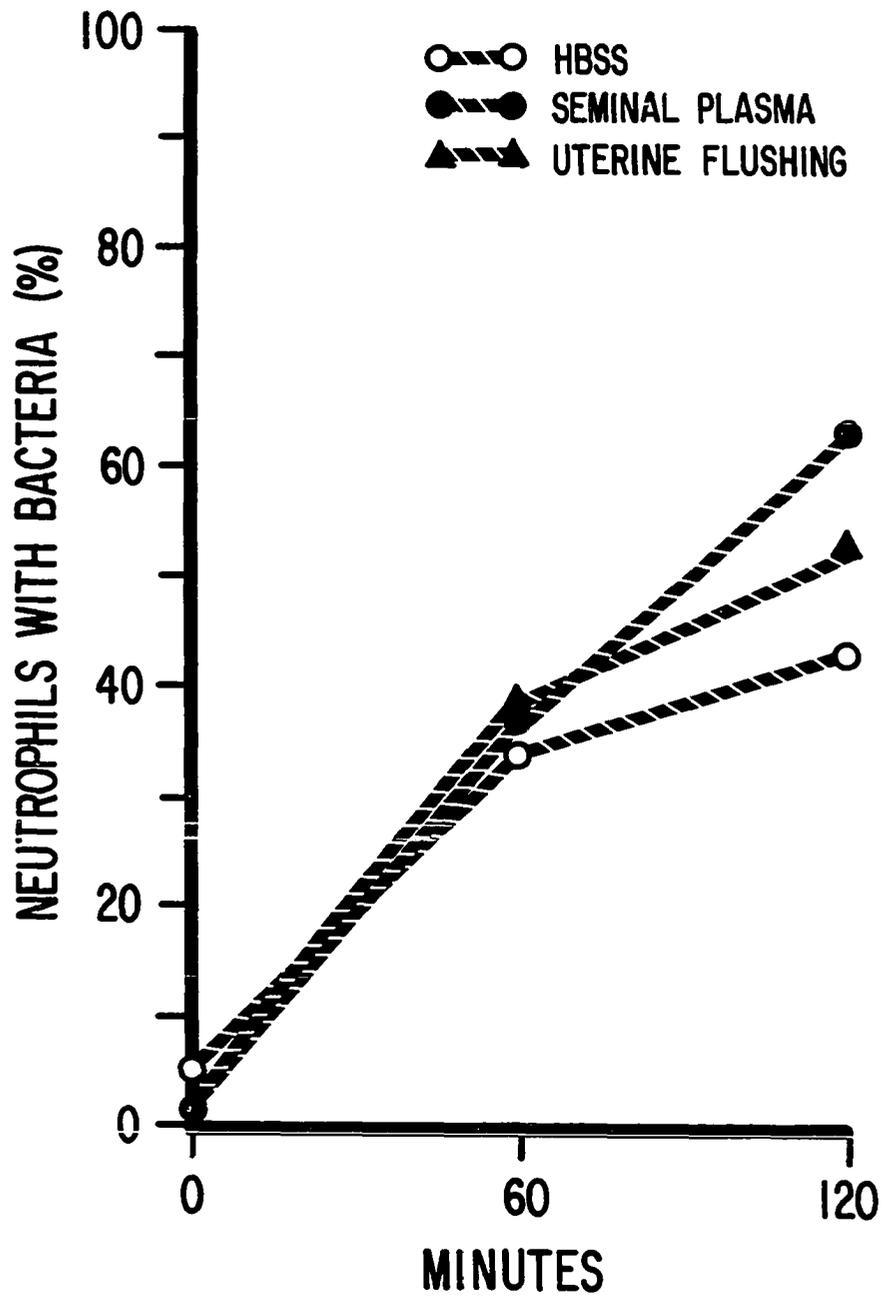
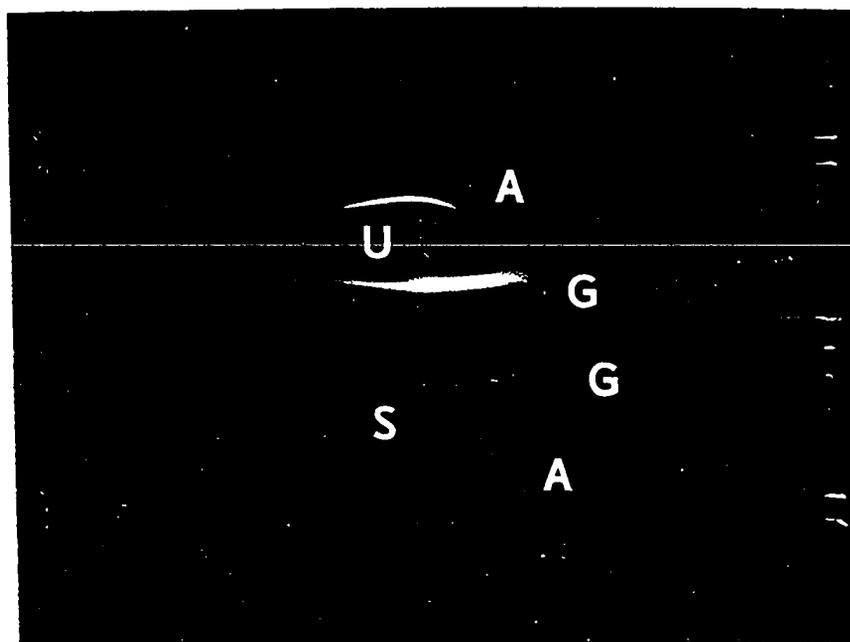


Fig. 7. Immunelectrophoresis pattern. Precipitation line of IgG(G) and IgA(A) can be seen for uterine flushing (UF) and Seminal plasma (S)



DISCUSSION

Neutrophil phagocytosis of CEMO is slower than for E. coli and this may reflect a mechanism by CEMO to avoid neutrophil ingestion. A lower number of CEMO than E. coli were phagocytized at each time period in each medium. A similar in vitro observation and conclusion has been reported previously with CEMO in serum (7). A discrepancy with this conclusion appears to occur with E. coli in the presence of neutrophils (Fig. 2). E. coli numbers gradually increased with time. These E. coli values are artificially high and result from the rapid proliferation of E. coli in each medium. Other observations like the actual number phagocytized, bacterial numbers per neutrophil, and the percent neutrophils with bacteria were always greater for E. coli than for CEMO. Thus, CEMO may avoid host defenses by being slowly phagocytized.

Seminal plasma and uterine flushings did not enhance phagocytosis or intracellular killing of CEMO. We demonstrated immunoglobulin in seminal plasma and uterine flushings and showed the type present could enhance phagocytosis and killing of E. coli but not CEMO. Improved neutrophil phagocytosis and killing has been reported for CEMO in the presence of serum antibody (7). These observations may provide partial explanation as to why CEMO remains limited to a surface infection and does not seem to disseminate systemically (87, 96). Differences in E. coli killing ability between neutrophils in uterine flushings and seminal plasma may result from a lower concentration of immunoglobulins in the uterine flushings.

Seminal plasma may promote the growth of CEMO. Seminal plasma has been reported to increase virulence of CEMO in horses (128). Seminal plasma did not decrease phagocytosis or enhance intracellular survival in vitro but CEMO numbers increased more in seminal plasma than with other media. Neutrophil function was not affected by seminal plasma since E. coli was phagocytized and killed more rapidly in this medium than in the others. This suggests that seminal plasma may enhance CEMO multiplication, rather than alter the neutrophil's ability to phagocytize and kill CEMO.

Equine neutrophils are able to kill phagocytized CEMO. This is supported by our observation of a marked decrease in the number of intracellular CEMO colony-forming units after 60 minutes of incubation. Intracellular killing of CEMO by equine neutrophils has been reported previously (7). Neutrophils killed CEMO as rapidly and efficiently in HBSS as in other media. Killing of E. coli by neutrophils was initially best with seminal plasma and uterine flushings. With time, more E. coli than CEMO were killed in each medium. This suggests that the neutrophil has the ability to kill CEMO and that the various media did not affect killing ability.

We observed a residual number of viable CEMO in neutrophils in each medium. This is not likely a result of altered neutrophil killing ability since a similar effect was not observed in previous experiments when neutrophils were in serum (7). Further, this rapid killing with a residual bacterial population was not observed with E. coli. The

residual intraneutrophil CEMO may represent a unique subpopulation of CEMO.

EFFECT OF CONTAGIOUS EQUINE METRITIS ORGANISM (CEMO) AND
TWO CEMO LIPOPOLYSACCHARIDES ON EQUINE NEUTROPHILS

ABSTRACT

Equine neutrophils were combined with the contagious equine metritis organism (CEMO), or two CEMO lipopolysaccharides (LPS-a;LPS-p) to evaluate the effect of these agents on neutrophil morphology and function. All agents inhibited random migration of equine neutrophils. CEMO and LPS-p (25 μ g) were chemotactic stimuli for neutrophils. Neutrophils exposed to each of the lipopolysaccharides had fewer granules; whereas, those exposed to CEMO had more granules than controls (PBS). Neutrophil iodination was elevated with 10 and 25 μ g LPS-a but not significantly altered by LPS-p or CEMO. Staphylococcus aureus (S. aureus) ingestion was increased by LPS-p at the 25 μ g level whereas LPS-a consistently decreased S. aureus ingestion. Nitroblue tetrazolium (NBT) reduction was significantly increased with LPS-p (25 μ g). These results suggest that the effects of CEMO on equine neutrophils may be partially a result of CEMO cell wall lipopolysaccharides.

INTRODUCTION

Contagious equine metritis is a venereal disease of horses caused by a Gram negative coccobacillus. The organism is oxidase-, phosphatase-, and catalase-positive, microaerophilic and does not require hematin or nicotinamide for growth (127). The contagious equine metritis organism (CEMO) was proposed to be classified in the genus *Hemophilus* (127) but this has not been generally accepted (8).

The disease is characterized by a severe mucopurulent vaginal exudate (84). Neutrophils are present in the uterine and cervical lamina propria in the acute stages (87). High numbers of extracellular and moderate numbers of intraneutrophilic CEMO can be seen in the vaginal exudate. We have shown that CEMO is slowly phagocytized but rapidly killed once ingested by equine neutrophils (7). We suggested CEMO altered the neutrophil at the local site of ingestion but did not inhibit neutrophil killing functions.

Endotoxins found in the cell wall of many Gram negative bacteria, are composed of lipopolysaccharides (LPS) and protein. Endotoxins have been demonstrated to have many roles in disease processes (13, 73) including alteration of neutrophil phagocytosis (16), oxidative metabolism (66, 83), and migration (37, 49, 114, 115).

The purpose of this study was to evaluate neutrophil function after exposure to CEMO and lipopolysaccharides isolated from CEMO. The neutrophil functions evaluated were 1) oxidative metabolism, 2) migration

(directed and random), 3) phagocytosis, and 4) myeloperoxidase- H_2O_2 halide antimicrobial system. The morphological features of neutrophils that were evaluated included 1) granule number, 2) nuclear, phagosome, and total neutrophil area.

MATERIALS AND METHODS

Animals. Six adult horses were included in this study. Blood was taken from horses for each test on each experimental day. All horses remained clinically healthy throughout the course of the experiments.

Neutrophils. Neutrophils were harvested from the blood of each animal as previously described (6). Blood was collected and allowed to sediment for 20 minutes at room temperature. Subsequent processing was conducted at 4° C. Erythrocytes were lysed with hypotonic ammonium chloride in tris buffer. Neutrophils were separated from other leukocytes by centrifugation on two successive Ficoll-Hypaque density gradients.

Bacteria. A streptomycin-susceptible strain of CEMO (78M3056) was used. This culture was originally isolated in a Kentucky outbreak of CEM and was obtained from Dr. B. Blackburn, Diagnostic Bacteriology Laboratory, National Veterinary Service Laboratories, Ames, Iowa. The CEMO used was three in vitro passes from the virulent field isolate. This isolate remains virulent and retains a capsule after 5 in vitro passes.

The stock cultures of bacteria were placed in glycerol and stored at -60° C. For each experiment, CEMO was cultured in brucella broth with 0.02% cysteine hydrochloride (GIBCO Laboratories, Grand Island, N.Y.) at 37° C, with continuous rotation for one day.

Broth cultures were centrifuged at 1,500 x g for 25 minutes and suspended in Hanks' balanced salt solution with 0.1% gelatin. Viable

bacteria were counted and standardized by absorption spectrophotometry to 5×10^9 per ml. This standard concentration was confirmed for each experiment by titration and plating CEMO on Eugon chocolate blood agar with 10% horse blood.

Lipopolysaccharide (LPS) isolation. Extraction of CEMO LPS was first attempted using the phenol-chloroform-petroleum ether method (36); negligible amounts of LPS were recovered. The cells were subsequently treated by the hot aqueous phenol method (140). The phenol soluble lipopolysaccharide from the hot aqueous method was isolated following a previously reported procedure (92). Extractions were repeated twice, extracts were pooled, concentrated to 20% original volume, lyophilized, and resuspended in distilled water to give a three percent solution by weight.

After deoxyribonuclease and ribonuclease treatment at pH 7.2 with 0.025 M magnesium sulfate in 0.1 M tris buffer for 18 hours at 22° C, the extracts were centrifuged at 105,000 x g for 4 hours at 4° C. The pellets were resuspended in distilled water and the centrifugation was repeated. Impurities having a molecular weight less than 50,000 Daltons were removed by ultrafiltration dialysis. The extracts were evaluated, following previously reported procedures for protein (12), (Biorad, Bio-Rad Laboratories, scan with UV light), hexose-heptose (145), 2 keto 3 deoxyoctamate (KDO) (53), fatty acids (67), and nucleic acids (136). The extracts were lyophilized and stored at 4° C. Phenol phase LPS is referred to as LPS-p and the aqueous phase LPS is referred to as LPS-a.

Electron microscopic evaluation of LPS. Evaluation of LPS was done following a previously reported procedure (111). Each LPS extract was suspended in distilled water and atomized onto copper grids. Samples were stained with uranyl acetate and examined with an electron microscope at 60 KV.

Anti-CEMO-LPS antibody production. Antisera were produced in eight adult New Hampshire red chickens by weekly intravenous inoculations of LPS-a (25 μ g), LPS-p (25 μ g), or formalinized CEMO (1×10^9). Preinoculation sera samples were collected to detect any naturally occurring anti-CEMO-LPS. Chickens were exanguinated on the fourth week of inoculation. Sera were filter sterilized and stored at -65° C.

Limulus amebocyte lysate assay. The limulus lysate (Etoxate, Sigma Chemical Co.) assay was conducted in triplicate as previously described (147). Shigella flexneri LPS (I-3255) (Etoxate, Sigma Chemical Co.) was used as a positive control. After one hour incubation, the tubes were inverted and examined for the presence of a firm gel. A negative reaction was indicated when a firm gel was not observed.

Gel diffusion precipitin test. This test was conducted as previously described (44). LPS was placed in the center well and anti-LPS was placed in the outside wells.

Neutrophil function tests. Two concentrations of LPS (10 & 25 μ g) were used in the iodination test and S. aureus phagocytosis test. Chemotaxis and random migration tests were conducted using 25 μ g of each LPS. All tests with CEMO were conducted using 5×10^9 viable CEMO.

Iodination was done using a modification of a previously reported procedure (58). Neutrophils, NaI, ^{125}I , and preopsonized zymosan (Sigma Chemical Co.) were suspended in Earle's balanced salt solution (EBSS) with one of the lipopolysaccharides, CEMO, or phosphate buffered saline (PBS). Tubes containing this mixture were rotated at 37° C for 20 minutes. The reaction was terminated by adding cold 10% trichloroacetic acid to the tubes. Tubes were centrifuged, the precipitate was collected, and counts per minute of radioactivity in the precipitate determined in a gamma counter. Results were recorded as nmoles of NaI/ 10^7 neutrophils/hour and are given as percent of control (PBS).

Neutrophil ingestion of S. aureus was measured following a previously reported procedure (100). Briefly, a ^{125}I , iododeoxyuridine labeled coagulase positive S. aureus was combined with anti-S. aureus serum, equine neutrophils, and LPS, CEMO, or PBS and incubated with gentle rocking for 45 minutes at 37° C. Lysostaphin was added to lyse extracellular S. aureus, tubes were centrifuged, and the gel pellet collected. Percent ingestion was estimated by determining counts per minute of radioactivity in the cell pellet using a gamma counter with results expressed as percent of control (PBS).

Nitroblue tetrazolium (NBT) reduction by neutrophils was evaluated following a previously reported procedure (100). NBT, neutrophils, and preopsonized zymosan (Sigma Chemical Co.) were combined in EBSS. Tubes were incubated for 10 minutes at 37° C. The reaction was stopped with N-ethylmaleimide, pyridine was added to precipitate formazan, and an optical density (OD) at 580 nm was determined spectrophotometrically.

Results were obtained as $OD/50 \times 10^6$ neutrophils/10 minutes in 5.0 ml pyridine expressed as percent of control (PBS).

Random migration tests were conducted following a modification of a previously reported assay (77). Briefly, agar (0.8%) was buffered at pH 7.2 with bicarbonate and added to 10% equine serum and 1% antibiotic-actinomycin solution. Plates were incubated for 24 hours at 37° C with 10% CO₂. The area of migration was determined using semiautomated computerized morphometry (Bioquant II, R & M Biometrics). Areas were expressed as mm² migrated.

Chemotaxis assays were done following a previously reported procedure (77). Agar was prepared as for the migration assays. Three 2.4 mm wells were cut into the agar and ten microliters of neutrophils (50×10^6 /ml) were placed in the center well, 10 µg of each LPS, or 5×10^9 CEMO was placed in the outer wells and PBS was placed in the inner wells. Plates were incubated in 10% CO₂ at 37° C for 24 hours. Migration was stopped by adding cold glutaraldehyde to the petri plates. Length from the leading edge of neutrophil migration to the periphery of the central area of the well was measured using a semi-automated computerized measuring device (Bioquant II, R & M Biometrics). Distance traveled was expressed as mm.

Neutrophil morphometry. Neutrophils from two horses were combined with opsonized zymosan (Sigma Chemical Co.) and LPS-a (25 µg), LPS-p (25 µg), 5×10^9 CEMO, or PBS. Two samples from each horse for each test material were incubated as described for iodination. Cells were processed for transmission electron microscopy by fixation in 2.5%

glutaraldehyde, post fixed in 1.0% osmium tetroxide, dehydrated in graded alcohols, cleared in propylene oxide, infiltrated and embedded in epoxy resin (Epon 812, Shell Chemical Co.). One section was cut from each sample at 70 nm and stained with uranyl acetate and lead citrate. Twelve micrographs from each sample were made at a magnification of 24,000. Morphometry was done using semiautomated computerized morphometry (Bioquant II, R & M Biometrics). Measurements were conducted for total cell, nuclear, and zymosan containing phagolysosome areas. Values were expressed as μm^2 . Numerical density was determined for neutrophil granules and expressed as granules/ μm^2 total cell and granules/ μm^2 cytoplasm.

Statistical analysis. Statistical analysis consisted of the Student T test for paired comparisons and the analysis of variance for multiple mean comparisons.

RESULTS

Lipopolysaccharide characteristics. The yield of LPS-a and LPS-p were approximately 0.3 to 0.4 percent of the original bacterial cell dry weight. LPS-a and LPS-p had hexoses and heptoses as well as two fatty acids with retention times equivalent to a 3-hydroxylated fourteen carbon fatty acid and a saturated nonhydroxylated sixteen carbon fatty acid standard. LPS-a contained 5.0% heptose and 0.7% hexose, and LPS-p contained 3.6% heptose and 0.7% hexose. Both lipopolysaccharides contained keto-deoxy-octanate (KDO). LPS-p contained 0.54% and LPS-a 0.44% KDO. Protein and nucleic acids were not demonstrated in either LPS preparation.

Limulus lysate gelation occurred with 15 picograms LPS-a and 30 picograms LPS-p. With the Shigella flexneri LPS reference, a soft gel was observed by 2 hours at the 25 picogram level. Gelation was not observed when other assay reagents were incubated with the amebocyte lysate.

The CEMO lipopolysaccharides were morphologically different. LPS-a was filamentous and branched. The filaments were 6 to 11 nm in diameter, varied in length, and were associated with spherical or oblong structures. The spherical structures were 4 to 11 nm in diameter and were clustered at varying distances along the filaments. Many regions of the filaments were not associated with these structures (Fig. 1). LPS-p was primarily spherical and ovoid shaped with few filamentous structures. These structures varied in diameter from 8 nm to 62 nm (Fig. 2).

Fig. 1. Aqueous phase lipopolysaccharide (LPS-a). (F) filaments with clusters of (S) spherical and (O) oval structures. Bar = 0.5 μm

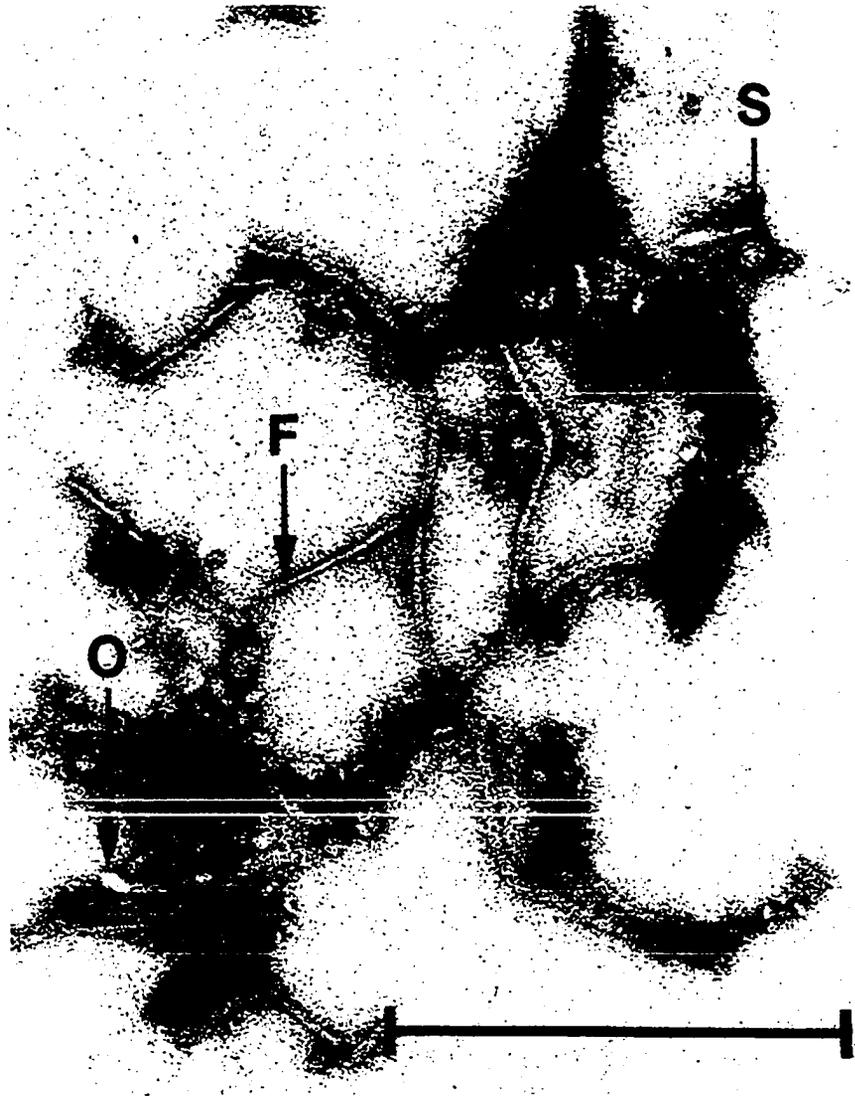
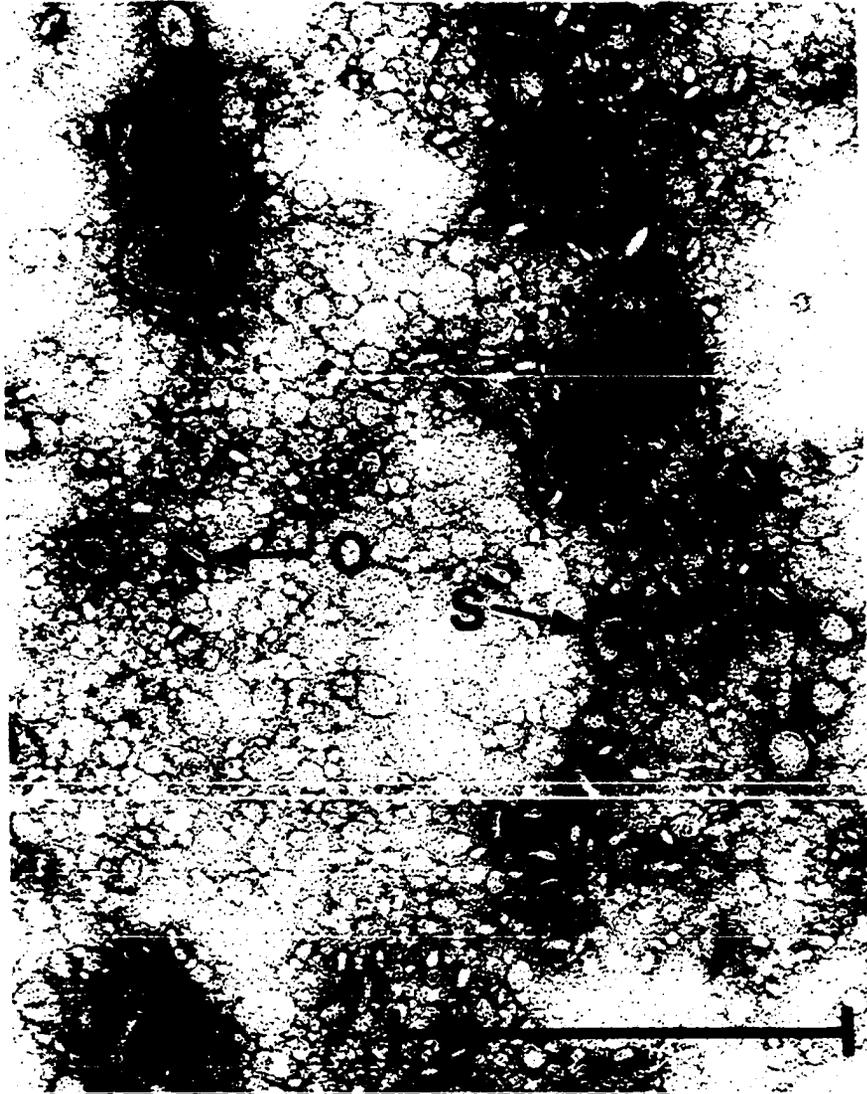


Fig. 2. Phenol phase lipopolysaccharide (LPS-p). (S) spherical and (O) oval structures. Bar = 0.5 μm



Chicken anti-CEMO and anti-LPS-a formed precipitin lines to LPS-a but not LPS-p after 24 hours of incubation. Chicken anti-LPS-p formed precipitin lines with LPS-p and LPS-a after 24 hours incubation.

Random migration and chemotaxis. Whole CEMO, LPS-a and LPS-p significantly ($p < 0.05$) inhibited random migration of equine neutrophils as compared to phosphate buffered saline (PBS). The least random migration occurred in the presence of whole CEMO (Table 1). Both the whole organism and LPS-p had significantly ($p < .05$) greater chemoattractant qualities than the LPS-a or PBS (Table 1).

Staphylococcus aureus (S. aureus) ingestion. Although there were no statistically significant differences among LPS-a, LPS-p, CEMO and PBS in the ability to alter neutrophils ingestion of S. aureus, LPS-a consistently decreased the ingestion of S. aureus at both dosage levels. LPS-p at the high dosage level consistently increased the ingestion of S. aureus by equine neutrophils (Fig. 3).

Iodination. The effect, on neutrophil iodination, of CEMO, LPS-a and LPS-p at different dose levels are demonstrated in Figure 4. Both levels of LPS-a significantly ($p < .05$) increased iodination and this effect was dose related. High dosage LPS-a increased iodination 35 to 45% and low dose increased iodination 5 to 20%. Whole CEMO inhibited iodination 4 to 18%.

Nitroblue tetrazolium (NBT) reduction. LPS-p at the high dose level and whole CEMO increased NBT reduction. The LPS-p increase ranged from 6 to 18% and was significantly ($p < 0.05$) different from the control.

Table 1. Neutrophil migration under Agarose^a

Test material ^b	Random migration (mm ²)	Chemotaxis (mm)
PBS	22.0 ± 0.51	2.1 ± 0.1
CEMO	3.3 ± 0.25	2.7 ± 0.2
LPS-a (25 µg)	8.7 ± 2.5	2.2 ± 0.1
LPS-p (25 µg)	6.4 ± 1.9	2.8 ± 0.3

^a(mean +/- S.E.M.).

^bPBS = phosphate buffered saline.

CEMO = contagious equine metritis organism.

LPS-a = aqueous soluble lipopolysaccharide.

LPS-p = phenol soluble lipopolysaccharide.

Fig. 3. Mean values of Staphylococcus aureus ingestion by equine neutrophils (means \pm S.E.M.)

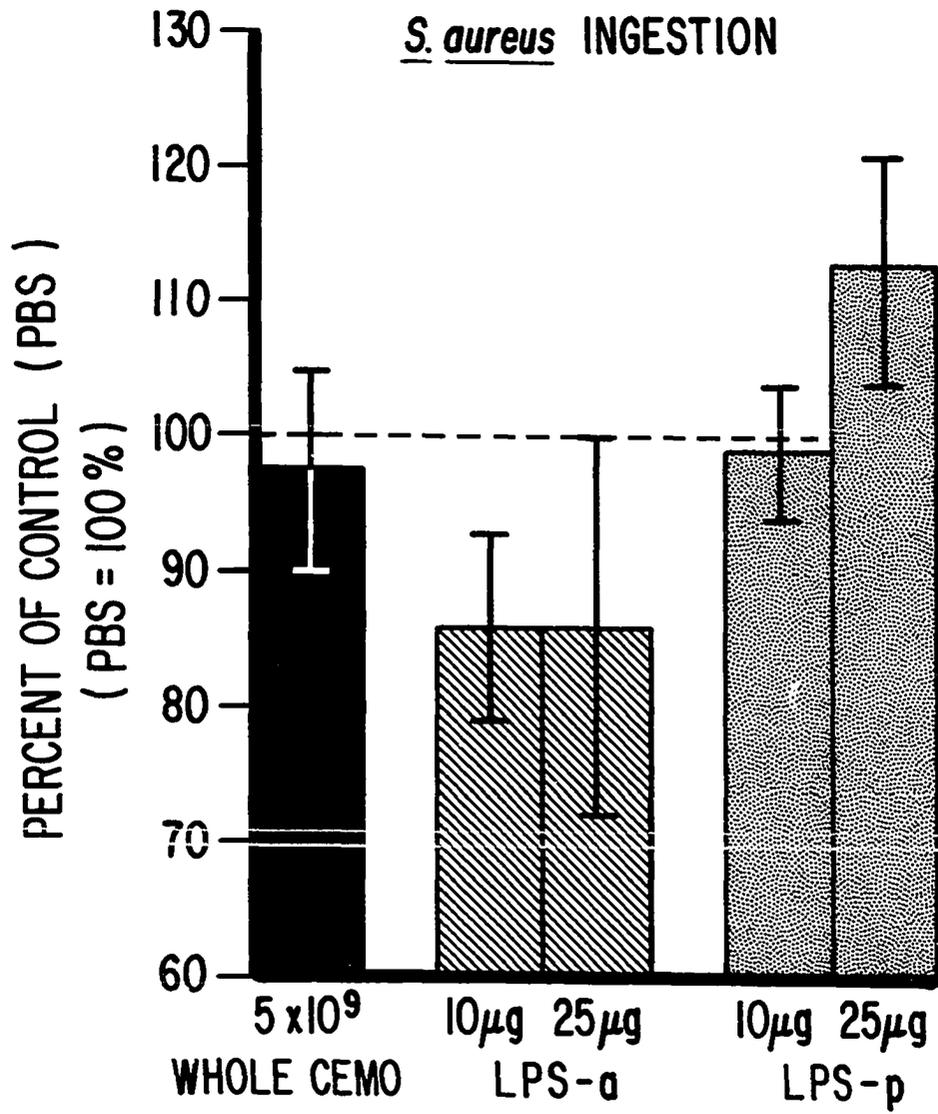
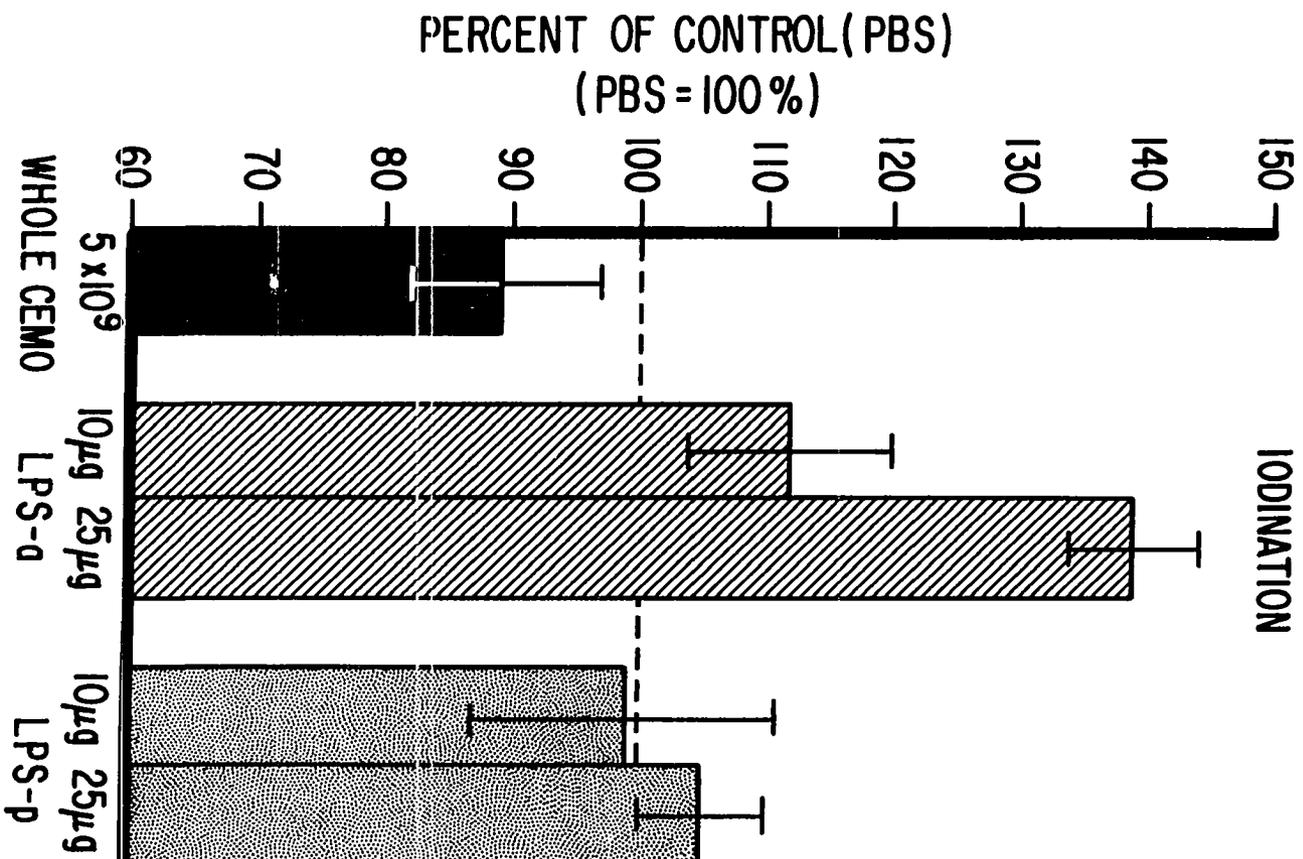


Fig. 4. Mean values of iodination by equine neutrophils (means \pm S.E.M.)



Whole CEMO consistently ($p < 0.15$) increased NBT reduction. The other materials tested did not significantly alter NBT reduction (Fig. 5).

Neutrophil morphology. Neutrophils exposed to whole CEMO consistently had more granules per square micron of cytoplasm than did neutrophils in the presence of the other test materials (Table 2). Neutrophils in the presence of LPS-a (25 μg) had fewer granules per square micron of cytoplasm than did neutrophils in the other test materials. Zymosan area per area of cytoplasm was similar for PBS, whole CEMO, and LPS-p (25 μg).

Zymosan-containing phagolysosomes occupied 14 to 17% of the neutrophil cytoplasm. The phagolysosomes were frequently located near the center of the neutrophil with other organelles distributed near the cell periphery. Primary and secondary granules were juxtaposed to and surrounded zymosan phagosomes. When a layer of microfilaments encircled phagosomes, granules were located around these cytoskeletal elements. Phagolysosomal membranes were undulant and loosely apposed to the opsonized zymosan particle. The space between the phagolysosomal membrane and the zymosan particle frequently contained flocced, electron dense material. The center of the opsonized-zymosan particle was electron dense (zymosan) and was circumscribed by an electron lucent zone (serum opsonins). The electron lucent zone consisted of multiple laminated fibrils concentrically arranged about the zymosan core. Most of the opsonized zymosan particle was composed of this electron lucent zone (serum opsonins). These particles seldom had evidence of fragmentation or dissolution (lysosomal digestion).

Fig. 5. Mean values of nitroblue tetrazolium reduction by equine neutrophils (means \pm S.E.M.)

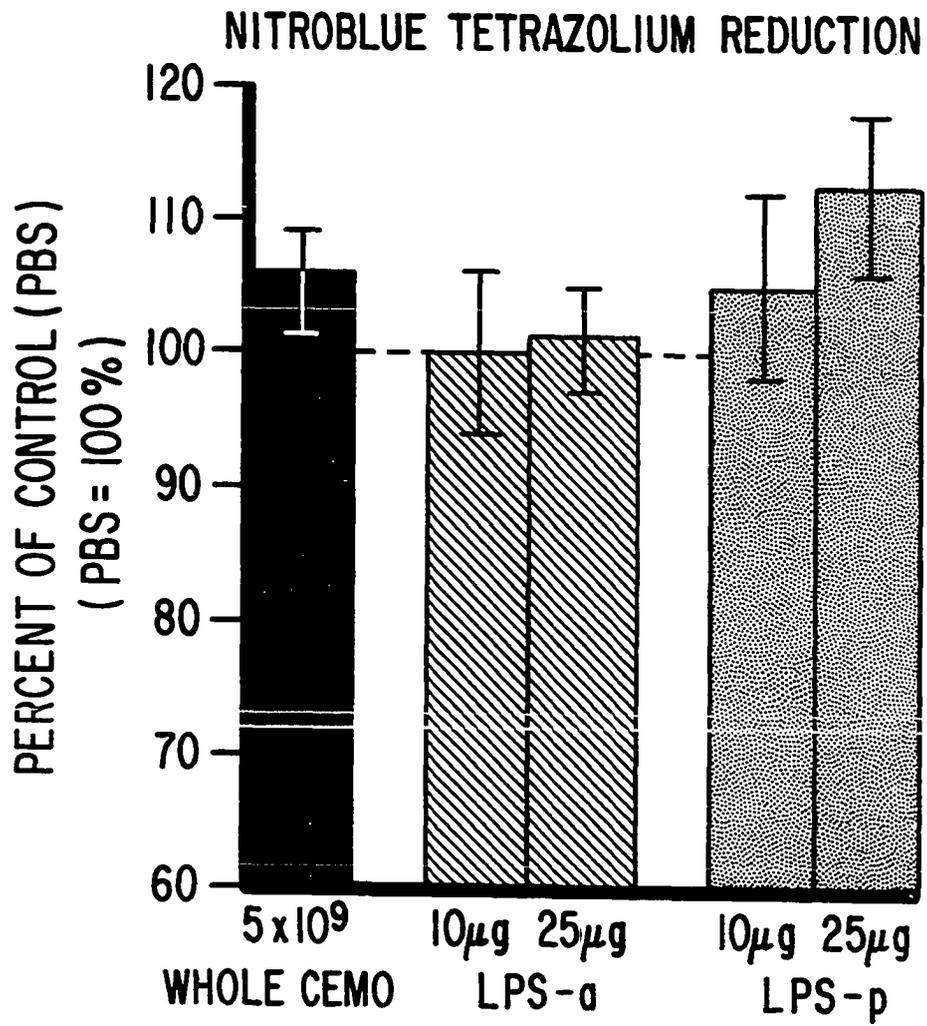


Table 2. Morphometry of neutrophils containing Zymosan^a

Test material ^b	Neutrophil				Zymosan	
	Total cell area (μm^2)	Nucleus area (μm^2)	Granule number	Granules/cytoplasm area (number/ μm^2)	Zymosan phagolysosome area (μm^2)	Zymosan area/cytoplasm area
PBS	41.1 ± 1.6	2.9 ± 0.3	66.4 ± 4.5	2.08	6.3 ± 0.5	0.16
Whole CEMO	42.9 ± 1.9	3.6 ± 0.4	80.2 ± 8.8	2.46	6.8 ± 0.4	0.17
LPS-a (25 μg)	41.0 ± 1.9	2.7 ± 0.3	55.7 ± 11.3	1.69	5.4 ± 0.4	0.14
LPS-p (25 μg)	37.7 ± 2.0	2.8 ± 0.3	53.5 ± 1.05	1.83	5.8 ± 0.5	0.16

^a values are means +/- S.E.M.

^b PBS = phosphate buffered saline.

CEMO = contagious equine metritis organism.

LPS-a = aqueous soluble lipopolysaccharide.

LPS-p = phenol soluble lipopolysaccharide.

Microfilaments were seen near the membrane of zymosan-phagosomes in neutrophils of each treatment group. Microfilaments were focally distributed about the phagosomes and occasionally formed an electron dense layer which encircled the phagosome. Microfilaments were also located near the plasma membrane and frequently observed in cellular projections and pseudopodia.

Multivesicular bodies were seen in approximately 50% of the PBS treated neutrophils (Fig. 6), usually in the peripheral cytoplasm near the plasma membrane. They were occasionally fused with the cell membrane and some were opened to the extracellular environment. Multivesicular bodies had either electron lucent or electron dense matrices; those with electron lucent matrices were most frequent.

A granular flocculated cytoplasm was seen in approximately 50% of the neutrophils in the two LPS treated groups. Thirty-three percent of the neutrophils in the presence of whole CEMO and 10% of the neutrophils in PBS had this cytoplasmic change.

Neutrophils in each treatment group contained both primary and secondary granules and segmented nuclei. The nucleus composed 7 to 12% of the total cell cytoplasm. The plasma membranes were undulant and had multiple pseudopodia (Fig. 7). Golgi lamellae were flattened, elongated, and centrally located. Coated vesicles were occasionally seen juxtaposed to the Golgi lamellae. Lipid droplets were infrequently seen but when present were near phagosomes.

Fig. 6. Multivesicular body (M) in a neutrophil exposed to CEMO lipopolysaccharide

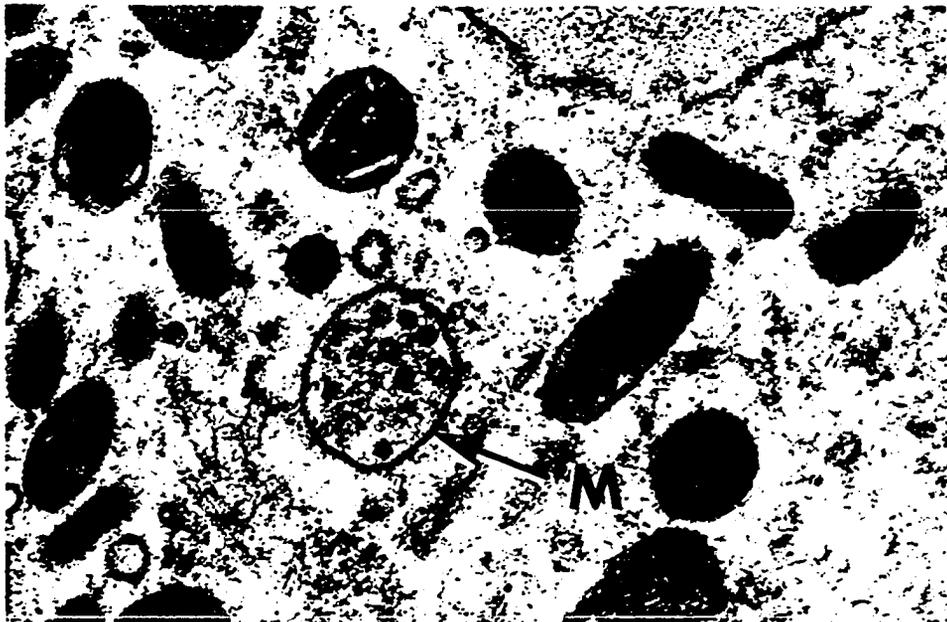
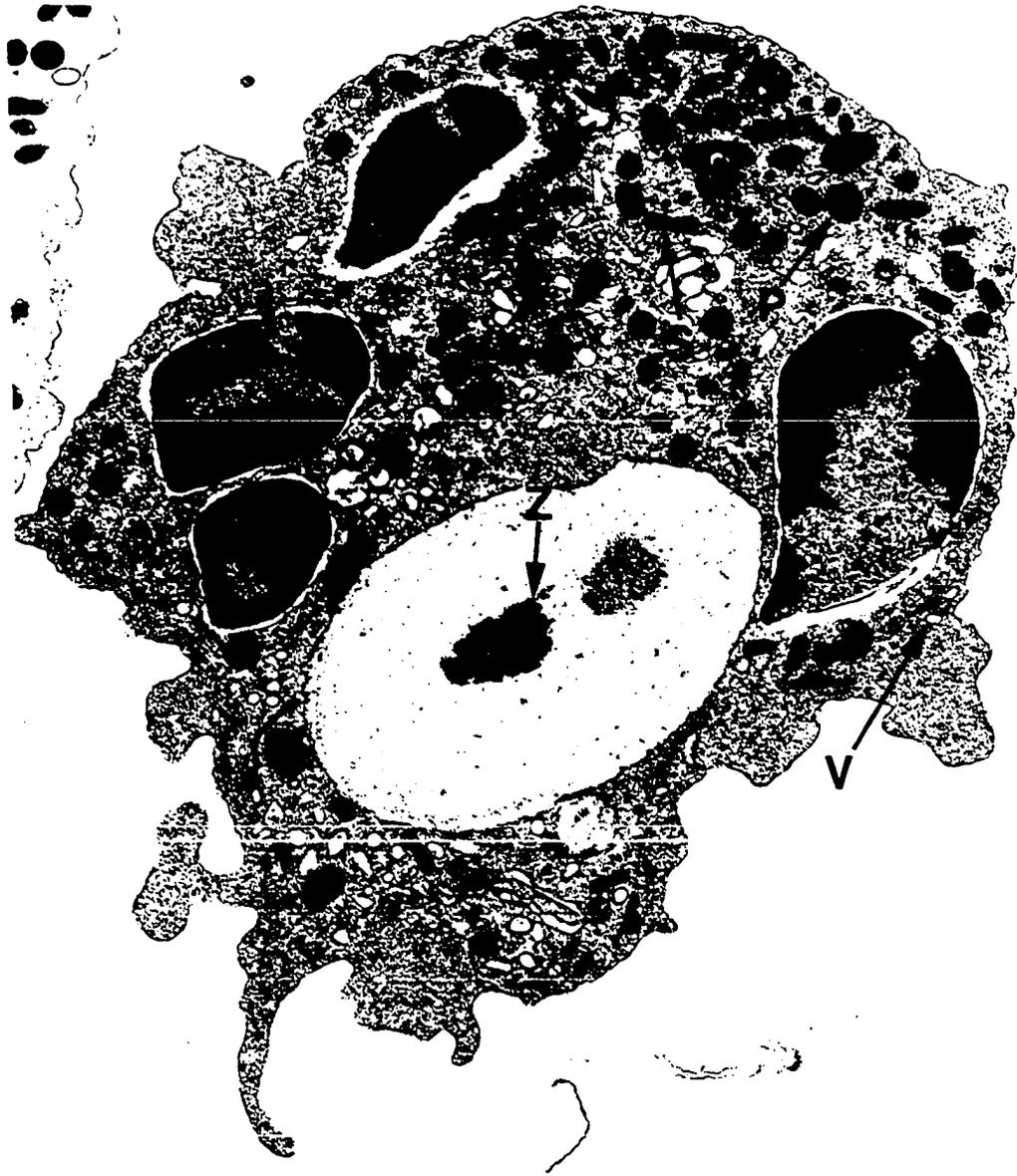


Fig. 7. Equine neutrophils. Abbreviations: (Z) opsonized zymosan within phagolysosomes, (P) primary and (S) secondary granules are randomly distributed throughout the cytoplasm, and (V) vacuoles near phagolysosomes



DISCUSSION

Contagious Equine Metritis Organism (CEMO) lipopolysaccharides increase degranulation and may alter oxidative metabolism and myeloperoxidase activity of equine neutrophils. The iodination reaction is dependent upon ingestion, oxidative metabolism, degranulation, and myeloperoxidase activity of neutrophil primary granules (58). An increase in the iodination reaction would require an increase in any or all of these steps. LPS-p may increase iodination by increasing neutrophil ingestion, oxidative metabolism, and degranulation. This is supported by the effect of LPS-p on S. aureus ingestion, NBT reduction and neutrophil granule number. LPS-a probably caused the increase in iodination by increasing degranulation since S. aureus ingestion was actually inhibited and NBT reduction was not significantly altered. Since LPS-a increased iodination more than LPS-p and both caused neutrophils to degranulate, it is possible that LPS-a also increases myeloperoxidase activity. LPS of other bacteria have been shown to increase the activity of some lysosomal enzymes (71, 72, 140) as well as increase neutrophil degranulation (141).

CEMO is killed rapidly once ingested by equine neutrophils (7). Myeloperoxidase is important in a bacteriocidal mechanism used by the neutrophil (58), however, some pathogenic bacteria are able to modify this mechanism and thus modify the host's defense capabilities (22). Since CEMO components (LPS) increase neutrophil degranulation, this may

provide a partial explanation for the rapid intracellular killing of CEMO by equine neutrophils.

Whole CEMO and its lipopolysaccharides inhibit random migration of equine neutrophils. We did not establish if this inhibition was a result of a cytotoxic or noncytotoxic mechanisms. Endotoxins stimulate release of migration inhibiting factors from neutrophils (37, 131) with some factors working by noncytotoxic mechanisms (37). Since some neutrophil functions were increased after exposure to CEMO or its lipopolysaccharides, it is likely these CEMO components or any migration inhibiting factors they made induce work by noncytotoxic mechanisms.

The chemoattractant effect of whole CEMO may be a result of its endotoxins. Whole CEMO attracted neutrophils the maximal distance detectable by our system. LPS-p also had strong chemoattractant effects but no significant chemoattraction could be detected with LPS-a. Endotoxins have been demonstrated to interact with complement to generate chemoattractant agents (114, 115) and endotoxin alone has been demonstrated to attract mouse macrophages (134). Our system had heat inactivated equine serum in the agarose so it is possible CEMO endotoxin is chemoattractive without complement or it uses a heat stable component of serum to form a neutrophil chemoattractant.

LPS-a and LPS-p may or may not be related lipopolysaccharides of the CEMO cell wall. Antisera to the whole CEMO cross reacted only with LPS-a and not LPS-p. LPS-p may be deeper in the outer cell membrane, or not be a dominant antigenic determinant when CEMO is intact. Further, LPS-p seems to be more hydrophobic than LPS-a since it was extracted in

the phenol phase. In the synthesis of LPS in Enterobacteriaceae, polysaccharides are added to lipid A before being transferred to the outer cell membrane (19). If a similar synthetic route occurs in CEMO, LPS-p may be similar to LPS-a but deeper in the cell wall and thus have less polysaccharide content. Finally, LPS-p may have been generated as a result of the chemical extraction procedure and is actually a fragment of LPS-a. This is suggested by the structural similarity of the spheres in the two preparations and the serologic reactivity of these two lipopolysaccharides. Previous reports using different extraction methods have demonstrated distinct biological activities for LPS from the same organism (33, 71, 73, 89). Although both lipopolysaccharides were derived by aqueous phenol extraction, functionally, they act differently by inducing different neutrophil responses in certain neutrophil function tests.

The inhibition of phagocytosis seen with CEMO may be a result of a cell wall LPS. This is supported by the inhibition of S. aureus ingestion by LPS-a. Previous reports have demonstrated CEMO is slow to be phagocytized (7). It was speculated this was a local effect and did not alter the neutrophils ability to phagocytize other bacteria. Our S. aureus ingestion results provide direct support of this speculation and further suggest this inhibition may be a result of a cell wall LPS.

Degenerative changes seen in the cytoplasm of LPS treated neutrophils may be a result of release of secondary granule enzymes into the cell. LPS has been demonstrated to induce lysosomal enzyme release from neutrophils (141). The same mechanisms involved in extracellular enzyme

release may also cause intracellular release thus leading to cell degeneration. LPS induced cell degeneration is also suggested by the increased presence of multivesicular bodies in the LPS-treated neutrophils. The exact origin and function of multivesicular bodies is not well established but they may be involved in autophagocytic processes. Multivesicular bodies have also been demonstrated to contain acid phosphatases and are thus a unique form of lysosome (34, 79). Lipopolysaccharides have been demonstrated to increase certain cellular enzymes (13) and this LPS induced enzyme increase may occur in equine neutrophils by increasing multivesicular bodies.

GENERAL DISCUSSION AND SUMMARY

For in vitro neutrophil function to be evaluated with precision and accuracy, there is a need for the cell to be intact functionally. Results of the first study suggest in vitro isolation procedures of horse neutrophils should be conducted at 4° C to achieve an intact cell. Neutrophils isolated at 4° C had total cell granule numbers found in control neutrophils. Microtubules are involved with neutrophil degranulation (46), and it is possible to control microtubule polymerization/depolymerization using different temperatures (81). Thus, isolation of equine neutrophils should be accomplished at 4° C if neutrophil functions are to be adequately evaluated.

CEMO is slow to be phagocytized by equine neutrophils but once ingested is rapidly killed. This suggests CEMO is an extracellular pathogen. Further, the slow uptake may represent a virulence factor used to avoid certain host defenses. Several possible mechanisms may explain how CEMO inhibits phagocytosis. The data suggests the mechanism involves local events at the site of bacterium-neutrophil contact. This is supported by the observations that neutrophils containing CEMO are able to phagocytize other CEMO as well as Staphylococcus aureus. CEMO may be utilizing its extracellular capsule (122) to slow phagocytosis by the neutrophil. Capsules of other bacteria have been demonstrated to have a similar role (22).

Antibody and complement opsonize CEMO, thus improving uptake by equine neutrophils. Antibody and complement have been demonstrated to

improve phagocytosis in vitro while the in vivo role of antibody alone is not clearly established (47). These studies suggest CEMO uptake is enhanced to a greater degree by antibodies from serum (primarily IgG) than by antibodies from genital secretions (primarily IgA). These observations may provide partial explanation for CEMO infection being limited to the surface of the genital tract.

The effects of CEMO on equine neutrophils may be partially a result of CEMO lipopolysaccharides (LPS). The importance of LPS from Gram negative bacteria has been demonstrated in various diseases (13, 73). The biochemical effect of LPS on various mammalian cells and tissues has also been established (13). CEMO LPS affects equine neutrophil degranulation, random migration, chemotaxis, and oxidative metabolism. In the natural disease, there is an extensive neutrophil reaction with minimal tissue damage (87). The results suggest that some of this inflammatory response may be a result of CEMO lipopolysaccharides.

The studies in this dissertation have allowed for a better understanding of the neutrophil-bacteria interaction in contagious equine metritis. Since the predominant amount of work was conducted in vitro, important questions remain to be evaluated. First and foremost, can these results be demonstrated to apply to an in vivo situation? Would mucosal immunization be the best route of vaccination to prevent clinical disease? Would subunit vaccines (capsular components) provide better protection than bacterins? Would nonspecific neutrophil function modulators (hyaluronic acid) be a better therapeutic approach in treating this disease than using antibiotics or vaccines?

BIBLIOGRAPHY

1. Acland, H. M., and R. M. Kenney. 1982. Progression of lesions and recovery of organisms in contagious equine metritis in mares. Paper presented to the American College of Veterinary Pathologists, Atlanta, Georgia. Available from the Secretary-Treasurer of the American College of Veterinary Pathologists.
2. Arp, L. H., and N. F. Cheville. 1981. Interaction of blood-borne Escherichia coli with phagocytes of spleen and liver in turkeys. *Am. J. Vet. Res.* 42:650-657.
3. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. I. Histochemical staining of bone marrow smears. *J. Cell Biol.* 39:286-298.
4. Bainton, D. F., J. L. Ulliyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J. Exp. Med.* 134:907-934.
5. Beinestock, J., and A. D. Befus. 1980. *Mucosal Immunology.* *Immunology* 41:249-270.
6. Bertram, T. A., and F. L. Coignoul. 1982. Morphometry of equine neutrophils separated at different temperatures. *Vet. Pathol.* 19:534-543.
7. Bertram, T. A., F. L. Coignoul, and A. E. Jensen. 1982. Phagocytosis and intracellular killing of the contagious equine metritis organism by equine neutrophils in serum. *Infect. Immun.* 37:1241-1247.

8. Biberstein, E. L., and K. Zinnemann. 1982. International Committee on Systematic Bacteriology. Subcommittee on the taxonomy of *Hemophilus*. *Int. J. Syst. Bacteriol.* 32:244-245.
9. Björkstén, B. 1979. The effect of human colostrum on neutrophil function. *Pediat. Res.* 3:737-741.
10. Bourne, J., and T. Newby. 1981. Mucosal immunity. *Inpractice* 3:5-11.
11. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21:77-89.
12. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
13. Bradley, S. G. 1979. Cellular and molecular mechanisms of action of bacterial endotoxins. *Ann. Rev. Microbiol.* 33:67-94.
14. Cheville, N. F. 1976. *Cell Pathology*. The Iowa State University Press, Ames, Iowa.
15. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leukocytes. I. Observations on requirements and consequences of particle ingestion. *J. Exp. Med.* 111:667-679.
16. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leukocytes. II. The influence of lipopolysaccharide endotoxin. *J. Exp. Med.* 111:689-704.
17. Corbel, M. J., and R. A. Brewer. 1980. Antibodies to *Haemophilus equigenitalis* in bovine sera. *Vet. Rec.* 106:35.

18. Crowhurst, R. C. 1977. Genital infections in mares. *Vet. Rec.* 100:476.
19. Davis, B. D., and W. B. Wood. 1980. Host-parasite relations in bacterial infections. Page 559 in B. D. Davis, R. Dulbecco, H. N. Eisen, H. S. Ginsburg, eds. *Microbiology*. 3rd edition. Harper and Row Publishers, Hagerstown.
20. Dawson, F. L., J. A. Benson, and P. Croxton-Smith. 1978. The course of serum antibody development in two ponies experimentally infected with contagious equine metritis. *Equine Vet. J.* 10: 145-147.
21. Delesse, M. A. 1847. Procédé mécanique pour déterminer la composition des roches. *C. R. Acad. Sci. (Paris)* 25:545-551.
22. Densen, P., and G. L. Mandell. 1980. Phagocytes strategy vs. microbial tactics. *Rev. Infect. Dis.* 2:817-838.
23. Donahue, J. M., T. W. Swerzek, and B. J. Smith. 1978. Laboratory diagnosis of contagious equine metritis. *Am. Assoc. Vet. Lab. Diag.* 21:497-506.
24. Duncan, J. R., and K. W. Prasse. 1977. *Veterinary Laboratory Medicine*. The Iowa State University Press, Ames, Iowa.
25. Dutta, S. K., M. K. Bumgardner, J. C. Scott and A. C. Myrup. 1981. Separation and identification of equine leukocyte populations and subpopulations. *Am. J. Vet. Res.* 42:1037-1039.
26. Eaglesome, M. D., and M. M. Garcia. 1979. Contagious equine metritis: A review. *Can. Vet. J.* 20:201-206.

27. Edwards, P. R., and W. H. Ewing. 1962. Identification of enterobacteriaceae. Burgess Publishing Company, Minneapolis, Minnesota.
28. Fales, W. H., B. O. Blackburn, R. S. Youngquist, W. F. Braun, L. K. Schlater, and L. G. Morehouse. 1979. Laboratory methodology for the diagnosis of contagious equine metritis in Missouri. *Am. Assoc. Vet. Lab. Diag.* 22:187-198.
29. Fantone, J. C., and P. A. Ward. 1982. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am. J. Pathol.* 107:397-418.
30. Fearon, D. T. 1980. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte and monocyte. *J. Exp. Med.* 152:20-30.
31. Fernie, D. S., I. Batty, P. D. Walker, H. Platt, M. E. Mackintosh, and D. J. Simpson. 1980. Observations on vaccine and post-infection immunity in contagious equine metritis. *Res. Vet. Sci.* 28:362-367.
32. Ferrante, A., and Y. H. Thong. 1980. Simultaneous preparation of mononuclear and polymorphonuclear leukocytes from horse blood on Ficoll-Hypaque medium. *J. Immunol. Methods* 34:279-285.
33. Fong, J. S. C., and R. A. Good. 1971. Prevention of the localized and generalized Schwartzman reaction by anticomplementary agent, cobra venom factor. *J. Exp. Med.* 134:642-655.

34. Friend, D. S. 1969. Cytochemical staining of multivesicular body and Golgi vesicles. *J. Cell Biol.* 41:269-279.
35. Fukushi, Y. 1981. The role of intravesicular polymorphonuclear leukocytes in experimental cystitis. *Invest. Urol.* 18:471-474.
36. Galanos, C., O. Luderitz, and O. Westphal. 1969. A new method for the extraction of R-lipopolysaccharide. *Eur. J. Biochem.* 98:245-249.
37. Geotzl, E. J., and K. F. Austen. 1972. A neutrophil-immobilizing factor derived from human leukocytes. I. Generation and partial characterization. *J. Exp. Med.* 136:1564-1580.
38. Glasser, L. 1977. Effect of storage on normal neutrophils collected by discontinuous-flow centrifugation leukapheresis. *Blood* 50:1145-1150.
39. Goren, M. B. 1977. Phagocyte lysosomes: Interaction with infectious agents, phagosomes, and experimental perturbations in function. *Annu. Rev. Microbiol.* 31:507-533.
40. Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291-302.
41. Griffin, F. M., J. A. Griffin, and S. C. Silverstein. 1976. Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. *J. Exp. Med.* 144:788-809.

42. Griffin, F. M., and S. C. Silverstein. 1974. Segmental response of the macrophage plasma membrane to a phagocytic stimulus. *J. Exp. Med.* 139:323-336.
43. Guidry, A. S., M. J. Paape, and R. E. Pearson. 1977. The effect of local immunization of mammary gland on phagocytosis and intracellular killing of Staphylococcus aureus. *J. Dairy Sci.* 60:135.
44. Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. 1972. Fowl cholera: Gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. *Avian Dis.* 16:925-936.
45. Henning, A. 1956. Bestimmung der Oberfläche beliebig geformter Körper mit besonderer Anwendung auf Körperhaufen im mikroskopischen Bereich. *Mikroskopie* 11:1-10.
46. Hoffstein, S., I. M. Goldstein, and G. Weissman. 1977. Role of microtubule assembly in lysosomal enzyme secretion from human polymorphonuclear leukocytes. *J. Cell Biol.* 73:242-256.
47. Horwitz, M. A. 1982. Phagocytosis of microorganisms. *Rev. Infect. Dis.* 4:104-123.
48. Hughes, K. L., J. D. Bryden, and F. McDonald. 1978. Equine contagious metritis. *Aust. Vet. J.* 54:101.
49. Issekutz, A. C., and W. D. Biggar. 1977. Influence of serum derived chemotactic factors and bacterial products on human neutrophil chemotaxis. *Infect. Immun.* 15:212-220.
50. Jain, N. C. 1970. A comparative cytochemical study of leukocytes of some animal species. *Folia Haemat.* 94:49-63.

51. Jain, N. C. 1968. Alkaline phosphatase activity in leukocytes of some animal species. *Acta Haemat.* 39:51-59.
52. Jones, T. C., and J. G. Hirsch. 1971. The interaction in vitro of Mycoplasma pulmonis with mouse peritoneal macrophages and L cells. *J. Exp. Med.* 133:231-259.
53. Karkhauis, Y. D., J. Y. Zeltner, J. J. Jackson, and D. J. Carlo. 1978. A new improved microassay to determine 2-keto-3-deoxyoctanate in lipopolysaccharide of Gram negative bacteria. *Anal. Biochem.* 85:595-601.
54. Khaleel, S. A., P. Z. Allen, and R. M. Kenny. 1978. Immunological observations on stallion sperm and seminal plasma including differential localization of immunoglobulins on the sperm head. Pages 287-292 in *Equine Infectious Diseases IV. The Journal of Equine Medicine and Surgery Suppl. I. Veterinary Publications, Inc.*
55. Kimberg, D. V., A. V. Loud, and J. Wiener. 1968. Cortisone-induced alterations in mitochondrial function and structure. *J. Cell Biol.* 37:63-79.
56. Kistler, G. S., R. B. Caldwell, and E. R. Weibel. 1967. Development of fine structural damage to alveolar and capillary lining cells in oxygen-poisoned rat lungs. *J. Cell Biol.* 32:605-628.
57. Klebanoff, S., and R. A. Clarke. 1978. *The Neutrophil.* 1st ed. Elsevier, Amsterdam.

58. Klebanoff, S. J., and R. A. Clark. 1977. Iodination by human polymorphonuclear leukocytes: A re-evaluation. *J. Lab. Clin. Med.* 89:675-686.
59. Knowles, R. C. 1978. Epidemiologic and regulatory aspects of contagious equine metritis (CEM). *Proc. Annu. Conv. Am. Assoc. Equine Pract.* 24:287-290.
60. Kolmer, J. A. 1939. Preserved citrated blood "banks" in relation to transfusion in the treatment of disease with special reference to the immunologic aspect. *Am. J. Med. Sci.* 197:442-452.
61. Lane, T. A. 1978. Granulocyte concentrate preservation: 6°C versus room temperature. *Transfusion* 18:394-400.
62. Latimer, K. S., and K. W. Prasse. 1982. Neutrophilic movement of a Basenji with Pelger-Huët anomaly. *Am. J. Vet. Res.* 3:525-527.
63. Laxdal, T., R. P. Messner, R. C. Williams, and P. G. Quie. 1968. Opsonic agglutinating, complement fixing antibodies in patients with subacute bacterial endocarditis. *J. Lab. Clin.* 71:638-653.
64. Lignicres, R. 1980. Observations sur l'apparition de quelques foyers de métrite contagieuse équine en France. *Bull. Acad. Vét. Fr.* 53:323-332.
65. Lukehart, S. A., and J. N. Miller. 1978. Demonstration of the in vitro phagocytosis of Treponema pallidum by rabbit peritoneal macrophages. *J. Immunol.* 121:2014-2024.
66. Matula, G., and P. Y. Paterson. 1971. Spontaneous in vitro reduction of nitroblue tetrazolium by neutrophils of adult patients with bacterial infection. *N. Engl. J. Med.* 285:311-317.

67. Mayberry, W. R. 1980. Hydroxy fatty acids in *Bacteriodes* species: D-(-)-3 hydroxy-15-methylhexadecanoate and its homologs. *J. Bacteriol.* 143:582-587.
68. McCall, C. E., I. Katayama, R. S. Cotran, and M. Finland. 1969. Lysosomal and ultrastructural changes in human "toxic" neutrophils during bacterial infection. *J. Exp. Med.* 129:267-293.
69. McCullough, J. 1980. Liquid preservation of granulocytes. *Transfusion* 20:129-137.
70. McCullough, J., S. J. Benson, E. J. Yunis, and P. G. Quie. 1969. Effect of blood bank storage on leucocyte function. *Lancet* 2: 1333-1337.
71. McGivney, A., and S. G. Bradley. 1978. Effects of bacterial endotoxins in lysosomal enzyme activities of human cells in continuous culture. *J. Reticuloendothel. Soc.* 23:223-230.
72. McGivney, A., and S. G. Bradley. 1977. Enhanced lysosomal enzyme activity in mouse cells treated with bacterial endotoxin in vitro. *Soc. Exp. Biol. Med.* 155:390-394.
73. Morrison, D. C., and R. J. Ulevitch. 1978. The effects of bacterial endotoxins on host mediation systems. *Am. J. Pathol.* 93:526-617.
74. Mumme, J., and R. Ahlswede. 1978. Nachweis von *Haemophilus equigenitalis* im Zervixtupfer einer Warmblutstute. *Dtsch. Tieraerztl. Wochenschr.* 86:257-259.
75. Murphy, P. 1976. *The Neutrophil*. Plenum Publishing Corporation, New York.

76. Muschel, L. H. 1960. Serum bacteriocidal action. Ann. N.Y. Acad. Sci. 88:1265-1272.
77. Nelson, R. D., R. L. Simmons, and P. G. Quie. 1976. Chemotaxis under agarose: A method for measurement of spontaneous migration and chemotaxis of polymorphonuclear leukocytes and monocytes from human peripheral blood. Pages 663-675 in In vitro methods in cell mediated and tumor immunity. Academic Press Publications, New York.
78. Nessi, P., and J. Frei. 1977. Leucocyte energy metabolism. Enzyme 22:176-182.
79. Novikoff, A. B., E. Essner, and N. Quintana. 1964. Golgi apparatus and lysosomes. Fedn. Proc. 23:1010-1022.
80. Oliver, J. M. 1978. Cell biology of leukocyte abnormalities: Membrane and cytoskeletal function in normal and defective cells. Am. J. Pathol. 93:220-259.
81. Olmstead, J. B., and G. G. Borisy. 1973. Microtubules. Annu. Rev. Biochem. 42:507-540.
82. Paape, M. J., R. E. Pearson, and W. D. Schultze. 1978. Variation among cows in the ability of milk to support phagocytosis and in the ability of polymorphonuclear leukocytes to phagocytize Staphylococcus aureus. Am. J. Vet. Res. 39:1907-1910.
83. Park, B. H., and R. A. Good. 1970. NBT test stimulated. Lancet 2:616.

84. Pearson, R. E., S. P. Sahu, A. H. Dardiri, and F. W. Wilder. 1978. Contagious equine metritis: Clinical description of experimentally induced infection. *J. Am. Vet. Med. Assoc.* 173: 402-404.
85. Pfaller, W., W. M. Fischer, N. Strieder, H. Wurnig, and P. Deetjen. 1975. Morphologic changes of cortical nephron cells in potassium-adapted rats. *Lab. Invest.* 31:678-684.
86. Pitre, J., M. F. Legendre, and G. Voisin. 1979. Informations et réflexions sur le diagnostic microbiologique et serologique de la métrite coccobacillaire des équidés. *Prat. Vét. Equine* 11:11-26.
87. Platt, H., J. G. Atherton, and D. J. Simpson. 1978. The experimental infection of ponies with contagious equine metritis. *Equine Vet. J.* 10:153-159.
88. Platt, H., J. G. Atherton, D. J. Simpson, C. E. D. Taylor, R. O. Rosenthal, D. F. J. Brown, and T. G. Wreghitt. 1977. Genital infection in mares. *Vet. Rec.* 101:20.
89. Polak, L., and J. L. Turk. 1969. Suppression of the hemorrhagic component of the Schwartzman reaction by anticomplement serum. *Nature* 223:738-739.
90. Powell, D. G. 1981. Contagious equine metritis. Pages 161-182 in C. E. Cornelius and C. F. Simpson, eds. *Advances in veterinary science and comparative medicine.* Academic Press, Inc., New York.

91. Powell, D. G. 1977. Epidemiological aspects of contagious equine metritis. Paper presented to special scientific meeting on contagious equine metritis, Cambridge, England. Available from British Equine Veterinary Association, Cambridge, England.
92. Raff, R. A., and R. W. Wheat. 1968. Carbohydrate composition of the phenol-soluble lipopolysaccharide of Citrobacter freundii. J. Bacteriol. 95:2035-2043.
93. Reed, W. P. 1975. Serum factors capable of opsonizing shigella for phagocytosis by polymorphonuclear neutrophils. Immunology 28:1051-1059.
94. Reid, I. M. 1980. Morphometric methods in veterinary pathology: A review. Vet. Pathol. 17:522-543.
95. Ricketts, S. W., and P. D. Rossdale. 1979. Endometrial biopsy findings in mares with contagious equine metritis. J. Reprod. Fertil., Suppl. 27:355-359.
96. Ricketts, S. W., P. D. Rossdale, and C. A. Samuel. 1978. Endometrial biopsy studies of mares with contagious equine metritis 1977. Equine Vet. J. 10:160-166.
97. Ricketts, S. W., P. D. Rossdale, N. J. Wingfield-Digby, M. M. Falk, R. Hopes, M. D. N. Hunt, and C. K. Peace. 1977. Genital infection in mares. Vet. Rec. 101:65.
98. Roantree, R. J., and L. A. Rantz. 1960. A study of the relationship of the normal bacteriocidal activity of human serum to bacterial infection. J. Clin. Invest. 39:72-81.

99. Ross, G. D., C. L. Jarowski, E. M. Rabellino, and R. J. Winchester. 1978. The sequential appearance of Ia-like antigens and two different complement receptors during the maturation of human neutrophils. *J. Exp. Med.* 147:730-744.
100. Roth, J. A., and M. Kaberle. 1981. Evaluation of bovine polymorphonuclear leukocyte function. *Immunol. Immunopathol.* 2: 157-174.
101. Rowley, D. 1971. Endotoxins and bacterial virulence. *J. Infect. Dis.* 123:317-327.
102. Rowley, D. 1954. The virulence of strains of Bacterium coli for mice. *Br. J. Exp. Pathol.* 35:528-538.
103. Sahu, S. P. 1981. Contagious equine metritis: Effect of vaccination on control of the disease. *Am. J. Vet. Res.* 42:45-48.
104. Sahu, S. P., and A. H. Dardiri. 1980. Contagious equine metritis: Isolation and characterization of the etiologic agent. *Am. J. Vet. Res.* 41:1379-1382.
105. Sahu, S. P., R. E. Pierson, and A. H. Dardiri. 1980. Contagious equine metritis: Effect of intrauterine inoculation of contagious equine metritis agent in pony mares. *Am. J. Vet. Res.* 41:5-9.
106. Schalm, O. W., N. C. Jain, and E. J. Carroll. 1975. *Veterinary hematology.* 3rd ed. Lea and Febiger, Philadelphia.

107. Schliwa, M., K. B. Pryzwansky, and U. Euteneuer. 1982. Centrosome splitting in neutrophils: An unusual phenomenon related to cell activation and motility. *Cell* 31:705-717.
108. Schrek, R. 1943. Studies in vitro on the physiology of normal and cancerous cells. *Arch. Pathol.* 35:857-872.
109. Scully, C. M. 1980. Comparative opsonic activity of Streptococcus mutans in oral fluids, and phagocytic activity of blood, crevicular, and salivary polymorphonuclear leukocytes in rhesus monkeys. *Immunology* 39:101-107.
110. Senff, L. M., W. D. Dawyer, and R. A. Hack. 1977. Effect of gonococci on nitroxide spin-labeled membranes of human blood cells (abstract # D48). Page 77 in Abstracts of Annual meetings of the American Society for Microbiology. American Society of Microbiology, Washington, D.C.
111. Shands, J. W., J. A. Graham, and K. Nath. 1967. The morphologic structure of isolated bacterial lipopolysaccharides. *J. Mol. Biol.* 25:15-21.
112. Shreeve, J. E. 1978. Preliminary observations on x-factor growth requirement of the bacterium responsible for CEM. *Vet. Rec.* 102:20.
113. Silverstein, S. C. 1977. Endocytotic uptake of particles by mononuclear phagocytes and the penetration of obligate intracellular parasites. *Am. J. Trop. Med. Hyg.* 26 (Suppl.): 161-169.

114. Snyderman, R., H. Gewurz, S. E. Mergen Hagen. 1968. Interaction of the complement system with endotoxic lipopolysaccharides. Generation of a factor chemotactic for polymorphonuclear leukocytes. *J. Exp. Med.* 128:259-275.
115. Snyderman, R., H. S. Shin, J. K. Phillips, H. Gewurz, and S. E. Mergenhagen. 1969. A neutrophil chemotactic factor derived from C'5 upon interaction of guinea pig serum with endotoxin. *J. Immunol.* 103:413-422.
116. Stjernhalm, R. L., and R. C. Manek. 1970. Carbohydrate metabolism in leukocytes. XIV. Regulation of pentose cycle activity and glycogen metabolism during phagocytosis. *J. Reticuloendothel. Soc.* 8:550-571.
117. Stossel, T. P. 1974. Phagocytosis (Second of Three parts). *New Eng. J. Med.* 290:774-780.
118. Stossel, T. P., and J. H. Hartwig. 1976. Interactions of actin, myosin, and a new actin binding protein of rabbit pulmonary macrophages. II. Role in cytoplasmic movement and phagocytosis. *J. Cell Biol.* 68:602-619.
119. Stossel, T. P., and J. H. Hartwig. 1975. Interaction between actin, myosin, and an actin-binding protein from rabbit alveolar macrophages. Alveolar macrophage myosin magnesium-adenosine triphosphatase requires a cofactor for activation by actin. *J. Biol. Chem.* 25:5706-5712.

120. Sugimoto, C., Y. Isayama, M. Kashiwazaki, T. Fujikura, and K. Mitani. 1980. Detection of Haemophilus equigenitalis, the causal agent of contagious equine metritis in Japan. Natl. Inst. Anim. Health Q. (Jpn.) 20:118-119.
121. Sugimoto, C., E. Miyagawa, E. Mitani, M. Nakazawa, and Y. Isayama. 1982. Cellular fatty acid composition of Haemophilus equigenitalis. J. Clin. Microbiol. 15:791-794.
122. Swaney, L. M., and S. S. Breese. 1980. Ultrastructure of Haemophilus equigenitalis, causative agent of contagious equine metritis. Am. J. Vet. Res. 41:127-132.
123. Swerczek, T. W. 1978. The first occurrence of contagious equine metritis in the United States. J. Am. Vet. Med. Assoc. 173: 405-407.
124. Tainturier, D. J., C. F. Delmas, and H. J. Dabernat. 1981. Bacteriological and serological studies of Haemophilus equigenitalis, agent of contagious equine metritis. J. Clin. Microbiol. 14:355-360.
125. Targowski, S. P. 1976. Separation of mononuclear leukocytes and polymorphonuclear leukocytes from equine blood. Can. J. Comp. Med. 40:285-290.
126. Taylor, C. E. D. 1979. A recently recognized venereal disease of horses and its causative organism. J. Infect. 1:81-86.

127. Taylor, C. E., R. O. Rosenthal, D. F. Brown, S. P. Lapage, L. R. Hill, and R. M. Legros. 1978. The causative organism of contagious equine metritis: Proposal for a new species to be known as Haemophilus equigenitalis. Equine Vet. J. 10:136-144.
128. Timoney, P. J., J. F. McArdle, P. J. O'Reilly, and J. Ward. 1978. Infection patterns in pony mares challenged with the agent of contagious equine metritis 1977. Equine Vet. J. 10:148-152.
129. Timoney, P. J., and D. G. Powell. 1982. Isolation of the contagious equine metritis organism from colts and fillies in the United Kingdom and Ireland. Vet. Rec. 20:478-482.
130. Timoney, P. J., J. Ward, and P. Kelly. 1977. A contagious genital infection of mares. Vet. Rec. 101:103.
131. Udaka, K., and K. Udaka. 1972. A migration inhibiting substance for PMN-leukocytes and its role in inflammation. Fed. Proc. 31: 657 (Abstr).
132. Vaerman, J. P., and J. Ferin. 1974. Local immunological response in the vagina, cervix, and endometrium. Acta Endocr. 78(S194):281-305.
133. VanFurth, R., T. L. Van Zwet, and P. C. Leijh. 1978. In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. Pages 32.1-32.19 in D. M. Wier, ed. Handbook of Experimental Immunology. Vol. 2. Blackwell Scientific Publications, Oxford, England.

134. Verghese, M. W., and R. Snyderman. 1982. Endotoxin (LPS) stimulates in vitro migration of macrophages from LPS-resistant mice but not from LPS-sensitive mice. *J. Immunol.* 128:608-612.
135. Vosti, K. L., and E. Randall. 1970. Sensitivity of serologically classified strains of Escherichia coli of human origin to the serum bacteriocidal system. *Am. J. Med. Sci.* 259:114-119.
136. Warburg, O., and W. Christian. 1942. Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* 310:384-421.
137. Ward, H. K., and J. F. Enders. 1933. An analysis of the opsonic and tropic action of normal and immune sera based on experiments with the pneumococcus. *J. Exp. Med.* 57:527-547.
138. Watanabe, I., S. Donahue, and N. Hoggatt. 1967. Method for electron microscopic studies of circulating human leukocytes and observations on their fine structure. *J. Ultrastruct. Res.* 20:366-382.
139. Weibel, E. R. 1969. Stereological principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* 26:235-302.
140. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Pages 83-91 in R. L. Whistler, J. BeMiller, and M. L. Wolfram, eds. *Methods of carbohydrate biochemistry*. Vol. IV. Academic Press, New York.
141. White, R. R., and E. H. Montgomery. 1977. Exocytosis of polymorphonuclear leukocyte lysosomal contents induced by dental plaque. *Infect. Immun.* 16:934-937.

142. Wiener, J., A. V. Loud, D. V. Kimberg, and D. Spiro. 1968. A quantitative description of cortisone-induced alterations in the ultrastructure of rat liver parenchymal cells. *J. Cell Biol.* 37:47-61.
143. Wingfield-Digby, N. J. 1978. The technique and clinical application of endometrial cytology in mares. *Equine Vet. J.* 10: 167-170.
144. Wintrobe, E. 1975. *Clinical Hematology*. 7th ed. Lea and Febiger, Philadelphia.
145. Wright, B. B., and P. A. Rebers. 1972. Procedure for determining heptose and hexose in lipopolysaccharides. Modification of the cysteine-sulfuric acid method. *Anal. Biochem.* 49:307-319.
146. Wu, W. G., and S. Marcus. 1963. Humoral factors in cellular resistance. I. The effects of heated and unheated homologous and heterologous sera on phagocytosis and cytopepsis by normal and "immune" macrophages. *J. Immunol.* 91:313-322.
147. Yin, E. T., C. Galanos, S. Kinsky, R. A. Bradshaw, S. Wessler, O. Luderitz, and M. E. Sarmiento. 1972. Picogram-sensitive assay for endotoxin gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharides and lipid A from Gram negative bacteria. *Biochem. Biophys. Acta* 261:284-289.
148. Yin, H. L., and T. P. Stossel. 1979. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium dependent regulatory protein. *Nature* 281:583-586.

149. Zavy, M. T., F. W. Bazer, and D. C. Sharp. 1978. Nonsurgical technique for collection of uterine fluid from mare. *J. Anim. Sci.* 47:672-676.
150. Zipursky, A., E. J. Brown, and J. Bienenstock. 1973. Lack of opsonizing potential of 11S human secretory IgA. *Proc. Soc. Exp. Biol. N. Y.* 142:181-184.

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APPENDIX: DETAILS OF SELECTED SOLUTIONS AND PROCEDURES

Ammonium chloride-Tris buffer.

SOL#1 11.25 grams Trizma base in 500 ml H₂O
Adjust pH to 7.65 with 1 N HCl

SOL#2 41.5 grams Ammonium chloride in 5 liters H₂O

- Use 4500 ml SOL#2
500 ml SOL#1
Adjust pH 7.2 with 1 N HCl

Sonication.

- A seven-inch conical stainless steel sonifier tip was placed into each tube.
- Sonifier was used with a frequency setting of 3 and a direct current amperage of 3-4.
- Sonifier was used at above settings for 15 seconds in each tube.
- Branson Sonic Power, Branson Instruments Incorporated, Danbury, CT.

Phenol:chloroform:petroleum-ether lipopolysaccharide extraction procedures.

- 25 grams bacteria + 100 ml phenol:chloroform:petroleum-ether (in ratio of 2:5:8), stir 10 minutes at 4^o C.
- Centrifuge at 4,000xg for 15 minutes at 4^o C and filter supernatant fluid through Whatman #1 paper.
- Repeat step #1 and #2 twice and combine supernatants.
- Evaporate petroleum-ether and chloroform in a rotary evaporator, then transfer to glass centrifuge bottles and add H₂O until precipitate forms.
- Centrifuge at 1,600xg at 10 minutes for 10^o C, and decant supernatant.

- Wash precipitate 3 times with 25 ml of 80% phenol, wiping bottle dry with filter paper after each decantation of the supernatant (8,000xg for 10 minutes at 10° C).
- Wash precipitate 3 times with ether (8,000rpm for 15 minutes at 10° C) and then dry in vacuum desiccator over CaCl₂.
- Resuspend in 25 ml H₂O, place in 45° C H₂O bath for 10 minutes, and place in vacuum desiccator for two hours.
- Shake sample for 5 minutes at room temperature, centrifuge at 105,000xg for 4 hours at 4° C, and dissolve pellet in 20 ml H₂O.
- Centrifuge 1,600xg for 10 minutes at 4° C and discard undissolved material.
- Lyophilize supernatant.

Modified Westphal procedure.

- 20 grams bacteria + 350 ml 68° C H₂O, then add 350 ml 90% 68° C phenol.
- Stir suspension for 30 minutes (vigorous), then cool to 10° C.
- Centrifuge at 8,000xg for 45 minutes at 10° C and collect aqueous layer.
- Reheat sample to 68° C, add 350 ml 68° C H₂O, stir, centrifuge, and collect aqueous layer.
- Dialyze combined aqueous layers against water.
- Reduce volume by rotary evaporator to 100 ml, then lyophilize.
- Resuspend to 3% solution in water and centrifuge at 105,000xg for 4 hours at 4° C.
- Resuspend pellet in water and repeat centrifugation.

- Resuspend pellet in H₂O and add equal volume of 0.1M Tris buffer with 0.25M Magnesium sulfate (pH 7.2), add 0.25 mg Deoxyribo nuclease I and 0.25 mg Ribonuclease A and 2 drops toluene.
- Allow to stand 24 hours at room temperature.
- Remove toluene by bubbling N₂ gas through suspension.
- Remove nucleotides by ultracentrifugation or by ultrafiltration dialysis.
- Lyophilize remaining pellet or suspension.

Phenol-soluble lipopolysaccharide extraction procedure.

- 20 grams bacteria + 350 ml 68° C H₂O, then add 350 ml 90% 68° C phenol.
- Stir for 30 minutes (vigorous), and cool to 10° C.
- Centrifuge at 8,000xg for 45 minutes at 10° C and collect aqueous phase.
- Reheat sample to 68° C, add 350 ml 68° C H₂O, stir, centrifuge, and collect aqueous phase as for Westphal aqueous lipopoly-saccharide extraction; collect phenol phase.
- Add 350 ml phenol to remaining material, heat, stir and centrifuge.
- Collect phenol phase and dialyze combined phenol phases against H₂O.
- Filter to remove coarse residue, centrifuge 8,000xg for 60 minutes at 10° C, and concentrate supernatant fluid by rotary.
- Lyophilize and resuspend in water to 3%, centrifuge at 105,000xg for 4 hours at 4° C.

- Resuspend pellet in water, check for nucleic acid absorbance; if present, then add equal volume of 0.1M Tris buffer with 0.25M Magnesium sulfate (pH 7.2), add 0.25 mg Deoxyribonuclease I and 0.25 mg Ribonuclease A and 2 drops toluene.
- Allow to stand 24 hours at room temperature.
- Remove toluene by bubbling N₂ gas through suspension.
- Remove nucleotides by ultracentrifugation or by ultrafiltration dialysis.
- Lyophilize sample.