THE EFFECTS OF *PASTEURELLA HAEMOLYTICA* LIPOPOLYSACCHARIDE ON BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS IN CELL CULTURE

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July, 1989

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PREFACE

I take this opportunity to express my sincere appreciation to all those who have aided me with my research. First, I thank my graduate committee, Dr. Roger Panciera, Dr. Anthony Confer, Dr. Derek Mosier, Dr. Kenneth Clinkenbeard, and Dr. Robert Fulton. Drs. Confer, Clinkenbeard, and Mosier were my primary sources of research ideas which were invaluable. I appreciate Dr. Panciera's help, and I hope that I have learned from his non-nonsense, objective approach to problem-solving. I thank Dr. Fulton for his support at critical times.

I appreciate all the technical help I have received, especially from Dr. Kathy Kocan, Rene Simons, Janet Durham, and Sharon Oltjen. A special thanks is given to June Willis for rushing some thin sections through the electron microscopy laboratory at the last minute. I appreciate Sherl Holesko for her prompt attention to my typing needs.

I thank my parents for the values they instilled in me and for relieving me of responsibilities at Stafford which made possible the continuation of my education.

I am indebted to my wife, Charlotte, for her physical, spiritual, emotional, and monetary support during my pursuit of higher education. I apologize to her and my children for any suffering they may have endured due to lack of my attention.

This research was funded in part by grants No. 85-CRSR-2-2618 and 86-CRSR-2-2880 from the U.S. Department of Agriculture. Additional fund-

ing came from the University Center for Energy Research, the University Center for Water Research, and the College of Veterinary Medicine.

I thank my heavenly Father for the gift of a good mind. I hope that He is continually glorified through my life and accomplishments.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Bovine pneumonic pasteurellosis, commonly called shipping fever, is a severe respiratory disease which primarily affects young, stressed cattle. It remains a major source of economic loss to the cattle industry despite extensive efforts to control it by the producers, animal health industry, and veterinary profession (Jensen, 1968; Church and Radostits, 1981).

Pneumonic pasteurellosis has a multifactorial etiology which includes physical stressors such as transport, commingling, surgeries, and nutrition, and viral infections such as infectious bovine rhinotracheitis, parainfluenza-3 (PI-3), bovine viral diarrhea, and bovine respiratory syncytial virus (Rosenquist, 1984; Wikse, 1985). The most severe disease and loss result from a bacterial pneumonia which may be either primary or secondary. The most common cause is Pasteurella haemolytica A1 (Schiefer et al., 1978).

The pathogenesis of pneumonic pasteurellosis is incompletely understood. Pasteurella haemolytica is believed to be carried in low numbers in the nasal passages of normal cattle. Following the stress of transport, commingling, or respiratory viral infection, a marked increase in nasal colonization commonly occurs (Frank and Smith, 1983; Frank et al., 1986). Recently, fimbriae have been demonstrated on P. haemolytica and

have a potential but uncertain role in bacterial adherence to nasal mucosa (Morck et al., 1988). Grey and Thomson (1971) demonstrated P. haemolytica organisms in aerosol droplets within tracheal air of calves with detectable colonization of the nasal cavities. These droplets were of sufficiently small size (≤5 microns) to bypass mucociliary clearance and be deposited in the lung. Therefore, infective droplets have been considered to be the major mechanism of bacterial deposition in the lung following nasal colonization.

Inhaled organisms are rapidly cleared from the lungs of normal calves (Lillie and Thomson, 1972). Therefore, it is likely that successful infection of the lung requires that pulmonary clearance be compromised. Lopez et al. (1976) demonstrated defective pulmonary clearance of P. haemolytica in calves following PI-3 viral infection. Gilka et al. (1974a) demonstrated defective pulmonary clearance caused by pulmonary edema and inconsistently by hydrocortisone, but not following PI-3 viral infection. However, in the latter instance the PI-3 virus-infected calves had significant pre-existing antibody to P. haemolytica that may have enhanced clearance.

Pulmonary alveolar macrophages (PAM) are the primary means of bacterial clearance from the alveoli and respiratory bronchioles (Goldstein et al., 1974). Reduced pulmonary clearance is, therefore, most likely the result of impaired PAM function. Several studies have demonstrated decreased phagocytosis, impaired intracellular killing, and other alterations in PAM function resulting from viral infections (Forman and Babiuk, 1982; Hesse and Toth, 1983; Trigo et al. 1985). Physical stressors which have been associated with pneumonic pasteurellosis cause increased plasma cortisol concentration; glucocorticoids

reduce phagocytic and bactericidal capabilities of macrophages and also inhibit antibody responses, selected neutrophil functions, and lymphocyte responses (reviewed in Roth, 1984).

Recently, considerable attention has been directed toward mechanisms by which P. haemolytica can impair lung defense mechanisms. Pasteurella haemolytica excretes a leukotoxin which is toxic to bovine leukocytes and to PAM. It also impairs phagocytosis and production of leukocyte chemotactic factors by the PAM (Markham and Wilkie, 1980; Markham et al., 1982). Leukocytes exposed to P. haemolytica culture supernates manifested an initial increase in chemiluminescence, indicating phagocytic stimulation, followed by a rapid cessation of activity. These effects were caused by a heat-labile factor believed to be leukotoxin (Richards and Renshaw, 1986). Fimbriae and an extensive glycocalyx, demonstrable on P. haemolytica from experimentally infected calves, have been proposed to be another potential inhibitory factor in pulmonary clearance through inhibition of phagocytosis (Morck et al., 1988). Additionally, lipopolysaccharide (LPS), commonly called endotoxin, caused pulmonary edema and markedly reduced pulmonary clearance of P. haemolytica in calves (Gilka et al., 1974a). In vitro experiments with P. haemolytica LPS demonstrated alterations in selected bovine leukocyte functions including reduced phagocytic activity in polymorphonuclear leukocytes at certain LPS doses (Confer and Simons, 1986). Endotoxemia caused variable effects on PAMs in other species. In the dog reduced PAM adherence, increased hydrogen peroxide production, reduced bacterial phagocytosis, and reduced bacterial killing were seen. In the rat there was enhanced PAM adherence, reduced chemotactic response, and increased hydrogen peroxide, neutrophil chemotactic factor, and inter-

leukin-1 (IL-1) production without apparent effect on bacterial phagocytosis (Jacobs et al., 1986; Christman et al., 1988).

The lesions of pneumonic pasteurellosis are those of an acute, fibrinous pleuropneumonia and include serofibrinous exudation into the alveoli, alveolar septal edema, microvascular thrombosis, hemorrhage, and inflammatory cellular infiltration followed by extensive alveolar necrosis (Schiefer et al., 1978; Allan et al., 1985). These lesions strongly implicate vascular damage as an early event in the disease because: (1) the loss of large plasma proteins, such as fibrinogen, and hemorrhage would require a loss of vascular integrity; and (2) exposure of the subendothelial matrix is a major mechanism of microvascular thrombosis via activation of the intrinsic clotting mechanism and stimulation of platelet adherence (Jensen et al., 1976; Slauson and Cooper, 1982; Breider et al., 1987). The proposed mechanisms of vascular damage in pneumonic pasteurellosis include direct damage due to P. haemolytica toxins, especially LPS, and secondary damage due to the inflammatory response (Breider et al., 1988).

Endothelial Cells

Characterization

Endothelial cells (EC) line the lumens of the entire circulatory system including the lymphatic channels. They are of mesodermal origin, form a single layer of flattened, polygonal cells, and are of three types, continuous, fenestrated, and EC of high endothelial venules. Regional differences in EC morphology are most apparent in the endothelial junctions. Junctions in the continuous type of endothelium vary from that of the arterioles, which have a continuous network of tight junc-

tions surrounded by large gap (communicating) junctions, to that of the capillaries and venules, which have no gap junctions. The fenestrated type is found in several visceral organs (reviewed in Thorgeirsson and Robertson, 1978; Ryan and Ryan, 1982). Endothelium of high endothelial venules, is more cuboidal to columnar and is involved in the lymphocyte emigration into secondary lymphoid organs and chronic inflammatory sites (Harlan, 1985). Experimental evidence indicates that these EC direct lymphocyte homing by specific cell surface molecules, "vascular addressins" (Streeter et al., 1988). Capillary EC tend to be more attenuated than arterial EC with the periphery of many cells having a thickness of less than 0.1 um. Plasmalemmal vesicles are also characteristic features of EC. They are found in higher density in capillaries and many communicate with the luminal cell surface thereby greatly increasing the surface area (Ryan and Ryan, 1982).

Cell Culture

Much of the recent advancement in the understanding of EC function has resulted from the development of EC culture methods. Jaffe et al. (1973a) and Gimbrone et al. (1974) were instrumental in the development of techniques for EC isolation, culture, and identification from human umbilical vein endothelium. Later, Booyse et al. (1974) developed the techniques for isolation and culture of bovine aortic EC. Subsequently, these techniques were adapted to the isolation of bovine pulmonary artery EC (Ryan et al., 1978). Further refinements in techniques have resulted in the isolation of bovine pulmonary artery EC without the use of enzymes and the ability to clone individual EC (Ryan et al., 1980; Gajdusek and Schwartz, 1982). Clotting Factor VIII antigen, which is

normally present in EC, is used as an identifying marker. It can be demonstrated by indirect immunofluorescent microscopy and is shared only by platelets and megakaryocytes (Jaffe et al., 1973b; Thorgeirsson and Robertson, 1978).

Normal Cell Functions

Endothelial cells were once considered to be relatively inert and to primarily function as a semipermeable, nonthrombogenic physical barrier between the blood and the underlying tissues. Increasingly, evidence indicates that EC have a wide range of metabolic activities which are normally antithrombogenic (Fishman, 1982). Endothelial cells respond to a variety of stimuli by specific alterations in function, metabolism, and structure which may profoundly affect the pathogenesis and outcome of a disease process. These responses include increases and decreases in normal functional activities, the induction of new functions, and de novo synthesis of molecules (Cotran, 1987).

The normal functional activities of the endothelium include the transport of solutes, the metabolism or clearance of blood-borne products, and the production of various bioactive products. The transport mechanisms of EC are poorly understood. Physiological data predicts a two-pore model for the transport of solutes with pore radii of 50-80 and 200-250 angstroms (Taylor and Granger, 1983). Plasmalemmal vesicles and transendothelial channels are most commonly purported to be responsible for solute transport in continuous endothelium such as in the lung. There is considerable experimental evidence to both support and deny the occurrence of vesicular transport (Simionescu et al., 1982; Shea and Raskova, 1983; Bundgaard, 1983). Transendothelial channels have been

demonstrated and may represent a fusion of luminal and abluminal vesicles (Simionescu et al., 1982). However, these channels have not been demonstrated conclusively in the lung and their density may be inadequate to account for the normal volume of pulmonary transendothelial solute transport (Gil, 1983). The pulmonary endothelium is selectively permeable to anionic proteins which is compatible with the positive charges lining the walls of both vesicles and transendothelial channels (Simionescu, 1982; Taylor and Granger, 1983). In contrast to vesicular or channel transport, water is apparently transported across the entire surface of the EC. The effects of disease on endothelial transport have not been reported, but evidence indicates that endothelial vesicles do not play a determining role in pulmonary edema (Chinard and DeFouw, 1983).

The pulmonary endothelium occupies a unique niche in the circulatory system because the entire blood volume has intimate exposure to it prior to entering the systemic circulation. Therefore, the pulmonary endothelium can regulate the entry of numerous hormones and mediators into the systemic circulation. Much experimentation has studied simply the removal of products during pulmonary circulation, so the relative regulatory contribution of EC versus circulating and fixed leukocytes and platelets in the pulmonary circulation is often unknown. Endothelial cells effectively remove serotonin and partially remove norepinephrine from pulmonary circulation by an active transport mechanism (Nicholas et al., 1974). Other biogenic amines, histamine and epinephrine, are not processed (reviewed in Ryan and Ryan, 1982). Prostaglandins (PG) of the E and F series and thromboxane A_2 are cleared during pulmonary passage, but prostaglandins of the A and B series and prostacyclin are not

cleared. Endothelial cells account for the uptake of PGE and $PGF_{1\alpha}$ but not for their metabolism (Ryan and Ryan, 1982). Isolated, perfused rat lung metabolized the leukotrienes, LTC_4 to LTD_4 and LTE_4, whereas LTB_4 was not metabolized (Harper et al., 1984). The adenine nucleotides are efficiently degraded and removed from the pulmonary circulation by dephosphorylation accomplished by phosphatases located along the plasmalemmal vesicles. Adenosine is then removed by EC in an energy-requiring process (reviewed in Ryan and Ryan, 1982).

Several hormones are also removed or metabolized in the pulmonary microcirculation. Aldosterone is removed from circulation (Sulza et al., 1983). Cortisol and cortisone are removed, and cortisone is converted to cortisol, some of which is returned to the circulation (Nicholas and Kim, 1975). Bradykinin is inactivated by pulmonary EC. Angiotensin I is converted to angiotensin II by angiotensin-converting enzyme, the latter located along the luminal surface of the pulmonary endothelium (Ryan and Ryan, 1982).

Normal endothelium also actively prevents thrombosis. The EC remove $PGF_{1\alpha}$, thromboxane A_2, adenine nucleotides, serotonin, bradykinin, and angiotensin I, all of which promote platelet aggregation (Thorgeirsson and Robertson, 1978). Heparan sulfate proteoglycans on the surface of EC inhibit the conversion of prothrombin to thrombin (Buonassisi and Colburn, 1982). Endothelial cells synthesize and release several plasminogen activators that are active in fibrinolysis (Levin and Loskutoff, 1982). Heparin-like molecules, antithrombin III, and thrombomodulin are also anticoagulant endothelial surface molecules (Stern et al., 1985). In addition, EC also continuously release prostacyclin, a potent inhibitor of platelet aggregation (Ryan and Ryan, 1982). Conversely, EC

normally synthesize several procoagulant factors including Factor VIII antigen, von Willebrand factor, Factor V, and tissue factor (Jaffe et al., 1973b; Brox et al., 1984; Stern et al., 1985).

Endothelial cells have been shown to normally synthesize and secrete a variety of additional substances. These include a complex surface glycocalyx, several components of basement membrane, a vasodilator known as endothelium-derived relaxing factor, and the C_3 complement component (Luft, 1966; Jaffe et al., 1976; Warren et al., 1987; Vanhoutte, 1988).

Functional Changes Induced by Various Stimuli

Endothelial cells respond to a variety of stimuli by functional changes. These include selective inhibition or amplification of normal baseline metabolic functions or the de novo expression of normally undetectable ones. The concurrent stimulation of several functional and structural changes in EC is a process known as endothelial activation.

Several stimuli cause an increase in procoagulant factors to be produced by the EC. Colucci et al. (1983) have demonstrated increased production and cell-surface expression of tissue factor in human umbilical vein EC following exposure to endotoxin. Also, thrombin, IL-1 and tumor necrosis factor (TNF) cause increased tissue factor activity in cultured EC (Brox et al., 1984; Bevilacqua et al., 1986). Stimuli such as thrombin or histamine cause increased release of von Willebrand factor (de Groot et al., 1984; Hamilton and Sims, 1987). An inhibitor of tissue plasminogen activator which would inhibit clot lysis, is released *in vivo* and in cultured EC following exposure to IL-1 or endotoxin (Emeis and Kovistra, 1986). Tumor necrosis factor causes similar effects in cultured EC (Cotran, 1987). Physical trauma to endothelial monolayers

promotes platelet adherence and aggregation (Grabowski, 1987). Platelet activating factor, a strong stimulator of platelet adherence and aggregation, is released after exposure of EC to IL-1, TNF, thrombin, histamine, bradykinin, LTC_4 or LTD_4, vasopressin, angiotensin II, or von Willebrand factor (reviewed in Zimmerman et al., 1987).

In addition to an increased release of procoagulant factors, several stimuli also cause a decrease in the normal anticoagulant products of EC. Interleukin-1 and TNF decrease endothelial surface thrombomodulin, thereby markedly inhibiting the anticoagulant effects of protein S and protein C (Naworth and Stern, 1986; Naworth et al., 1986; Esmon, 1987). In addition to increased secretion of an inhibitor of tissue plasminogen activator, IL-1 and thrombin cause decreased endothelial secretion of tissue plasminogen activator (Levin and Loskutoff, 1982; Bevilacqua et al., 1986b).

In contrast to the above effects, a number of the same stimuli cause increased prostacyclin synthesis and release from EC. These stimuli include IL-1, TNF, thrombin, histamine, bradykinin, ADP, ATP, phospholipase C, and endotoxin (Lollar and Owen, 1980; Rossi et al., 1985; Meyrick, 1986; Resink, 1987; de Nucci, 1988). Prostacyclin is a potent inhibitor of platelet aggregation and considered to be one of the major anticoagulant effectors secreted by EC (reviewed in Wallis and Harlan, 1986). However, in activated EC, the overall balance of the functional alterations is tipped towards coagulation. Interleukin-1 causes fibrin deposition on the surface of apparently intact endothelium *in vivo* in the rabbit (Naworth and Stern, 1986). Similar fibrin clot formation on the surface of cultured EC is caused by endotoxin exposure (Stern et al., 1985).

Other substances which are released by appropriately stimulated EC include endothelium-derived relaxing factor. This factor, which Palmer et al. (1987) showed to be nitric oxide, is released from EC stimulated with bradykinin, vasopressin, ADP, ATP, arachidonic acid, or phospholipase C (de Nucci et al., 1988). Endothelium-derived relaxing factor acts synergistically with prostacyclin to inhibit platelet aggregation (Radomski et al., 1987). Additionally, EC stimulated by treatment with phorbol myristate acetate or by phagocytosis of fixed platelets, lipid particles, or polystyrene microspheres have markedly increased release of reactive oxygen metabolites which could enhance local inflammatory reactions (Dorog et al., 1988).

Potential Immunologic Functions

Endothelial cells are also capable of a wide range of functional changes that indicate their participation in immune reactions. Under normal culture conditions, EC express major histocompatibility complex class I antigens (Pober and Gimbrone, 1982). The cytokines, gamma-interferon and TNF, cause increased expression of class I antigen and are synergistic. Interferon alpha and beta also stimulate increased class I antigen expression on EC, but they are not synergistic with gamma-interferon (Zapierre et al., 1988). Further, EC express class II major histocompatibility complex antigens when stimulated by gamma-interferon. Other cytokines have not demonstrated this effect, and alpha and beta interferon strongly inhibit the gamma-interferon induction of class II antigens (Pober et al., 1983; Zapierre et al., 1988). Endothelial cells have the ability to phagocytize bacteria and other particles, and phagocytosis stimulates the production of reactive oxygen metabolites (Vann

and Procter, 1987; Dorog et al., 1988). The above observations indicate that EC have the necessary mechanisms whereby they can process and present antigen in an immunologic reaction.

In addition to the major histocompatibility complex antigens, EC express an additional lymphocyte binding molecule, ICAM-1. The expression of ICAM-1 is markedly increased by IL-1, TNF, and gamma-interferon (Pober et al., 1986). Endothelial cells stimulated by IL-1 or TNF, but not gamma-interferon, express newly synthesized IL-1 at their surface. This could be important for activating T-lymphocytes bound to foreign antigen-major histocompatibility complex on the endothelial surface (Kurt-Jones et al., 1987). Tumor necrosis factor and gamma-interferon also induce morphologic changes in EC causing them to become more plump and to have widened intercellular gaps (Stolpen et al., 1986). The preceding observations led Pober (1988) and colleagues to propose the following theory of EC participation in immune inflammation. The relevant T-cells react with appropriately presented foreign antigen and subsequently release lymphotoxin (TNF) and gamma-interferon. These then activate local venular EC causing increased class I and class II antigen expression. The activated EC becomes an antigen presenting-cell and binds T-cells specific for the eliciting antigen and, via signals such as membrane IL-1, stimulate increased T-cell cytokine release causing amplification of the effect. Simultaneously, increased ICAM-1 expression promotes adhesion of additional nonspecific lymphocytes and monocytes. Morphologic changes in EC facilitate extravasation of inflammatory cells and may contribute to leakage of macromolecules into the perivascular tissue.

Endothelial Injury - In Vitro Studies

Several biologically relevant substances are directly cytotoxic to cultured EC. Bacterial LPS from Escherichia coli and Salmonella sp. causes marked bovine EC detachment and increased chromium-leakage, an indicator of cell lysis. These effects were not seen in EC from humans, goats, or dogs or in bovine aortic smooth muscle cells (Harlan et al., 1983). E. coli LPS also causes increased nuclear pyknosis and lactate dehydrogenase (LDH)-leakage from BPAEC (Meyrick et al., 1986). E. coli LPS and lipid A, but not the lipid A-related compound, lipid X (2,3 diacyl-glucosamine-1-PO_4), induce morphologic changes, cell-detachment, and LDH-leakage from bovine aortic EC. The morphologic changes include loss of confluency with a majority of the cells having bleb-like structures over most of their surface. In addition, the cells contain large vacuoles, dense bodies, and pyknotic nuclei (Gartner et al., 1988). Other substances that cause direct injury to EC as determined by detachment and/or the release of large internal markers include hydrogen peroxide, hyperoxia, phagocytosis of Staphylococcus aureus, and sulfhydryl amino acids (probably mediated by hydrogen peroxide) (Wall et al., 1980; Bowman et al, 1983; Chopra et al., 1987; Vann and Procter, 1987).

Several substances damage EC monolayers grown on gel-coated polycarbonate filters causing an increased permeability to water or large molecules. E. coli LPS causes an increased permeability of BPAEC monolayers to radiolabeled albumin and an increased hydraulic conductance (Meyrick et al., 1986). Human endothelial monolayer permeability to radiolabeled albumin is also increased by thrombin and hyperoxia (Del Vecchio et al., 1987; Phillips et al,. 1977).

In vitro, EC cytotoxic effects are either caused or augmented by

inflammatory cells. Several studies have suggested that adherence of neutrophils to EC is important in the initiation of endothelial injury (Sacks et al., 1978). Neutrophils have been stimulated to adhere to EC by numerous chemotactic factors including C5a, formyl-methionyl-leucyl-phenyalanine, and LTB_4 (Gimbrone et al., 1984; Tonnesen et al., 1984). Phorbol diesters, LPS, IL-1, and TNF also promote neutrophil adhesion to human EC by effects that are independent for both cell types (Gamble et al., 1985; Schleimer and Rutledge, 1986). Subsequent experiments demonstrated that neutrophil adhesion is dependent on the CDw18 complex on neutrophils and an induceable surface molecule of EC called "endothelial-leukocyte adhesion molecule-1" (ELAM-1) (Pohlman et al., 1986; Bevilacqua et al., 1987).

Under certain conditions, neutrophils cause cytotoxic effects in EC. Neutrophils stimulated by phorbol esters, chemotactic peptides, or C5a produce cytotoxic changes in human EC. These changes are markedly enhanced by low concentrations of LPS (1-10 ng/ml) or by preincubation of the EC with IL-1 or TNF (Smedley et al., 1986; Varani et al., 1988). The LPS-enhanced cytolysis is not inhibited by oxygen radical scavengers, occurs with neutrophils which do not produce hydrogen peroxide, but is inhibited by specific elastase inhibitors (Smedley et al., 1986). Trace amounts of LPS enhances chemoattractant-induced secretion of elastase by neutrophils (Fittschen et al., 1988). Additionally, Harlan et al. (1985) demonstrated that stimulated neutrophils damaged EC by a mechanism independent of oxygen radicals. This contrasts with earlier studies which indicated that neutrophils kill EC by hydrogen peroxide or oxygen radicals (Sacks et al., 1978; Martin, 1984).

Leukocyte migration through an intact endothelial layer has often

been proposed to contribute to increased vascular permeability (Wittels et al., 1974). However, complement-stimulated neutrophil migration and lymphokine-stimulated lymphocyte migration across bovine pulmonary arterial intimal explants do not increase permeability. Lymphokine-stimulated neutrophil transmigration does cause increased permeability suggesting that the nature of the chemoattractant and the leukocyte type are both important in stimulating increased permeability (Meyrick et al., 1987).

Interleukin-2-activation of human lymphocytes causes increased adherence to and lysis of both human and bovine EC. This effect is not isolated to a specific lymphocyte type since it occurs in large granular and small agranular lymphocytes along with $CD4^+$ and $CD8^+$ T-cells and non-T subpopulations (Damle et al., 1987).

Recent work by Suttorp and colleagues (1985a, 1985b) demonstrated that the pore-forming bacterial toxins, staphylococcal α-toxin and Pseudomonas aeruginosa cytotoxin, insert into EC and cause a potassium efflux and a calcium influx. The pores produced were of a specific size being permeable to sucrose but not to inulin or dextran. Both toxins stimulated arachidonic acid metabolism which resulted in increased prostacyclin production. However, lysis of EC was not seen. More recent experiments demonstrated similar effects with E. coli hemolysin (Seeger and Suttorp, 1987). Clinkenbeard et al. (1989a, 1989b) demonstrated that P. haemolytica leukotoxin affects bovine lymphoma cells causing potassium efflux and calcium influx. A functional pore diameter slightly less than that of sucrose was demonstrated. Similarities between leukotoxin and the aforementioned bacterial pore-forming toxins warrant further investigation into its possible effects on EC.

Pulmonary Vascular Injury in Experimental Disease and Pneumonic Pasteurellosis

Most of the experimental data pertaining to vascular injury and relevant to this study has been derived from animal models of the adult respiratory distress syndrome in humans. Since many of these models involve either direct endotoxin infusion or the putative mediators of endotoxemia, they will be discussed later. Included here will be the observations and experimental production of vascular lesions in pneumonic pasteurellosis.

Vascular lesions have often been cited in descriptions of the pathology of naturally occurring pneumonic pasteurellosis. Tweed and Edington (1930) described blood and lymph vessels being filled with a coagulated material. Graham (1953) reported histological findings indicating that shipping fever pneumonia began with a severe congestion of the lung followed by hemorrhage and a fibrinous exudation into the alveoli. Blood vessel thrombosis was reported in about one-half of these cases. Blood clots and thrombosis of pulmonary venules and arterioles along with thrombosis of alveolar septal capillaries have been associated with areas of infarction, hemorrhage, and inflammatory cellular infiltration in pneumonic pasteurellosis (Jensen et al., 1976; Scheiffer et al., 1978).

Experimental pneumonia produced by intratracheal or aerosol exposure *P. haemolytica* causes sequential vascular-related changes. As early as 4 hours post-exposure, a mild alveolar edema was seen (Gilka et al., 1974a). Fibrinous exudation into the alveoli with moderate neutrophil infiltration was reported 6 to 18 hours post-exposure (Friend et al., 1977; Breider et al., 1988). Allan et al. (1985) reported alveolar

flooding edema with inflammatory cells or extensive networks of fibrin and intra-alveolar hemorrhage caused by disrupted, hyperemic alveolar capillaries by 2 days post-exposure. By 6 days post-exposure, focal necrosis which contained thrombosed blood vessels was common. A purulent vasculitis affected a few blood vessels.

The importance of neutrophils in the pathogenesis of P. haemolytica vascular lesions has been examined. Slocombe et al. (1985) found that 1- to 3-week-old calves inoculated intratracheally with P. haemolytica developed severe lesions of fibrinous pneumonia, whereas neutrophil-depleted calves developed only infrequent alveolar lesions consisting of edema, hemorrhage, and a few neutrophils. In contrast, Breider et al. (1988) found in 130 - 230 kg calves that while lesions in neutrophil-depleted calves were less severe, lesions indicative of vascular damage were extensive. The lesions included interlobular and intra-alveolar edema with diffuse alveolar hemorrhage, exudation of fibrin, and occasional small arterial thrombosis.

Endotoxin

Endotoxin is a biologically active substance that is a component of the gram-negative bacterial cell wall. It consists primarily of LPS with associated protein complexes. LPS contains a hydrophobic lipid portion, termed lipid A, which is covalently bound to the central acidic hetero-oligosaccharide core. The core is bound to a hydrophilic heteropolysaccharide chain containing the specific repeating O-antigen units. Most of the toxic effects of endotoxin are caused by the lipid A portion of the LPS molecule. LPS has an affinity for a variety of cell membrane components including phospholipids, glycoproteins, and glycosphingolipids.

Most of the membrane interactions of LPS described to date involve non-specific insertions of the hydrophobic portion of the molecule into the membrane phospholipids. A few specific membrane interactions have been described and a lipoglycoprotein receptor has been identified on human red blood cells (Springer et al, 1974). Few of the biochemical mechanisms of endotoxin activity have been adequately established, and specific interactions have not been described in EC. Animal species and strain, bacterial species and strain, and method of endotoxin isolation and purification can affect the responses to endotoxin. Therefore, many apparently contradictory results exist in the literature. The biological actions of endotoxin are myriad with virtually every organ system being affected in the endotoxemic animal (reviewed in Morrison and Ulevitch, 1978; Bradley, 1979; Haeffner-Cavaillon et al, 1985; Morrison, 1985). Because of the immensity of the literature dealing with endotoxins, this review will primarily include the pulmonary effects of endotoxin and putative mediators of those effects.

Pulmonary Pathophysiologic Effects Due to Endotoxin

The pathophysiology of the pulmonary responses to endotoxemia have been studied most extensively in the sheep. Intravenous ^{125}I-labeled *E. coli* LPS is rapidly cleared from the circulation and predominantly localized in the lung. In sheep, the half-life of LPS in the circulation was 2.38 minutes with 77.6% of the recovered dose in the lung. In contrast, in the rat the half-life and pulmonary recovery were 12.39 minutes and 2.02% respectively. The rat is highly resistant to the pulmonary effects of endotoxin. The high degree of pulmonary localiza-

tion in the sheep was attributed to uptake by the pulmonary intravascular macrophages, but uptake by the pulmonary endothelium could not be ruled out (Warner et al., 1988). Similarly, in calves, over 50% of the injected radioactivity of ^{51}Cr-labeled Pseudomonas LPS remained in the lungs 3 and 6 days after injection. ^{51}Cr-labeled LPS was 90% cleared from the blood in 1 minute and 99% cleared in 3 minutes (Maxie et al., 1974a). Similar results were obtained with ^{3}H-labeled LPS although clearance from circulation was slower (75% at 1 minute, 80% by 3 minutes) and tissue retention was less (Maxie et al., 1974b).

Intravenous injections of lethal doses of E. coli LPS in unanesthetized sheep causes progressive changes consisting of early persistently increased pulmonary resistance with decreased dynamic compliance followed by pulmonary edema, respiratory failure, and death (Esbenshade et al., 1982). Brigham et al. (1979) described a biphasic pulmonary reaction to LPS consisting of an early phase of pulmonary hypertension followed by a long late phase of increased pulmonary vascular permeability. This biphasic response has been consistently reported and the separation of the phases is useful in terms of functional and structural changes and pathogenetic mechanisms.

Intravenous infusion of a single dose of E coli LPS into sheep (0.25 - 2 ug LPS/kg body weight) causes a 3- to 4-fold increase in pulmonary arterial pressure that peaks between 30 minutes and 1 hour post-infusion, but remains elevated above baseline for several hours. Concurrent with the increased pressure is a marked increase in protein-poor pulmonary lymph flow resulting in a marked increase in lung lymph protein clearance (Brigham et al., 1979; Demling et al., 1981; Snapper et al., 1983a). The increased lymph flow and protein clearance exceeded

that which could be attributed to increased microvascular pressure (Brigham et al., 1979).

Lung mechanics are also markedly altered during the hypertensive phase of endotoxemia in the sheep. Dynamic lung compliance markedly decreases, lung resistance to airflow markedly increases, and the alveolar-to-arterial oxygen gradient markedly increases (Esbenshade et al., 1982; Snapper et al., 1983a). The functional residual capacity decreases (Esbenshade et al., 1982).

Temporally coincident with the hypertensive phase is a marked decrease in circulating leukocytes. This is primarily due to a decrease in neutrophils, but mononuclear cells also decrease significantly (Snapper et al., 1983b; Warner et al., 1988). A marked hypoxemia also accompanies the first phase of endotoxemia (Esbenshade et al., 1982).

The permeability phase begins 2 to 4 hours post-infusion and is heralded by a steadily increasing pulmonary lymph protein clearance. This is characterized by a markedly increased lung lymph flow (5 to 6 times baseline) with an increasing lung lymph to plasma protein concentration ratio (Brigham et al., 1979; Demling et al., 1981; Esbenshade et al., 1982). The magnitude of these changes indicates a marked increase in vascular permeability. Molecular sieving of proteins less than 100 angstroms molecular radius occurs but the molecular radius of lost proteins is increased (Brigham et al., 1979). The lymph to plasma protein concentration ratio remains at or slightly above baseline but less than 1:1 indicating that the vascular membrane is not freely permeable to proteins and the vascular permeability is not due to gross vascular defects (Brigham et al, 1979; Demling et al., 1981). Pulmonary

arterial pressure remains mildly elevated to near normal during the permeability phase (Demling et al., 1981; Esbenshade et al., 1982).

The decrease in dynamic lung compliance and increase in lung resistance to airflow is moderate during the early permeability phase, but deteriorates as pulmonary edema develops pre-terminally (Esbenshade et al., 1982). Functional residual capacity improves early but drops precipitously as pulmonary edema develops (Esbenshade et al., 1982; Snapper et al., 1983a). The hypoxemia and increased alveolar to arterial oxygen-gradient persists throughout the permeability phase with sublethal doses of LPS (Snapper et al., 1983a).

Other functional changes accompany the permeability phase of endotoxemia. The airway responsiveness to an inhaled bronchoconstrictor (histamine) increases (Hutchinson et al., 1983). Pulmonary vasoconstriction in response to hypoxia is significantly reduced (Hutchinson et al., 1985). Demling et al. (1980) demonstrated an increase in lymph lysosomal enzymes that correlated well with increased vascular permeability. Plasma lysosomal enzymes increased, but correlated poorly with vascular permeability. They also demonstrated that 24 hours after a sublethal dose of LPS, measured parameters were essentially normal with the exception of a moderate leukocytosis.

Continuous administration of low doses (9 - 24 ng kg^{-1} hr^{-1}) of E. coli LPS to sheep causes a hyperdynamic state with increased cardiac output, decreased peripheral resistance, and mild elevations in pulmonary artery pressure. In contrast, the larger single dose of endotoxin described above results in a hypodynamic state with decreased cardiac output and marked pulmonary artery hypertension. Lung lymph flow also increases in the continuous low dose model and is similar to that seen

in the permeability phase of the single infusion model. Neutropenia at 1.5 hours followed by neutrophilia at 4 - 24 hours is similar to that in the single dose model (Traber et al., 1988). Repeated doses of 0.5 ug LPS/kg 3 times a week for 10 to 14 weeks results in a chronic, persistent pulmonary hypertension (more than 50% above baseline). Pulmonary vasoreactivity to hypoxia and a PGH_2 analog are also decreased (Meyrick and Brigham, 1986).

Studies of endotoxemia in calves are few. Olson and Brown (1985) reported that calves exposed to 4 ug of E. coli LPS/kg/hour for 5 hours had changes similar to those described in the sheep models. The pulmonary artery pressure and pulmonary vascular resistance were markedly elevated within 30 minutes and remained elevated for the 5 hour test. The cardiac index was markedly depressed throughout the endotoxemia. The alveolar-to-arterial oxygen gradient was markedly elevated by 30 minutes but returned to normal after 1.5 hours (cf. sheep). Neutrophil, lymphocyte, and platelet counts were markedly depressed throughout the study. Lung extravascular thermal volume, an indicator of extravascular lung water, was increased after 2 hours exposure. Margolis et al. (1987) demonstrated similar changes in pulmonary arterial pressure and cardiac output in calves infused with E. coli endotoxin. They also demonstrated that plasma thromboxane increased from 30 to 60 minutes post-infusion and prostacyclin increased after 3 hours.

Pulmonary Structural Alterations Due to Endotoxin

Meyrick and Brigham (1983) examined the histologic changes in sequential peripheral lung biopsies of open-chested, anesthetized sheep following a single infusion of E. coli LPS (1.25 ug/kg infused over 30

minutes). Fifteen minutes following the start of endotoxin infusion, a 3-fold increase in neutrophils was seen in capillaries and small vessels with about equal numbers of lymphocytes. Disgorged neutrophil-specific and azurophilic granules were found in the capillary lumen. By 30 minutes, some granulocyte disruption was accompanied by more numerous free specific granules. There was mild interstitial edema. Transendothelial-migrating neutrophils were seen in the pulmonary capillaries of some but not all sheep. By 1 hour, the changes seen at 30 minutes were more severe. Additionally, some EC were more electron dense than normal and had increased pinocytotic vesicles. Infrequently capillary lumens contained fibrin. Type I pneumocytes were occasionally sloughed leaving areas of exposed alveolar basement membrane. Lymphatics were dilated. From 2 to 4 hours, the ultrastructural appearance of the lung was similar to that seen at 1 hour. In addition, foci of capillary disruption were seen, and the endothelium of small veins and arteries had returned to normal. Occasionally intracapillary monocytes had phagocytosed red cells and cellular debris.

Warner et al. (1988) described the ultrastructural changes following a bolus injection of ^{125}I-labeled E. coli in sheep (0.8 ug/kg). Within 10 minutes after injection, they described vascular congestion with increased platelets and surface structural changes in pulmonary intravascular macrophages, platelet phagocytosis, and platelet adherence. These changes in the intravascular macrophages were considered to be evidence of activation. By 60 minutes, many capillaries were occluded with neutrophils, platelets, and fibrin. Severe EC injury and interstitial edema were described.

Intrinsic Mediators of Endotoxemia

Tumor Necrosis Factor (Cachectin). Tumor necrosis factor (TNF) is synthesized and secreted by macrophages after stimulation by endotoxin. TNF may be detected in the circulation of rabbits within 1 hour after endotoxin administration (Beutler et al., 1985a,b). The role of TNF in mediating endotoxic effects is indicated by experiments which showed that mice are protected from the lethal effects of endotoxemia by specific polyclonal antibodies to TNF (Beutler et al., 1985c). Similarly, passive immunization of baboons with neutralizing monoclonal antibody to TNF protects them from septic shock (Tracey et al., 1987). Recombinant human cachectin produces signs similar to endotoxic shock when injected into rats in biologically relevant amounts (Tracey et al., 1986). Recombinant TNF also causes pulmonary edema and increased vascular permeability in guinea pigs which is indistinguishable from that of endotoxin (Stephens et al., 1988).

In contrast, TNF is a doubtful mediator of endotoxin-induced neutrophil emigration in the rabbit dermal model. Repeated endotoxin injections into the same site results in tachyphylaxis. IL-1 stimulates cross-tachyphylaxis to endotoxin-induced neutrophil emigration, but TNF does not (Cybulsky et al., 1988a). Additionally, only when IL-1 and TNF are co-injected is the cellular infiltrate comparable to that induced by endotoxin (Movat et al., 1987). However, TNF induces IL-1 production in the rabbit *in vivo* and in macrophages and EC *in vitro* (Dinarello et al., 1986; Libby et al., 1986). TNF also mediates several additional responses which cause neutrophil adherence to EC and intravascular hypercoagulability (see Endothelium).

Interleukin-1. Endotoxin is a potent inducer of IL-1 synthesis and secretion by primarily mononuclear phagocytes. A similar effect has been seen in neutrophils, EC, and smooth muscle cells (reviewed by Cybulsky et al., 1988b). Compelling evidence implicates IL-1 as the principal mediator of endotoxin-induced neutrophil emigration into the rabbit dermis. Endotoxin, synthetic lipid A and IL-1 are essentially equipotent in inducing neutrophil emigration (Cybulsky et al., 1988a). Endotoxin and IL-1 exceed the potency of TNF in inducing neutrophil emigration by several orders of magnitude (Cybulsky et al., 1988a, Wankowicz et al., 1988). The kinetics of endotoxin- and IL-1-induced neutrophil emigration are similar. IL-1-induced emigration is first detectable 30 minutes after injection, peaks at about 1.5 hours, and declines to a low level by 3.5 hours. The endotoxin-induced emigration follows a nearly identical pattern but is delayed 30 minutes in initiation and in the peak response. Presumably, the 30 minute delay in initiation of the IL-1-induced response is the time required for endothelial expression of neutrophil adhesion molecules, whereas the additional 30 minutes required for the endotoxin-induced effect is the time required for endotoxin-induced IL-1 secretion to occur (Cybulsky et al., 1988a). Finally, IL-1 and endotoxin exhibit cross-tachyphylaxis, whereas TNF and neutrophil chemoattractants do not (Cybulsky et al., 1988a).

Like TNF, recombinant IL-1 induced pulmonary lesions similar to those produced by endotoxin (Goldblum et al., 1988). Partially purified native IL-1 can be substituted for the preparative and/or provoking dose of endotoxin in the local Shwartzman-reaction (Beck et al., 1986). However, subsequent studies using recombinant cytokines demonstrated that IL-1 and TNF are both required and act synergistically for preparation

of the Shwartzman-like reaction (Movat et al., 1987). Submaximal doses of IL-1 and TNF also synergize in neutrophil emigration in the rabbit dermal model (Wankowicz et al., 1988). Because endotoxin is a potent inducer of both TNF and IL-1 synthesis, it is likely that these two cytokines act synergistically or additively in mediating many of the effects of endotoxin.

Complement. The lipid A portion of LPS can directly activate the classical complement pathway and the polysaccharide and heterooligosaccharide portions can activate the alternate pathway (reviewed in Morrison and Ulevitch, 1978). However, the results of *in vivo* experiments are unclear and often contradictory concerning the role of complement in mediating endotoxemia. For example, From et al. (1970) demonstrated an abrogation of the initial (2-5 minutes) endotoxin-induced hypotensive effect in complement-depleted dogs with no effect on the subsequent progressive hypotension and mortality. Garner et al. (1974) demonstrated no hypotensive effect and complete protection from endotoxic lethality in complement-depleted dogs. May et al. (1972) found that complement-depleted or C4-deficient guinea pigs had an accelerated mortality due to endotoxin. Subsequent studies have failed to resolve these apparent conflicts (reviewed in Fine, 1985). In sheep, infusion of complement-activated plasma results in neutrophil sequestration in the pulmonary circulation and a biphasic physiologic response. However, the initial pulmonary hypertension and leukopenia occur and resolve much faster than that induced by endotoxin. Likewise, the increase in lung permeability is less pronounced, and within 2 hours the complement-induced lesions have resolved (Meyrick and Brigham, 1984).

Arachidonic Acid Metabolites. Endotoxin infusion into sheep caused increased concentrations of both the cyclooxygenase and lipoxygenase products of arachidonic acid metabolism with consistently higher concentrations in the pulmonary lymph than in the plasma (reviewed in Brigham, 1985). Apparently, thromboxane A_2 is the mediator of the early pulmonary hypertension following bolus injection of endotoxin. Inhibitors of cyclooxygenase and thromboxane synthesis effectively inhibit the thromboxane A_2 release after endotoxin injection in sheep and calves and prevent the pulmonary hypertension but not the later increase in vascular permeability (Winn et al., 1983; Margolis et al., 1987). The release of prostacyclin is temporally correlated with the increase in vascular permeability, but direct cause and effect relationships are not established (Brigham, 1985). Prostacyclin infusion decreases the lung lymph flow in sheep during endotoxemia, and this decrease is associated with decreased pulmonary artery pressure (Smith et al., 1982). Prostacyclin infusion also reduces platelet aggregation and the release of thromboxane A_2 in the thrombin-induced vascular injury model in sheep (Malik et al., 1985). These mechanisms may be involved in the prostacyclin-induced attenuation of lymph flow in endotoxemia and could indicate that prostacyclin release during endotoxemia is a protective mechanism. PGE_2 and $PGF_{2\alpha}$ are also released into the pulmonary circulation during endotoxemia. Both have pulmonary vasoconstrictor properties that result in increased microvascular hydrostatic pressure, but do not increase vascular permeability to protein. Therefore, their mediator effects in endotoxemia are uncertain (reviewed in Malik et al., 1985).

The role of lipoxygenase products of arachidonic acid metabolism in mediating endotoxin effects is also uncertain. 5-HETE- and 12-HETE-

release into the plasma and lung lymph correlate with increased permeability, but no cause and effect relationship has been established (Brigham, 1985). LTB_4 infusion into sheep causes a transient increase in pulmonary artery pressure and lung lymph flow with an associated marked neutrophil sequestration in the lungs (Malik et al., 1985). LTC_4 and LTD_4 cause a transient pulmonary hypertension which is inhibited by thromboxane synthestase inhibitors (Malik et al., 1985). Lipoxygenase inhibitors decrease the vascular permeability due to arachidonic acid injection in isolated rabbit lungs, but bolus injection of leukotrienes do not cause increased permeability, Therefore, a cause and effect relationship could not be established (Seeger et al., 1987).

Platelet Activating Factor. Platelet activating factor (PAF) can be detected in the circulation of rats in less than 10 minutes after endotoxin injection. Injection of PAF mimics the early hypotensive effect of endotoxin in the rat. However, PAF-induced effects occur in 30 seconds, whereas endotoxin-induced effects require 2-3 minutes. This delay is considered to be the time required for PAF secretion. A PAF antagonist is capable of reversing endotoxin-induced hypotension (Doebber et al., 1985).

Platelet activating factor is secreted by EC in response to IL-1, thrombin, and leukotrienes. It was postulated that this may be an important mediating factor in many types of vascular injury primarily through the effect of PAF on leukocytes and platelets (Zimmerman et al., 1987). These experiments provide preliminary evidence that PAF may be a mediator of several endotoxin-induced effects.

Neutrophils. Injection of a single bolus of endotoxin into sheep

causes a rapid decline in circulating neutrophils and a 3-fold increase in peripheral lung neutrophils increasing to a 6-fold increase by 4 hours after injection (Meyrick and Brigham, 1983). Lung lymph lysosomal enzymes, most likely from sequestered leukocytes, are elevated in the early phase of endotoxemia, persist throughout the permeability phase, and correlate to lung lymph flow (Demling et al., 1981). Neutrophil depletion markedly reduces endotoxin-induced alterations of airway mechanics and vascular permeability (Hinson et al., 1983; Heflin and Brigham, 1981). However, the endotoxin-induced increases in vascular permeability in neutrophil-depleted sheep remain significant. In vitro observations indicate that endotoxin has direct injurious effects on endothelium which are enhanced by neutrophils. Therefore, though neutrophils intensify the lung injury due to endotoxin, they are not the sole mediators of the response (Brigham and Meyrick, 1984). Additionally, zymosan-activated plasma causes vascular sequestration of neutrophils similar to that induced by endotoxin, but the magnitude and duration of effects are much less (Meyrick and Brigham, 1984).

Endotoxin effects on neutrophils may enhance their damaging effects in the pulmonary vasculature. Pretreatment of neutrophils with LPS (10 ng/ml) causes them to release much greater quantities of superoxide anion, myeloperoxidase, and lysozyme than untreated neutrophils when subsequently exposed to immune complexes or chemoattractants (Guthrie and Johnston, 1982). Similarly, pretreatment of neutrophils with LPS enhances chemoattractant-induced elastase secretion (Fittscher et al., 1988). Release of proteases and superoxides causes endothelial damage in vitro (see Endothelium) and likely mediates the neutrophil-induced vascular damage.

Conclusion

Bacterial endotoxin produces pronounced pulmonary vascular effects in small ruminants and calves. The similarity of the hemodynamic effects and vascular lesions seen with experimental pneumonic pasteurellosis to those of endotoxemia have led to numerous implications of the importance of endotoxin in pneumonic pasteurellosis. The reported use of P. haemolytica endotoxin in any lung injury model is extremely rare. The action of endotoxin on the pulmonary vasculature combines direct effects with the complex interactions of multiple, intrinsic mediators. Bovine pulmonary artery EC have been used as an in vitro model to more specifically study some of the mechanisms of endotoxin-induced vascular injury in a less complex setting, and many of the findings have subsequently been confirmed or supported in vivo.

This research attempts to define potential pathogenic mechanisms of vascular injury in bovine pneumonic pasteurellosis. It will test the hypothesis that P. haemolytica LPS is a factor in the pathogenesis of the vascular lesions of pneumonic pasteurellosis. Cultured bovine pulmonary artery EC will be the test model. The objectives of the research are to examine the effects of P. haemolytica LPS on BPAEC by assays of cytotoxicity as well as morphologic and functional alterations in the EC.

CHAPTER II

THE DIRECT EFFECTS OF *PASTEURELLA HAEMOLYTICA* LIPOPOLYSACCHARIDE ON BOVINE PULMONARY ENDOTHELIAL CELLS *IN VITRO*

Introduction

Bovine pneumonic pasteurellosis (shipping fever) is a disease of major economic importance to the cattle industry (Jensen, 1968). The etiology is complex and involves the interaction of stress with viral and bacterial infections (Jericho, 1979; Rehmtulla and Thomson, 1981; Yates, 1982). *Pasteurella haemolytica* biotype A, serotype 1 causes the severe, fibrinous pleuropneumonia that is responsible for the high mortality and economic loss (Schiefer et al., 1978; Frank, 1979; Rehmtulla and Thomson, 1981). The pneumonic lesions include early pulmonary edema, extensive serofibrinous exudation into the alveoli, and neutrophil sequestration and emigration (Gilka et al., 1974b; Allan et al., 1985). The disease spreads aggressively throughout the lungs and results in widespread necrosis with vascular and lymphatic thrombosis (Schiefer et al., 1978; Jericho, 1979).

In pneumonic pasteurellosis, the early appearance of pulmonary edema followed by fibrinous exudation indicates that vascular damage is an important pathogenetic factor (Gilka et al., 1974b). Endotoxin [bacterial lipopolysaccharide (LPS)] has been implicated as a potential factor in causing this vascular damage (Slocombe et al., 1984; Allan et al.,

1985; Breider et al., 1988). Experimentally, intravenous infusion of endotoxin caused increased vascular permeability in sheep (Meyrick and Brigham, 1983). In vitro, the LPS of Escherichia coli and Salmonella typhosa were directly toxic to bovine pulmonary arterial endothelial cells (BPAEC) (Harlan et al., 1983; Meyrick et al., 1986).

This study was designed to examine the direct effects of LPS purified from P. haemolytica A1 on cultured BPAEC. These effects were evaluated by measuring several indicators of cell injury or lysis and by morphologic examination.

Materials and Methods

Lipopolysaccharide Preparation

Pasteurella haemolytica A1 LPS was prepared by phenol-water extraction (Wesphal and Jann, 1965; Confer and Simons, 1986). The resulting LPS at a concentration of 5.2 mg/ml (dry weight) contained 168 ug of 2-keto, 3-deoxyoctanate/ml, and had 6.8 x 10^5 EU/ml (130 EU/ug) of endotoxin activity as determined by a chromogenic Limulus amebocyte assay.[a]

Endothelial Cells

Monolayer cultures of BPAEC[b] were maintained in 75 cm^2 tissue culture flasks in Medium 199 with Earle's salts[c] containing 4.35 g $NaHCO_3$/l, 10^{-5} M thymidine,[c] 50 ug gentamicin[c]/ml, 5 ug fungizone[c]/ml, 0.2 M glutamine,[c] and 10% heat-inactivated fetal bovine serum (FBS)[d]

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[a]Whittaker MA Bioproducts, Inc., Walkersville, MD.
[b]CCl-209, American Type Culture Collection, Rockville, MD.
[c]Gibco Laboratories, Inc., Grand Island, NY.
[d]Hyclone Laboratories, Logan, UT.

(Complete medium). Cell cultures were incubated at 37 C in 5% CO_2, and medium was changed twice weekly. For each experiment, BPAEC were transferred to 24-well tissue culture plates[e] at a concentration of 3.5×10^4 cells per well and grown to confluence (6-7 days). Cultures were used at passages 16 through 19.

Lactate Dehydrogenase (LDH) Leakage Assay

The complete medium was removed from confluent monolayers of BPAEC in 24-well plates and replaced with 500 ul of RPMI 1640 medium[c]/well containing 10% FBS and from 0.001 to 10 ug of LPS/ml, no LPS (negative control), or 1% Triton X-100 (positive control). Each treatment was tested in triplicate or quadruplicate. The plates were incubated at 37C in 5% CO_2 for 0.5 to 24 hours. At the predetermined times, the medium was removed and centrifuged for 5 minutes at 290 xg. Fifty ul of the supernatant were assayed for LDH activity using a spectrophotometric method[f,g] (Clinkenbeard et al., 1989). Percent LDH-leakage was calculated by the following formula:

$$\text{LDH activity (IU/L) in supernatants of:} \quad \frac{\text{(test medium-control medium)}}{\text{(1\% Triton medium-control medium)}} \times 100$$

Chromium-Leakage Assay

BPAEC monolayers in 24-well plates were labeled with ^{51}Cr[h] by incubation for 1 hour at 37 C in complete medium containing 50 uCi of activity of ^{51}Cr/ml. Following a triple rinse in phosphate-buffered saline

[e]Nunclon Multidish, A/S Nunc, Kamstrup, Denmark.
[f]LD-L, Sigma Diagnostics, St. Louis, MO.
[g]System 5 Analyzer, Gilford Instrument Laboratories, Inc., Oberlin, OH.
[h]ICN Radiochemicals, Irvine, CA.

solution (PBSS), the wells received 500 ul of complete medium containing 0, 0.1, or 10 ug of LPS/ml or 1% Triton X-100. The plates were incubated at 37C for 1 to 24 hours. Each treatment was tested in triplicate. At the predetermined times, the medium was removed and centrifuged at 290 xg for 5 minutes. Counts per minute (cpm) were determined for 200 ul of each supernatant using an automated gamma counter[i]. Percent ^{51}Cr-leakage was calculated by the same formula as that used for percent LDH-leakage using cpm in medium supernatants rather than LDH activity.

Cell-Detachment Assay

Complete medium was removed from confluent monolayers of BPAEC in 24-well plates and replaced with 1.0 ml of complete medium containing from 0.001 to 10 ug of LPS/ml or no LPS. After an 18 hour incubation at 37C in 5% CO_2, the medium was removed. The wells were rinsed in PBSS until free cells were not seen by light microscopy. The remaining cells were released by incubation in 0.05% trypsin and 0.53% EDTA until release was complete as determined by light microscopy. Total volume in each well was brought up to 1.0 ml with PBSS containing 10% FBS to halt the trypsin activity. The cell suspensions were agitated and counted directly without dilution using a hemacytometer. In a separate experiment, the monolayers were exposed to 1 ug of LPS/ml for periods of 0.5 to 8 hours and examined by the same method. Percent detachment was calculated by the following formula:

$$\frac{\text{(number of cells in control well - number of cells remaining)}}{\text{(number of cells in control wells)}} \times 100$$

[i]Gamma 5500, Beckman Instruments, Inc., Fullerton, CA.

Statistical Analyses

Means and standard errors of the means (SEM) were calculated. The samples were tested for equal variances using the F-test. Experimental means were compared to control means using appropriate Student's t tests (Steele and Torrie, 1980). Results were considered significant at $p < 0.05$.

Scanning Electron Microscopy (EM)

BPAEC monolayers were grown to confluence on coverslips within 24-well plates and exposed to 1 ug of LPS/ml in RPMI 1640 medium. At 2, 4, 8, and 24 hours, the monolayers were fixed *in situ* with 2.5% glutaraldehyde for one hour. The fixed cells were coated by the osmium-thiocarbohydrazide procedure of Malik and Wilson (1975), dehydrated in serial ethanol rinses, and critical-point dried by ethanol-CO_2 solvent exchange.[j] The coverslips were mounted on aluminum stubs and viewed with a scanning electron microscope[k] with the specimen tilted at a 45 degree angle to the electron beam.

Results

Exposure of BPAEC monolayers to *P. haemolytica* LPS caused a dose- and time-dependent release of LDH (Table I). Significant LDH-leakage first became detectable after 4 hours exposure to LPS concentrations of 0.1 ug/ml or greater. The percent-release increased throughout the 24-hour test period and also was detectable with an LPS concentration of

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[j] Samdri Pvt 3 Critical Point Dryer, Tousimis Research Corp., Rockville, MD.

[k] JSM 35-U, Jeol Ltd., Tokyo, Japan.

TABLE I

LDH-LEAKAGE FROM CULTURED BOVINE ENDOTHELIAL CELLS FOLLOWING EXPOSURE TO PASTEURELLA HAEMOLYTICA LPS

LPS Concentration[a]	Percent LDH-Leakage[b]			
	2 hours	4 hours	8 hours	24 hours
0.001	0 (2)	0 (2)	1 ± 1 (2)	21 ± 21 (2)
0.01	1 ± 1 (3)	3 ± 1 (3)	12 ± 5 (3)[c]	29 ± 15 (3)[c]
0.1	0 (3)	4 ± 2 (3)[c]	25 ± 6 (3)[d]	38 ± 11 (3)[d]
1.0	0 (3)	8 ± 2 (4)[d]	31 ± 4 (4)[d]	66 ± 16 (4)[d]
10.0	1 ± 1 (3)	9 ± 2 (4)[c]	33 ± 3 (4)[d]	57 ± 1 (4)[d]

[a]ug/ml.
[b]Expressed as the mean ± SEM.
Parentheses = Number of experiments.
[c]Significant leakage ($p < 0.05$) in more than one-half of experiments (i.e., 2 of 3 or 3 of 4).
[d]Significant leakage ($p < 0.05$) in all experiments.
LDH = Lactate dehydrogenase.
LPS = Lipopolysaccharide.

TABLE II

COMPARISON OF ^{51}Cr- AND LDH-LEAKAGE FROM CULTURED BOVINE ENDOTHELIAL CELLS FOLLOWING LPS EXPOSURE

	LPS Concentration[a]	Percent Leakage[b]		
		2 hours	4 hours	24 hours
^{51}Cr-leakage	0.1	ND	10 ± 10 (2)	59 ± 6 (2)
	10.0	0 (2)	15 ± 14 (2)	61 ± 18 (2)
LDH-leakage	0.1	0 (3)	4 ± 2 (3)	38 ± 11 (3)
	10.0	1 ± 1 (3)	9 ± 2 (4)	57 ± 11 (4)

[a]ug/ml.
[b]Expressed as the percent leakage ± SEM.
Parentheses = Number of experiments.
LDH = Lactate dehydrogenase.
LPS = Lipopolysaccharide.

0.01 ug/ml at 8 and 24 hours. The maximal dose-response occurred at 1 and 10 ug/ml with no significant differences between the two doses. Mean positive control values were 102 (±3) IU/L and mean negative control values were 33 (±2) IU/L which were within the linear limits of the test (data not shown).

Leakage of ^{51}Cr from BPAEC monolayers following exposure to P. haemolytica LPS followed a dose- and time-dependent pattern similar to that for LDH-leakage (Table II). Significant ^{51}Cr-leakage was first detectable following 4 hours of exposure and reached a value of 61% (±18) at 24 hours with an LPS concentration of 10 ug/ml. Significant differences were not detected between percent ^{51}Cr-leakage and percent LDH-leakage at any time or dose tested.

Cell-detachment was not detectable following 18 hours exposure to LPS at 0.001 ug/ml or 0.01 ug/ml, but was 29% (±4) at 0.1 ug/ml, 81% (±2) at 1 ug/ml, and 89% (±2) at 10 ug/ml. In a separate experiment, exposure to 1 ug of LPS/ml caused no significant release by 1 hour, 23% (±3) by 2 hours, 85% (±4) by 4 hours, and 95% (±2) by 8 hours.

Examination of normal BPAEC monolayers by scanning EM revealed a mounded surface morphology. Numerous distinct fissures were present along the cell junctions because of shrinkage during fixation and processing (Figure 1). The surface of each endothelial cell had several large depressions (up to 2 um in diameter) and numerous small depressions (0.1 to 0.2 um in diameter) (Figure 2). After 1 hour of LPS exposure, many of the cells contained foci of rarefaction that had rounded, raised margins and mildly depressed centers (Figures 3,4). The numbers of large depressions similar to those seen in normal cells were normal to decreased. After 2 hours of LPS exposure, cells had retracted

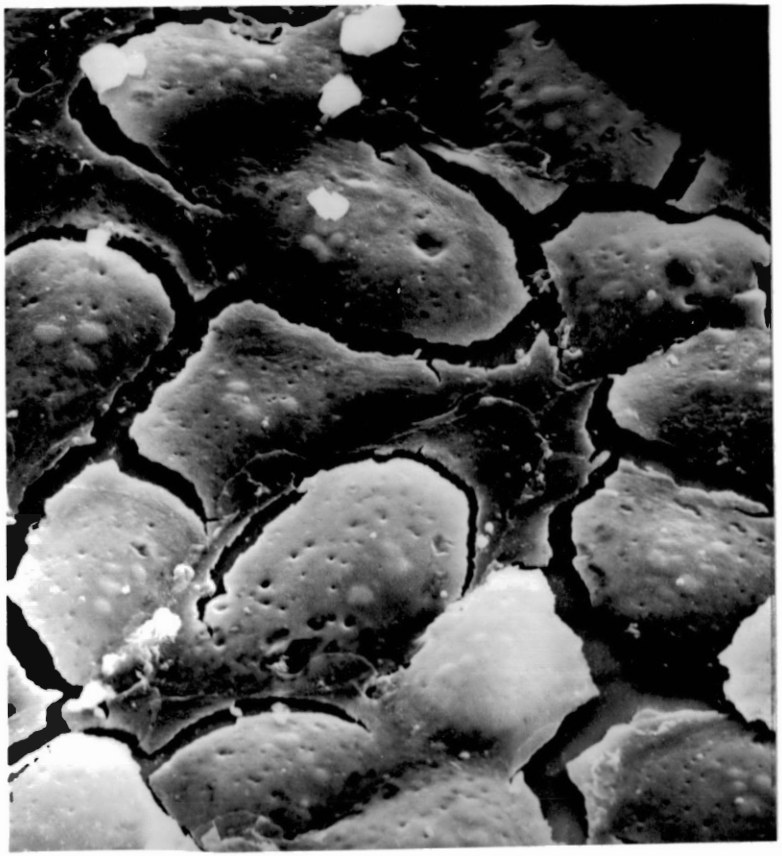

Figure 1. Normal endothelial monolayers with rounded cell morphology and pitted surfaces. The sharp fissures along cell junctions are processing artifacts. x940

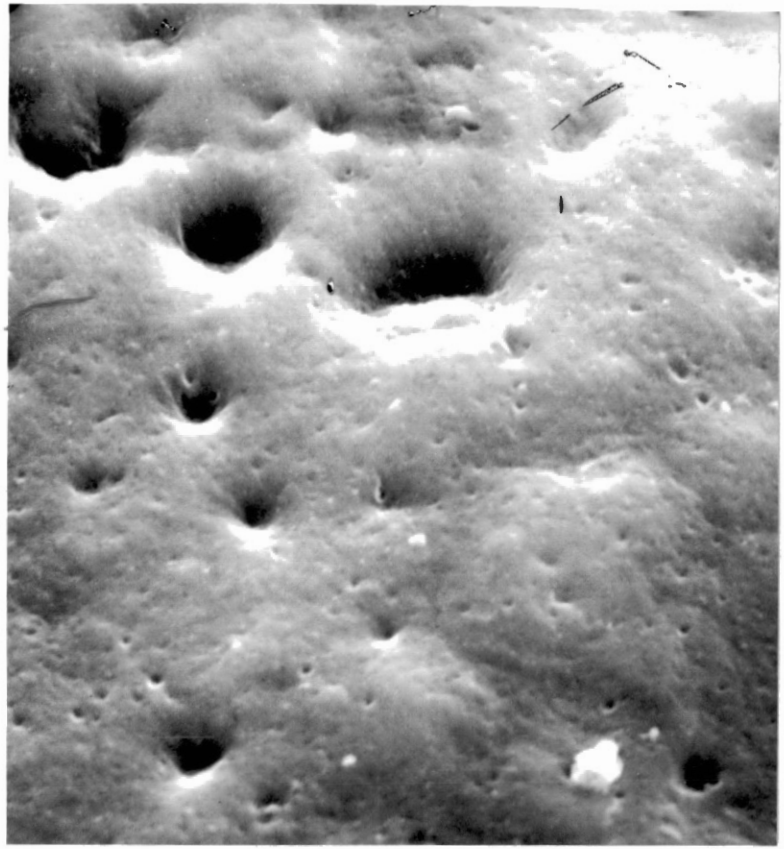

Figure 2. Normal endothelial cells surface with large (up to 2 um diameter) and small (0.1 to 0.2 um diameter) depressions. x9400

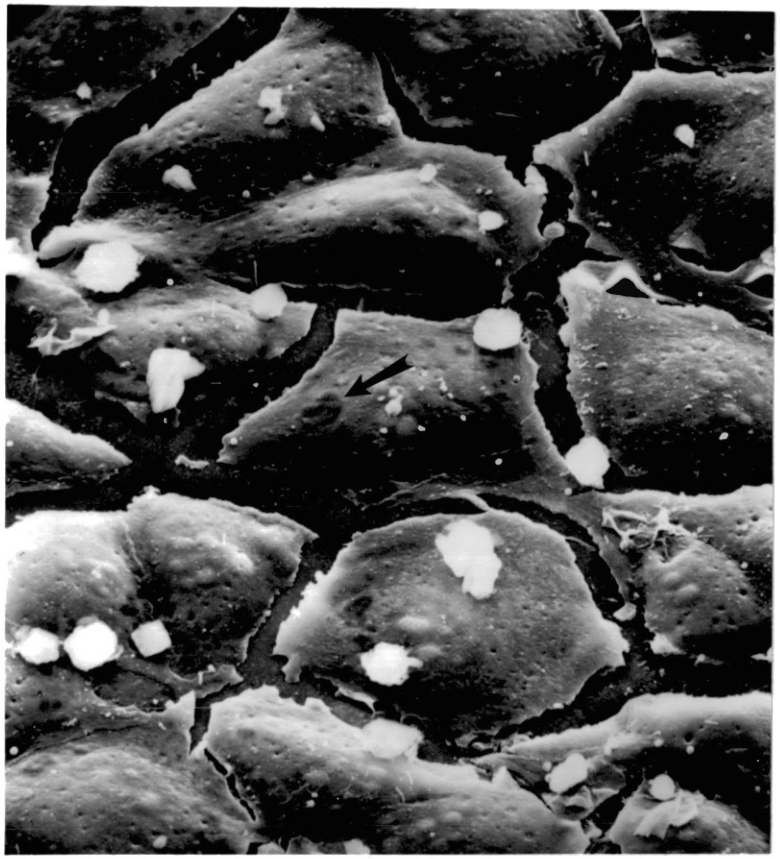

Figure 3. Endothelial monolayer after a 1 hour exposure to 1 ug of LPS/ml. Morphologic changes consist of appearance of rarefied areas (arrow) and a decrease in large surface pits. x940

Figure 4. Rarefied area on the surface of endothelial cell after a 1 hour exposure to 1 ug of LPS/ml. Note raised, rounded margin (arrow). x9400

and had fine cytoplasmic projections streaming from their edges along the surface of the glass (Figure 5). Mild to marked cytoplasmic blebbing and ruffling of the cell surface was most pronounced along, but not limited to the edges of the cells (Figure 6). Many cell surfaces had grape cluster-like appearance caused by retraction and bleb formation. The changes observed at 4 hours were similar to, but much more extensive than those seen at 2 hours; most of the cells had detached. By 8 hours, cell detachment was essentially complete.

Discussion

Pasteurella haemolytica LPS caused severe dose- and time-dependent damage to BPAEC monolayers. Following 2 hours of exposure to LPS, there were marked morphologic changes consisting of cell-retraction and surface bleb formation along with significant cell-detachment. However, ^{51}Cr- and LDH-leakage was not detectable at this time. Detachment apparently preceded the formation of membrane defects which allowed the release of large internal molecules. In our study, LDH- and ^{51}Cr-leakage were equivalent indicators of severe membrane damage. Determination of LDH-leakage was simpler, yielded more consistent results, and avoided radiation hazards; therefore, LDH-leakage was the method of choice for these experiments.

Our results were similar to those previously described in which BPAEC were exposed in vitro to LPS from other bacteria (Harlan et al., 1983; Meyrick et al., 1986). Whereas direct cytotoxic effects of LPS have been demonstrated in bovine endothelial cells (EC) in vitro, the EC of the human, goat, and canine were resistant to LPS cytotoxicity (Harlan et al., 1983). These findings suggest that endotoxin may be a

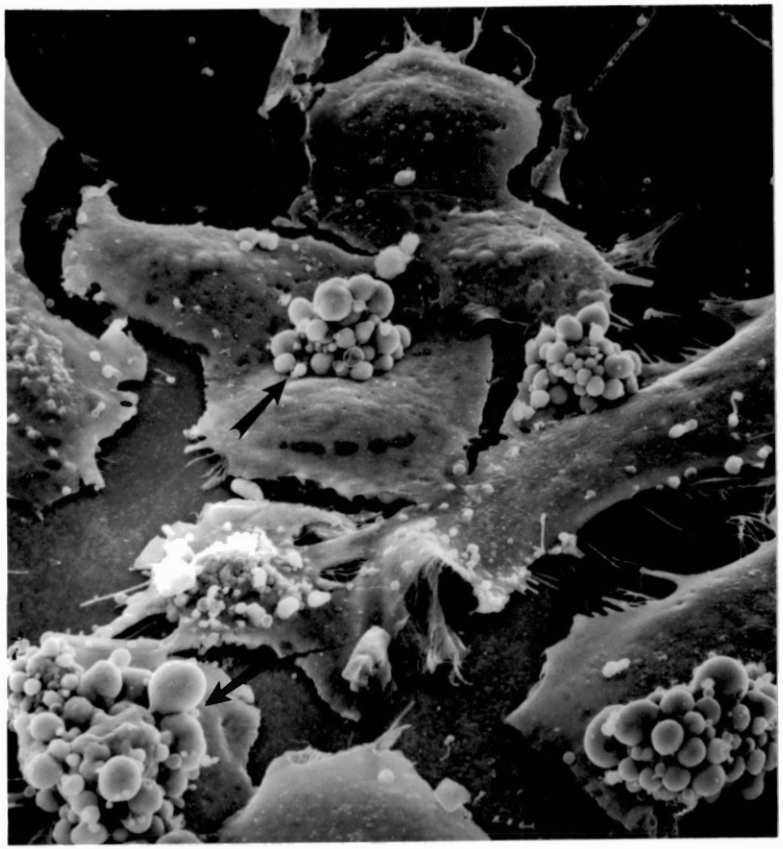

Figure 5. Endothelial monolayer after a 2 hour exposure to 1 ug of LPS/ml. Cell retraction is marked. Several cells are rounded with severe bleb formation (arrows). x940

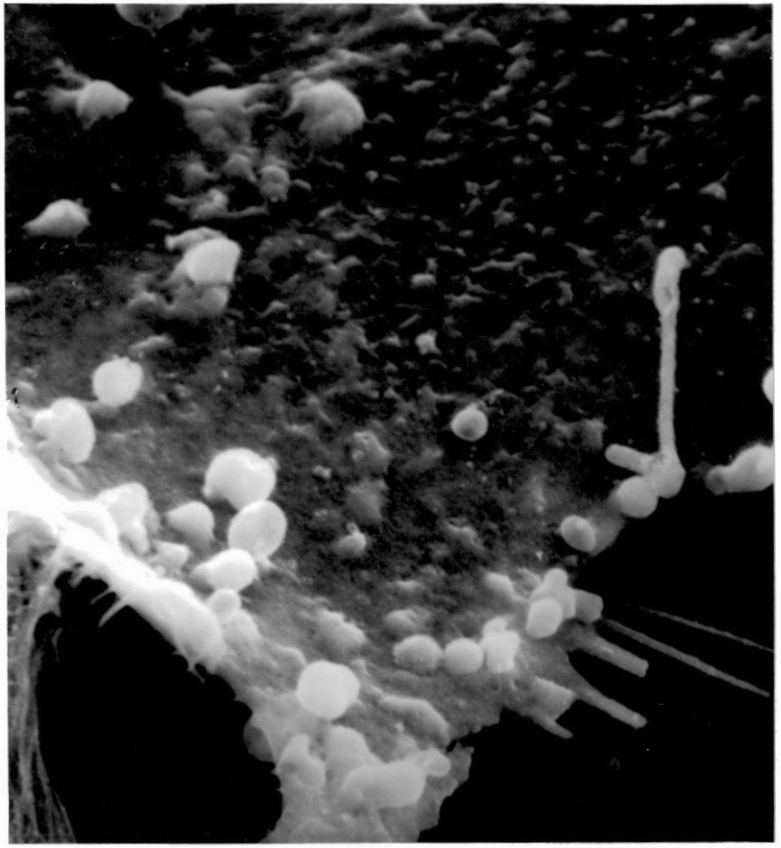

Figure 6. Endothelial cell surface after a 2 hour exposure to 1 ug of LPS/ml. Bleb formation is most pronounced along the edge and surface is ruffled. Fine cytoplasmic projections are streaming from the edge along the surface of the glass. x5400

more important factor in causing vascular lesions in gram negative infections in the bovine than in several other species.

A recent study demonstrated that the lesions of bovine pneumonic pasteurellosis were caused by both neutrophil-independent and -dependent mechanisms (Breider et al., 1988). Endotoxin is potentially involved in both mechanisms. Endotoxin-mediated, neutrophil-independent mechanisms could arise through the endotoxin-mediated release of interleukin-1 (IL-1) and tumor necrosis factor (TNF) from monocytes and macrophages (Cybulsky et al., 1988). Both IL-1 and TNF activate EC, which in turn promote local hypercoagulability of the blood by expressing an overall increase in procoagulant and decrease in anticoagulant activities (Bevilacqua et al., 1985; Bevilacqua et al., 1986; Cotran, 1987). Endotoxin may also amplify this local response by promoting the synthesis and release of additional IL-1 by the EC (Libby et al., 1986). The demonstrated direct toxic effects of P. haemolytica LPS for bovine EC would enhance vascular damage, platelet adhesion, leakage of serum proteins into the alveoli, and activation of the intrinsic clotting mechanism (Slauson and Cooper, 1982). These mechanisms may be important in inducing the thrombosis and fibrinous exudation seen in pneumonic pasteurellosis.

Arachidonic acid metabolites are also likely mediators of some of the pulmonary vascular effects of endotoxin. Thromboxane A_2 causes an early increase in pulmonary arterial pressure and lung lymph flow in experimental endotoxemia (Casey et al., 1982; Brigham , 1985; Malik et al., 1985). However, this effect is transient and may have little influence on the outcome of endotoxemia (Casey et al., 1982; Margolis et al., 1987). An increase in plasma and lung lymph levels of prostacyclin

is associated with a later and more prolonged decrease in pulmonary arterial pressure and increased lung lymph flow and protein clearance (Gunther et al., 1982; Malik et al., 1985; Ogletree et al., 1986). Endothelial cells are considered to be the major source of prostacyclin, and release is stimulated by both endotoxin and IL-1 (Malik et al., 1985; Rossi et al., 1985; Meyrick 1986). The lipoxygenation products, 5- and 12-hydroxyeicosatetraenoic acid (5- and 12-HETE) and leukotrienes (LT) have been implicated as mediators of the vascular permeability associated with endotoxemia (Brigham, 1985; Ogletree et al., 1986). Experimental pneumonic pasteurellosis in newborn calves induced hemodynamic alterations similar to those caused by endotoxemia, especially those associated with prostacyclin and 5- and 12-HETE, which further emphasizes their potential importance in the disease (Alnoor et al., 1986). However, the cause and effect relationship between arachidonic acid metabolites and endotoxin-induced vascular damage remain unclear.

Endotoxin also may be important in neutrophil-dependent mechanisms of damage to the bovine lung. Endotoxin-induced IL-1 and TNF release could promote leukocyte adhesion to EC through expression of specific leukocyte-adhesion proteins on the surface of EC (Bevilacqua et al., 1985; Gamble et al., 1985; Bevilacqua et al., 1987). Also IL-1 acts as a chemotactic factor for neutrophils (Sander et al., 1984). Additionally, endotoxin acts directly on neutrophils to promote vascular adhesion (Haslett et al., 1987). Various lipoxygenase products of arachidonic acid metabolism, especially LTB4, are potent chemotaxins and probably contribute to the neutrophil-dependent effects (Malik et al., 1985). Although P. haemolytica LPS is not directly toxic to bovine leukocytes, it can enhance certain leukocyte functions (Confer and Simons, 1986).

Therefore, the direct effects and the mediation of the effects of endotoxin by IL-1, TNF, and arachidonic acid metabolites may be important factors in both neutrophil-dependent and -independent mechanisms.

In conclusion, P. haemolytica A1 LPS is capable of causing direct damage to bovine pulmonary arterial EC in vitro. This strongly implies a potential role for endotoxin in the pathogenesis of vascular lesions in pneumonic pasteurellosis. This is not the only potential mechanism for the action of LPS in producing lesions in the disease, nor is LPS the only factor produced by P. haemolytica that is likely to play an important role. Continued research into other mechanisms and interactions involved in pneumonic pasteurellosis are needed to further elucidate its pathogenesis and to devise logical approaches for its prevention and treatment.

Summary

Bovine pulmonary artery EC in cell culture were exposed to LPS purified from P. haemolytica serotype A1. This resulted in severe membrane damage which caused a time- and dose-dependent leakage of lactate dehydrogenase that was first detected 4 hours after exposure and reached a maximum of 67% after 24 hours exposure to 1 ug of LPS/ml. Mean release of ^{51}Cr followed a similar pattern and reached a maximum of 61% following 24 hours exposure to 10 ug of LPS/ml. Morphologically, EC responded to LPS by marked cell membrane retraction, the formation of numerous cytoplasmic blebs, and ruffling of the cell membrane. Subsequently, the cells became rounded and detached. Cell-detachment reached a mean of 95% following 8 hours exposure to 1 ug of LPS/ml. These studies demonstrate that P. haemolytica LPS is capable of causing

direct damage to BPAEC which may be important in the pathogenesis of bovine pneumonic pasteurellosis.

CHAPTER III

PASTEURELLA HAEMOLYTICA LIPOPOLYSACCHARIDE-INDUCED ARACHIDONIC ACID-RELEASE FROM AND NEUTROPHIL ADHERENCE TO BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

Introduction

Bovine pneumonic pasteurellosis is a disease having major economic impact on the North American cattle feeding industry (Jensen, 1968). The lesions of bovine pneumonic pasteurellosis, most commonly caused by Pasteurella haemolytica A1, indicate that vascular damage is an important early event in the pathogenesis of the disease. These lesions include alveolar edema, serofibrinous exudation into the alveoli, hemorrhage, microvascular thrombosis, and endothelial cell (EC) swelling (Jensen et al., 1976; Breider et al., 1988). Endotoxin, or bacterial lipopolysaccharide (LPS), has frequently been cited as a potential causative factor of the vascular damage (Jensen et al., 1976; Slocombe et al., 1984; Allan et al., 1985; Breider et al., 1988).

There is little direct evidence for the involvement of endotoxin in pneumonic pasteurellosis. However, indirect evidence is sufficient to warrant investigation. In sheep, Escherichia coli LPS causes pulmonary vascular lesions which are similar to, but less severe than, the early lesions of pneumonic pasteurellosis (Meyrick and Brigham, 1983; Warner et al., 1988). E. coli endotoxemia in calves causes similar effects to

those seen in the sheep relative to hemodynamics and lung mechanics with evidence of pulmonary permeability edema (Olson and Brown, 1985). Experimental P. haemolytica pneumonia in newborn calves also causes hypoxemia, neutropenia, and decreased vascular response to hypoxia, which are similar to those in the sheep endotoxin model (Hutchinson et al., 1985; Alnoor et al., 1986). However, differences exist including decreases in pulmonary artery pressure and pulmonary vascular resistance in the calf pneumonia versus increases in the endotoxemic sheep (Esbenshade et al., 1982; Alnoor et al., 1986). Intravenously administered P. haemolytica LPS causes increased plasma levels of the arachidonic acid (AA) metabolites, thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$, and prostaglandin $F_{2\alpha}$, in sheep similar to that seen with E. coli LPS (Demling et al., 1981; Emau et al., 1984)

In our laboratory, the effects of LPS extracted from P. haemolytica A1 have been examined using bovine pulmonary artery endothelial cells (BPAEC) in cell culture. We found direct LPS-induced toxicity resulting in cell detachment, severe morphologic alterations, and evidence of cell lysis (Chapter II). The study reported herein was designed to investigate P. haemolytica LPS-induced functional alterations in BPAEC by effects on neutrophil adherence and AA-release. These alterations may be indicative of endothelial activation, an important event in the inflammatory response (Cotran, 1987), which may amplify or mediate the vascular damage of pneumonic pasteurellosis.

Materials and Methods

Lipopolysaccharide

Lipopolysaccharide was extracted from P. haemolytica A1 by a hot

phenol-water method (Westphal and Jann, 1965; Confer and Simons, 1986). The resulting LPS contained 32.3 ug of 2-keto,3-deoxyoctanate/mg dry weight and had an endotoxin activity of 1.3×10^5 EU/mg as determined by a chromogenic Limulus amebocyte lysate assay.[a] Protein content was less than 0.1 ug/ml.

Endothelial Cells

Bovine pulmonary artery endothelial cells were obtained commercially[b] or isolated using a modification of previously described techniques (Ryan et al., 1980). Briefly, pulmonary arteries were obtained from calves at a local abattoir. They were suspended in cold phosphate buffered saline solution (PBSS) containing gentamycin (100 ug/ml) and transported on ice. The arteries were washed 3 times then suspended in PBSS with gentamycin for 1 hour at 4C. After rewarming the solution and arteries in a 37C water bath, the arteries were slit open and the luminal surface lightly scraped once with a sterile scalpel blade. The cells were suspended in Medium 199 with Earl's salts and bicarbonate[c] containing thymidine[c] (10^{-5} M), getamincin[c] (50 ug/ml), fugizone[c] (5 ug/ml), glutamine[c] (0.2 M), and 10% conditioned medium (complete medium) with 20% heat-inactivated fetal bovine serum (FBS).[d] The cells were pelleted at 1000 xg, 10 minutes, 4C. They were resuspended in complete medium with 20% FBS and transferred to a 25 cm^2 tissue culture flask. After 2 days incubation at 37C, 5% CO_2, the cells were harvested with

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[a]Whittaker MA Bioproducts, Inc., Walkersville, MD.
[b]ATCC, CCl-209, American Type Culture Collection, Rockville, MD.
[c]Gibco Laboratories, Inc., Grand Island, NY.
[d]Hyclone Laboratories, Logan, UT.

trypsin/EDTA, counted, and transferred to 96-well tissue culture plates[e] at a concentration of about 5 cells/well. Pure cultures of BPAEC were chosen based on the typical cobblestone morphology and later verified by positive indirect immunofluorescent staining for Factor VIII-related antigen.[e] The EC were grown in progressively larger tissue culture plates up to 75 cm^2 tissue culture flasks (usually requiring 3 passages). The concentration of FBS subsequently was reduced to 15% then 10% on successive passages.

Monolayers of both lines of BPAEC were maintained in 75 cm^2 tissue culture flasks in complete medium with 10% FBS. All cultures were incubated at 37C in 5% CO_2 and medium was changed twice weekly. The commercial cell-line was used at passages 17 through 20. The primary cell-line was used at passages 7 and 8.

Arachidonic Acid-Release Assay

Confluent monolayers of BPAEC in 24-well plates[f] were labeled with ^{3}H-arachidonic acid (AA)[g] by the addition of 0.2 uCi AA/well in 1 ml of complete medium with 10% FBS and incubation at 37C in 5% CO_2 for 48 hours (Suttorp et al., 1985b). The radiolabeled monolayers were washed 3 times in medium 199. Complete medium with 10% FBS with or without inhibitors was added (400 ul/well) and incubated for 15 minutes at 37C. The inhibitors included hydrocortisone[h] (1 mg/ml) and indomethacin[h] (5 uM to 5mM[i]). Four ul of a 100-fold concentration of LPS were added to

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[e]Behring Diagnostics, La Jolla, CA.
[f]Nunclon, A/S Nunc, Kamstrup, Denmark.
[g]New England Nuclear, Boston, MA.
[h]Sigma LD-L, Sigma Diagnostics, St. Louis, MO.
[i]In complete media at 37C, 5 mM indomethacin is a saturated solution and, therefore, the molarity is approximate.

each well. Baseline controls received no LPS. The treated monolayers were incubated at 37C in 5% CO_2 for up to 8 hours. Following incubation, the medium was removed and centrifuged in a microcentrifuge[j] for 2 minutes at 10,000 rpm. A scintillation fluor[k] was added to 300 ul of the supernatant and counts per minute (cpm) of radioactivity were determined.[l]

Neutrophil Isolation

Neutrophils were isolated from fresh, citrated bovine blood by differential sedimentation and hypotonic lysis (Clinkenbeard et al., in press). The blood was centrifuged at 700 xg, 20C for 45 minutes. The plasma and buffy coat were removed and discarded. Hank's balanced salt solution without calcium, magnesium, and bicarbonate (HBSS)[c] was added [1:1 (vol:vol)] to the remaining packed cells. A 2:1 (vol:vol) quantity of lysing buffer (7.4 ml of 0.2 M NaH_2PO_4, 19.0 ml of 0.2 M Na_2HPO_4, 373.6 ml water, pH 7.2) was added to the packed cells in HBSS, gently agitated, and centrifuged at 700 xg, 20C, 15 minutes. The pellet was resuspended in 3 ml HBSS. Six ml of lysing buffer were added with gentle agitation. Three ml of a restorative buffer (2.7% NaCl in lysing buffer) were added, and the cells were pelleted at 700 xg, 20C, 15 minutes. The cells were resuspended in 1 ml HBSS or 1 ml RPMI 1640[c], counted, diluted to 10^7 cells/ml, and held at room temperature for use later the same day (2-3 hours). A differential count was performed to assess purity. This procedure consistently yielded over 90% neutrophils.

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[j]Surspin, Model 7040, Helena Laboratories, Beaumont, TX.
[k]Ecolite, ICN Biomedicals Inc., Irvine, CA.
[l]Tri-Carb 300, Packard Instrument Co., Laguna Hills, CA.

Neutrophil Adherence Assay

Neutrophil adherence to BPAEC was assessed by the rose bengal stain method (Gamble and Vadas, 1988). Briefly, 10^4 BPAEC/well were placed in 96-well, flat-bottom dishes[f] and grown for 24 hours establishing a subconfluent monolayer. The media was removed from selected wells and replaced with 200 ul of inhibitors in medium 199 with 5% FBS. Inhibitors were also added to aliquots of neutrophils and the BPAEC and neutrophils were incubated for 15 minutes at 37C. Inhibitors used included cycloheximide[h] (10 ug/ml), actinomycin D[h] (10 ug/ml), and polymyxin B[h] (10 ug/ml). After incubation, the media was removed from each well and replaced with 200 ul of medium 199 with 5% FBS or the same containing various doses of LPS with or without inhibitors. Concurrently, various doses of LPS were added to appropriate aliquots of neutrophils in HBSS. Neutrophils and BPAEC were incubated for 1 or 2 hours at 37C. Neutrophils were centrifuged for 5 minutes, 2,000 rpm[j], and resuspended in medium 199 with 5% FBS. To each well of BPAEC, 5 x 10^5 neutrophils were added and coincubated for 30 minutes, 37C. Media and nonadherent neutrophils were removed and each well was rinsed once in medium 199 with 5% FBS. Rose bengal stain[h] (100 ul, 0.25% in PBSS) was added to each well at room temperature for 5 minutes. All wells were washed 3 times in PBSS and examined with a light microscope for a visual estimate of adherence. Fifty percent ethanol in PBSS (200 ul/well) was added and gently agitated at room temperature for 30 minutes. Optical density at 570 nm wavelength (OD_{570}) was determined with an ELISA reader.[m] Results were reported as increased optical density (OD_{570} test - OD_{570} of wells

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[m]EIA Reader, EL307, Bio-Tek Instruments, Inc., Burlington, VT.

containing BPAEC only). The inhibition index was calculated by the following formula:

$$(1 - \frac{(OD_T - OD_C)}{(OD_L - OD_C)}) \times 100$$

where OD_T is the optical density reading of the individual inhibitor test, OD_C is the mean optical density of the unstimulated control, and OD_L is the mean optical density of the LPS-stimulated neutrophil adherence without the inhibitor. Since this assay was not quantitative, the number obtained was relative to, but not identical to, the percent inhibition of LPS-induced neutrophil adherence resulting from the effect of the inhibitor.

Statistics

The arachidonic acid-release experimental data were analyzed for unequal variance by the F-test. The means were compared by the appropriate Student's t test. The neutrophil adherence data were analyzed by the least significant difference test; only pre-planned comparisons were considered (Steele and Torrie, 1980). Results were considered significant if $P \leq 0.05$.

Results

Arachidonic Acid-Release

Labeling of BPAEC monolayers with 0.2 mCi of ^{3}H-AA per well for 48 hours resulted in 75 + 1.5% incorporation of the radioactivity. Release of radioactivity from the labeled monolayers after exposure to *P. haemolytica* LPS was time- and LPS dose-dependent (Tables III and IV). Release

TABLE III

^{3}H-ARACHIDONIC ACID-RELEASE FROM ENDOTHELIAL CELLS FOLLOWING EXPOSURE TO *P. HAEMOLYTICA* LIPOPOLYSACCHARIDE

Test	1 hour[a]	2 hour[a]	4 hour[a]	8 hour[a]
LPS[b]	2009 ± 279 (12)	4408 ± 386 (12)[e]	6292 ± 432 (13)[e]	11865 ± 948 (13)[e]
Indomethacin[c]	1772 ± 176 (10)	2629 ± 285 (10)[f]	3057 ± 523 (9)[f]	6220 ± 835 (9)[f]
Hydrocortisone[d]	1832 ± 191 (8)	3953 ± 493 (8)[e]	7369 ± 964 (9)[e]	9119 ± 958 (8)[e]
Control	1437 ± 149 (13)	2612 ± 198 (13)	2612 ± 305 (13)	5085 ± 305 (11)

[a]Results are expressed in mean counts per minute ± SEM (n).
[b]*P. haemolytica* LPS 0.1 ug/ml.
[c]Indomethacin, 5 mM, with 0.1 ug LPS/ml.
[d]Hydrocortisone, 1 mg/ml, with 0.1 ug LPS/ml.
[e]Significant release compared to baseline ($p < 0.05$).
[f]Significant inhibition of release compared to LPS ($p < 0.05$).
LPS = lipopolysaccharide.

TABLE IV

DOSE-RESPONSE OF P. HAEMOLYTICA LPS-INDUCED ^{3}H-ARACHIDONIC ACID-RELEASE FROM ENDOTHELIAL CELLS

Dose (ug LPS/ml)	4 hours[a]	8 hours[a]
10	8501 ± 109 (4)[b]	9803 ± 2685 (3)
1	8252 ± 368 (4)[b]	9000 ± 1257 (5)[b]
0.1	8229 ± 147 (4)[b]	9588 ± 937 (7)[b]
0.01	5880 ± 404 (4)	6685 ± 545 (6)[b]
0.001	5029 ± 139 (4)	5500 ± 579 (5)
0	4825 ± 243 (3)	4517 ± 760 (4)

[a]Results are expressed as mean counts per minute ± SEM (n).
[b]Significant release compared to control.
LPS = Lipopolysaccharide.

was consistently elevated after one-hour exposure to 0.1 ug LPS/ml, averaging 1.4 times the baseline, and increased to 2.3 times the baseline after 8 hours. Significant release was detectable with a LPS dose of 0.01 ug/ml and was maximal with a dose of 0.1 ug/ml. The ^{3}H-AA-release was inhibited by 5 mM indomethacin[i] but not by hydrocortisone (1 mg/ml). Also, indomethacin doses of 500 uM to 5 uM were ineffective in inhibiting release (data not shown).

Neutrophil-Adherence Assays

Neutrophil-adherence to BPAEC subconfluent monolayers was increased

after exposure of either cell type or both to P. haemolytica LPS (Figure 7). Neutrophils were generally more sensitive than BPAEC to LPS-induced neutrophil-adherence. The minimum dose causing increased adherence was 0.1 ug LPS/ml on neutrophils (4 of 5 experiments) and 1 ug LPS/ml on BPAEC (4 of 5 experiments). Treatment of both cell types resulted in increased adherence over treatment of either cell type individually (3 of 4 experiments). Essentially no difference in LPS dose-response was seen by increasing the LPS exposure time from 1 to 2 hours, and no difference in dose-response was seen between the commercial BPAEC-line and the cell-line established in this laboratory.

The effects of various inhibitors on the LPS-induced neutrophil-adherence to BPAEC are shown (Table V). Treatment of either neutrophils or BPAEC with either polymyxin B, cycloheximide, or actinomycin D markedly inhibited neutrophil adherence to BPAEC.

Discussion

P. haemolytica LPS caused a time- and dose-dependent release of radioactivity from BPAEC which had incorporated ^{3}H-AA. This suggests LPS-induced EC activation with the release of AA metabolites, such as prostaglandins, thromboxanes, or lipoxygenase products, or damage to BPAEC resulting in the release of unaltered AA. The release was inhibited by 5 mM indomethacin^{i} but not inhibited by 5 - 500 uM indomethacin or 1 mg hydrocortisone/ml.

Previous experiments examining the effects of thrombin on EC indicated that the release of radioactivity from EC which had incorporated tritiated AA was highly correlated to prostaglandin-release (Lollar and Owen, 1980). However, under altered conditions, i.e., in the presence of

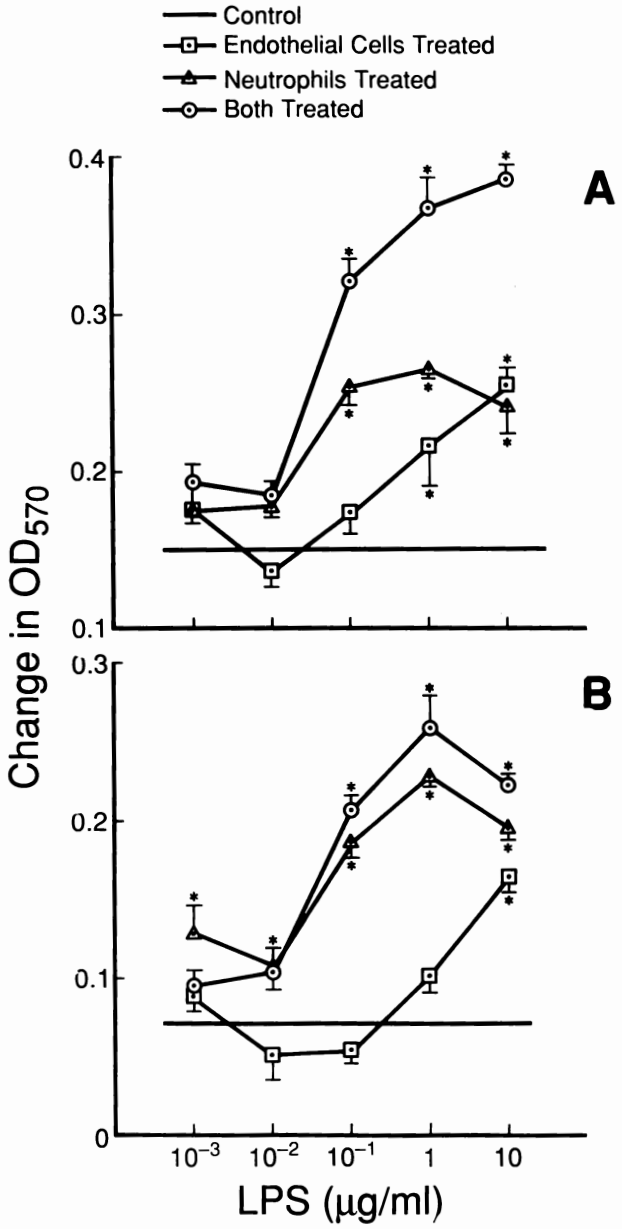

Figure 7. P. haemolytica LPS-induced increase in optical density at 570 nm wavelength caused by adherent neutrophils stained with Rose Bengal.
A. Endothelial cell-line established in our laboratory. LPS treatment time was 2 hours.
B. Commercial endothelial cell-line. LPS treatment time was 1 hour.
*Significant difference from control, $p < 0.05$.

TABLE V

INHIBITION OF LPS-INDUCED NEUTROPHIL ADHERENCE TO BPAEC

Cell-Type Treated	Inhibition Index[a] Cycloheximide (10 ug/ml)	Actinomycin D (10 ug/ml)	Polymyxin B (10 ug/ml)
Neutrophil	93 ± 20	106 ± 17	72 ± 16
BPAEC	132 ± 16	94 ± 17	60 ± 12

[a]Expressed as mean inhibition index ± standard error of the mean, n=16.
LPS = Lipopolysaccharide.
BPAEC = Bovine pulmonary artery endothelial cells.

0.1% albumin, up to 90% of the AA was released unaltered. The release of prostaglandins, but not AA, was inhibited by 20 uM indomethacin. In contrast, the release of AA-radioactivity from EC exposed to Pseudomonas aeruginosa cytotoxin in the presence of albumin was highly correlated to prostacyclin-release (Suttorp et al., 1985). Further studies of the thrombin-induced release of arachidonate from EC defined three enzymes, phospholipase A_2, a triglyceride lipase, and phospholipase C, which were involved in the release. Arachidonate was subsequently liberated from the cells either unchanged or as an AA-metabolite, predominantly prostacyclin (Thomas et al., 1986).

In this experiment, the failure of hydrocortisone to inhibit AA-release indicated that the release did not involve phospholipase A_2 (Blackwell et al., 1978). Furthermore, failure of 5 uM indomethacin to inhibit the AA-release indicated that the released product was not a

cyclooxygenase metabolite (Moncada et al., 1976). Together, these findings would be inconsistent with a prostaglandin-release, but would indicate the release of AA by another mechanism. This mechanism may involve either activation of one of the aforementioned enzymes or a direct LPS-cell membrane interaction. We have previously shown that LPS causes severe damage to the BPAEC membrane resulting in the release of lactate dehydrogenase (Chapter II). The inhibition of AA-release by 5 uM indomethacin[i] was most likely independent of cyclooxygenase inhibition. Comparable concentrations of indomethacin have had stabilizing effects on red blood cell membranes and lysosomal membranes, and similar effects may be responsible for the inhibition reported herein (Brown et al., 1971; Ignaro, 1971).

Pasteurella haemolytica LPS also stimulated neutrophil-adherence to EC by independent effects on both cell types. The effect was dose-dependent and required protein synthesis and mRNA transcription in both cell types. Inhibition by polymyxin B indicated that the increased adherence was caused by the lipid A portion of the LPS molecule.

Neutrophils are probably important in the pathogenesis of pneumonic pasteurellosis. Vascular sequestration of neutrophils was a prominent early event in experimental disease (Slocombe et al., 1984). Additionally, neutrophil-depletion of calves resulted in either moderate or marked decrease in the severity of pulmonary lesions in experimental pneumonic pasteurellosis (Slocombe et al., 1985; Breider et al., 1988). Similarly, neutrophil-depletion resulted in an attenuation of the vascular permeability seen in endotoxemia in sheep (Heflin and Brigham, 1981). In vitro experiments with human EC revealed that an induceable surface molecule, ELAM-1, was responsible for neutrophil adherence. Expression

of ELAM-1 was induced by E. coli LPS and various cytokines and was inhibited by actinomycin D and cycloheximide (Bevilacqua et al., 1985; Bevilacqua et al., 1987). Neutrophil adhesion to human EC also occurred when neutrophils were stimulated by E. coli LPS, complement fragments, or tumor necrosis factor, but these neutrophil-dependent effects were not inhibited by cycloheximide or actinomycin D (Tonnesen et al., 1984; Gamble et al., 1985; Haslett et al., 1987). A mechanism involving the CDw18 complex on the neutrophil was demonstrated in LPS-induced neutrophil adherence to human EC (Pohlman et al., 1986). Therefore, the data reported herein suggests a similar mechanism involved in the EC-mediated neutrophil adherence induced by P. haemolytica LPS acting on BPAEC as that induced by E. coli LPS acting on the human EC. However, the neutrophil-mediated adherence may involve different mechanisms requiring de novo protein synthesis in the bovine neutrophil.

In conclusion, this study provides additional evidence of the potential involvement of LPS in the pathogenesis of pneumonic pasteurellosis. Neutrophil adherence could be essential for the neutrophil infiltration seen in pneumonic pasteurellosis and P. haemolytica LPS was capable of causing adherence in vitro by independent effects on both cell types. Arachidonic acid release from BPAEC, whether it indicates the release of AA metabolites or, more likely, the release via other mechanisms, was also induced by P. haemolytica LPS. Both of these findings may indicate EC activation which would have greater ramifications in the inflammatory and immune responses of pneumonic pasteurellosis. Further research is needed to verify endothelial activation by P. haemolytica LPS and its relevance to pneumonic pasteurellosis.

CHAPTER IV

PASTEURELLA HAEMOLYTICA LIPOPOLYSACCHARIDE-INDUCED CYTOTOXICITY IN BOVINE PULMONARY ARTERY ENDOTHELIAL MONOLAYERS: INHIBITION BY INDOMETHACIN

Introduction

Pneumonic pasteurellosis (shipping fever) is a severe respiratory disease of young cattle. The etiology is multifactorial and includes physical stressors as well as viral and bacterial infections. The most common and severe disease results from pneumonia caused by *Pasteurella haemolytica* A1 (Schiefer et al., 1978; Wikse, 1985). The lesions of pneumonic pasteurellosis, which include serofibrinous exudation, hemorrhage, microvascular thrombosis, and endothelial cell (EC) swelling, indicate that vascular damage is an important pathogenetic factor (Jensen et al., 1976; Breider et al., 1988).

Endotoxin, or bacterial lipopolysaccharide (LPS), is often cited as a potential causative factor of the vascular damage of pneumonic pasteurellosis (Jensen et al., 1976; Slocombe et al., 1984; Breider et al., 1988). There is little direct evidence for LPS's pathogenic role in pneumonic pasteurellosis. However, intravascular *Escherichia coli* LPS causes pulmonary vascular lesions in sheep similar to, but less severe than, those of pneumonic pasteurellosis (Meyrick and Brigham, 1983). Changes in lung mechanics and hemodynamics in calves given intravenous

E. coli LPS are sufficiently similar to those changes in sheep to indicate that E. coli LPS may cause similar lesions in both species (Olson and Brown, 1985). Also, LPS from P. haemolytica and E. coli cause similar increases in plasma prostanoids and serotonin when administered intravenously in sheep (Emau et al., 1984; Brigham, 1985). Therefore, indirect evidence justifies the investigation of LPS in pneumonic pasteurellosis.

We have shown that P. haemolytica LPS induces direct toxic effects in bovine pulmonary artery endothelial cells (BPAEC). The indicators of toxicity included cell detachment, morphologic alterations, and release of large internal molecules indicating cell membrane damage (Chapter II). The study reported herein was designed to further evaluate the LPS-induced morphologic alterations in BPAEC and to determine if the cytotoxic effects could be inhibited, thereby suggesting potential mechanisms of LPS-induced BPAEC cytotoxicity.

Materials and Methods

Endothelial Cells

Monolayer cultures of BPAEC[a] were maintained in 75 cm^2 tissue culture flasks in Medium 199 with Earle's salts[a] containing 4.35 g $NaHCO_3$/l, 10^{-5} M thymidine,[b] 50 ug gentamicin[b]/ml, 5 ug fungizone[b]/ml, and 0.2 M glutamine,[b] with 10% conditioned media and 10% heat-inactivated fetal bovine serum (FBS)[c] (Complete medium). Cell cultures were incubated at 37 C in 5% CO_2, and medium was changed twice weekly. Mono-

[a]CCl-209, American Type Culture Collection, Rockville, MD.
[b]Gibco Laboratories, Inc., Grand Island, NY.
[c]Hyclone Laboratories, Logan, UT.

layers of BPAEC were grown in 24-well plates[d] for the lactate dehydrogenase-leakage and transmission electron microscopy experiments and were grown in 25 cm^2 tissue culture flasks for phase-contrast light microscopy. Cell cultures used for scanning electron microscopy were grown on 7 mm diameter coverslips which were rendered pyrogen-free by dry heat (200C, 4 hours) and placed in 24-well plates.

Lipopolysaccharide

Lipopolysaccharide was extracted from P. haemolytica A1 by a hot phenol-water method (Westphal and Jann, 1965; Confer and Simons, 1986). The resulting LPS contained 32.3 ug of 2-keto,3-deoxyoctanate/mg dry weight and had an endotoxin activity of 1.3×10^5 EU/mg as determined by a chromogenic Limulus amebocyte lysate assay.[e] Protein content was less than 0.1 ug/ml.

Lactate Dehydrogenase (LDH)-Leakage

Confluent BPAEC monolayers were pulse-treated with LPS and selected monolayers were either pulse or continuously treated with inhibitors according to the following protocol. All cultures were grown to confluency in 24-well plates. The monolayers were washed once with RPMI without phenol red,[b] containing 5% FBS (RPMI/F). Each well received 500 ul RPMI/F with or without inhibitors. The plates were incubated for 30 minutes (37C, 5% CO_2). The medium was removed and each well received 500 ul of the same medium (RPMI/F with or without inhibitors respectively) which, with exception of the control wells, also contained 1 ug of

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[d]Nunclon Multidish, A/S Nunc, Kamstrup, Denmark.
[e]Whittaker MA Bioproducts, Inc., Walkersville, MD.

LPS/ml. After an additional 30 minutes incubation (37C, 5% CO_2), all wells were washed 3 times and 500 ul of medium was replaced in each well. The wash and replacement medium consisted of RPMI/F with or without inhibitors. Incubation was continued at 37C in 5% CO_2. After incubation, the medium was removed, centrifuged, and the supernatant tested for LDH-activity by a spectrophotometric method as previously described (Chapter II). Statistical analysis of results was performed on preplanned comparisons by Student's t tests (Steele and Torrie, 1980). Results are reported as mean ± standard error of the mean.

Inhibitors

Several chemicals were examined for inhibitory effects on the P. haemolytica LPS-induced LDH-leakage from BPAEC. The protein synthesis inhibitor, cycloheximide[f] (10 ug/ml); the mRNA transcription inhibitor, actinomycin D[f] (4 ug/ml); the cytoskeletal inhibitors, cytochalasin B[f] (3 ug/ml) and colchicine[f] (1 uM); the acid vesicle inhibitors, chloroquine[f] (0.2 mM) and ammonium chloride[f] (10 mM); the energy inhibitor, 2-deoxyglucose[f] (50 mM) with sodium azide[f] (5 mM); hydrocortisone[f] (1 mg/ml); indomethacin[f] (5-5000 uM); and polymyxin B[f] (10 ug/ml) were used. Percent inhibition was determined by the formula:

$$(1 - \frac{I-C}{L-C}) * 100$$

in which I is the LDH-leakage (IU/L) from BPAEC exposed to inhibitor and LPS, L is the LDH-leakage from BPAEC exposed to LPS, and C is the control LDH-leakage from BPAEC exposed to neither LPS nor inhibitor.

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[f]Sigma Chemical Co., St. Louis, MO.

Phase Contrast Microscopy

An inverted phase contrast microscope[g] was prewarmed in a 37C 5% CO_2 incubator. Confluent monolayers of BPAEC in 25 cm^2 tissue culture flasks were washed once in complete medium, and the medium was replaced with 4 ml of complete medium containing 1 ug of LPS/ml. A field suitable for viewing was selected, and a time equals zero photomicrograph was taken. The cell culture was left undisturbed at 37C in 5% CO_2 and photomicrographs were taken at on-half hour intervals.[h] Indomethacin-treated monolayers were preincubated with 5 mM indomethacin[i] in complete medium for 15 minutes, 37C, 5% CO_2. Sufficient LPS was added to make the medium 1 ug of LPS/ml and photomicrographs were taken as before.

Scanning Electron Microscopy

Confluent BPAEC monolayers were grown on 7 mm diameter coverslips in 24-well plates. All wells were rinsed in complete medium. Selected wells were exposed to 5 mM indomethacin[h] in complete medium for 15 minutes at 37C in 5% CO_2 with the remaining wells receiving complete medium only. After incubation, LPS was added with swirling to the appropriate wells to give a final concentration of 1 ug of LPS/ml. Incubation was at 37C in 5% CO_2 for 1, 2, 4, and 8 hours. The monolayers were fixed in situ for 1 hour in 2.5% glutaraldehyde and were dehydrated in serial ethanol rinses. The coverslips were removed then critical point dried by ethanol-CO_2 solvent exchange[j], mounted on aluminum studs, and sputter-

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[g]CK2, Olympus Optical Co., LTD, Tokyo, Japan.
[h]PM-10AD 16mm-cine, Olympus Optical Co., LTD, Tokyo, Japan.
[i]In complete medium at 37C, 5 mM indomethacin is a saturated solution and, therefore, the molarity is approximate.
[j]Samdri Pvt3 Critical Point Dryer, Tousimis Research Corp, Rockville, MD.

coated with gold-palladium.[k] The specimens were examined with a scanning electron microscope[l] with the specimen tilted at a 45 degree angle to the electron beam.

Transmission Electron Microscopy

Confluent BPAEC monolayers were washed once then 5 mM indomethacin[i] in complete medium was placed in selected wells with the remainder receiving complete medium only. The monolayers were incubated for 15 minutes at 37C in 5% CO_2. LPS (1 ug/ml) was added to wells with and without indomethacin. Incubation was at 37C in 5% CO_2 for predetermined time periods minus 5 minutes. The monolayers were removed by scraping with a rubber policeman, suspended in their original medium, and transferred to 1.5 ml microcentrifuge tubes. The cells were pelleted in a microcentrifuge[m] at 2000 rpm for 5 minutes. The supernatant was removed and the cell pellets were fixed in 1% osmium tetroxide in cacodylate buffer for 1 hour. The pellets were rinsed in cacodylate buffer, dehydrated through serial alcohol rinses and propylene oxide, then embedded in DER.[n] Sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope.[o]

Results

LDH-Leakage

The effects on LPS-induced LDH-leakage was examined in the presence

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[k]Hummer II, Technics Inc., Alexandria, VA.
[l]JSM 35-U, Jeol Ltd., Tokyo, Japan.
[m]Surspin, Model 7040, Helena Laboratories, Beaumont, TX.
[n]Electron Microscopy Sciences, Fort Washington, PA.
[o]Jeol 100CXII, Jeol LTD, Tokyo, Japan.

of several inhibitory substances (Table VI). Inhibition of LDH-leakage was demonstrated in the presence of polymyxin B (10 ug/ml) and indomethacin (5 mM). Further experiments determined that a concentration of indomethacin of 500 uM or greater was required to inhibit the LPS-induced LDH-leakage from BPAEC (Table VII).

TABLE VI

THE EFFECTS OF INHIBITORY CHEMICALS ON THE LPS-INDUCED LEAKAGE OF LDH FROM BPAEC[a]

Inhibitor	LDH-Leakage (IU/L)	
	Continuous[b]	Pulse[c]
Cycloheximide (10 ug/ml)	25.9	26.5
Actinomycin D (4 ug/ml)	26.1	24.9
Cytochalasin B (3 ug/ml)	24.9	22.4
Colchicine (1 uM)	24.6	23.3
Chloroquine (0.2 mM)	40.0[d]	27.6
Ammonium Chloride (10 mM)	22.0	24.0
2-Deoxyglucose (50 mM) with Sodium Azide	23.8	29.7
Hydrocortisone (1 mg/ml)	24.2	26.1
Indomethacin (5 mM)	NA[e]	14.7
Polymyxin B (10 ug/ml)	12.5	12.6

[a]Results are from one of three replicates. All were pulse-exposed to LPS (1 ug/ml) for 30 minutes, then LDH-leakage determined after 6 hours. LPS resulted in LDH-leakage of 24.8 ± 0.5 (n=3). Control release was 14.5 ± 0.2 (n=3).

[b]Inhibitor remained in medium for 6-hour duration.

[c]Inhibitor was removed 30 minutes after LPS-exposure.

[d]Increased LDH-leakage was caused by 0.2 mM chloroquine alone.

[e]5 mM indomethacin interfered with test assay.

TABLE VII

INHIBITION OF LPS-INDUCED LDH-LEAKAGE FROM BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS BY INDOMETHACIN

Indomethacin Concentration (um)	Percent Inhibition[a]	
	Continuous[b]	Pulse[c]
5000[d]	NA[e]	89 ± 6[f]
500	88 ± 3[f]	65 ± 11[f]
50	8 ± 6	0
5	0	10 ± 6

[a]Expressed as mean percent inhibition ± standard error (n=4). LPS concentration was 1 ug/ml.
[b]Indomethacin remained for duration of incubation, 6 hours.
[c]Indomethacin was removed 30 minutes after LPS exposure (see Materials and Methods).
[d]Saturated at 37C, molarity is approximate.
[e]5000 uM indomethacin in assay supernatant interfered with LDH assay.
[f]Significant difference from control, $p < 0.05$.

Phase Contrast Microscopy

By phase-contrast light microscopy BPAEC retraction and detachment was seen within 1.5 hours after exposure to LPS and became progressively greater through 16 hours. Sequential photomicrographs revealed that the retraction and detachment occurred rapidly with the cells appearing essentially normal then retracted and out of the plane of focus one-half hour later (Figure 8). Cell detachment resulted in severe defects in the integrity of the monolayer leaving large gaps between adjacent cells.

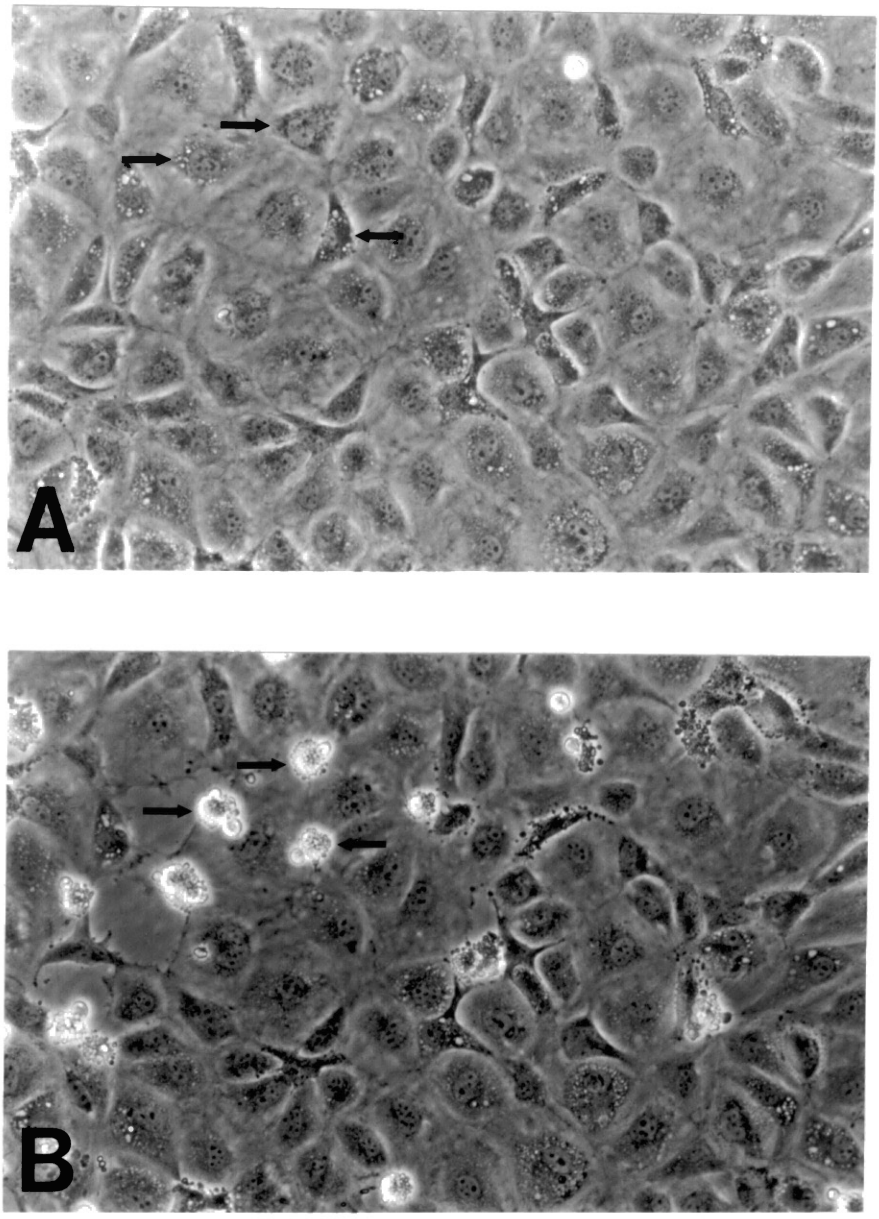

Figure 8. Phase contrast photomicrographs of BPAEC monolayers after exposure to P. haemolytica LPS.

A. After 1 hour.
B. After 1.5 hours.
Arrows denote cells which are apparently normal (A) and severely retracted with blebs 0.5 hours later (B).
Original magnification 200x.

Indomethacin treatment markedly reduced but did not completely halt cell detachment (Figure 9). In LPS- and indomethacin-treated BPAEC, cell detachment initially occurred 5 hours after LPS-exposure and was moderate by 16 hours after exposure. In contrast to the LPS-treated cells, the indomethacin- and LPS-treated cells appeared to spread and undermine the detaching cell, and the monolayer integrity was preserved for 10-12 hours after LPS-exposure.

Scanning Electron Microscopy

By scanning electron microscopy, the normal endothelial monolayer was formed by polygonal to angular cells with a mounded surface. The nucleus was surrounded by a zone of large membrane pits or pores. The periphery of the cell was thin and flat and tightly adjoined neighboring cells with occasional, small gaps along the junctions (Figure 10). After 1 hour of exposure to LPS, the junctional gaps were more frequent and enlarged. Occasional cells had numerous, small blebs over their surfaces. After 2 hours of LPS-exposure, contracted cells which had severe, large, membrane bleb formation were common. These retracting cells left large defects in the monolayer integrity. Adjacent cells often appeared essentially normal (Figure 11). Similar changes affected more cells with time resulting in retraction with membrane bleb formation then detachment. By 8 hours after LPS-exposure, detachment was marked (Figure 12).

Indomethacin pretreatment resulted in a marked reduction of the LPS-induced effects. After 2 hours of LPS-exposure, the indomethacin-treated monolayers were indistinguishable from controls (Figure 13). Between 4 and 8 hours after exposure mild morphologic changes were seen

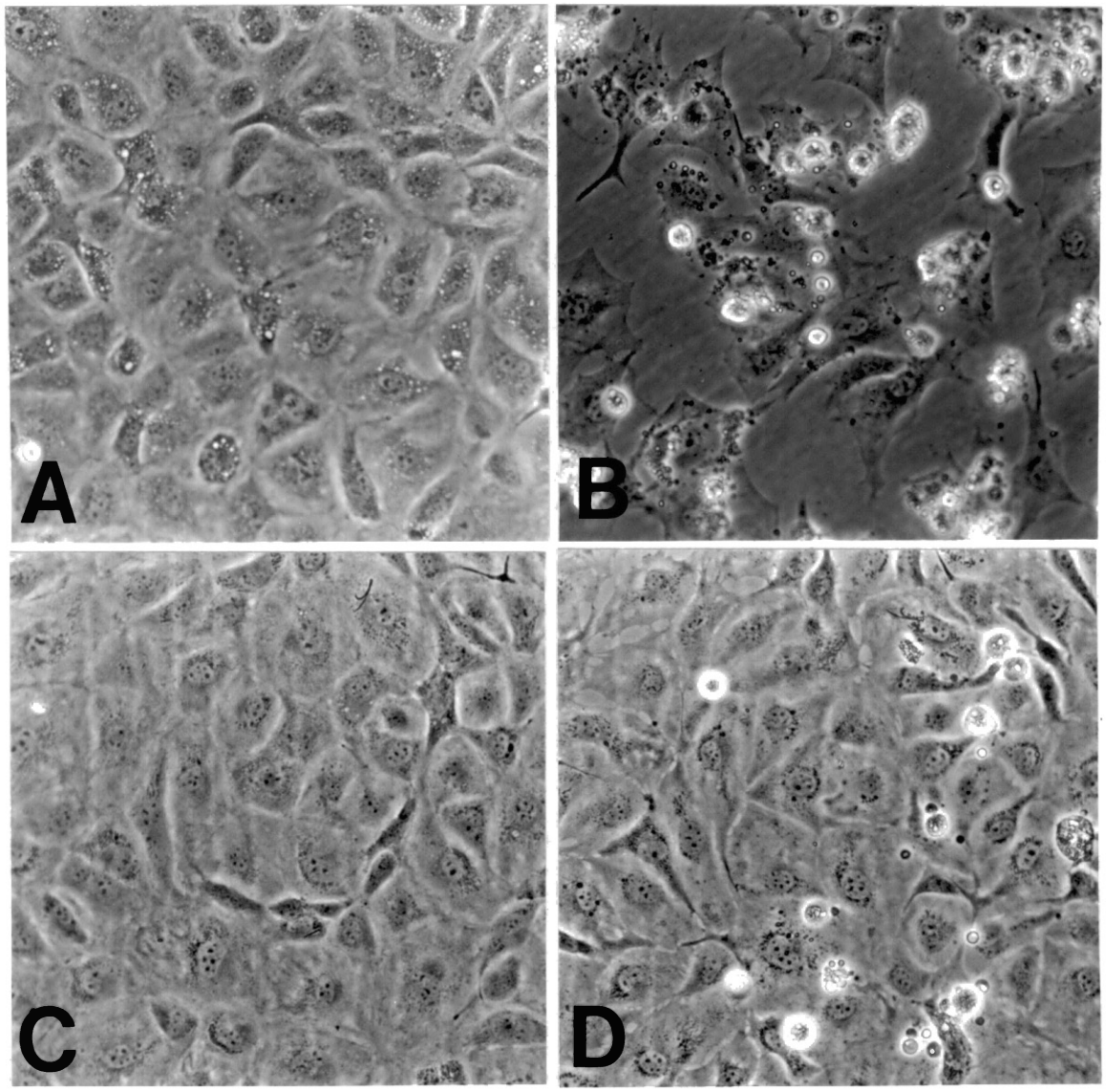

Figure 9. Phase contrast photomicrographs of BPAEC monolayers after exposure to LPS demonstrating the protective effect of indomethacin.

A. LPS-treated BPAEC at 0 hours.
B. LPS-treated BPAEC after 8 hours.
C. Indomethacin- and LPS-treated BPAEC at 0 hours.
D. Indomethacin- and LPS-treated BPAEC at 8 hours.
Note marked inhibition of cell retraction with preservation of the monolayer integrity.
Original magnification 200x.

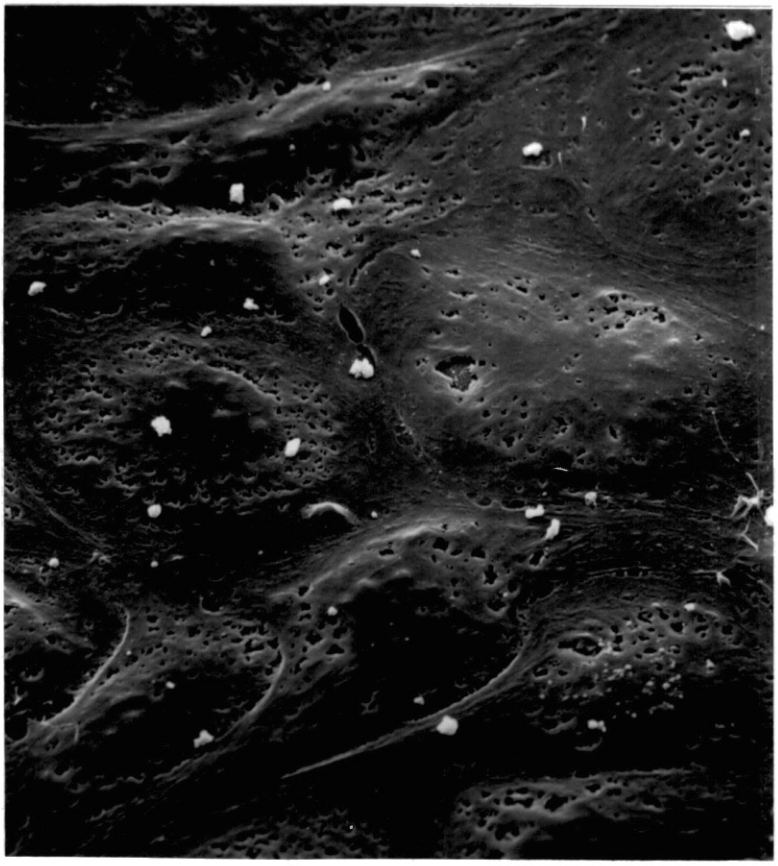

Figure 10. Scanning electron micrograph of control BPAEC monolayer comprised of polygonal cells with mounded surface and perinuclear pits or pores. Original magnification 940x.

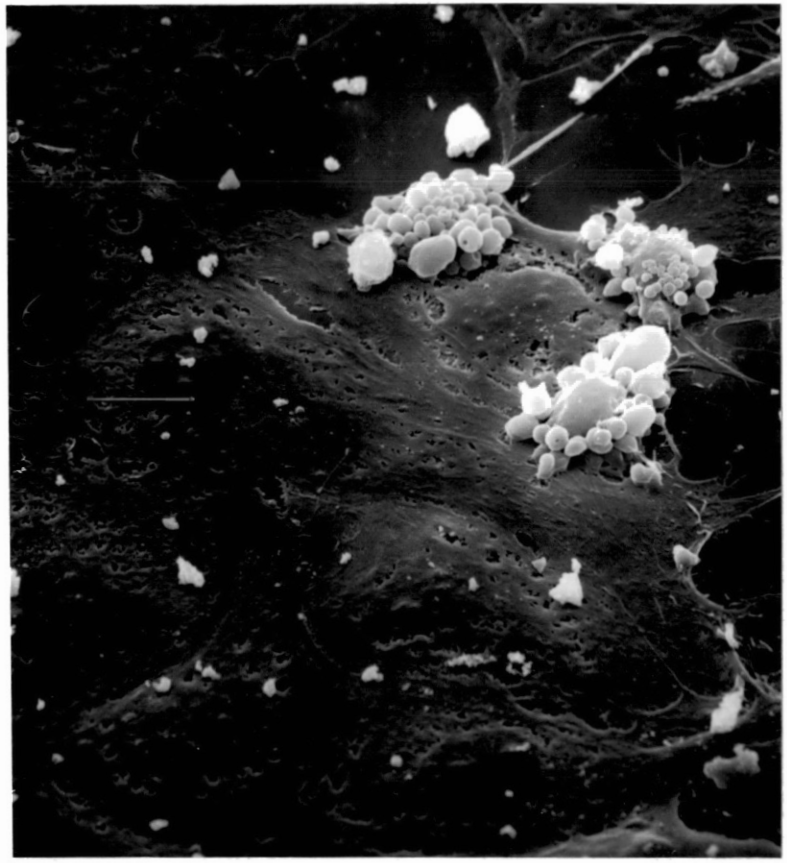

Figure 11. Scanning electron micrograph of BPAEC after 2 hours exposure to P. haemolytica LPS. Essentially normal monolayer is adjacent to cells which have severely retracted with marked membrane bleb formation.
Original magnification 940x.

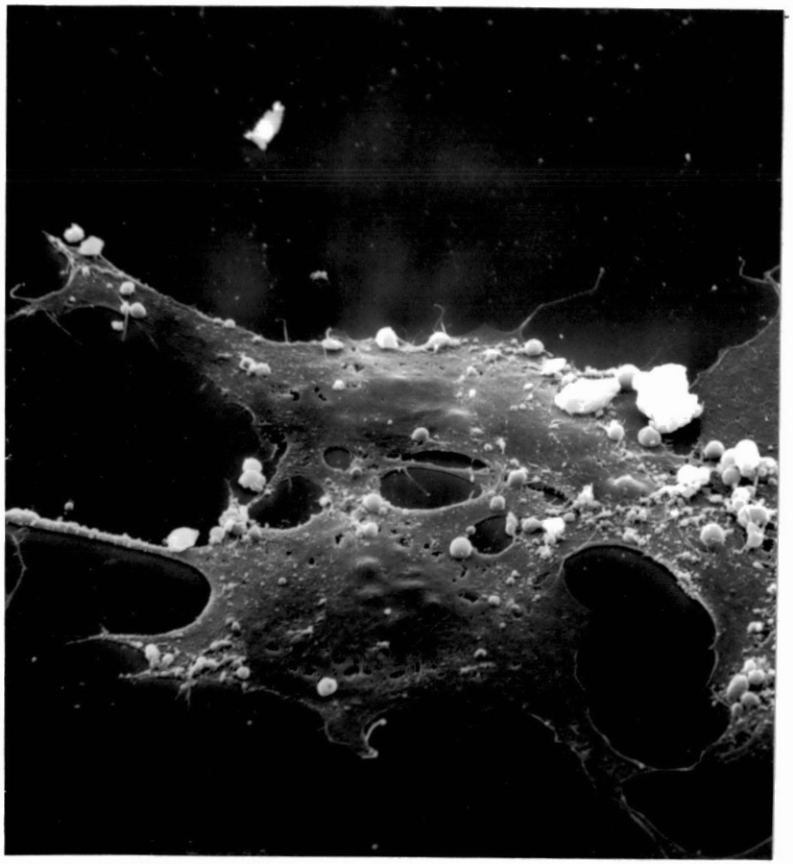

Figure 12. Scanning electron micrograph of BPAEC after 8 hours exposure to P. haemolytica LPS. Occasional cells remain with variable degrees of alteration separated by expanses of empty glass.
Original magnification 940x.

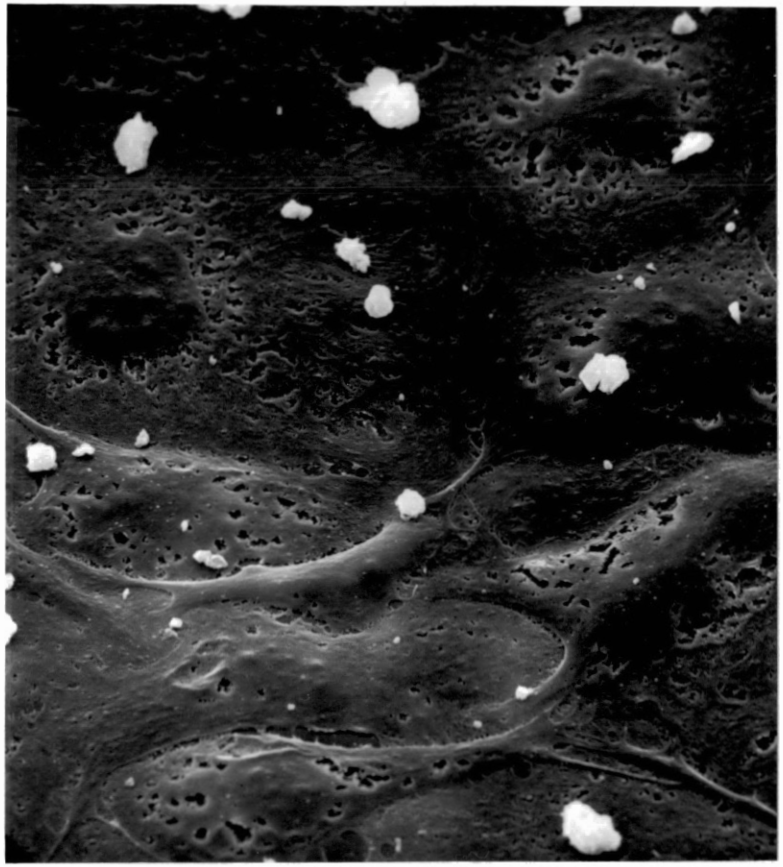

Figure 13. Scanning electron micrograph of indomethacin-treated BPAEC after 2 hours exposure to P. haemolytica LPS. Indistinguishable from control.
Original magnification 940x.

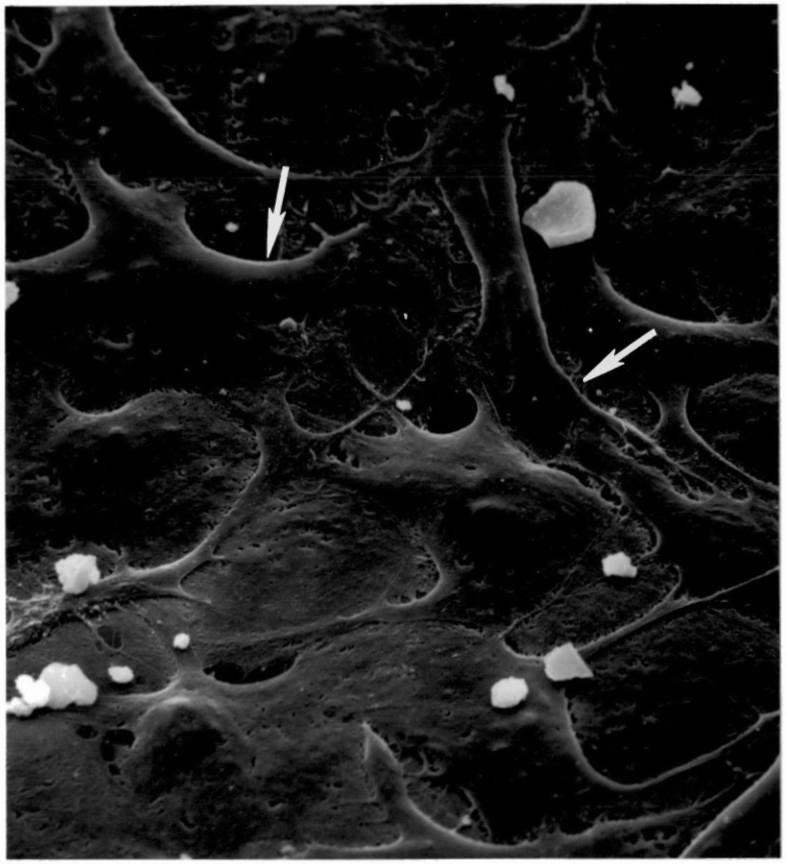

Figure 14. Scanning electron micrograph of indomethacin-treated BPAEC after 8 hours exposure to LPS. Note increased angularity of cells, some of which have raised apparently undermined edges (arrows).
Original magnification 940x.

in the EC (Figure 14). Some of the cells became more stellate in appearance and cell processes lying between or overlying adjacent cells were prominent. The edges of some of the cells were rounded and raised with the adjacent cell undermining that edge. However, the monolayer integrity was preserved.

Transmission Electron Microscopy

By transmission electron microscopy, the normal BPAEC monolayer was a uniform population of cells which were polygonal to elongate depending on the plane of the cut (Figure 15). Membranes of adjacent cells were closely apposed and occasional areas of membrane interdigitation were seen. Plasmalemmal vesicles or caveolae were moderately abundant along the cell junctions and cell surfaces. The cytoplasm was well supplied with mitochondria and rough endoplasmic reticulum (RER). Multivesicular bodies were common (5 or less/cell). Occasionally, autophagosomes with myelin figures were present. Nuclei were oval and centrally located and the nuclear membrane was usually, but not consistently, invaginated.

After 30 minutes of exposure to P. haemolytica LPS, the majority of cells remained essentially normal; however, some exhibited extensive changes. These included moderately to severely swollen mitochondria, moderate dilatation of the RER, rarefied cytoplasm, and occasional karyolytic nuclei.

After 1 hour of LPS exposure, the cellular changes were marked (Figure 16). All cells remained joined into a monolayer but had a mosaic pattern of alternating light, relatively normal, and dark cells. The light cells were the predominant cell type and had extensively dilated RER and markedly swollen mitochondria. The cytoplasm was electron

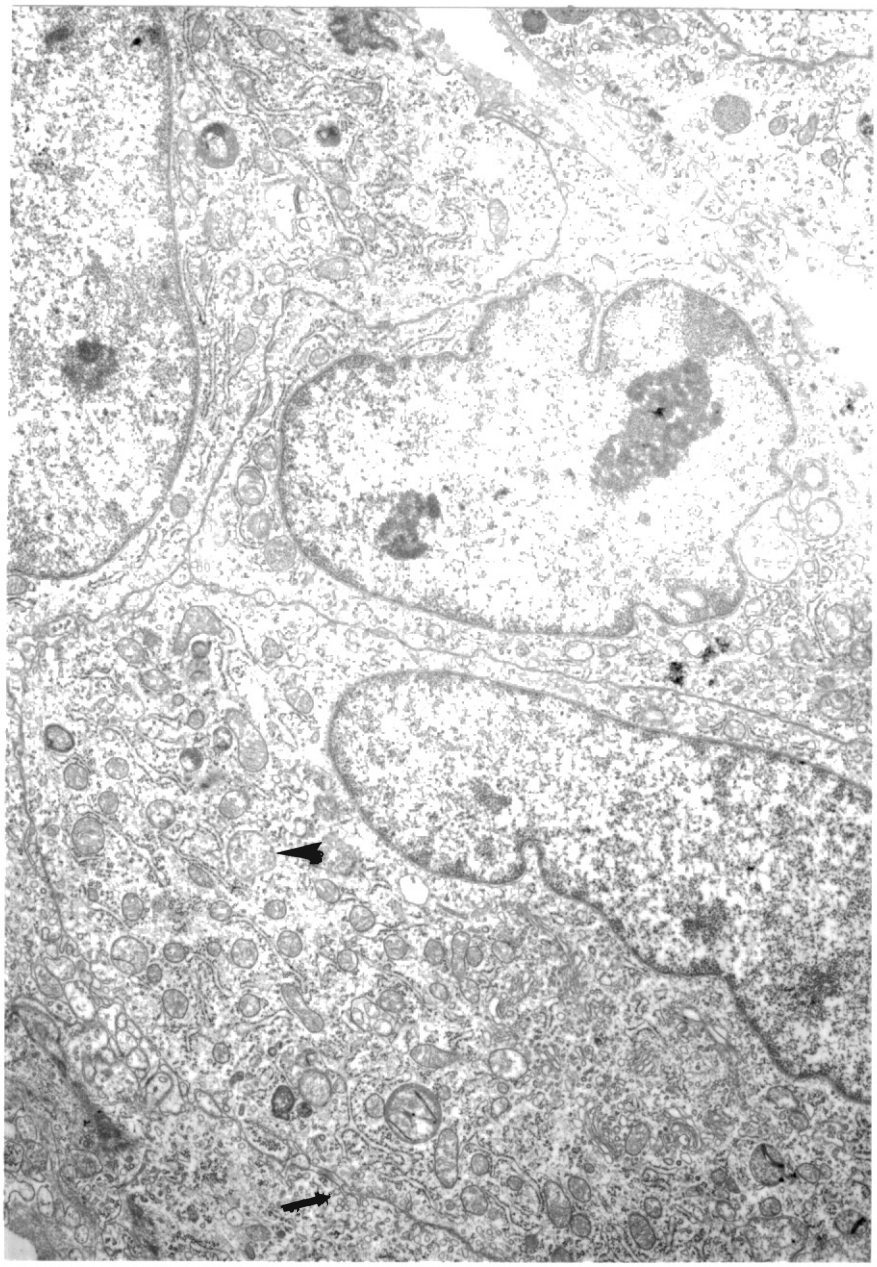

Figure 15. Transmission electron micrograph of normal BPAEC monolayer. All junctions are closely apposed with occasional interdigitations. Nuclei are smooth to multiply indented. Plasmalemmal vesicles are plentiful (arrow) and multivesicular bodies are common (arrowhead).
Original magnification 4800x.

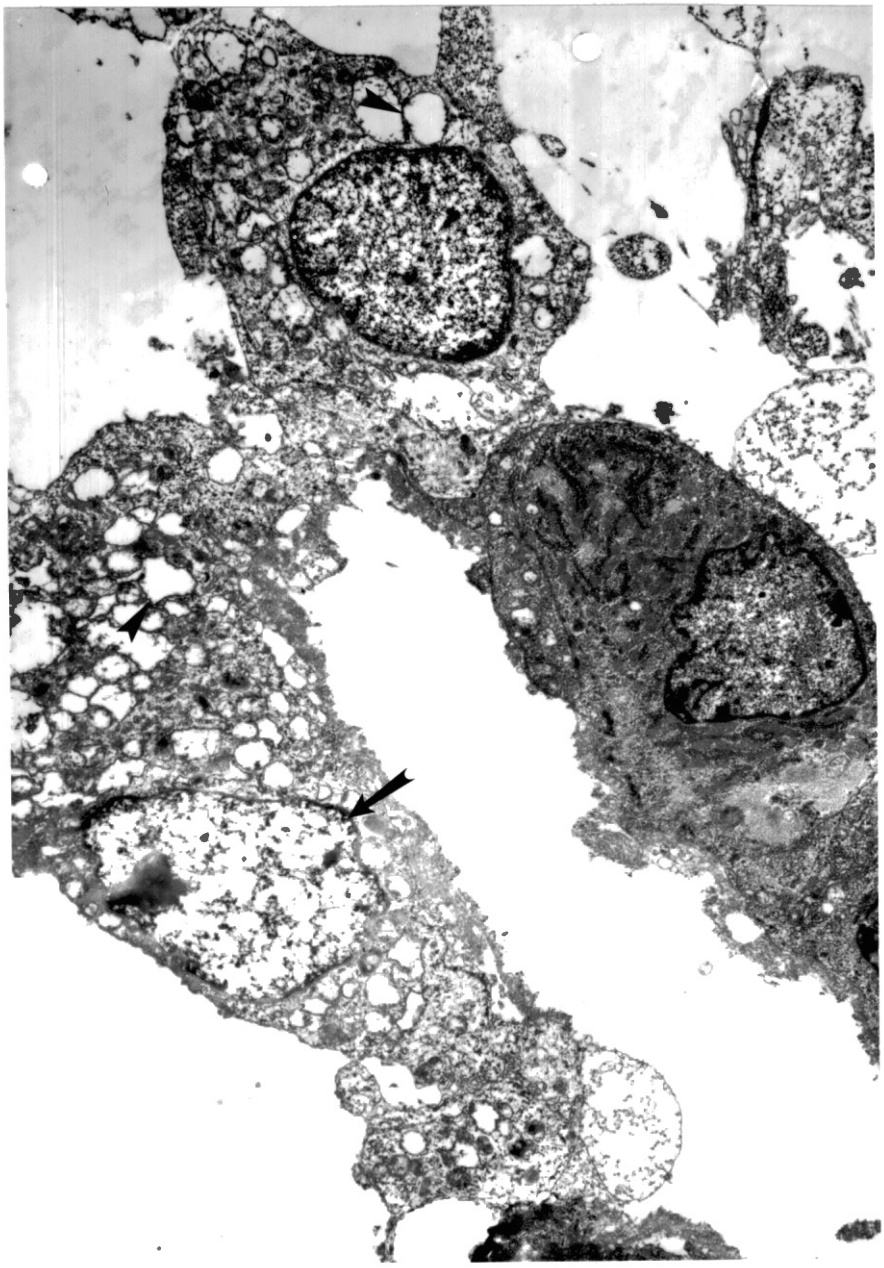

Figure 16. Transmission electron micrograph of BPAEC after 1 hour exposure to LPS. Note the alternating pattern of light and dark cells. Light cells have severely dilated RER (arrowheads) and karyolysis (arrow). Original magnification 2900x.

lucent. Nuclei were enlarged and karyolytic. The outer nuclear membrane was wavy resulting in irregular dilatations of the nuclear envelope. The cytoplasmic membrane had an irregular contour and contained many indistinct foci. The dark cells had dense, granular cytoplasm with multifocally condensed intermediate filaments. The RER was minimally to mildly dilated. Mitochondria were relatively normal. The nuclei were crenated, but the nuclear membranes were distinct and normal. The cytoplasmic membrane was distinct and predominantly smooth with occasional projections or outpocketings. More normal cells were the minority and were characterized by sporadic mitochondrial swelling and dilated RER.

After 2 hours of exposure to LPS, multifocal disruptions of the monolayer were common. Dilatations or gaps in cell junctions were pronounced (Figure 17). The dark cells were more numerous and many had become rounded with an indistinct membrane. Nuclear crenation was more severe. A few cells were completely detached from surrounding cells and exhibited extensive formation of membrane-bound blebs. Many of the blebs contained organelles. The centers of these cells were dense due to karyorrhectic and cytoplasmic debris. The light cells remained the predominant cell type with changes similar to those described at 1 hour. Relatively normal cells were infrequent.

After 4 hours of LPS exposure, the cell junctions were markedly compromised resulting in severe fragmentation of the monolayers (Figure 18). Dark cells, which were unattached and had severe cytoplasmic bleb formation, were common (Figure 19). The cell membrane of the light cells were markedly irregular with pseudopodia, clefts, and membrane-bound blebs occurring frequently.

Treatment of BPAEC with indomethacin inhibited some, but not all,

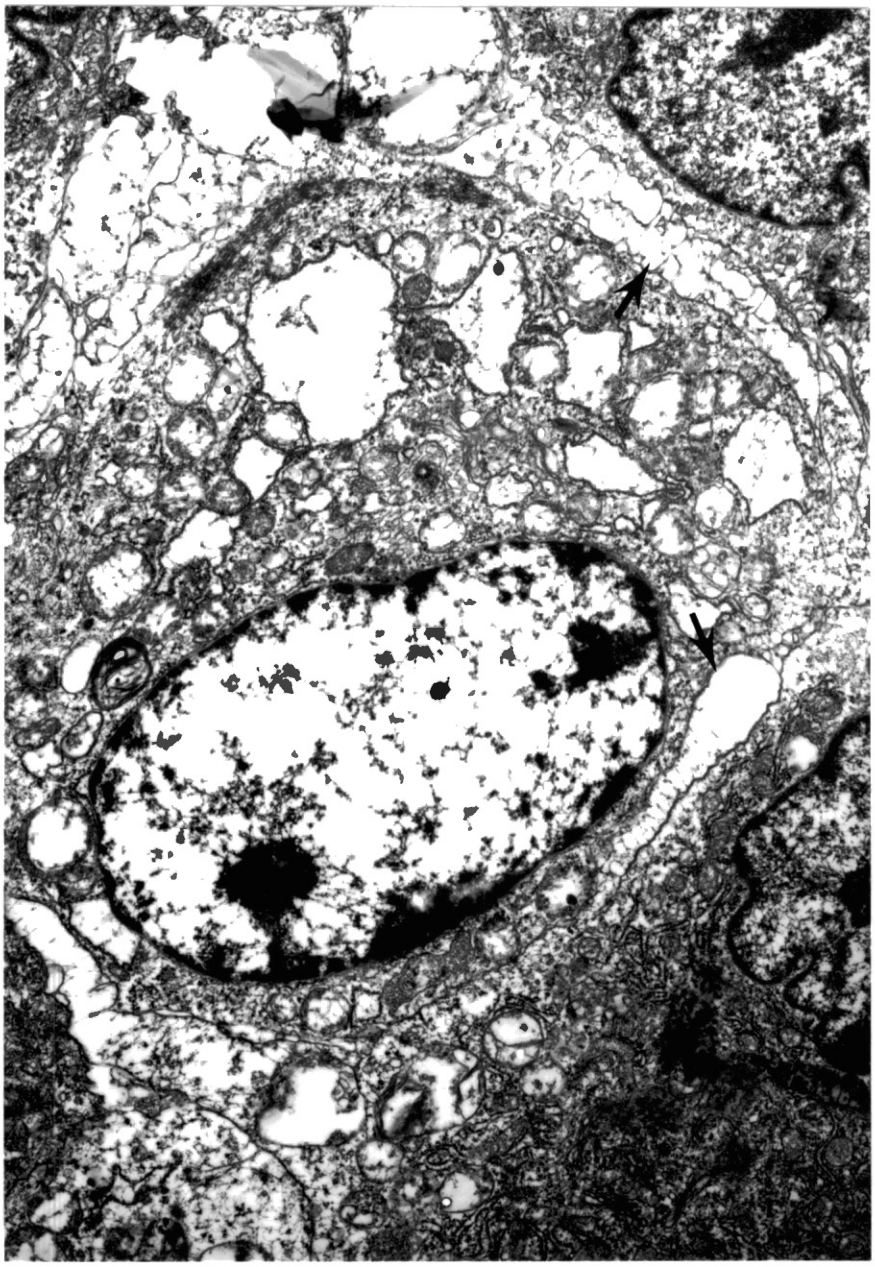

Figure 17. Transmission electron micrograph of BPAEC after 2 hours exposure to LPS. Separations of cell junctions are prominent (arrows). Marked mitochondrial swelling and dilatation of RER is seen with karyolysis.
Original magnification 5800x.

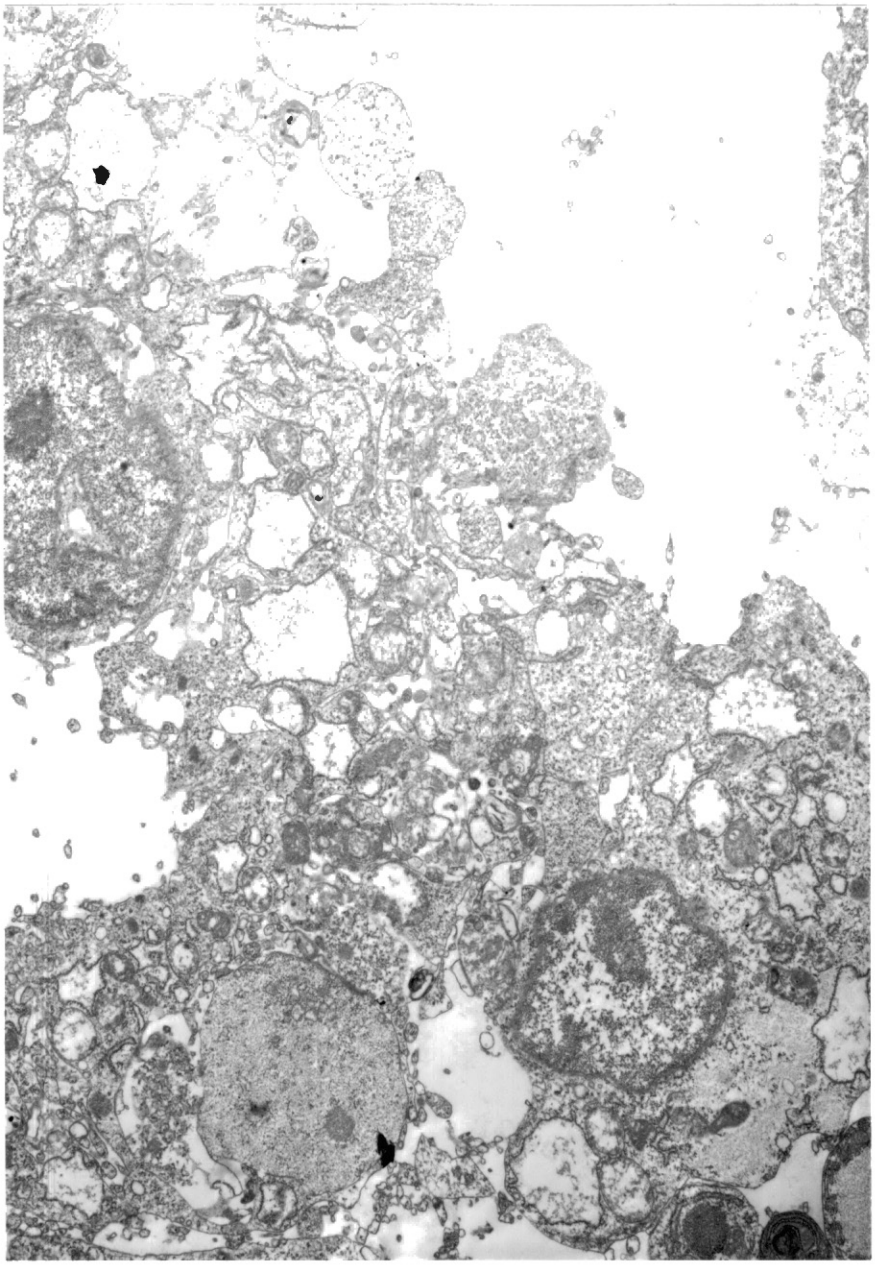

Figure 18. Transmission electron micrograph of BPAEC after 4 hours exposure to LPS. Extreme distortion of the cell outline is due to marked cytoplasmic membrane irregularity.
Original magnification 3600x.

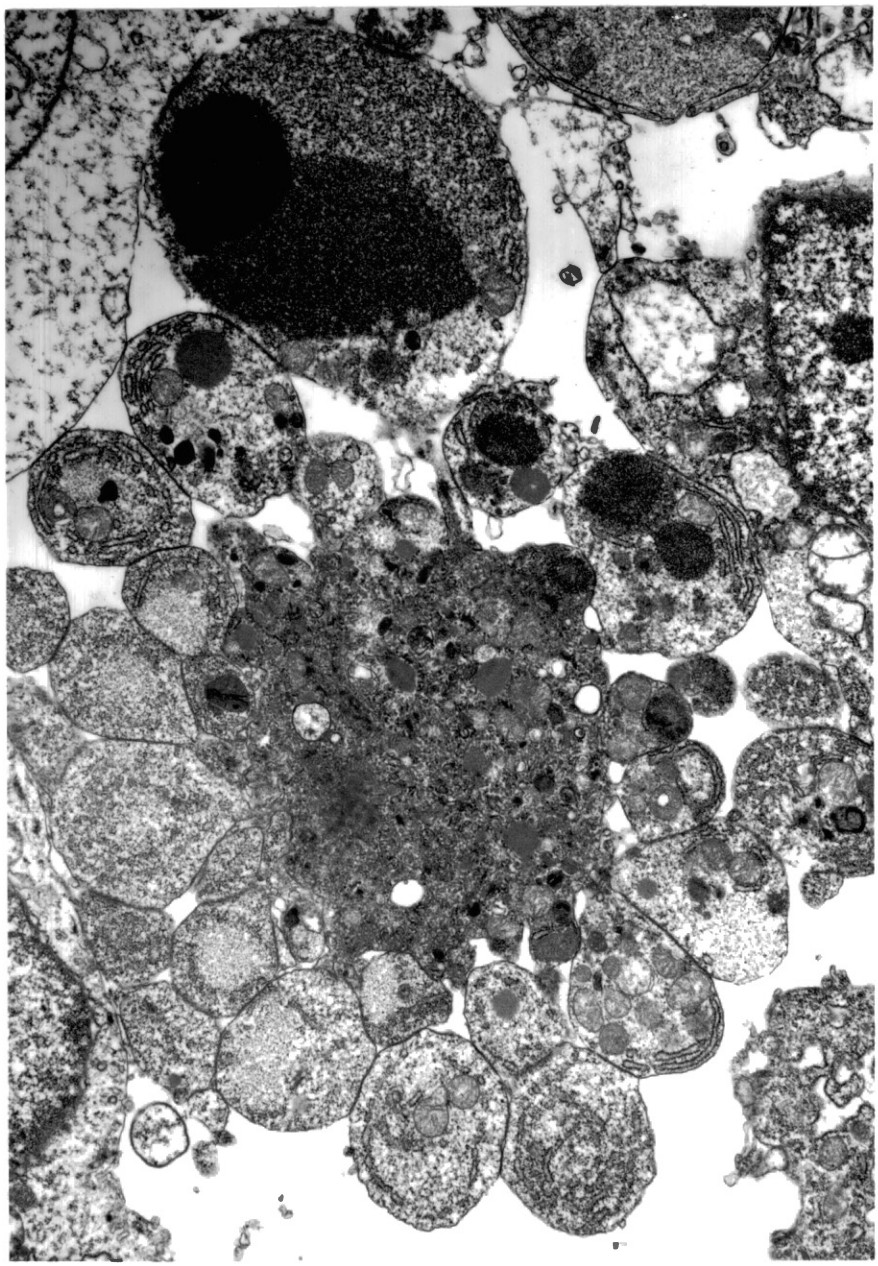

Figure 19. Transmission electron micrograph of BPAEC after 4 hours exposure to LPS. The cell is detached from the monolayer and has marked formation of membrane-bound blebs, many of which contain recognizable organelles. Nuclear detail is completely lost.
Original magnification 5800x.

of the LPS-induced changes (Figure 20). Dilatation of the RER, mitochondrial swelling, and mitochondrial condensation were seen. However, the cytoplasmic membrane remained distinct with an even contour. All junctions remained tightly apposed. The nuclei and nuclear membrane remained normal after 4 hours LPS-exposure. Control BPAEC, treated with indomethacin only, contained a moderate decrease in the cytoplasmic electron density with occasional mild dilatation of the RER. The mitochondrial were normal. The changes were mild compared to those in BPAEC treated with both LPS and indomethacin.

Discussion

As in our previous studies, Pasteurella haemolytica LPS induced marked morphologic alterations and LDH-leakage in BPAEC (Chapter II). In the present study, a striking feature of LPS-induced cytotoxicity was that individual EC varied markedly in their susceptibility and response. As early as one-half hour after LPS-exposure, a few cells had marked changes observed by transmission electron microscopy, whereas after 16 hours, a few normal appearing cells remained as determined by phase contrast microscopy. A typical morphologic reaction of BPAEC to P. haemolytica LPS might be summarized as follows. Within a few minutes to several hours after exposure to LPS, the EC reacts with dilatation of the RER and mitochondrial swelling. Soon after, separations form in the cell junctions. These progressively enlarge while the cell membrane becomes increasingly irregular in contour. At some critical point the cell contracts forming numerous cytoplasmic membrane-bound blebs. The contraction leaves a large defect in the monolayer and results in the cell detachment.

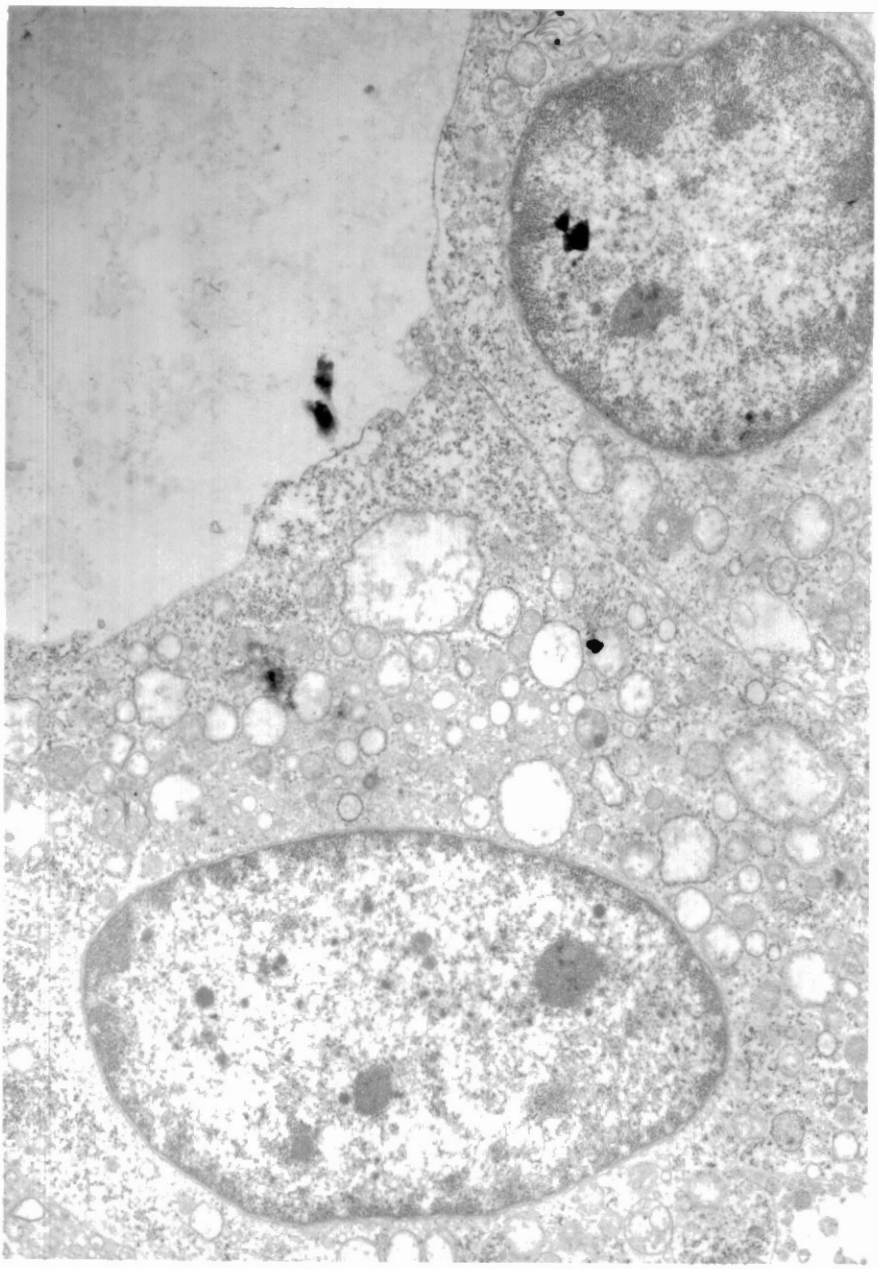

Figure 20. Transmission electron micrograph of indomethacin-treated BPAEC after 2 hours exposure to LPS. Nuclei are normal. The cell junctions are closely apposed. Mitochondrial swelling and dilatation of the RER are seen.
Original magnification 3600x.

Variations in this sequence were seen. A prominent variation involved the formation of the dark cell with a condensed, granular cytoplasm. A dark cell/light cell phenomenon has been described that was attributed to alterations in cellular hydration, with excessive dehydration yielding dark cells (reviewed in Ghadially, 1982). The alterations in hydration were attributed to fixation artifacts, the physiologic state of hydration at the time of fixation, or cellular pathology rendering the cell more susceptible to dehydration during tissue processing. In the present experiments, the dark cells were likely pathological, because they were not present in control monolayers. The response of the nucleus was also quite variable. Karyorrhexis, nuclear crenation, and nuclear swelling with karyolysis were seen. However, the P. haemolytica LPS-induced morphologic alterations were nonspecific lesions of cellular degeneration and did not, of themselves, suggest specific mechanisms of LPS cytotoxicity.

Indomethacin at high concentrations (500 uM or greater) inhibited LDH-leakage from BPAEC and also inhibited many of the morphological changes. The monolayer integrity of untreated BPAEC exposed to P. haemolytica LPS was compromised within 1.5 hours with large defects where cells had retracted and detached. However, indomethacin-treated monolayers retained closely apposed cell junctions with few gaps for up to 12 hours after LPS-exposure. The major effects of indomethacin appeared to involve preservation of the nucleus and the cytoplasmic membrane and prevention of the cytoplasmic membrane bleb formation. Prevention of LDH-leakage also indicates preservation of cell membrane integrity (Chopra et al., 1987). Also, the dark cells were not seen in the indomethacin-treated LPS-exposed BPAEC. However, swelling of the mitochon-

dria and dilatation of the RER were similar in indomethacin-treated and -untreated, LPS-exposed BPAEC.

The mechanism of the indomethacin inhibition of LPS-induced morphologic alterations and LDH-leakage in this study is uncertain. Indomethacin apparently did not block LPS-binding because intracellular changes, i.e., dilatation of the RER and mitochondrial swelling, were similar in the indomethacin-treated and -untreated cells, and these changes were not attributable to a direct effect of indomethacin. Additionally, Schorer et al. (1985) found that 5 mM indomethacin did not inhibit E. coli LPS- or lipid A-induced tissue factor production in EC further indicating that LPS-binding is not inhibited. Indomethacin is primarily used as an inhibitor of the cyclooxygenase pathway of arachidonic acid metabolism. However, the concentrations of indomethacin required in the present study were much higher than those described as required for specific cyclooxygenase inhibition (Moncada et al., 1976). Therefore, cyclooxygenase inhibition is not likely to be the primary mechanism involved in the present study.

Indomethacin was reported to stabilize red blood cell membranes to hyperthermic lysis, and stabilization required concentrations similar to those reported herein (Brown et al., 1971). Morphologic evidence in the present study supports membrane stabilization as a mechanism, because membrane changes, both cytoplasmic and nuclear, were inhibited despite changes in organelles. The indomethacin-induced membrane changes, which required relatively high concentrations compared to cyclooxygenase-inhibition, may be due to lysosomal stabilization, protein stabilization, or inhibition of enzymes such as diglyceride lipase, phospholipase A_2, or protein kinase (Grant et al., 1970; Iguarro, 1971; Catalan et

al., 1980; Franson et al., 1980; Rittenhouse-Simmons, 1980). Additionally, indomethacin has a calcium-antagonistic effect in EC which inhibits cell-retraction (Northover, 1977). This may have prevented the LPS-induced retraction and possibly the bleb formation reported herein.

Despite extensive effort, the mechanisms of LPS-induced toxicity are poorly understood. Past reports of LPS-induced cytotoxicity in BPAEC have demonstrated inhibition only with inhibitors of LPS-binding, such as polymyxin B (Harlan et al., 1983). This report demonstrates that indomethacin inhibits the LPS-induced effects of the leakage of LDH, loss of monolayer integrity, and morphologic alterations in the cell membrane and nucleus of BPAEC.

In conclusion, P. haemolytica LPS induces morphologic alterations in BPAEC in cell culture which result in loss of monolayer integrity. If similar effects occur in vivo, they might be important factors in the exudation of fibrin and microvascular thrombosis seen in pneumonic pasteurellosis. Inhibition of some of the LPS-induced effects in BPAEC may provide clues for further investigation into the thus far elusive nature of LPS toxicity.

CHAPTER V

SUMMARY AND CONCLUSIONS

Fibrinous pneumonia caused by Pasteurella haemolytica A1 is the most frequent and serious consequence of pneumonic pasteurellosis (shipping fever) of young cattle. The lesions indicate that vascular damage is an important, early event in the pathogenesis of pneumonic pasteurellosis. Endotoxin [bacterial lipopolysaccharide (LPS)] has often been implicated as an important causative factor of the vascular damage. This research was designed to test the hypothesis that P. haemolytica LPS is a factor in the pathogenesis of the vascular lesions of pneumonic pasteurellosis. The test model employed was bovine pulmonary artery endothelial cells (BPAEC) in cell culture.

The results reported in this work demonstrate LPS-induced cytotoxicity in BPAEC. LPS resulted in time- and dose-dependent cell detachment and LDH- and ^{51}Cr-leakage. The leakage of LDH and ^{51}Cr indicates cell membrane defects large enough to permit the loss of large internal molecules and is widely regarded as evidence of cell lysis. Morphologic studies demonstrated that LPS induced severe changes in BPAEC. Individual cells were variable in their sensitivity to LPS, but, with some exceptions, followed a predictable series of morphologic changes. Swelling of the mitochondria and dilatation of the rough endoplasmic reticulum were followed by progressively enlarging gaps in the cell junctions. At some critical point, marked cell retraction with severe membrane bleb

formation was followed by detachment. Arachidonic acid was also liberated from LPS-treated BPAEC in a time- and dose-dependent manner. This release probably was an index of cell membrane damage and not due to increased prostanoid synthesis. The LDH-leakage, morphologic alterations, and arachidonic acid-release were inhibited by indomethacin at a concentration of 0.5 to 5 mM. In addition, LPS induced increased neutrophil adherence to BPAEC by independent effects on both cell-types. Increased adherence was prevented by treatment of either cell-type by inhibitors of mRNA transcription or protein synthesis.

The findings reported herein provide direct evidence that LPS may be involved in the pathogenesis of the vascular lesions of pneumonic pasteurellosis. Endothelial cell swelling reported in experimental P. haemolytica pneumonia is compatible with the LPS-induced morphologic changes seen in BPAEC. Furthermore, retraction or sloughing of endothelial cells (EC) as herein reported could cause increased vascular permeability leading to pulmonary edema, the loss of large plasma proteins such as fibrinogen, and hemorrhage. Exposure of the basement membrane by such retraction or sloughing could result in platelet adherence, activation of the intrinsic clotting mechanism, and intravascular thrombosis. All these effects have been reported in experimental and natural pneumonic pasteurellosis and could be induced by a direct effect of LPS on pulmonary EC.

Increased LPS-induced neutrophil-adherence to BPAEC has broad implications in the pathogenesis of pneumonic pasteurellosis. Obviously, neutrophil-adherence could be a key factor in the vascular sequestration and infiltration of neutrophils seen in P. haemolytica pneumonia. Additionally, increased neutrophil adherence, through an effect on EC which

requires de novo protein synthesis, is evidence of EC activation. Endothelial activation may play a key role in the local inflammatory and immune responses through the promotion of local hypercoagulability of the blood, the release of vasoactive substances and cytokines, the adherence and emigration of nonspecific inflammatory cells, and the presentation of antigen with recruitment of antigen-specific lymphocytes. Endothelial activation has not yet been conclusively demonstrated in this model, but continued experimentation is anticipated.

The indomethacin-inhibition of LDH-leakage, arachidonic acid-release, and morphologic changes is the first reported inhibition of the cytotoxic effects of LPS in BPAEC that may involve a mechanism other than inhibition of binding. The morphologic evidence indicates that LPS binding occurs because LPS continues to induce many effects in cytoplasmic organelles in indomethacin-treated BPAEC, but the effects on the cell membrane and possibly the nuclear membrane are prevented. This is compatible with the reported effects of indomethacin resulting in membrane preservation and is supported by the prevention of LPS-induced arachidonic acid-release and LDH-leakage reported herein. Additionally, indomethacin may prevent LPS-induced BPAEC retraction by a calcium antagonistic effect as has been demonstrated in smooth muscle cells. These results do not indicate that indomethacin would be of value in treating bovine endotoxemia, since the concentration required for the described effects are unattainable *in vivo*. However, indomethacin may provide a useful tool for studying the mechanisms of LPS-toxicity.

Finally, caution should be exercised against overinterpretation of the results of these studies because *in vivo* proof is yet to be demonstrated. Nevertheless, these studies have provided evidence that LPS can

cause effects in EC which could be responsible for many of the vascular lesions of pneumonic pasteurellosis.

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VITA

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