LIPOPOLYSACCHARIDE INDUCED APOPTOSIS OF A BOVINE PULMONARY ENDOTHELIAL CELL LINE

by

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Abstract

Haemophilus influenzae type b (Hib) was found to induce gross morphological changes and cell death in both bovine brain endothelial cells (BBEC) and a bovine pulmonary endothelial cell line (CPAE). The causative agent was identified as lipopolysaccharide (LPS), an integral component of the outer membrane of gram-negative bacteria. The cytotoxic effect of LPS was demonstrated by the MTT and LDH release assays and was dose and time dependent. Similar cytotoxicity was seen using LPS from *Escherichia coli* and *Salmonella typhimurium*. Other cell lines tested, including human and mouse endothelial cells, were resistant to LPS. The presence of serum was essential for LPS mediated cell death. Antibodies against CD14, a macrophage/monocyte LPS receptor, blocked LPS-induced endothelial cell death. While CD14 is not found on endothelial cells, a soluble form is found in serum, sCD14. The involvement of sCD14 in endothelial cell death was confirmed by immunodepleting sCD14 from serum. The mode of cell death was investigated to distinguish between necrosis and apoptosis, two processes that are biochemically and morphologically distinct. DNA fragmentation characteristic of apoptosis was observed, and the occurance of apoptosis was confirmed by transmission electron microscopy. Potential LPS-induced signal transduction events were investigated for their role in apoptosis of CPAE cells. Calcium, nitric oxide and inositol phosphates did not appear to be involved in cell death, and inhibitors blocking protein synthesis, kinases, vacuolar acidification, microtubule rearrangements, and GTP proteins had no effect. In contrast, inhibitors of tyrosine phosphatases (vanadate/H_2O_2) and microfilament assembly (cytochalasin D) blocked cell death. Other studies were initiated to determine whether LPS acts by mimicking ceramide, as LPS and ceramide share structural similarity. Ceramide is a second messenger in the sphingomyelin pathway, and has been implicated in the process of apoptosis. Ceramide induced apoptosis in CPAE cells, but the mechanism appeared to be morphologically and kinetically different to that of LPS-induced apoptosis.

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AP	alkaline phosphatase
ARDS	adult respiratory distress syndrome
BAPTA/AM	1,2-bis-(2-aminophenoxy)ethane-N,N,N',N' tetraacetic acid acetoxy methylester
BBEC	bovine brain microvascular endothelial cells
bp	base pairs
BPI	bactericidal/permeability increasing protein
BSA	bovine serum albumin
BVDV	bovine viral diarrhea virus
CAPK	ceramide-activated protein kinase
CAPP	ceramide-activated protein phosphatase
CHO	chinese hamster ovary cells
COS-1	monkey kidney fibroblast-like cell line
CPAE	bovine pulmonary artery endothelial cell line
DIC	disseminated intravascular coagulation
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(b-aminoethyl ether)N,N,N',N'-tetraacetic acid
ELAM	endothelial leukocytes adhesion molecule
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GPI	glycosylphosphatidylinositol
H_2O_2	hydrogen peroxide
HDL	high density lipoproteins
HeLa	human cervix epitheliod cell line
Hib	*Haemophilus influenzae* type b
HL-60	human leukemia cells
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
ICE	interleukin-1b-converting enzyme
IFN	interferon
IL	interleukin
J774A.1	mouse monocyte-macrophage cell line

JNK	c-Jun N-terminal kinase
KDO	3-deoxy-D-manno-octulosonic acid
LBP	LPS binding protein
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPS	lipopolysaccharide
LRR	leucine rich repeats
L-NMMA	N^G-monomethyl-L-arginine
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MDCK	canine kidney epithelial-like cell line
MEM	minimal essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	nitro blue tetrazolium
NO	nitric oxide
NOS	nitric oxide synthase
PAF	platelet activating factor
PBS	phosphate buffered saline
PBS^{++}	phosphate buffered saline containing 0.5 mM Ca^{2+} and Mg^{2+}
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
sCD14	soluble CD14
SD	standard deviation
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
TNF	tumour necrosis factor
U937	human monocyte-like cell line
VCAM	vascular-cell adhesion molecule

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Dedication

There is but one serious philosophical problem,

and that is suicide.

Albert Camus, *The Myth of Sisyphus*

Showing that the Absurd is present in death as well as in life,

and introducing Hope.

Chapter 1 Introduction

1.1 Lipopolysaccharide

Over a hundred years ago, a heat stable nonsecreted toxin was discovered in gram-negative bacteria. Richard Pfeiffer, a student of Robert Koch, found that heat inactivated lysates of *Vibrio cholera* injected into guinea pigs caused fever and other pathological symptoms (Pfeiffer, 1892). At the same time Eugenio Centanni at the University of Bologna made a similar discovery using *Salmonella typhi* (reviewed by Rietschel and Brade, 1992). These cell-associated toxins were called endotoxins to distinguish them from the previously identified actively secreted bacterial exotoxins, and were found to be characteristic of all gram-negative bacteria. Fifty years after their discovery, endotoxins were found to consist of carbohydrates and lipids rather than amino acids, and were therefore given the name lipopolysaccharide (LPS). The chemical nature of LPS was completely elucidated in 1983 when the structure of the lipid A anchor was described (Rietschel *et al.*, 1983; Takayama *et al.*, 1983; Imoto *et al.*, 1983).

LPS is found in the outer leaflet of the outer membrane of gram-negative bacteria (Fig 1.1). LPS forms an integral part of the outer membrane structure and acts as a barrier against heavy metals, lipid disrupting agents, and larger molecules. The highly variable outermost carbohydrate portion of LPS also protects bacteria by binding complement at a safe distance from the membrane (Joiner, 1988). It has been estimated that one bacterial cell contains approximately 3.5 x 10^6 LPS molecules, which in the case of *Escherichia coli* accounts for 75% of the outer leaflet, with the remaining 25% consisting of transmembrane proteins (Rietschel *et al.*, 1993).

1.1.1 LPS structure

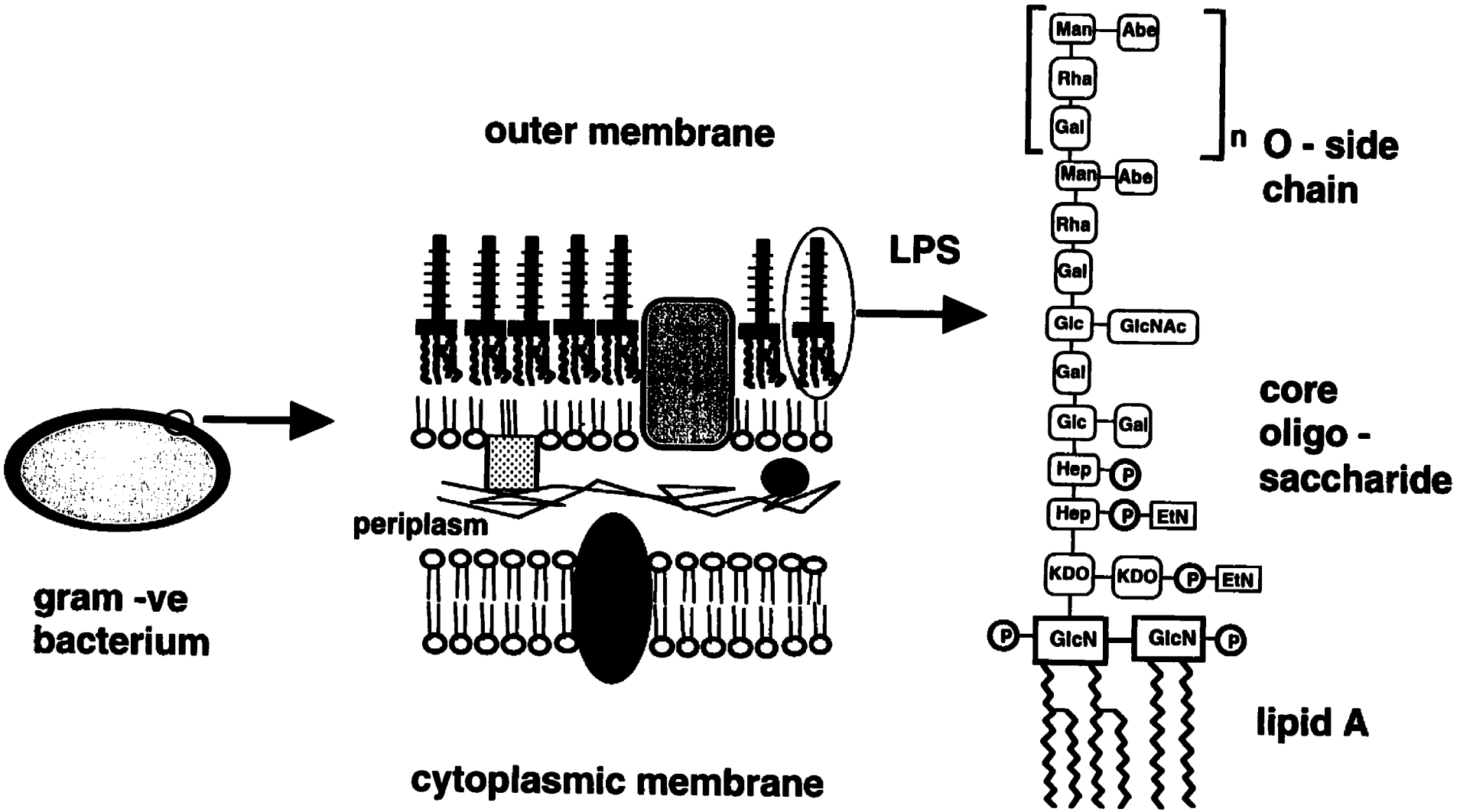
The LPS of various bacterial families share a common architecture, consisting of a polysaccharide and a phosphorylated glucosamine-disaccharide with covalently linked acyl

groups, called lipid A (Fig 1.1). The outermost portion, the O-specific chain, is a polymer of up to 50 repeating oligosaccharide units which are unique for a bacterial species or serotype. This region is not absolutely required for bacterial growth or survival *in vitro*. Mutants lacking the O-specific chain and varying parts of the core are called rough mutants, distinguishable from the wild type or smooth organisms by the altered appearance of colonies on agar. These bacteria are not commonly found in blood infections (bacteremia), because they are easily lysed by complement. Several gram-negative pathogenic bacteria, such as *Haemophilus influenzae* and *Neisseria meningitidis*, lack the O-specific chain and have developed additional methods to evade host defenses. The biological importance of the outer region is illustrated by comparing circulatory half lives. The half life of smooth LPS injected into mice is in the order of hours, while that of rough LPS is only minutes (Chedid *et al.*, 1966). The middle portion of LPS, the core oligosaccharide, consists of an inner and outer core and is less variable. The outer core is made up of hexoses, chiefly glucose, galactose and N-acetyglucosamine. The inner core contains heptoses linked to phosphates and phosphoethanolamines, and a unique sugar, 3-deoxy-D-manno-octulosonic acid (KDO). At least a portion of this inner core is necessary for bacterial viability (Raetz *et al.*, 1991). The structure of the section which is anchored in the bacterial membrane, lipid A, is relatively conserved. It is this portion of the LPS molecule which is responsible for its pathogenic effects, as was demonstrated by experiments using synthetic lipid A (Galanos *et al.*, 1984). Variations in the number, position, length, and degree of saturation of the fatty acid chains affect the toxicity of lipid A.

1.1.2 Biological effects of LPS

LPS has important clinical relevance because it has a direct role in the pathogenesis of gram-negative bacterial infections. Microbial products in the bloodstream trigger sepsis, which is characterized by fever, chills, tachycardia (increased heart rate), tachypnea (increased respiratory rate) and altered mental status. If hypotension and signs of inadequate perfusion

Figure 1.1 LPS structure. LPS is found in the outer leaflet of the outer membrane of gram-negative bacteria. The outermost portion, the O-side chain, if present, consists of repeated oligosaccharide units. The core oligosaccharide contains hexoses and heptoses and a unique sugar, KDO. The portion anchored in the membrane, lipid A, consists of a phosphorylated glucosamine-disaccharide with covalently linked acyl groups. While the same basic structure of LPS is found in all gram-negative bacteria, many variations are seen in the type, position, and number of sugars, the presence and position of phosphates, and the length, position and degree of saturation of the fatty acid chains. Illustrated here is the basic proposed structure of *Salmonella typhimurium* LPS. Abbreviations: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; Hep, L-*glycero*-D-*manno*heptose; KDO, 3-deoxy-D-*manno*octulosonic acid; EtN, ethanolamine; P, phosphate.


outer membrane
LPS
periplasm
gram -ve
bacterium
cytoplasmic membrane
Man
Abe
Rha
Gal
n
O - side
chain
Man
Abe
Rha
Gal
Glc
GlcNAc
Gal
core
oligo -
saccharide
Glc
Gal
Hep
P
Hep
P
EtN
KDO
KDO
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EtN
P
GlcN
GlcN
P
lipid A

of cells and organs develop, the condition is then termed septic shock (Dal Nogare, 1991). While septic shock can be caused by fungal and gram-positive bacterial products, gram-negative bacteria account for approximately half of all cases and are associated with higher levels of mortality (Corriveau and Danner, 1993). The pathogenesis of septic shock is complex, with numerous cellular and humoral targets, and a plethora of resulting mediators and downstream effects. LPS triggers the inflammatory response as well as both the coagulation and complement cascades. Clinical symptoms such as fever and vasodilation can lead to disseminated intravascular coagulation (DIC), hypotension, adult respiratory distress syndrome (ARDS), multiple organ failure and eventually death (Glauser *et al.*, 1991). There are over 200,000 cases of septic shock in the U.S. every year, and the mortality rate is as high as fifty percent, despite modern medicine and supportive techniques (Dal Nogare, 1991).

Evidence identifying LPS as the causative agent in gram-negative septic shock is compelling but primarily circumstantial. Animals injected with purified LPS develop symptoms very similar to sepsis (Coalson *et al.*, 1979). Human volunteers injected with low doses of LPS (4 ng/kg) developed many of the characteristics, in a mild form, of septic shock (Suffredini *et al.*, 1989). In a tragic but informative incident a laboratory worker self-administered a high dose of purified *Salmonella minnesota* LPS (1 mg). She consequently developed the full clinical manifestations of septic shock, showing that LPS alone is sufficient even in a single dose (Taveira *et al.*, 1993).

The pathogenic effects of LPS are caused by microgram quantities, which can enter the blood from several sources. Many infections are due to normal flora which take advantage of a breach in host defense, such as a perforated bowel or major burn. LPS can be released by live bacteria during log phase growth or under sub optimal growth conditions. It is also released after bacterial death and disintegration, for example as a result of antibiotic treatment (Tesh and Morrison, 1988). In addition to antibiotic development, medical progress has contributed to the number of cases of sepsis, most of which occur in hospitals, in other

ways. For example, both immunosuppression and the use of invasive medical devices such as catheters are associated with septic shock.

Most bacterial exotoxins directly injure eukaryotic cells by disrupting vital functions such as membrane integrity, signal transduction pathways, or protein synthesis. In the case of LPS however, the harmful effects are largely caused by the activation of the host immune system. This reaction can be beneficial at low doses, eradicating the invading organism. However, at higher levels of LPS the host response can be too vigorous and lead to shock and death. LPS stimulates host cells (mainly macrophage/monocytes, but also endothelial cells, smooth muscle cells, and neutrophils) to produce and release numerous mediators. These include the pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF), bioactive lipids such as platelet activating factor (PAF), and reduced oxygen species such as nitric oxide (NO) (Schletter *et al.*, 1995). High levels of these mediators can initiate the various cascades which lead to shock.

1.1.3 LPS receptors

LPS interacts with many different cell types, and in the last ten years numerous potential LPS receptors have been identified on these cells (reviewed by Lynn and Golenbock, 1992). The leukocyte integrin CD18 binds to LPS in its membrane associated form. CD18 binds many ligands involved in cell-cell and cell-matrix interactions, and was shown to be not important in LPS activation of cells (Wright *et al.*, 1990). Later it was found that CHO cells transfected with CD11c/CD18 acquire a serum-independent LPS responsiveness, but this reaction is slow and less sensitive when compared to CHO cells transfected with CD14, needing approximately ten fold more LPS for similar levels of NF-kB translocation (Ingalls and Golenbock, 1995). LPS also binds to scavenger receptors. These receptors, also known as the acetyl low density lipoprotein (LDL) receptor, play a role in cholesterol metabolism and the pathogenesis of artherosclerosis. Further work suggests that rather than being involved in LPS signalling, the scavenger receptor may actually have a role

in the detoxification of LPS *in vivo* (Hampton *et al.*, 1991). A number of other LPS-binding proteins have been identified using photo affinity cross-linking techniques, but have not been well characterized. One, a 70 kDa protein, was later identified as contaminating albumin from tissue culture media or serum which had become associated with the cells (Dziarski, 1994). The best characterized LPS receptor, and the only one shown to be conclusively involved in the activation of cells, is CD14.

1.2 CD14

1.2.1 Membrane CD14

CD14 was first identified as a marker for myeliod differentiation. A series of monoclonal antibodies specific for monocytes and macrophages recognized a 55 kDa cell surface glycoprotein, which was designated as CD14 (Bazil *et al.*, 1987). CD14 maps to chromosome 5 within a region containing a cluster of genes coding for growth factors and receptors (Goyert *et al.*, 1988). CD14 is anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage rather than the more common transmembrane anchor (Haziot *et al.*, 1988). GPI anchors are complex glycolipids which are highly conserved in eukaryotic cells. A number of mammalian cell-surface proteins use GPI anchors as a means of attachment to the plasma membrane (Englund, 1993). This group includes ectoenzymes, complement regulatory factors and cell adhesion molecules. It is still not known why some proteins are GPI-anchored rather than containing the more common transmembrane domain. It has been shown however that GPI-linked molecules differ from transmembrane proteins in cell surface distribution, lateral mobility, and lipid environment. Mammalian GPI anchors are found in membrane microdomains rich in glycolipids, sphingolipids, and cholesterol (Brown and Rose, 1992). Since they are associated with the outer leaflet only, they are attached to the cell loosely compared to transmembrane proteins.

CD14 is mainly found on cells of the myelomonocytic lineage. It is absent in early progenitor cells and generally increases with maturation to monocytes. Recent work looking

at epitope expression and LPS-binding capacity of CD14 on monocytes during maturation suggests that there are at least three different forms of CD14, and these may reflect different stages of cell maturation (Pedron *et al.*, 1995). Blood monocytes with both high and low levels of CD14 are found, and different levels are seen in various types of macrophages. Anti-CD14 staining is also detected on neutrophils, granulocytes, B cells, and mammary cells (Lynn and Golenbock, 1992). Work in a murine model system found CD14 gene expression in a number of other cell types including epithelial cells (Fearns *et al.*, 1995). Recently Wanatabe *et al.* (1996) detected CD14 gene expression in human gingival fibroblasts. In both epithelial and fibroblast cells CD14 was detected in the cells, but surface expression was not established.

1.2.2 CD14 is a receptor for LPS

A function for CD14 was established by Wright *et al.* (1990), who showed that it acted as a receptor for LPS. They found that anti-CD14 antibodies blocked the interaction between macrophages and LPS coated erythrocytes. Originally CD14 was thought to bind a complex of LPS and a serum protein, LPS-binding protein (LBP). LBP is a 60 kDa glycoprotein found in normal serum at low levels and rising over 100-fold to 50 μg/ml during an acute phase response in rabbits (Tobias *et al.*, 1986). It is found at higher levels in normal human sera, 5 to 10 μg/ml (Tobias *et al.*, 1992), sufficient to enhance the effects of LPS on macrophages (Mészáros *et al.*, 1994). LBP binds specifically to the lipid A moiety of LPS (Tobias *et al.*, 1989). Anti-LBP antibodies can block the LPS potentiating effect of serum (Schumann *et al.*, 1990), and protect mice against LPS (Gallay *et al.*, 1993b). Recently it has been shown that LBP is not absolutely essential for the binding of LPS and CD14, but that it acts as a catalyst transferring LPS to CD14, and is not part of the final complex (Hailman *et al.*, 1994; Tobias *et al.*, 1995). LPS is a highly amphipathic molecule, and aqueous solutions contain a mixture of aggregated and disaggregated LPS. The rate of solution is very slow, and it is the monomeric form of LPS which is the most biologically active (Takayama *et al.*,

Figure 1.2 LPS activation of macrophages via CD14. The current model of LPS activation of macrophages via CD14 is shown here. LPS is released into the blood where it binds to serum proteins including LPS binding protein (LBP). LBP is thought to act as a catalyst, possibly by releasing LPS from aggregates, and transferring it to CD14 on the cell surface. Since CD14 is GPI anchored, it is postulated that after binding LPS it interacts with an as yet unidentified co-receptor or receptors, which then triggers intracellular signalling.

LBP
LPS
LBP
CD14
?
IL-1
TNF
CD14
MΦ
LPS
aggregates
bacteria

1995). LBP may act by freeing LPS monomers from aggregates, which can then bind to CD14 (Fig 1.2). LPS can also interact with other serum proteins such as albumin (Takayama *et al.*, 1990), septin (Wright *et al.*, 1992), and high density lipoproteins (HDL) (Ulevitch *et al.*, 1981). The binding of LPS to HDL is also catalyzed by LBP and results in its neutralization (Wurfel *et al.*, 1994). LBP has further been shown to transfer LPS from sCD14 to reconstituted HDL particles (Wurfel *et al.*, 1995). The ultimate response to LPS *in vivo*, cell activation or LPS neutralization, may depend on the relative amounts of these, and possibly other, serum components.

Evidence confirming that CD14 is of paramount importance for the response of cells to LPS is expanding. Transfection of human CD14 into Chinese hamster ovary fibroblasts transfers macrophage-like responsiveness to these normally unresponsive cells (Golenbock *et al.*, 1993). Similarily, the murine pre-B cell line 70Z/3 transfected with CD14 responds to 10,000-fold lower LPS concentrations than the parental cell line (Lee *et al.*, 1992). Mice overexpressing human CD14 are hypersensitive to LPS (Ferrero *et al.*, 1993), while CD14 deficient mice are "exquisitely" insensitive to LPS (Haziot *et al.*, 1995). Recent work suggests that CD14 mediated signalling in leukocytes can be triggered by whole bacteria, and is not dependent on LPS shedding (Katz *et al.*, 1996). Grunwald *et al.* (1996) propose a novel CD14-dependent phagocytosis pathway for gram-negative bacteria in monocytes. It is important to note that in the absence or blockage of CD14, LPS can still activate cells, but a much higher dose is required (Kitchens *et al.*, 1992; Lynn *et al.*, 1993).

Characterization of the CD14 cDNA showed that it contains 10 leucine-rich repeats (LRRs) (Ferrero *et al.*, 1990). LRRs are short sequence motifs present in a diverse group of proteins with different functions and cellular locations (Kobe and Deisenhofer, 1994). All are involved in protein-protein or protein-lipid interactions, and about half are known to take part in signal transduction pathways. However, a truncated form of CD14 consisting of only 152 amino acids at the amino terminal, lacking 7 of the 10 LRRs, is still active (Juan *et al.*, 1995). Another paper suggested that only 65 amino acids at the N-terminal are important for LPS

binding (Viriyakosol and Kirkland, 1995). Further characterization of CD14 should provide additional insights into its mode of action.

1.2.3 LPS signal transduction via membrane CD14

It is still unclear how CD14, lacking a cytoplasmic domain, transmits signals leading to cell activation. Other GPI anchored molecules have been found to have signalling capability, such as Thy-1 in T cells and LFA-3 in monocytes (reviewed by Robinson, 1991). A number of GPI anchored proteins including CD14 can be co-precipitated with protein kinase activity (Stefanova *et al.*, 1991). The protein tyrosine kinase (PTK) $p56^{lyn}$ can be co-precipitated with CD14 in monocytes, and is activated along with PTKs $p58^{hck}$ and $p59^{c\text{-}fgr}$ (Stefanova *et al.*, 1993). Protein tyrosine phosphorylation occurs in monocytes following activation by LPS (Weinstein *et al.*, 1991), and this is mediated by CD14 (Weinstein *et al.*, 1993). Several of these proteins are isoforms of mitogen-activated protein (MAP) kinase (ERK1 and ERK2) (Weinstein *et al.*, 1992). LPS can also activate the c-Jun N-terminal kinase (JNK) (Hambleton *et al.*, 1996) and the p38 MAP kinase (Han *et al.*, 1994). Thus, LPS can stimulate three distinct MAPK subfamilies. Gradually, events in the signalling pathways triggered by LPS in macrophages are being discovered (reviewed by Schletter *et al.*, 1995; DeFranco *et al.*, 1995).

Early events remain unclear however. Following binding of LPS, CD14 may interact with an unidentified transmembrane protein or act directly by somehow inserting LPS into the membrane or otherwise aiding its uptake. The importance of the GPI anchor was questioned in a paper which showed that when CD14 was converted to an integral membrane protein it mediated the same cellular events as the normal GPI anchored form (Lee *et al.*, 1993). Studies looking at the intracellular fate of LPS have been few and mainly inconclusive (reviewed by Risco and DaSilva, 1995). Using immunogold labeling and transmission electron microscopy in both macrophages and pneumocytes, LPS aggregates could be detected in the cytoplasm, phagosomes, mitochondria, and eventually in the nucleus where it

accumulated (Risco *et al.*, 1991). However, an extremely high amount of LPS (0.5 mg/ml) was used in this study.

1.2.4 Soluble CD14

A soluble form of CD14 (sCD14) was initially detected in cellular supernatants and serum by its ability to block the binding of anti-CD14 antibodies to cells (Maliszewski *et al.*, 1985). It is also found in the urine of nephrotic patients (Bazil *et al.*, 1986). sCD14 lacks the GPI anchor and is fairly abundant, found in normal serum at a concentration of 2-6 μg/ml (Grunwald *et al.*, 1992). There are two forms of sCD14 in serum, differing in their electrophoretic mobility, with apparent molecular masses of 48 and 56 kDa. They also have different sensitivity to endoglycosidases and peptide maps, and appear to be released from the cell by different mechanisms (Labeta *et al.*, 1993). A number of different mechanisms have been suggested to account for the release of sCD14. Bazil and Strominger (1991) report a protease dependent shedding of CD14, while other evidence suggests that at least two mechanisms are involved, one of which is protease independent (Bufler *et al.*, 1995). Another group described a mechanism involving endocytosis followed by exocytosis resulting in the release of membrane bound CD14, as well as evidence that sCD14 can be released by mechanisms which do not involve cleavage of the membrane bound form (Durieux *et al.*, 1994). It is also possible that CD14 is released by a cell associated phospholipase D, a pathway described for other GPI anchored proteins. The link between the mode of release and the different soluble isoforms is still not completely understood, however a model has been proposed in which the smaller form is derived from the membrane by proteolytic cleavage while the larger form is directly released from an intracellular source (Durieux *et al.*, 1994; Landmann *et al.*, 1995). A functional difference between the two sCD14 isoforms has not been detected, although one study found the larger form predominated at the higher levels of sCD14 associated with gram-negative septic shock, while sera from healthy volunteers only contained the smaller form (Landmann *et al.*, 1995).

1.3 Endothelial cells

The majority of the work in this thesis was done using primary isolate bovine brain endothelial cells (BBEC) and a bovine pulmonary endothelial cell line (CPAE). Endothelial cells are of particular interest in studies concerning the effects of LPS. They are considered to be the major ultimate target of LPS-induced events, and endothelial cell damage accounts for much of the pathology of septic shock (Morrison and Ulevitch, 1979; Petersdorf and Dale, 1980).

1.3.1 Function of endothelial cells

In the human body there are more than 10^{12} endothelial cells lining the inside of blood vessels, covering a surface area of over 100 m^2 (Jaffe, 1987). Found at the strategic interface between blood and tissues, endothelial cells not only form the structural basis of blood vessels, but also play a major role in many metabolic functions (Dzau *et al.*, 1993). These include hemostasis, coagulation, thrombolysis, control of vascular tone, antigen presentation, and basement membrane and growth factor synthesis. In addition, endothelial cells regulate the trafficking of circulating cells, and activated endothelial cells play a critical role in pathological conditions such as inflammation, tumor angiogenesis, and wound healing.

Endothelial cells are a highly heterogeneous population, varying not only from organ to organ, but also in different vessel calibers within an organ (McCarthy *et al.*, 1991). Large vessel endothelium differs from microvascular endothelium in several ways. They control vasoconstriction and vasodilation, blood pressure and other physiological parameters, but are not involved in neovascularization processes or blood-tissue exchange of nutrients and oxygen. Microvascular endothelium has been divided into three phenotypes based on morphology: continuous, fenestrated, and discontinuous. These morphological differences correlate with vascular permeability (Risau, 1995). Endothelial cells with unique features have been further studied, such as the brain microvascular endothelial cells forming the blood-brain barrier. These continuous endothelial cells precisely control substances entering

or leaving the brain due to complex tight junctions, minimal pinocytic activity, and specific carrier systems transporting nutrients, metabolites and hormones. The phenotype of an endothelial cell is controlled by its microenvironment, where determinants such as shear stress, type of neighboring cell and extracellular matrix all can have an effect. Interestingly, the microenvironmentally regulated differentiation of endothelial cells is reversible. Cultured endothelial cells lose organ specific characteristics over time, which has been a barrier in their study and in the identification of site-specific markers (Augustin *et al.*, 1994).

Normal endothelial cells are metabolically active and have a low turnover rate. Under certain circumstances they become activated, and proliferate or become adhesive for blood cells. This is seen for example during angiogenisis and inflammation. Different cytokines activate distinct programs of activation. IL-1 and TNF are well studied activators of the endothelial cell inflammatory reaction. They cause changes in hemostasis, vascular tone and permeability, and the recruitment of other cells due to the induction of adhesion molecules. Endothelial cells are also important producers of cytokines, which include IL-1, IL-6, IL-8, and numerous hematopoietic growth factors (Mantovani *et al.*, 1992).

1.3.2 Effects of LPS on endothelial cells

Endothelial cell injury has been implicated as a principal element in the pathology of septic shock in humans (Morrison and Ulevitch, 1979; Petersdorf and Dale, 1980). Shock occurs when insufficient oxygen and nutrients reach vital organs. In the case of septic shock this results from a combination of peripheral vasodilation and endothelial cell injury resulting in the peripheral pooling of blood. Injection of LPS into experimental animals results in endothelial cell damage (Stewart and Anderson, 1971; Gerrity *et al.*, 1975; 1976; Reidy and Bower, 1977; Hansson *et al.*, 1985; Kang and Williams, 1991; Penn and Chisolm, 1991). These *in vivo* studies do not distinguish between injury caused by LPS directly and injury caused by LPS-generated inflammatory mediators. The clinical manifestations of gram-negative septicemia result primarily from excessive stimulation of the host's immune system

by LPS, and many of the mediators released have well characterized effects on endothelial cells (reviewed by Mantovani and Dejani, 1989). For example, TNF elicits a plethora of responses from endothelial cells resulting in leukocyte recruitment and adhesion, changes in antithrombotic properties, stimulation of prostacyclin production leading to vasodilation, and the production of other cytokines and chemotactic factors. Oxygen free radicals such as superoxide are produced by LPS-stimulated neutrophils and is thought to be involved in endothelial cell damage in the lung (Varani and Ward, 1994).

Advances in tissue culture techniques allowed the study of LPS effects on cultured endothelial cells. Harlan *et al.* (1983a) showed that *Escherichia coli* and *Salmonella minnesota* and *typhosa* LPS directly cause the death of bovine endothelial cells *in vitro* in the absence of other cells. The same cytotoxic effect is seen using *Pasteurella haemolytica* LPS (Paulsen *et al.*, 1989) and *Haemophilus influenzae* LPS (Langford *et al.*, 1991). Meyrick and coworkers demonstrated that sheep pulmonary endothelial cells are also sensitive to LPS (1989). However, some species to species variability is seen in *in vitro* studies, as no evidence of similar cytotoxicity is detected in human umbilical vein (HUVEC), goat aortic, and canine vena cava endothelial cells (Harlan *et al.*, 1983b). HUVECs respond to LPS in a non-lethal fashion, with increased expression of tissue factor (Stern *et al.*, 1985), intercellular adhesion molecule 1 (ICAM-1) (Dustin *et al.*, 1986), endothelial-leukocyte adhesion molecule (ELAM-1) (Bevilacqua *et al.*, 1987), IL-1 and IL-6 (Jirik *et al.*, 1989), IL-8 and vascular-cell adhesion molecule 1 (VCAM-1) (Pugin *et al.*, 1993b).

1.4 Cell death

While it was clear that LPS treated bovine endothelial cells die, nothing was known about the specific mode of cell death The last twenty years has seen significant advances in the study of cell death, resulting in the establishment of two major categories, necrosis and apoptosis. In reviewing the literature for a recent review on the topic, Dr E. Faber was moved to comment that "there is no field of basic cell biology and cell pathology that is more

confusing and more unintelligible than is the area of apoptosis versus necrosis." (Faber, 1994). This confusion is due in part to the use and misuse of death-related terms. The term necrosis, dating back to ancient Greek texts, was traditionally used to describe an advanced stage of tissue breakdown, such as seen in cases of gangrene. A number of other terms were proposed over the years to describe specific types of cell death, only some of which are used today (reviewed by Majno and Joris, 1995). Elegant drawings showing clearly apoptotic cells were published by Flemming in 1885, who called the process chromatolysis. Many aspects of what is now called apoptosis were observed since that time, and in 1971 it was documented in full by John Kerr, who showed a sequence of changes that cells underwent during liver atrophy. Originally termed shrinkage necrosis, the name apoptosis was later selected, from the Greek meaning "dropping or falling off", as in leaves from a tree (Kerr *et al.*, 1972). Currently, the two categories of cell death, necrosis and apoptosis, are distinguished based on biochemical and morphological differences (Wyllie *et al.*, 1980). There are lingering debates on the use of these terms, and numerous cases which defy easy categorization. In combination with the recent explosion in knowledge related to cellular events during apoptosis, this will probably lead to an expansion and redefinition of the current terminology.

1.4.1 Necrosis

Necrosis is the name currently used to describe non-apoptotic cell death. It has been argued that accidental cell death is a more accurate term, but the shorter and trendier name will likely persist, and the original meaning of necrosis will be altered. Necrosis occurs when cells die from extreme and abrupt injury, such as physical or chemical trauma, hyperthermia or ischaemia. It usually involves direct or indirect damage of the plasma membrane, resulting in a disruption of ionic gradients. The permeability of the plasma membrane can be increased by changes in its structure, for example by complement or viral lysis, or by obstruction of cation pumps, as in the case of hypoxia and respiratory toxins. As transmembrane ion gradients are dissipated, ion-regulated functions are arrested, ATP levels become exhausted,

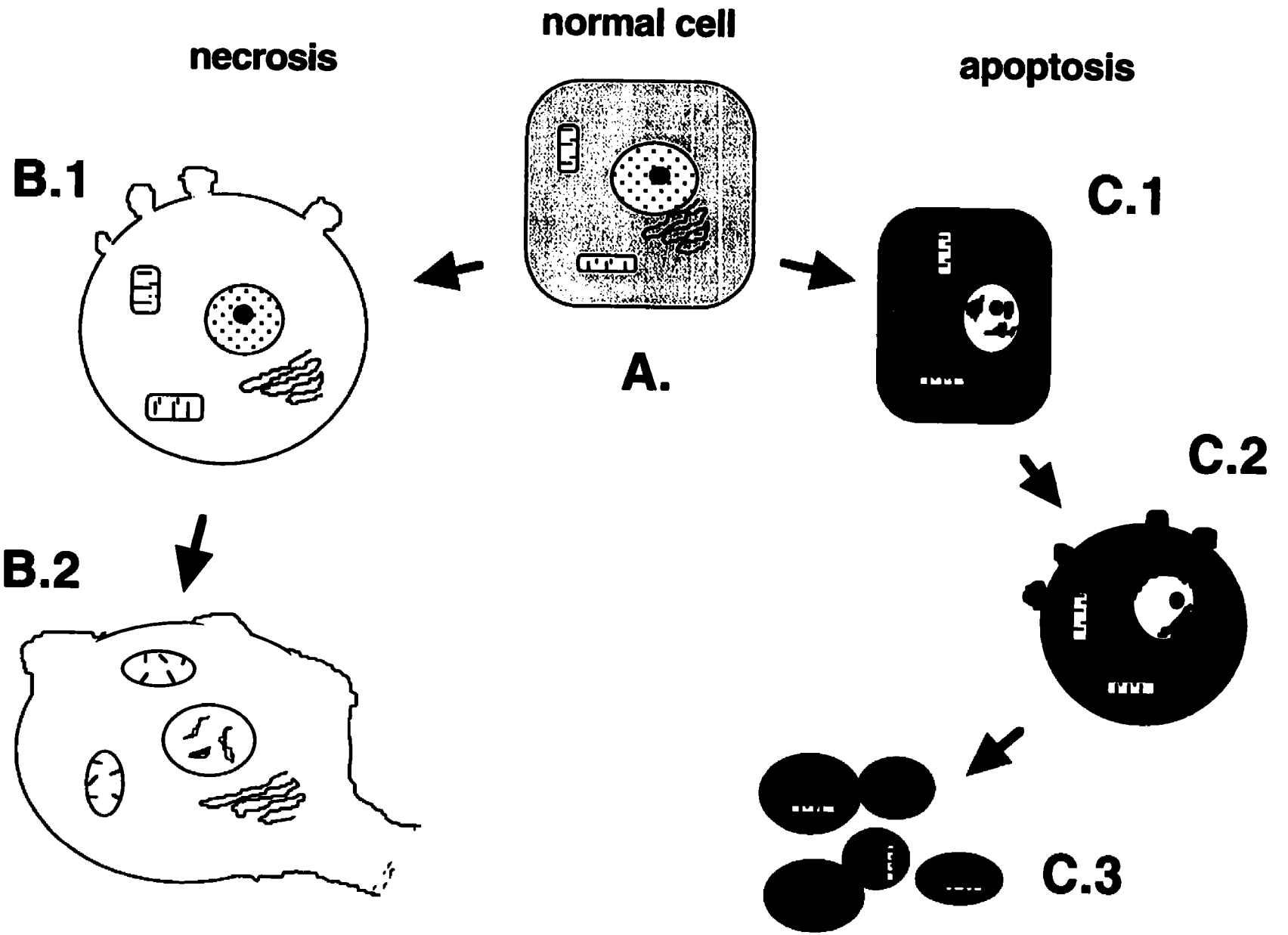
and water enters the cell, causing cell swelling. This characteristic swelling has inspired a recent suggestion that the process be renamed "oncosis", derived from *Ónkos* meaning swelling, a term first coined in 1910 (Recklinghausen), and that the term necrosis be reserved for the final dissolution of the cell, common to both death pathways (Majno and Joris, 1995). Necrosis is a passive process, which can occur at 4°C, and usually affects cells in groups.

Morphologically, necrosis is characterized by early membrane blebbing, which precedes changes in permeability, followed by reversible cell swelling, mitochondrial condensation and nuclear chromatin flocculation (Fig 1.3, B.1). Finally the cell enters the irreversible phase indicated by extreme swelling of mitochondria, development of plasma membrane ruptures, the release of lysosomal hydrolases, and ultimately cell disintegration (Fig 1.3, B.2). The nucleus remains mainly intact throughout the process, and DNA is degraded into fragments of irregular size in the later stages. Following cell rupture and the release of cell contents, local inflammation may occur.

1.4.2 Apoptosis

Apoptosis is a process in which cells die in a controlled manner, in response to specific stimuli, following an intrinsic program. The terms apoptosis and programmed cell death are often used interchangeably. Programmed cell death originally refered to situations where cells are programmed to die at a fixed time. In most, but not all cases, the morphology of this death indicates apoptosis. Some authors began to use the term programmed cell death in reference to the cascade of intracellular events that lead to apoptosis, leading to the current confusion. Since it was first described, apoptosis has been identified in many different circumstances. Cell mediated cytotoxic mechanisms, either antibody dependent or by T cells and natural killer cells, occurs by apoptosis. Other examples include the death of cells during embryogenisis and metamorphosis, the death of cells with short life spans (such as neutrophils), and the deletion of autoreactive T cells during maturation (Cohen, 1993). Apoptosis can also be seen as a result of exposure to poison, and recently it has been seen to

Figure 1.3 Morphology of cell death. Cells die by one of two pathways, necrosis or apoptosis. A normal cell is indicated by (A), containing mitochondria and other organelles and a nucleus with diffused chromatin and a distict nucleolus. In the early stages of necrosis (B.1) membrane blebbing and cell swelling is seen. Nuclear contents flocculate, and the cell continues to swell along with organelles such as mitochondria (B.2) and eventually the cell ruptures, releasing cell contents. An early feature of apoptosis is cell shrinkage and chromatin clumping (C.1). Organelles remain unchanged, while membrane budding may occur and the chromatin condenses along the nuclear membrane forming characteristic cresents (C.2). Finally the cell breaks apart into membrane bound apoptotic bodies which may contain intact organelles and fragments of the nucleus (C.3).


normal cell
necrosis
apoptosis
B.1
A.
C.1
C.2
B.2
C.3

be triggered by a number of viruses including HIV and bacteria (Zychlinsky, 1993). In addition, some cytotoxic agents have been shown to cause apoptosis at low doses and necrosis at higher levels (Lennon *et al.*, 1991).

Cells undergoing apoptosis display characteristic morphological changes. Early stages include shrinking of the cell and the clumping of the chromatin (Fig 1.3, C.1). In some cases extensive budding (blebbing) of the cytoplasmic membrane is seen. The chromatin condenses along the inside of the nuclear membrane, forming characteristic crescent-shaped areas of dense DNA. Organelles such as mitochondria remain intact (Fig 1.3, C.2). Eventually the nucleus fragments along with the cell to form membrane bound apoptotic bodies that contain nuclear material, organelles, and condensed cytoplasm (Fig 1.3, C.3). No cellular material is released and therefore an inflammatory response is not provoked. Cells undergoing apoptosis *in vivo* are rapidly recognized and engulfed by both professional and non-professional phagocytes (Savill *et al.*, 1993). *In vitro* apoptotic bodies continue to exclude vital dyes for some time before finally disintegrating, a process termed secondary necrosis.

The most consistent biochemical feature of apoptosis is the degradation of genomic DNA, which occurs in a specific pattern producing DNA fragments that are in multiples of 180-200 base pairs (bp) (Wyllie *et al.*, 1984). This is the length of a DNA strand wrapped around the histone octomer in a nucleosome, which suggests that the chromatin is being cleaved at the linker DNA between adjacent nucleosomes. This DNA fragmentation occurs at an early stage, before changes in viability or membrane permeability are seen, and is irreversible. There have been cases reported where the morphological characteristics of apoptosis are present but the DNA ladder cannot be detected (Tomei *et al.*, 1993). There is still some specific DNA degradation in these cases, resulting in large fragments of 50 or 300 kilo bp (Oberhammer *et al.*, 1993). Another general feature of apoptosis is the redistribution of plasma membrane phosphatidylserine. Normally found in the inner leaflet, the externalization of this phospholipid is an early event in apoptosis, and once exposed it is

thought to be involved in the recognition of apoptotic cells by phagocytes (Fadok *et al.*, 1992; Martin *et al.*, 1995b).

The explosion in apoptosis research, which started in the early 90's, continues unabated (Wick, 1994; Cohen, 1995). Much has been learned about the pathways involved in apoptotic signalling. Receptors such as Fas (Apo-1/CD95) (Trauth *et al.*, 1989; Yonehara *et al.*, 1989) and the TNF receptor (TNFR1) (Tartaglia *et al.*, 1993) were found to be involved in the induction of apoptosis, and other proteins with similar death domains have been identified. Ced-3, a *Caenorhabditis elegans* gene which codes for a cysteine protease with strong homology to mammalian interleukin-1β-converting enzyme (ICE), is required for apoptosis (Yuan *et al.*, 1993). ICE cleaves pro-IL-1β to produce the active form (Thornberry *et al.*, 1992). A large family of ICE-related proteases has been identified, and a unified nomenclature has recently been proposed, combining a "c", denoting the presence of a cysteine at the active site, and "aspase", indicating the requirement for an aspartic acid at the cleavage site, to give "caspase" (Alnemri *et al.*, 1996). In addition, numerous specific substrates and inhibitors have been discovered for the various caspases, and their central role in the regulation and execution of apoptosis is rapidly being elucidated (reviewed by Martins and Earnshaw, 1997; Martin and Green, 1995; Kumar, 1995). Recently proteins which link receptors to caspases have been identified. FADD (Fas associated through death domain protein, or MORT1) binds to Fas and interacts directly with caspase-8, also called FLICE (FADD-like ICE, or MACH) (Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1995; Muzio *et al.*, 1996; Boldin *et al.*, 1996). Similar adaptor proteins have been found for the TNF receptor, but the link to proteases of the caspase family is less direct, possibly involving FADD (Hsu *et al.*, 1995; Hsu *et al.*, 1996).

The *Bcl-2* (B-cell lymphoma) gene was identified as an inhibitor of apoptosis (Hockenbery *et al.*, 1990). Many Bcl-2 homologues have been found and they appear to interact to control apoptosis, some preventing apoptosis like Bcl-2 while others promote apoptosis, for example Bax and Bad (reviewed by Hale *et al.*, 1996). The action of these

molecules has lately been further elucidated (summarized by Golstein, 1997). Bcl-2 is found in the outer mitochondrial membrane where it prevents the release of cytochrome c into the cytosol which would in turn initiate apoptosis (Yang *et al.*, 1997; Kluck *et al.*, 1997). The determination of the structure of another anti-apoptotic family member, Bcl-xL, has lead to the suggestion that these proteins interact to form pore structures with variable permeability (Muchmore *et al.*, 1996). Numerous transcription factors such as myc, fos, jun and p53 have also been implicated in apoptosis (reviewed by Williams and Smith, 1993). The list of inducers and inhibitors of apoptosis grows daily, and overlaps considerably (reviewed by Wertz and Hanley, 1996). Cell-free models of apoptosis have been developed, and will be very useful in the study of specific events (Solary *et al.*, 1993; Lazebnik *et al.*, 1993; Newmeyer *et al.*, 1994).

1.5 Objectives

Initially, the observation was made that *Haemophilus influenzae* caused marked morphological changes in bovine brain endothelial cells (BBEC). Further work identified the causative bacterial agent as LPS and showed that cell death was the end result. The same cytotoxic effect of LPS was seen with a bovine pulmonary endothelial cell line (CPAE) (Patrick *et al.*, 1992). The main aim of this study was to further characterize this LPS-induced bovine endothelial cell death. The first step was to quantitate and characterize the cell death using cytotoxicity assays (Chapter 3). The initial focus was on extracellular events, due to the observation that the presence of serum was necessary for cell death. This resulted in the identification of a serum protein involved in LPS-mediated endothelial cell cytotoxicity (Frey *et al.*, 1992) (Chapter 4). Another direction of study was to determine the mode of cell death, distinguishing between death by apoptosis or by necrosis (Chapter 5). Many approaches were used to try and identify other factors involved in signalling events triggered by LPS (Chapter 6). Finally, work was done to address the theory that LPS acts by mimicking ceramide, an attractive theory resulting from the observations that i) ceramide is an emerging key player in

one of the signal transduction pathways leading to apoptosis, ii) LPS shares strong structural similarity to ceramide, and iii) LPS can activate the ceramide activated protein kinase (CAPK) (Joseph *et al.*, 1994) (Chapter 7).

Chapter 2 Materials and Methods

2.1 Cells

2.1.1 Bovine endothelial cells

Bovine brain microvascular endothelial cells (BBEC) were isolated from the cerebral cortex of freshly slaughtered cows as described by Bowman *et al.* (1983). Briefly, leptomeninges, ependymal tissue and large vessels were removed before the minced tissue was digested in 0.5% dispase (Boehringer Mannheim). Microvessels were isolated by centrifugation in 15% dextran (Sigma) and further digested with 1 mg/ml collagenase/dispase (Boehringer Mannheim). Endothelial cells were separated from other cells on a Percoll gradient (Sigma). Purity was confirmed by positive immunofluorescent staining with anti-factor VIII antibodies. BBEC were grown on bovine fibronectin (Sigma) treated plates, and maintained in medium 199 (M199, ICN Biomedical), 10% horse serum (Hyclone Laboratories), 25 mM HEPES, 10 mM $NaHCO_3$, 20 µg/ml endothelial cell-growth supplement (Sigma), 100 µg/ml heparin (Sigma), 100 µg/ml penicillin, and 100 µg/ml streptomycin.

Bovine pulmonary artery endothelial cells (CPAE, ATCC #CCL 209) were obtained from ATCC (Rockland, MD). CPAE is an endothelial cell line derived from the main stem pulmonary artery of a young cow, by enzyme treatment of artery lumen scrapings. This cell line tests positive for non-cytopathic bovine viral diarrhea virus (BVDV) (Bolin *et al.*, 1994). CPAE were maintained in minimal essential media (MEM, Gibco) with 16% FCS (Hyclone or Gibco) and antibiotics at 37°C with 5% CO_2. CPAE cells were obtained at passage # 12, and were used from passage # 14 to 30. The growth rate of these cells decreased at higher passages, and large multinucleated cells began to appear. A subcultivation ratio of 1:2 to 1:4 was used, as CPAE cells did not grow without cell-cell contact. Stock CPAE cells were stored at -130°C in MEM containing 20% FCS and 5% DMSO at approximately $1x10^6$

cells/ml. On two occasions, lab stocks of CPAE were tested for mycoplasma contamination using a kit from Boehringer Mannheim and were negative.

2.1.2 Additional cell lines

Other cell lines used in this work were: HeLa (epitheliod carcinoma, human, ATCC CCL 2), COS-1 (kidney, fibroblast-like, monkey, ATCC CRL 1650), MDCK (epithelial-like kidney, canine), L-cells (fibroblast-like, connective tissue, mouse, ATCC CCL 1), J774A.1 (monocyte-macrophage, mouse, ATCC TIB 67), Endo-D1 (mouse endothelial cells, a gift from Hermann Ziltner, BRC, UBC), HUVEC (umbilical cord vein endothelial cells, human, ATCC CRL 1730), and U937 (monocyte-like, histiocytic lymphoma, human, ATCC CRL 1593).

2.2 Sera

Fetal calf serum (FCS) was purchased from Gibco and Hyclone. CPAE cells grew better in FCS from Gibco. When grown in the presence of Hyclone FCS, a higher background of dying cells was seen. This caused problems with visual observations of cytotoxicity, and especially disrupted detection of induced DNA fragmentation by producing a high background signal. Horse serum was from Hyclone. Macaque monkey serum was a gift from A. Smith at the University of Washington, WA. Rabbit and mouse sera were obtained at UBC animal facilities. Human serum was pooled from healthy volunteers. Human serum depleted for complement factors C3, C5, and B was a gift from D. Devine (University of British Columbia).

2.3 Reagents and chemicals

2.3.1 Lipopolysaccharide

Haemophilus influenzae type b (Hib, ATCC 10211) LPS was isolated by magnesium ethanol precipitation, a method effective in extracting both smooth and rough LPS (Darveau

and Hancock, 1983). To assess the purity of the final product SDS gel electrophoresis was used. Gels were silver stained to identify the LPS and Coomassie brilliant blue stained to check for protein contamination. *Escherichia coli* O217:B8 LPS (L3129) and *Salmonella typhimurium* LPS (L6511) were prepared by phenol extraction and obtained from Sigma. LPS was prepared as a 1 mg/ml stock in distilled water, and from this a working stock of 10 μg/ml was prepared in MEM or calcium-free MEM. Sonication of stock and working LPS solutions (Bransonic 12 sonicating water bath, 2 min), resulted in more consistent results, especially when preparing serial dilutions of LPS. The stock solutions were stored at 4°C.

2.3.2 Anti-CD14 antibodies

Purified monoclonal antibodies 3C10 (ATCC TIB228; isotype IgG2b) (VanVoorhis *et al.*, 1983), 60b (Todd *et al.*, 1982), and 26ic (Todd *et al.*, 1982), were gifts of S. Wright (Rockefeller University, New York). AML 2-23 (IgG2b) was purchased from Medarex, West Lebanon, NH. MY4 was obtained from Coulter Immunology, Hialeah, FL (IgG2b). Anti-ovine CD14 (which cross reacts with bovine CD14) was from Serotec (mouse IgG1, clone # VPM 65, IgG1).

2.3.3 Other antibodies

Rabbit antisera against bovine IFN-α, IFN-γ, IL-2, and TNF, were a kind gift from A. Potter, at Veterinary Infectious Disease Organization (VIDO) in Saskatoon, Saskatchewan. The neutralization capacity of these antisera were tested in the following assays: TNF; cytotoxicity assay using WEHI 164 cells, subclone 13, INF-α and IFN-γ; anti-viral VSV (vesicular stomatitis virus) assay, IL-2; proliferation assay using concanavalin A treated blast cells. Salmonella O antisera (Group B factors 1, 4, 5, 12) was from DIFCO. Anti-phosphotyrosine 4G10 (mouse monoclonal IgG2bk) was purchased from Upstate Biotechnology Incorporated (UBI).

2.3.4 Miscellaneous reagents and chemicals

Polymyxin B, proteinase K, prestained protein molecular weight standards, 100 base pair DNA ladder, and goat anti-mouse IgG (H+L) alkaline phosphatase conjugated, were all from Gibco. Arginase from bovine liver (Sigma, 205 units/mg) was dissolved in MEM pH 9.5 at 10 mg/ml (2.05 units/µl). N^G-Monomethyl-L-arginine (MW 248.3, Calbiochem) was prepared as a 100 mM stock in distilled H_20. BAPTA/AM was prepared in DMSO at 10 mM and stored at -20°C protected from the light. Thapsigargin was obtained from Calbiochem. Emetine (5 mg/ml) and cyclohexamide (10 mg/ml) from Sigma were prepared in dH_2O and stored at -20°C. Actinomycin D (Sigma) stock was prepared at 1 mg/ml DMSO. Staurosporine, H7, W7, and sodium orthovanadate were from Sigma. Genistein was obtained from UBI. Okadaic acid was from Gibco/BRL. Pertussis toxin and inactive pertussis toxin were obtained from Connaught. Cholera toxin (Calbiochem) was prepared as a 1 mg/ml stock in 50 mM Tris, 200 mM NaCl, 1 mM EDTA. Nocodazole (Sigma) was prepared at 10 mg/ml in DMSO. Cytochalasin D was from Sigma. Phalloidin conjugated to fluorescein isothiocyanate (FITC) was purchased from Molecular Prodes, Inc. Momensin and bafilomycin A1 were from Sigma, while ammonium chloride was obtained from BDH. Natural ceramide (Type III from bovine brain, MW 565.97) and C2-ceramide (N-acetyl-D-sphingosine, MW 341.5) were from Sigma. Ceramide was dissolved in ethanol:dodecane (98:2) at 10 mM, and C2-ceramide was prepared at 20 mM in DMSO. Both stocks were stored at -80°C. Prior to use, reagents were diluted in MEM.

2.4 Viability and cytotoxicity assays

2.4.1 MTT viability assay

Viability assays were performed using the tetrazolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, #M2128, Sigma). This assay has many advantages including minimal cell manipulation, rapidity, convenience of quantification, and the avoidance of radioactive reagents used in other common assays

measuring changes in viability. The MTT assay detects living but not dead cells using a quantitative colorimetric assay which measures mitochondrial dehydrogenase activity in viable cells (Mosmann, 1983). MTT is reduced to an intensely coloured insoluble formazan by the cleavage of the tetrazolium ring and the binding of a hydrogen atom.

The MTT solution was made up in phosphate buffered saline (PBS) at 5 mg/ml and filtered to remove undissolved particles. Unused portions were stored protected from light for up to two weeks at 4 °C. Cells were seeded in 96 well plates (Falcon) at density of 2 $x10^5$ cells/ml, 200 µl/well and used within 2 to 4 days. Prior to experiments, cells were washed 3 times with PBS pre-warmed to 37°C containing 0.5 mM calcium and magnesium (PBS^{++}), or up to 6 times in experiments when the absence of FCS was critical. The outer wells of the 96 well plates were not used due to erratic results obtained in preliminary experiments, and replicate samples were non-randomly dispersed over the plate. Samples of interest were added in MEM to a final volume of 100 µl and the cells incubated at 37 °C with 5% CO_2. Visual checks of cytotoxicity were made periodically. At the end of the incubation period, 10 µl of the MTT solution was added per well, and the cells were incubated a further 2 hours. At this point the formazan crystals in the cells were dissolved by the addition of 150 µl solubilization buffer per well (20% SDS, 50% dimethyl formamide, pH 4.7) pre-warmed to 37°C, combined with vigorous mixing. The plate was then read at 590 nm on a microtitre plate reader (BioRad model 3550). Appropriate blanks were prepared to account for the effects of serum and other factors which may affect the assay.

This assay measures cell metabolic activity and a decrease in activity, compared to controls, is considered to represent cell death. Percent decreased metabolic activity was calculated by dividing the OD of treated wells by the OD of untreated control cells and multiplying by a hundred. With certain cell lines (e.g. L cells, Cos-1) the incubation period following MTT addition was reduced to 1 hour, due to overproduction of formazan resulting in solubility problems after 2 hours.

2.4.2 LDH release cytotoxicity assay

Cytotoxicity assays done measuring the release of lactate dehydrogenase (LDH) were performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780). LDH is a stable cytosolic enzyme released upon cell lysis. Released LDH from culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells (Nachlas *et al.*, 1960).

CPAE cells were seeded in the same manner as described for the MTT assay. Following three washes with PBS^{++}, solutions were added in MEM lacking phenol red (Gibco, 320-1200AJ) to a final volume of 100 µl. Cells incubated in culture media alone were used to measure spontaneous LDH release. Other control cells were incubated in 90 µl media to which 10 µl lysis buffer was added 45 min prior to the end of the incubation to measure total LDH release. At the end of the incubation period 50 µl from each well was moved to a new 96 well plate. Substrate buffer (50 µl/well) was then added to the new plate, and the samples kept in the dark at room temperature for approximately 30 min depending on colour development. Stop solution (50 µl/well) was then added, and the OD of the samples determined using a microtitre plate reader at 490 nm. Blanks consisted of the appropriate media to control for LDH contained in serum. Percent cytotoxicity was calculated by subtracting the OD of untreated cells (spontaneous LDH release) from both the sample OD (various treated cells) and the total OD (lysed cells), and then dividing the corrected sample OD by the corrected total OD and multiplying by a hundred.

Some experiments measured both MTT conversion and LDH release from the same cells. In these experiments 50 µl was removed for LDH detection as usual, and the volume was then returned to 100 µl by the addition of 50 µl MEM. The MTT reagent was added and incubated for a further 2 hours as usual, while the rest of the LDH assay was performed.

Due to the high variability in the percent decreased metabolic activity or cell death obtained between experiments, results from different days could not be quantitatively

compared. Results shown in figures are from representative experiments. Experiments were performed at least twice, usually three or more times, and results were qualitatively similar unless otherwise mentioned.

2.5 Depletion of soluble CD14 from serum

A 1-ml column of anti CD14-Sepharose (60b) was equilibrated with PBS and 2 ml of normal human serum was passed over at a flow rate of 0.05 ml/min. Control serum was passed over an identical column with an irrelevant isotype matched (anti-CD18) antibody. Depletion of sCD14 from serum was measured using a capture ELISA. ELISA Terasaki plates were coated with another anti-CD14 monoclonal antibody, AML2-23, and then blocked with FCS. Dilutions of human serum were incubated with the plates for 60 min at 21°C, then with biotinylated 3C10 to detect CD14 for 60 min at 21°C. Retention of biotin was measured by adding alkaline phosphatase conjugated streptavidin in a final step. The material bound to the anti-CD14 column was eluted with 0.1 mM triethylamine, pH 11.5.

2.6 DNA fragmentation detection

Cells were grown overnight in 100 mm dishes and washed with pre-warmed PBS^{++}. Appropriate reagents were added, and the cells incubated at 37°C 5% CO_2 for varying lengths of time prior to DNA isolation using a modification of the method of Sellins and Cohen (1987). Medium containing non-adherent cells and cell fragments was removed and pooled with a subsequent PBS wash. This was centrifuged (10 min, 228 g, Beckman GPKR) and the resulting pellet lysed in TE buffer (10 mM Tris pH 7.5, 1 mM EDTA) containing 0.2% Triton X-100. Adherent cells were also treated with lysis buffer and the two lysed samples pooled. DNA associated with the nucleus was removed by centrifugation (10 min, 16000 g). Proteinase K was added to the resulting supernatant at a final concentration of 200 µg/ml and incubated (1 h, 55°C). DNA was precipitated using isopropanol and ammonium acetate overnight at -20°C followed by centrifugation (20 min, 16000 g). After drying, pellets were

resuspended in TE with 50 μg/ml RNase and electrophoresed in a 2% agarose gel. DNA in the gel was visualized by ultraviolet fluorescence after staining the gel with ethidium bromide.

2.7 Transmission electron microscopy

Transmission electron microscopy (TEM) studies were used to confirm the occurrence of apoptosis. In the first attempt, the cells were treated with LPS, then scraped and pelleted before fixing in 2.5% glutaraldehyde. For the second attempt, samples were fixed in 2.5% glutaraldehyde before being gently collected by scraping and pelleting and finally embedded in 4% agarose. In both cases the samples were then postfixed with 1% OsO_4, followed by 2% uranyl acetate, and subsequent dehydration in a graded series of ethanol solutions. Embedding in Spurr Low-viscosity medium preceded sectioning and restaining in 2% uranyl acetate and Reynold's lead citrate. Samples were viewed in an electron microscope (model 400; Philips, Mahwah, NJ).

2.8 Fluorescent and phase microscopy

Apoptotic morphology was also examined using fluorescent and phase-contrast microscopy. CPAE were seeded on coverslips in 24 well plates. Prior to use the coverslips were cleaned by boiling for 10 min in 0.1 N HCl, followed by rinsing in distilled water and ethanol and finally autoclaved. Following treatment, cells were fixed on ice for 7 min using 100% methanol that has been cooled to -20°C. Residual methanol was removed with a PBS wash, and nucleic acids were then stained with acridine orange (2 μg/ml) or ethidium bromide (10 μg/ml) for 30 minutes at room temperature. Actin filaments were stained for 30 minutes at room temperature using FITC conjugated phalloidin at a 1:500 dilution of stock (200 units/ml) solution. Cells that had lifted off the coverslip were studied by first spinning them down onto poly-L-lysine treated coverslips (10 min, 228 g), before fixing and staining as above. Coverslips were coated by incubation with a 1:50 dilution of 0.1% w/v poly-L-lysine

(Sigma) overnight at 4°C. Finally, coverslips were washed with PBS and mounted on slides for examination using a Zeiss Axioskop microscope.

2.9 Western blotting

2.9.1 Preparation of cell lysates

Protein samples for western immunoblot analysis were prepared by lysing cells in a 50 mM Tris pH 7.5 buffer containing 1% Triton X-100, 0.4 mM sodium vanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 1 mM sodium fluoride. The Triton X-100 insoluble fraction consisting of cytoskeleton and nuclear components was collected by centrifugation at 16000 g. A portion of the soluble fraction containing cytosolic and membrane proteins was removed for protein determination. Protein concentration was determined using the bicinchoninic acid assay (BCA, Sigma), using bovine serum albumin (BSA) as a standard.

2.9.2 SDS-PAGE and western immunoblot analysis

Samples were mixed with concentrated sodium dodecyl sulfate (SDS) reducing buffer, to a final concentration of 50 mM Tris-HCL pH 6.8, 2% SDS, 0.1% bromophenol blue and 10% glycerol. These samples were boiled for 5 minutes before storage at -20°C. Dithiothreitol (DTT) was added to the samples prior to separation at a final concentration of 100 mM. The samples were separated on polyacrylamide gels according to the methods of Laemmli (1970). Proteins were electrophoretically transferred to nitrocellulose (AB-S 83, Scheicher and Schuell, Keene, NH) using the Biorad Mini Protean II system. Relative protein loading amounts were checked by staining the blots with Ponceau S (0.2%). The membrane was blocked with 5% BSA 0.2% Tween in PBS overnight at 4°C, followed by a 30 minute room temperature incubation with gentle shaking. The membrane was washed once in PBS before incubation with primary antibody diluted in PBS with 0.5% BSA for one hour at room temperature. The anti-phosphotyrosine mouse monoclonal antibody, 4G10, was used at a

dilution of 1:2000. The membrane was then washed 5 times in PBS for 5 minutes before incubation with the secondary antibody, goat anti-mouse IgG coupled to alkaline phosphatase (AP) at a 1:2000 dilution for a further 1 hour at room temperature with gentle shaking. The membrane was then washed as before, with an additional wash in AP substrate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCL pH 9.5). Finally colour development was carried out using 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Gibco) and 0.25 mg/ml nitro blue tetrazolium (NBT, Gibco) in AP substrate buffer. The reaction was stopped after 10 to 60 minutes by washing in large amounts of water.

2.10 Inositol phosphate assay

Production of inositol phosphates (IPs) was determined as described by Ruschowski *et al.* (1992). Briefly, cells were seeded in 60 mm dishes for 4 hours after which the medium was replaced with MEM containing 1% FCS and 10 μCi [3H]myo-inositol (184 Ci/mmol, Amersham Life Science) and incubated overnight. Cells were washed once with PBS before the addition of HEPES-saline containing 10 mM LiCl for 15 minutes. This was then replaced with fresh HEPES-saline containing 6.25% FCS alone or with 100 ng/ml LPS. After various times, cells were washed with cold PBS and scraped into 1 ml PBS. Cells were then added to 4 ml of 2:1 methanol:chloroform, mixed, and incubated at room temperature for 30 minutes. Following the addition of 0.125 ml EDTA, 1.2 ml chloroform and 1 ml H_2O samples were vortexed and centrifuged at 500 g for 20 minutes. The aqueous phase was loaded onto a 1 ml Dowex anion exchange column (AG 1-X8 resin, Bio-Rad Laboratories) pre-washed with 20 ml of 5 mM inositol. The columns were washed with 20 ml 5 mM inositol and 20 ml 5 mM borate/60 mM Na formate, before IPs were eluted with 1 M ammonium formate in 20 ml 0.1 M formic acid. The production of IPs was determined by counting the eluant of each sample diluted in Ready Safe liquid scintillation cocktail (Beckman).

2.11 Data imaging

Negatives (TEM, phase/fluorescent microscopy, and photographed westerns) were scanned (Agfa Studio and Epson ES-1200C Scanners) into Adobe Photoshop version 3.0.4, where they were labeled before printing with a Hewlett Packard Laser Jet 5MP and a Mitsubishi S3600-30U colour printer. Confocal laser scanning images were analysed using NIH Image version 1.60. Projections made in NIH Image were saved in a TIFF format, then imported into Adobe Photoshop version 3.0.4.

Chapter 3 LPS is directly cytotoxic to bovine endothelial cells

3.1 LPS causes visual cytotoxic effects on BBEC and CPAE

Initially the effect of live *Haemophilus influenzae* type b (Hib) on primary cultures of bovine brain endothelial cells (BBEC) was studied (Patrick *et al.*, 1992). The endothelial cells rounded up and membrane perturbations appeared on the cell surface after incubation with Hib for two hours. This effect was duplicated both with heat killed Hib and overnight bacterial broth passed through a 0.22 μm pore filter (Nucleopore). The evidence of a heat stable secreted bacterial product pointed towards LPS, which is unaffected by boiling, and is shed from Hib. This hypothesis was confirmed in two ways. Bacterial filtrates incubated with polymyxin B, a cationic antibiotic which binds to the lipid A portion of LPS and inactivates it (Morrison and Jacobs, 1976), did not disrupt the BBEC monolayers. In addition, purified LPS isolated from Hib caused identical cytotoxic effects.

These experiments were repeated using the bovine pulmonary endothelial cell line, CPAE. Identical morphological changes were seen in both cell types, and CPAE cells were routinely used in further experiments due to the difficulties obtaining BBEC. Untreated monolayers of CPAE cells contained elongated cells and a few round cells (Fig 3.1 A, B). Following treatment with 100 ng/ml LPS, cells were seen which were rounding up and blebbing (Fig 3.1 C, arrow heads). At a higher magnification the extensive membrane pertubations were seen in some LPS treated CPAE cells (Fig 3.1 D, arrow heads). This effect was first seen approximately two hours after the addition of LPS, and over time more cells were affected. Isolated cells throughout the monolayer were affected, rather than cells in groups. The blebbing cells eventually detached from the substratum.

3.2 LPS-induced reduced metabolic activity measured using the MTT assay

Several lines of evidence suggested that the LPS was cytotoxic to CPAE and BBEC cells. Morphological criteria and trypan blue staining indicated that the cells were dead. In

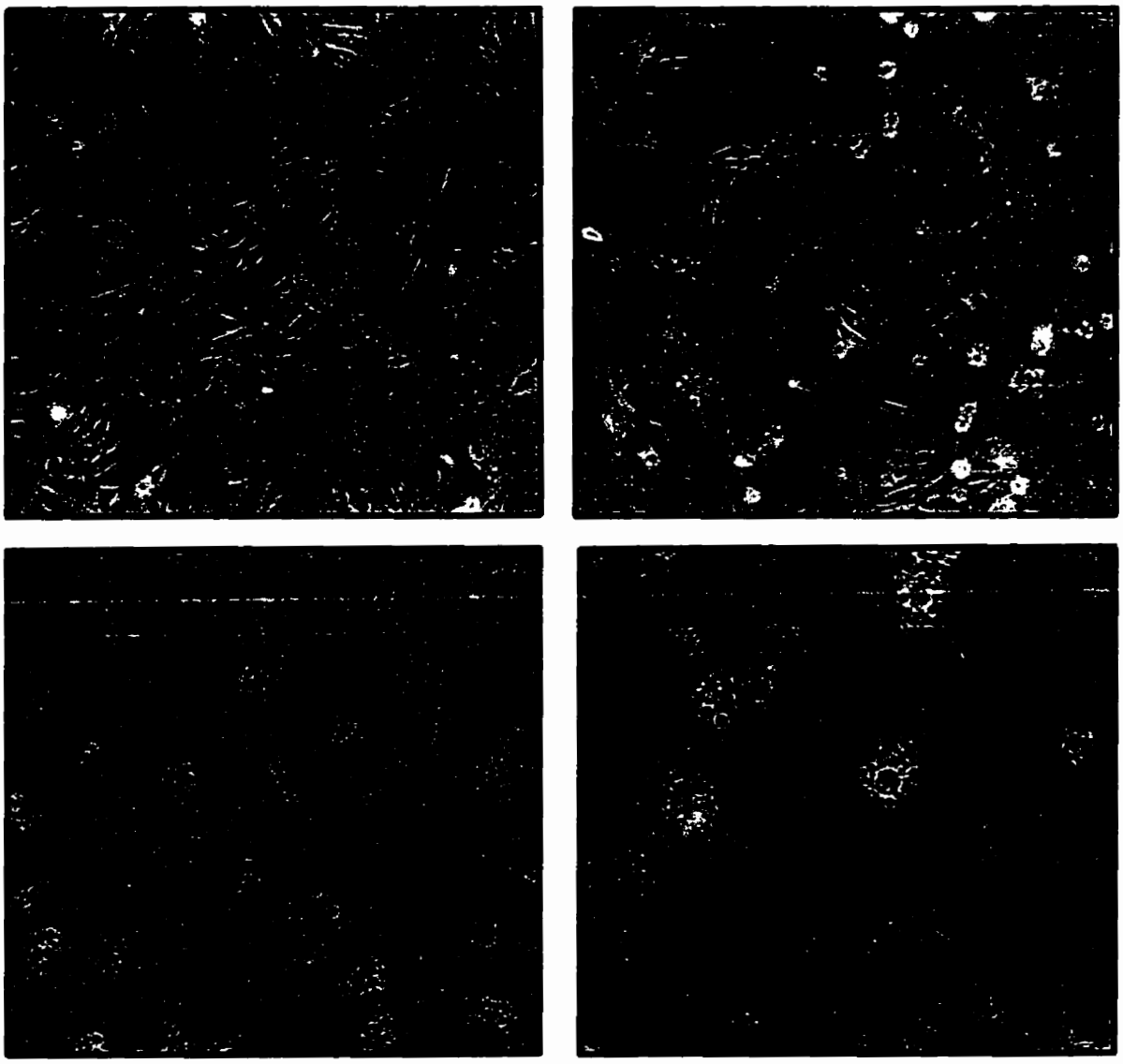


Figure 3.1 Phase contrast micrographs of control and LPS treated CPAE cells. Morphological effects of LPS on CPAE cells. (A, B) Untreated CPAE cells. (C, D) CPAE cells treated with 100 ng/ml LPS for 3 hours. Arrow heads indicate typical afflicted cells. (A, C) Low magnification, x70. (B, D) Oil immersion, x340.

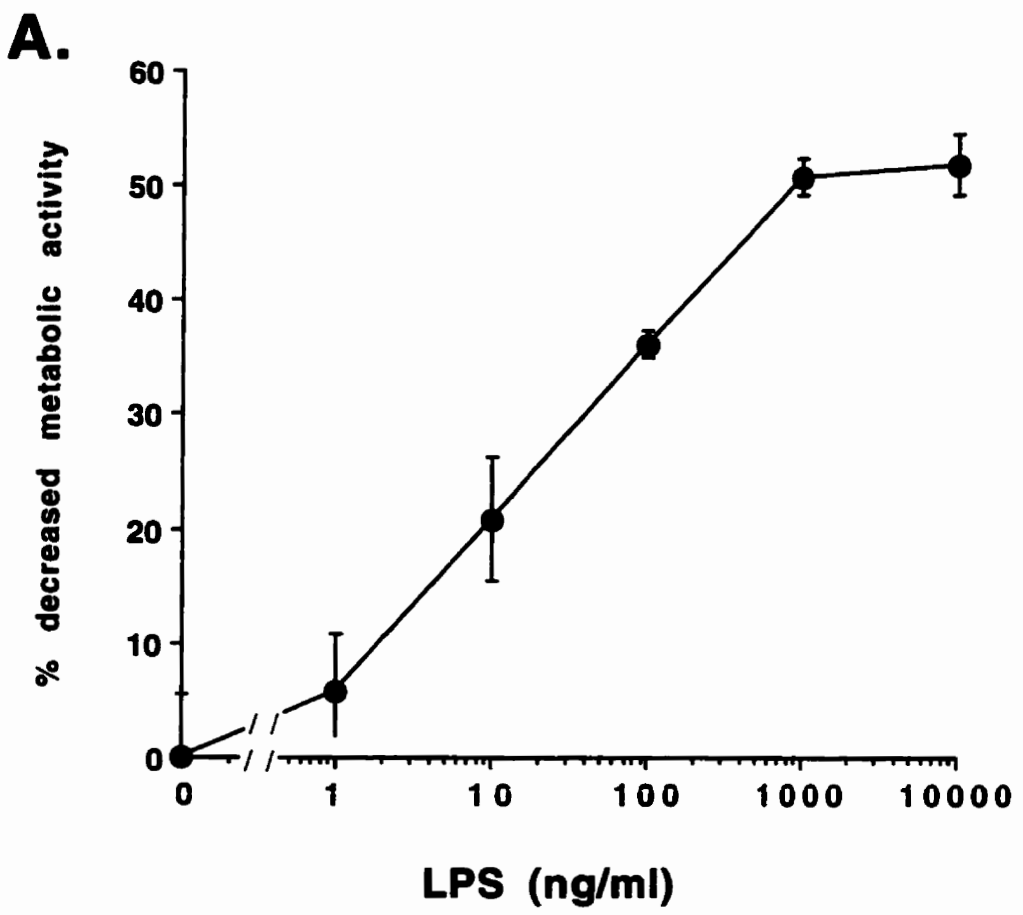
addition, cells which became detached following LPS treatment could not be reseeded. In order to quantitiate the LPS-induced cell death the MTT assay was employed, an assay which detects metabolic acitivity. A decrease in activity, compared to untreated controls, is universally considered to represent cell death. Using this method, it was shown that an overnight incubation with LPS had a dose dependent cytotoxic effect on CPAE cells, with measurable death starting to occur at 1 ng/ml and increasing with LPS concentration until it reached a maximum at about 1 μg/ml (Fig 3.2 A). The LPS-induced CPAE cell death also increased over time, first detectable at 4 hours and then plateauing out at 24 to 36 hours (Fig 3.2 B). From these results, an LPS concentration of 100 ng/ml and an incubation time of overnight (approximately 18 hours) was chosen for future experiments. The time was chosen because an overnight incubation gave a dependable, clearly measurable level of cell death, which was not consistently the case with shorter, for example 6 hour, incubations, and lengths of time in between were impractical. The LPS concentration of 100 ng/ml was chosen because it was mid-range, below saturation levels, and induced morphological changes in sufficient cells to be easily perceived by phase contrast microscopy after 2 to 3 hours. In addition, this concentration is within the physiological range of LPS measured *in vivo*, at the higher levels correlated with shock development (Brandtzaeg *et al.*, 1989; Doran, 1892).

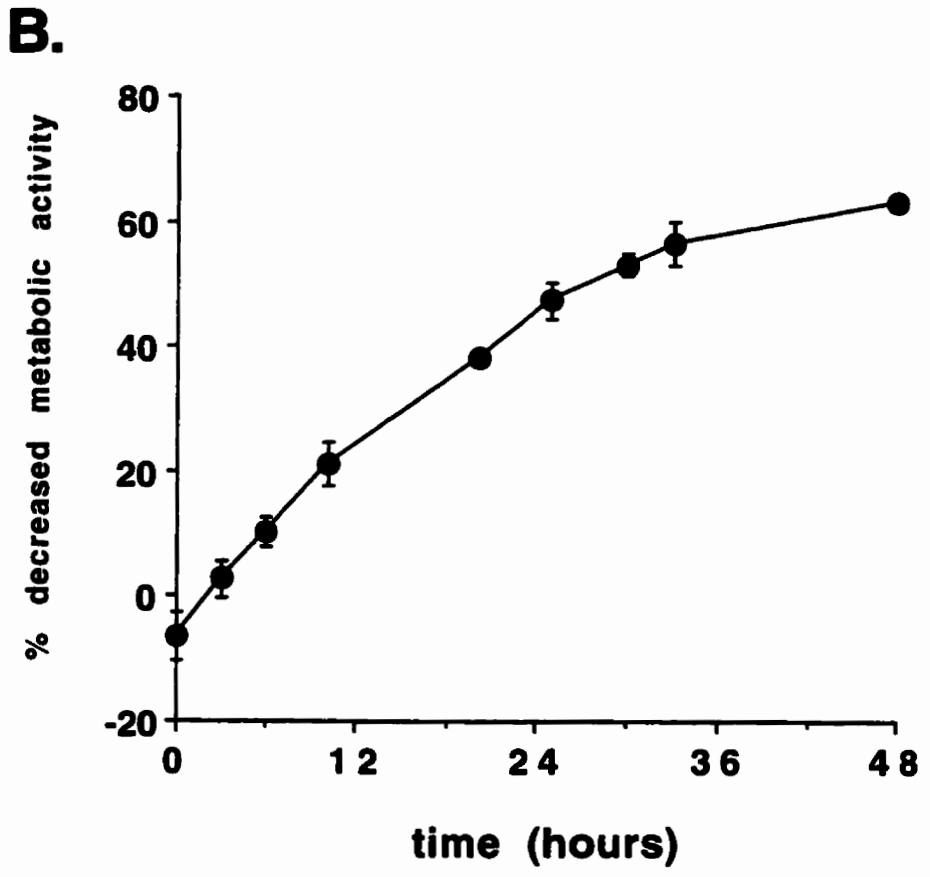
The amount of decreased cell metabolic activity measured using the MTT assay after overnight incubation with 100 ng/ml LPS varied from 25 to 80% between experiments. One possible explanation for this heterogeneity is the physical nature of LPS and the resulting difficulties in achieving completely reproducible dilutions. The age of the CPAE cells could also have an effect. Generally, cells of low passage number tended to give lower levels of cell death. This was not unexpected since the growth rate of CPAE cells would be greater with a lower passage number, allowing for dead cells to be replaced by actively replicating survivors. Another possible source of variability could be the serum. Human serum from different sources and donors potentiated varying levels of LPS-induced cell death. FCS was

more constant, and consistently gave a higher level of cell death than human serum when used at the same concentration on the same day. At no point, either with increasing LPS concentration or incubation time, was 100% cell death seen, either visually, or by measurement with the MTT assay.

It should be noted that there are several factors which need to be considered in the evaluation of experiments using any assay measuring tetrazolium salt reduction. While orginally considered a specific measure of the activity of the mitochondrial respiratory chain, it is becoming increasingly evident that other electron donating systems can reduce tetrazolium salts (Berridge and Tan, 1993). The specific contribution of these other systems is not known. There are also a number of conditions which can influence the reduction of tetrazolium salts. These include the level of glucose in the culture medium, the pH of the medium, and other factors (Marshall *et al.*, 1995). Certain compounds, such as the kinase inhibitor genistein, inhibit cell growth while enhancing the reduction of tetrazolium salts, which can result in misleading readings (Pagliacci *et al.*, 1993). Most of these variables were controlled by appropriate blanks and controls, and usually only presented a problem during longer incubations spanning several days.

Figure 3.2 Cytotoxic effect of LPS on CPAE cells measured with the MTT assay. Time and dose response experiments, measuring cell viability with the MTT assay. (A) LPS dose response. CPAE cells were incubated for 18 hours with the indicated concentration of LPS and 6.25% FCS in MEM. (B) Time course. CPAE cells were incubated for the indicated times with 100 ng/ml LPS and 6.25% FCS in MEM. Values are the average of four samples +/- SD. Results shown are from representative experiments, experiments were repeated ten (A) or three (B) times.

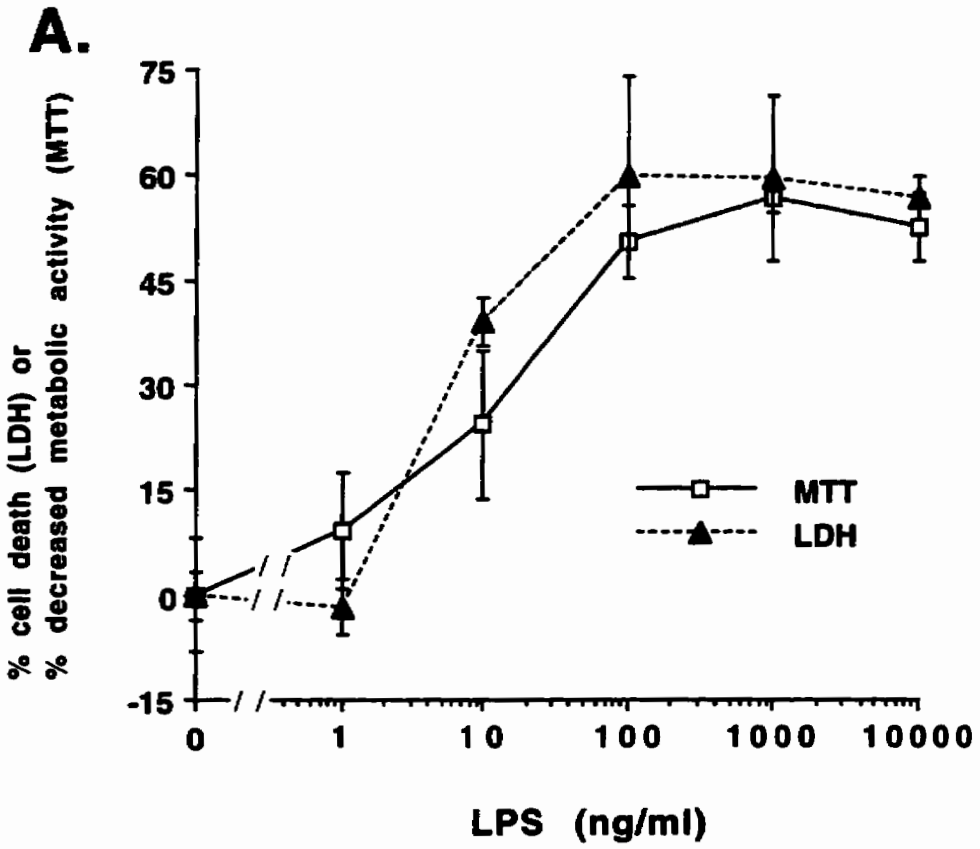

A.
% decreased metabolic activity
60
50
40
30
20
10
0
0
1
10
100
1000
10000
LPS (ng/ml)

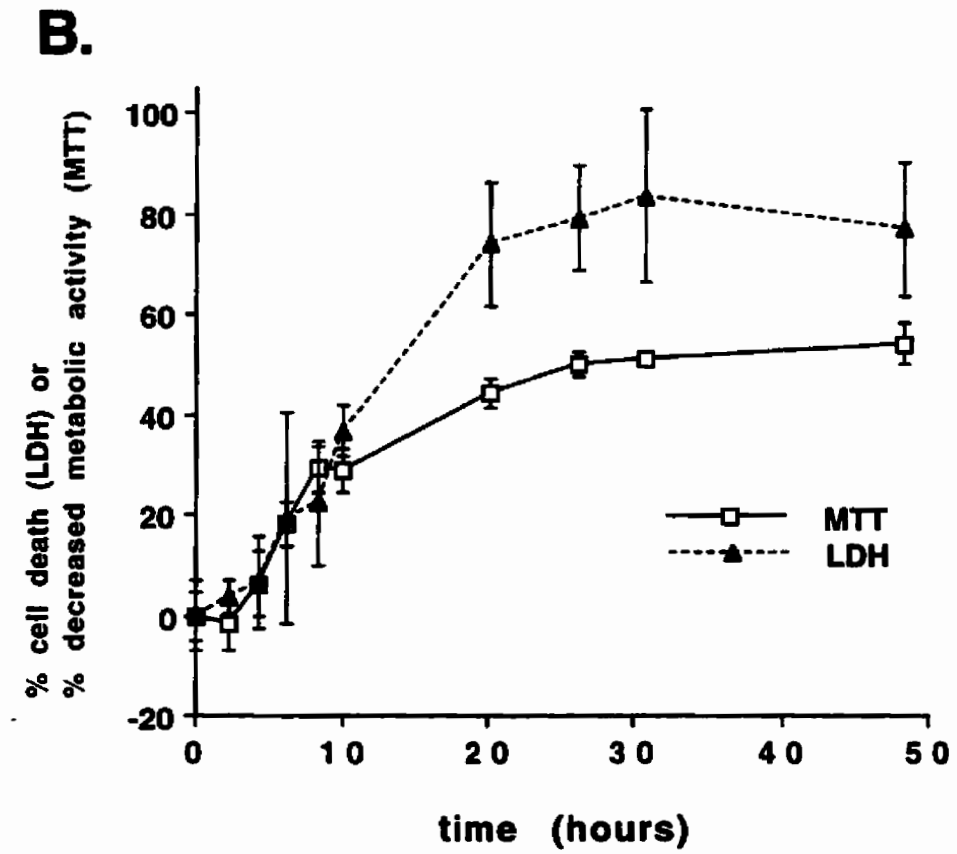

B.
% decreased metabolic activity
80
60
40
20
0
-20
0
12
24
36
48
time (hours)

3.3 LPS-induced cytotoxicity measured using the LDH release assay

To confirm results from the MTT assay, a cytotoxicity assay was performed which measures the release of lactate dehydrogenase (LDH) from cells. LDH is a stable cytosolic enzyme that is released upon cell lysis. This assay is therefore a direct measure of cell death, as compared to the MTT assay which measures cell viability. Experiments measuring LDH release are comparable to the commonly used ^{51}Cr release cytotoxicity assay (Korzeniewski and Callewaert, 1983). Experiments measuring LPS-induced cytotoxicity using both the MTT and the LDH release assays gave very similar measures of cell death and decreased metabolic activity (Fig 3.3 A). Results shown are not statistically different at the .01 level of significance using the Student's t test. One exception was the measurement of LPS-induced cell death over time, where results from the two assays were equivalent only for the first 10 hours (Student's t test, .025 level of significance). After this time the amount of decreased metabolic activity measured using the MTT assay started to level off while the amount measured by LDH release continued to rise and leveled off approximately 30% higher (Fig 3.3 B). This could be due to surviving CPAE cells proliferating over time, which would affect the MTT measurement, but not the LDH levels. Since the MTT assay was both simpler and cheaper, it was utilized as the main measure of cell death, except in cases where compounds were known to interfere with the MTT assay, or to confirm MTT results.

Figure 3.3 Cytotoxic effect of LPS on CPAE cells measured with the MTT and LDH release assays. Time and dose response experiments, comparing decreased cell viability measured with the MTT assay and increased cell death measured with the LDH release assay. (A) LPS dose response. CPAE cells were incubated for 18 hours with the indicated concentration of LPS and 6.25% FCS in MEM. (B) Time course. CPAE cells were incubated for the indicated times with 100 ng/ml LPS and 6.25% FCS in MEM. Values are the average of four samples +/- SD. Results shown are from representative experiments, experiments were repeated three times.


A.
% cell death (LDH) or
% decreased metabolic activity (MTT)
75
60
45
30
15
0
-15
0
1
10
100
1000
10000
MTT
LDH
LPS (ng/ml)


B.
% cell death (LDH) or
% decreased metabolic activity (MTT)
100
80
60
40
20
0
-20
0
10
20
30
40
50
MTT
LDH
time (hours)

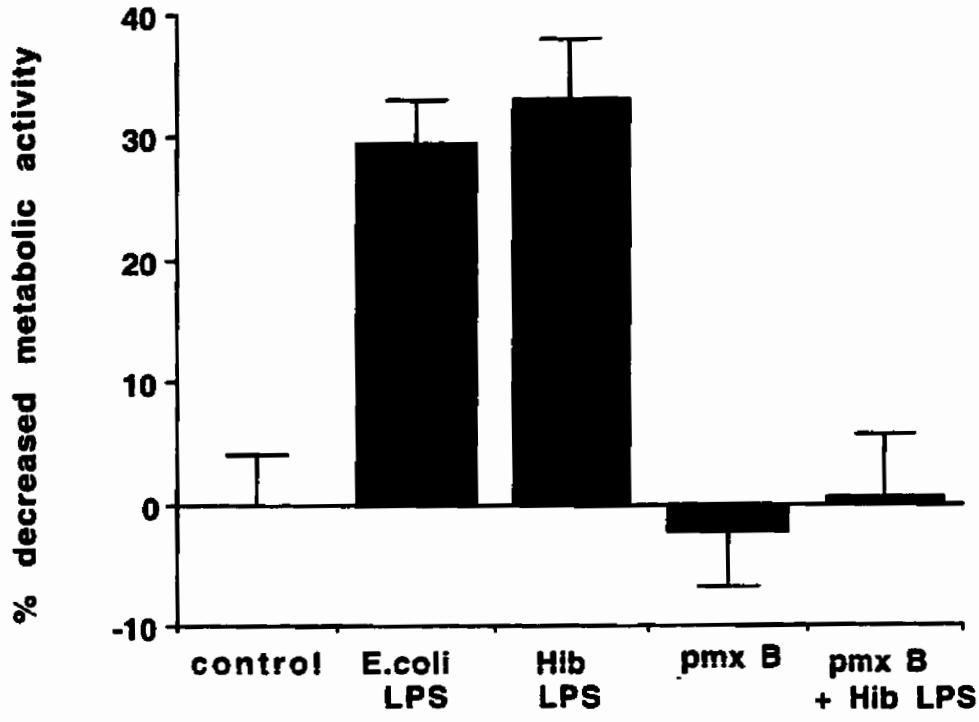
3.4 Other LPS and cell lines

The initial experiments with BBEC and CPAE cells were done using LPS from *Haemophilus influenzae*. This LPS lacks the outer O-specific chain. LPS from other gram-negative species were assayed to see if they also caused cell death in CPAE cells. LPS isolated from *Escherichia coli* O217:B8 and *Salmonella typhimurium* gave almost identical dose response curves, comparable to levels obtained with *Haemophilus influenzae* LPS (Fig 3.4). Results shown for different types of LPS within an experiment are not statistically different at the .025 a level of significance using the Student's t test. These LPS contain an O-specific chain, and are refered to as smooth (S)-LPS. Due to the commercial availability of *E. coli* LPS, this LPS was routinely used for further experiments. The ability of the cationic antibiotic polymyxin B to block LPS-induced CPAE cell death is also shown in Fig 3.4. Polymyxin B binds to the lipid A portion of LPS, resulting in its inactivation. Alone, polymyxin B had no deleterious effects on CPAE cells up to concentration of 20 μg/ml as measured by the MTT assay (data not shown).

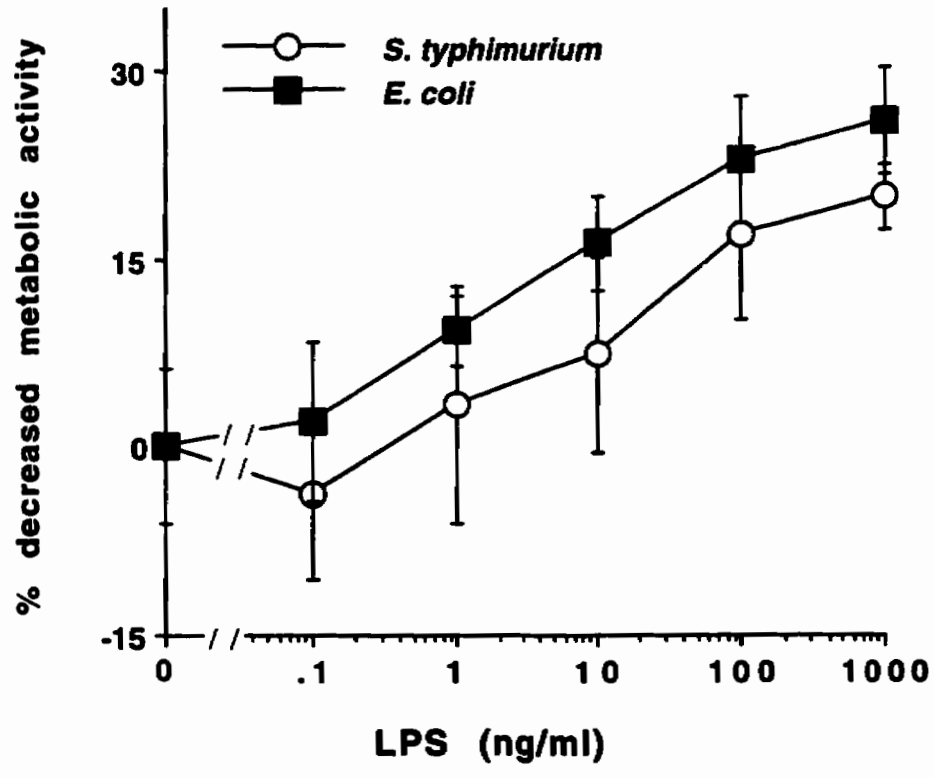
Other mammalian cells lines were tested to determine if they were also sensitive to LPS-induced cytotoxicity. These cell lines included two other types of endothelial cells, human umbilical vein endothelial cells (HUVEC), and a mouse endothelial cell line (Endo-D1). The viability of these cells was unaffected by LPS up to a concentration of 10 μg/ml. Combining 100 μg/ml LPS with an extended incubation of 26 hours resulted in some measurable cell death in both cell types. However, this was less than 20% and no visual signs of cell death were seen. Numerous non-endothelial cell lines were also examined. LPS caused no measurable cell death in the human epitheloid cell line HeLa, epithelial-like Madin-Darby canine kidney cells (MDCK), fibroblast-like monkey kidney Cos-1 cells, or mouse fibroblast L cells. Monocyte-like mouse J774A.1 cells showed decreased viability when treated overnight with LPS, although no cell blebbing or other visible signs of death were detectable.

Figure 3.4 Cytotoxic effect of LPS from different gram-negative bacterial species. LPS from *Haemophilus influenzae* (Hib), *Escherischia coli* (*E.coli*) and *Salmonella typhimurium* (*S. typhimurium*) were assayed for their ability to cause CPAE cell death. Decreased metabolic activity was measured after an overnight incubation using the MTT assay. (A) Comparison of *E. coli* and Hib LPS. CPAE cells were incubated in MEM containing 6.25% FCS alone (control), or with 100 ng/ml *E. coli* or Hib LPS. The blocking effect of the antibiotic polymyxin B (pmx B, 5 μg/ml) is also shown. Polymyxin B alone caused no cell death, and completely blocked the cytotoxic effect of 100 ng/ml Hib LPS. (B) Comparison of *E. coli* and *S. typhimurium* LPS. CPAE cells were incubated in MEM containing 6.25% FCS and the indicated amount of either *E. coli* or *S. typhimurium* LPS. Values are the average of four samples +/- SD. Results shown are from representative experiments, experiments were repeated at least three times.

A.


% decreased metabolic activity
40
30
20
10
0
-10
control
E.coli LPS
Hib LPS
pmx B
pmx B + Hib LPS

B.


% decreased metabolic activity
S. typhimurium
E. coli
30
15
0
-15
0
.1
1
10
100
1000
LPS (ng/ml)

3.5 Discussion

LPS caused distinct morphological changes in both CPAE and BBEC cells, including cell rounding and fragmenting. This was identified as cell death using several methods, including two diverse cytotoxicity assays. One measures cell viability by detecting mitochondrial activity (MTT assay), while the other directly detects cell death by measuring a cytosolic protein released upon cell lysis (LDH release assay). Sensitivity to LPS was not restricted by the vessel caliber of the source of the endothelial cells, since both brain microvessel and large vessel pulmonary endothelial cells were affected. This agrees with a study by Arditi *et al.* (1993), which found LPS-induced cytotoxicity in primary isolates of bovine brain microvascular, pulmonary and aortic endothelial cells. Nor was there any apparent difference in the response to LPS between the primary cultured brain cells and the pulmonary cell line, eliminating the possibility that this was a cell line artifact. In addition, much of the previous work showing LPS-induced endothelial cytotoxicity was performed with primary cultured cells (Harlan *et al.*, 1983a; Paulsen *et al.*, 1989; Langford *et al.*, 1991; Meyrick *et al.*, 1989).

There is a clear discrepancy in the sensitivity to LPS of endothelial cells from different species. We found that human and mouse endothelial cells were unaffected by LPS, while others have shown that canine, goat and human endothelial cells are resistant (Harlan *et al.*, 1983b). It is conceivable that the resistance of human endothelial cells to LPS is a particular trait of the umbilical vein cells routinely used. One study found that human glomeral endothelial cells are killed by LPS at the same level as bovine arterial endothelial cells, while HUVEC cells are totally resistant (Raghu *et al.*, 1986). However, it has been recently shown that human brain microvessel endothelial cells are activated rather than killed by LPS (Arditi *et al.*, 1995). Sheep are the only other species studied with endothelial cells sensitive to LPS at a level comparable to bovine endothelial cells (Meyrick *et al.*, 1989).

The variability in sensitivity between the animal species, to LPS measured *in vitro*, may be related to disparities seen *in vivo*. Major differences have been seen in animals used

as models of endotoxemia (reviewed by Redl *et al.*, 1993). Rodents are often used due to their small size, but high levels of LPS are necessary. Rabbits are more sensitive, and sheep are even more so, and they are commonly used for studies on the effects of LPS on the lung. Pigs are commonly used as a model for human sepsis, but there are considerable differences in their reaction to LPS. Chimpanzees share similar LPS sensitivity to humans, while baboons are highly insensitive. Cows have not been used in *in vivo* testing of LPS, so their sensitivity is not known.

The source of LPS is not critical, as very similar results were obtained with LPS from *H. influenzae* , *S. typhimurium*, and *E. coli*. This was not unexpected since the lipid A portion of LPS is thought to be responsible for most of the cytopathic effects of LPS and its structure is relatively conserved between gram-negative species. Polymyxin B inactivates LPS by binding to lipid A, and as it blocked the cytotoxic effect of LPS on the bovine endothelial cells it suggests that this region of LPS is involved. Langford *et al.* (1991) found that purified LPS from mutant *H. influenzae* strains differing in LPS core structure all cause bovine endothelial cell death. While no clear correlation between the cytotoxicity and virulence of the mutants is seen, the avirulent mutant I69 is considerably less cytotoxic than the parental strain. This mutant has a minimal core structure, consisting of a single phosphorylated KDO residue (Helander *et al.*, 1988). It is possible that the reduction in cytotoxicity of I69 LPS was due to solubility problems or accelerated detoxification by serum.

Chapter 4 Involvement of soluble CD14 in LPS mediated endothelial cell death

4.1 Necessity of serum for LPS-induced cytotoxicity

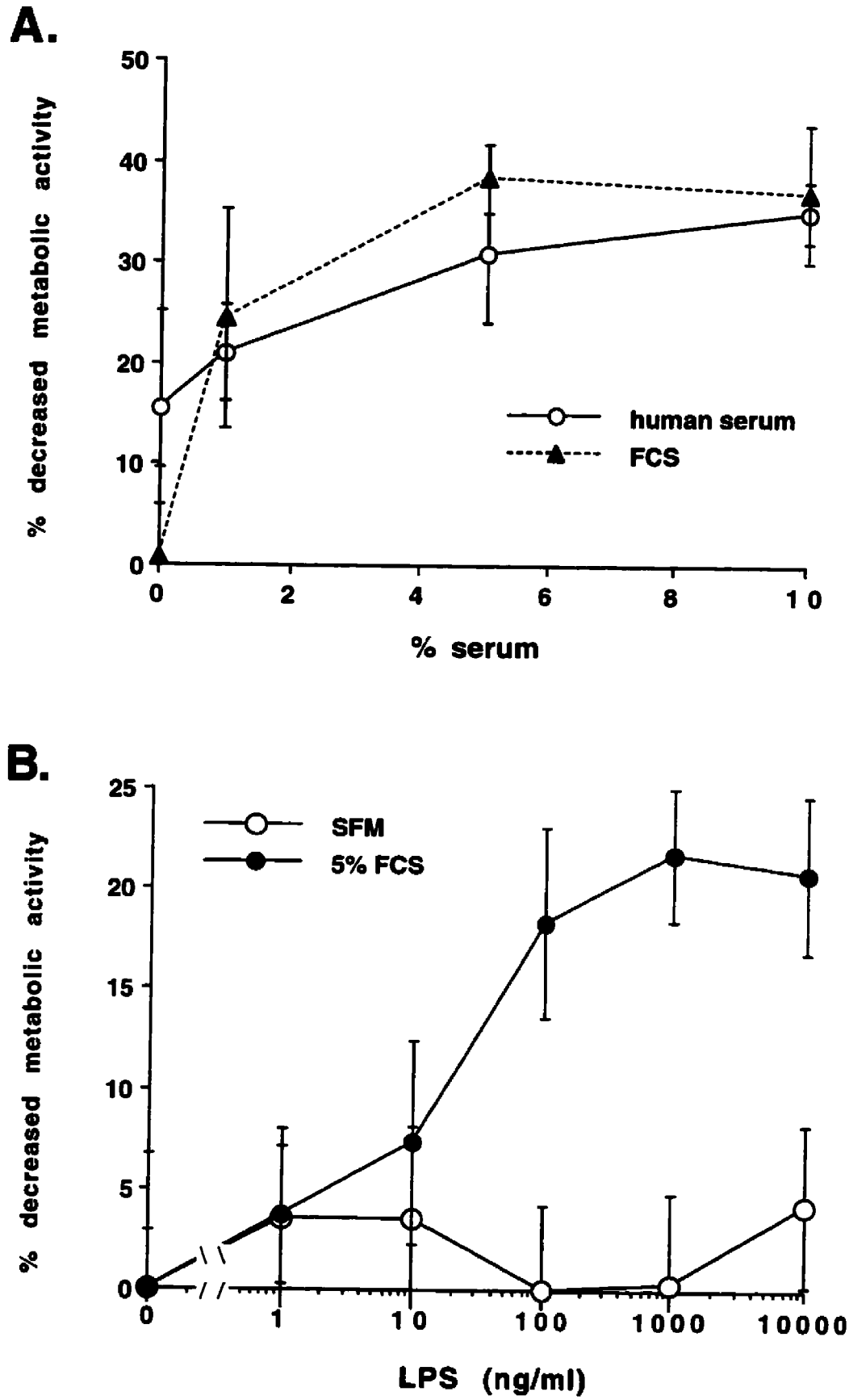
Horse serum was present in all the initial BBEC experiments, and neither Hib nor purified LPS had any cytotoxic effect in its absence. When human, bovine, or fetal calf serum (FCS) was substituted for horse serum, Hib LPS once again caused BBEC cell death. This serum requirement was further studied using the CPAE cells. LPS-induced cell death was observed with all types of sera assayed, which included, in addition to those mentioned above, porcine, mouse, rabbit, goat, and monkey serum. Using the MTT assay, the serum effect was titered in CPAE cells using human and fetal calf sera. Detectable cell death occurred at serum concentrations of 1%, and the effect increased with serum concentration until it appeared to level off at 5 to 10% serum (Fig 4.1 A). Serum interferes with the MTT assay at higher concentrations, and human serum especially gave exceedingly high blank OD reading at concentrations greater than 20%, making cell death measurements at higher serum levels unreliable.

LPS dose response experiments in the presence and absence of FCS demonstrated that no cell death occurred in the absence of serum up to a LPS concentration of 10 μg/ml (Fig 4.1 B). Interestingly, at one point LPS-induced cytotoxicity was seen in serum-free medium containing bovine serum albumin (BSA, Sigma), which was included as a protein carrier in some experiments. An earlier batch of BSA did not interfere in the assay. This might indicate varying levels of other contaminating serum proteins such as LBP and sCD14 in commercially available BSA which could have been responsible for this effect.

4.2 Characterization of the active component in serum

LPS can trigger the alternative pathway of complement activation (Morrison and Kine, 1977). To examine whether complement played a role in LPS-induced endothelial cell death, serum was heated to 56°C for 30 minutes, a common practice used to inactivate the

Figure 4.1 Effect of serum on LPS-induced CPAE cell death. (A) Effect of serum concentration. CPAE cells were incubated in MEM containing 100 ng/ml LPS and the indicated amount of either FCS or human serum overnight. (B) Cytotoxic effect of LPS in the presence and absence of serum. CPAE cells were incubated in either serum-free MEM (SFM) or MEM containing 5% FCS along with increasing amounts of LPS for 18 hours. Cytotoxicity was measured using the MTT assay. Values are the average of four samples +/- SD. Results shown are from representatative experiments, similar experiments were performed at least twice.

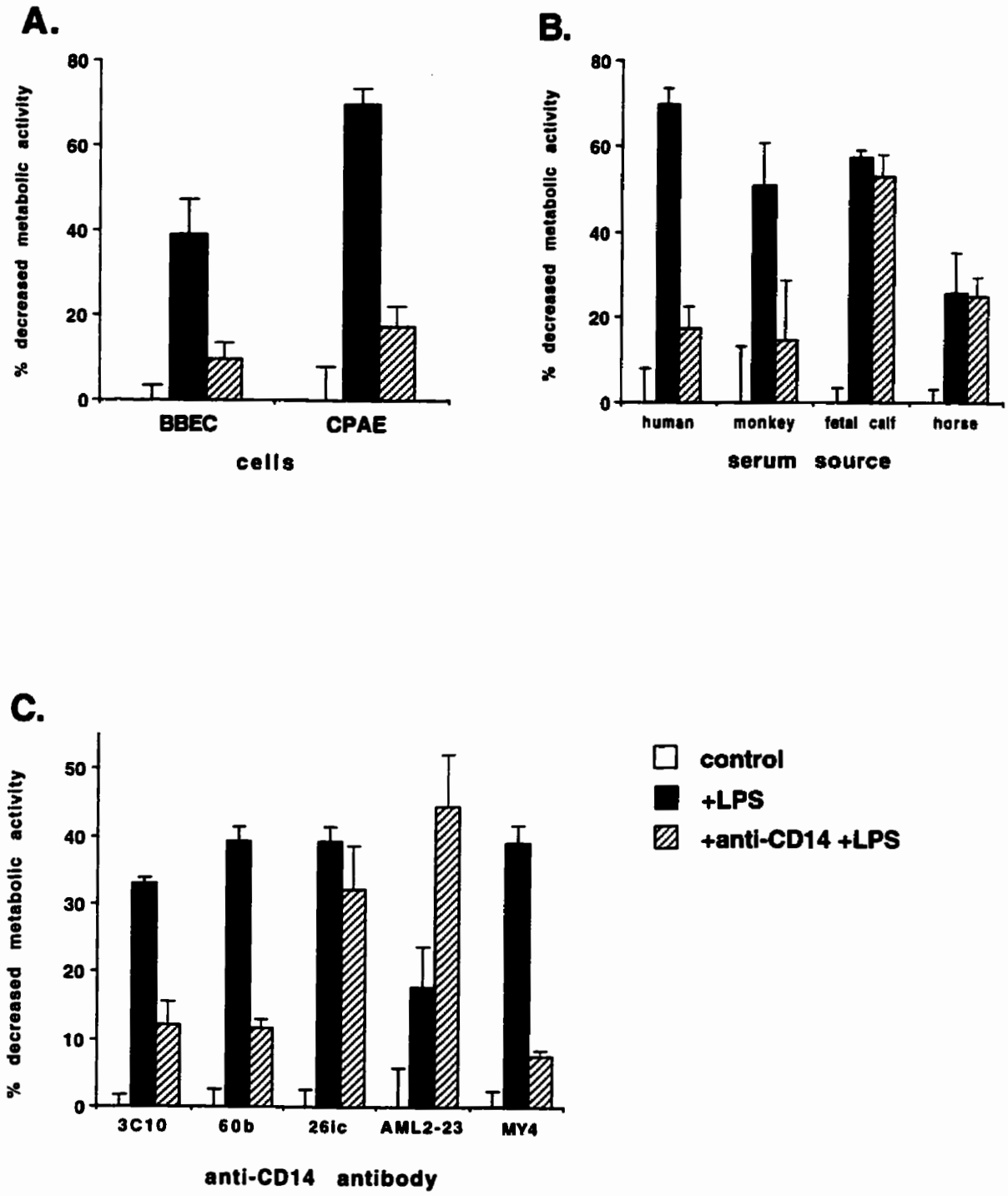

A.
% decreased metabolic activity
50
40
30
20
10
0
0
2
4
6
8
10
% serum
human serum
FCS
B.
% decreased metabolic activity
25
20
15
10
5
0
0
1
10
100
1000
10000
LPS (ng/ml)
SFM
5% FCS

complement system. This had no effect on LPS-induced cytotoxicity. In addition, there was no loss of cytotoxicity using human serum specifically depleted for complement factors C5, C3, and B. The Centricon 30 system was used to separate serum components by size with a molecular cutoff of 30 kDa. The LPS-induced cytotoxic effect was seen only in the fraction containing serum components larger than 30 kDa and was lost in the fraction consisting of components 30 kDa or smaller.

A possible candidate for the active moiety in serum was suggested by the identification of CD14 as a receptor for LPS on monocytes (Wright *et al.*, 1990). While CD14 has not been detected on the surface of endothelial cells, it is found in serum (Maliszewski *et al.*, 1985). This soluble form is fairly abundant in human serum (approximately 5 μg/ml), but had no known function at the time. To determine if sCD14 was involved in the cytotoxic effects of LPS on the bovine endothelial cells, we used anti-CD14 antibodies to block cytotoxicity. The mouse monoclonal antibody, 3C10, directed against human CD14, blocks human monocyte activation by LPS (Wright *et al.*, 1990). We found that it was also capable of blocking LPS-induced cell death in both BBEC and CPAE cells at a concentration of 2 μg/ml or greater, when added to cells incubated with serum 30 minutes prior to LPS (Fig 4.2 A). This blocking effect was seen when human serum was used, but was not seen when horse, fetal calf, bovine, or rabbit serum were used, demonstrating the species specificity of the antibody (Fig 4.2 B). To see if there was any species cross reactivity, macaque monkey serum was tested. All 9 samples of serum from different monkeys supported LPS-induced cell death. The anti-CD14 antibody, 3C10, could also protect CPAE cells in macaque monkey serum (Fig 4.2 B).

Other anti-CD14 monoclonal antibodies directed against the human antigen were assayed to confirm that inhibition was due to blocking CD14 (Fig 4.2 C). 26ic, which binds to CD14 but does not block its interaction with LPS (Wright *et al.*, 1990), did not block LPS-induced CPAE death up to a concentration of 20 μg/ml. Another, 60b, which also blocks macrophage activation by LPS, protected CPAE cells against LPS mediated cytotoxicity.

Figure 4.2 Blocking effect of anti-CD14 antibodies. (A) Blocking effect with both endothelial cell types. CPAE and BBEC cells were incubated in MEM containing 6.25% human serum. Cells were incubated in medium alone (control), with 100 ng/ml LPS (+LPS), or pre-treated 30 min with 5 μg/ml 3C10 before the addition of LPS (+anti-CD14 +LPS). (B) Blocking effect in various sera. CPAE cells were incubated as described above in MEM containing 6.25% of the indicated serum. (C) Effect of different anti-CD14 antibodies. CPAE cells were incubated in 6.25% human serum and pre-incubated 30 min with the indicated anti-CD14 antibody prior to the addition of 100 ng/ml LPS. Antibodies were used at a final concentration of 2 (3C10, 60b), 5 (MY4, AML2-23) or 20 μg/ml (26ic). Decreased metabolic activity was measured after an overnight incubation using the MTT assay. Values are the average of four samples +/- SD. Figures are combined from results from separate experiments, and are representative of experiments repeated twice (A, BBEC results) or at least three times.


A.
% decreased metabolic activity
80
60
40
20
0
BBEC
CPAE
cells
B.
% decreased metabolic activity
80
60
40
20
0
human
monkey
fetal calf
horse
serum source
C.
% decreased metabolic activity
50
40
30
20
10
0
3C10
60b
26ic
AML2-23
MY4
anti-CD14 antibody
control
+LPS
+anti-CD14 +LPS

Two commercially available anti-human CD14 antibodies were also used. One, MY4, blocked endothelial cell death almost completely at 5 μg/ml in human serum, but appeared more species specific than 3C10 since it had no blocking effect in macaque monkey serum. The other, AML2-23, did not protect the CPAE cells from LPS, and caused considerable cytotoxicity on its own after an overnight incubation. Eventually anti-ovine CD14 antibodies became available, which cross-reacted with bovine but not human macrophages. As expected, these antibodies blocked the LPS effect in fetal calf and bovine sera, but had no effect in human serum (data not shown).

A number of the antibodies and other compounds used in this work contain sodium azide as a preservative agent. It was important therefore to determine whether azide itself was toxic to CPAE cells. Experiments showed that azide had no detrimental effects on CPAE cells in an overnight incubation up to a concentration of 0.01%, well above the levels resulting from diluted antibodies. The presence of azide also had no effect on LPS-induced cytotoxicity, eliminating the possibility that azide accompanying anti-CD14 antibodies was responsible for blocking cell death rather than the antibodies.

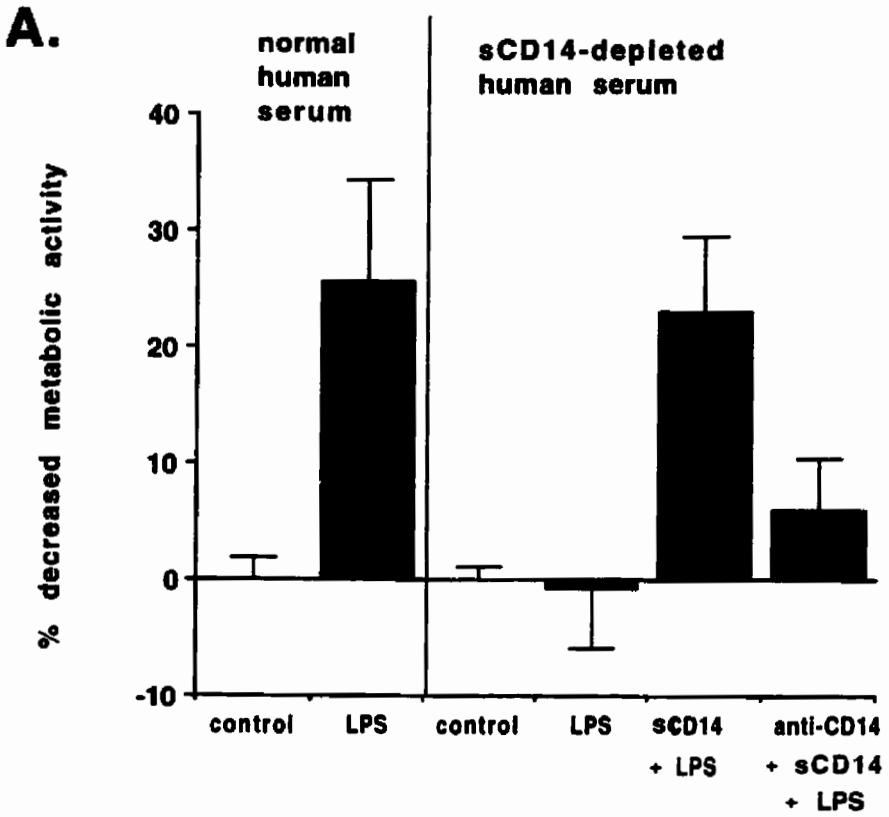
4.3 sCD14 immunodepleted serum

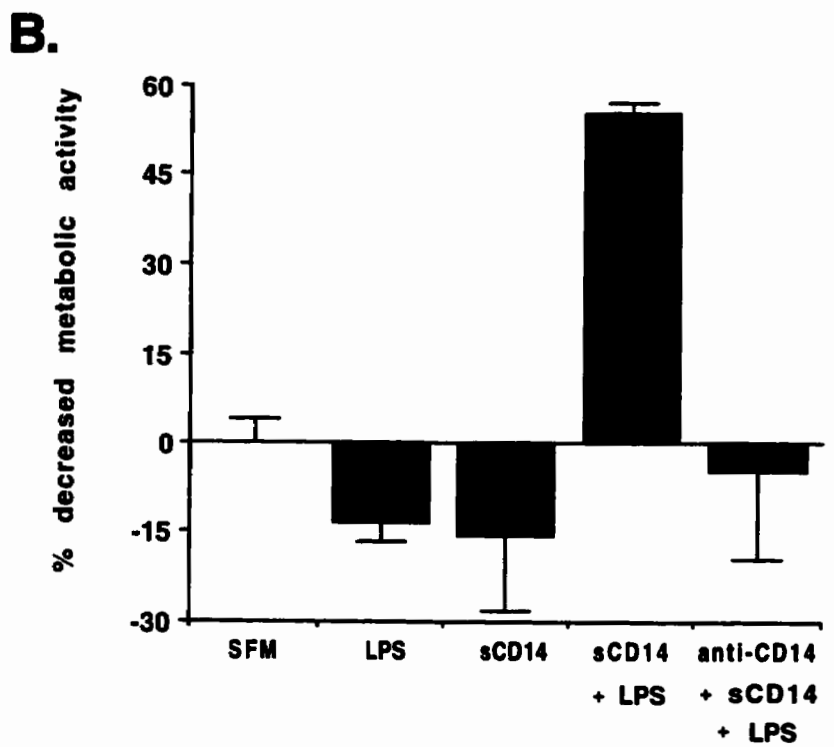
The involvement of CD14 in LPS-induced CPAE cell death was shown by the blocking effect of anti-CD14 antibodies. It was possible however that there was CD14 on the surface of the endothelial cells, and that the presence of serum was necessary for another factor, such as LPS binding protein (LBP). Two lines of inquiry indicated that CPAE cells did not possess membrane-bound CD14. Binding of anti-CD14 antibodies to the endothelial cells was not detectable by ELISA using a sensitive fluorescence plate reader (S.D. Wright, personal communication), and pretreatment of CPAE with 3C10 did not decrease their sensitivity to LPS added in a subsequent incubation. The species specificity of the anti-CD14 antibodies also argued that the source of CD14 was the serum, since the species specificity of the blocking antibodies matched the species of the serum and not that of the cells.

In order to confirm the role of sCD14, human serum was depleted of sCD14 by affinity chromatography on anti-CD14-Sepharose. Serum depleted of sCD14 in this way failed to support a cytotoxic response of CPAE to LPS (Fig 4.3 A). The depleted serum was fully capable of supporting responses of CD14-bearing cells to LPS, such as the production of TNF by macrophages. The possibility of contaminating anti-CD14 antibodies in the depleted serum was excluded by this result, since they would block macrophage activation if present. This was also confirmed by ELISA using wells coated with sCD14 and anti-mouse IgG secondary antibodies (Frey *et al.*, 1992). This sensitive assay indicated that the final concentration of anti-CD14 antibodies was < 0.002 μg/ml in assays containing sCD14 depleted serum. The ability of depleted serum to support a response of endothelial cells to LPS was restored by the readdition of sCD14 (4 μg/ml) to the depleted plasma, and this could again be blocked with the anti-CD14 antibody, 3C10 (Fig 4.3 A).

The material eluted from the anti-CD14 column appeared as a doublet at 55 kDa in SDS gels, the expected size for sCD14. The ability of this material to support LPS induced cytotoxicity on its own was examined. Neither sCD14 nor LPS alone had a cytotoxic effect on CPAE cells when incubated in serum-free conditions, but in combination they resulted in considerable cell death (Fig 4.3 B). This cytotoxicity could be blocked with the anti-CD14 antibody 3C10. However, the purity of the eluted material is not certain, therefore it is not possible to conclude that sCD14 is sufficient to promote LPS-induced cell death in this particular case. A small amount of LBP, undetectable on a protein gel, may be present and involved in inducing cell death.

Figure 4.3 Effect of LPS in soluble CD14 depleted serum. (A) sCD14 depleted serum. CPAE cells were incubated in 6.25% control or sCD14 depleted human serum. LPS (100 ng/ml), sCD14 (approximately 4 μg/ml), and anti-CD14 antibody 3C10 (5 μg/ml, pre-incubated 30 min) were added where indicated. (B) sCD14 in SFM. CPAE cells were incubated in serum-free MEM (SFM), along with the indicated compounds at the concentrations given above. Cytotoxicity was measured after an overnight incubation using the MTT assay. Values are the average of four samples +/- SD. Results are from representative experiments, experiments were performed twice (A) or three times (B).


A.
normal human serum
sCD14-depleted human serum
% decreased metabolic activity
40
30
20
10
0
-10
control
LPS
control
LPS
sCD14 + LPS
anti-CD14 + sCD14 + LPS


B.
% decreased metabolic activity
60
45
30
15
0
-15
-30
SFM
LPS
sCD14
sCD14 + LPS
anti-CD14 + sCD14 + LPS

4.4 Discussion

The necessity of serum for LPS-induced cytotoxic effects was seen previously. Meyrick *et al.* (1986) found that at nanogram levels LPS had no effect on bovine pulmonary endothelial cells in the absence of serum, but caused considerable cell death in the presence of 10% FCS. At higher LPS doses (in the microgram range) they did see cytotoxicity in the absence of serum, and at 10 μg/ml the level of cell death was the same with or without serum. At this dose of LPS, neither visual nor measurable CPAE cell death was detected in the absence of serum in my system. This inconsistency could be due to the sonication of LPS which would in theory result in smaller and more uniform micelles. It is possible that unsonicated LPS at higher concentrations may be found in larger aggregates that can interact with cells independent of serum.

I have shown that sCD14 is necessary for the cytotoxic response of bovine pulmonary endothelial cells to LPS (Frey *et al.*, 1992). Similarly, other groups subsequently showed that other cells lacking membrane CD14 use the soluble form to respond to LPS. Human umbilical vein endothelial cells show increased expression of endothelial-leukocyte adhesion molecule 1 (ELAM-1) after treatment with LPS, and the astrocytoma cell line U373 synthesizes IL-6 in response to LPS, both reactions being dependent on the presence of sCD14 (Frey *et al.*, 1992).

Our findings demonstrating a role for sCD14 in the LPS-induced activation of cells lacking membrane CD14 were later confirmed and extended by several other groups. Pugin *et al.* (1993a) showed that sCD14 is necessary for the activation of human endothelial and epithelial cells by LPS. Haziot *et al.* (1993) used recombinant sCD14 to show that it is sufficient to mediate the response of human endothelial cells to LPS, but that this effect is enhanced by the presence of LBP. Similar work with bovine endothelial cells confirmed the role of recombinant sCD14 alone, but found little effect with added LBP (Arditi *et al.*, 1993). Another group found that sCD14 mediates the activation of the transcription factor NF-κB in human endothelial cells (Read *et al.*, 1993). Further work linking sCD14 to human

endothelial cell activation or bovine endothelial death has since been published (Von Asmuth *et al.*, 1993; Goldblum *et al.*, 1994; Golenbock *et al.*, 1995; Noel *et al.*, 1995; Ishii *et al.*, 1995; Yang *et al.*, 1996).

Following the discovery of CD14 as an LPS receptor on macrophages and monocytes, it was suggested that sCD14 could be used as a therapeutic agent to neutralize LPS (Maliszewski, 1991). Evidence that sCD14 can mediate the activation of cells by LPS suggests the opposite. In addition, septicemia occurs despite the natural high levels of sCD14 found in blood. Several recent studies have looked at the levels of sCD14 in clinical situations. Levels of sCD14 are elevated in polytraumatized and severely burned patients (Kruger *et al.*, 1991). Soluble CD14 levels in septic patients are significantly higher in those with multiple organ failure (MOF), suggesting that it may reflect the degree of pathophysiology (Endo *et al.*, 1994). Another group also found that high levels of sCD14 are associated with increased mortality in gram-negative septic shock (Landmann *et al.*, 1995). Interestingly, elevated levels of sCD14 are also found to be associated with non-gram-negative sepsis (Landmann *et al.*, 1996). These results do not preclude the possibility that mononuclear cells shed CD14 to down-regulate their response to LPS, and high levels of sCD14 merely indicate a serious infection but do not actively play a role. The actual *in vivo* relevance of LPS activation of cells via sCD14 compared to membrane bound CD14 is not known, although sCD14 accounts for over 99% of the total CD14 content of blood. The original hypothesis that sCD14 could be used to neutralize LPS was recently supported by a study showing that high levels of recombinant sCD14 inhibits LPS-induced mortality in mice (Goyert and Haziot, 1995).

The identification of sCD14 in human serum as a necessary factor for LPS-induced CPAE cell death, combined with the fact that sera from many other species could support the same cytotoxic effect, suggested that sCD14 was present in serum from other species. CD14 was originally identified on human cells (Haziot *et al.*, 1988), and was characterized in mice soon after (Setoguchi *et al.*, 1989). The presence of CD14 on porcine (Ziegler-Heitbrock *et*

al., 1994), and bovine (Yang *et al.*, 1995) macrophages has been reported, and recently monkey and baboon CD14 was sequenced (DeGraw *et al.*, 1995). In addition, a LPS-binding protein has been detected in bovine serum, with similar characteristics to human and rabbit LBP (Khemlani *et al.*, 1992; Khemlani *et al.*, 1994; Horadagoda *et al.*, 1994; Horadagoda *et al.*, 1995).

The source of serum, and hence sCD14, had little effect on the cytotoxic effects of LPS on the CPAE cells, suggesting that the putative LPS/CD14 receptor is therefore not species specific. An interesting study was performed taking advantage of a pharmacological difference between murine and human phagocytes (Delude *et al.*, 1995). The lipid A analog, lipid IVA, acts as a LPS antagonist in human phagocytes, while in mouse phagocytes it behaves as a LPS agonist. Murine and human cell lines were transfected with either murine or human CD14 cDNA expression vectors. These transfectants display sensitivities to lipid IV that reflect the sensitivities of the macrophages of similar species and were independent of the source of CD14 cDNA.

In summary, I have demonstrated the requirement of serum for LPS-induced CPAE cell death. A necessary factor in serum was identified as the soluble form of the membrane LPS receptor, CD14. This identification helped to explain how cells lacking the membrane bound form of CD14 could react to LPS. It may be that both soluble and membrane anchored CD14 act in a similar fashion, interacting with the same putative transmembrane protein leading to intracellular signalling, and possibly sharing other downstream elements.

Chapter 5 LPS induces apoptosis in CPAE cells

5.1 Introduction

While it was clear that CPAE cells die following incubation with LPS, the mode of death was not known. The two assays (MTT and LDH) used to measure cell death do not distinguish between apoptosis and necrosis. The MTT assay, which measures a mitochondrial dehydrogenase, would clearly detect necrotic death since mitochondrial swelling and destruction occur. And while mitochondria remain morphologically unchanged during apoptosis, it is thought that their function is affected at an early stage (Kroemer *et al.*, 1997; Zamzami *et al.*, 1996; Marchetti *et al.*, 1996). LDH release would occur both during necrosis and following secondary necrosis in apoptotic cell death. Morphologically, using normal phase microscopy, it is difficult to distinguish between necrotic cell blebbing and apoptotic cell budding. The fact that LPS treated CPAE cells were affected singly rather than in groups suggested the mechanism of apoptosis. Not much was known about the role of apoptosis in pathogenic processes. For these reasons, we examined the mode of cell death of LPS-treated CPAE cells.

5.2 DNA fragmentation

The hallmark biochemical event in apoptosis is DNA cleavage between nucleosomes which produces DNA fragments of specific lengths, which can be analyzed by agarose gel electrophoresis. This phenomenon is fairly easily detected in cell populations unless the level of apoptosis is low, in which case radioactively labeled DNA can be used to increase sensitivity. To look for the occurrence of apoptosis in LPS treated CPAE cells, elaborate DNA purification methods were initially tried, resulting in smears from both LPS-treated and control CPAE cells. Finally, a simpler method was adopted which only isolates extranuclear DNA, as the DNA fragments produced during apoptosis can diffuse out of the nucleus (Sellins and Cohen, 1987). Using this method, it was found that DNA isolated from LPS

treated CPAE cells produced a 200 bp ladder in a 2% agarose gel stained with ethidium bromide (Fig 5.1). A modification to the original method involved the collection of non-adherent cells by centrifugation. Characteristic DNA fragments were isolated from both adherent and non-adherent LPS treated cells, and combining both samples gave the most distinct results. DNA fragmentation was observed in CPAE cells treated with LPS concentrations of 10 ng/ml or more after 3 hours (Fig 5.1 A). Time course experiments indicated that DNA fragmentation was first detectable between 2 and 2.5 hours after the addition of 100 ng/ml LPS (Fig 5.1 B). After LPS treatment for 18 hours the resulting DNA ladder was less distinct, and after 24 hours resembled a smear. CPAE cells that were killed via necrosis did not exhibit DNA fragmentation, in fact no extranuclear DNA was isolated from these cells. Necrosis was induced by complement killing using antiserum raised against CPAE cell membranes, or by repeated freeze/thaw cycles, standard methods known to result in cell death by necrosis (Duvall and Wyllie, 1986). No extranuclear DNA was isolated from untreated cells during short incubations, however over time a faint DNA ladder would appear. This background apoptosis became detectable around 12 hours when cells were incubated with 5% serum, and earlier, within 6 to 8 hours, when cells were incubated in serum-free conditions. Levels of background apoptosis also depended on the passage number of the cells, increasing as the cells aged, and was affected by variability in the growth medium and the source of FCS.

No DNA fragmentation was seen after 4 hours in cells incubated in serum-free medium with up to 10 μg/ml LPS (data not shown). As expected, DNA fragmentation was detected in CPAE cells incubated with LPS (100 ng/ml) and serum from several different species (human, bovine, porcine, rabbit, goat, and horse) (Fig 5.2 A). The involvement of sCD14 in LPS-induced CPAE apoptosis was verified by the addition of the anti-human CD14 antibody, MY4 (5 μg/ml), which completely blocked DNA fragmentation in CPAE cells incubated with 5% human serum and 100 ng/ml LPS (Fig 5.2 B). The blocking effect of human specific CD14 antibody, MY4, was seen only when human serum was used. In

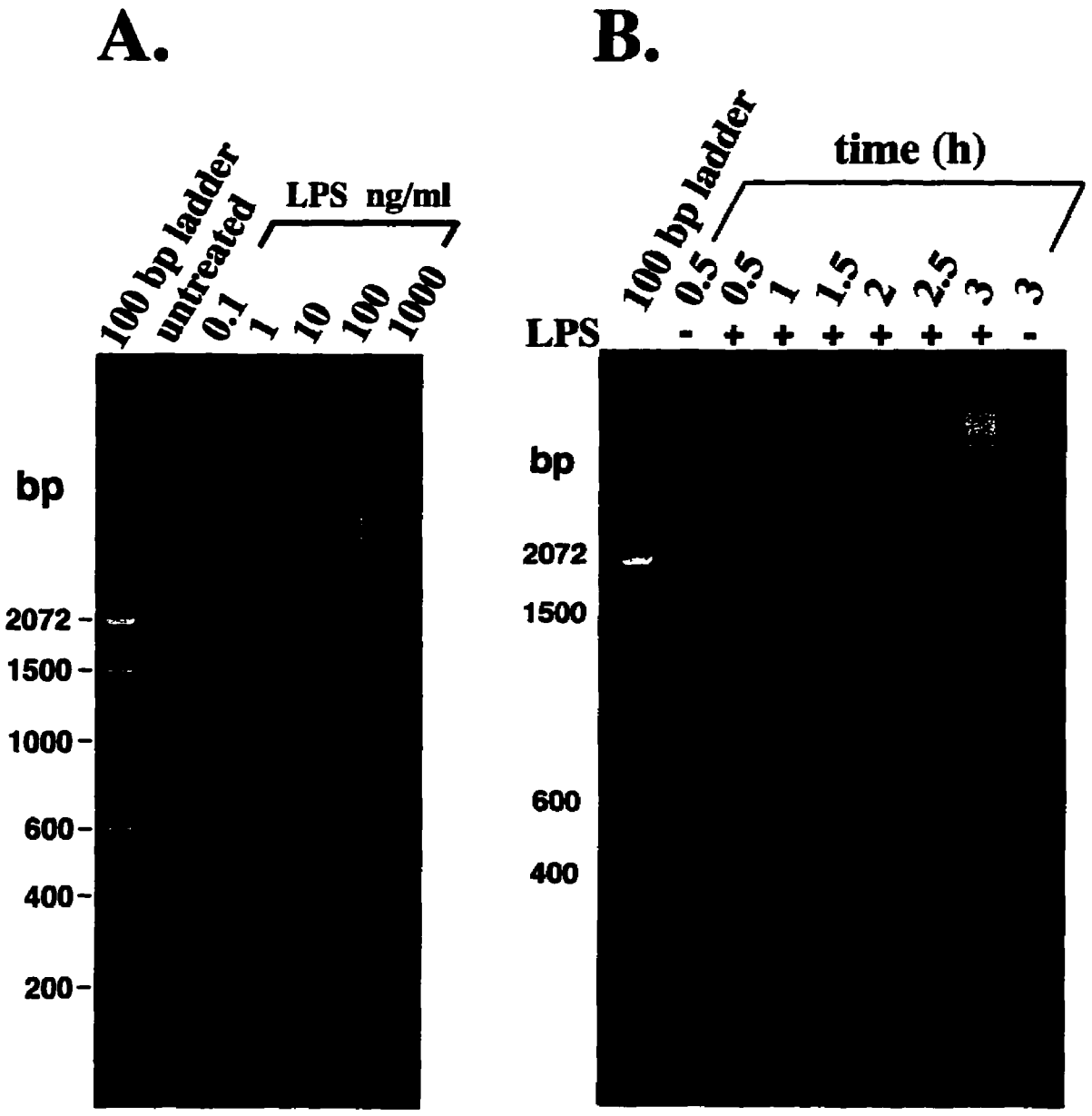


Figure 5.1 LPS-induced DNA fragmentation in CPAE cells. (A) Dose effect. CPAE cells were incubated in MEM with 5% FCS and the indicated amount of LPS for 3 hours. (B) Time course. Cells were incubated for the indicated time with or without 100 ng/ml LPS in MEM containing 5% FCS. At the end of the incubation periods, cells were lysed and extranuclear cellular DNA was isolated and subjected to gel electrophoresis in a 2% agarose gel.

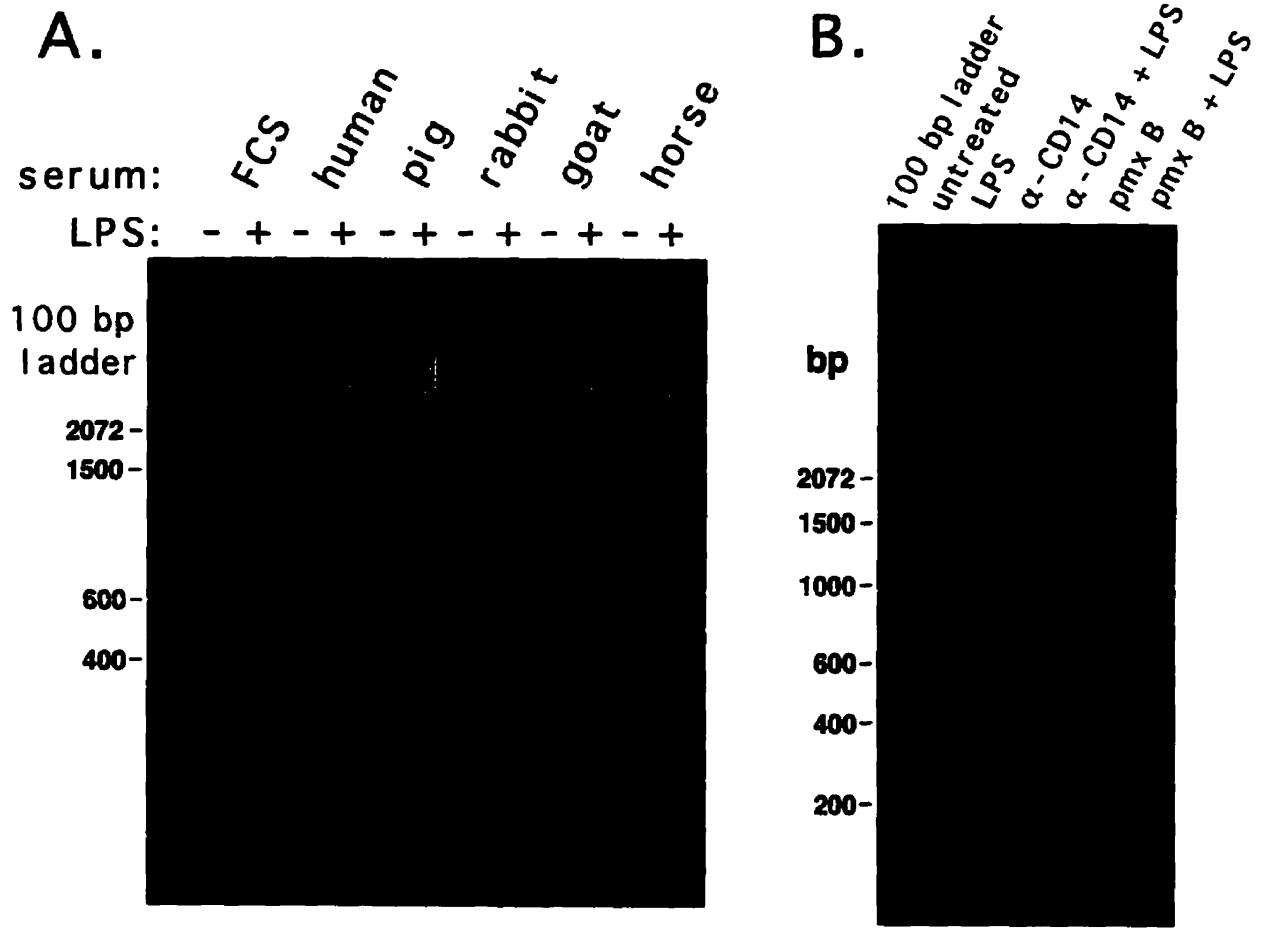


Figure 5.2 Effect of serum on LPS-induced DNA fragmentation in CPAE cells. (A) Serum from different species. CPAE cells were incubated in MEM containing 5% serum from the indicated species in the presence and absence of 100 ng/ml LPS for 3 h. (B) Blocking effect of anti-CD14. CPAE cells were incubated in MEM with 5% human serum, to which LPS (100 ng/ml), anti-CD14 antibody MY4 (α-CD14, 5 μg/ml, 30 min pre-incubation), or polymyxin B (pmx B, 5 μg/ml, 30 min pre-incubation) was added, and cells incubated for a further 3 hours. At the end of the incubation periods, cells were lysed and extranuclear cellular DNA was isolated and subjected to gel electrophoresis in a 2% agarose gel.

addition, DNA fragmentation was blocked with 5 μg/ml polymyxin B, the antibiotic which binds and inactivates LPS (Fig 5.2 B).

5.3 Fluorescent microscopy

In order to confirm the occurrence of apoptosis, cells were examined using fluorescent and phase contrast microscopy. LPS treated CPAE cells showed characteristic apoptotic morphological changes such as cell rounding, membrane pertubations, and the formation of apoptotic bodies, which were visible using phase contrast microscopy (Fig 3.1 D). When DNA intercalating dyes such as acridine orange and ethidium bromide were used, condensed chromatin and nuclear fragmentation could be seen. After a 3 hour treatment with 100 ng/ml LPS most CPAE cells appeared normal, with scattered cells showing signs of apoptosis, rounding up (Fig 5.3 A; arrow) and membrane budding (Fig 5.3 C; arrow) as seen using phase contrast microscopy. The corresponding micrographs of ethidium bromide stained cells demonstrated the difference between normal and apoptotic nuclei (Fig 5.3 B, D, E). Normal nuclei were uniformly stained with 2 to 6 distinct intensely stained nucleoli. In the nucleus of apoptotic cells brightly stained DNA was found along the margins of the nucleus (Fig 5.3 B; arrow, E). LPS treated monolayers also contained cells which appeared morphologically to be undergoing mitosis, an example is shown in Fig 5.3 of a cell in anaphase (A, B; arrowhead). At any given point only a few cells (less than 0.1%) could be found which showed clear signs of apoptotic nuclear condensation. This is most likely due to the transient nature of this stage. Over time however, an increasing number of cells in the later stage of apoptosis, fracturing into apoptotic bodies and staining brightly and uniformly for DNA, could be found.

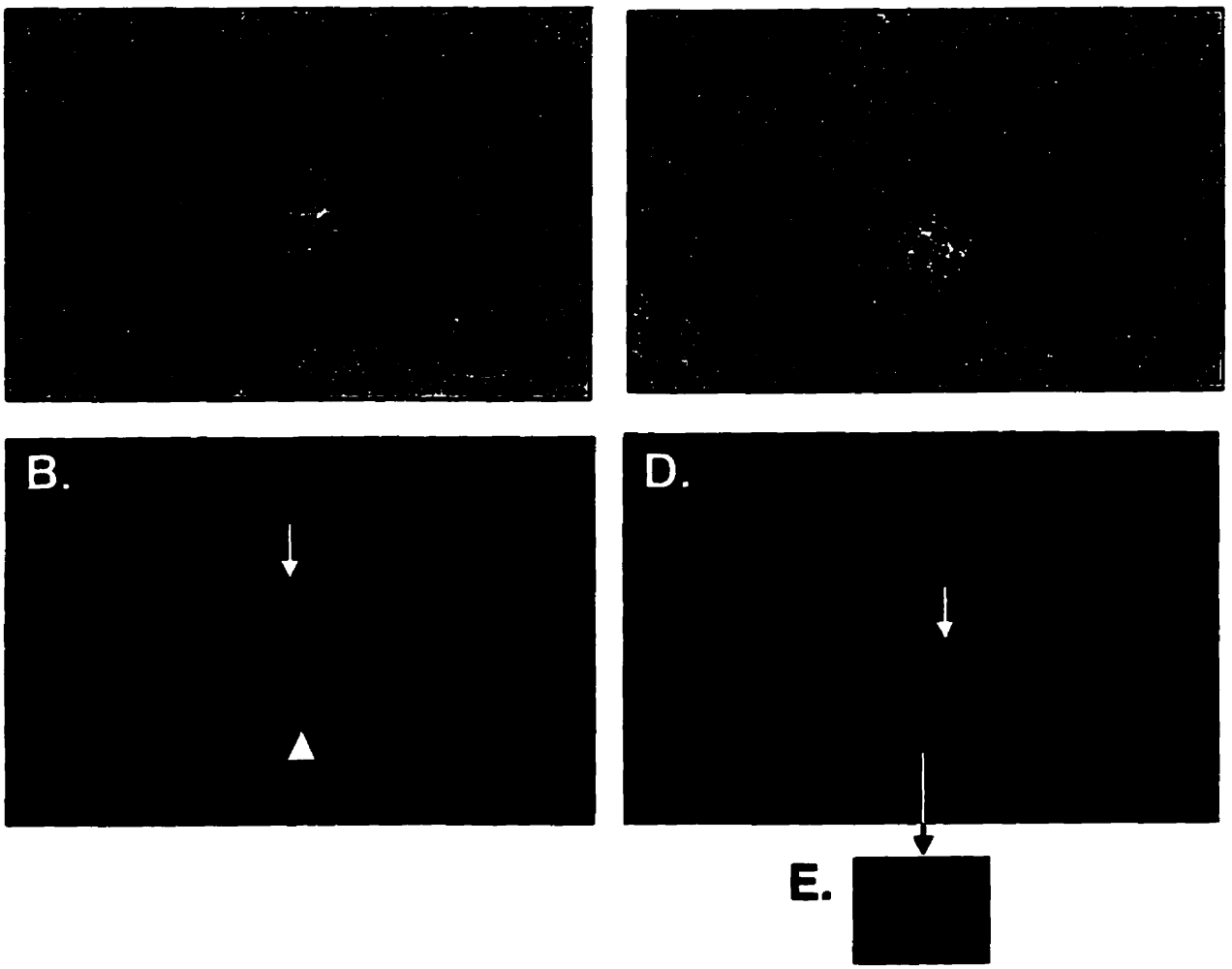


Figure 5.3 Apoptotic morphology by phase and fluorescent microscopy. Morphology of LPS treated (100 ng/ml, 3 hours) CPAE cells using phase contrast microscopy (A, C) and fluorescent microscopy of ethidium bromide stained cells (B, D, E). Arrows in (A, B) indicate position of an apoptotic nucleus seen in (B), while arrowheads point to a cell undergoing mitosis. (C) Another apoptotic cell (arrow) surrounded by normal looking cells. The corresponding fluorescent photographs focus on the normal nuclei (D) or on the apoptotic nucleus (E). Magnification x290 (A, B), x280 (C, D, E).

The fluorescent micrographs of LPS treated CPAE cells are not very clear or detailed. This is due in part to the high background of the DNA stains used and the relatively low resolution of the microscope, compounded by the difficulties encountered when trying to visualize details within a rounded cell. A more specific DNA dye such as Hoechst 33342 would have given clearer results, had the appropriate filter been available. An attempt at confocal laser scanning microscopy was made to overcome the depth problem, and a sharper picture was seen (Fig 5.4). Images were collected using a BIO-RAD MRC-600 confocal laser scanning microscope using BIO-RAD COSMOS software. The entire acridine orange stained apoptotic cell is shown in (A), still attached but rounding up and budding extensively. (B) and (C) are side views of the cell, from the side and bottom with respect to (A). While surrounding normal appearing cells were only 4 μm or so in height, as can be seen from the cells on either side of the apoptotic cell in (C), the apoptotic cell extended above the surface 14 μm. Shown in (D) is a 1 μm slice of the upper portion of the figure shown in (A), showing the nucleus starting to break apart with the DNA staining brightly along the nuclear membrane.

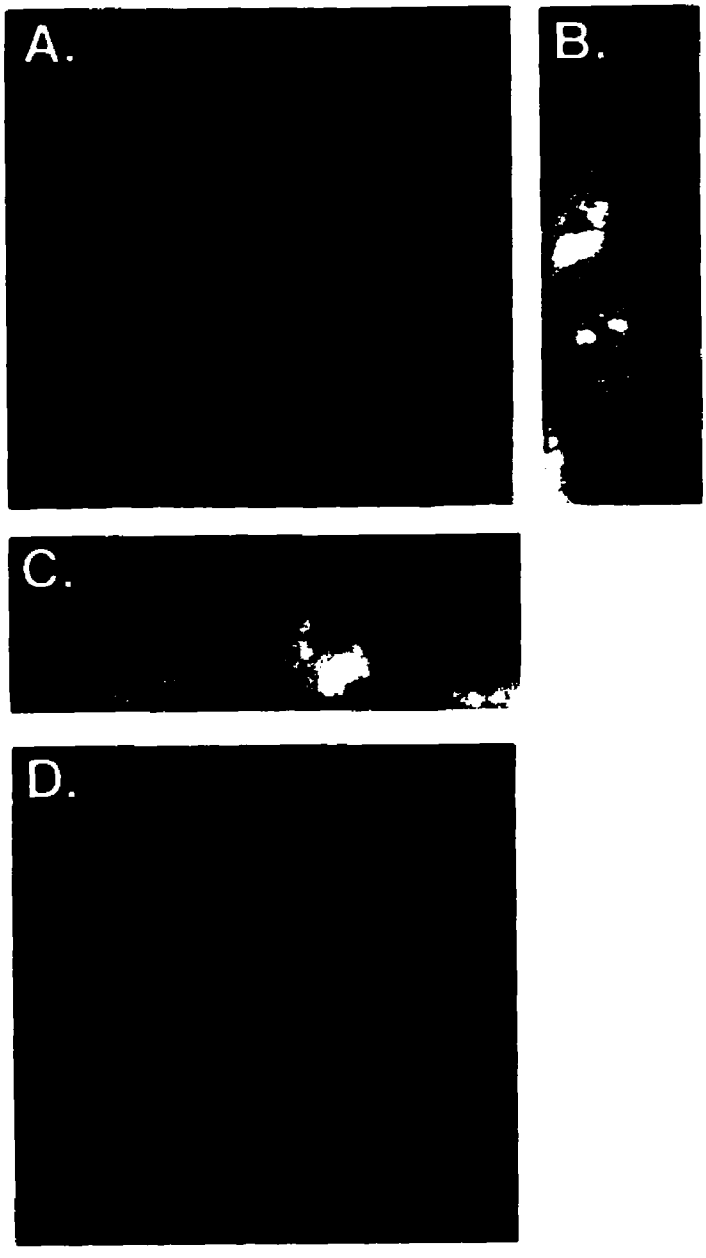


Figure 5.4 Apoptotic morphology using confocal microscopy. Confocal micrograph of LPS treated (100 ng/ml, 3 hours) CPAE cell stained with acridine orange. (A) The entire cell compiled from 88 0.2 μm sections, showing extensive budding, fragmenting nucleus and remaining cell attachment, along with neighbouring cells. This figure is shown along the X/Y axis, looking down the Z axis. (B) Side view of cell from right side of (A), shown along the Y/Z axis, looking down the X axis. (C) Side view of cell from the bottom of (A), shown along the X/Z axis, looking down the Y axis. (D) A 1 μm section (5 x 0.2 μm sections) of the upper portion of the cell shown in (A), showing the DNA condensing at the margins of the nucleus. Magnification x4000.

5.4 Transmission electron microscopy

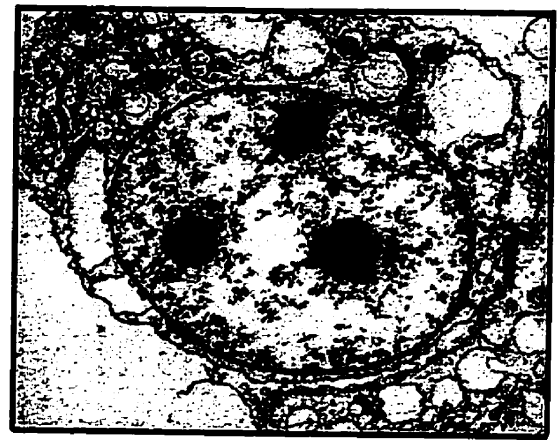
Since results looking for apoptotic morphology using fluorescent staining were to some extent ambiguous, it was decided to try the classical method of transmission electron microscopy (TEM) to confirm the mode of cell death. In the first attempt, LPS treated (100 ng/ml, 4 hours) and control cells were scrapeded off and spun down before fixing. This method resulted in a relative high proportion of apoptotic cells, mainly in the later stages (Fig 5.5 F), but also many showing the clear nuclear condensation characteristic of apoptosis (Fig 5.5 C, D, E). Unfortunately untreated CPAE cells did not survive this method intact. Most control cells ruptured, and the remainder contained huge vacuoles and no remaining mitochondria or other recognizable organelles (Fig 5.5 A). The nuclear structure was maintained however, showing diffuse chromatin and distinct nucleoli (Fig 5.5 B). Apoptotic cells appeared to be resistant to the conditions which destroyed the normal cells, possibly due to cell shrinkage.

In a susequent experiment, cells were fixed before gently being scraped. This method was successful in maintaining untreated cell morphology. Normal CPAE cells were long and narrow and the nucleus contained diffuse chromatin and distinct nucleoli (Fig 5.6 A). CPAE cells treated for 3 hours in media containing 6.25% FCS and 100 ng/ml LPS were found at different stages of apoptosis, which were not found in control cells. Following the addition of LPS, nuclear chromatin coalesced and condensed along the nuclear membrane, a characteristic of apoptosis (Fig 5.6 B). In addition, the cell cytoplasm was denser, vacuolization occurred, and the cells rounded up. Some LPS treated CPAE cells also exhibited characteristic later stages of apoptosis. As the process continued the nuclei came apart and the cells fragmented into membrane-bound apoptotic bodies which contained a variety of cytoplasmic organelles and darkly staining nuclear fragments (Fig 5.6 C).

Figure 5.5 Transmission electron microscopy of LPS treated CPAE cells (method 1). CPAE cells were treated with 100 ng/ml LPS for 4 hours before being prepared for TEM, using the first method (Chapter 2.7). (A) Untreated cell. (B) Normal nuclei. (C, D, E) LPS treated cells with chromatin condensed along the inside of the nuclear membrane, forming characteristic crescents. (F) LPS treated cell in a later stage of apoptosis, fragmenting into apoptotic bodies. Magnification x2500 (A), x4410 (B, C), x8000 (D), x3000 (E, F).

A.

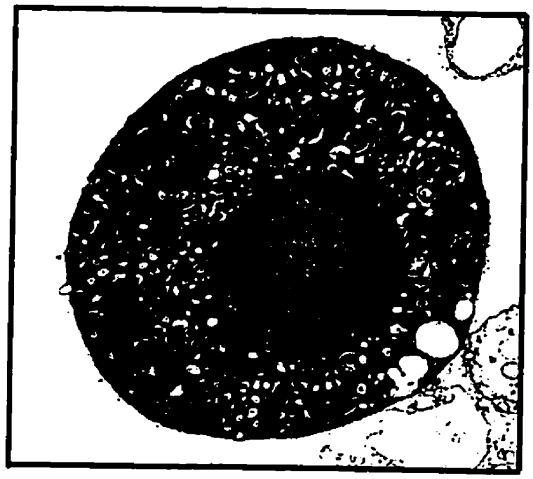
B.



C.

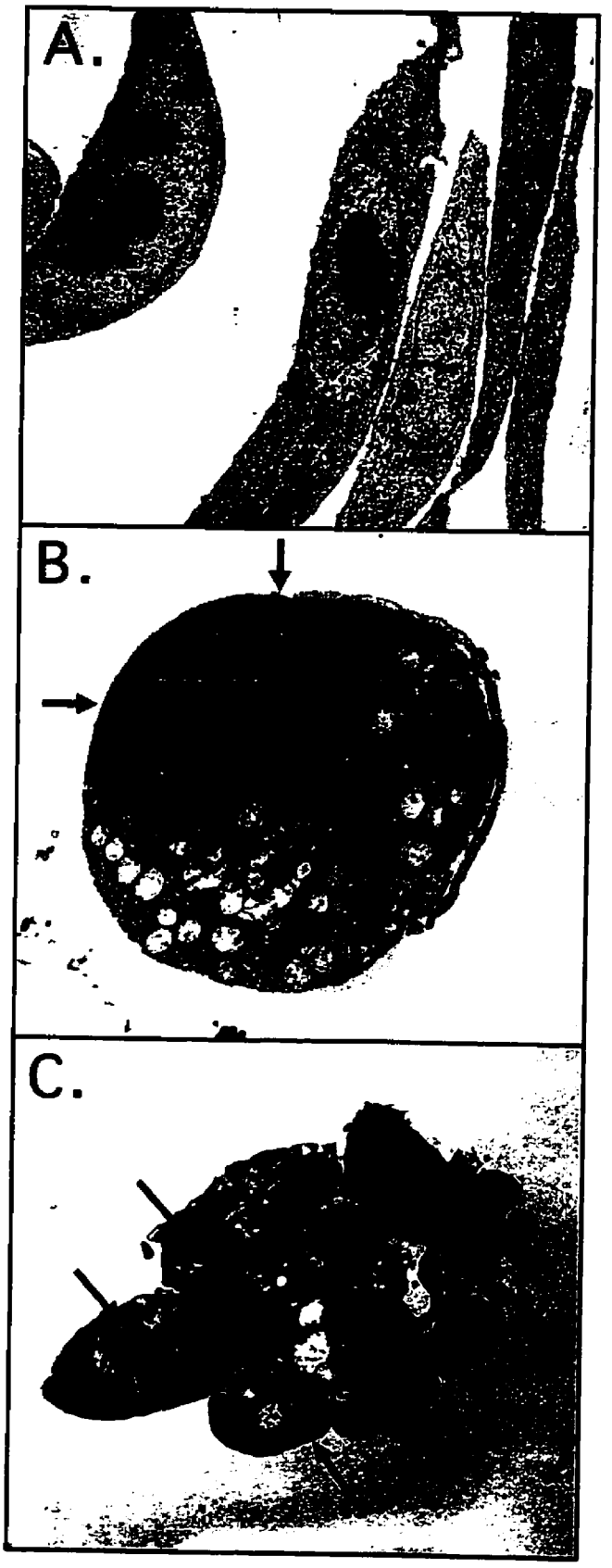
D.

E.



F.

Figure 5.6 Transmission electron microscopy of LPS treated CPAE cells (method 2). CPAE cells were prepared for TEM using the second method (Chapter 2.7). (A) Control CPAE cells. (B) Cell treated with 100 ng/ml LPS for 3 hours showing condensed cytoplasm and perinunclear chromatin aggregation (arrows). (C) LPS treated cell in a later stage of apoptosis, breaking up into apoptotic bodies (light arrow) containing darkly staining nuclear material (arrows). Magnification x3150 (A), x9450 (B), x4000 (C).


A.
B.
C.

A single LPS treated CPAE cell showing characteristic apoptotic morphology was seen in a series of TEM sections (Fig 5.7). All four sections show perinuclear chromatin aggregation, and other nuclear features such as several nucleoli can be seen in the sections. This is not unexpected, as nucleolus structure is known to be preserved throughout the apoptotic process (Falcieri *et al.*, 1994). In additon, part of the lower right of the nucleus can be seen starting to break away (C, D).

A CPAE cell which may be functioning as a non-professional phagocyte engulfing apoptotic bodies was seen in the sample of LPS treated cells (Fig 5.8). This non-apoptotic cell with a normal nucleus contains what appears to be two previously engulfed apoptotic bodies (arrow heads), and has membrane perturbations resembling pseudopodia in the vicinity of one of two external apoptotic bodies (arrows). The ability to phagocytose apoptotic bodies has previously been demonstrated in endothelial cells in the mouse liver (Dini *et al.*, 1995).

CPAE cells with another type of distinctive morphology were seen in LPS-treated TEM samples. These cells, with no defined nucleus but containing several small and very distinct dark-stained areas, were identified as mitotic cells (Fig 5.9 A, B). As the monolayer was reduced by the loss of cells by apoptosis, the survivors divided (C) and would eventually fill in the spaces. Hansson *et al.* (1985) found an equal increase of both cell death and replication when studying the endothelium of LPS treated rats, thus leading to no increase in vessel denudation.

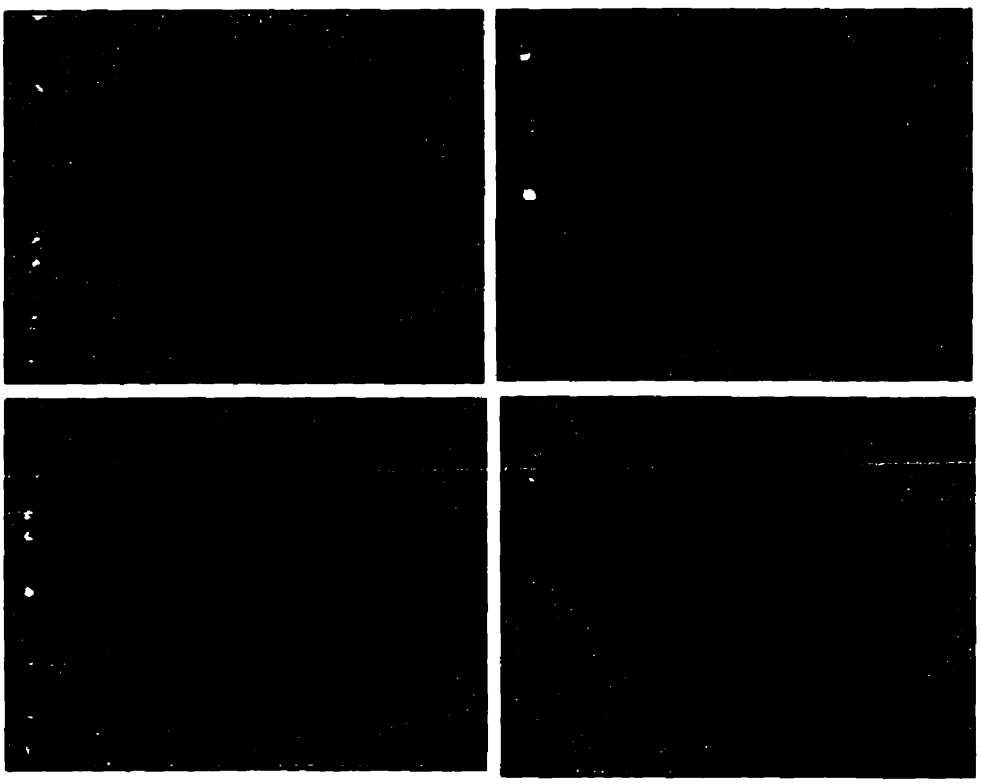


Figure 5.7 Series of TEM micrographs of apoptotic CPAE cell. The same LPS treated (100 ng/ml, 3 hours) CPAE cell showing characteristic apoptotic morphology was seen in a series of TEM sections (A to D). All four sections show perinunclear chromatin aggregation, and other nuclear features such as several nucleoli can be seen at different stages in the sections. Part of the nucleus can be seen fragmenting in the lower right of sections (C and D). Magnification x6300.

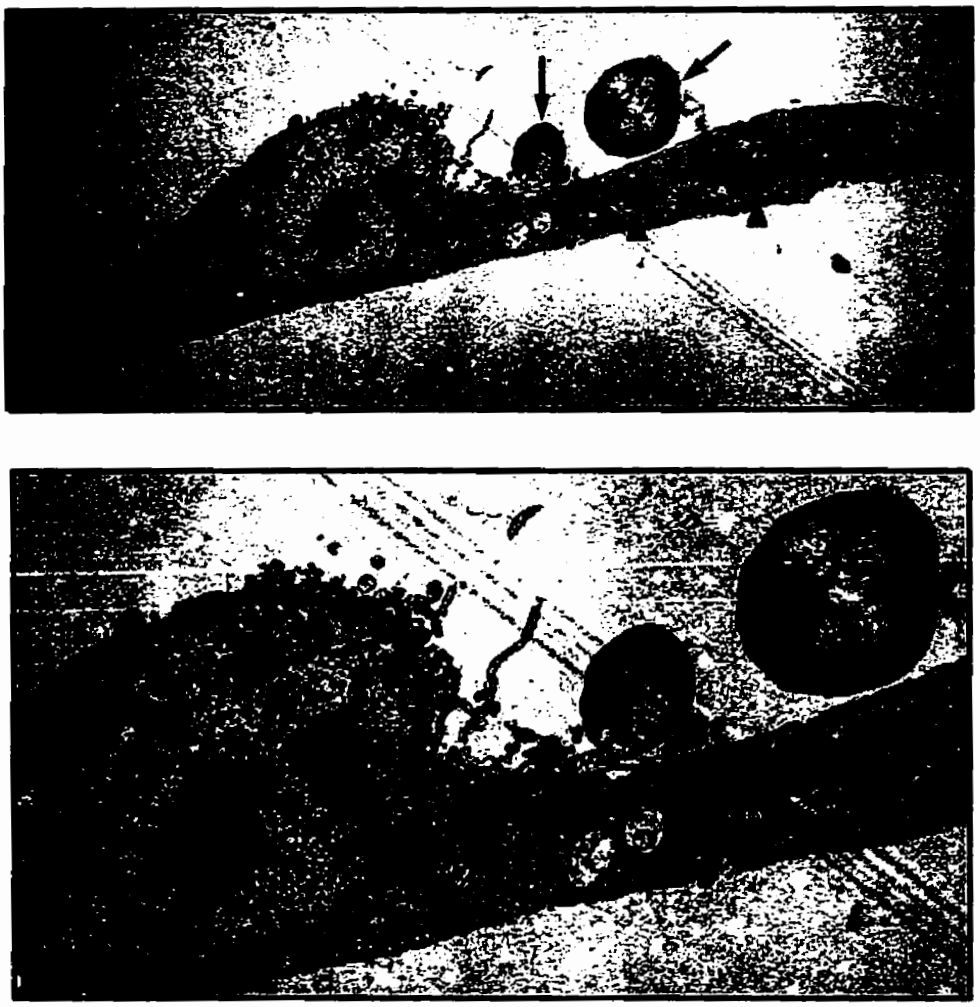


Figure 5.8 Transmission electron microscopy of phagocytic CPAE cell. Non-apoptotic CPAE cell from LPS treated (100 ng/ml, 3 hours) monolayer which may be acting as a non-professional phagocyte. The nucleus appears normal and there are membrane perturbations on the surface near external apoptotic bodies (A, arrows). Two areas inside the cell resemble engulfed apoptotic bodies (A, arrow heads). (B) enlargement of (A). Magnification x3750 (A), x9500 (B).

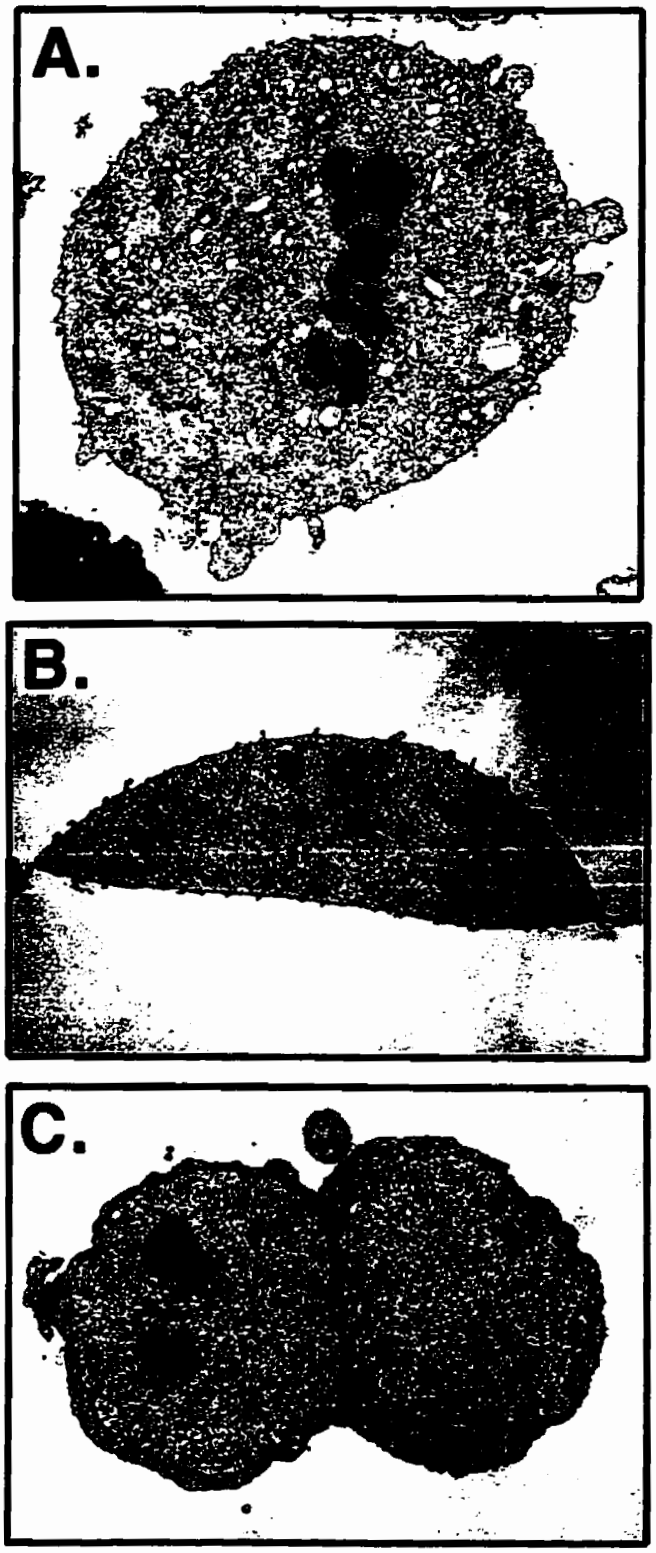


Figure 5.9 Transmission electron microscopy of mitotic LPS treated CPAE cells. CPAE cells from LPS treated monolayer undergoing mitosis. (A, B) early stage, (C) cell actively dividing. Magnification x3250 (A), x3150 (B), x8000 (C).

5.5 Discussion

Disease is the result of biological process gone wrong. In the case of apoptosis, disease can be due to either an inhibition or an increase in the occurance of normal apoptotic cell death (reviewed by Barr and Tomei, 1994; Thompson, 1995). Cancers and autoimmune disorders are a result of increased cell survival, which can be due either to an increase of proliferation or a decrease of apoptosis. Neurodegenerative disorders and ischemic injury are associated with an increase in apoptosis. Recently the importance of apoptosis in infectious diseases has become apparent (Zychlinsky, 1993). Several viruses have developed a mechanism to block apoptosis in infected cells, while in the case of HIV infection it is thought that CD4+ cells are depleted via apoptosis.

Bacterial pathogens have also been found to be able to induce apoptosis (reviewed by Chen and Zychlinsky, 1994). Numerous bacterial toxins can trigger apoptosis, and these mainly fall into two categories. One group consists of pore-forming proteins, such as the *Staphylococcus aureus* α-toxin (Jonas *et al.*, 1994) and *E. coli* hemolysin (Jonas *et al.*, 1993). At higher concentrations these toxins cause cell death by necrosis. Another group of apoptosis inducing bacterial toxins act by inhibiting protein synthesis. Examples of these are *Shigella dysenteriae* shiga toxin (Keenan *et al.*, 1986) and *Pseudomonas aeruginosa* exotoxin A (Morimoto and Bonavida, 1992). Other apoptosis-inducing bacterial toxins include the Staphylococcal enterotoxin B (SEB) (Jenkinson *et al.*, 1989), and the *Bordetella pertussis* adenylate cyclase hemolysin (Khelef *et al.*, 1993). *Shigella flexneri* induces apoptosis in macrophages (Zychlinsky *et al.*, 1992), and the *ipaB* gene product has been identified as the causative agent (Zychlinsky *et al.*, 1994b). Macrophages invaded by *Salmonella typhimurium* have been found to undergo apoptosis (Lindgren *et al.*, 1996; Monack *et al.*, 1996). The study of apoptosis in bacterial pathogenesis is a relatively new field, but will likely expand greatly in the near future.

We have shown that LPS treated CPAE cells die by apoptosis. The discovery that LPS can induce apoptosis is the first non-protein bacterial product that has been shown to do

so. This was demonstrated using both morphological and biochemical criteria. It is important to verify the occurance of apoptosis, as there is still some questions about the specificity of some assays. Both the detection of DNA cleavage (Enright *et al.*, 1994; Bicknell and Cohen, 1995), and the related TUNEL assay have been associated with death by necrosis recently (Levin, 1995). This has not hindered some groups, such as Maeda *et al.* (1995), from claiming that LPS caused apoptosis in sheep endothelial cells, based merely on the results of DNA fragmentation. This fragmentation was seen after 22 h with 250 ng/ml LPS, possibly in the absence of serum, althought this was not clear from the text. It is curious that the morphological changes seen in the cells, described as cell swelling and blebbing, were used to confirm the occurance of apoptosis, while they are usually associated with necrosis. Other work has also linked LPS with endothelial cell apoptosis. While LPS alone does not cause cell death in porcine endothelial cells, when an 18 hour LPS (200 μg/ml) treatment is followed by induction of the heat shock response, death occurs and this has been identified as apoptosis (Buchman *et al.*, 1993). Again, only DNA fragmentation was used as a criterion, and the presence or absence of serum in the assays was not clear. Another group found that LPS can induce apoptosis in sheep endothelial cells grown on plastic. This death could be prevented by culturing the cells on collagen, laminin, fibronectin, or integrin ligands, suggesting an important role for the extracellular matrix (Hoyt *et al.*, 1995; Hoyt *et al.*, 1996). However, in our system the bovine brain endothelial cells grown on fibronectin were sensitive to LPS.

Whether apoptosis is primarily a protective response by the host, or a method developed by pathogens for their own survival and spread, is not yet understood. Apoptosis is however increasingly being identified as a protective response to pathogens, with the suicide of damaged or infected cells enhancing the survival of the multicellular organism as a whole (Williams, 1994). When *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) infected macrophages were killed by necrosis or apoptosis it was found that only death by apoptosis resulted in BCG killing (Molloy *et al.*, 1994). There would appear to be a definite advantage

of an apoptotic mechanism versus necrosis for endothelial cells which react to LPS *in vivo*. Damaged cells dying via apoptosis release no cellular material, thereby minimizing inflammatory responses. Cells undergoing apoptosis *in vivo* are rapidly recognized and engulfed by phagocytes (Savill *et al.*, 1993). The lack of inflammatory response resulting specifically from the death of endothelial cells *in vivo* may be a minor element during gram-negative sepsis, when there is a extensive systematic inflammatory response. However, it may be that one fewer source triggering the host's immune system can make a critical difference in the final outcome.

Chapter 6 LPS signal transduction in CPAE cells

6.1 Introduction

Much of the research into the action of LPS has focused on the interaction of LPS with cells of the myelomonocytic lineage. In recent years many intracellular signalling proteins have been identified which are activated/provoked downstream of the initial contact between LPS and these cells (see chapter 1.2.3). Much less is known about the signalling triggered by LPS in endothelial cells. A better understanding of the events leading to cell death may help to identify a possible site for therapeutic intervention. Several directions were attempted to study LPS-induced events in CPAE cells. Some work was originally done looking at the extracellular events, but this was abandoned due to competition from large, well equipped labs with access to recombinant sCD14 and LBP. Other undertakings focused on intracellular signals triggered by LPS. A variety of intracellular events were examined, selected by the availability of techniques and reagents, and by results from related models of apoptosis.

This chapter illustrates the dangers and disadvantages of measuring cell death while using compounds with a range of cytotoxic effects of their own. An inhibitor could well block the LPS-induced signalling pathway, but if it triggers cell death by itself, this could be difficult to establish. Another major problem in the study of LPS-induced signalling in CPAE cells is the nature of apoptosis. Active cell death can be divided into two processes; a condemned or latent phase where the cell recognizes a signal and becomes committed to die, and the execution phase, containing all the hallmark features of apoptosis (Earnshaw, 1995). While the execution stage can be very rapid, in the order of minutes, the condemned phase can last for hours or even days. The duration of this phase is also very variable even in synchronized clonal populations, and as a result an aliquot removed from experimental cultures contains cells in various stages of apoptosis: condemned, execution and secondary necrosis. This makes the detection of any transient signalling events very difficult.

6.2 Extracellular events

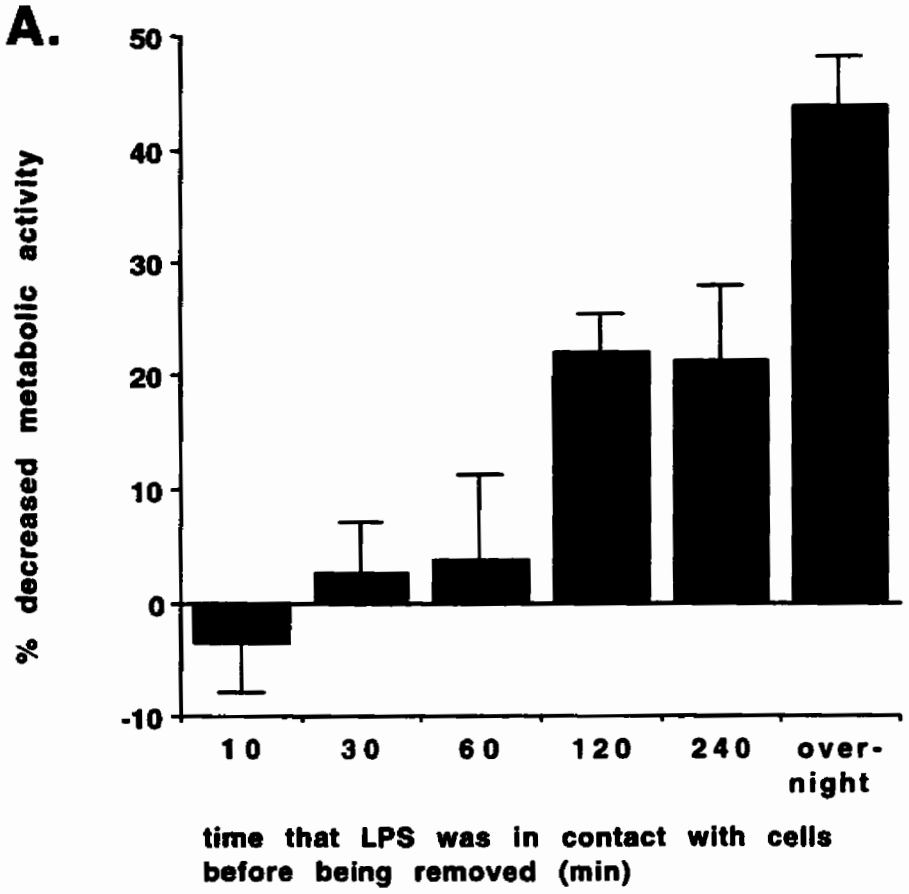
6.2.1 Kinetics of LPS-induced CPAE cell death

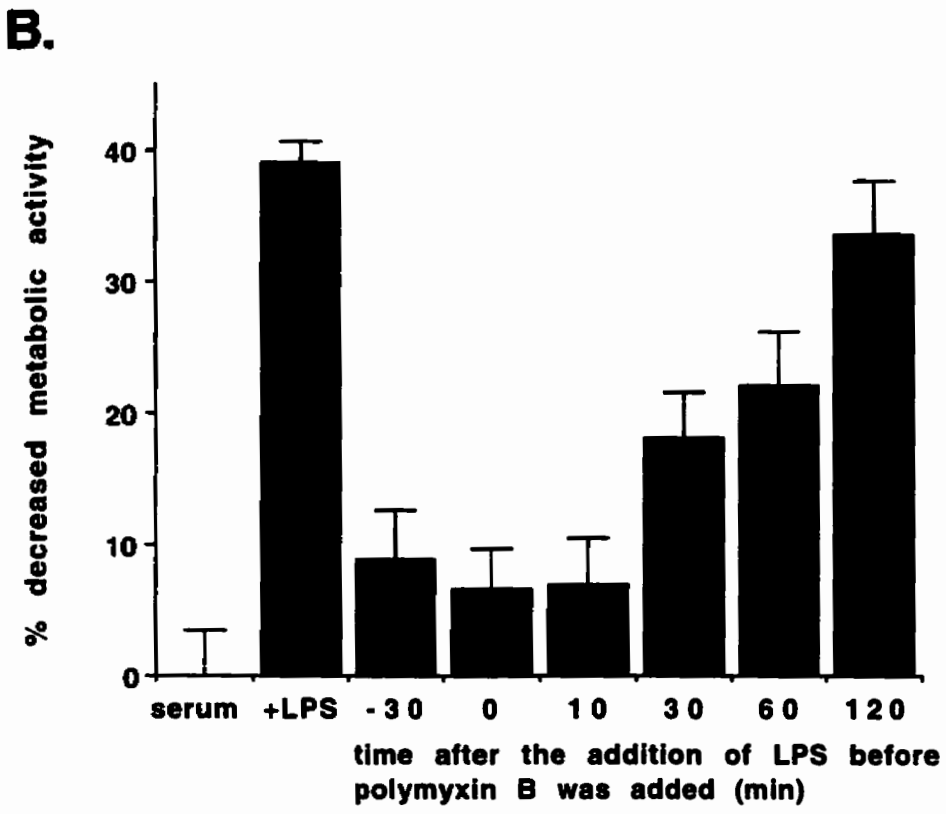
In order to determine the kinetics of the LPS-induced cell death, two approaches were used. In the first, LPS (100 ng/ml) was added to CPAE cells incubated in MEM containing 6.25% FCS at 37°C and removed at various times by aspiration. The cells were then washed 4 times with MEM and further incubated in new MEM with 6.25% FCS. The cells were observed over time for visible signs of cytotoxicity, and then left overnight and cell death measured using the MTT assay (Fig 6.1 A). Two hours after the addition of LPS, morphological signs of cell death were seen in cells incubated 1 hour or more with LPS, but not in cells where LPS had been removed after 10 or 30 minutes. However, some cell budding was detectable after 3.5 hours in cells which had been incubated with LPS for 30 minutes. Increasing the amount of time the cells were incubated with LPS resulted in increasing visual evidence of cytotoxicity. Although visible signs of death occurred in cells incubated 30 and 60 minutes with LPS, the level of cell death was not high enough to be detected using the MTT assay. Cells treated with LPS for 2 hours gave a level of cell death approximately half of those treated overnight with LPS.

The second approach was to add polymyxin B at different times before and after the addition of LPS, and incubate the cells overnight before measuring cytotoxicity using the MTT assay (Fig 6.1 B). When polymyxin B was added before, at the same time, or 10 minutes after LPS, cytotoxicity was completely blocked. When added 30 minutes later, some cell death was still seen, and when polymyxin B was added 2 hours after LPS, levels of cell death were almost equivalent to those seen in the absence of the antibiotic.

These experiments fit a model where LPS first interacts with serum protein(s) such as LBP and sCD14. This occurs within the first half hour, after which it starts to interact with cells. Much of the initial signalling would appear to occur within a few hours, while the steady increase in measurable cell death over time (Fig 3.2 B), could be explained by the variable length of the condemned phase during apoptosis. The difference in the levels of cell

Figure 6.1 Kinetics of LPS action. (A) LPS removal. CPAE cells were incubated in MEM containing 6.25% FCS in the presence or absence of 100 ng/ml LPS. After the indicated times, the media was removed, the cells washed 4 times with MEM, and new MEM containing 6.25% FCS added. After a further overnight incubation, cell death was measured using the MTT assay. (B) Polymyxin B addition. Cells were incubated as above, and polymyxin B (5 μg/ml) was added at the indicated times. After a further overnight incubation, cell death was measured using the MTT assay. Values are the average of four samples +/- SD. Results shown are from representative experiments, experiments were repeated three times.


A.
% decreased metabolic activity
50
40
30
20
10
0
-10
10
30
60
120
240
over-
night
time that LPS was in contact with cells
before being removed (min)


B.
% decreased metabolic activity
40
30
20
10
0
serum
+LPS
-30
0
10
30
60
120
time after the addition of LPS before
polymyxin B was added (min)

death compared to maximal levels seen after two hours treatment using the two methods (LPS removal and polymyxin B addition), may be due to serum proteins bound to LPS which interfere with polymyxin B binding and inactivation of LPS.

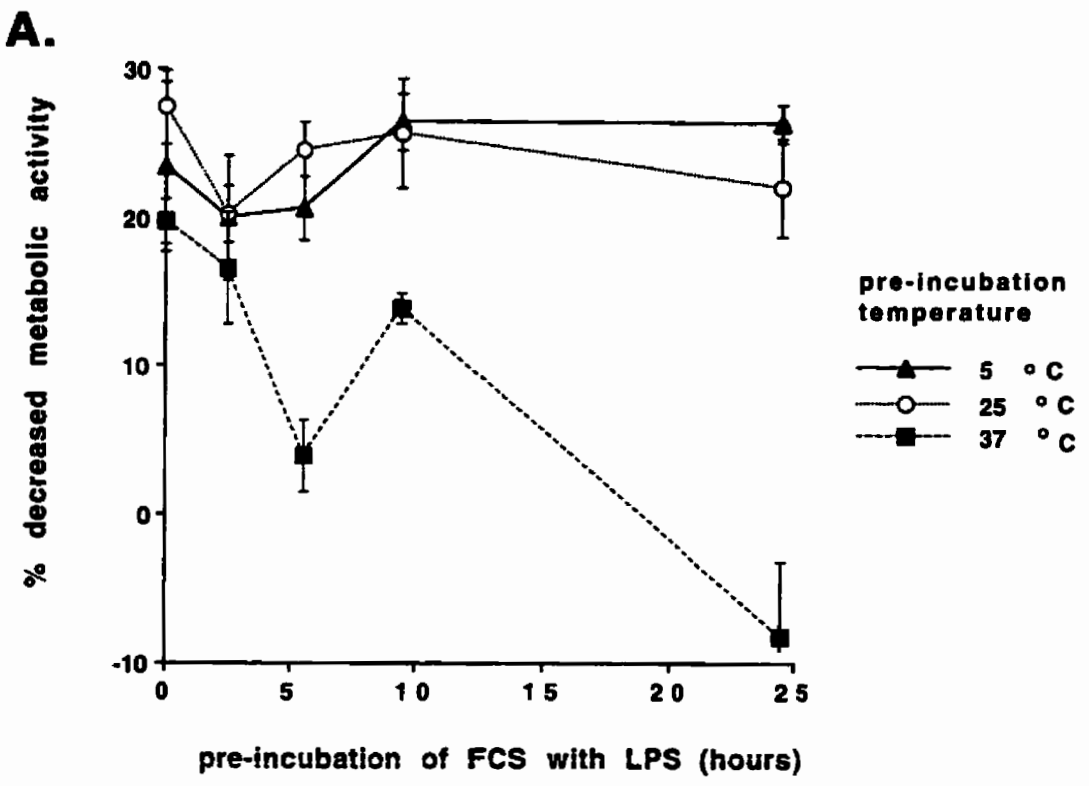
6.2.2 Interaction between LPS and serum

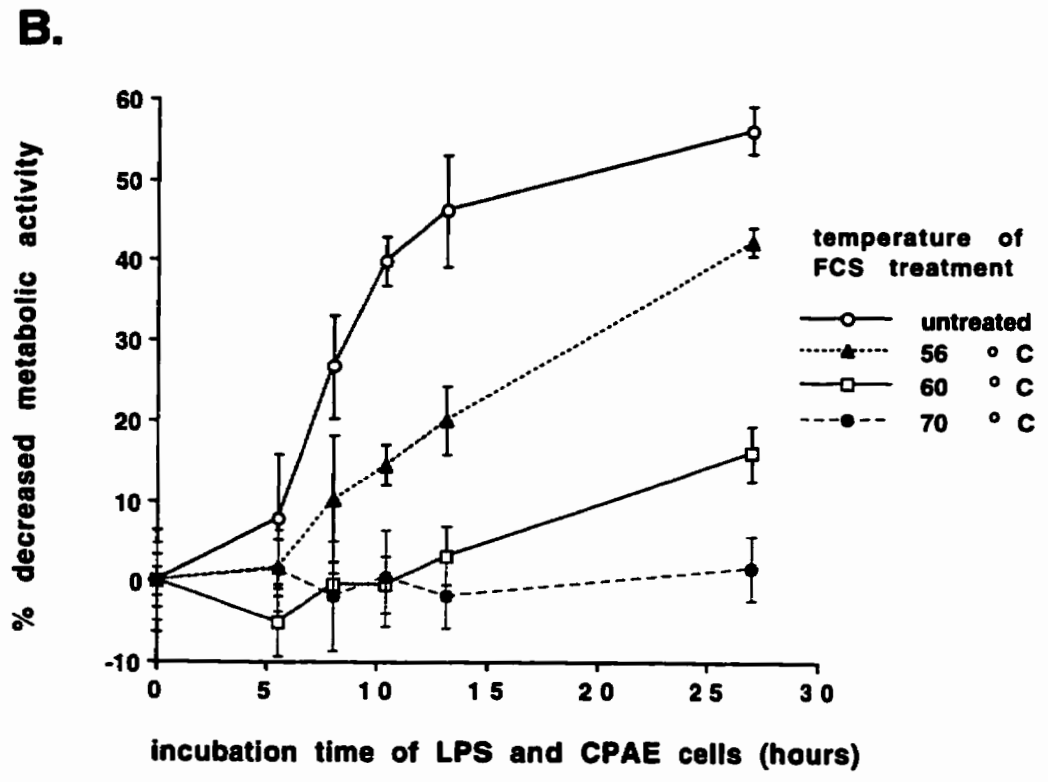
It had previously been suggested that LPS was inactivated by incubation with serum, probably by interacting with lipid elements in the blood (Skarnes *et al.*, 1958; Ulevitch and Johnston, 1978; Johnson *et al.*, 1977; Emancipator *et al.*, 1992). To look at this possibility using this system, LPS (100 ng/ml) was pre-incubated with FCS (6.25%) in MEM for varying lengths of time at 37°C, room temperature (25 +/- 2°C), and at 5°C. These samples were then added to CPAE cells and incubated overnight, after which cell death was measured using the MTT assay (Fig 6.2 A). A decrease in the amount of cell death was seen with increasing pre-incubation time at 37°C, until no death was detectable using LPS/FCS pre-incubated 24 hours. No change was seen with LPS/FCS pre-incubated at room temperature or at 5°C. No decrease of cell death was seen when LPS and FCS were incubated separately at 37°C for 24 hours (data not shown).

In other studies, FCS was heated at different temperatures for 30 minutes, and the cytotoxic effect of LPS in this sera was measured by the MTT assay over time (Fig 6.2 B). It was found that in untreated sera cell death started around 5 hours, and increased until it plateaued at about 12 hours. In the presence of serum heated to 56°C cells died more slowly, but after 27 hours almost reached the level of untreated serum. There was a much smaller level of death at that time in serum heated to 60°C, and none at all in serum treated at 70°C.

The factor in serum affected by heating may be LBP, since LBP is known to be heat sensitive. Human LBP is stable at 56°C and inactivated at 59°C, while mouse LBP loses its activity between 53 and 56°C (Gallay *et al.*, 1993). Since this work was completed, LBP

Figure 6.2 Interaction between LPS and serum. (A) LPS inactivation by serum. LPS (100 ng/ml) and FCS (6.25%) were incubated together for varying lengths of time at 5, 37°C and room temperature (25°C). They were then added to CPAE cells and cytotoxicity measured after an overnight incubation using the MTT assay. (B) Effect of heat treated FCS. FCS was heated for 30 minutes at 56, 60, and 70°C. CPAE cells were incubated for varying lengths of time in MEM containing 6.25% treated or control FCS and 100 ng/ml LPS. Cytotoxicity was measured using the MTT assay. Values are the average of three samples +/- SD. Results shown are from representative experiments, experiments were repeated three (A) or two (B) times.


A.
% decreased metabolic activity
30
20
10
0
-10
0
5
10
15
20
25
pre-incubation of FCS with LPS (hours)
pre-incubation temperature
5 °C
25 °C
37 °C


B.
% decreased metabolic activity
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
incubation time of LPS and CPAE cells (hours)
temperature of FCS treatment
untreated
56 °C
60 °C
70 °C

has been identified in bovine serum (Khemlani *et al.*, 1992). The heat sensitivity of bovine LBP was examined by heating both serum and purified LBP, and the results are very similar to those described above (Meszaros *et al.*, 1995). These results would fit with experiments done using recombinant sCD14 and LBP, which found that while LBP is not necessary for the action of sCD14 and LPS, it did act to accelerate the process (Haziot *et al.*, 1993; Arditi *et al.*, 1993).

6.2.3 Cytokines

The release of cytokines is a key event in the development of sepsis. Most of the prominent cytokines are thought to be released by cells of the myelomonocytic lineage, such as monocytes and neutrophils. These cytokines include tumor necrosis factor (TNF) α, interleukin-1 (IL-1), and interferon (IFN) γ (Dofferhoff *et al.*, 1991). Endothelial cells also release a number of important cytokines, such as IL-1, IL-6, IL-8, and numerous colony stimulating factors (CSFs) when stimulated with LPS (Mantovani *et al.*, 1992). It is possible that LPS exerts its lethal effect on CPAE cells by stimulating the release of an autocrine cytokine rather than acting directly. The effects of IL-6 on bovine endothelial cells have been studied (Maruo *et al.*, 1992), and while it increases permeability by rearranging actin filaments and changing the cell shape, no evidence of toxicity is seen.

TNF has many known effects on endothelial cells including cytotoxicity. Robaye *et al.* (1991) showed that primary culture bovine pulmonary endothelial cells undergo apoptosis when treated with TNF. Studies comparing bovine endothelial cells from two sources found that cells cultured from the main pulmonary artery are sensitive to TNF-α while lung microvascular endothelial cells are resistant (Meyrick *et al.*, 1991). TNF-α also results in LDH release from cultured bovine brain endothelial cells (Terada *et al.*, 1992). Another group found that TNF-α enhances the cytotoxic effects of LPS on bovine endothelial cells, while only causing modest cytotoxicity on its own (Sharma *et al.*, 1992). *In vivo*, rabbits infused with TNF-α demonstrate pulmonary vascular endothelial cell damage (Goldblum *et*

al., 1989). The ability of endothelial cells to produce TNF has been debated and it is generally thought that they do not produce it (Pober and Cotran, 1990). Several studies detected no TNF following LPS treatment of endothelial cells (Libby *et al.*, 1986; Seifert *et al.*, 1991). However another group did measure TNF release from LPS treated bovine endothelial cells (Akarasereenont *et al.*, 1995).

Rabbit antiserum raised against bovine TNF was used to determine whether this cytokine was involved in LPS-induced CPAE cell death. Antibodies against a number of other bovine cytokines were available (IFN-α, IFN-γ, and IL-2), and were also tested. These antisera are effective in a neutralization assay at a dilution of 1:100 to 1:500 (A. Potter, VIDO, personal communication, see section 2.3.3 for details). At these concentrations, the antibodies were added to CPAE cells in the absence and presence of LPS. After 3 hours LPS-induced cell death was evident in all samples, and the IL-2 antisera was cytotoxic on its own. Following an overnight incubation, measurement of cell death using the MTT assay confirmed these results (Fig 6.3). Further experiments looked at the effects of raising the dilution of antisera to 1:25, except for anti-IL-2 which was decreased to 1:1000. No changes were seen, except the anti-TNF antisera caused measurable cell death at this concentration, but without visual signs of apoptosis.

The lack of blocking using the anti-TNF antiserum suggests that, even if produced by CPAE cells, TNF is not involved in LPS-induced cell death. The reason for the cell death resulting from the 1:25 dilution of the anti-TNF antiserum is unclear. The cytotoxic effect of the anti-IL-2 antiserum is also difficult to explain, but is probably not due to the removal of a vital growth factor. IL-2 is produced by T cells and acts as a growth factor for T cells, B cells and natural killer cells (Smith, 1988). IL-2 is not known to interact with endothelial cells, nor is it commonly found in serum. A proper study of the role of cytokines was hindered by the lack of available antibodies against bovine cytokines, especially those known to be released by LPS treated endothelial cells. Antisera against bovine IL-1 would be especially interesting to test in this system as it is produced by LPS treated endothelial cells and has many effects

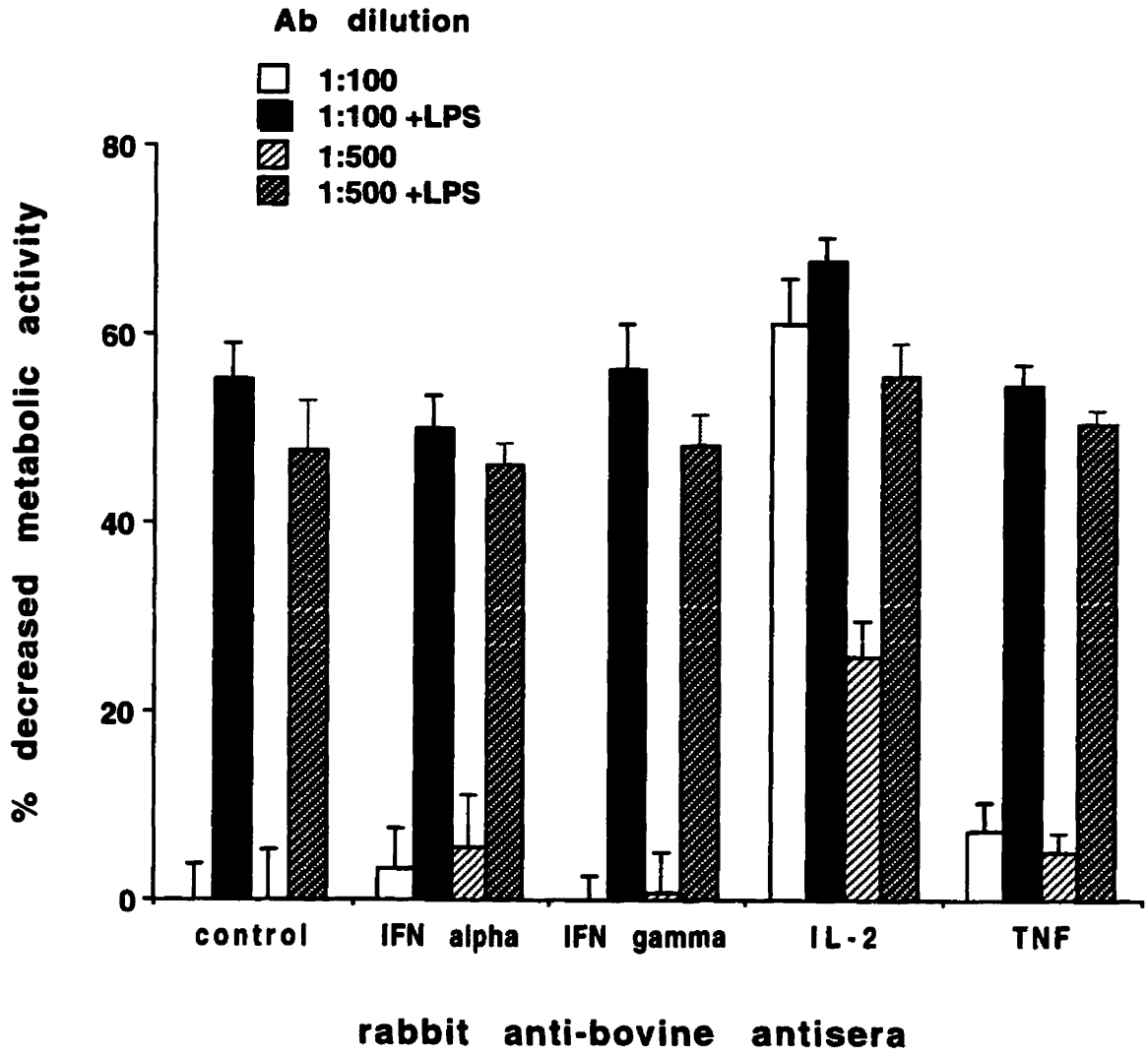


Figure 6.3 Anti-bovine cytokine antisera. Rabbit anti-bovine cytokine antisera at a 1:100 or 1:500 dilution was added to CPAE cells incubated in MEM containing 6.25% FCS and in the presence or absence of 100 ng/ml LPS. Cytotoxicity was measured after an overnight incubation using the MTT assay. Values are the average of four samples +/- SD. Results shown are from representative experiments, experiments were repeated three times.

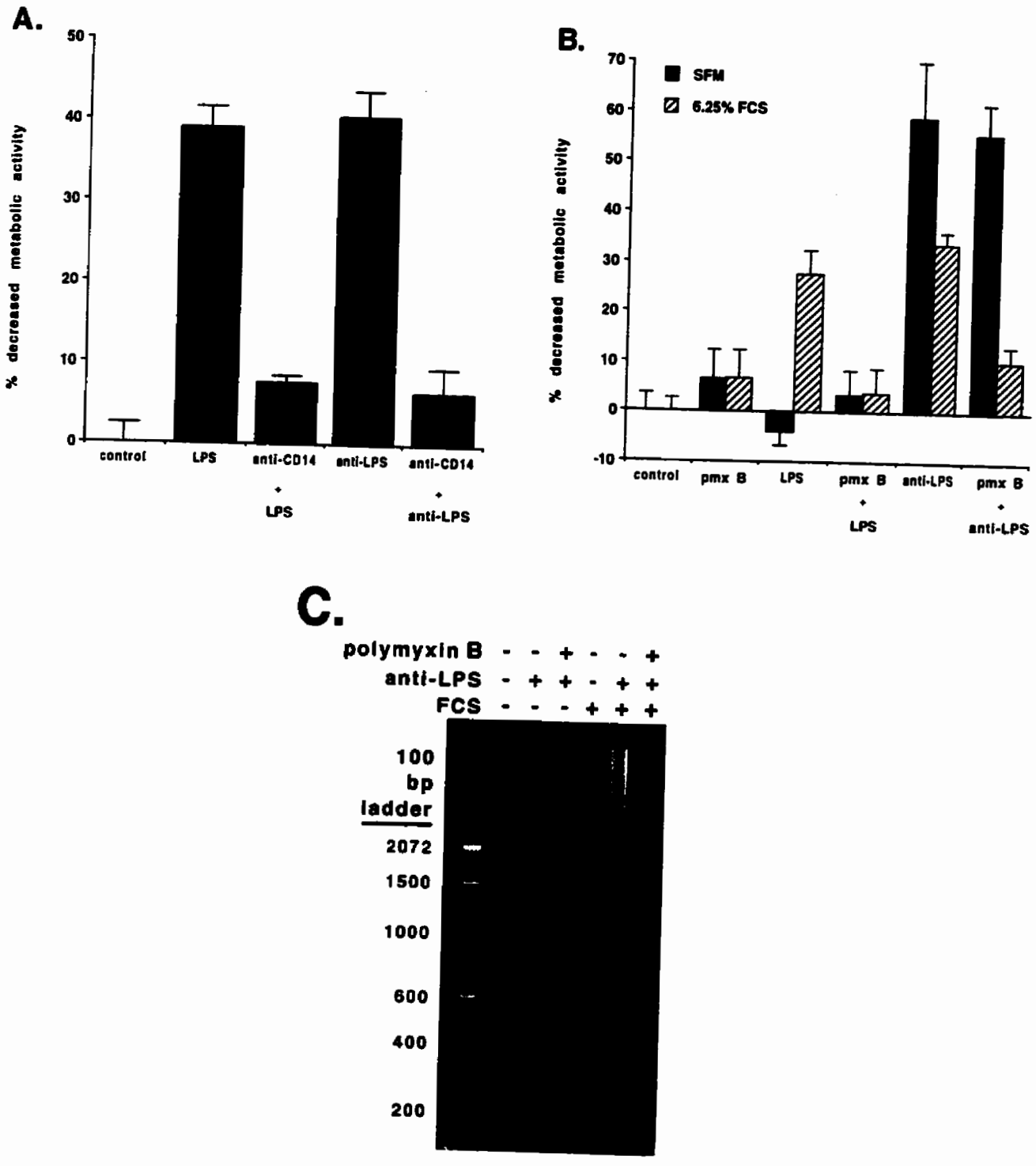
which overlap with those of TNF. The use of other methods which directly detect or measure cytokine production, such as PCR or ELISA, could also yield useful information for the further study of the possible role of cytokines in LPS-induced CPAE apoptosis.

6.2.4 Anti-LPS antiserum

Polyclonal anti-*Salmonella typhimurium* LPS (O-chain) antiserum has been used extensively in the lab for immunofluorescent studies of *S. typhimurium* interactions with host cells (Garcia-del Portillo and Finlay, 1995). This antiserum was assayed to see whether it would block the LPS-induced CPAE cell death. Surprising, not only did it not protect the cells from *S. typhimurium* LPS, the antiserum had a cytotoxic effect on its own in the absence of LPS. Cell death was still considerable when the antiserum was diluted to 1:10,000. Further experiments with the anti-LPS antisera showed that it behaved much like LPS. Anti-LPS antiserum killing could be blocked in human serum with anti-CD14 antibodies (Fig 6.4 A), and could be blocked by polymyxin B in the presence of FCS. A major difference was the ability of the anti-LPS antiserum to cause CPAE cell death in serum-free media, and the inability of polymyxin B to block this cell death (Fig 6.4 B). Later, with the development of the DNA fragmentation assay, it was confirmed that the anti-LPS antiserum caused apoptosis in the presence of serum, and necrosis in its absence (Fig 6.4 C).

There are several possible explanations for the cytotoxic effect of the anti-LPS antiserum. First, it could be due to contaminating LPS. It is surprising that it is so potent, resulting in the same amount of cell death at a 1:2000 dilution as 100 ng/ml LPS. This would suggest that the original antiserum contains approximately 400 μg/ml LPS, or that there is less LPS but that it is in a very active state. It is possible that the binding of the antibodies to the O chain of LPS results in the increased exposure of the biologically active lipid A portion. Anti-idiotype antibodies mimicking LPS structure is another possibility. It should be possible to distinguish between these two models using the heat stable properties of LPS. However, experiments in which both LPS and anti-LPS antiserum were heated to 100°C gave

Figure 6.4 Cytotoxic effect of anti-*Salmonella* LPS antiserum. (A) Blocking effect of anti-CD14 antibody. CPAE cells were incubated in MEM containing 6.25% human serum and the indicated compounds: LPS (100 ng/ml), anti-CD14 antibody MY4 (5 μg/ml), anti-LPS antiserum (1:1000). (B) Cytotoxic effect in serum-free medium. CPAE cells were incubated in MEM alone (SFM) or MEM with 6.25% FCS and the indicated compounds at the concentrations given above and polymyxin B (pmx B, 5 μg/ml). Cytotoxicity was measured after an overnight incubation using the MTT assay. Values are the average of four samples +/- SD. (C) DNA fragmentation. CPAE cells were incubated in MEM alone or with 5% FCS with the indicated compounds at the concentrations given above. After 4 hours, cells were lysed and extranuclear cellular DNA was isolated and subjected to gel electrophoresis in a 2% agarose gel. Representative results are shown, similar experiments were repeated at least three times.


A.
% decreased metabolic activity
50
40
30
20
10
0
control
LPS
anti-CD14
anti-LPS
anti-CD14
+
LPS
+
anti-LPS
B.
% decreased metabolic activity
SFM
6.25% FCS
70
60
50
40
30
20
10
0
-10
control
pmx B
LPS
pmx B
+
LPS
anti-LPS
pmx B
+
anti-LPS
C.
polymyxin B - - + - - +
anti-LPS - + + - + +
FCS - - - + + +
100
bp
ladder
2072
1500
1000
600
400
200

conflicting results. In some cases LPS was still cytotoxic after heating as expected, but in other experiments it was knocked out. In each case however, the anti-LPS antiserum gave results identical to LPS, suggesting the presence of contaminating LPS. More work would need to be done to determine the exact mechanism of cytotoxicity caused by the anti-LPS antiserum, especially in serum-free conditions.

It is interesting to note however, that since this effect was seen, results have appeared from clinical trials using anti-LPS antibodies. While early laboratory studies were promising, none of the antibodies tested show any clear efficacy in human clinical trials, except possibly in some specific patient subsets (reviewed by Danner and Natanson, 1995). In one case a trial was stopped early due to excessive mortality in the antibody treated group (McCloskey *et al.*, 1994), agreeing with previous work with the same antibody using a canine model of septic shock which indicated that it is harmful (Quezado *et al.*, 1993). The antibody in this case is a monoclonal antibody of IgM class directed against the lipid A portion of LPS (Teng *et al.*, 1985), and it is therefore questionable whether it is comparable to polyclonal anti-serum raised against the O-chain of LPS.

6.3 Intracellular signalling pathways

6.3.1 Calcium

Calcium ions play a key role in signalling events in many cellular processes. There has been much speculation regarding the role of calcium in cell death (reviewed by Trump and Berezesky, 1995). Disturbances in calcium homeostasis are thought to play a role in necrosis (Fawthorp *et al.*, 1991). In addition, many studies show an increase in intracellular free Ca^{2+} preceeding apoptosis, and in most cases the source of Ca^{2+} was identified as extracellular rather than a release from internal stores (reviewed by Martin *et al.*, 1994). However, in other systems no increase in calcium is detected, and there are several examples of apoptosis occuring in calcium free conditions (Lennon *et al.*, 1992; Lee *et al.*, 1993).

The possible involvement of calcium in LPS-induced CPAE cell death was investigated through several avenues. First, the source of external calcium was removed by incubation of cells in calcium-free MEM. Cells incubated in this media rounded up slightly, but remained attached. The loss of external calcium had no inhibitory effect on LPS-induced death as measured by the MTT assay. In fact, the level of LPS-induced cell death in calcium-free media was consistently slightly higher than that found in normal MEM in experiments done on the same day. The addition of the calcium chelator EDTA or EGTA (200 μM) to normal MEM also had no effect on LPS-induced CPAE death (Fig 6.5 A). The combination of calcium-free medium and chelator resulted in many cells lifting and dying after an overnight incubation, but the additional cytotoxic effect of LPS could still be clearly detected.

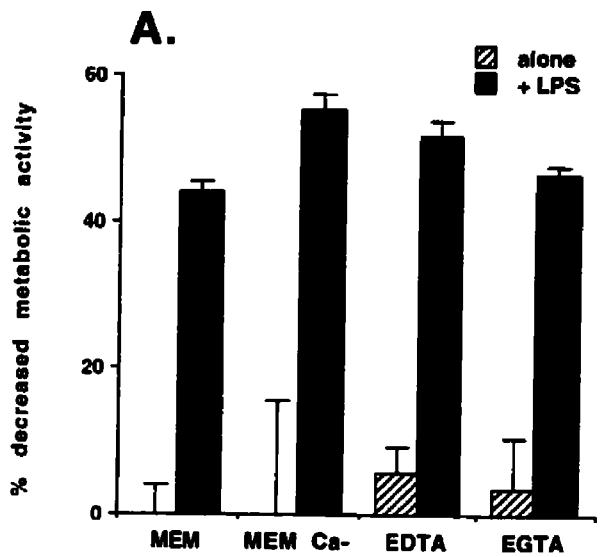
As a consequence of these experiments, stock (10 μg/ml) LPS was routinely prepared in calcium-free MEM. It was found that stock LPS prepared in calcium-free MEM resulted in a slightly quicker and higher level of cell death, despite being diluted 1:100 in normal MEM when added to cells. This is possibly due to changes in the supramolecular structure of LPS clusters in the absence of calcium. Divalent cations stabilize the outer membrane by cation bridging (Seydel *et al.*, 1993), and magnesium concentration has a strong effect on the supramolecular structure of LPS (Brandenburg *et al.*, 1992).

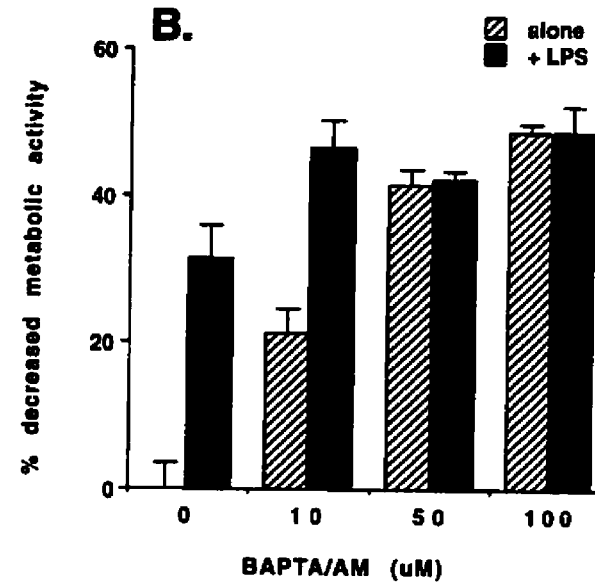
Two inhibitors that alter intracellular calcium levels were also assayed. BAPTA/AM is the membrane permeable form of BAPTA, a calcium chelator. Once it enters cells, it is hydrolyzed by cytosolic esterases and is trapped. At lower BAPTA/AM concentrations (10, 50 μM) visible signs of LPS-induced CPAE death were still apparent, while at a higher concentration (100 μM) cells were completely rounded and no additional LPS effects were seen. After an overnight incubation, BAPTA/AM caused considerable cell death on its own as measured using the MTT assay. At the higher BAPTA/AM concentrations cytotoxic levels were the same in the absence or presence of LPS , while at 10 μM there was an additive effect (Fig 6.5 B).

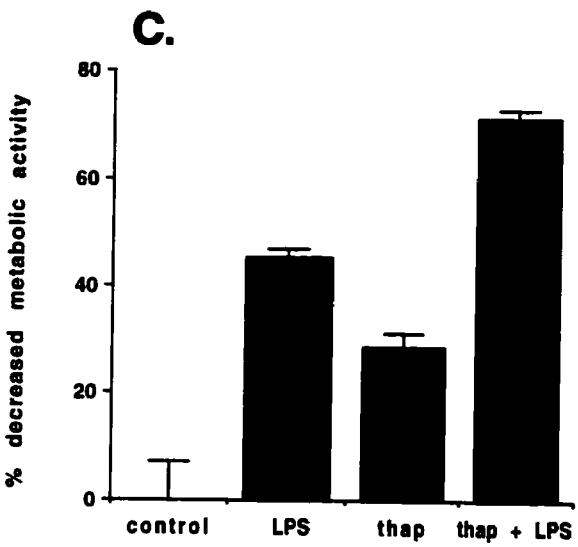
Thapsigargin alters cytosolic calcium concentrations by releasing internal Ca^{2+}- stores into the cytosol. Thapsigargin is a cell-permeant sesquiterpene lactone that empties internal stores of calcium by inhibiting endoplasmic reticular Ca^{2+}-ATPase. Cells were pre-incubated with 2 μM thapsigargin in calcium-free MEM for 1 hour before the addition of LPS (Missiaen *et al.*, 1994). After 3 hours no effect was seen with thapsigargin alone, while it appeared to enhance LPS-induced cell death. After an overnight incubation cells incubated with thapsigargin showed visible signs of cytotoxicity such as cell rounding and blebbing. This was measurable by the MTT assay, and additive to LPS-induced cell death (Fig 6.5 C). When these experiments were repeated to look for DNA fragmentation, no apoptosis was detectable at 3 hours using thapsigargin alone, but in combination with LPS it resulted in a much stronger DNA ladder compared to LPS alone (Fig 6.5 D). The enhanced cell death induced by LPS in calcium-free medium could also be detected by DNA fragmentation analysis (Fig 6.5 D).

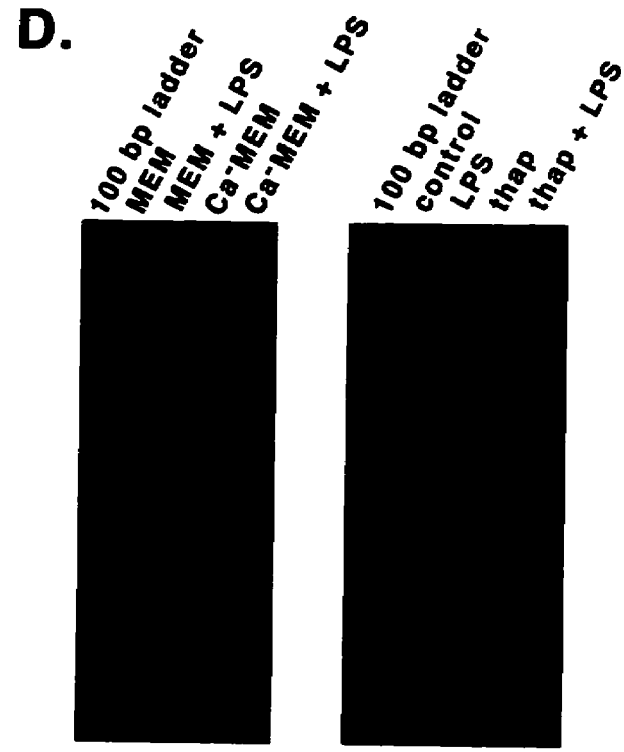
These results do not point to an essential role for calcium in LPS-induced CPAE cell death. Certainly an extracellular source of calcium is not necessary, nor are intact internal stores. However, these results must be interpreted cautiously, as a disruption of normal calcium states can lead to cell death on its own, as was clearly shown with BAPTA/AM and thapsigargin.

Figure 6.5 Effect of calcium on LPS mediated CPAE cell death. (A) External calcium. CPAE cells were incubated with 6.25% FCS in MEM, Ca^{2+} free MEM (Ca-), or MEM containing 200 µM EDTA or EGTA in the presence or absence of 100 ng/ml LPS. (B) BAPTA/AM. CPAE cells were pre-incubated for 1 hour with the indicated amount of BAPTA/AM in Ca^{2+} free MEM before the addition of 6.25% FCS and 100 ng/ml LPS. (C) Thapsigargin. CPAE cells were pre-incubated for 1 hour with 2 µM thapsigargin (thap) in Ca^{2+} free MEM before the addition of 6.25% FCS and 100 ng/ml LPS. After an overnight incubation cytotoxicity was measured using the MTT assay. Values are the average of four samples +/- SD. (D) DNA fragmentation. Left gel: cells were incubated in MEM or Ca^{2+} free MEM with 5% FCS in the presence or absence of 100 ng/ml LPS. Right gel: cells were preincubated 1 hour with 2 µM thapsigargin in MEM containing 5% FCS before the addition of 100 ng/ml LPS to appropriate samples. After 3 hours cells were lysed and extranuclear cellular DNA was isolated and subjected to gel electrophoresis in a 2% agarose gel. Results shown are from representative experiments, experiments were repeated three (A, C) or two (B, D) times.


A.
alone
+ LPS
% decreased metabolic activity
60
40
20
0
MEM
MEM Ca-
EDTA
EGTA


B.
alone
+ LPS
% decreased metabolic activity
60
40
20
0
0
10
50
100
BAPTA/AM (uM)


C.
% decreased metabolic activity
80
60
40
20
0
control
LPS
thap
thap + LPS


D.
100 bp ladder
MEM
MEM + LPS
Ca-MEM
Ca-MEM + LPS
100 bp ladder
control
LPS
thap
thap + LPS

6.3.2 Nitric oxide

Another signalling molecule thought to play a role both in sepsis and endothelial cell signalling is nitric oxide (NO). Nitric oxide is synthesized via the oxidation of arginine by a family of synthases (NOS), which are either constitutive (cNOS) or inducible (iNOS). Constitutive NOS transiently produces low amounts of NO (pico, nanomolar) and is involved in physiological regulation such as the maintenance of blood vessel tone. Inducible NOS results in higher levels of NO (nano, micromolar) for prolonged periods of time and plays a role in host defense, cytotoxicity and inflammation (Clancy and Abramson, 1995). Endothelial cells contain both types of synthase, and NO release is detected in bovine aortic endothelial cells following LPS treatment (Salvemini *et al.*, 1989; Salvemini *et al.*, 1990), while another group found that LPS inhibits NO production in the same type of endothelial cells (Myers *et al.*, 1992).

To determine whether NO was involved in LPS-induced CPAE cell death two approaches were used. One was to eliminate extracellular sources of arginine. This was done by treating normal MEM (126 mg arginine/L) with arginase. The pH of MEM was raised to 9.5 with KOH, treated with sufficient arginase to eliminate all arginine at 37°C for 35 minutes, the pH was then re-adjusted to 7.2 with HCl, and the medium was filtered (0.2 μM). Free arginine in the serum was removed by dialysis (autoclaved dialysis tubing, BRL, cutoff 12,000-14,000 MW). The levels of LPS-induced cell death in this arginine-free medium were identical to those measured in normal medium. The other approach was to use a specific inhibitor of NO synthesis (Palmer *et al.*, 1988), N^G-monomethyl-L-arginine (L-NMMA). Recommended concentrations of this inhibitor, 0.4 to 1 mM, pre-incubated with the cells for 1 hour, had no effect on LPS-induced CPAE cell death. Nor did a higher dose of 2.5 mM either alone or in combination with arginine-free media.

These results suggest that NO has no role in LPS-induced CPAE cell death. Recent work by Melzig and Loose (1995) confirm this conclusion, using both the inhibitor L-NMMA and glucocorticoid dexamethasone, which inhibits the induction of NO synthase. In a model

of LPS-induced bovine endothelial barrier dysfunction, no blocking effect was seen using a different NO synthesis inhibitor, N-nitro-L-arginine methyl ester (Berman *et al.*, 1993). These results are not unexpected, as constitutive NO is not found at levels high enough to kill cells, and inducible NO takes longer than a few hours to have an effect. Another group did find that L-NMMA and dexamethosone are effective in blocking LPS-induced endothelial cell death (Palmer *et al.*, 1992). However they were using porcine endothelial cells, not normally considered sensitive to LPS, and very high doses of LPS. Even at a LPS concentration of 10 μg/ml only 23% of the cells were dead after 24 hours.

6.3.3 Tyrosine phosphorylated proteins

Protein phosphorylation is a major control mechanism in cell function. Many receptors contain a tyrosine kinase activity which, when activated, and can trigger a complex phosphorylation cascade. Proteins involved in signalling can interact through SH2 domains which recognize and bind to phosphorylated tyrosines. Furthermore, the activity of many proteins is controlled by the phosphorylation/dephosphorylation of specific residues. Considering the significant role of protein phosphorylation in signalling, is therefore probable that it is also involved in the control of apoptosis. Another indication for a role for protein phosphorylation in apoptosis is the fact that modulators of protein phosphorylation, such as the phosphatase inhibitor okadaic acid and the broad kinase inhibitor staurosporine, are potent inducers of apoptosis. A number of phosphoproteins, as well as kinases and phosphatases, are implicated in apoptosis (reviewed by Gjertsen and Døskeland, 1995). The phosphorylation of tyrosine residues is less profuse than that of serine or threonine residues, which makes the detection of tyrosine phosphorylated proteins possible using western blots probed with specific anti-phosphotyrosine antibodies, and this method was used to examine LPS-induced CPAE cell death.

Early attempts to find tyrosine phosphorylation/dephosphorylation events in LPS treated CPAE cells looked at the entire population of treated cells. No differences were seen

in anti-phosphotyrosine western blots comparing LPS treated and untreated cells at time points ranging from 1 minute to 2 hours. At any given point however only a small percentage of cells show visible signs of apoptosis. Therefore another approach was used, collecting the apoptotic cells which had lifted after 3 hours of treatment and analyzing their phosphotyrosine profile. At first no LPS-induced phosphorylation events were seen. However a further modification to the method was introduced, in case a phosphorylation event was transient. The floating cells were repeatedly collected as they lifted off, approximately every 20 minutes. Using this method, in the insoluble fraction of the LPS-treated lifted cells a novel band of ca. 92 kDa was seen (Fig 6.6, arrow head). This band could be detected faintly in LPS treated non-adherent cells collected after 4 hours (LB), but was much stronger in samples from LPS treated floating cells collected every 20 minutes (LA). Another band running at the position of the 105 kDa marker was reduced in intensity in the detergent insoluble fraction of both LPS-treated floating cells compared to control cells. This band was seen in both detergent soluble and insoluble fractions in all samples from adherent cells, and is missing from the soluble fraction of all samples from non-adherent cells. No differences were seen in the phosphotyrosine profiles of LPS treated and control adherent cells. Samples containing the 92 kDa phosphoprotein were run on a 8% SDS-PAGE gel with unlabeled protein markers in order to determine the size more accurately.

Since the 92 kDa phosphoprotein was seen in cells which had already lifted, a step coinciding with the formation of apoptotic bodies, it is likely not involved in early signalling events. However, as it was not seen or very faint in LPS treated non-adherent cells collected after 3 hours, it would appear to be phosphorylated transiently. The 92 kDa phosphoprotein was not detected in CPAE cells treated with C2-ceramide or staurosporine, compounds which also cause apoptosis in CPAE cells. This suggests that the phosphorylation of this protein is not generally involved in the process of apoptosis, however the lack of detection may have been due to differences in the timing of events during apoptosis triggered by different agents. Two possible candidate tyrosine phosphorylated proteins with homologous size are members

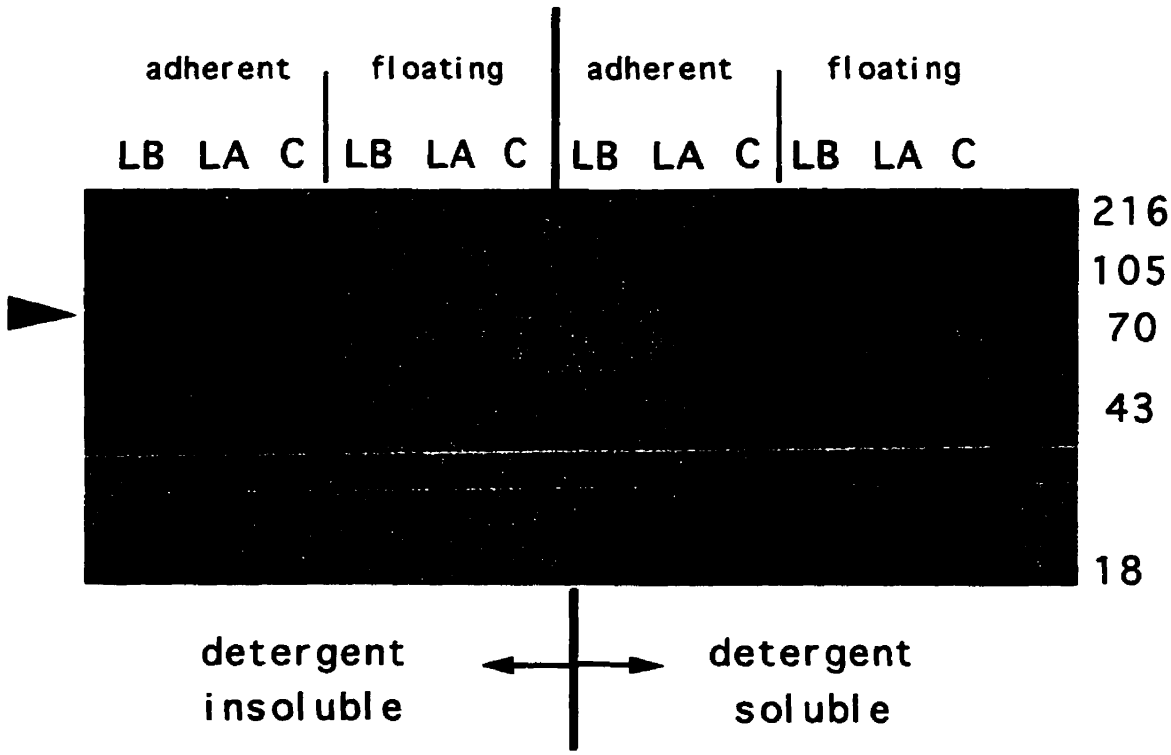


Figure 6.6 Tyrosine phosphoprotein profile of LPS treated CPAE cells. CPAE cells were incubated in MEM containing 5% FCS alone (control cells, C), or including 100 ng/ml LPS (LPS-treated, L). Cells were incubated for 4 hours before floating cells were collected (C, LB), or non-adherent cells were collected every 20 minutes by centrifugation (LA) from 2 to 4 hours and pooled later. After samples were prepared, 25 μg of protein was loaded in each well of a 12% SDS-PAGE gel. After transfer to nitrocellulose, the blot was probed with the anti-phosphotyrosine antibody, 4G10. Similar results were seen in five separate experiments.

of the STAT (signal transducers and activators of transcription) family of transcription factors. STAT1α (p91) and STAT3 (p92) are tyrosine phosphorylated and migrate to the nucleus following treatment of cells with various growth factors and cytokines (Leaman *et al.*, 1996). However, in one system STAT3 was recently implicated as part of an anti-apoptotic response (Fukada *et al.*, 1996). Much more work would have to be done to determine the identity of the 92 kDa phosphoprotein, and to determine its possible role in LPS-induced signalling in CPAE cells.

There are no obvious candidate proteins for the reduced 105 kDa band. However, under the conditions used (12% SDS-PAGE gel, pre-labeled protein markers) it was difficult to determine the size accurately. If the size was underestimated, a potential molecule could be the 125 kDa focal adhesion kinase (FAK). FAK is a nonreceptor protein tyrosine kinase which is autophosphorylated and activated in response to integrin-mediated signals (reviewed by Hanks and Polte, 1997). Recent studies have implicated FAK in cell survival (reviewed by Meredith andSchwatz, 1997), and reduced levels of phosphorylated FAK were seen in human endothelial cells after apoptosis was induced by the removal of growth factors (Yang *et al.*, 1996).

Robaye *et al.* (1994) analyzed the apoptotic death of bovine aortic endothelial cells triggered by TNF and cycloheximide using two-dimensional electrophoresis. They found that 2D gels of P^{32} labeled cells undergoing apoptosis had 23 spots of increased intensity and 2 of decreased intensity. Two of the major spots of increased intensity were approximately the size of the LPS-induced 92 kDa band. Recently Arditi *et al.* (1995) showed that LPS stimulates the tyrosine phosphorylation of mitogen activated protein kinases (MAPK) p44, p42, and p41 in both human and bovine brain microvessel endothelial cells. A possible reason that these phosphoproteins were not detected in LPS treated CPAE cells, despite similar methods, could be due to differences between primary cultured cells and cell lines. It is intriguing that LPS-induced MAP kinase stimulation was found in endothelial cells from

two species with very different reactions to LPS (death vs activation), as well as in macrophages with yet another response (Weinstein *et al.*, 1992).

6.3.4 Inositol phosphate production

Some external signals detected by surface receptors result in the hydrolysis of phosphatidylinositol-4,5-bisphosphate, an inositol lipid located in the plasma membrane, resulting in the release of two intracellular second messengers. One, diacylglycerol (DAG) activates protein kinase C (PKC), while the other, inositol 1,4,5-triphosphate (IP3), mobilizes calcium stores (Berridge, 1987). To determine whether this pathway was triggered during LPS-induced CPAE cell death, levels of inositol phosphates (IPs), including IP3, were measured following LPS treatment. Cells were prelabeled with [^{3}H]myoinositol and the levels of radiolabled IPs was measured over time following LPS addition. An increase from approximately 5000 to 8000 cpm was seen, but the same increase was also measured in cells treated only with serum. This serum-induced flux occured between 10 and 30 minutes, and the levels were still high at 60 minutes. Incubation of the cells with serum during the overnight labeling eliminated any increase in IP levels following LPS or serum addition, however the incorporation levels of [^{3}H]myoinositol were very low under these conditions. Labeling in serum-starved conditions followed by incubation in serum for 4 hours prior to the addition of LPS resulted in good incorporation but no detectable IP flux. It is possible that the unsynchronized nature of apoptosis, in which only a small portion of cells are at the execution stage at any given time, resulted in small overlapping IP fluxes which were below detection levels. On the other hand, the DAG/PKC pathway is implicated in growth regulation and protection from apoptosis (Hannun and Obeid, 1995), and therefore it is not unexpected to find that was not involved in cell death.

6.4 Inhibitors

6.4.1 Protein synthesis inhibitors

As recently as 5 years ago, the involvement of protein synthesis was considered to be a characteristic of apoptosis. This conclusion was based on early models of apoptosis in which protein synthesis inhibitors could block cell death (reviewed by Schwartzman and Cidlowski, 1993). However, over time more and more exceptions were found. Not only were systems identified where protein synthesis inhibitors do not block apoptosis, but also cases where they could induce apoptosis on their own. Three protein synthesis inhibitors were used to see in which category LPS-induced CPAE apoptosis belongs. Cycloheximide alone was cytotoxic in an overnight incubation down to a concentration of 0.1 μg/ml, but at all concentrations tested the addition of LPS resulted in a higher level of cell death. In a DNA fragmentation assay 1 and 5 μg/ml cycloheximide caused DNA laddering after 3.5 hours, and enhanced LPS-induced laddering. The two other protein synthesis inhibitors, actinomycin D (1 to 10 μg/ml) and emetin (0.1 to 1 μg/ml), were also found to be toxic and had no blocking effect on LPS-induced CPAE cell death (data not shown).

6.4.2 Kinase inhibitors

A number of kinase inhibitors were tested to determine whether protein phosphorylation was involved in LPS-induced death. Two protein kinase C (PKC) inhibitors, staurosporine and H7, were used. Genistein, a protein tyrosine kinase (PTK) inhibitor, and W7, a calmodulin inhibitor were also tested. None of these products exhibited any blocking effect on LPS-induced CPAE apoptosis, as determined using visual criteria, by measuring cytotoxicity using the MTT assay, and by DNA fragmentation detection. Staurosporine and genistein both caused DNA fragmentation on their own. Genistein acts by binding to the ATP binding site of PTKs, and has the drawbacks of considerable toxicity and non-specific inhibition of serine/threonine kinases (Levitzki, 1990). Staurosporine nonspecifically inhibits protein kinases including PTK, PKC, and protein kinase A (PKA), by an unknown

mechanism involving binding to a specific site (Ruegg and Burgess, 1989). Staurosporine is a potent inducer of apoptosis (Weil *et al.*, 1996), but may act through a mechanism other then protein tyrosine kinase inhibition (Azuma *et al.*, 1995), and by a pathway independent of PKC inhibition (Raff *et al.*, 1993). The antibiotic polymyxin B also acts as a PKC inhibitor, competing with the phosphotidylserine cofactor (Mazzei *et al.*, 1982; Casnellie, 1991). As neither staurosporine nor H7 blocked LPS-induced CPAE cell death, it would seem that the effectiveness of polymyxin B (Fig 3.4) is due to its LPS binding capacity, and not a result of PKC inhibition.

Another class of protein tyrosine kinase inhibitor binds to the substrate binding site. An example of this is the tyrphostins, one of which, tryphostin B46, was found to attenuate the cytotoxic effects of LPS on bovine aortic endothelial cells (Melzig and Loose, 1995). Another tyrosine kinase inhibitor, herbimycin A, has long been known to inhibit LPS-induced tyrosine phosphorylation in macrophages (Weinstein *et al.*, 1991), and was recently shown to block MAP kinase activation in LPS treated human and bovine brain endothelial cells, as well as blocking LDH release in the bovine endothelial cells (Arditi *et al.*, 1995). Herbimycin A is a potent and selective inhibitor of many tyrosine kinases which acts by binding irreversibly to active sulfhydryl residues near the ATP sites (Uehara and Fukazawa, 1991), and along with the tryphostins, would be interesting to examine in this system.

6.4.3 Sodium vanadate

Meredith *et al.* (1993) found that human endothelial cells underwent apoptosis when grown in suspension, deprived of extracellular matrix interactions. This death could be blocked by the protein phosphotyrosine phosphatase inhibitor sodium orthovanadate. They used concentration of 100 μM for 18 hours, while in our lab a combination of 1 mM vanadate and 2 mM hydrogen peroxide (H_2O_2) pre-incubated for 15 minutes and then removed is routinely used (Volberg *et al.*, 1992). Vanadate has been shown to act in synergy with H_2O_2,

but their exact mechanism is not known. It is thought that orthovanadate is oxidized in the presence of H_2O_2 to pervanadate and can then readily enter cells (Howarth and Hunt, 1979).

The long pre-incubation period in the first method resulted in high background death in control CPAE cells, but a reduced pre-incubation period (8 hours) resulted in no blocking of LPS-induced death. The higher concentration of vanadate in combination with H_2O_2 caused cells to slowly contract, displaying prominent nuclei, and eventually lift. No visible signs of LPS-induced apoptosis could be detected in cells pre-treated with vanadate/H_2O_2, and no DNA laddering was seen after 3 hours. In an overnight incubation, the blocking ability of the 15 minute treatment with vanadate/H_2O_2 appeared to be reversed, and LPS-induced CPAE cell killing was seen. However it should be noted that little or no contaminating RNA was seen in DNA samples from cells treated with vanadate/H_2O_2, as is commonly found in all samples. It is possible that this treatment was fairly destructive to the cells, and may only block apoptosis by causing death by necrosis. Proteins extracted from vanadate/H_2O_2 treated cells (3 hours) and examined by anti-phosphotyrosine western blots showed that this treatment resulted in high levels of tyrosine phosphorylated proteins in CPAE cells.

A recent paper (Yang *et al.*, 1996) looked at the role of tyrosine phosphatase regulation in endothelial cell apoptosis. They found that human endothelial cells undergoing apoptosis after removal of growth factors showed a general dephosphorylation of cellular proteins, and that apoptosis could be blocked using orthovanadate at a concentration of 50 μM. While protein tyrosine phosphatases (PTP) have been implicated in numerous cases of apoptosis by the use of inhibitors, very few specific PTPs have been identified (reviewed by Gjertsen and Døskeland, 1995). The classic receptor-like PTP CD45 is expressed at higher levels in T-cells udergoing apoptosis (Alexander *et al.*, 1993), while B cells lacking CD45 are more susceptible to apoptosis (Ogimoto *et al.*, 1994). The PTP FAP-1 interacts with the cytosolic domain of the Fas receptor and counteracts Fas-induced apoptosis (Sato *et al.*, 1995).

Vanadate has many effects on cells in addition to blocking protein tyrosine dephosphorylation (Gordon, 1991). One of these is an increase in cellular pH (Cassel *et al.*, 1984). Recently it has been shown that intracellular acidification appears to be associated with apoptosis, possibly in order to activate a DNase which is involved in producing fragments during apoptosis (Rebollo *et al.*, 1995). Vanadate may therefore block apoptosis or at least the formation of characteristic DNA fragments by counteracting the drop in cellular pH. In addition, H_2O_2 alone provokes other responses in cells, including the stimulation of MAP kinases activity (Stevenson *et al.*, 1994), effects which should be taken into consideration when interpreting this data.

To detemine whether phosphotyrosine phosphatases are truly involved in LPS-induced apoptosis more specific and less toxic inhibitors would be useful. However, while more than 30 protein tyrosine phosphatases have been characterized to date, the number of specific inhibitors is much more limited. Zinc chloride is used as a general phosphotyrosine phosphatase inhibitor (Gordon, 1991). Another possibility is phenylarsine oxide, a protein tyrosine phosphatase inhibitor which can block apoptosis in eosinophils and neutrophils (Yousefi *et al.*, 1994). Inhibitors targeting other phosphatases could also be assayed. A role for serine/threonine dephosphorylation in a late stage of apoptosis is suggested by experiments using the specific inhibitor calyculin A (Luo and Kessel, 1996). The phosphatase inhibitor okadaic acid also blocks apoptosis triggered by a number of inducers (Song *et al.*, 1992).

6.4.4 Vacuolar acidification

A collection of specific inhibitors were used to determine whether vacuolar acidification is necessary for the action of LPS on CPAE cells. This might be the case if LPS is taken up by the cells rather than signalling through a surface receptor. The inhibitors tested were: ammonium chloride (20 mM), methylamine (5 mM), chloroquine (10 μM), momensin (20 μM), and bafilomycin A1 (1 μM). These agents render MDCK and CHO cells resistant to

diptheria toxin, which indicates that endosomal acidifiation is blocked (Finlay and Falkow, 1988). While none of these inhibitors were toxic to CPAE cells at the concentrations used in an overnight assay, all except bafilomycin A1 caused marked morphological changes in the cells. Ammonium chloride and methylamine especially caused cells to fill with vacuoles. However, all showed morphological indications of LPS-induced death in addition to the effects they caused alone, and levels of cell death as measured by the MTT assay were not affected (data not shown).

6.4.5 GTP-binding regulatory proteins

The potential role of GTP binding proteins (G proteins) in LPS-induced signalling was examined using bacterial toxins. G proteins contain subunits which, when bound to GTP, either stimulate (α_S) or inhibit (α_i) the production of cyclic AMP (cAMP) by adenyl cyclase. Cholera toxin covalently modifies α_S-GTP, while pertussis toxin ribosylates α_i-GDP, in both cases leading to an increase of cAMP. These toxins were pre-incubated with cells for 3.5 hours before the addition of LPS (Eason *et al.*, 1992). No blocking of LPS-induced cell death was observed with pertussis toxin (or its inactive control) at 100 ng/ml nor with cholera toxin at 100 ng/ml (data not shown).

6.4.6 Nocodazole

Not much is known at the moment about the role of cytoskeletal proteins in the process of apoptosis. Nocodazole is a potent and specific microtubule inhibitor (Parczyk *et al.*, 1989). CPAE cells were treated with 10 µg/ml nocodazole for 2 hours before the addition of LPS (Finlay *et al.*, 1991). Three hours later nocodazole treated cells were slightly rounded, but LPS-induced cell budding was still very evident. The occurrence of apoptosis was confirmed by detection of DNA fragments (data not shown).

6.4.7 Cytochalasin D

While studying the morphology of LPS treated CPAE cells using phase and fluorescent microscopy, an attempt was made to stain actin filaments using labeled phalloidin., a mushroom toxin which binds to actin filaments (Cooper, 1987). This was done in order to see cell structure in addition to the nuclear structure stained with acridine orange or ethidium bromide. Typical results are seen in figure 6.7, showing the same cells in phase contrast (A), stained with phalloidin (B), and acridine orange for reference (C). Normal cells contained a diffuse network of actin throughout the cell (Fig 6.7 B, arrow head), but LPS treated cells undergoing apoptosis stained very differently (Fig 6.7 B, arrow). These cells had a single point of brightly stained actin in the cell, usually in the vicinity of the nucleus or remnants of the nucleus.

To further study the role of actin in LPS-induced apoptosis the microfilament inhibitor cytochalasin D was used. Cytochalasins are fungal metabolites which bind to the barbed end of actin filaments, inhibiting both the association and dissociation of actin subunits (reviewed by Cooper, 1987). CPAE cells were pre-incubated with 0.1 to 1 μg/ml cytochalasin D for 1 hour (Finlay *et al.*, 1991). At a concentration of 1 μg/ml, CPAE cells underwent a dramatic morphological change. Cells rounded up while still remaining stretched out. This effect, called cell arborization, has been well described as a result of cytochalasin D treatment (Schliwa, 1982). The effect was dose dependent, with cells incubated with 0.1 μg/ml appearing almost normal. When LPS was added following pre-incubation with cytochalasin D, no visual characteristics of LPS-induced apoptosis were seen, except at 0.1 μg/ml where the number of budding cells was reduced compared to LPS treated cells. In an overnight incubation cytochalasin D was fairly toxic on its own, but its effect was not additive to the LPS-induced cell death (Fig 6.8 A). When the incubation time was reduced to 8 hours, the cytotoxic effect of cytochalasin D was minimal, and a reduction in the LPS-induced killing was evident (Fig 6.8 B). The level of cell death was not reduced completely to zero however, a result which was confirmed by DNA fragmentation assays, which showed that cytochalasin

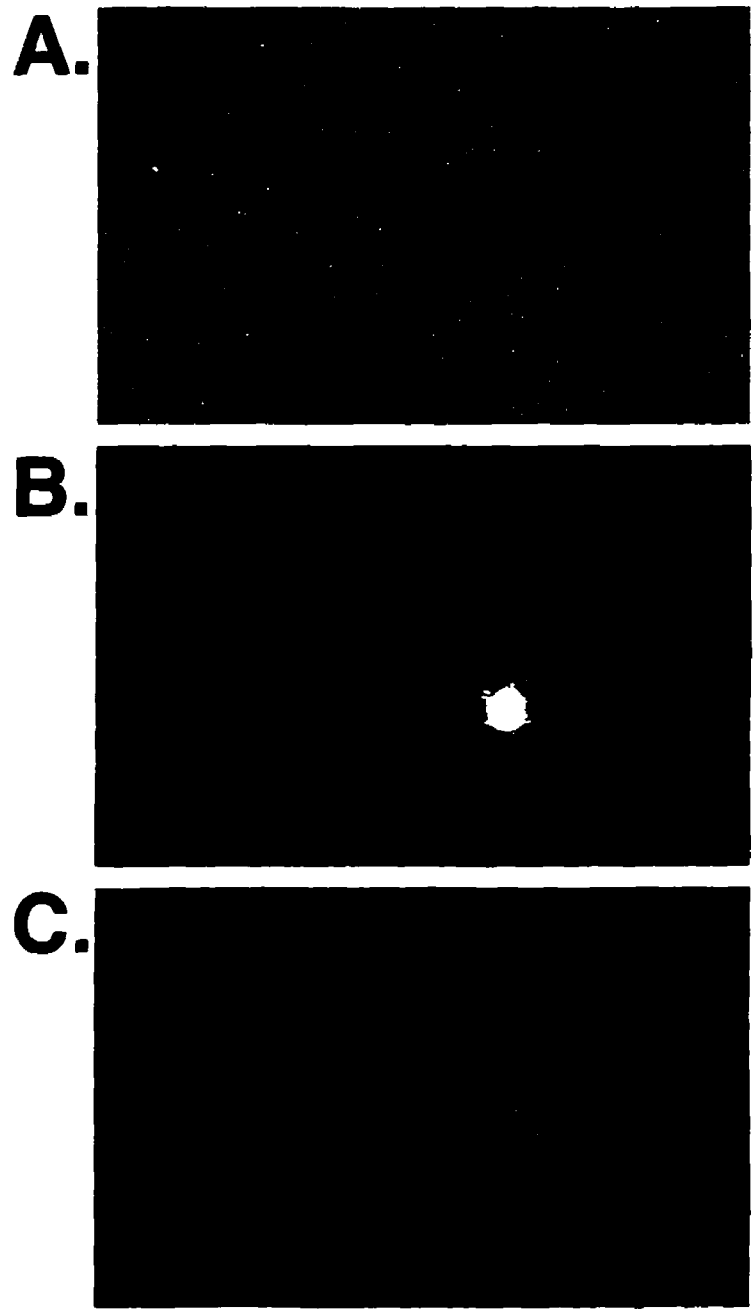
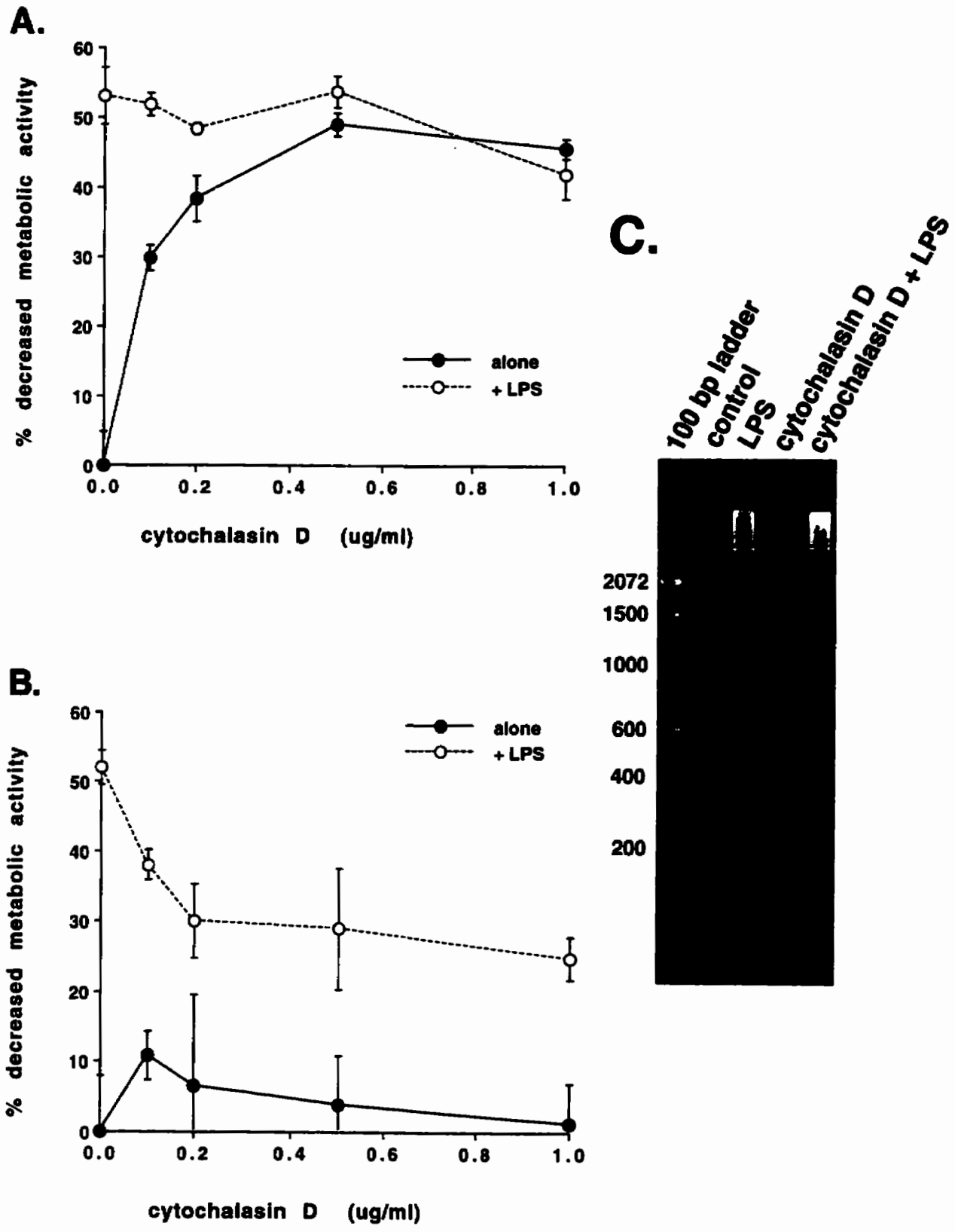


Figure 6.7 LPS-induced actin rearrangement. The same field of LPS treated (100 ng/ml, 3 hours) CPAE cells is shown by phase contrast microscopy demonstrating cell morphology (A), and fluorescent microscopy following staining with FITC conjugated phalloidin indicating actin filaments (B), and acridine orange showing location of nucleic acids for reference (C). Arrow indicates a cell undergoing apoptosis, while the arrow head points to a cell with normal morphology. Magnification x500.

Figure 6.8 Effect of cytochalasin D on LPS mediated CPAE cell death. (A) CPAE cells were incubated in MEM containing 6.25% FCS and the indicated concentration of cytochalasin D (1 hour pre-incubation) in the presence or absence of 100 ng/ml LPS. Cytotoxicity was measured after an overnight incubation using the MTT assay. Values are the average of four samples +/- SD. (B) Cells were treated as above, with the incubation period reduced to 8 hours. (C) CPAE cells were incubated in MEM containing 5% FCS and the indicated compounds; LPS (100 ng/ml), cytochalasin D (1 μg/ml, pre-incubated 45 min). After 3 hours cells were lysed and extranuclear cellular DNA was isolated and subjected to gel electrophoresis in a 2% agarose gel. Results shown are from representative experiments, experiments were repeated three (A, B) or two (C) times.


A.
% decreased metabolic activity
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
alone
+ LPS
cytochalasin D (ug/ml)
B.
% decreased metabolic activity
alone
+ LPS
cytochalasin D (ug/ml)
C.
100 bp ladder
control
LPS
cytochalasin D
cytochalasin D + LPS
2072
1500
1000
600
400
200

D (1 μg/ml) reduced the LPS-induced ladder, but did not completely eliminate it (Fig 6.8 C). In fact, cytochalasin D alone triggered apoptosis in CPAE cells, detectable after about 6 hours by the formation of DNA fragments.

Cytochalasins are one of many compounds which have been found to both block and induce apoptosis. Work with cytochalasin B and E showed that they can prevent the formation of apoptotic bodies in HL-60 cells undergoing apoptosis, but have no effect on DNA fragmentation (Cotter *et al.*, 1992). In another system they both induce DNA fragmentation (Kolber *et al.*, 1990). Cytochalasin D has been reported to inhibit the LPS-induced TNF production in macrophages, indicating that microfilament reoerganization may be important in the LPS signalling pathway in these cells (Shinji *et al.*, 1993). More recently it was shown that cytochalasin D blocked neutrophil activation by LPS, along with other evidence suggesting that LPS internalization and early endosome fusion are required for signal transduction in that system (Detmers *et al.*, 1996). It would be interesting to ascertain whether cytochalasin D prevents apoptosis in CPAE cells by preventing LPS uptake. However, it is also possible that cytochalasin D blocks LPS-induced signalling in CPAE cells merely by triggering a form of apoptosis with an extended condemned period.

6.5 Miscellaneous

6.5.1 Heat shock

Heat shock can both induce and inhibit apoptosis in different systems (Ghibelli *et al.*, 1992; Samali and Cotter, 1996). In the case of porcine endothelial cells, neither LPS nor heat shock alone will induce apoptosis, however in combination they do (Buchman *et al.*, 1993). CPAE cells were incubated at 42.5°C for one hour (in the absence of 5% CO_2). Cells were then incubated with or without LPS for a further 4 hours. The heat shock treatment did not reduce the LPS-induced DNA fragmentation, nor did it cause DNA fragmentation by itself (data not shown).

6.5.2 Dimethyl sulfoxide

In porcine endothelial cells treated with LPS and heat shock, the resulting apoptosis can be blocked by antioxidant agents such as dimethyl sulfoxide (DMSO), which blocks DNA fragmentation in that system at a concentration of 1% (Abello *et al.*, 1994). This was not the case in my system, where DMSO showed no signs of blocking LPS-induced CPAE cell death, and caused considerable DNA fragmentation on its own at concentrations of 2% and higher (data not shown).

6.5.3 Temperature

The development of LPS-induced apoptosis at different temperatures was studied. The levels of DNA fragmentation after 3 hours were not affected when cells were incubated at 37°C in normal atmosphere as compared to 5% CO_2. No changes in cell morphology or DNA fragmentation were seen in cells incubated at 4°C as expected since apoptosis is an active process, nor was any evidence seen at 18°C. Cells incubated at 30°C exhibited reduced but definite visual signs of apoptosis, although no DNA fragmentation was detected after 3 hours. This suggests that apoptosis can occur at 30°C, but at a slower rate. The binding of LPS to CD14 was found to be uneffected by temperature over the range of 4 to 37°C (Kirkland *et al.*, 1993), although temperature is known to affect LPS supramolecular structure (Brandenburg *et al.*, 1992). The interaction of LPS/sCD14 with cells and downstream signalling events may occur at a reduced rate at 30°C.

6.5.4 LPS-resistant CPAE cells

Since a level of CPAE cell death of 100% is never seen after treatment with LPS even at high concentrations, the question was raised regarding the possible presence of a resistant subpopulation. To address this question, CPAE cells were repeatedly treated overnight with 100 ng/ml LPS in MEM containing 5% FCS and the survivors re-seeded. After two rounds, a definite decrease in the amount of cell death was seen. After five rounds no cell death was

visible following LPS addition. To eliminate the possibility that resistance was somehow due to repeated incubations in a lower serum concentration and re-seeding, the selection was repeated with control cells treated identically except for the LPS. The resulting LPS treated cells were completely resistant to LPS at all concentrations tested, while mock treated cells gave identical results to untreated CPAE (all points except one not statistically different at a 0.1 level of significance using the Student's t test) (Fig 6.9). These results were later confirmed using the DNA fragmentation assay. Other known apoptosis inducing agents were assayed to see if resistance to LPS was linked to resistance to other agents. Staurosporine (1 μM), genistein (250 μM), and DMSO (4%) were used, all of which caused apoptosis in CPAE cells after 4 hours. Intriguingly, only staurosporine, known to cause apoptosis in almost all cells tested (Weil *et al.*, 1996), caused DNA fragmentation in the LPS resistant cells.

LPS treated cells remained completely resistant to LPS after several weeks of continuous passage in the absence of LPS, suggesting that resistance is not due to the development of a transient tolerance. It has long been known that animals repeatedly injected with LPS become non-responsive (Beeson, 1946). However, it was later determined that this tolerance is mediated by macrophages and lasts only 48 hours in mice (Freudenberg and Galanos, 1988) and 96 hours in rats (He *et al.*, 1992).

A possible source of the resistant cells could be contamination with another cell line which is not killed by LPS. HeLa cells, which have a history of overtaking slower growing cell lines (Lavappa *et al.*, 1976), are routinely used in this laboratory, and were briefly cultured at the same time as CPAE. To check if the resistant cells were still endothelial cells antibodies against factor VIII, a specific endothelial cell marker, were used in an immunofluorescent assay. Unfortunately, neither resistant nor parental CPAE stained with this antibody, a result confirmed by a later edition of the ATCC catalogue which mentioned that CPAE had lost this antigen. Polyclonal rabbit antisera raised against CPAE cytoplasmic membranes was also used, and both the parental and resistant CPAE cells stained strongly, while HeLa cells were very faint.

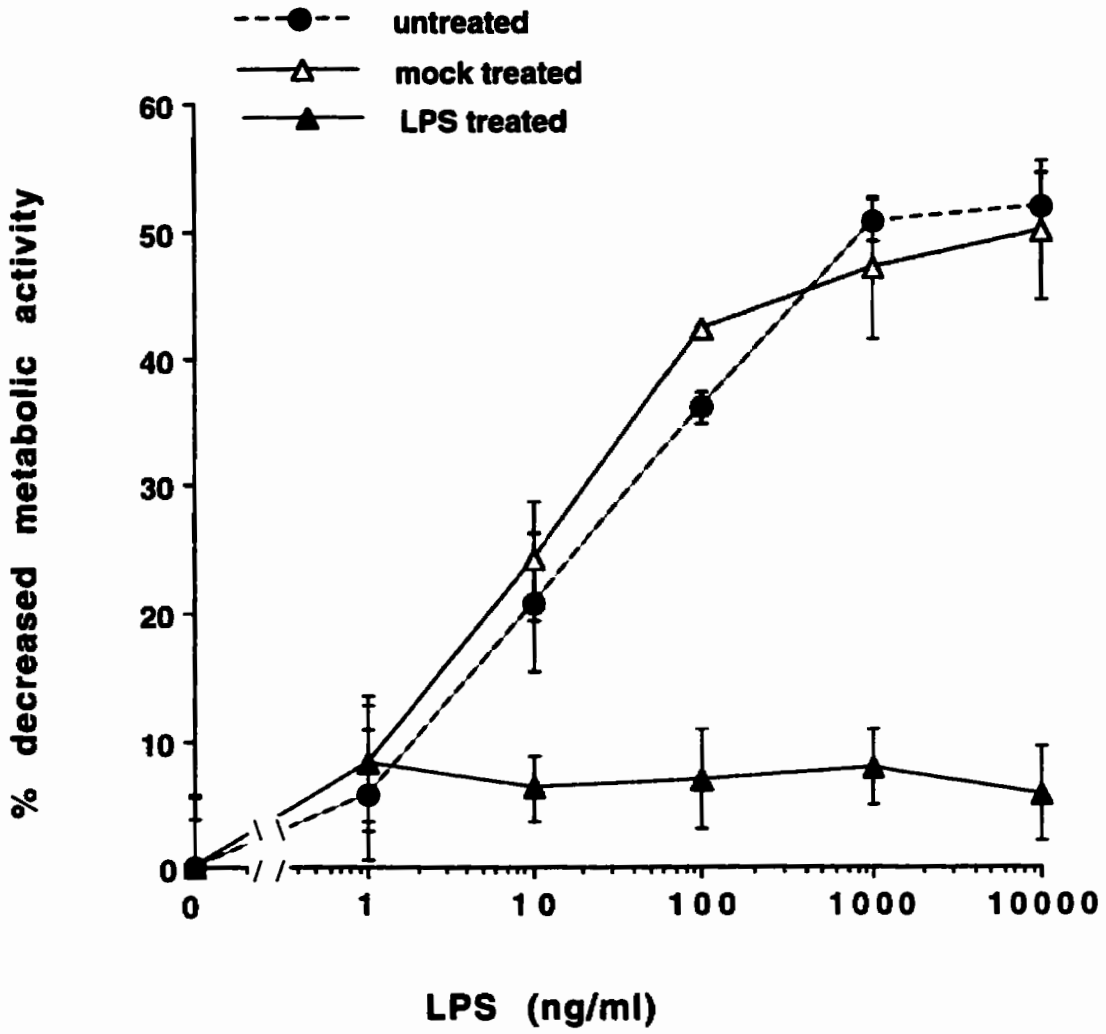


Figure 6.9 LPS resistant CPAE cells. Untreated, mock treated, and LPS treated CPAE cells were incubated in MEM containing 6.25% FCS and increasing concentrations of LPS. Cytotoxicity was measured using the MTT assay following an overnight incubation. Values are the average of four samples +/- SD. Results shown are from a representative experiment, similar experiments were repeated three times.

Further work with the LPS resistant cells was hindered by the lack of a pure line of LPS sensitive cells for comparison. Also, if more work was to be done with these cells it would be important to establish conclusively that they are in fact endothelial cells, perhaps by measuring angiotensin converting enzyme activity, for which CPAE are known to be positive. Recently an interesting paper was published in which similarly produced resistant and sensitive cells were compared (Gottschalk *et al.*, 1995). A subline of WEHI-231 cells was generated which was resistant to anti-IgM induced apoptosis. These cells remained sensitive to other triggers of apoptosis, and a comparison of gene expression and signal transduction events only revealed a reduction of ceramide production as a possible reason for resistance. It would be interesting to determine whether the LPS resistant subline of CPAE shares the same responses to previously tested reagents, such as the cytotoxic response to the anti-IL-2 antisera seen in section 6.2.3.

Table 1: Summary table for chapter 6

Functions studied	Specific compounds used (1)	Effect on LPS killing (2)	Cytotoxicity alone (3)
Cytokines	antibodies against bovine: TNF, IFN-α, IFN-γ, IL-2	-	+/- (?)
Calcium	Ca^{2+} free MEM, EGTA/EDTA, BAPTA/AM, thapsigargin	-	+/- (?)
Nitric oxide	arginine-free MEM, L-NMMA	-	-
Protein synthesis	cycloheximide, emetin, actinomycin D	-	+ (A)
Kinases -PKC, PTK	staurosporine, genistein, W7, H7	-	+/- (A)
Phosphatases -tyrosine	vandate/H_2O_2	+	+ (?)
Vacuolar acidification	ammonium chloride, methylamine, chloroquine, momensin, bafilomycin A1	-	-
GTP proteins -$G\alpha_s$, $G\alpha_i$	cholera toxin, pertussis toxin	-	-
Cytoskeleton -microtubules	nocadazole	-	-
Cytoskeleton -microfilaments	cytochalasin D	+	+ (A)
Heat shock	42.5°C, 1 hour	-	-
Antioxidants	DMSO	-	+ (A)

(1) Compounds used in these studies of LPS-mediated CPAE cell death. For concentrations and conditions, see Materials and Methods, and text.

(2) Effect of compounds on LPS-mediated CPAE cell death: (-) no effect, (+) blocking effect.

(3) Cytotoxic effect of compounds alone: (-) no cytotoxic effect, (+) cytotoxic, (+/-) some but not all compounds in this class caused cell death. Mode of cell death was either identified as apoptosis (A) or not determined (?).

6.6 Discussion

it is a tale,
Told by an idiot, full of sound and fury,
Signifying nothing.

W. Shakespeare (Macbeth)

The many varied factors which induce apoptosis give rise to the same end event, triggering the DNases that cleave DNA and the cell machinery which affects the morphological changes characteristic of apoptosis. However, it has become clear that the initial signalling pathways which ultimately lead to apoptosis are very diverse. Numerous different aspects of signal transduction were examined in the study of LPS-induced CPAE cell death, and many of the results are summarized in Table 1. Most of the results were discussed in the individual sections, and will be only briefly mentioned here. The majority of the signalling events studied did not appear to have a role in LPS-induced cell death. However, many of the compounds employed were cytotoxic on their own, and therefore the results are difficult to conclusively interpret. In addition, negative results must be cautiously interpreted, only indicating that under the given circumstances no effect was seen. As the signals triggering cell death are further studied, evidence for redundant pathways leading to death is increasingly being found. It is possible that LPS activates more than one such pathway, and that blocking only one has no effect.

Two compounds appeared to successfully block the development of apoptosis in LPS treated CPAE cells, cytochalasin D and vandate/H_2O_2. This could indicate a role for microfilaments and protein tyrosine phosphatases in LPS triggered cell death. However, as previously mentioned in the appropriate sections, there are several other possibilities such as non-specific effects and the cytotoxic nature of the compounds used, which need to be examined further. As mentioned in individual sections in this chapter, several aspects examined deserve further study. The preliminary results showing a 92 kDa tyrosine phosphorylated protein in LPS treated CPAE cells might aid in the further study of the effects of compounds which also cause cell death. If it is found that this protein is phosphorylated

specifically by LPS treatment, it could be used to distinguish between death induced by LPS and by other compounds.

Chapter 7 Comparison of LPS and ceramide induced CPAE cell death

7.1 Introduction

The sphingomyelin cycle was first described by Okazaki *et al.* (1989), who found that vitamin D3 caused the hydrolysis of sphingomyelin. The enzyme which initiates the pathway, neutral sphingomyelinase, is a phospholipase C concentrated in the plasma membrane (Kolesnick, 1991). Sphingomyelin is the most abundant sphingolipid in almost all mammalian cells, accounting for about 10% of cellular phospholipids, and is preferentially concentrated in the outer leaflet of the plasma membrane (Hanada *et al.*, 1993). Ceramide serves as a second messenger in the sphingomyelin pathway, released by the hydrolysis of membrane sphingolipids, especially sphingomyelin. Ceramide can then readily redistribute across the membrane bilayer (Lipsky and Pagano, 1985). Several intracellular targets of ceramide have recently been described. The first was a 97 kDa proline directed serine/threonine protein kinase which was named ceramide-activated protein kinase (CAPK) (Mathias *et al.*, 1991; Joseph *et al.*, 1993). Another target, ceramide-activated protein phosphatase (CAPP), belongs to the phosphatase class 2A (PP2A) family of serine/threonine protein phosphatases, and is activated by ceramide (Dobrowsky and Hannun, 1992; Hannun, 1994). CAPP is inhibited by okadaic acid, a potent inhibitor of the PP2A family. It has also been suggested that ceramide may directly trigger PKCζ, but little is known about this potential pathway (Lozano *et al.*, 1994). Preliminary results showed that ceramide binds to and activates protein kinase c-Raf leading to the subsequent activation of the MAPK cascade (Pfeilschifter *et al.*, 1996). The understanding of the signalling pathways of sphingolipids is still very fragmented, and complicated by the fact that there are at least 10 common forms of ceramide and many other sphingolipid molecules potentially involved in signalling (Hannun and Linardic, 1993; Hakomori and Igarashi, 1995).

Early studies with ceramide were hindered by cytotoxicity. These effects were similar to those produced by TNFα, suggesting that ceramide might also trigger apoptosis. This was

confirmed by Obeid *et al.* (1993) who showed that cermide causes apoptosis in U937 cells. A direct link to TNFα was identified when it was seen that TNFα stimulates sphingomyelin hydrolysis (Kim *et al.*, 1991; Dressler *et al.*, 1992), induces ceramide production and results in the stimulation of CAPK (Liu *et al.*, 1994). Further evidence suggests that it is the 55 kDa TNF receptor which is involved (Wiegmann *et al.*, 1992; Yanaga and Watson, 1992). The structurally homologous death receptor Fas is also linked to the sphingomyelin pathway (Cifone *et al.*, 1994; Gill *et al.*, 1994; Tepper *et al.*, 1995). Ceramide production has been recently identified in many other cases of apoptosis, including the response of WEHI 231 cells to irradiation, corticosteroids and anti-immunoglobbin (Quintans *et al.*, 1994), and in daurorbicin induced apoptosis (Bose *et al.*, 1995). Ionizing radiation generates ceramide and initiates apoptosis (Haimovitz-Friedman *et al.*, 1994), and ceramide-initiated signalling is required for several other types of stress-induced apoptosis (Verheij *et al.*, 1996). On the other hand, it is important to note that ceramide production is linked to other physiological functions, such as growth inhibition, cell cycle arrest, cell proliferation, and differentiation (Kolesnick and Fuks, 1995; Saba *et al.*, 1996). Interestingly, the cosmetic industry saw the potential of ceramide before it dawned on the scientific community, producing Ceramide Time Complex Capsules (Elizabeth Arden) which promise "a younger tomorrow". This treatment reduces lines and wrinkles, possibly by inducing premature apoptosis in the upper layers of skin cells.

Cells respond to LPS, IL-1 and TNF in a similar fashion, and these stimuli use common signalling elements. Joseph *et al.* (1994) looked to see whether LPS also uses the sphingomyelin pathway. They found that both LPS and lipid A stimulate CAPK in HL-60 cells and neutrophils. LPS does not cause sphingomyelin hydrolysis however, and therefore stimulates CAPK without generating ceramide. Using molecular modeling they identified a strong structural similarity between ceramide and a region of lipid A. This led to the suggestion that LPS may act by mimicking ceramide (Wright and Kolesnick, 1995). This theory was further supported when it was shown that macrophages from mice hypo-

responsive to LPS (CH3/HeJ, *Lps*^{d}) fail to respond to ceramide or sphingomyelinase (Barber *et al.*, 1995). Macrophages from LPS-responsive mice (C3H/OuJ, *Lps*^{n}) on the other hand show enhanced expression of LPS inducible genes when treated with ceramide, suggesting that the *Lps* gene is involved in both LPS and ceramide signalling.

It was decided to investigate the possibility that LPS could be triggering apoptosis in CPAE cells by mimicking ceramide. As the downstream elements in the ceramide pathway are still incompletely characterized and few tools to study them are available, it was decided to first compare LPS and ceramide induced death. Natural ceramide contains long chain saturated or monounsaturated fatty acids and is poorly soluble in aqueous solutions. Synthesized cell permeable ceramide analogues which retain the biological activity of ceramide are commonly used (Dobrowsky *et al.*, 1993). C2-ceramide, used in this work, differs from natural ceramide by the replacement of the 18 carbon acyl chain by a 2 carbon acyl group (Hannun *et al.*, 1993).

7.2 Ceramide-induced cytotoxicity

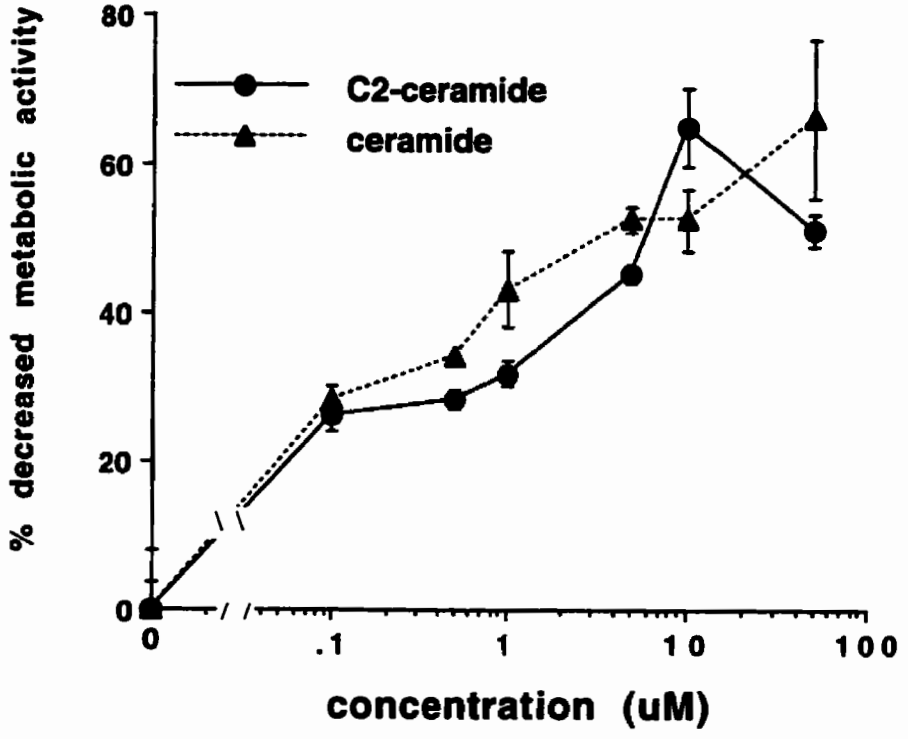
The first step in investigating the possible role of the sphingomyelin pathway in LPS mediated cell death was to determine whether ceramide itself could cause cell death in CPAE cells. Natural ceramide and a cell permeant analogue C2-ceramide cause apoptosis in U937 cells when an ethanol:dodecane (98:2) carrier is included at a 1:200 dilution (Ji *et al.*, 1995b). Eventually, the conditions were found where ceramide caused CPAE cell death as measured by the MTT assay. These conditions included the presence of ethanol:dodecane carrier in which the ceramide was first diluted and mixed before further dilution in MEM, and very vigorous mixing in the MEM (5 minutes) in the complete absence of serum. The same inhibiting effect of serum was later seen by Sweeney *et al.* (1996). This could be due to binding by serum proteins and the resulting hinderance or inactivation of ceramide. In addition it was found that the cytotoxic effects of ceramide were considerably reduced when dilutions were carried out in Costar microfuge tubes, as compared to dilutions done in Falcon

conical tubes. The reasons for this is not clear, as both containers are made of polypropylene. The cell-permeant analogue, C2-ceramide, was more dependable than the natural ceramide, most likely due to the differences in solubility, and was therefore preferably used. The ethanol:dodecane carrier alone occasionally caused some cytotoxicity in overnight incubations as measured by the MTT assay, but usually had little or no measurable effect after 5 or 6 hours.

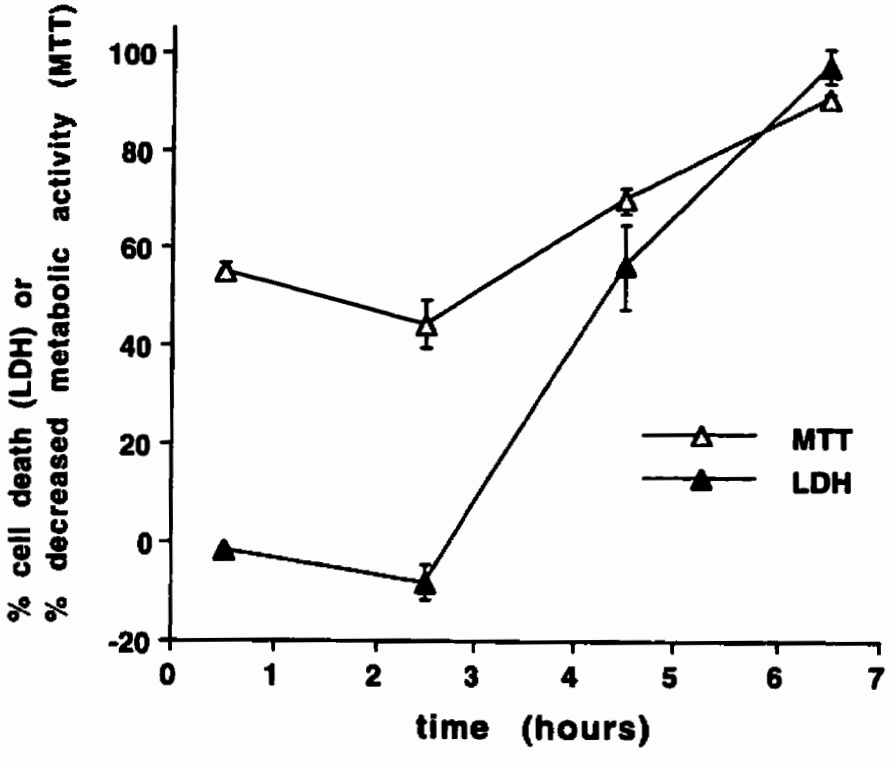
Natural ceramide and C2-ceramide gave almost identical dose response curves, starting to level off at about 10 μM (Fig 7.1 A). When cytotoxicity was measured over time however, unexpected results were seen. High cell death was measured at the shortest time point, even when ceramide was added just before the addition of the MTT reagent, and the following incubation shortened from 2 to 1 hour. In addition, this percent cell death did not increase appreciably over time up to 4 hours, while no visible signs of cell death were seen by phase-contrast microscopy until 2 to 3 hours, and by 4 hours the effect was marked. This suggested either a technical problem with the assay, or an extremely quick shutdown of mitochondrial function following ceramide treatment. To test the validity of the assay, LDH release was also measured. The two assays gave very similar results in a C2-ceramide dose response experiment (data not shown). When cell death was measured over time however, the LDH release results conformed more to the visual observations, only indicating measurable cell death after 3 to 4 hours. By 6 hours the levels of cell death as measured by the two assays were the same (Fig 7.1 B). These results could be due to the differences in the methods of measurement. The MTT assay measures a mitochondrial dehydrogenase, and mitochondria are thought to be early targets in apoptosis (Kroemer *et al.*, 1997; Zamzami *et al.*, 1996; Marchetti *et al.*, 1996). LDH release however would only occur during secondary necrosis, when apoptotic bodies finally rupture.

Figure 7.1 Cytotoxic effect of ceramide and C2-ceramide. (A) Dose response. CPAE cells were incubated in MEM containing a 1:200 dilution of an ethanol:dodecane (98:2) carrier and ceramide or C2-ceramide at the indicated concentrations. Cytotoxicity was measured using the MTT assay following a 5.5 hour incubation. (B) Time course. CPAE cells were incubated in MEM containing a 1:200 dilution of an ethanol:dodecane (98:2) carrier and 10 μM C2-ceramide for the indicated times. Cytotoxicity was measured using both the MTT and the LDH release assay. Values are the average of four samples +/- SD. Results shown are from representative experiments, experiments were repeated four (A) or three (B) times.

A.


% decreased metabolic activity
80
60
40
20
0
C2-ceramide
ceramide
0
.1
1
10
100
concentration (uM)

B.


% cell death (LDH) or
% decreased metabolic activity (MTT)
100
80
60
40
20
0
-20
MTT
LDH
0
1
2
3
4
5
6
7
time (hours)

7.3 Ceramide-induced apoptosis

Once the conditions for ceramide-induced cell death were determined, the next step was to look for DNA fragmentation to confirm that the mode of death was apoptosis. Characteristic DNA ladders were isolated from CPAE cells treated for 5 hours with 10 μM C2-ceramide, but not at 1 μM or lower (Fig 7.2 A). Further experiments showed that DNA fragmentation also occurred using 10 μM ceramide (Fig 7.2 B), and could be detected after treatment with 5 μM C2-ceramide and ceramide (data not shown). Despite the rapid onset of cell death as measured by the MTT assay, DNA laddering was detectable only after 4 hours. This was unexpected, since in the case of LPS-induced apoptosis DNA fragmentation could be detected after 2 hours when only a small fraction of cells were starting to show signs of apoptosis. In contrast, after 4 hours almost all ceramide treated cells had lifted, and still only a modest DNA ladder was seen. Longer incubations were hindered by the increase in background apoptosis occuring in CPAE cells incubated in the absence of serum.

The morphology of C2-ceramide (10 μM) treated CPAE cells were examined for confirmation of apoptosis. After 2.5 hours many cells in the treated monolayer had rounded up (Fig 7.3 A). Even at higher magnification, details in these rounded cells were difficult to detect, both in phase contrast (B) and in the matching acridine orange stained fluorescent micrograph (C). Some cells showed signs of condensed chromatin (C, arrow), and cells appearing to break apart into apoptotic bodies were also seen (C, arrowhead). While these photographs were admittedly obscure, they were comparable to published proofs of apoptosis. In addition, the ability of ceramide and C2-ceramide to induce apoptosis is well documented (Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Ji *et al.*, 1995b; Martin *et al.*, 1995), and so no further work was done to confirm the mode of cell death.

Figure 7.2 DNA fragmentation in ceramide and C2-ceramide treated CPAE cells. (A) C2-ceramide dose effect. CPAE cells were incubated in MEM alone or containing a 1:200 dilution of an ethanol:dodecane (98:2) carrier and increasing amounts of C2-ceramide. (B) C2-ceramide and ceramide. Cells were incubated as above and with 10 μM ceramide or C2-ceramide. Cells incubated with 5% FCS and 100 ng/ml LPS are included as a positive control. After 5.5 hours, cells were lysed and extranuclear cellular DNA was isolated and subjected to gel electrophoresis in a 2% agarose gel.

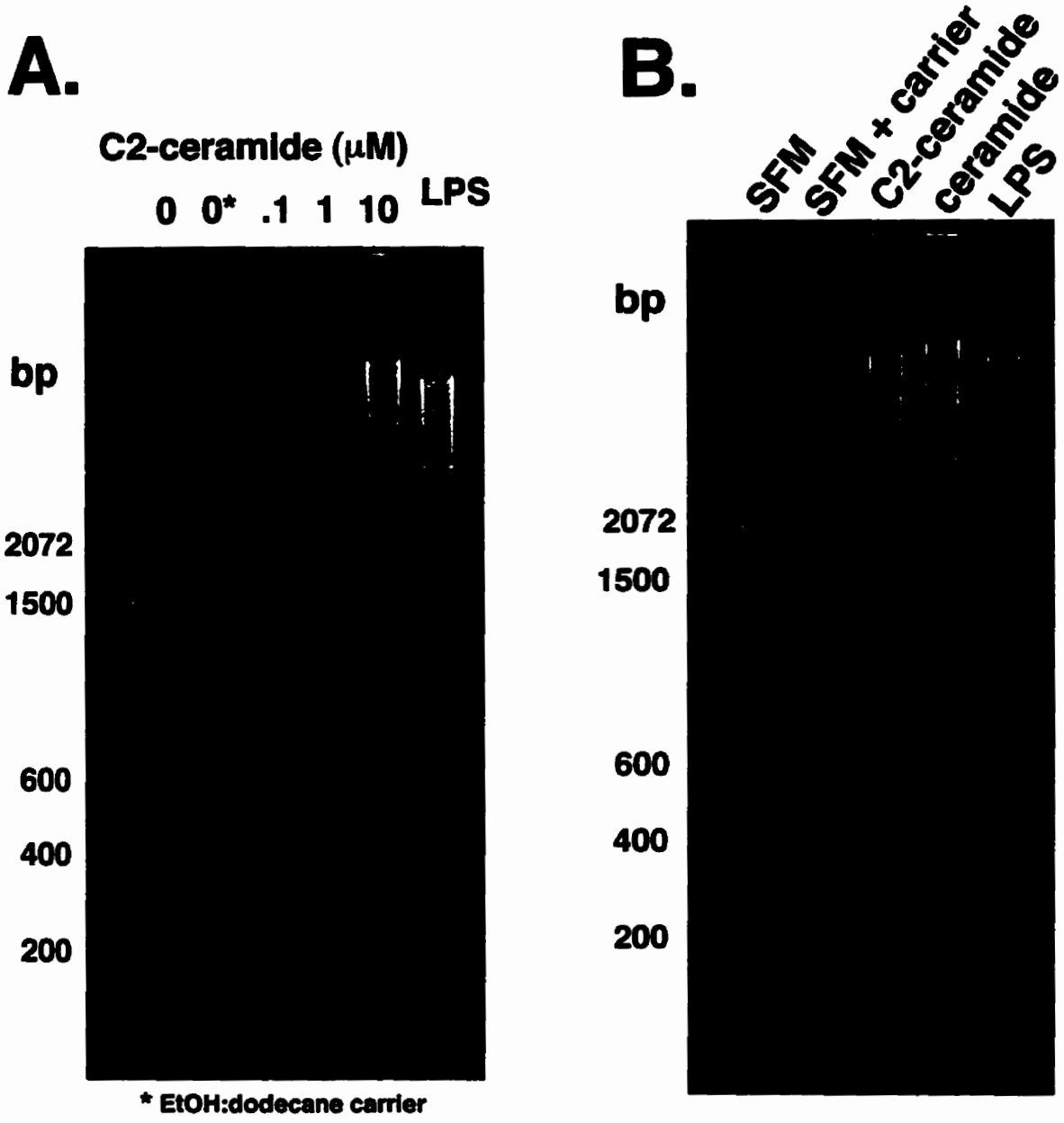
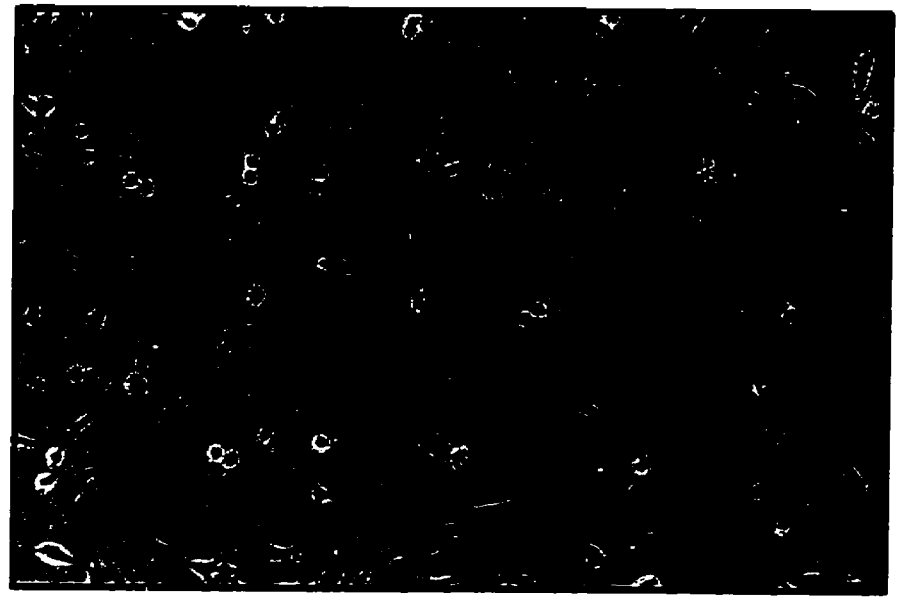
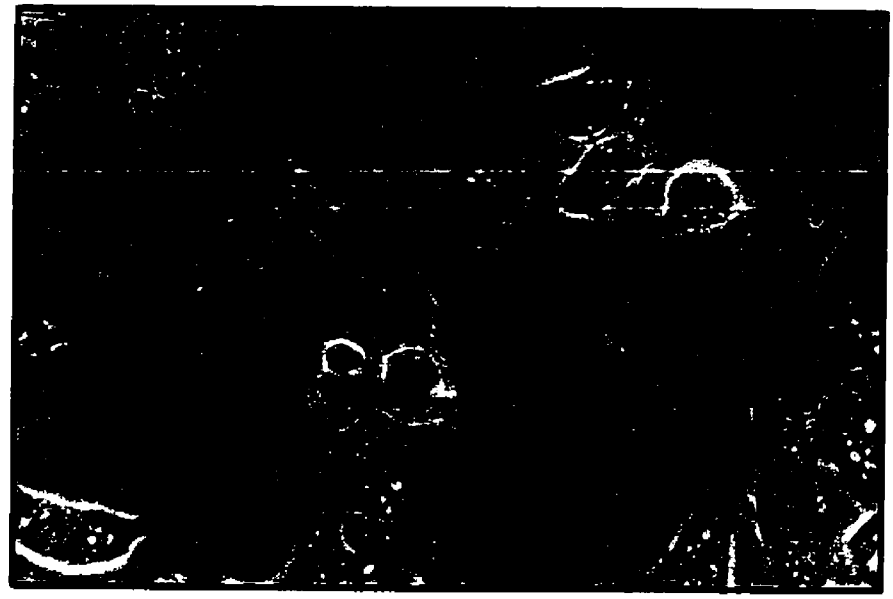
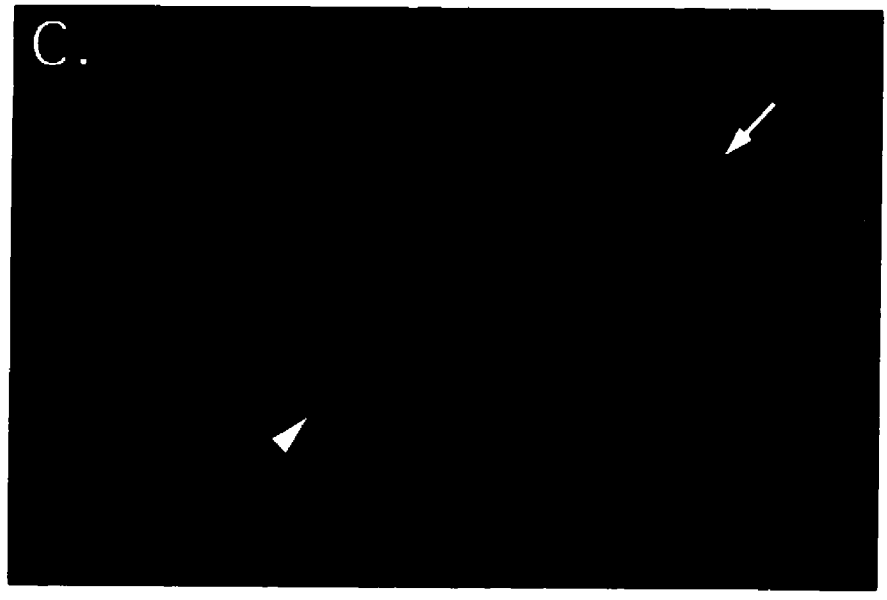

A.
C2-ceramide (μM)
0 0* .1 1 10 LPS
bp
2072
1500
600
400
200
* EtOH:dodecane carrier
B.
SFM
SFM + carrier
C2-ceramide
ceramide
LPS
bp
2072
1500
600
400
200

Figure 7.3 Morphology of C2-ceramide treated cells by phase contrast and fluorescent microscopy. CPAE cells were incubated in MEM with 1:200 ethanol:dodecane (98:2) carrier and 10 μM C2-ceramide for 2.5 hours. (A) Phase contrast micrograph, magnification x20. (B) Phase contrast micrograph, magnification x100. (C) Corresponding fluorescent micrograph of acridine orange stained cells, magnification x100. Arrow points to possible apoptotic cell with condensed chromatin. Arrow head indicates cell which appears to be breaking up into apoptotic bodies. Magnification x65 (A), 320 (B, C).






C.

7.4 Comparison of ceramide and LPS induced CPAE cell death

7.4.1 Visual comparison

Once ceramide-induced apoptosis in CPAE cells was confirmed, it was compared to LPS-induced apoptosis. Several morphological differences between LPS and ceramide treated cells were seen (Fig 7.4). LPS treated cells started to bud and eventually lifted off as a clump of apoptotic bodies. Cells treated with ceramide on the other hand rounded up first, lifted off, and then started to break apart into apoptotic bodies. Another major difference was that while only a small proportion of CPAE cells underwent apoptosis in response to LPS at any given time, their reaction to ceramide appeared more synchronous. Almost half of a ceramide treated monolayer rounded up and lifted off between 2 to 3 hours, while most of the remaining adherent cells lifted within 4 to 5 hours. In addition, LPS treated CPAE cells which were not showing signs of undergoing apoptosis appeared normal, while all ceramide treated cells which had not rounded up or started to break apart were full of vacuoles.

7.4.2 Okadaic acid

The phosphatase inhibitor okadaic acid, the causative agent of shellfish poisoning, is a potent inhibitor of protein phosphatase 1 and 2A (Hardie *et al.*, 1991). *In vitro* 1 nM okadaic acid totally inhibits PP2A, but in intact cells a higher concentration is necessary. Okadaic acid blocks the ceramide activated protein phosphatase (CAPP), inhibits c-myc down-regulation by ceramide and TNF-α (Wolff *et al.*, 1994), and can block several forms of apoptosis (Song *et al.*, 1992). Okadaic acid is also a potent inducer of apoptosis in many different systems (Bøe *et al.*, 1991; Davis *et al.*, 1994; Gjertsen and Dφskeland, 1995).

Okadaic acid (1 to 1000 nM, 1 hour pre-incubation) however failed to block either LPS or ceramide/C2-ceramide induced apoptosis in CPAE cells (data not shown). At 100 nM okadaic acid did not cause DNA fragmentation in CPAE cells after 6 hours. These results did not distinguish between the two inducers of apoptosis, but indicated that ceramide did not signal through CAPP to induce apoptosis in CPAE cells. However, it is still possible that

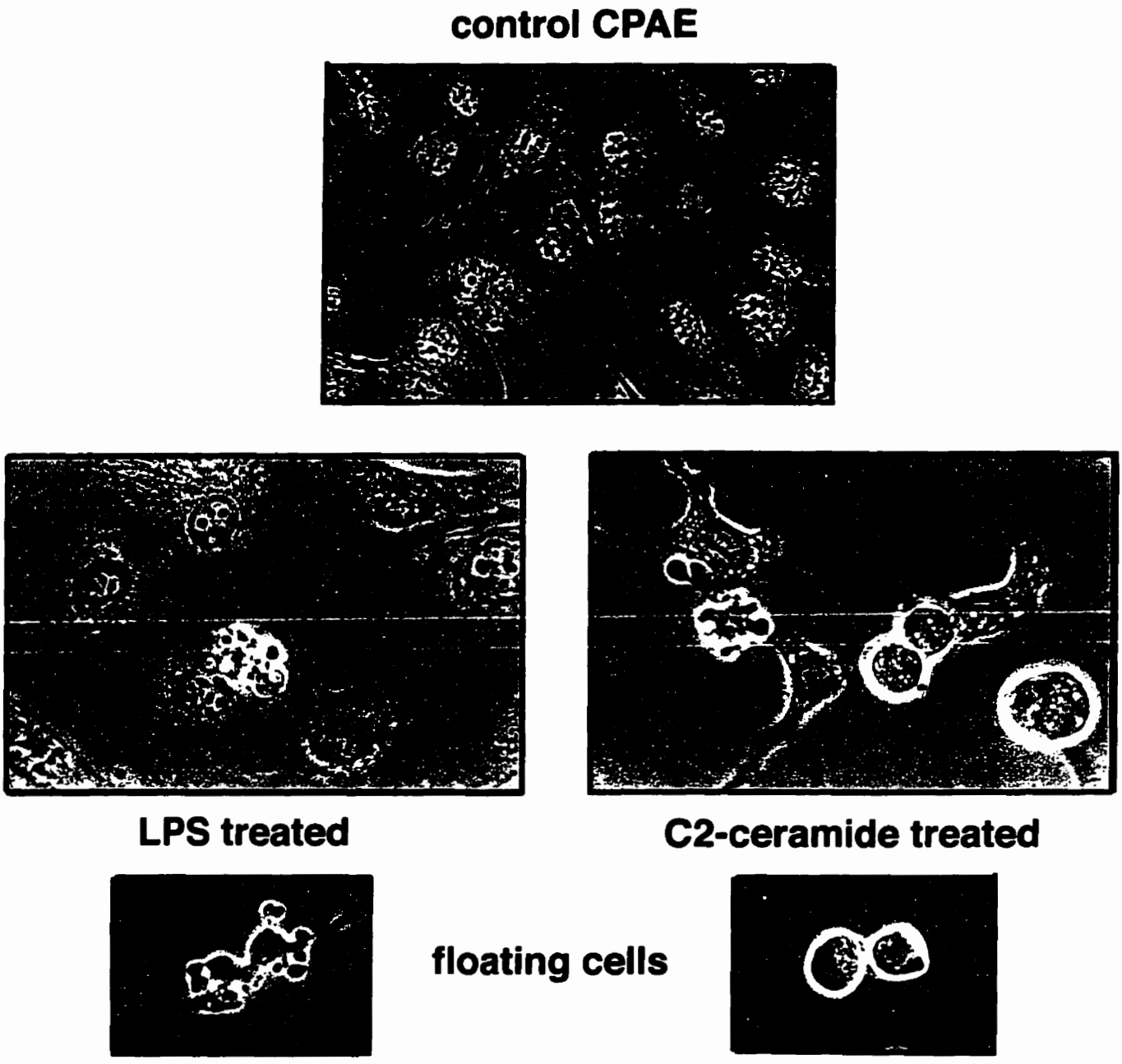


Figure 7.4 Morphology of LPS and C2-ceramide treated CPAE cells. Phase contrast micrographs of untreated and LPS (100 ng/ml, 4 hours, in MEM containing 5% FCS) or C2-ceramide (10 μM, 2.5 hours, in MEM containing 1:200 ethanol:dodecane (98:2) carrier) CPAE cells. Control CPAE cells are shown in the top panel, middle panels show adherent treated cells, and bottom panels show typical non-adherent treated cells spun down onto poly-L-lysine treated coverslips. Magnification x250.

ceramide induced signalling in CPAE cells proceeded via CAPK, or by a third as yet undiscovered pathway.

7.4.3 Cytochalasin D

Another indication that LPS and ceramide act through separate pathways is the fact that cytochalasin D appeared to block LPS-induced apoptosis but had no effect on ceramide-induced death. However a complication arose from the fact that cytochalasin D caused apoptosis in CPAE cells on its own after 6 hours. This resulted in the situation where the cytochalasin D blocking of LPS-induced apoptosis was detectable by DNA fragmentation analysis after 3 hours, at which point LPS-induced cytotoxicity was not yet detectable by the MTT assay. In the case of ceramide-induced apoptosis the lack of blocking by cytochalasin D could be measured at 3 hours using the MTT assay, but was not clear in the DNA ladder assay at 6 hours due to background apoptosis triggered by cytochalasin D alone.

7.4.4 LPS resistant cells

Another attempt to distinguish between the two systems was done using the LPS resistant strain of CPAE. These were CPAE cells which were repeatedly treated with LPS and the survivors re-seeded until no cell death was visible following LPS addition. These cells were completely resistant to LPS at all concentrations tested, but were equally (if not even slightly more) susceptible to ceramide-induced killing. This was shown using both the MTT and the LDH release assays, as well as DNA fragmentation detection (data not shown). It is possible however that the difference in susceptibility was due to a block in an upstream event in the LPS resistant cells. For example, the LPS resistant cells might be deficient in a mechanism for the uptake of LPS, a mechanism not required by ceramide.

7.4.5 Phosphotyrosine profile

A further indication that LPS acted through a pathway distinct from that of ceramide was differences in the profile of tyrosine phosphorylated proteins. LPS treated CPAE cells showed a band of ca. 92 kDa in an anti-phosphotyrosine western blot, which was found only in the insoluble fraction of cells which have lifted (chapter 6.3.3). This band was not seen in any fraction of C2-ceramide treated cells collected in an identical fashion. Other distinct differences were seen in both the soluble and insoluble fractions of LPS and C2-ceramide treated cells in P-Tyr blots (Fig 7.5). A phosphorylated insoluble protein of ca 105 kDa was found in untreated and LPS treated adherent cells, as well as in non-adherent LPS treated cells. It was fainter in C2-ceramide treated adherent cells, and missing in C2-ceramide treated floating cells (arrow). In addition there was a wide band at ca 120 kDa which is seen only in the soluble fraction of C2-ceramide treated cells (arrow head). The 92 kDa protein phosphorylated by the addition of LPS (Fig 6.6) was not visible on this blot due to differences in the washing conditions used in developing the blots. The addition of the detergent Tween-20 (0.1%) to the PBS resulted in a more definite 120 kDa band, but also reproducibly resulted in the disappearance of the 92 kDa band. Interestingly, under these conditions the 105 kDa band, which is absent in the insoluble fraction of LPS-treated non-adherent cells in figure 6.6, can clearly be seen in the same fraction here.

Little information has been found to date on tyrosine phosphorylated proteins downstream of ceramide. A 23 kDa nuclear protein was tyrosine phosphorylated in U937 cells treated with ceramide, C2-ceramide, and TNFα (Ji *et al.*, 1995a). In myeloid Hl-60 cells ceramide activated MAPK (Raines *et al.*, 1993; Yao *et al.*, 1995). Verheij *et al.*, (1996) did not detect MAPK activation in C2-ceramide treated bovine endothelial cells, while LPS caused MAPK phosphorylation in bovine endothelial cells (Arditi *et al.*, 1996).

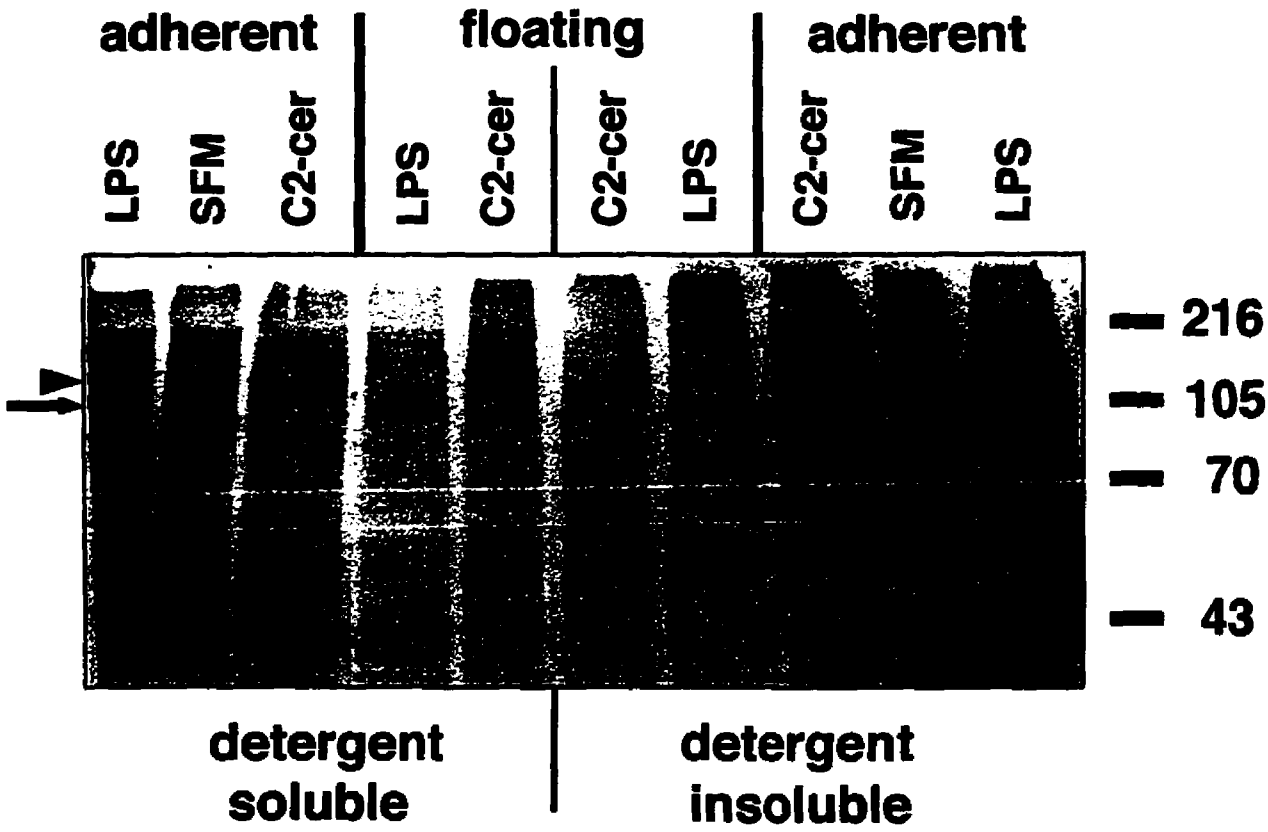


Figure 7.5 Tyrosine phosphoprotein profile of LPS and C2-ceramide treated cells. CPAE cells were treated with 100 ng/ml LPS in MEM containing 5% FCS, or 10 μM C2-ceramide in MEM with ethanol:dodecane (98:2) carrier. Non-adherent cells were collected every 20 minutes by centrifugation for 4.5 hours and samples pooled later, while adherent cells were collected at the end of the incubation. After samples were prepared, 25 μg of protein was loaded in each well of a 12% SDS-PAGE gel. After transfer to nitrocellulose, the blot was probed with the anti-phosphoyrosine antibody, 4G10. Profiles for cells incubated in SFM and media containing 5% FCS were identical, and only that for SFM is shown.

7.5 Discussion

There were a number of indications that LPS and ceramide work through different pathways. There were several morphological differences between LPS and ceramide treated CPAE cells, as well as variations in their phosphotyrosine profiles. There were also interesting kinetic differences seen. A DNA ladder could be isolated from LPS treated cells within 2 to 3 hours, however a much longer incubation of 8 to 12 hours was needed for measurable cytotoxicity. In ceramide treated cells however death appeared to be much quicker, especially as measured by the MTT assay, and even the LDH release assay gave a measurable reading after 4 hours. DNA fragmentation was not detectable until approximately 5 hours following ceramide addition. Since DNA laddering is considered an early event in apoptosis, it is possible that while ceramide treated cells were rapidly metabolically inactivated, the process of apoptosis was not triggered until the cells lifted off. It has been shown in other cells that lack of attachment can lead to apoptosis (Meredith *et al.*, 1993; Boudreau *et al.*, 1995). Another possibility is that the condemned phase of ceramide-induced apoptosis was longer than that of LPS-induced apoptosis.

The theory that LPS may act by mimicking ceramide is tantalizing, however some fundamental questions remain. For example, while ceramide can "flip-flop" across lipid bilayers and therefore could interact with cytoplasmic elements from the inner plasma membrane, it is less obvious how LPS could do so. LPS contains a large hydrophilic polysaccharide which could not easily cross a lipid bilayer. In addition, recent work by Barber *et al.* (1996) demonstrates that stimulating the ceramide pathway only partially mimics LPS-induced responses in macrophages. The comparison of LPS and ceramide induced apoptosis in CPAE cells would suggest that there are major differences in their actions, and therefore does not warrant further study. However, while these results suggest that LPS does not act simply by mimicking ceramide, there still may be a connection between the two. LPS may trigger a series of events, which lead to the production of ceramide and finally apoptosis.

It is also possible that the results obtained by the addition of exogenous ceramide is not comparable to endogenously produced ceramide.

Chapter 8 Final Discussion

In summary, this thesis describes experiments examining the effects of LPS on bovine endothelial cells. LPS was cytotoxic to both primary isolate bovine brain endothelial cells (BBEC) and a bovine pulmonary endothelial cell line (CPAE). A serum protein, sCD14, was necessary for this LPS-induced cytotoxicity, and the mode of LPS treated CPAE cell death was apoptosis. Studies investigating signal transduction in LPS treated CPAE cells identified numerous elements which did not appear to be involved in cell death (e.g. NO, calcium, protein synthesis, microtubules), and certain which might be involved (microfilaments, tyrosine phosphatases). There were major differences between LPS and ceramide induced CPAE cell death, suggesting that LPS did not simply act by directly mimicking ceramide. These discoveries may have implications for the understanding of the fate of endothelial cells *in vivo* during sepsis and septic shock.

There are a number of potential directions for the continuation of this project, in addition to follow-up experiments previously mentioned. Testing the relevance of this system *in vivo* would be extremely difficult for several reasons. First, the species specificity of endothelial cell sensitivity to LPS would necessitate excessively large model animals. In addition, it would be very difficult to isolate the direct effects of LPS on endothelial cells from the indirect effects. Another obvious field of inquiry is the identity of the LPS/sCD14 receptor. The search for this putative receptor has been ongoing in many labs for several years now, but to date no promising candidates have been put forward. A biotinylated LPS with retained biological activity has recently been described (Luk *et al.*, 1995), which may be useful in identifying receptor(s). However, while this LPS binds to monocytes and endothelial cells, it binds to endothelial cells in the absence of serum, suggesting that sCD14 is not involved in this interaction.

Several other possible lines of inquiry stem from novel discoveries in the field of apoptosis. Recently the importance of proteases in apoptosis has been identified, to the extent

of being hailed as a "pharmaceutical Holy Grail" (Whyte and Evan, 1995). Inhibitors of proteases are able to block apoptosis in several systems (Nakajima and Henkart, 1994; Sarin *et al.*, 1993). In addition, a number of protease inhibitors can block the cytotoxic effect of LPS on bronchial epithelial cells (Koyama *et al.*, 1995). A high level of LPS (100 μg/ml) is needed for cell death in this system however, and the mode of death is not established. In the original paper showing that LPS caused bovine endothelial cell injury *in vitro* however, Harlan *et al.* (1983a) tried a number of protease inhibitors to no effect.

A specific inhibitor for ICE (caspase-1) would be interesting to try in the system of LPS-induced CPAE cell apoptosis. As mentioned previously, ICE is a protease which activates pro-IL-1β by cleavage at two sites. When *Shigella* invades macrophages it induces apoptosis and the release of IL-1β (Zychlinsky *et al.*, 1994a). Intracellular *Shigella* releases a virulence factor, IpaB, which binds to ICE, and the addition of specific inhibitors which block the protease activity of ICE abolished both apoptosis and IL-1β release (Chen *et al.*, 1996). LPS treated bovine endothelial cells produce IL-1 (Akarasereenont *et al.*, 1995), therefore ICE is probably activated. Whether ICE or IL-1 is necessary for CPAE cell death could be established using specific ICE inhibitors and antibodies against IL-1. It is interesting to note that IL-1ß signals through the sphingomyelin pathway in EL4 cells (Mathias *et al.*, 1993). It may be that LPS, rather than mimicking ceramide, causes IL-1 production which then in turn activates the sphingomyelin pathway.

Another interesting direction which could be pursued is the possible connection between LPS-induced apoptosis and the cell cycle. The response of CPAE cells to LPS is unsynchronized, with only a small proportion of cells undergoing apoptosis at any time point. This may indicate that there is a link to a specific stage in the cell cycle during which the cells are susceptible to LPS. Many forms of apoptosis appear to be linked to specific steps in the cell cycle (reviewed by Meikrantz and Schlegel, 1995), while others are independent and can occur at any phase (Lindenboim *et al.*, 1995). The link can be detected using flow cytometry to simultaneously determine the stage of the cell cycle and the viability (live, necrotic, or

apoptotic) of cells (Belloc *et al.*, 1994). Alternatively, cells can be arrested at various stages of the cell cycle using specific inhibitors and the incidence of LPS-induced apoptosis determined.

As the mechanisms involved in apoptosis are more fully understood, the question of why bovine endothelial cells are sensitive to LPS while most human cells are resistant may be resolved. It may be due to a difference in the number of triggers necessary to induce apoptosis, some cells being more "primed" for death. For example, while LPS alone does not have a cytotoxic effect on human endothelial cells, when co-incubated with the protein synthesis inhibitor cycloheximide or actinomycin D significant cytotoxicity results (Harlan *et al.*, 1983b). Similar results were seen with porcine endothelial cells, where treatment with LPS followed by the induction of the heat shock response caused cell death (Buchman *et al.*, 1993). The heat shock response was triggered by incubation at 45°C for 90 min, 80 μM sodium arsenite, or 50 μg/ml cycloheximide. Interestingly, when similar experiments were tried with endothelial cells of mouse origin, no cell death was seen (Abello and Buchman, 1994). If human endothelial cells are also sensitive to LPS in combination with heat shock, to date not examined, this would have interesting implications considering conditions found *in vivo* during sepsis and septic shock.

A recent study did find that LPS alone (probably in the presence of serum) could induce apoptosis in human endothelial cells (Haendeler *et al.*, 1996). Using HUVEC and an LPS dose of 1 μg/ml, they found a modest increase in DNA fragmentation (less than three fold) over untreated cells after an extended (18 hour) incubation. To visualize this fragmentation extracted DNA had to be radioactively labeled, and at such low levels of cell death, it is not surprising therefore that I found nothing using the less sensitive MTT assay. Haendeler *et al.* made the interesting discovery that the LPS-induced increase in apoptosis correlated with a reduction of Bcl-2, the cell death inhibitor. In addition, an upregulation of the homologue Bax, which heterodimerizes with Bcl-2 and promotes apoptosis (Oltvai *et al.*, 1993), was also seen. The changes in Bcl-2 and Bax levels and resulting apoptosis could be

blocked by vitamin C and E. Similar studies would be interesting using the more LPS sensitive bovine endothelial cells, assuming the anti-human antibodies used to detect Bax and Bcl-2 by western blot analysis cross react with the bovine homologues, since no changes were detectable in Northern blot analysis.

As its role in septic shock has been revealed, LPS has inevitably acquired a negative reputation. However, there are two counterbalancing points to keep in mind. First, LPS can have beneficial effects. At low doses LPS initiates the series of events leading to a self-limiting inflammatory response, ridding the body of the invading organism. It can also result in a transient state of LPS tolerance, during which a higher and normally dangerous dose of LPS has no effect (Proctor *et al.*, 1986). In addition, during this tolerant stage animals are protected from other forms of assault, such as lethal irradiation (Smith *et al.*, 1966). The second point is that unlike most pathogenic factors, LPS is vital for gram-negative bacterial viability. It has not evolved over time specifically to manipulate host defenses, but rather has been found in its present form long before animals with any form of immune system existed. It is our reaction to LPS which has evolved to its present state and must therefore have some survival advantage despite its excesses (Legrand, 1990). Lewis Thomas in *The Lives of a Cell* wrote "When we sense lipopolysaccharide, we are likely to turn on every defense at our disposal; we will bomb, defoliate, blockade, seal off, and destroy all the tissues in the area.", concluding that "All of this seems unnecessary, panic driven."

Sepsis and septic shock are serious conditions whose incidence are rising and mortality remains high despite modern treatments. A wide variety of approaches for the prevention and treatment of septic shock are being attempted. These include antibodies against LPS and several of the cytokines it induces, such as TNF, as well as soluble receptors for these cytokines. Synthetic and natural lipid A analogues have been discovered which are non-toxic and may block the effects of LPS. Host proteins that interact with LPS such as LBP and the neutrophil granule protein, bactericidal/permeability increasing protein (BPI), are being studied in the hope that they can lead to an effective treatment. Homologous to LBP,

BPI also binds to LPS, but leads to its neutralization (Dentener *et al.*, 1993). Arditi *et al.*, (1994) found that BPI protected bovine brain microvessel endothelial cells from LPS-induced injury. Reconstituted HDL particles, which bind and neutralize LPS, attenuate the effects of LPS in animal models of endotoxemia (Levine *et al.*, 1993; Parker *et al.*, 1995; Hubsch *et al.*, 1995). So far, despite promising early animal work, none of these treatments have succeeded in clinical trials. Part of the problem lies with the complex nature of sepsis, and also with the lack of early detection and delays in the determination of the aetiological agent. Many of the treatments work only when applied before or concurrently with LPS exposure, and are ineffective once the early mediators have been triggered.

The discovery of non-toxic forms of LPS has helped identify elements of LPS necessary for virulence in addition to serving as a possible new therapy. For example, deacylated LPS from *Salmonella typhi* does not activate HUVEC cells (Pohlman *et al.*, 1987), nor does LPS from *Bacteriodes fragilis* (Magnuson *et al.*, 1989). In both cases the non-toxic forms of LPS also inhibited activation by normal LPS, and it has since been shown that inactive LPS analogues bind CD14 (Kitchens *et al.*, 1992). However, a recent study using heat killed gram-negative bacteria found that *B. fragilis* can activate HUVEC cells in a sCD14 dependent fashion (Noel *et al.*, 1995). These studies demonstrate the often overlooked importance of other bacterial cell membrane components, not contained in purified LPS solutions but probably playing a role in *in vivo* interactions with cells. Significant differences are found between the biological activity of serum released and phenol-water extracted LPS (Tesh and Morrison, 1988).

CD14 has not been a focus for exploitation as a target for therapy, although a recent study found that antibodies against CD14 have a protective effect in LPS treated cynomolgus monkeys (Leturcq *et al.*, 1996). The blockage of the CD14 pathway might have a therapeutic application extending beyond just gram negative septic shock. Recently, the list of ligands which bind to CD14 has grown. An unidentified component of gram-positive *Staphylococcus aureus* stimulates neutrophils and monocytes following binding to CD14 (Kusunoki *et al.*,

1995). Anti-CD14 antibodies block the reaction of cells to lipoarabinomannan from *Mycobacterium tuberculosis* (Zhang *et al.*, 1993), manuronic acid polymers from *Pseudomonas* (Espevik *et al.*, 1993), soluble peptidoglycan fragments from *S. aureus* (Weidemann *et al.*, 1994), chitosans from arthropods (Otterlei *et al.*, 1994), rhamnose-glucose polymers from *Streptococcus mutans* (Soell *et al.*, 1995), and insoluble cell walls from several gram-positive species (Pugin *et al.*, 1994). This involvement in the response to a broad range of pathogens, has led to the suggestion that CD14 may act as a "pattern recognition" receptor recognizing bacterial envelope components (Pugin *et al.*, 1994).

The exact role of sCD14 and the best way to exploit it for therapy also remains controversial. High levels of recombinant sCD14 protect mice from LPS (Goyert and Haziot, 1995), while in humans high levels correlate with poor prognosis in sepsis patients (Landmann *et al.*, 1995). Soluble CD14 is capable of activating macrophages lacking membrane CD14 (Schutt *et al.*, 1995), and may have a role in the activation of normal neutrophils and macrophages (Hailman *et al.*, 1996), while at the same time the addition of recombinant sCD14 blocks the production of TNF by macrophages (Haziot *et al.*, 1994). Landmann *et al.*, (1996) hypothesize that sCD14 may act as an initial trigger when LPS concentrations are high, or alternatively become important later when myeloid cells are adapted to LPS and downregulate their response. It is also possible that sCD14 is necessary to activate cells in the extravascular space in the absence of myeloid cells.

The effects of LPS are widespread and very complex, as recent attempts to find a cure for septic shock has shown. Therapies aimed at one mediator or event have little effect, rather like plugging only one hole in a sieve, while methods aimed against the direct cause itself, LPS, are ineffective, possibly due to the speed with which LPS initiates the immune cascade. It may be true that the main event in sepsis is the activation of myeloid cells and their subsequent production of cytokines such as TNF and IL-1 (Pugin *et al.*, 1993). However, as these events are eventually addressed and controlled or blocked, other effects of LPS may increase in importance. It is therefore essential to understand the interaction of LPS with

other cell types, especially endothelial cells whose preservation is vital for health. In addition, LPS treated CPAE cells are an interesting and useful model of apoptosis.

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IMAGE EVALUATION
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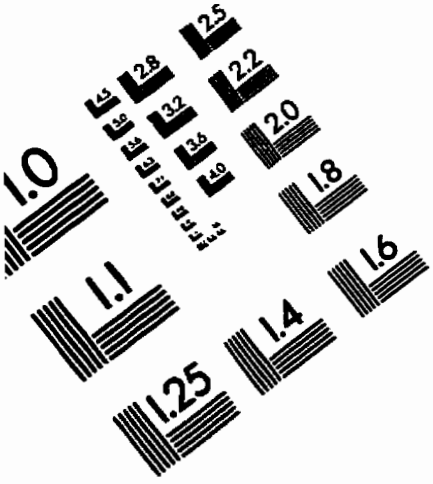

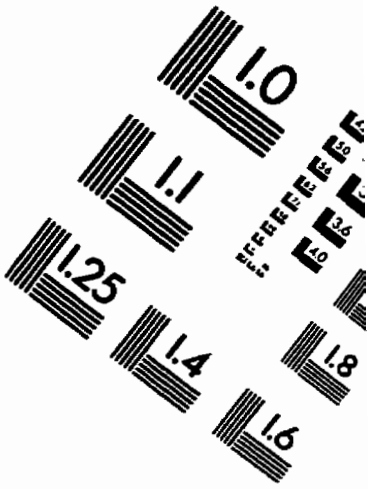

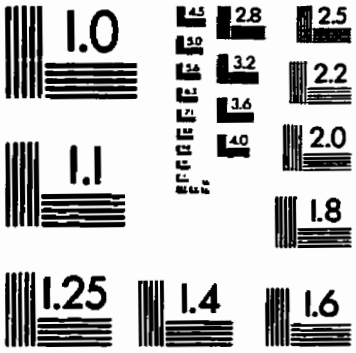

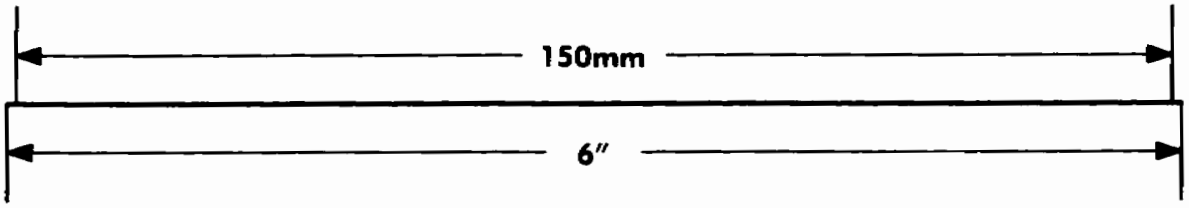

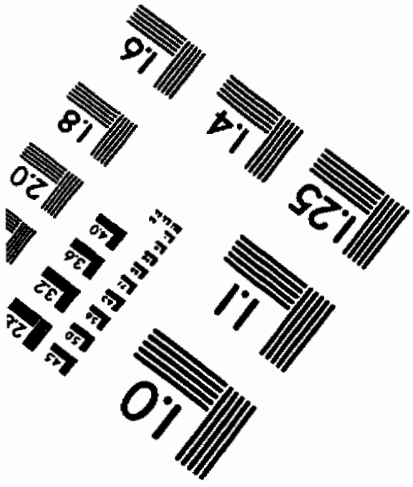

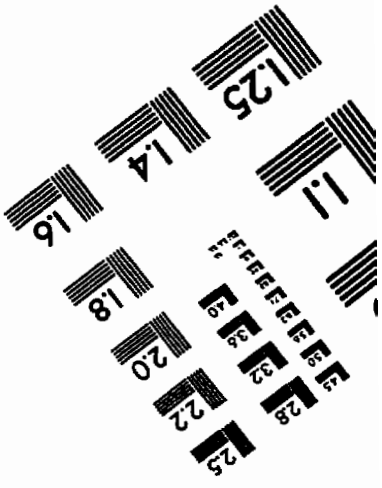

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