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I am submitting herewith a dissertation written by David F. Dean entitled "LPS-Induced Signal Transduction in Bovine Alveolar Macrophages". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Major Professor

We have read this dissertation and
recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

ENDOTOXIN-INDUCED SIGNAL TRANSDUCTION IN BOVINE ALVEOLAR MACROPHAGES

A Dissertation

Presented for the

Doctor of Philosophy Degree

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David F. Dean

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DEDICATION

This dissertation is dedicated to the faculty, staff, and students of the University of Tennessee, College of Veterinary Medicine.

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ABSTRACT

Alveolar macrophages are the major contributors to fibrin deposition in the inflamed bovine lung through enhanced expression of tissue factor (tissue thromboplastin). Bacterial endotoxin (LPS) is known to enhance tissue factor expression in alveolar macrophages but the cellular biochemical pathways by which this occurs are not known. We have investigated the receptor-mediated intracellular events which occur in bovine alveolar macrophages stimulated by LPS using tissue factor expression as the measurable functional endpoint. We examined these events using LPS alone or in combination with a concentrated bovine serum fraction shown to have LPS-enhancing activity. Alveolar macrophages were obtained by volume-controlled bronchopulmonary lavage of healthy Holstein calves. Tissue factor expression was quantitated by the use of a amidolytic assay. Receptor dependency was investigated through the use of a anti-CD14 monoclonal antibody. Inhibition by pertussis toxin was used to investigate the role of G-proteins and the involvement of Protein Kinase C was evaluated using three known inhibitors of this important signalling kinase. Changes in intracellular Ca^{2+} and pH were monitored by using Ca^{2+}- and pH-sensitive dyes with changes in fluorescence intensities measured by spectrophotometry. The role of tyrosine kinase activation was investigated using the known tyrosine kinase inhibitor, Herbimycin A. LPS-induced tyrosine phosphorylation was characterized by antiphosphotyrosine immunoblotting, MAP

kinase co-migration analysis, and performance of a MAP kinase activity assay.

The data obtained provides evidence for both CD14-dependent and -independent signalling pathways in LPS-stimulated macrophages and that while the CD14-independent pathway may involve a pertussis toxin-sensitive G-protein, signalling through a protein tyrosine kinase may be common to both pathways. Tyrosine kinase activation results in the phosphorylation of a solitary 30 kDa protein which may be a novel MAP Kinase. While activation of Protein Kinase C appears to be a critical step in the signalling pathway utilized by LPS resulting in enhanced expression of tissue factor by alveolar macrophages, LPS stimulation failed to result in mobilization of intracellular Ca^{2+} stores or alter cytoplasmic pH.

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INTRODUCTION

SIGNAL TRANSDUCTION

The general question of how extracellular signals modulate cell behavior is central to cell biology today. Macrophages are of particular interest since they play a central role in host defense against both infectious agents and neoplasia. Macrophage function is regulated by extracellular signals which stimulate the execution of various complex functions. Unlike the neutrophil, our understanding of the mechanisms of signal transduction in macrophages is in its infancy. The mechanisms by which macrophages respond to stimuli represent a sequence of events beginning with the cell surface recognition stimulus.

Binding of ligand leads to transmembrane signalling to the cytosolic face of the plasma membrane followed by communication of the signal to the site(s) at which the response is engendered. In the generation of superoxide anion, the response takes place in the plasma membrane and thus transmembrane and intracellular signalling may be synonymous (Babior, 1984). In the induction of changes in the functional phenotype of the macrophage, signal transduction involves the conveyance of information to the nucleus leading to gene activation and gene repression (Riches et al, 1988).

Cell Surface Receptors

All water-soluble signalling molecules, as well as some lipid-soluble ones, bind to specific membrane receptor proteins on the surface of the target cells they influence. These receptor proteins bind the signalling molecule (the ligand) with

high affinity and convert this extracellular event into one or more intracellular signals that alter the behavior of the target cell. Cross talk between receptors occurs both at the level of coupling proteins and intracellular effectors, resulting in fine-tuning of cellular responses (Crystal and West, 1991). Unlike intracellular receptors for steroid and thyroid hormones, cell-surface receptors do not regulate gene expression directly. Instead, they relay a signal across the plasma membrane, and the influence they exert on events in the cytosol or nucleus generally depends on the production of new intracellular signals (Hollenberg, 1991). There are at least three know classes of cell-surface receptor proteins: (a) channel-linked, (b) G-protein-linked, (c) catalytic. Most cell-surface receptor proteins belong to one of these three classes, which are defined by the transduction mechanism used.

Channel-linked receptors are transmitter-gated ion channels involved mainly in rapid synaptic signalling between electrically excitable cells. This type of signalling is mediated by a small number of neurotransmitters that transiently open or close the ion channel to which they bind, briefly changing the ion permeability of the plasma membrane and thereby the excitability of the postsynaptic cell (Barnard, 1992).

Catalytic receptors, when activated by their ligand, operate directly as enzymes. Almost all of the known catalytic receptors are transmembrane proteins with a cytoplasmic domain that functions as a tyrosine-specific protein kinase (Ullrich and Schlessinger, 1990). Among this type are well-characterized receptors

for at least five polypeptide hormones and growth factors, namely, epidermal growth factor, insulin, platelet-derived growth factor (PDGF), and macrophage colony-stimulating growth factor (Crystal and West, 1991).

G-protein-linked receptors indirectly activate or inactivate a separate plasma membrane-bound enzyme or ion channel. The interaction between the receptor and the enzyme or ion channel is mediated by a third protein, called a GTP-binding regulatory protein (G-protein) (Lismaa and Shine, 1992). The G-protein-linked receptors usually activate a chain of events that alters the concentration of one or more small intracellular signalling molecules, referred to as intracellular or second messengers. These second messengers act in turn to alter the behavior of yet other target proteins in the cell (Taylor, 1990). The study of G-proteins is particularly germane to the subject of signal transduction pathways in leukocytes, and these molecules will be discussed in detail in the following section.

G-proteins

G-proteins are a class of heterotrimeric, membrane-associated proteins that couple the activation of receptors to stimulation of effector enzymes and ion channels (Weiss et al, 1988). Even though the primary messengers are many, the number of known receptors coupled to effectors was recently listed as 91 (Hepler and Gilman, 1992). G-protein interaction with receptor is predicted to occur within the cytoplasmic domain of the receptor, although the exact contact site required for binding and activation are presently unknown (Birnbaumer, 1990).

In their inactive state, heterotrimeric G-proteins are a complex of α, β, and γ subunits in which the guanine nucleotide binding site of the α subunit is occupied by GDP. When an extracellular signal in the form of a ligand binds to a receptor, the ligand-receptor complex catalytically acts as a guanine nucleotide release factor (GRF), causing the α subunit to release GDP. The G-protein with an "empty" guanine nucleotide binding site on its α subunit has an increased affinity for the receptor. However, the complex is transient because the high concentration of intracellular GTP drives binding of GTP onto the α subunit. Binding of GTP causes the G-protein to dissociate into α and $\beta\gamma$ subunits. The free GTP-bound α subunit can now activate a downstream effector (Bomsel and Mostov, 1992; Hepler and Gilman, 1992; Spiegel, 1992; Lismaa and Shine, 1992).

Effectors include adenyl cyclase, the cGMP-specific phosphodiesterase of photoreceptor cells, phospholipases of the C and A_2 type, and, as of recently, various classes of ionic channels (Kaziro et al, 1991). As a consequence of ion channel modulation, occupancy of G-protein-coupled receptors by primary messengers leads not only to changes of intracellular second messengers (i.e., cyclic nucleotides, inositol phosphates, diacylglycerol, arachidonic acid, and calcium), but also of the cell's membrane potential, which itself is a potent regulator of cellular function (Birnbaumer, 1990).

In the next step, the G_α subunit will spontaneously hydrolyze its bound GTP to GDP. Consequently, the G_α reassociates with the $\beta\gamma$ subunit, returning the system to the beginning of the cycle (Bomsel and Mostov, 1992).

The α subunits are thought to provide the principal specificity to each type of G-protein. About 20 varieties of α subunits are known, some having well-defined specificities for particular receptors and effectors (Simon et al, 1991). In contrast, there is less heterogeneity in the β and γ subunits and only recently have differences in their functions been investigated (Lefkowitz, 1992). The long-held view is that the role of $\beta\gamma$ is to complex with and inactivate the α subunit. However, there is now considerable evidence indicating that $\beta\gamma$ also acts on effectors (Jelsema and Axelrod, 1987; Logothetis et al, 1987; Ito et al, 1992; Tang and Gilman, 1991; Federman et al, 1992; Blumer and Thorner, 1991; and Pitcher et al, 1992).

Although there are as yet no useful drugs that have G-proteins as a target, many G-proteins are the targets of two relevant bacterial toxins: cholera toxin (CTX) and pertussis toxin (PTX). These toxins are enzymes that covalently modify specific G_α subunits by transferring the ADP-ribose moiety from NAD onto arginine (CTX) or cysteine (PTX) of the G_α subunit (ADP-ribosylation reaction), (Birnbaumer and Brown, 1990). Modification of G_α by CTX constitutively activates these proteins (by inhibiting their GTPase activity), whereas modification by PTX prevents receptor-mediated activation of G-proteins (Hepler and Gilman, 1992). Because of their G-protein specificity, and because of the effect they have on G-protein function, CTX and PTX are powerful tools to investigate possible involvement of a G-protein in a cellular response.

Protein Tyrosine Kinases

As stated previously, one class of cell surface receptors are those which have intrinsic tyrosine kinase activity. Other types of receptors (such as the receptor for IL-2), which have no intrinsic tyrosine kinase activity, bind to and activate intracellular tyrosine kinases when they interact with their ligands (Aderem, 1993). Intracellular tyrosine kinases are often referred to as protein tyrosine kinases (PTK) to distinguish them from receptor tyrosine kinases (RTK). Protein tyrosine kinase activation has recently been associated with binding of ligand or antibody to certain cell-surface proteins that are anchored to the membrane by glycophosphatidylinositol (GPI) (Thomas and Samuelson, 1992). However, it is not known whether the PTKs interact directly with the glycolipid anchor of the GPI-linked antigens (these cell surface molecules lack intracellular domains), or through interaction with other proteins (Stefanova et al, 1991). Whatever the mechanism of linkage between receptor and enzyme, PTK-mediated phosphorylation of "tyr" residues triggers conformational changes in regulated proteins which alters their properties, leading to the physiological responses that are evoked by particular agonists (Aderem, 1993). The diverse actions of different agonists are largely explained by the pleiotropic actions of the protein kinases and phosphatases that they regulate, and by the presence (or absence) in cells of the particular target proteins on which they act (Cohen, 1992).

Inositol Phospholipid Metabolism

It is now clear that membrane phospholipids are not merely structural components of the membrane, but are dynamic participants in lipid-protein interactions essential to cell activation. The breakdown and resynthesis of membrane phospholipids occurs in response to stimulation, and these receptor-mediated events are now known to be important in transmembrane signalling. Thus, the concept of a phosphoinositol cycle has emerged in which receptor-ligand binding initiates a series of changes leading to phospholipid breakdown and the generation of biologically important intermediates followed by eventual resynthesis of the original membrane phospholipids. The turnover of membrane phospholipids is the major transducing event in this important messenger system.

Inositol-containing phospholipids are ubiquitous components of all eukaryotic cells and constitute 2 to 8% of the total phospholipid (Irvine, 1992). Phosphatidylinositol (PI) accounts for over 80% of the total phosphatidylinositols. Agonist-induced turnover of PI can occur both at the plasma membrane and in the endoplasmic reticulum (ER) (Bansal and Majerus, 1990).

A role for inositol phospholipids in signal transduction was first suggested in 1953, when it was found that some extracellular signalling molecules stimulate the incorporation of radioactive phosphate into PI (Hokin and Hokin, 1953). It was later shown that this incorporation results from the breakdown and subsequent resynthesis of inositol phospholipids triggered by a receptor coupled to a G-protein which activated an enzyme called phospholipase C (PLC)(Liscovitch, 1992). The

inositol phospholipids found to be the most important in signal transduction were two phosphorylated derivatives of PI, PI-phosphate (PIP) and PI-bisphosphate (PIP_2), which are thought to be located mainly in the inner leaflet of the plasma membrane. Although PIP_2 is less plentiful in animal cell membranes than PI, it is the hydrolysis of PIP_2 that matters most (Irvine, 1992).

The chain of events linking extracellular signals to intracellular responses via PIP_2 breakdown begins with the binding of the signalling molecule to its receptor in the plasma membrane (Hardin, 1992). More than 25 different receptors have been shown to utilize the inositol-phospholipid transduction pathway (Liscovitch, 1992). An activated receptor in-turn activates a G-protein that then activates the phospholipase C (Berridge, 1993; Hardin, 1992). In less than a second, this enzyme cleaves PIP_2 to generate two products: inositol trisphosphate (IP_3) and diacylglycerol (DAG). At this step the signalling pathway splits into two branches.

IP_3 is a small water-soluble molecule that releases calcium from the "calcium-sequestering compartment" (Putney et al, 1989). The organelle from which IP_3 releases calcium was originally believed to be a component of the endoplasmic reticulum, but more recently it has been suggested that the calcium comes from a novel IP_3-responsive organelle, termed the "calciosome" (Berridge, 1993). The mechanism by which IP_3 releases calcium appears to involve its interaction with a specific membrane receptor (Irvine, 1992). Binding of IP_3 to its receptor increases calcium efflux from the intracellular pool by opening a calcium

channel, which may be closely associated with the receptor (Putney et al, 1989).

While the IP_3 produced by hydrolysis of PIP_2 is increasing the concentration of calcium in the cytosol, the other cleavage product of PIP_2, DAG, is exerting quite different effects (Irvine, 1992). It has two potential signalling roles. It can be further cleaved to release arachidonic acid, which can be used in the synthesis of prostaglandins and related signalling molecules; or, more important, it can activate a specific protein kinase, which can then phosphorylate a number of proteins with different functions in the target cell (Bansal and Majerus, 1990).

The enzyme activated by DAG is called protein kinase C (PKC) because it is calcium dependent. The DAG produced by receptor activation, together with the phospholipid phosphotidylserine in the cytoplasmic half of the plasma membrane, binds to PKC, thereby increasing the affinity of the enzyme for calcium so that PKC becomes active at the usual low concentration of calcium in the cytosol (Berridge, 1987). In many cells, however, it seems likely that PKC is normally activated by the cooperative effect of DAG and an increase in cytosolic calcium brought about by IP_3 (Liscovitch, 1992). An important aspect of the activation process appears to be a physical translocation of the enzyme from the cytosol into the membrane, which might be the role of calcium. This calcium-mediated translocation into the membrane may then serve to "prime" the enzyme to the stimulatory action of DAG (Berridge and Irvine, 1989). When activated by DAG and calcium, PKC transfers the terminal phosphate group from ATP to specific serine and threonine residues on target proteins that vary depending on the cell

(Liscovitch, 1992). The activation of PKC is transient because within seconds, DAG is phosphorylated to form phosphatidate or further cleaved to arachidonic acid.

The hallmark of the inositol lipid signalling system is that there is a bifurcation in the flow of information. The dual signal hypothesis concerns the way in which the IP_3/calcium and DAG/PKC signal pathways cooperate with each other to control a whole host of cellular processes. In many cells the two pathways appear to act synergistically (Berridge, 1987). It is proposed that the IP_3/calcium pathway plays a major and direct role in initiating cellular responses (Berridge, 1993). The DAG/PKC pathway may also contribute directly to the final response, but its predominant role appears to be as a modulator of either the calcium-signalling pathway (homologous interactions) or of other signal pathways (heterologous interactions) (Berridge and Irvine, 1989).

Calcium Signalling

Calcium was the first intracellular regulator to be discovered. Despite the subsequent discovery of several other second messengers including cyclic nucleotides, products of inositol hydrolysis, and products of tyrosine phosphorylation, calcium still appears to be a centrally important activator in almost all cell types (Rink and Merritt, 1990).

The concentration of free calcium in the cytosol of any cell is extremely low, whereas its concentration in the extracellular fluid and in the calciosome is high

(Lytton and Nigam, 1992). Thus there is a large gradient tending to drive calcium into the cytosol across the plasma membrane and the membrane of the calciosome (Missiaen, Taylor, and Berridge, 1991). When a signal transiently opens calcium channels in either of these membranes, calcium rushes into the cytosol, dramatically increasing the local calcium concentration and activating calcium-sensitive response mechanisms in the cell (Rink and Merritt, 1990).

For this signalling mechanism to work, the concentration of calcium in the cytosol must be kept low, and this is achieved in several ways. All eucaryotic cells have a calcium-ATPase in their plasma membrane that uses the energy of ATP hydrolysis to pump calcium out of the cytosol (Alberts, 1989). A calcium pump in the membrane of the calciosome also plays an important part in keeping the cytosolic calcium concentration low (Lytton and Nigam, 1992). Calcium is stored in the lumen of the calciosome loosely bound to a calcium-binding protein called calsequestrin, which has a low affinity but high capacity for calcium (Lytton and Nigam, 1992).

Two pathways of calcium signalling have been defined, one is used mainly by electrically active cells and the other used by almost all eucaryotic cells. The first of these pathways has been particularly well studied in nerve cells, in which depolarization of the plasma membrane causes an influx of calcium into the nerve terminal, initiating the secretion of neurotransmitter; the calcium enters through voltage-gated calcium channels that open when the plasma membrane of the nerve terminal is depolarized by an invading action potential (Nargeot, 1991). In

the second, and more ubiquitous pathway, the binding of extracellular signalling molecules to cell-surface receptors causes the release of calcium from the calciosome; the event at the cell surface are coupled to the opening of calcium channels in the internal membrane through the previously described second messenger, IP_3 (Taylor and Marshall, 1992).

Calmodulin is a protein which has been found in all animal and plant cells that have been examined (Rink and Merritt, 1990). Calmodulin functions as a multipurpose intracellular calcium receptor, mediating most calcium-regulated processes. It has four high-affinity calcium-binding sites; and it undergoes a large conformational change when it binds calcium (Schulman and Lou, 1989). The binding of calcium to calmodulin induces calmodulin to bind to various target proteins in the cell and thereby alter their activity (Lytton and Nigam, 1992).

Among the targets regulated by calcium-calmodulin complexes are many enzymes and membrane transport proteins. Prominent among these are calcium/calmodulin-dependent protein kinases (Ca^{2+}-kinases), which phosphorylate serine and threonine residues on proteins (Schulman and Lou, 1989). Recently, a broad-specificity Ca^{2+}-kinase has been identified; called calcium/calmodulin-regulated "multifunctional" kinase, it may mediate many of the actions of calcium in mammalian cells (Schulman and Lou, 1989).

Protein Kinase C

Protein kinase C (PKC) is an important regulatory enzyme discovered by Nishizuka and colleagues (Takai et al, 1977). The enzyme is ubiquitously distributed, having been found in virtually every tissue assayed (Nishizuka, 1986). Its broad distribution suggests that it plays an important role in the control or modulation of many different biological processes (Nishizuka, 1989). For example, PKC is thought to play a role in the regulation of the endocrine, exocrine, immune, and inflammatory systems (Rando, 1988).

PKC is now known to be a large family of proteins with multiple subspecies that have subtle individual characteristics (Coussens, 1986). Biochemical and immunohistochemical studies suggest that the PKC subspecies may be differently located in particular cell types and with limited intracellular localizations (Asaoka et al, 1992) . Presumably, each member has discrete functions in the processing and modulation of various physiological and pathological responses to external signals (Coussens et al, 1986).

One of the most interesting aspects of the biology of PKC is its mechanism of activation. As expected, the activity of the kinase is strongly regulated. Under quiescent conditions, the enzyme is cytoplasmic and inactive (Rando, 1988). When stimulated, the kinase is translocated to the membrane where it can actively phosphorylate its substrates at selected threonine or serine residues (Rando, 1988). The mechanism of activation of PKC is novel. The DAG binds to the protein, causing it to be translocated to the membrane. Here, in the presence of

a phospholipid such as phosphatidylserine (PS) and low calcium concentrations, the kinase becomes activated. The calcium concentrations required are submicromolar to micromolar, which are in the physiological range (Asoaka et al, 1992). PKC can also be activated in the absence of DAG, but in the presence of PS, when the calcium levels are in the millimolar range. Thus, the specific role of DAG is to increase the affinity of the kinase for calcium (Rando, 1988).

Since the kinase is activated at physiological calcium concentrations, and concentrations of phospholipid do not vary on a time scale relevant to kinase regulation, DAGs are usually considered to be the physiological regulators of the enzyme (Rando, 1988).

Phorbol esters can also directly stimulate PKC and have been used extensively. The dose-response curve for PMA activation of PKC is similar to the saturation curve for PMA binding to the receptor, as are the structure-activity relationships for enzyme activation when compared with receptor binding, suggesting that PKC may act as a phorbol receptor (Burns and Bell, 1991). Furthermore, when the enzyme is maximally stimulated by either PMA or DAG alone, the addition of the other activator does not lead to additional stimulation, indicating that both of these agents acted by a common mechanism (Nishizuka, 1989).

When activated by DAG and calcium, PKC transfers the terminal phosphate group from ATP to specific serine or threonine residues on target proteins that vary depending on the cell (Mochly-Rosen et al, 1991). In some cells the activation of

PKC increases the transcription of specific genes (Alberts, 1989).

Signal Transduction and Control of Gene Transcription

During the past 30 years, most of the activity in the field of signal transduction has concentrated on the identification of hormones and growth factors, receptors for these molecules, second messengers, enzymes that generate second messengers, and the mechanisms that control their activity. Only recently has it been feasible to approach what appears to be the final step in signal transduction, the control of transcription factor activity. An important step in this progression has been the identification of cis-responsive nuclear proteins (transcription factors) that are responsible for modulation of gene expression in response to various extracellular signals (McMahon and Monroe, 1992). With the recent identification and cloning of several transcription factors it has been possible to examine the mechanisms by which signalling pathways control the activity of these transcription regulators (Karin, 1991).

Modulation of transcription factor activity is most commonly achieved by changes in the phosphorylation state of the transcription factor (Herschman, 1989). Phosphorylation affects transcription factor activity at several distinct levels (Karin, 1991). It can modulate their intracellular location by controlling their association with other proteins, have both negative and positive effects on their DNA-binding activity, and modulate the activity of their transcriptional activation domains (Karin and Smeal, 1992). In addition to phosphorylation, protein-protein interactions also

have an important role in mediating crosstalk at the nuclear level between different signalling pathways (Karin and Smeal, 1992; Karin, 1991).

BACTERIAL ENDOTOXIN

Structure and Chemical Nature

Bacterial endotoxin is an intrinsic component of the cell wall of Gram-negative bacteria, is released from both actively growing and dead bacteria, and is toxic for mammals and most vertebrates (Raetz et al, 1991). The name (endotoxin) is derived from its intimate association with the cell wall, in contrast to exotoxins, which are released by bacteria into the extracellular environment (LeGrand, 1990). The biologically active and toxic chemical component of endotoxin is lipopolysaccharide, and the terms endotoxin and bacterial lipopolysaccharide (LPS) are often used synonymously (Freidman et al, 1990). There are numerous varieties of LPS which differ in chemical structure depending on the species of Gram-negative bacteria (Raetz, 1990). Gram-negative bacteria have a trilaminar cell wall composed of an inner layer, middle peptidoglycan layer, and an outer layer. Bacterial LPS is a component of the outer-most portion of the outer layer (Raetz, 1990).

LPS is a macromolecule composed of three distinct regions: a lipid region, (lipid A), and a covalently bound heteropolysaccharide region consisting of a core polysaccharide and a O-specific polysaccharide (Rietschel and Brade, 1992). Lipid A is composed of a glucosamine disaccharide with 1,4 substitutions by phosphate

groups and is responsible for most of the deleterious effects of LPS (Raetz, 1990).

The Biologic and Cellular Effects of LPS

There is great species variation with regard to sensitivity to LPS. The following list of species is in descending order of sensitivity to LPS: humans, cattle, horses, rabbits, dogs, swine, guinea pigs, cats, rats, mice, and nonmamalian species the least sensitive (Morrison and Ryan, 1987).

The biologic activity of LPS include the following: hypotension, blood clotting abnormalities, pyrogenicity, induction of plasminogen activator, increased adhesiveness by both leukocytes and endothelial cells, initial leukopenia followed by leukocytosis, complement activation, bone marrow necrosis, platelet aggregation, induction of tumor necrosis, and the induction of synthesis of tumor necrosis factor (TNF) and interleukin-1 (IL-1) as well as other cytokines and mediators of inflammation (LeGrand, 1990).

Within the last decade it has become increasingly apparent that LPS is not directly responsible for many of the sequelae observed during endotoxemia. LPS-induces the synthesis and secretion of a multitude of potent mediators and cytokines from other cell types and it is these mediators which are actually responsible for many of the LPS-induced cellular and organismal pathology (Rietschel and Brade, 1992). Arguably, the two most important of these, TNF and IL-1, are synthesized by macrophages in response to LPS stimulation. The diversity of the mediators that can be mobilized by endotoxin has made it difficult

to define a unifying hypothesis for the mechanism(s) of action of endotoxin. It has also confounded attempts to provide specific therapy for endotoxin-related disease.

Lipopolysaccharide Binding Protein and CD14

Lipopolysaccharide binding protein (LBP) is an acute phase protein that has recently been characterized in rabbits and humans, and its presence is also indicated in mice, rats, subhuman primates, and pigs (Ulevitch, 1993). LBP isolated from human and rabbit serum is a 60-kDa glycoprotein present in normal serum at <0.5 μg/ml and rising up to 50 μg/ml after an acute-phase response (within 24 hours) (Schumann et al, 1990).

Recent data suggest two functions for LBP, opsonization of LPS-bearing particles and increasing the sensitivity of leukocytes to LPS. LBP serves as an opsonin by binding stably to Gram-negative bacteria or LPS-coated erythrocytes. The resulting LBP-coated particle can in turn be avidly bound by monocytes, macrophages, and neutrophils (Wright et al, 1989). LBP appears to bridge LPS-coated particles to macrophages by binding first to the LPS and then to macrophages. Pretreatment of LPS-coated erythrocytes with LBP enables binding to macrophages, but pretreatment of macrophages has no effect (Schumann et al, 1990). Moreover, macrophages do not recognize erythrocytes coated with LBP unless LPS is also added, thus suggesting that interaction of LBP with LPS results in a conformational change in LBP that allows recognition by macrophages (Raetz, C. et al, 1991).

Recognition of the presence of LPS is important for an efficient response to infection with Gram-negative bacteria. Thus, a principle function of LBP may be to enhance the ability of the host to detect LPS early in infection; release of cellular products would then enhance natural resistance mechanisms that combat infection (Schumann et al, 1990).

A receptor for LPS-LBP complexes was identified by allowing macrophages to spread on substrates coated with monoclonal antibodies directed against a variety of leukocyte antigens (Wright et al, 1990). Surface-bound antibodies against CD14, but not antibodies against other antigens, down-modulated the binding of erythrocytes coated with LPS-LBP complexes to macrophages. Addition of soluble antibodies directed against certain epitopes of CD14 also blocked binding of coated erythrocytes (Raetz et al, 1991).

CD14 is a 55-kDa glycoprotein originally described as a differentiation antigen of monocytes and macrophages. It has been shown to associate with membranes through a phosphoinositol-glycan anchor and expresses no cytoplasmic domain (Wright et al, 1990). Given its structure, a critical question is how ligation of CD14 by LPS-LBP complexes signals cells to produce cytokines or tissue factor. It is likely that additional proteins (or additional subunits of a receptor complex) are required for signal transduction (Ulevitch, 1993). An alternative model states that CD14 serves principally in the accumulation of LPS in or near the membrane and that signalling is caused by different molecules (Morrison, 1993). Moreover, LPS affects many different cell types and initiates a

broad spectrum of cellular responses with distinct time courses, further suggesting the existence of multiple receptors (Ziegler-Heitbrock and Ulevitch, 1993). In any case, the mechanism by which CD14 and LBP act represents an unanticipated departure from the usual mechanisms by which eukaryotic cells respond to other agonists, such as hormones or lymphokines. The binding of agonist (LPS) is mediated by a soluble protein rather than a membrane-bound protein, and only after the formation of a soluble complex does the LPS interact with a membrane-bound receptor (Raetz et al, 1991).

MACROPHAGES IN LUNG BIOLOGY AND DISEASE

Biology of Alveolar Macrophages

The alveolar macrophage (AM) is the pulmonary representative of the mononuclear phagocyte system. AMs are the most abundant nonparenchymal cell in the lung, dominating lymphocytes by a ratio of approximately 5-10 to 1 (Crystal, 1991). Importantly, AMs are mobile and thus can move to regions where they are needed (Holian and Scheule, 1990). AMs play a central role in maintaining normal lung structure and function through their capacity to scavenge particulates, remove macromolecular debris, kill microorganisms, function as an accessory cell in immune responses, recruit and activate other inflammatory cells, maintain and repair lung parenchyma, provide surveillance against neoplasms, and modulate normal lung physiology (Crystal, 1991).

To subserve its role in the lung, the functional capacity of the AM is broad.

Some of these processes occur within the cell (e.g., killing of microorganisms) and others occur in the environs surrounding the cell (e.g., presentation of antigens to T-lymphocytes). Many of these functions are turned off or operate at a low level in the resting state, but are up-regulated with activation (Brain, 1992). The AM is able to provide these functions by virtue of its mobility, ability to recognize external signals, capacity to phagocytize, and broad range of secretory products (Crystal, 1991).

Most studies aimed at deciphering the intracellular consequences of surface activation of mononuclear phagocytes have not been carried out on AM, particularly bovine AM. All available evidence suggests that the binding of ligands to specific cell-surface receptors results in a cascade of intracellular events through different paths, depending on the specific receptor. As in other mononuclear phagocytes and in neutrophils, the consequences of membrane receptor triggering of AM likely involves the various components of signal transduction described in the preceding section.

Alveolar Macrophage Procoagulant Activity

The lung is a major participant in hemostatic events, and the deposition of fibrin has been reported to be a significant early event in the pathogenesis of a wide variety of infectious and inflammatory lung diseases (Slauson, 1982).

Conversion of fibrinogen to fibrin in inflammatory reactions could be initiated in several ways that might involve either the intrinsic or extrinsic coagulation

pathways. Tissue factor, thromboplastin, or tissue thromboplastin III, is found in tissue rather that blood and is the major initiator of the extrinsic system. Tissue factor is an integral membrane glycoprotein. Most cell types studied in culture have manifested tissue factor activity as demonstrated by one- or two-stage clotting assays. Cells apparently fall into three categories with respect to tissue factor synthesis: some are constitutive produces of tissue factor (e.g., placental trophoblasts), some are nonproducers (e.g., lymphocytes, granulocytes, and platelets), and finally, some are inducible like monocytes, macrophages, and at least certain kinds of endothelial cells (Lyberg, 1984).

The extrinsic system begins with tissue factor existing in a sort of protected state within the plasma membrane. Upon cell injury (endothelial cells) or membrane perturbation (macrophages) it can be released into the circulation to form a complex with factor VII (proconvertin) in the presence of divalent calcium. The activity of the complex of tissue factor and proconvertin seems to be largely dependent on the concentration of tissue factor, although the subsequent enzymatic activity generated and the proteolytic activation of factor X via this extrinsic pathway resides in the activated proconvertin (VIIa) molecule. It is clear however, that proconvertin (VII) and its activated form (VIIa) have an obligate requirement for tissue factor in order to interact with their substrate proteins.

Mediation of fibrin deposition by macrophages has been shown to be important in a variety of situations including delayed-type hypersensitivity responses (Geczy and Meyer, 1982; Geczy et al, 1983; Ryan et al, 1987) the adult

respiratory distress syndrome (ARDS) (McGee and Rothberger, 1985; Idell et al, 1987), idiopathic pulmonary fibrosis and pulmonary sarcoidosis (Chapman et al, 1985), equine chronic obstructive pulmonary disease (COPD) (Grunig et al, 1988), lung bleomycin toxicity (Idell and Gonzalez et al, 1987), phorbol ester-induced acute pulmonary inflammation (Sitrin et al, 1987), BCG-induced granulomatous pneumonitis (Rothberger et al, 1984), oleic acid-induced lung injury in sheep (Idell et al, 1988), experimental alveolar damage in baboons (Idell et al, 1989), and hyperoxic pulmonary injury (Tipping et al, 1988). Extensive lung fibrin deposition may lead to alterations in pulmonary function (Basset et al, 1986; Slauson et al, 1976; Slocombe et al, 1984), retard the resolution of acute inflammation (Slauson et al, 1976; Rinaldo and Rogers, 1982), and promote the development of pulmonary fibrosis by providing a rich matrix-scaffolding for fibroblast and capillary ingrowth (Slauson, 1982; Rinaldo and Rogers, 1982; Spencer, 1975; Goldstein and Fine, 1986; Basset et al, 1986).

While bacterial endotoxin likely represents the major stimulus for macrophage procoagulant activity (PCA) at the site of infection, there are a myriad of other inflammatory mediators which may either directly stimulate PCA or modulate its induction (Kucey et al, 1991). Common to all stimuli is that they in one way or another react with the macrophage plasma membrane. This membrane perturbation can be accomplished by stimulant/cell membrane contact involving either specific receptors or more nonspecific interactions. Tissue factor production is a relatively early response of the macrophage to such stimuli, but is,

of course, far from the only result of such membrane perturbation (Lyberg, 1984).

The regulation of bovine alveolar macrophage PCA has been studied because of the intensely fibrinous nature of bovine acute lung injury due to *Pasteurella haemolytica*. Additionally, the abundance of potential agonists of alveolar macrophage PCA suggests a central role for bovine pulmonary alveolar macrophages in mediating lung fibrin deposition (Car et al, 1991). While LPS has been shown to induce bovine alveolar macrophage PCA in a dose-dependent manner (Car et al, 1988; Car et al, 1990), the cellular biochemical pathways by which LPS exerts this effect have not been well defined.

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PART 2

DETERMINATION OF THE EXPERIMENTAL PARAMETERS TO BE UTILIZED IN THE INVESTIGATION OF THE EFFECTS OF LPS ON TISSUE FACTOR EXPRESSION BY BOVINE ALVEOLAR MACROPHAGES

ABSTRACT

The data presented represents the results of preliminary experiments required prior to undertaking the planned dissertation research. In addition to the development and practice of experimental techniques, parameters such as optimal cell number per well (5×10^4), dosage range of LPS (0.01, 0.1, 1.0, and 10 µg/ml), length of incubation with LPS (4 h), and the identification of the most sensitive chromogenic substrate (S-2765) were established.

INTRODUCTION

Prior to undertaking a series of experiments aimed at dissecting the transmembrane and intracellular signalling pathways utilized by bacterial lipopolysaccharide (LPS) in bovine alveolar macrophage procoagulant induction, it was necessary to establish a set of experimental parameters such as cell number, LPS dosage range, length of incubation with LPS, and the relative sensitivity of two available chromogenic substrates, which would be utilized throughout the course of the study. Additional objectives of the preliminary experiments were to verify LPS dose-depended induction of tissue factor expression by alveolar macrophages and acquire expertise in the techniques of bronchopulmonary lavage, cell isolation/preparation, and in the performance of the amidoytic tissue factor assay.

MATERIALS AND METHODS

Bronchopulmonary Lavage

Pulmonary alveolar macrophages were harvested from 1 to 4 month-old, male Holstein-Frisian calves by volume-controlled bronchopulmonary lavage. Following the induction of anesthesia by intravenous Xylazine (0.11 mg/kg), the animals were intubated and anesthesia was maintained with a isoflurane/oxygen mixture delivered by an anesthetic machine. The calves were maintained in lateral recumbency. Apnea was induced by manual hyperventilation with pure oxygen. The anesthesia machine was disconnected and the lavage tubing connected to the endotracheal tube. The other end of the tubing was connected to a bottle containing a volume of sterile Hank's Balanced Salt Solution (HBSS-) equivalent to the functional reserve capacity (FRC) of the lung lobe to be lavaged. The FRC for the right lung of the calf to be lavaged was determined by using the following formula:

FRC (right lung) = body weight (kg) X 40 ml X 0.58

The volume of fluid was infused from a height of 30 to 40 cm above the level of the dependent lung. Following instillation of the fluid, the tubing was clamped, the drain tube immediately opened, and the lavage fluid removed from the lung by gravity-dependent drainage into a sterile 1 liter cold Erlenmeyer flask. Following performance of the lavage, calves were manually ventilated for several minutes with oxygen and then allowed to breath room air. Xylazine sedation was reversed by the intravenous administration of Yohimbine (0.15 mg/kg). The calves were

maintained in sternal recumbency until completely recovered. The majority of animals were ambulatory within 15 min. following completion of the procedure. The animals serving as a source of cells received the highest level of care and were housed in a P-2 facility approved by the USDA and AAALAC; the protocol for collection of cells was approved by the Animal Care and Concerns Committee of this institution.

Isolation of Alveolar Macrophages

The recovered bronchoalveolar lavage fluid was filtered through sterile gauze and centrifuged in 50 ml polypropylene tubes (200g for 10 min. at 4 °C). The pelleted cells were gently resuspended in cold HBSS-, and washed twice. Cells were resuspended to the desired final concentration in Delbecco's Minimum Essential Media (DMEM), utilized within 3 hours of collection, and kept on ice until assay. Cells were stained from cytospin preparations by Wright-Giemsa to determine proportions of leukocyte populations present. The total number of cells collected was determined by a hemocytometer. Counts of viable cells were obtained using the exclusion of trypan blue. Lung lavage cells were routinely 90 to 100% alveolar macrophages and greater than 95% viable.

Cell Treatment

Cells were suspended in 100 µl of DMEM and placed into the wells of a 96-well flat bottom tissue culture plate and incubated for 60 min. at 37°C in a humidified atmosphere at 5% CO_2 in air. Nonadherent cells were removed by

washing twice with 200 µl of warmed HBSS-.

Determination of Length of Incubation with LPS and Testing of the Relative Sensitivity of Two Chromogenic Substrates: Wells containing one of four different cell number (25, 50, 100, or 125 X 10^3) were incubated with LPS (1 µg/ml) for 1, 2, 3, 4, and 6 hr.

Determination of Cell Number and LPS Dosage: Wells containing one of five different cell number (6.25, 12.5, 25, 50, or 100 X 10^3) were incubated for 4 hours either in the absence of LPS or with 0.0001, 0.001, 0.01, 0.1, 1.0, or 10.0 µg/ml LPS.

All background values were subtracted before data analysis. None of the dosage levels used affected cellular viability as assessed by trypan blue exclusion following the last incubation period. Each treatment variable was done in triplicate and the experiments were repeated to confirm results. The results are expressed as means +/- SEM (standard error of the mean) of triplicate determinations. Analysis of variance was the method used to statistically analyze the data.

Measurement of Tissue Factor Expression

The expression of tissue factor by the cells was quantitated with a colorimetric assay based on digestion of one of two chromogenic substrates (S-2222 or S-2765) by factor X activated by tissue factor produced by alveolar macrophages. Following incubation with LPS, the cells were again washed with HBSS- and incubated for 1 h with 100 µl of a solution containing factors VII and

X and either S-2222 or S-2765. At the end of incubation, the plates were rapidly transferred into a Bio-Tek microplate reader and the optical density at 405 nm wavelength was quantitated with the degree of chromogenicity being directly proportional to the amount of tissue factor expressed. When log fold dilutions of rabbit brain thromboplastin were measured in both the prothrombin time assay (PTA) and the chromogenic tissue factor assay, the chromogenic assay produced a dose response curve markedly similar to that produced by the more conventional PTA.

Materials

Hank's Balanced Salt Solution was purchased from GIBCO Laboratories, Grand Island, NY, and DMEM was from Whittaker Bioproducts, Walkersville, MD. The bacterial endotoxin was derived from *Escherichia coli* (serotype 055:B5) and was obtained from Sigma Chemical Co., St. Louis, MO and contained 11,000 endotoxin units per microgram as determined with a chromogenic *Limulus* assay (QCL-1000, Whittaker Bioproducts, Inc., Walkersville, MD). The chromogenic substrates, N-α-Benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroanilide-dihydrochloride (S-2765) and N-αBenzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanaline hydrochloride (S-2222) were purchased from Kabi Pharmaceuticals, Franklin, OH. The coagulation factor concentrate, Proplex-T, was from Travenol Labs, Inc., Glendale, CA.

RESULTS AND CONCLUSIONS

Determination of the Length of Incubation with LPS and Testing of the Relative Sensitivity of Two Chromogenic Substrates

As demonstrated in **Figure 1**, maximum tissue factor expression occurred when the cells were incubated with LPS for 4 h. Also apparent in **Figure 1,** is that the use of the chromogenic substrate S-2765 consistently resulted in the measurement of a greater amount of optical density than did the substrate S-2222.

Determination of Cell Number and LPS Dosage

Figure 2 illustrates data which verifies LPS dose-dependent induction of tissue factor expression by alveolar macrophages. In addition, dosages of LPS less than 0.01 µg/ml were found to have a inconsistent stimulatory effect while a dose of 10 µg/ml consistently resulted in maximal stimulation of the cells. LPS at concentrations of 0.01, 0.1, 1.0, and 10.0 µg/ml provided a wide range of responses with tissue factor expression as the measurable functional endpoint.

As expected, the total quantity of tissue factor expressed was directly proportional to the number of cells in each well. Wells containing 5×10^4 cells produced a consistent incremental increase in the amount of tissue factor measured when stimulated with 0.01 to 10.0 µg/ml LPS. This cell number/well would be best suited for demonstrating either the stimulatory or inhibitory effect on tissue factor expression by experimental modulation of a particular component of the signal transduction pathway.

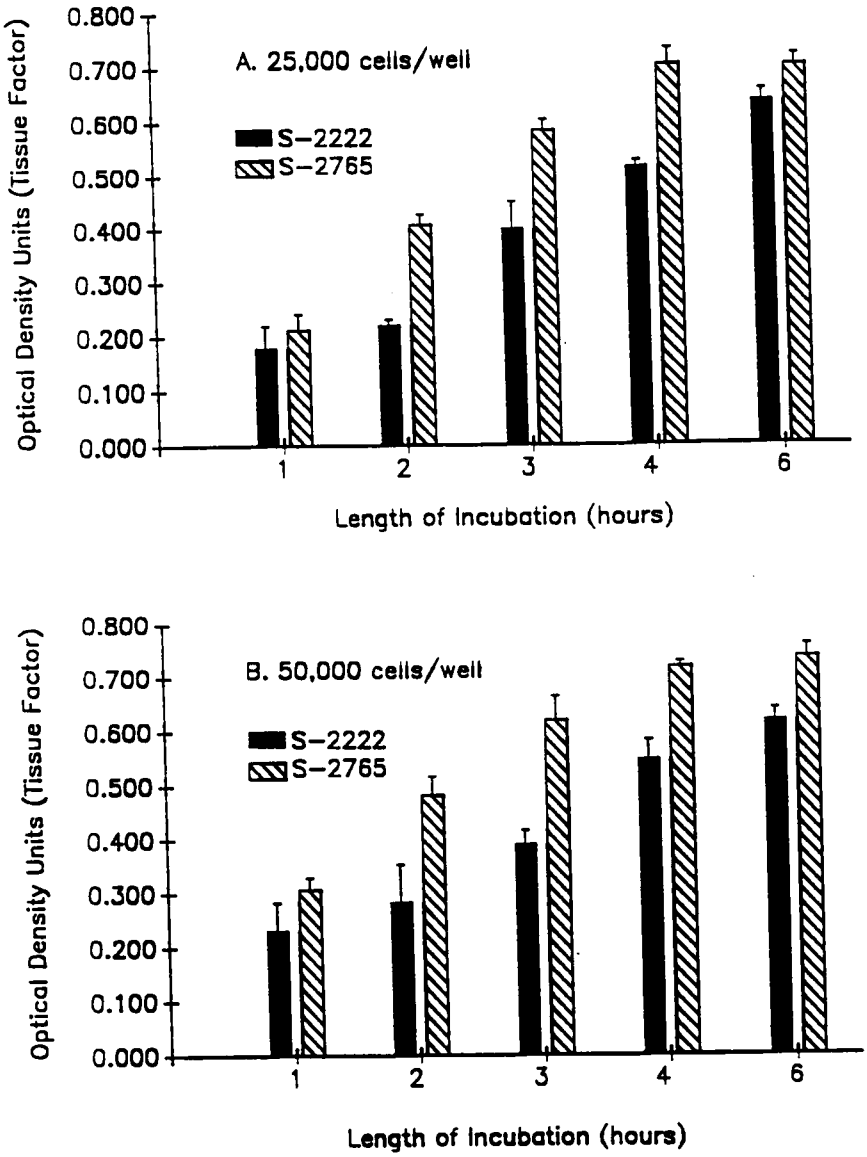
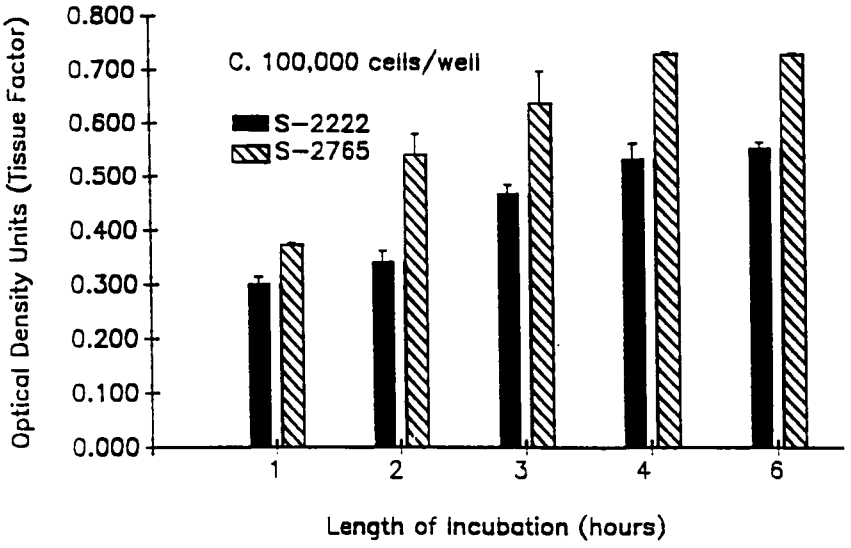


FIGURE 1 Determination of Length of Incubation with LPS and Testing of the Relative Sensitivity of Two Chromogenic Substrates. Wells containing either 25 (A), 50 (B), 100 (C), or 125 (D) X 10^3 cells were incubated with LPS (1 µg/ml) for 1, 2, 3, 4, and 6 hr. The results are expressed as means +/- SEM of triplicate determinations.


C. 100,000 cells/well
S-2222
S-2765
Optical Density Units (Tissue Factor)
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
1
2
3
4
6
Length of Incubation (hours)

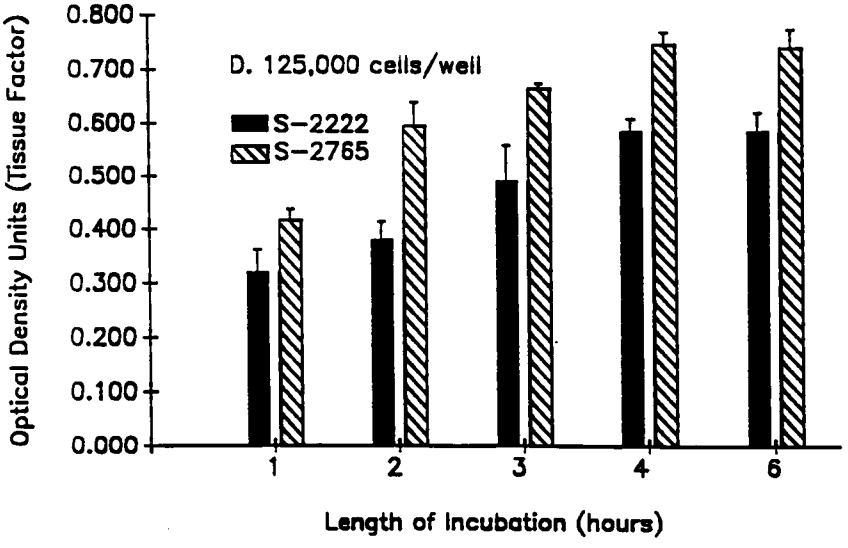

D. 125,000 cells/well
S-2222
S-2765
Optical Density Units (Tissue Factor)
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
1
2
3
4
6
Length of Incubation (hours)

FIGURE 1

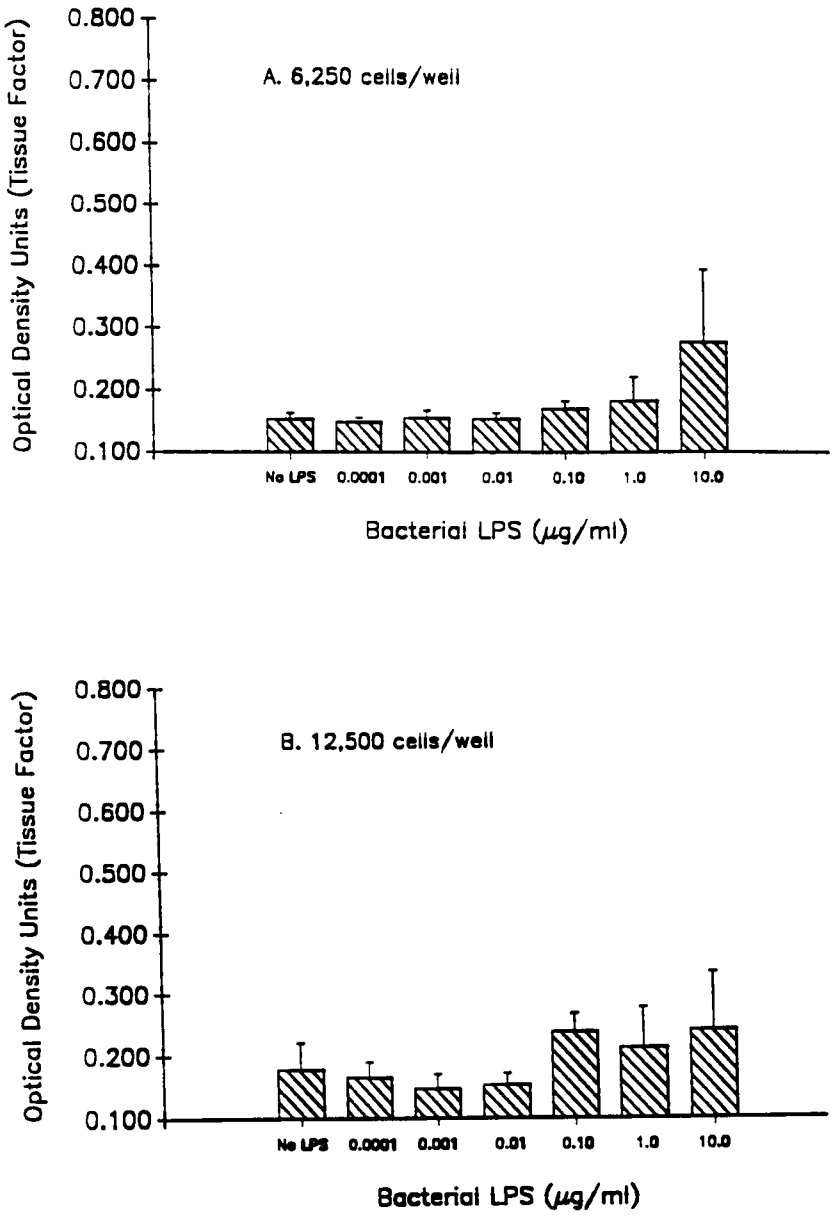


FIGURE 2 Determination of Cell Number and LPS Dosage. Wells containing either 6.25 (A), 12.5 (B), 25 (C), 50 (D), or 100 (E) X 10^3 cells were incubated for 4 h either in the absence of LPS or with 0.0001, 0.001, 0.01, 0.1, 1.0, or 10.0 μg/ml LPS. The results are expressed as means +/- SEM of triplicate samples.

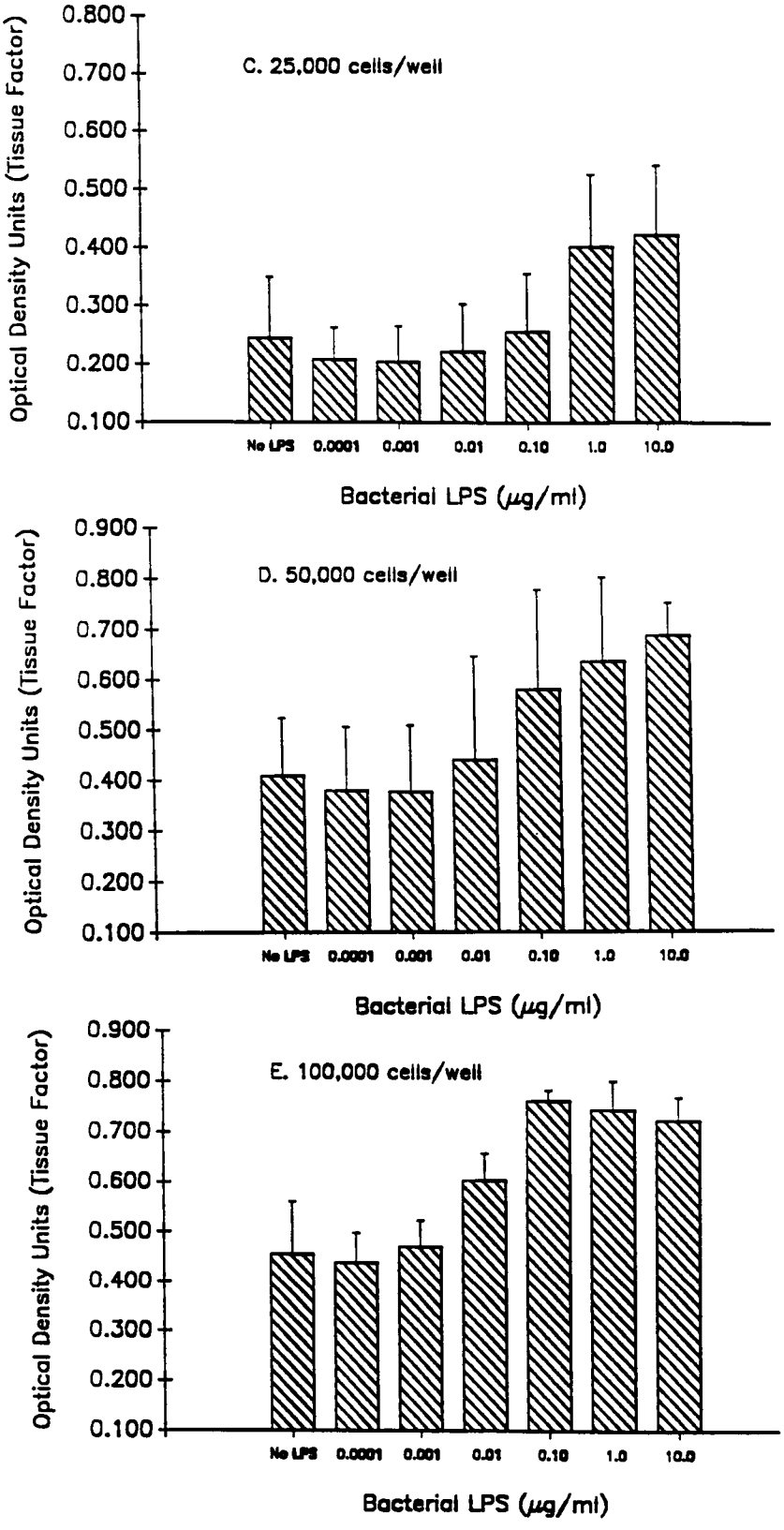

C. 25,000 cells/well
Optical Density Units (Tissue Factor)
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
No LPS
0.0001
0.001
0.01
0.10
1.0
10.0
Bacterial LPS (μg/ml)
D. 50,000 cells/well
Optical Density Units (Tissue Factor)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
No LPS
0.0001
0.001
0.01
0.10
1.0
10.0
Bacterial LPS (μg/ml)
E. 100,000 cells/well
Optical Density Units (Tissue Factor)
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
No LPS
0.0001
0.001
0.01
0.10
1.0
10.0
Bacterial LPS (μg/ml)

FIGURE 2

PART 3

ENDOTOXIN-INDUCED SIGNAL TRANSDUCTION IN BOVINE ALVEOLAR MACROPHAGES

ABSTRACT

Alveolar macrophages are the major contributors to fibrin deposition in the inflamed bovine lung through enhanced expression of tissue factor (tissue thromboplastin). Bacterial endotoxin (LPS) is known to enhance tissue factor expression in alveolar macrophages but the cellular biochemical pathways by which this occurs are not known. We have investigated the receptor-mediated intracellular events which occur in bovine alveolar macrophages stimulated by LPS using tissue factor (TF) expression as the measurable functional endpoint. We examined these events using LPS alone or in combination with a concentrated bovine serum fraction shown to have LPS-enhancing activity. Alveolar macrophages were obtained by volume-controlled bronchopulmonary lavage of healthy Holstein calves. Tissue factor expression was quantitated by the use of an amidolytic assay. Pretreatment of the macrophages with the anti-CD14 monoclonal antibody 60bd (20 μg/ml) resulted in inhibition of TF expression following stimulation 0.25, 0.5, or 1.0 ng/ml LPS combined with serum fraction 2 (500 ng/ml). Pretreatment with 60bd had no inhibitory effect when the cells were stimulated with LPS alone. Pretreatment of macrophages with pertussis toxin (PTX)(300 ng/ml) failed to have an inhibitory effect when the cells were stimulated with LPS combined with serum fraction 2, but resulted in a significant reduction in TF expression when the cells were stimulated with LPS (10 μg/ml) alone. Preincubation of the cells with the protein tyrosine kinase inhibitor, herbimycin A, resulted in profound inhibition of TF expression whether LPS was used in

combination with fraction 2, or alone. LPS-induced tyrosine phosphorylation was investigated further using western immunoblotting analysis. LPS was found to enhance tyrosine phosphorylation of a solitary 30 kDa polypeptide and the kinetics of phosphorylation varied depending upon whether the cells were stimulated with LPS alone (10 μg/ml) or with LPS (1ng/ml) in combination with serum fraction 2 (250 ng/ml). Maximum tyrosine phosphorylation occurred in 10 min when the cells were stimulated by LPS alone and occurred in 20 min when the cells were stimulated by LPS in combination with serum fraction 2. Results of Mitogen Activated Protein (MAP) kinase co-migration analysis and assay of MAP kinase activity provide circumstantial evidence that the 30 kDa polypeptide demonstrating enhanced tyrosine phosphorylation is a novel MAP kinase. The potential involvement of protein kinase C (PKC) was evaluated using 3 known inhibitors of this important signalling kinase: staurosporin (50 nM), chelerythrine (10 μM), and H-7 (100 μM). All three PKC inhibitors produced a marked reduction in the expression of TF by alveolar macrophages stimulated with either LPS or the known PKC agonist, PMA. Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and pH (pH_i) were monitored by using Ca^{2+}- and pH-sensitive fluorescent dyes with changes in fluorescence intensities measured by spectrophotometry. Stimulation by LPS failed to result in mobilization of intracellular Ca^{2+} stores or alter cytoplasmic pH. These data provide evidence for both CD14-dependent and -independent signalling pathways in LPS-stimulated macrophages and while the CD14-independent pathway may involve a PTX-sensitive G-protein, signalling through a herbimycin-

sensitive protein tyrosine kinase may be common to both pathways. The differential time-course of LPS-induced tyrosine phosphorylation between cells stimulated LPS alone or LPS in combination with serum fraction 2 further supports the notion that transmembrane signalling via CD14 requires interaction with additional associated proteins. The results of our experiments using 3 known inhibitors of PKC confirm previous reports implicating this enzyme in the pathway utilized by LPS resulting in enhanced expression of TF by bovine alveolar macrophages. In our examination of the effect of LPS on the levels of cytosolic calcium and pH, we found that stimulation of alveolar macrophages with high doses of LPS alone or low doses in combination with serum fraction 2, failed to effect either $[Ca^{2+}]_i$ or pH_i. (Supported by USDA Grant 90-37265 and the University of Tennessee Centers of Excellence).

INTRODUCTION

Monocytes and macrophages are important mediators of fibrin deposition (Geczy et al., 1983; Helin, 1986) and removal (Gerton et al., 1987) at both intravascular and extravascular sites and, together with extravasated humoral factors, combine to dictate the procoagulant or profibrinolytic extravascular milieu (Saksela et al, 1985; Ryan and Geczy, 1987; Chapman et al., 1988). Lung procoagulant activity present in alveolar fluid, largely tissue factor (coagulation factor III, tissue thromboplastin) from pulmonary alveolar macrophages (McGee, 1985) contributes to a microenvironment favoring deposition rather than removal of fibrin. Enhanced

procoagulant activity from alveolar macrophages has been shown to be important in a variety of acute and chronic inflammatory diseases occurring in both animals and man (Geczy, 1983; Ryan and Geczy, 1987; McGee and Rothberger, 1985; Geczy and Meyer, 1982; Idell et al., 1987; Chapman et al., 1985; Grunig et al., 1988; Idell et al., 1987; Sitrin et al., 1987; Rothberger et al., 1984; Idell et al., 1988; Idell et al., 1989; Tipping et al., 1988). Extensive lung fibrin deposition may lead to alterations in pulmonary function (Basset el al., 1986; Slauson et al., 1976; Slocombe et al., 1984), retard the resolution of acute inflammation (Slauson et al., 1976; Slocombe et al., 1984), and promote the development of pulmonary fibrosis by providing a rich matrix-scaffolding for fibroblast and capillary ingrowth (Slauson, 1982; Rinaldo and Rogers, 1982; Spencer, 1975; Goldstein and Fine, 1986; Basset et al., 1986). The expression of macrophage procoagulant activity has been shown to result largely from surface bound and secreted tissue factor (Goldstein and Fine, 1986) as well as other factors (Idell et al., 1988; Pitlick, 1975; Conway et al., 1989; Moon and Geczy, 1988).

Bacterial endotoxin or lipopolysaccharide (LPS) is an intrinsic component of the cell wall of Gram-negative bacteria and is released from dead to actively growing bacteria (Osborn et al., 1974; Galanos et al., 1977). The molecular structure and effects of LPS have been reviewed (Morrison and Ryan, 1987; Bradley, 1979). LPS has been shown to induce procoagulant activity in human monocytes (Rivers et al., 1984) and LPS from both *E. coli* 0111:B4 and *Pasteurella haemolytica* induces bovine alveolar macrophage procoagulant activity

in a dose-dependent manner (Car et al., 1988, 1990, 1991).

Almost all known macrophage activities in host defense involve responses that occur following interaction of a signal (ligand) with cell membrane receptors (Riches, et al., 1988). The best recent data from human and murine systems suggest that LPS may bind to multiple receptors, and that it may often be complexed in plasma with a lipopolysaccharide binding protein (LBP) (Tobias et al., 1992; Schumann et al., 1990) which subsequently permits the LPS:LBP complex to utilize a specific monocyte differentiation antigen and membrane receptor called CD14 (Schumann et al., 1992; Wright et al., 1990). Recent research has established the presence of and an important role for bovine serum factors with LPS-enhancing activity (Khemlani et al., 1992; Khemlani et al., 1993; Yang et al., 1993), and our signalling studies have utilized a concentrated bovine serum fraction which enhances a variety of LPS-induced effects similar to those observed in human and rabbit alveolar macrophages stimulated with LPS-LBP complexes (Martin et al., 1992).

The specifics of the transmembrane signalling pathways utilized by LPS resulting in increased expression of tissue factor by alveolar macrophages are largely unknown. Receptor occupancy in leukocytes is often, but not always, linked to the activation of certain guanine neucleotide regulatory proteins (G proteins) which functionally couple a wide array of receptors to biochemical effector systems that regulate various second messengers (Gilman, 1984; Lefkowitz and Carob, 1988; Stryer and Bourne, 1986; Hohen and Houslay, 1985;

Gilman, 1987; Neer and Clapham, 1988). Both stimulatory (G_s) and inhibitory (G_i) molecules play regulatory roles in cell activation (Stryer and Bourne, 1986; Cohen and Houslay, 1985; Gilman, 1987; Neer and Clapham, 1988). G-proteins can often be manipulated by pertussis toxin (PTX), which ADP-ribosylates and inactivates G_i (Ui et al., 1984). While little work on such systems has been reported for domestic animals, a pertussis toxin-sensitive G-protein resembling G_i and tentatively called G_n has been identified in bovine leukocytes (Gierschik et al., 1987). One target for the activated G-proteins in macrophages seems to be phospholipase C which, after activation, hydrolyses membrane phosphoinositols to generate two different intracellular signalling molecules, inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Hokin, 1985; Nomura et al., 1986; Berridge, 1985; Berridge, 1987). These important "second messengers" subsequently activate two independent but synergistic pathways which lead to cell effector events. IP_3 triggers a rise in cytosolic Ca^{2+} through release of Ca^{2+} from subcellular organelles and the rise in cytosolic Ca^{2+} in turn supports several processes (Hokin, 1985; Nomura et al., 1986; Berridge, 1985; Berridge, 1987). DAG directly activates the Ca^{2+}/phosphotidylserine-dependent protein kinase C (PKC) (Kuo and Turner, 1985; Kikkawa and Nishizuka, 1988; Nishizuka, 1986; Takai et al., 1985). Once activated, PKC catalyzes the activation by phosphorylation of a number of important endogenous proteins (Nishizuka, 1986; Takai et al., 1985); it seems likely that one or more of these phosphorylated proteins is related to tissue factor expression.

As stated previously, the specifics of the transmembrane signalling pathways utilized by LPS resulting in increased expression of tissue factor by alveolar macrophages are largely unknown. We have investigated the transmembrane and intracellular events that are triggered in macrophages by LPS including CD14-dependence, G-protein regulation, intracellular Ca^{2+} mobilization, protein kinase C dependency, and alteration of cytosolic pH. In addition, since protein tyrosine phosphorylation appears to be a major intracellular signalling event that mediates cellular responses, we examined whether LPS alters protein tyrosine kinase activity.

It is hoped that the knowledge acquired may contribute to bridging the gap between the well-known clinical effects of LPS and the cell biological and molecular means by which LPS exerts these effects, and could eventually lead to novel strategies for treatment or prevention of endotoxin-related diseases.

MATERIALS AND METHODS

Bronchopulmonary Lavage

Pulmonary alveolar macrophages were harvested from 1 to 4 month-old, male Holstein-Frisian calves by volume-controlled bronchopulmonary lavage. Following the induction of anesthesia by intravenous Xylazine (0.11 mg/kg), the animals were intubated and anesthesia was maintained with a isoflurane/oxygen mixture delivered by an anesthetic machine. The calves were maintained in lateral recumbency. Apnea was induced by manual hyperventilation with pure oxygen.

The anesthesia machine was disconnected and the lavage tubing connected to the endotracheal tube. The other end of the tubing was connected to a bottle containing a volume of sterile Hank's Balanced Salt Solution (HBSS-) equivalent to the functional reserve capacity of the lung lobe to be lavaged. The FRC for the right lung of the calf to be lavaged was determined by using the following formula:

FRC (right lung) = body weight (kg) X 40 ml X 0.58

The volume of fluid was infused from a height of 30 to 40 cm above the level of the dependent lung. Following instillation of the fluid, the tubing was clamped, the drain tube immediately opened, and the lavage fluid removed from the lung by gravity-dependent drainage into a sterile 1 liter cold Erlenmeyer flask. Following performance of the lavage, calves were manually ventilated for several minutes with oxygen and then allowed to breath room air. Xylazine sedation was reversed by the intravenous administration of Yohimbine (0.15 mg/kg). The calves were maintained in sternal recumbency until completely recovered. The majority of animals were ambulatory within 15 min. following completion of the procedure. The animals serving as a source of cells received the highest level of care; the protocol for collection of cells was approved by the Animal Care and Concerns Committee of this institution.

Isolation of Alveolar Macrophages

The recovered bronchoalveolar lavage fluid was filtered through sterile gauze and centrifuged in 50 ml polypropylene tubes (200g for 10 min. at 4 °C). The pelleted cells were gently resuspended in cold HBSS-, and washed twice.

Cells were resuspended to the desired final concentration in Delbecco's Minimum Essential Media (DMEM), utilized within 3 hours of collection, and kept on ice until assay. Cells were stained from cytospin preparations by Wright-Giemsa to determine proportions of leukocyte populations present. The total number of cells collected was determined by a hemocytometer. Counts of viable cells were obtained using the exclusion of trypan blue. Lung lavage cells were routinely 90 to 100% alveolar macrophages and greater than 95% viable.

Preparation of a Concentrated Bovine Serum Fraction (Fraction 2)

Preparation and use of a bovine serum fraction (fraction 2) rather than whole bovine serum allowed use of lower concentrations of serum proteins in these studies. Pooled bovine serum collected from normal, healthy cows was fractionated using Bio-Rex 70 chromatography media, as previously described for rabbit serum. Fifty ml of Bio-Rex resin was equilibrated with 41 mM NaCl in 50 Mm phosphate buffer, pH 7.3, containing 2 mM EDTA (phosphate-EDTA). Two hundred ml of bovine serum containing 5 mM EDTA, was run over the column at 60 ml/hr. The column was then washed with column equilibration buffer until the absorbance (280 nm) of the eluate was <0.2 absorbance units (AU). Washing was continued with 220 mM NaCl in phosphate-EDTA, again until the absorbance was <0.2; this was followed by a linear gradient formed from 60 ml each of 220 mM and 500 mM NaCl in phosphate-EDTA. Finally, the column was washed with 1 M NaCl in phosphate-EDTA. Pools of fractions were dialyzed *vs* 5 mM Hepes, pH

7.3, and concentrated approximately 10-fold using YM10 membranes in an Amicon ultrafiltration cell. Fraction 2 used in our current work corresponds to the column eluate obtained when washing with 220 mM NaCl.

Cell Treatment and Reagents - Tissue Factor Assay

5 X 10^4 cells were suspended in 100 µl of DMEM and placed into the wells of a 96-well flat bottom tissue culture plate and incubated for 60 min. at 37 °C in a humidified atmosphere of 5% CO_2 in air. Nonadherent cells were removed by washing twice with 200 µl of warmed HBSS-.

Anti-CD14 monoclonal antibody: The cells were pre-incubated with the anti-CD14 monoclonal antibody, 60bd (20 µg/ml), or IgG_1 isotype control for 30 min. prior to incubation for an additional 4 h with concentrations of 0.25, 0.5, or 1.0 ng/ml of *E. coli* LPS in combination with serum fraction 2 at a final concentration of 500 ng/ml. Alternatively, the cells were incubated with concentrations of 0.01, 0.10, 1.0, and 10 µg/ml of *E. coli* LPS in the absence of serum fraction 2.

Pertussis Toxin Inhibition: Following a 2 h pre-incubation with pertussis toxin (300 ng/ml), the cells were incubated for 4 h with various concentrations of LPS, either alone or in combination with serum fraction 2.

Herbimycin A Inhibition: Cells were pre-incubated for 2 h with Herbimycin A (1 µg/ml), prior to incubation for 4 h with LPS as described above.

Protein Kinase C Inhibition: The cells were preincubated for 30 min with 100 µl of one of three known inhibitors of protein kinase C: staurosporin (50 nM)

(Boehringer Manheim, Indianapolis, IN), chelerythrine (10 μM) (LC Services Corp., Woburn, MA), or H-7 (100 μM) (Molecular Probes, Eugene, OR). Following incubation with inhibitor, the cells were incubated an additional 4 h with either 4β-phorbol 12-myristate 13-acetate (PMA) 50 ng/ml, or 0111:B4 *E. coli* lipopolysaccharide at concentrations of 0.01, 0.10, 1.0, or 10.0 μg/ml.

Measurement of Tissue Factor Expression

The expression of tissue factor by the cells was quantitated with a colorimetric assay based on digestion of the chromogenic substrate S-2765 by factor X activated by tissue factor produced by alveolar macrophages. Following the 4 h incubation with LPS, the cells were again washed with HBSS- and incubated for 1 h with 100 μl of a solution containing factors VII and X and S-2765. At the end of incubation, the plates were rapidly transferred into a Bio-Tek microplate reader and the optical density at 405 nm wavelength was quantitated. The degree of chromogenicity being directly proportional to the amount of tissue factor expressed. When log fold dilutions of rabbit brain thromboplastin were measured in both the prothrombin time assay (PTA) and the chromogenic tissue factor assay, the chromogenic assay produced a dose response curve markedly similar to that produced by the more conventional PTA.

Control wells consisted of wells with untreated cells, macrophages exposed to monoclonal antibody or inhibitor in the absence of LPS, or with macrophages incubated with LPS in the absence of monoclonal antibody or inhibitor. All

background values were subtracted before data analysis. All initial LPS, monoclonal antibody and inhibitor dose levels used were chosen by dose-range testing from previous dose-response curves established in our laboratory with bovine alveolar macrophages. None of the dosage levels used affected cellular viability as assessed by trypan blue exclusion following the last incubation period. Each treatment variable was done in triplicate and the experiments were repeated to confirm results. The results are expressed as means +/- SEM (standard error of the mean) of triplicate determinations. Analysis of variance was the method used to statistically analyze the data.

Cell Treatment and Reagents - The Effect of LPS on Cytosolic Calcium and pH

Calcium: Alveolar macrophages were suspended at 5 X 10^7/ml in HBSS without Ca^{2+} and Mg^{2+} (HBSS-) and incubated at room temperature with 2.5 µM of the calcium-sensitive fluorescent dye fura-2/acetoxymethyl(AM) for 15 min. The suspension was then diluted 5-fold via the addition of 4 ml of HBSS- and incubated an additional 45 min to ensure adequate fura-2 loading. The cells were then centrifuged (200 g for 10 min) and resuspended to a concentration of 2.5 X 10^6/ml in HBSS-. After dye loading, 2 ml aliquots of the stock cell suspension were held within a thermostatted 1 cm^2 cuvette in a spectroflurometer at 37 °C with gentle stirring throughout the experiment. The various stimuli were added and fura-2 fluorescence emission at 510 nm was monitored with a Shimadzu fluorometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) at 335 nm and

360 nm excitations with the data expressed as the ratio of 335nm/360nm fluorescence.

pH Measurement: Alveolar macrophages were treated as above and loaded with 10 μM of the pH-sensitive fluorescent dye BCECF/AM. BCECF fluorescence was monitored for emission at 530 nm with 495 nm excitation.

Studies of Protein Tyrosine Phosphorylation (Western Blot Analysis)

Cell Stimulation and Lysis. All experiments were performed in serum-free medium. For each experimental sample, macrophages were suspended at 5 X 10^6/ml in DMEM in a sterile 5 ml siliconized glass cuvette at 37°C with stirring. The cells were incubated for various lengths of time with LPS either at a concentration of 1 ng/ml in combination with serum fraction 2 (100 ng/ml), or at 10 μg/ml in the absence of serum fraction 2. After stimulation, the cells (in a 700 μl aliquot) were pelleted by centrifugation (10,000 X g for 20 sec.) and the supernatant fraction was decanted. Cell pellets were washed with 700 μl of ice-cold PBS containing 1 mM Na_3VO_4. The cells were pelleted again and lysed in 80 μl of lysis buffer (50 mM Tris-HCl - pH 7.4, 1% Triton X-100, 137 mM NaCl, 5 mM EDTA, 1mM Na_3VO_4, 10% glycerol, 0.01 mM PMSF, 0.1 mM leupeptin, 2 mM β-mercaptoethanol, 20 mM β-glycerophosphate, and 10 μg/ml aprotinin). The lysates were incubated on ice for 10 min and the insoluble material was removed by centrifugation (10,000 X g for 8 min at 4°C). The supernatant fraction was collected and directly denatured by the addition of 3X denaturation buffer then

stored at -70 °C before analysis for MAP kinase activity.

Cell Fractionation Procedure. Following stimulation with LPS (10 µg/ml), a 1.5 ml aliquot of cells was pelleted by centrifugation and washed twice with ice cold PBS containing 1 mM Na_3VO_4. The cells were resuspended in 0.5 ml of PBS, Na_3VO_4, 0.5 mM EDTA, 0.1 mM Leupeptin, 0.01 mM PMSF, sonicated for 30 sec in a Vibracell bath sonicator at 0 °C, and centrifuged for 3 min at 6000 X g to pellet the nuclear and cell fragments. The resulting supernatant was centrifuged at 150,000 X g for 30 min. Following centrifugation, the membrane pellet was dissolved in 250 µl of a 1.5X denaturation buffer, while the cytosol fraction was diluted by one-third with 0.25 ml of 3X denaturation buffer.

Electrophoresis and Anti-phosphotyrosine Immunoblotting. Equivalent amounts of SDS denatured and β-mercaptoethanol reduced cell lysates were resolved by SDS-PAGE on 1.5-mm thick, 12% polyacrylamide gels run at constant current (30 mA/gel), for 2.5 h. The proteins were then electrotransferred from the gel to nitrocellulose membranes at 0.5 A for 18 h at 4 °C. After transfer, the membranes were rinsed with TRIS-HCl, NaCl, Tween-20 (TBST) at pH 8.0. The membranes were then covered with blocking buffer (1% BSA, 1% goat serum, 0.01% sodium azide, 50 ml TBST), and incubated at 37 °C for 1 h with shaking. The membranes were then washed twice with TBST (pH 8.0). Following washing, the membranes were incubated for 30 min at 37 °C with a 1:500 dilution of goat anti-mouse Ig-alkaline phosphatase conjugate in TBST. The membranes were washed twice with TBST and the phosphotyrosine-containing proteins were

detected by incubating the membranes in 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml nitro blue tetrazolium in 0.1 M $NaHCO_3$ and 10 mM $MgCl_2$, pH 9.8. Color development was continued for 15 to 45 min to produce the desired darkness and the reaction was terminated by rinsing the membrane in water.

MAP Kinase Activity Assay. Assays for MAP kinase activity in BG-1 cell lysates were performed by a modification of the method of Tobe (1991). Briefly, equal protein amounts (400 - 500 µg) of BG-1 detergent lysates were subjected to precipitation with agarose beads coated with monoclonal antibodies to rat ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in the presence of 1mM sodium orthovanadate. Immunoprecipitates were washed three times in immunoprecipitation buffer (10 mm Tris pH 7.5, 200 mM NaCl, 0.1% Triton X-100) followed by two washes in kinase buffer (40 mm Tris pH 7.5, 10 mM $MgCl_2$, 4 mM $MnCl_2$) then resuspended in 25 µl of kinase buffer supplemented with 0.5 mM DTT, 0.5 mM EGTA, 10 mM ATP, 5 µ Ci [γ ^{32}P] ATP (7000 Ci/mM, ICN, Irvine, CA), 8 µg/ml pKA inhibitor peptide (Sigma), and 0.4 mg/ml myelin basic protein (Gibco, BRL, Grand Island, NY). After incubation at room temperature for 20 minutes, the reactions were stopped by addition of 30 µl 2X SDS-PAGE sample buffer and centrifugation. Supernatants were separated on 14% Tris-glycine gels (Novex, San Diego, CA) fixed, dried, and subjected to autoradiography on Kodak XAR-5 film.

Materials

Hank's Balance Salt Solution was purchased from GIBCO Laboratories, Grand Island, NY, and DMEM was from Whittaker Bioproducts, Walkersville, MD. 60bd is an anti-human CD14 murine IgG_1 monoclonal antibody and was a generous gift from Dr. Robert F. Todd III, Division of Hematology and Oncology, University of Michigan Medical School, Ann Arbor, Michigan. 60bd was purified with an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, IL). The purified IgG_1 murine control antibody was purchased from Organon Teknika Corp., Malvern, PA. The bacterial endotoxin was derived from *Escherichia coli* (serotype 055:B5) and was obtained from Sigma Chemical Co., St. Louis, MO. and contained 11,000 endotoxin units per microgram as determined with a chromogenic *Limulus* assay (QCL-1000, Whittaker Bioproducts, Inc., Walkersville, MD). The chromogenic substrate, N-α-Benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroanilide-dihydrochloride (S-2765), was purchased from Kabi Pharmaceuticals, Franklin, OH. The coagulation factor concentrate, Proplex-T, was from Travenol Labs, Inc., Glendale, CA. Pertussis Toxin, Herbimycin A, and Yohimbine were purchased from Calbiochem, La Jolla, CA. Xylazine was from Miles Inc., Shawnee Mission, KS. The normal adult bovine serum was collected at our institution by jugular venipuncture from three clinically normal, adult bovine cows. Animals serving as blood donors received the highest level of care, and all protocols were reviewed and approved by the University Animal Care and Concerns Committee. Whole blood was allowed to clot for 1-2 h at 37 °C,

centrifuged (20 min, 1200 *g*, 22 °C), and re-centrifuged to ensure clarity, and finally frozen without preservative (-70 °C) until use. Sera used in our studies contained 7.9 g/dl of total protein. Bio Rex-70 resin was obtained from Bio-Rad Laboratories, Richmond, CA, and the YM10 filtration membranes were obtained from Amicon Corp., Danvers, MA.

RESULTS

The Effect of anti-CD14 Monoclonal Antibody on LPS-induced TF Expression

As demonstrated in **Figure 1**, pre-incubation of alveolar macrophages with the anti-CD14 MAB, 60bd (20 µg/ml) resulted in profound and linear inhibition of TF expression (P <.0001) following stimulation of the cells with either of 3 concentrations of LPS in combination with a fixed concentration of serum fraction 2 (500 ng/ml). 60bd reduced TF expression by 84% in cells stimulated with 0.25 ng/ml LPS, by 77% in those incubated with 0.5 ng/ml LPS, and by 69% in cells stimulated with 1ng/ml LPS. Pre-incubation of the cells with the IgG_1 isotype-specific control monoclonal antibody (20 µg/ml) failed to have a significant effect on expression of TF by the cells.

As seen in **Figure 2**, pre-incubation with 60bd had no significant effect on TF expression when the cells were stimulated with LPS in the absence of serum fraction 2. It was necessary to stimulate the cells with concentrations of LPS ranging from 0.01 to 10.0 µg/ml since using concentrations of 0.25 to 1.0 ng/ml in the absence of partially purified LBP failed to have a measurable effect on TF

expression.

Pertussis Toxin Inhibition of G-protein Signalling

Figures 3 & 4 represent the results obtained when alveolar macrophages were pre-incubated with PTX (300 ng/ml) prior to stimulation with either LPS in combination with serum fraction 2 or with LPS alone. Pre-incubation of alveolar macrophages with PTX failed to have a significant inhibitory effect on TF expression when the cells were stimulated with 0.25, 0.5, or 1.0 ng/ml LPS in combination with serum fraction 2 (500 ng/ml). However, when the cells were incubated with various concentrations of LPS in the absence of fraction 2, PTX reduced TF expression by 23 to 71% (P<0.0319).

The Effect of the Tyrosine Kinase Inhibitor, Herbimycin A, on LPS-induced TF Expression

Figure 5 illustrates the profound inhibitory effect of the tyrosine kinase inhibitor, Herbimycin A (1 µg/ml), on the expression of TF by alveolar macrophages following stimulation with LPS in combination with serum fraction 2 (P <.0001). The inhibition ranges from 86% at 0.25 ng/ml to 94% at 1.0 ng/ml. The effect of pre-incubation with Herbimycin A prior to stimulation with LPS in the absence of serum fraction 2 is demonstrated in **Figure 6**. Inhibition of tyrosine kinase in this system resulted in a significant reduction in the expression of TF by the cells ranging from 59% at 0.01 µg/ml to 96% at 0.1 µg/ml (P<.0001).

The Effect of Protein Kinase C Inhibition on LPS- and PMA-induced Tissue Factor Expression

Preincubation of the cells with known inhibitors of PKC resulted in marked inhibition of LPS- and PMA-induced enhancement of tissue factor expression. **Figure 7** depicts the results obtained when the cells were preincubated for 30 min with chelerythrin (10 μM) prior to stimulation with LPS. Pretreatment of the cells with chelerythrine resulted in a 58 to 156% reduction in tissue factor expression (P<0.0001). Pretreatment of the cells with H-7 (100 μM) resulted in inhibition ranging from 93 to 213% (P<0.0001) **(Figure 8)**. Procoagulant activity induced by the known PKC agonist, PMA (50 ng/ml), was reduced by 63% in cells pretreated with staurosporin (50 nM), by 111% with chelerythrine (10 μM), and by 98% with H-7 (100 μM) **(Figure 9)**.

The Effect of LPS on the Levels of Cytosolic Calcium ($[Ca^{2+}]_i$)

Platelet Activating Factor (PAF) (10^{-7} M) was used as a positive control in this study **(Figure 10a)** and typically stimulated $[Ca^{2+}]_i$ by 5-fold (19 nM to 120 nM). Stimulation of the cells with high doses of LPS alone **(Figure 10b)** or with LPS in combination with serum fraction 2 **(Figure 10c)** failed to affect $[Ca^{2+}]_i$.

The Effect of LPS on the Cytoplasmic pH (pH_i) of Alveolar Macrophages

PMA (50 ng/ml) was the positive control in the studies involving changes in intracellular pH and consistently resulted in a precipitous reduction of pH_i **(Figure 11a)**. Stimulation of the cells with LPS alone **(Figure 11b)** or with LPS in

combination with serum fraction 2 **(Figure 11c)** failed to affect pH_i.

LPS- Induced Tyrosine Phosphorylation as Shown by Anti-Phosphotyrosine immunoblotting

As seen in **figure 12**, LPS (10 µg/ml) rapidly increased tyrosine phosphorylation of a solitary Triton X-100-soluble protein with an apparent molecular mass of 30 kDa. Increased tyrosine phosphorylation was detectable by 5 min and reached a maximum by 10 min. When cells were stimulated by LPS (1 ng/ml) in combination with serum fraction 2, maximum tyrosine phosphorylation was observed to occur at 20 min **(figure 13)**.

Cell Fractionation and MAP Kinase Co-Migration Studies - Figure 14

When cytosolic and membrane-associated fractions were examined by anti-phosphotyrosine immunoblotting, a greater intensity of phosphorylation of the 30 kDa polypeptide was observed in the cytosolic fraction upon stimulation by LPS (10 µg/ml). When the cytosolic fraction was treated with both anti-phosphotyrosine antibody (PY-20) and a anti-rat MAP kinase polyclonal antibody, the anti-MAP kinase antibody stained a band coincident with that stained by PY-20. In addition, the anti-MAP kinase antibody clearly identifies two well-known MAP kinases with molecular weights of 41 and 44 kDa.

MAP Kinase Activity Assay

Anti-MAP kinase agarose beads used to capture MAP kinase activity assayed with Myelin Basic Protein (MBP) revealed a 38% increase in

phosphorylation of MBP substrate in cells treated with LPS (10 μg/ml) when compared to control (untreated) cells **(figure 15)**.

DISCUSSION

Our results confirm and extend earlier studies (Yang et al., 1993) showing that anti-CD14 monoclonal antibodies can be used to abrogate the enhanced expression of tissue factor by bovine alveolar macrophages stimulated by LPS in combination with serum factors, thus substantiating the importance of this receptor molecule in the signal transduction pathway utilized by LPS-LBP complexes resulting in enhanced expression of tissue factor by alveolar macrophages. Further, we have shown that anti-CD14 monoclonal antibody has no inhibitory effect on tissue factor expression when macrophages are stimulated with high doses of LPS in the absence of serum factors. This result suggests that although LPS-LBP binding to CD14 appears to mediate some biologic responses of macrophages, this pathway is not the only mechanism by which these cells respond to LPS. Concentrations of LPS greater than 10 ng/ml have been shown to stimulate monocytes or macrophages to secrete TNF-α and IL-1 under conditions in which LPS-LBP complexes are prevented from binding to CD14 by anti-CD14 antibodies (Wright, 1990; Kitchens et al., 1992; Couturier et al., 1992). In addition, monocytes from patients with paroxysmal nocturnal hemoglobinuria express little or no surface CD14, yet these cells respond to high concentrations of LPS (Couturier et al., 1992).

Our data and that of others cited above, which support the concept that stimulation by LPS may occur via CD14-dependent and -independent pathways raises several important questions such as: What is the relationship between CD14-dependent and -independent pathways? At present there are no data that address these issues. A critical question is how the ligation of CD14 by LPS-LBP complexes signals cells to produce tissue factor. CD14 is known to be one of several cell surface receptors which undergoes post-translational modification resulting in the addition of a glycophosphatidylinositol (GPI) anchor, which serves as its only means of attachment to the cell membrane (Ferguson and Williams, 1988). More than fifty such proteins have been described to date and they occur in a wide variety of eukaryotic cells. To date, eleven GPI-anchored proteins have been implicated in signalling events in hematopoietic cells: eight in T-cell activation and three in the initiation of metabolic changes in other leukocyte types (including CD14).

Exactly how GPI-linked molecules transduce activation signals is unclear (Robinson and Spencer, 1988). Most other signal transduction molecules are transmembrane polypeptides and can therefore interact with both extracellular ligands and cytoplasmic components. In contrast, GPI-anchored molecules associate only with the outer face of the lipid bilayer and must use a different signalling mechanism. How does binding of ligand to extracellular regions of CD14 cause intracellular biochemical changes, in the absence of direct contact with cytoplasmic components? The most plausible explanation is that additional

proteins (or additional subunits of a receptor complex) are required for signal transduction.

Two plausible models for a heterodimeric LPS receptor can be derived from what is known about the receptors for the cytokines IL-2 or IL-6. The IL-2R is a heterodimer (Hatakeyama et al., 1989; Mills et al., 1990) consisting of two ligand-binding subunits: the IL-2R α subunit which binds IL-2 with low affinity but does not transduce signals or mediate ligand internalization, and the IL-2R β subunit, which binds IL-2 with low affinity but does transduce signals and mediate ligand internalization. When both the alpha and beta subunits are present, high-affinity binding is observed (Hatakeyama et al., 1989) and the threshold stimulatory dose of IL-2 is substantially reduced. The receptor for IL-6 is a heterodimer consisting of an 80-kDa ligand-binding subunit and the gp130 subunit that does not bind ligand but does mediate transmembrane signalling (Hibi et al., 1990; Akira et al., 1990). Engagement of the 80-kDa subunit by IL-6 results in association of the ligand/receptor complex with gp130 and the initiation of transmembrane signalling.

An alternative model states that CD14 serves principally in the accumulation of LPS in or near the membrane and that signalling is caused by different molecules (Lund-Johansen, 1990). As GPI-linked molecules have high lateral mobility in the cell membrane (Low, 1988), they may be more easily perturbed or aggregated upon interaction with a specific ligand. This characteristic may be advantageous in the function of CD14 as an acceptor for LPS that transfers LPS with high affinity to another receptor on the membrane. Such additional LPS-

binding proteins have been identified (Ulevitch, 1993; Lynn and Golenbock, 1992) but their functional role and relationship to signalling via LBP/CD14-dependent pathways remain unclear. Our results and those of others cited which demonstrate that high levels of LPS can enhance certain macrophage functional endpoints (i.e., TNF-α production and TF expression) in the absence of LBP or CD14 support this concept. Clearly a major goal for future research will be to elucidate fully the additional proteins involved in recognition of LPS.

While much remains to be learned about receptors for LPS, even less is known about the early intracellular events that mediate LPS responses. We investigated the role of a pertussis-toxin sensitive G protein and herbimycin-sensitive protein tyrosine kinase in the early intracellular events that are triggered in bovine alveolar macrophages stimulated with LPS, either in combination with serum proteins or in their absence.

We found that pertussis toxin had no inhibitory effect on tissue factor expression when the cells were stimulated with LPS in combination with serum fraction 2 (i.e., CD14-dependent pathway), but produced a biologically and statistically significant inhibitory effect on cells incubated with LPS alone. This suggests that one or more alternative LPS-receptor molecules, exclusive of CD14, is coupled with a pertussis toxin-sensitive G protein, or, that CD14 transduces a stimulatory signal differently, depending upon whether the ligand (LPS) is bound to serum protein(s).

Several investigators have reported that LPS activates a pertussis toxin-

sensitive G protein (Jakway and DeFranco, 1986; Daniel-Issakani, 1989; Dziarski, 1989). However, if adenylate cyclase-coupled G_i protein were to be the initial target of LPS action, then it would be predicted that LPS should regulate intracellular cAMP levels. However, there is no unequivocal evidence that the critical action of LPS involves regulation of cAMP levels. It is evident that there is a family of G proteins, and pertussis toxin inhibits not only G_i but other members of this family as well. For example, pertussis toxin inhibits transducin, which activates cyclic GMP phosphodiesterase in retinal rods (Van Dop et al., 1984), coupling components involved in neurotransmitter-induced voltage-dependent ion channels in neural cells (Pfaffinger et al., 1984), histamine release by mast cells stimulated with compound 48/80 (Nakamur and Ui, 1983), and migration and lysosomal enzyme release in neutrophils (Okajima and Ui, 1985) and macrophages (Backlund et al., 1985) stimulated with the chemotactic ligand f-Met-leu-Phe or with leukotriene B_4. In mast cells, neutrophils, and macrophages, these stimuli appear to act not by regulating adenylate cyclase activity but rather by inducing hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) with subsequent mobilization of calcium into the cytoplasm (Nakamur and Ui, 1985; Okajima and Ui, 1985; Backlund et al., 1985). Thus, there are probably a number of different pertussis toxin-sensitive G_i-like coupling components. It is not clear which of these G_i-like molecules is responsible for the transmembrane signalling generated in response to LPS. However, it has been observed that LPS had no effect on PIP_2 hydrolysis or calcium mobilization in either WEHI-231 cells or $P388D_1$ cells (Masters et al.,

1985) or in normal B cells (Bijsterbosch et al., 1985). This is in contrast to the pertussis toxin-sensitive phosphoinoistide responses mentioned above. One model to explain these observations would be that LPS and chemotactic ligands such as f-Met-Leu-Phe activate functionally distinct G_i-like components. Thus, it appears that a given second messenger system can be coupled to different transmembrane coupling components in different cell types (Jakway and DeFranco, 1986).

It has been reported (Vistica et al., 1986) that pertussis toxin can activate T cells and macrophages. However, these effects require 2 to 10 µg/ml pertussis toxin, which is 6 to 30 times greater than the amount we found necessary to inhibit LPS-induced tissue factor expression.

In addition to G proteins, we investigated the involvement of a herbimycin-sensitive protein tyrosine kinase in the early biochemical events that are triggered in bovine alveolar macrophages stimulated by LPS. We found that pretreatment with herbimcyin A resulted in profound inhibition of TF expression in cells stimulated either with LPS alone or LPS in combination with serum fraction 2. These results suggest that protein tyrosine kinase activation is an important early signalling event in both CD14-dependent and -independent pathways resulting in enhanced expression of TF by alveolar macrophages. We utilized western blot analysis to show LPS-induced tyrosine phosphorylation of a single 30 kDa protein (pp30), and demonstrated a differential time course in phosphorylation of this protein when cells were stimulated with LPS alone (maximal phosphorylation

occurred at 10 min) *vs* that observed when cells were stimulated with LPS in combination with serum fraction 2 (maximal stimulation occurred at 20 min). Cell fractionation performed in conjunction with western immunoblotting revealed equal intensity of phosphorylation of the 30 kDa protein in cytosolic and membrane-associated fractions. Further, we provided evidence suggesting that pp30 may be a novel MAP kinase. Each of these results will be discussed separately.

Many receptors stimulate protein tyrosine phosphorylation following ligand binding, and this event is thought to be part of the signal transduction mechanism that mediates later cellular responses (Einstein et al., 1991). As stated above, CD14 has been shown to be a GPI-anchored cell surface receptor, and some GPI-anchored membrane glycoproteins are associated with protein tyrosine kinases (PTKs) (Thomas and Samelson, 1992; Stefanova et al., 1991) and this association may explain the signal-transducing capacity of these molecules. It is not known whether the PTKs interact directly with the glycolipid anchor of the GPI-linked antigens or through interaction with other proteins. Precise understanding of the associations between GPI-linked proteins and PTKs will require analysis of relevant glycolipid and protein-protein interactions.

Weinstein et al. (1991) found that *E. coli* K235 LPS increased tyrosine phosphorylation of several proteins in the RAW 264.7 murine macrophage cell line and in resident peritoneal macrophages from C3H/HeSNJ mice. Changes in tyrosine phosphorylation were detectable by 4 to 5 min., reached a maximum by 15 min. and declined after 30 to 60 min. Furthermore, they treated RAW 264.7

cells with herbimycin A resulting in inhibition of both LPS-stimulated tyrosine phosphorylation and LPS-stimulated release of arachidonic acid metabolites. They concluded that protein tyrosine phosphorylation is a rapid LPS-activated signalling event that may mediate release of arachidonic acid metabolites in RAW 264.7 cells (Weinstein, 1991).

To test whether macrophages possess a second mechanism for responding to high concentrations of LPS that is independent of CD14, Weinstein et al. (1993) pretreated macrophages with anti-CD14 monoclonal antibodies to block the CD14-dependent pathway and then exposed the cells to increasing doses of LPS. As expected, the induction of protein tyrosine phosphorylation stimulated by doses of LPS of 1 ng/ml or less was completely inhibited by the anti-CD14 antibodies. However, the extent of the inhibition diminished as the LPS concentration was raised to 10 ng/ml and very little inhibition was detected in cells treated with 1 µg/ml LPS. Thus, these investigators provide additional evidence for CD14-dependent and CD14-independent pathways of LPS-induced macrophage stimulation.

Because CD14-dependent and CD14-independent recognition of LPS appear to lead to the same functional responses by macrophages, it is attractive to hypothesize that protein tyrosine phosphorylation may be an intermediate signalling step that biochemically couples these LPS recognition mechanisms to the activation of functional responses (e.g., TF expression) by macrophages.

Given the growing evidence that MAP kinases are important signal

transduction components, we investigated the possibility that the tyrosine-phosphorylated 30 kDa protein identified in our LPS-stimulated macrophages demonstrated MAP kinase activity. We showed that MAP kinase activity is increased following LPS treatment, and that the 30 KDa protein co-migrates with a protein recognized by anti-MAP kinase antibody. Previously identified MAP kinases have molecular weights ranging from 41 to 63 KDa, thus our protein may represent a novel MAP kinase.

Weinstein et al. (1993) reported that LPS induces the tyrosine phosphorylation and activation of at least two MAP kinase isozymes. These isozymes had molecular weights of 41 and 44 kDa and most likely correspond to the two major MAP kinase isoforms seen in other cell types. Each of these proteins was reactive with anti-MAP kinase antibodies and comigrated with MAP kinase activity in fractions eluted from a MonoQ anion-exchange column. Thus, MAP kinases are the first identified substrates for LPS-induced tyrosine phosphorylation.

The precise function of MAP kinases in macrophages and in other cells is not yet known. Several proteins have been found to be efficient *in vitro* substrates of MAP kinases, and these may be indicative of MAP kinase function *in vivo*. For example, phosphorylation of microtubule-associated protein 2 by MAP kinases (Gotoh et al., 1991; Ray and Sturgill, 1987) may alter the cytoskeleton and could provide a molecular mechanism for the morphological changes induced by LPS in macrophages. Similarly, MAP kinases can phosphorylate and activate both the S6

ribosomal protein kinase (Chung et al., 1991; Sturgill, 1991) and the c-jun product (Pulverer et al., 1991), proteins that are involved in the regulation of translation and transcription, respectively. If MAP kinases phosphorylate these targets *in vivo*, these kinases may contribute to the altered expression of LPS-modulated proteins. Since many of the responses triggered by LPS in macrophages depend on transcription and translation, LPS activation of MAP kinases could be a critical part of the mechanism by which LPS induces responses in macrophages.

Despite the incomplete understanding of the role of MAP kinases in cells, our results and those of Weinstein (1993) suggest that these kinases could be very important targets of LPS action in macrophages and lend support to the hypothesis that induced protein tyrosine phosphorylation is part of the signal transduction pathway that mediates macrophage responses to LPS.

Our data clearly demonstrates a distinct difference in time required for maximal tyrosine phosphorylation between cells stimulated with LPS alone and those stimulated with LPS in combination with serum fraction 2. One possible explanation for the delayed response in the CD14-dependent system was eluded to earlier in the discussion. CD14, being a GPI-anchored membrane protein, may require interaction with transmembrane molecules that provide signalling activity. Thus, cellular activation thru CD14 may be a multistep process requiring several minutes to initiate intracellular events such as tyrosine phosphorylation.

Whether LPS-induced tyrosine phosphorylation results from altered activity of protein tyrosine kinases or protein tyrosine phosphatases remains to be

determined.

In our examination of the effect of LPS on the levels of cytosolic calcium ($[Ca^{2+}]_i$) we found that stimulation of alveolar macrophages with high doses of LPS alone or in combination with serum fraction 2 failed to affect $[Ca^{2+}]_i$. Our results are compatible with a recent report in which neither LPS nor LPS and LBP affected $[Ca^{2+}]_i$ in rabbit alveolar macrophages (Martin et al., 1992). The role of calcium ions in the mode of action of LPS is still debated. On one hand, it has been shown that LPS can stimulate the turnover of phosphatidylinositol (Prpic et al., 1987), thus generating inositol-1,4,5-trisphosphate, which in turn may release Ca^{2+} from intracellular stores. Furthermore, Ca^{2+} ionophores are able to mimic some of the actions of LPS (Drysdale et al., 1987; Finkel et al., 1987); finally, chelation of intracellular Ca^{2+} (Gorecka-Tisera et al., 1986; McLeish et al., 1989) or blocking of its entry (Wright et al., 1985) inhibits LPS-induced responses. All of these findings suggest that Ca^{2+} might play a role in monocyte/macrophage activation. On the other hand, studies aimed at directly evaluating variations in $[Ca^{2+}]_i$ have yielded inconclusive results. One study reported a minor increase in $[Ca^{2+}]_i$ at a single time point (McLeish et al., 1989). An additional report showed a modest and slow increase in $[Ca^{2+}]_i$ recorded in single cells, contrasts with a fast and massive increase elicited by lipid A (Prpic, 1987).

It is entirely possible that large increases in intracellular levels of calcium after incubation with LPS are not important to the long-term effects of LPS on cellular metabolism. It is worth noting that in some systems, only quite small

fluxes of $[Ca^{2+}]_i$ are needed to initiate full cellular responses (Abdel-Latif, 1986). On the other hand, LPS may exert its effects, or at least some of them, by mechanisms other than changes in intracellular levels of Ca^{2+}. This intriguing question requires further investigation.

In terms of second-messenger systems, LPS-stimulated inositolphospholipid hydrolysis has been observed in peritoneal macrophages (Prpic, 1987). In these studies the activation of phospholipase C (PLC), as measured by the formation of inositol trisphosphate, was rapid but rather modest in magnitude and significant PLC activation was shown to require relatively large doses (> 1 µg/ml) of LPS or lipid A. However, LPS does not stimulate detectable inositolphospholipid breakdown in many LPS-responsive cell types (Wang et al., Grupp and Harmony, 1985; Bijsterbosch et al., 1985). Thus, inositolphospholipid hydrolysis does not appear to be obligatory for many cellular responses to LPS.

The results of our experiments utilizing three known inhibitors of protein kinase C confirm previous reports (Car, 1990) implicating this important signalling kinase in the pathway utilized by LPS resulting in enhanced expression of TF by bovine alveolar macrophages.

As stated previously, the mechanism of activation of PKC is novel. Diacylglycerol (DAG) binds to the protein causing it to be translocated to the membrane. Here, in the presence of a phospholipid such as phosphatidlyserine and low calcium concentrations (i.e., physiologic range), the kinase becomes activated. The "conventional" mechanism of DAG generation is via the hydrolysis

of PIP_2 by phospholipase C resulting in the generation of both IP_3 and DAG. However, investigations into LPS-induced phosphoinositol hydrolysis (Prpic, 1987) have failed to produce data which definitively demonstrates significant and sustained PLC activation in LPS-stimulated macrophages. Since other phospholipases may be involved in cell activation (Cockcroft, 1984; Exton, 1988), Grove et al. (1990) investigated the effects of LPS on phospholipid metabolism in elicited mouse peritoneal macrophages. Their evidence suggests that LPS stimulates a sustained degradation of phosphatidylcholine (PC) via a specific phospholipase (Grove, 1990). A phosphatidylcholine-specific phospholipase C has been reported and can be activated by phorbol esters to degrade PC and produce phosphorylcholine and DAG (Besterman et al., 1986; Grove and Schimmel, 1982; Weinstein et al., 1980). The formation of DAG from PC breakdown represents a possible mechanism for the sustained activation of PKC in alveolar macrophages.

Interaction of macrophages with LPS and/or lipid A results in a characteristic pattern of protein phosphorylation (Riches et al., 1988; Hamiliton and Adams, 1987). Phosphorylation of 5 distinct proteins (pp28, pp33, pp38, pp67, pp103) has been reported to be induced or enhanced in LPS-treated macrophages. In brief, the patterns of phosphorylation induced by LPS and by PMA are similar (though not identical), and three of the common phosphoproteins yield virtually identical patterns of phosphopeptides when analyzed by limited proteolysis (Weiel et al., 1986). The evidence implying enhanced phosphorylation induced by LPS is mediated by PKC has been extensively discussed (Weiel et al., 1986).

Current dogma suggests that TF activity is recruited from an intracellular compartment or is synthesized *de novo* in response to a mitogenic stimulus (Hartzell et al., 1989). Zioncheck (1992) reports evidence for the *in vivo* phosphorylation of TF in response to PMA treatment. This result, taken together with the many examples of phosphorylation regulating intermediary metabolsim, suggests that TF may be regulated via phosphorylation *in vivo*. Furthermore, the cytoplasmic domains of both rat and human TF were rapidly phosphorylated after cells were treated with 10-100 nM PMA and this response was completely abolished by preincubating cells with staurosporine, a potent PKC inhibitor, prior to PMA treatment (Zioncheck, 1992). The functional significance of TF phosphorylation remains to be elucidated. Phosphorylation could act to indirectly modulate TF activity by altering internalization and degradation (Shin et al., 1991), or subcellular localization (Casanova et al., 1990). Phosphorylation of the cytoplasmic domains of various integral membrane proteins have also been shown to regulate protein-protein interactions with associated peripheral membrane proteins (Low et al., 1987; Hurley et al., 1989).

In our evaluation of the effect of LPS on intracellular pH (pH_i), we found that simulation of alveolar macrophages with high doses of LPS alone or in combination with serum fraction 2, failed to effect pH_i. Our results are compatible with a recent report in which neither LPS nor LPS and LBP affected pH_i in rabbit alveolar macrophages (Martin et al., 1992).

Within the acidic inflammatory milieu, macrophages must maintain their

cytoplasmic pH within a range conducive to optimal function. The pH_i of mammalian cells is closely regulated by a variety of ion transport systems (Roos and Baron, 1981). This tight control of pH_i is necessary because of the marked pH sensitivity of many intracellular enzymes. Maintenance of pH_i within a range conducive to optimal function is particularly problematic in the case of phagocytic leukocytes. The local microenvironment within which the phagocytic cells must effect their complex functions may threaten their ability to maintain a physiologic pH_i by two mechanisms: (1) the acidic extracellular milieu characteristic of inflammatory settings (Sawyer et al., 1991; Silver, 1978) induces cytoplasmic acid loading by passive diffusion of protons and (Sawyer et al., 1991) the numerous stimuli present in such environments result in cellular activation, generating increased amounts of metabolic acid.

Alveolar macrophages differ significantly from other phagocytic cells in terms of membrane transport properties. ATP-dependent proton pumps located in the plasma membrane of alveolar macrophages serve to extrude protons from the cytoplasmic space (Swallow et al., 1988; Swallow et al., 1990). These H^+ pumps are virtually quiescent under resting conditions but are activated by cytoplasmic acidification. It has been clearly shown that the plasma membrane of neutrophils manifests Na^+-H^+ antiporter activity (Grinstein and Furuya, 1986). The peritoneal macrophage (human) also differs from the alveolar macrophage in that it possesses significant Na^+-H^+ antiporter activity (Swallow et al., 1988). Thus major differences exist between the mechanisms for H^+ transport among cells that

otherwise share similar functions. This would suggest that the way in which cells differentiate results in site- and perhaps species-specific mechanisms for handling H^+ transport.

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APPENDIX

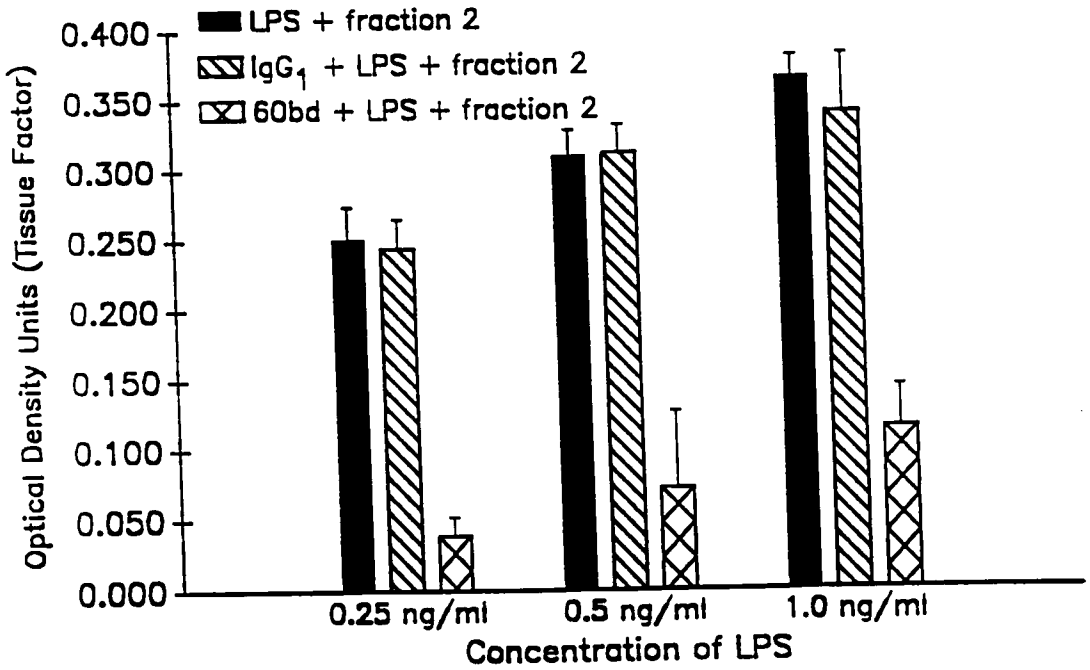

Figure 1 The effect of Anti-CD14 Monoclonal Antibody (MAb) on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS in Combination with Serum Fraction 2. Preincubation of cells with the anti-CD 14 MAb, 60 bd (20 µg/ml) for 30 min resulted in profound inhibition of TF expression (P<0.0001) following stimulation of the cells for 4 h with either of 3 concentrations of LPS in combination with a fixed concentration of serum fraction 2 (500 ng/ml). Preincubation of the cells with the IgG_1 isotype-specific control (20 µg/ml) MAb failed to have a significant effect on the expression of TF by the cells.

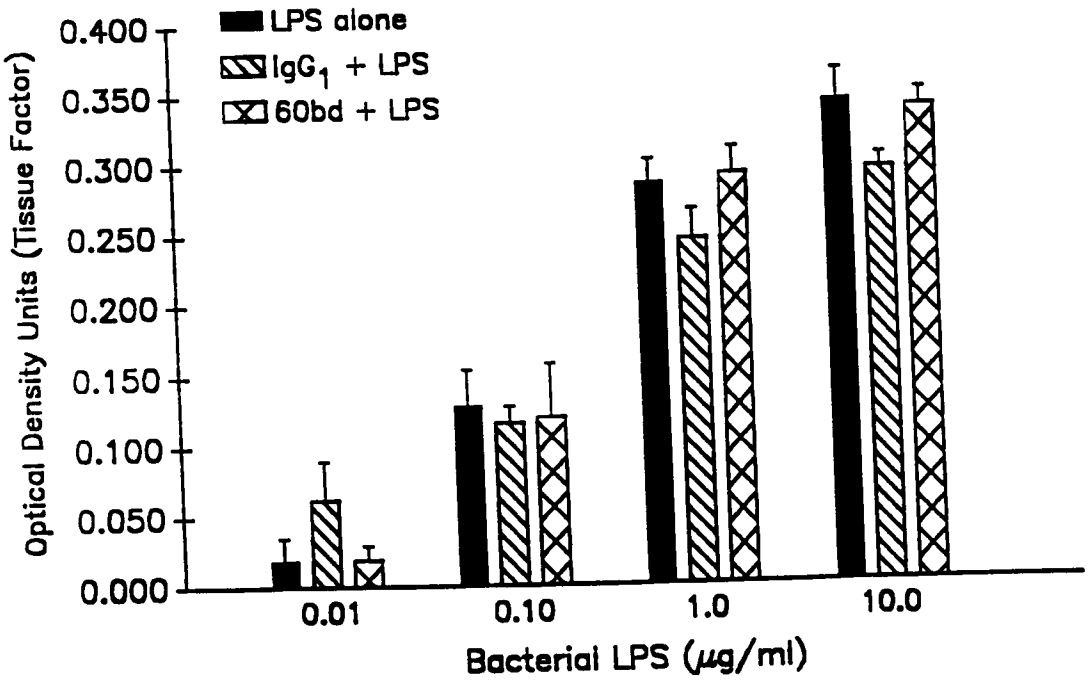

Figure 2 The Effect of Anti-CD14 MAb on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS in the Absence of Serum Fraction 2. Preincubation with 60bd for 30 min had no significant effect on TF expression when the cells were stimulated for 4 h with LPS in the absence of serum fraction 2.

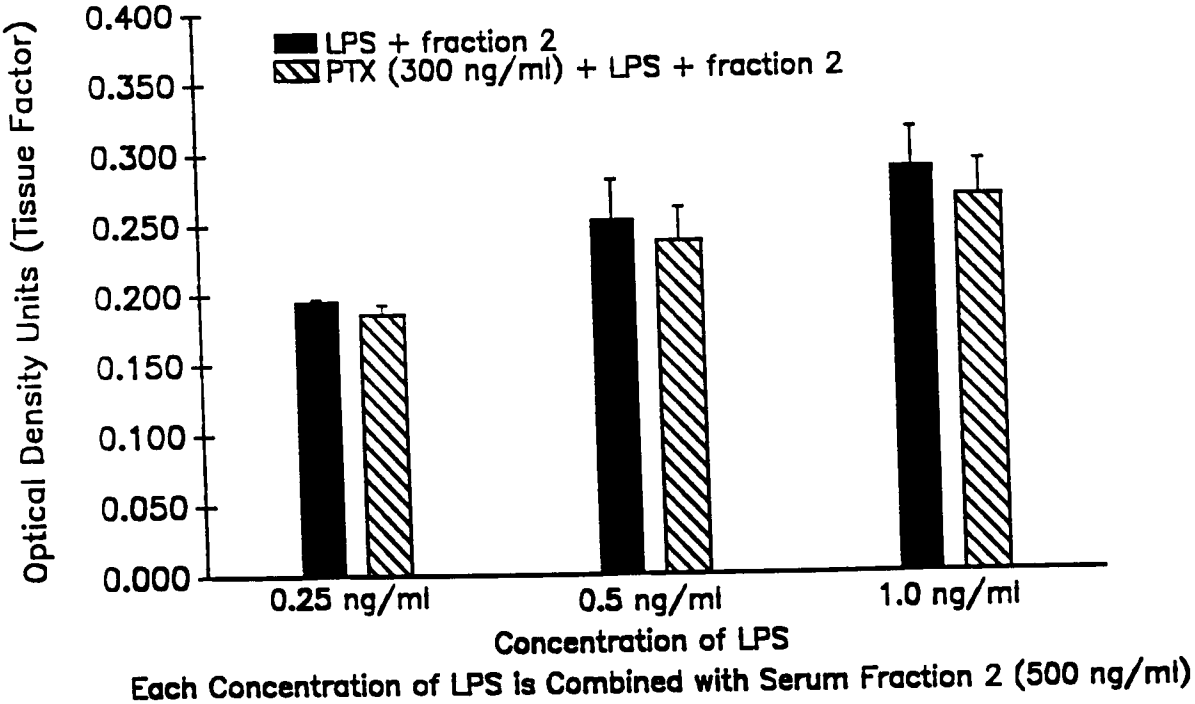

Figure 3 The Effect of Pertussis Toxin (PTX) on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS in Combination with Serum Fraction 2. Preincubation of cells for 2 h with PTX (300 ng/ml) failed to have a significant inhibitory effect on TF expression when the cells were stimulated with 0.25, 0.5, or 1.0 ng/ml LPS in combination with serum fraction 2.

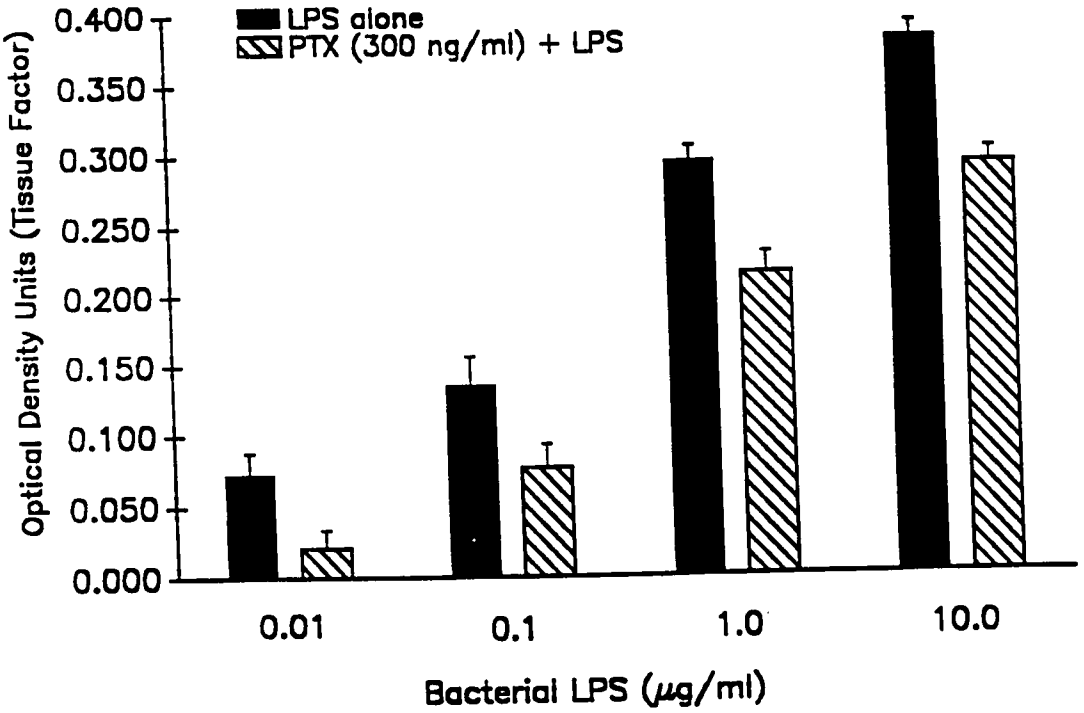

Figure 4 The Effect of PTX on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS in the Absence of Serum Fraction 2. When the cells were incubated with 0.01, 0.1, 1.0, or 10.0 μg/ml LPS in the absence of serum fraction 2, preincubation for 2 h with PTX (300 ng/ml) reduced TF expression by 23 to 71% (P<0.0319).

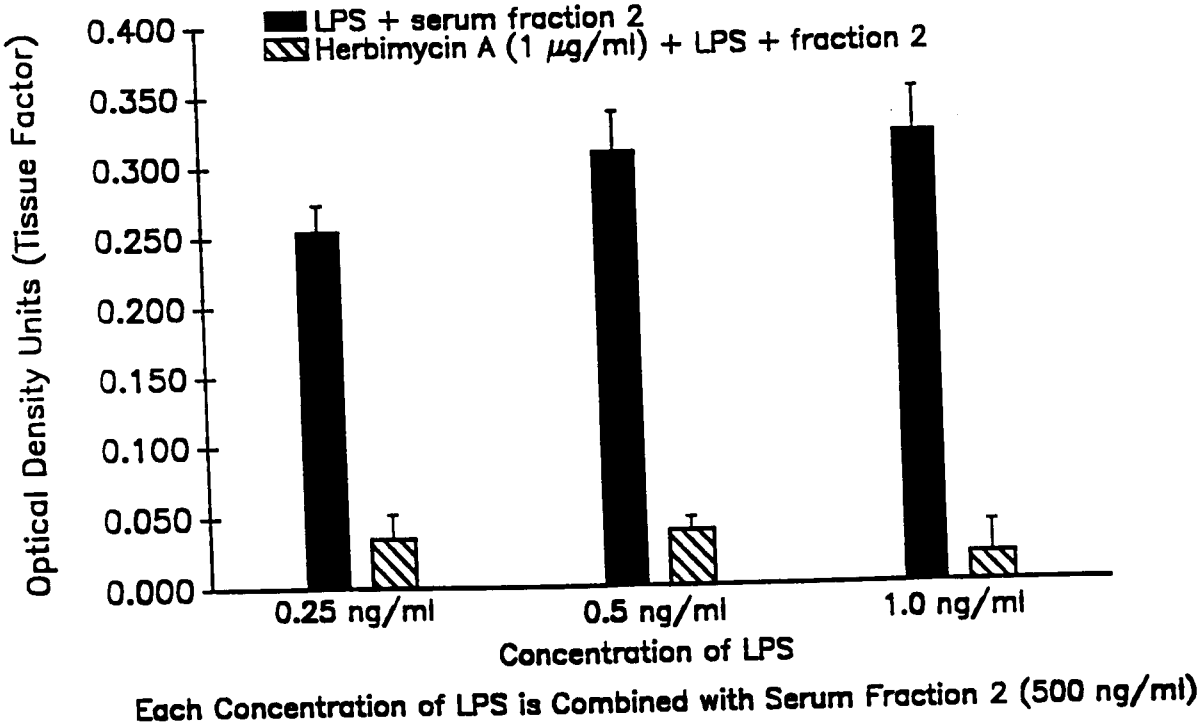

Figure 5 The Effect of Herbimycin A on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS in Combination with Serum Fraction 2. Preincubation for 30 min with herbimycin A (1 μg/ml) produced a profound inhibitory effect on the expression of TF by the cells following stimulation with 3 different concentrations of LPS in combination with a fixed concentration of serum fraction 2 (500 ng/ml). The inhibition ranges from 86% at 0.25 ng/ml to 94% at 1.0 ng/ml (P<0.0001).

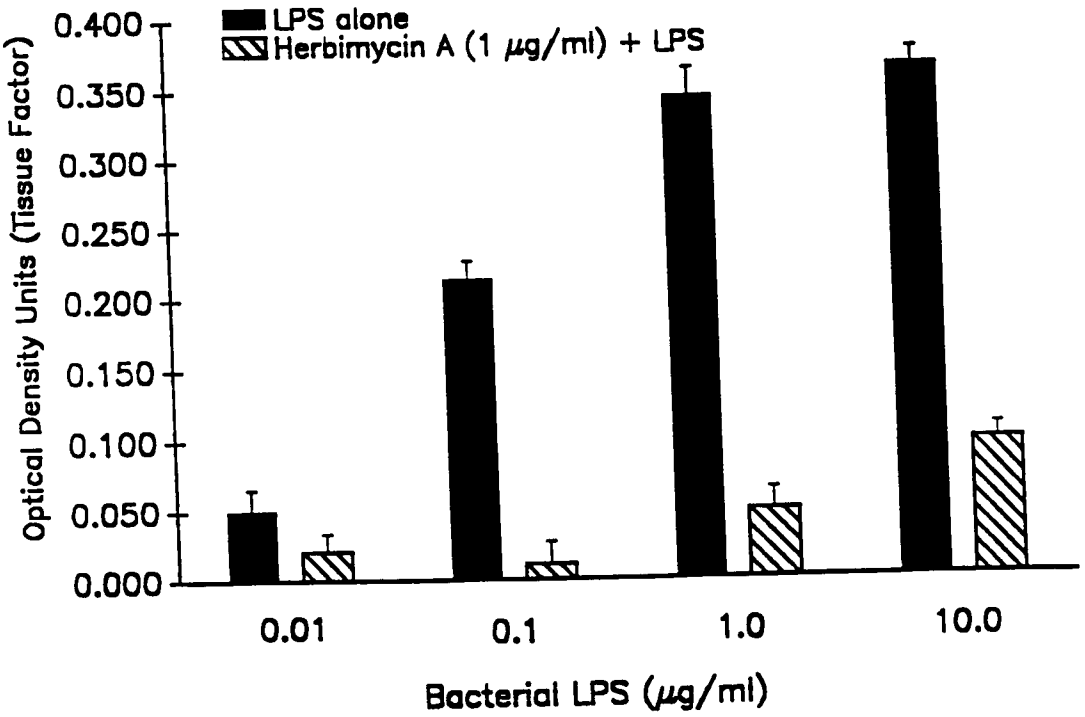

Figure 6 The Effect of Herbimycin A on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS in the Absence of Serum Fraction 2. Preincubation for 30 with herbimycin A (1 μg/ml) followed by incubation for 4 h with 0.01, 0.1, 1.0, or 10.0 μg/ml LPS in the absence of serum fraction 2, resulted in a reduction in the expression of TF ranging from 59% at 0.01 μg/ml to 96% at 0.1 μg/ml (P<0.0001).

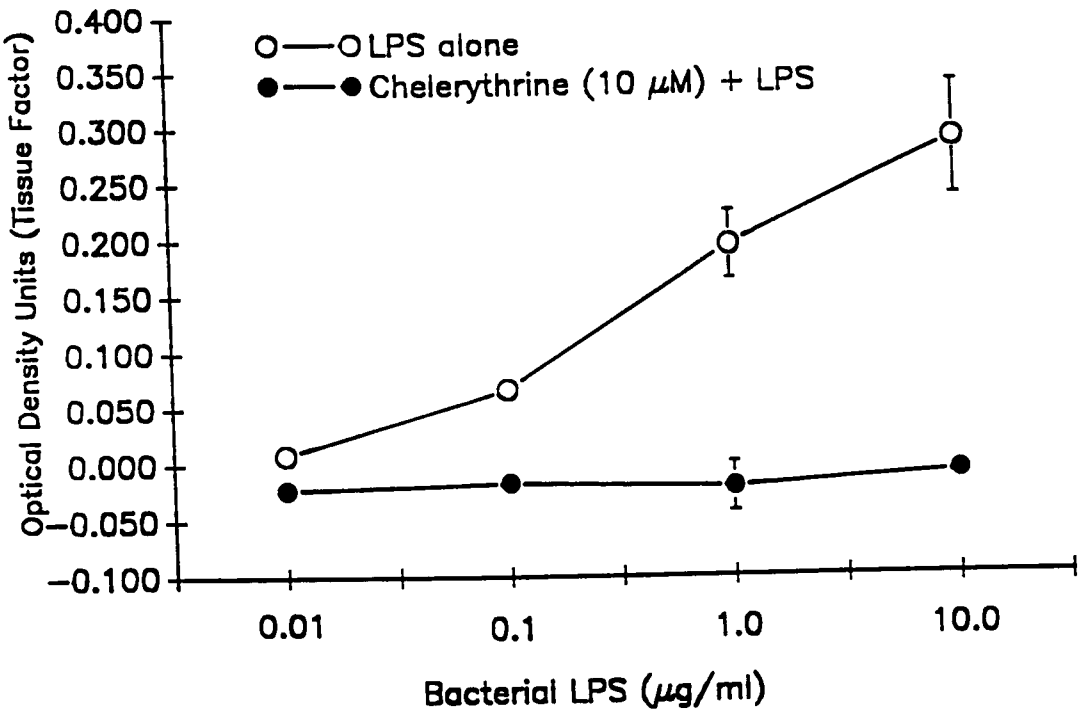

Figure 7 The Effect of Chelerythrine on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS. Preincubation of the cells for 30 min with chelerythrine (10 μM) prior to stimulation for 4 h with one of 4 concentrations of LPS resulted in a 58 to 156% reduction in TF expression ($P<0.0001$).

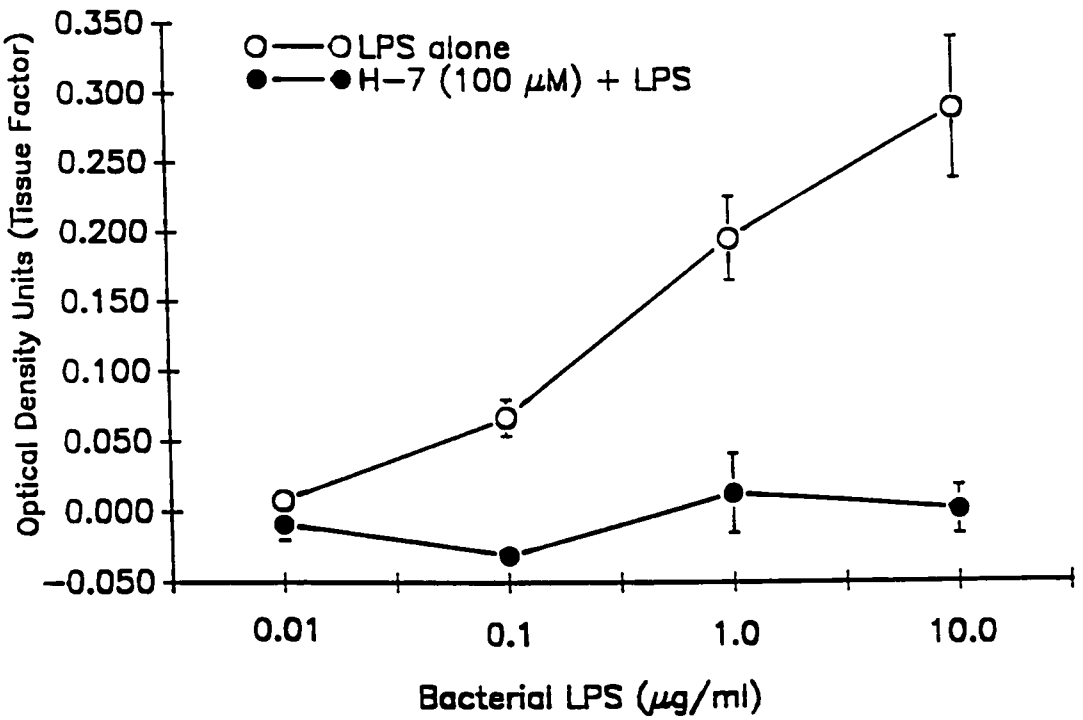

Figure 8 The Effect of H-7 on TF Expression by Bovine Alveolar Macrophages Stimulated by LPS. Pretreatment of the cells for 30 min with the PKC inhibitor H-7 (100 μM) resulted in inhibition of TF expression ranging from 93 to 213% (P<0.0001).

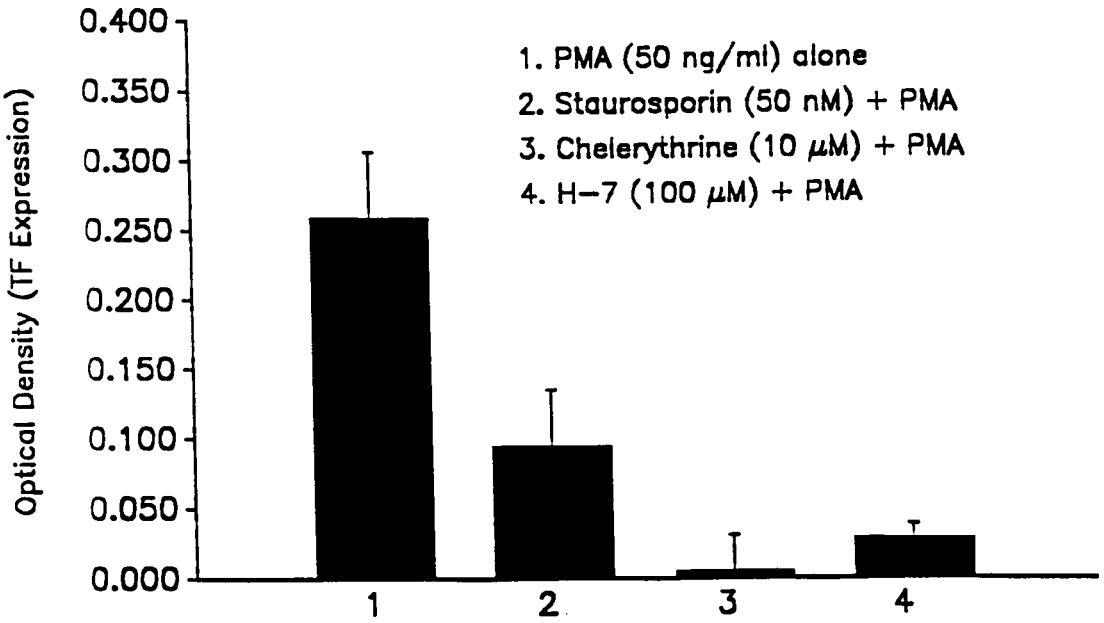

Figure 9 The Effect of Three Known Inhibitors of Protein Kinase C on TF Expression by Bovine Alveolar Macrophages Stimulated by PMA. Cells were preincubated for 30 min with one of three known inhibitors of protein kinase C prior to incubation for 4 h with PMA. Procoagulant activity induced by the known PKC agonist, PMA (50 ng/ml), was reduced by 63% in cells pretreated with staurosporin (50 nM), by 111% with chelerythrine (10 μM), and by 98% with H-7 (100 μM).

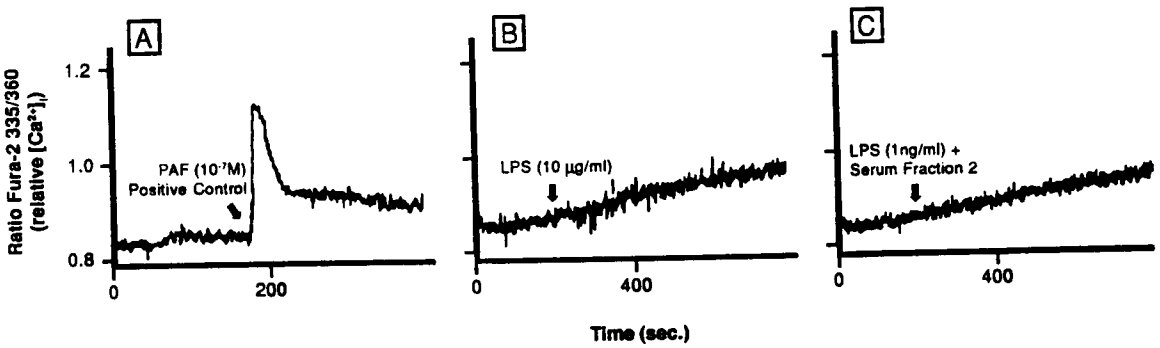

Figure 10 The Effect of LPS on Cytoplasmic Calcium ($[Ca^{2+}]_i$) in Alveolar Macrophages. The addition of Platelet activating factor (PAF)(10^{-7}M) to the medium **(a)** typically resulted in a 5-fold increase in $[Ca^{2+}]_i$ (19 µM to 120 µM). Stimulation of the cells with LPS alone (10 µg/ml) **(b)** or with LPS (1 ng/ml) in combination with serum fraction 2 (500 ng/ml) **(c)** failed to affect $[Ca^{2+}]_i$. The slow but stead rise in flourescence observed in **(b)** and **(c)** is due to leakage of dye from the cells which normally occurs under these experimental conditions.

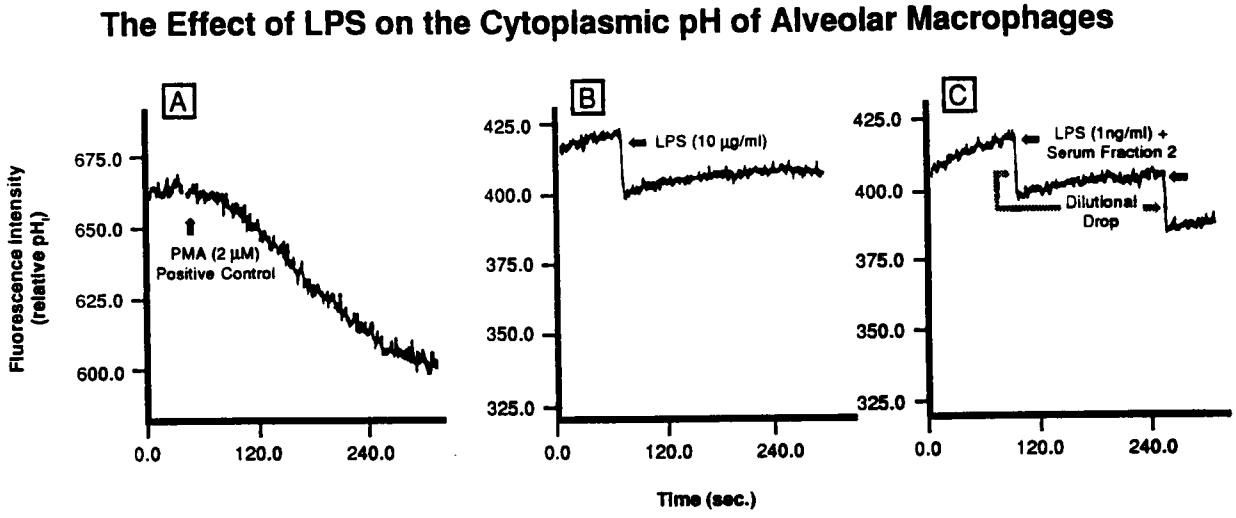

Figure 11 The Effect of LPS on the Cytoplasmic pH (pH_i) of Alveolar Macrophages. The addition of PMA (50 ng/ml) to the medium resulted in a precipitous reduction in pH_i **(a)**. Stimulation of the cells with LPS (10 µg/ml) alone **(b)** or with LPS 1ng /ml in combination with serum fraction 2 (500 ng/ml) **(a)** failed to affect pH_i.

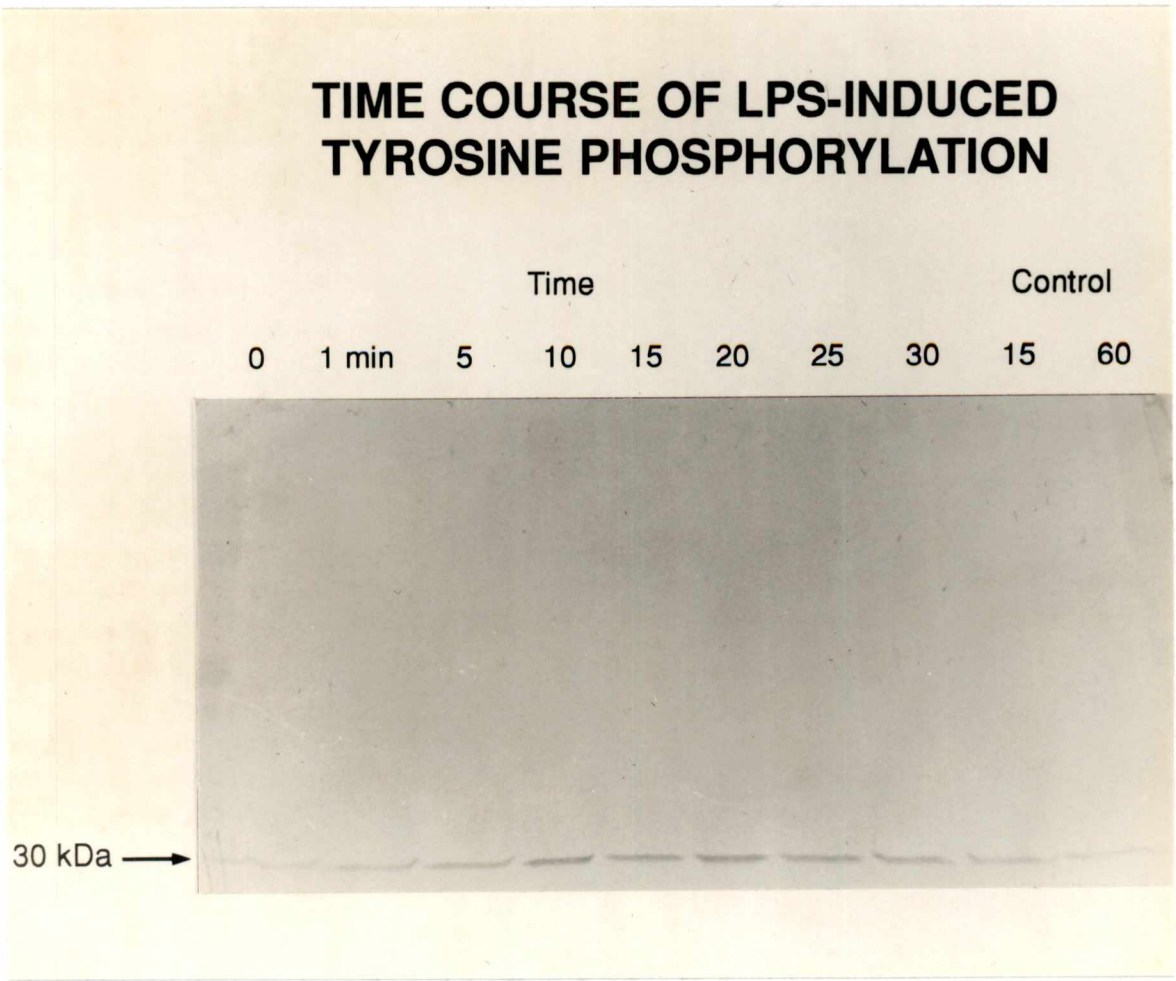

Figure 12 LPS-induced tyrosine phosphorylation as shown by anti-phosphotyrosine immunoblotting. LPS (10 µg/ml) rapidly increased tyrosine phosphorylation of a solitary polypeptide with a molecular mass of 30 kDa.

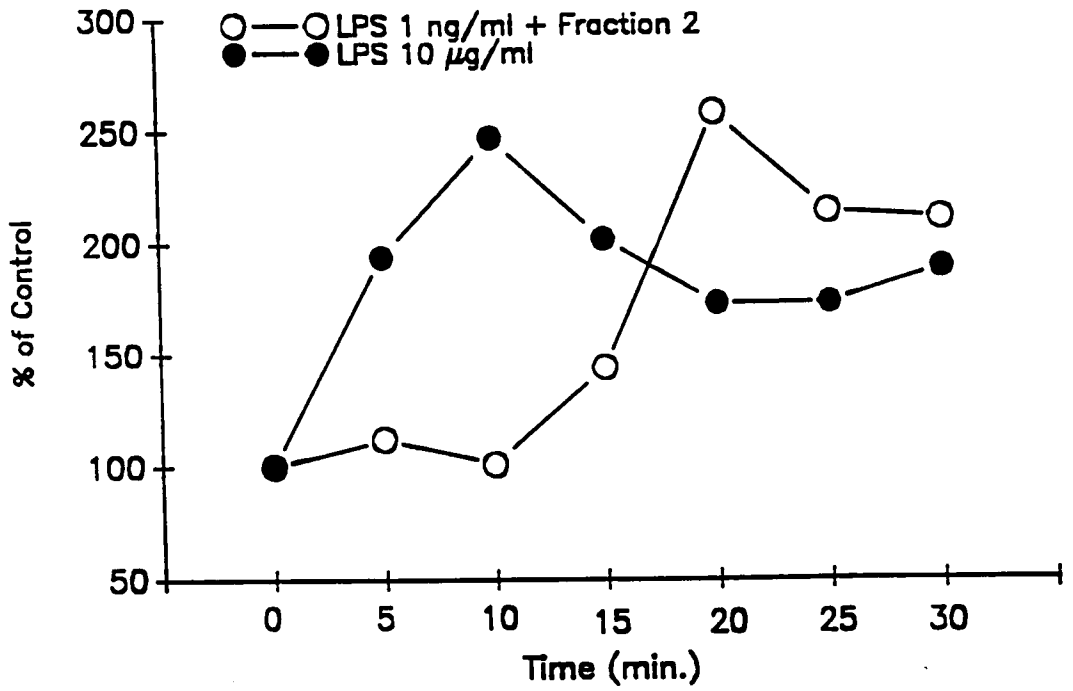

Figure 13 Comparison of time courses of tyrosine phosphorylation induced by LPS alone (10 µg/ml) and LPS (1 ng/ml) in combination with serum fraction 2 (250 ng/ml). Increased tyrosine phosphorylation was maximum in 10 min when cells were stimulated with LPS alone while maximum tyrosine phosphorylation occurred at 20 min when the cells were stimulated with LPS in combination with serum fraction 2.

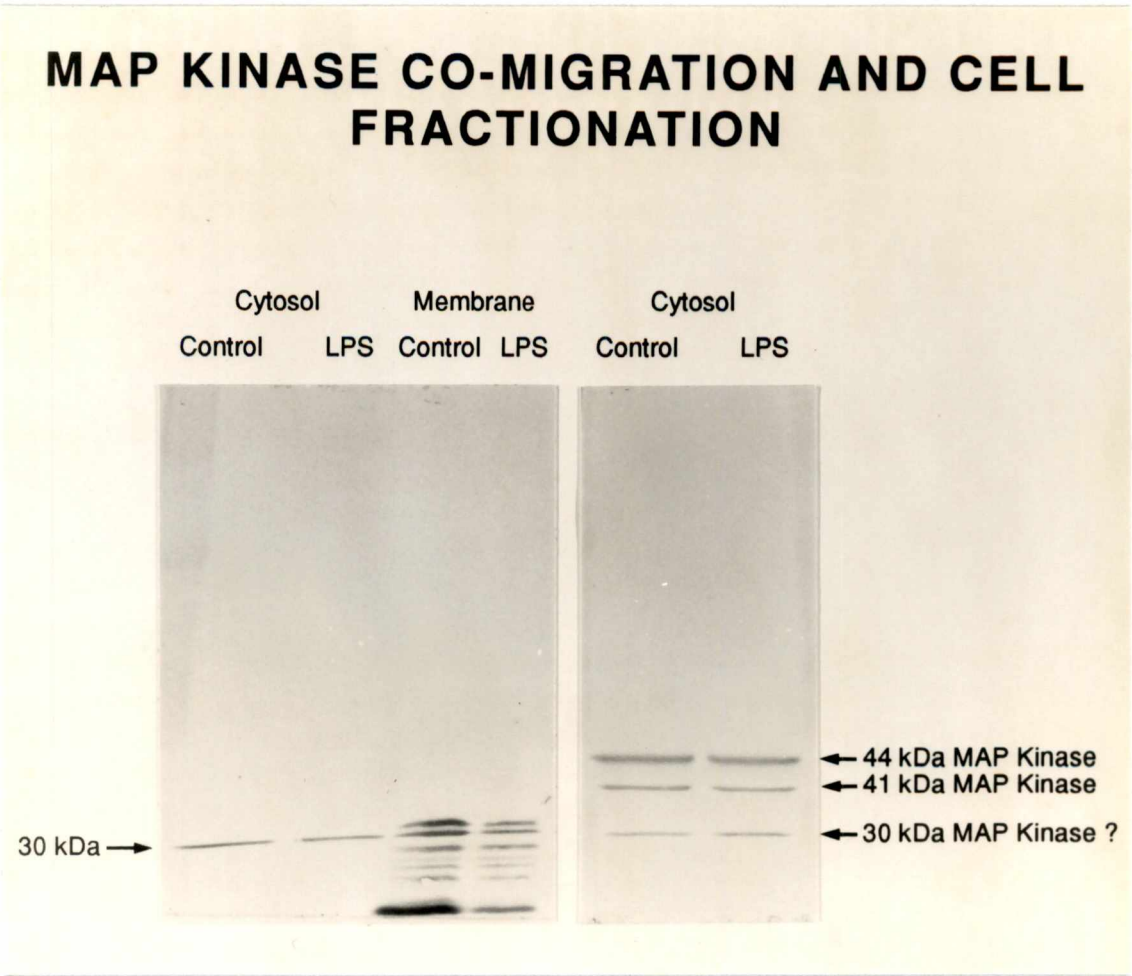

Figure 14 Cell fractionation and MAP kinase co-migration study. Stimulation by LPS (10 µg/ml) resulted in a higher intensity of tyrosine phosphorylation within the cytosolic fraction as compared to control. Application of anti-rat MAP kinase polyclonal antibody to the cytosolic fraction revealed comigration of the band stained with anti-phosphotyrosine antibody (PY-20) and the anti-MAP kinase antibody. Previously identified MAP kinases with molecular masses of 41 and 44 kDa were also stained by the anti-MAP kinases polyclonal antibody.

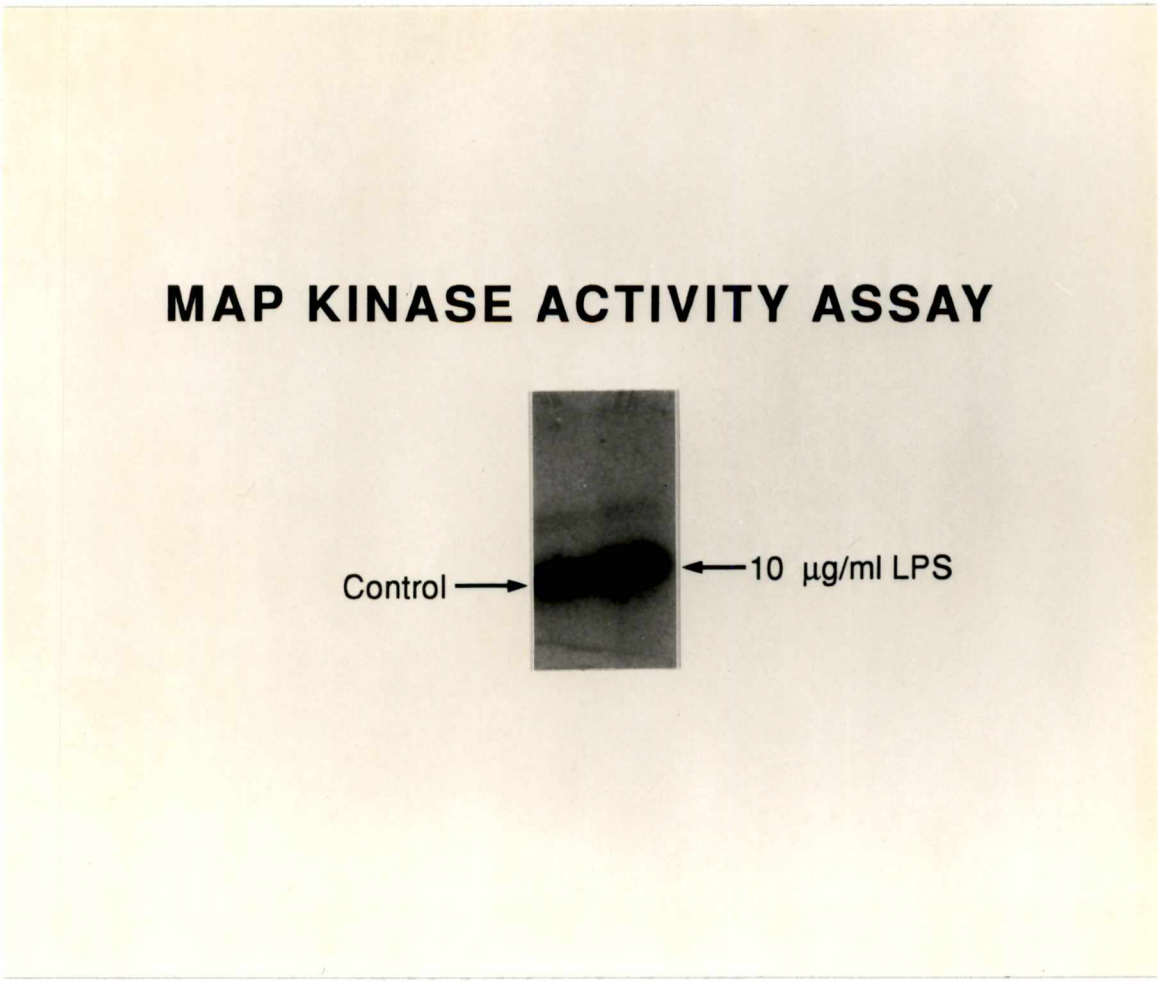

Figure 15 MAP kinase activity assay. Cell lysates were analyzed for MAP kinase activity using Myelin Basic Protein (MBP) as a substrate. There was 38% greater MAP kinase activity in the LPS-stimulated cells as compared to untreated cells.

VITA

David F. Dean was born in Washington D.C. on May 30, 1952. He attended primary and secondary schools in Rancho Palos Verdes, California. He received an Associate in Science degree in Animal Health Technology from Los Angeles Pierce College in 1979. In 1987 he received a Doctor of Veterinary Medicine degree from the University of Tennessee. Following two years as an associate in a companion animal practice in Greenville, South Carolina, he returned to the University of Tennessee to begin a residency training program in anatomic pathology and to pursue a Ph. D. in the Department of Pathobiology under the supervision of Dr. David O. Slauson. In the Spring of 1994 he completed residency training and received a Ph. D. in Comparative and Experimental Medicine.