Analysis of eicosanoids released from activated macrophages

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ANALYSIS OF EICOSANOIDs RELEASED
FROM ACTIVATED MACROPHAGES

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Presented in partial fulfillent of the requirements for
the degree of
Master of Science
University of Montana
1998

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4-30-98
Date
Macrophages are a major source of the eicosanoid derivatives of arachidonic acid, which mediate a number of reactions that have important biological functions. In this study, we developed a C18 minicolumn procedure for rapidly separating eicosanoid mixtures into prostaglandin, leukotriene, and arachidonic acid fractions. The procedure was then used to quantify eicosanoids released from macrophages (both human THP-1 cells and mouse peritoneal exudate cells) activated with different bacterial amphiphiles (lipopolysaccharide (LPS), lipoteichoic acid (LTA), and monophosphoryl lipid A), ceramide, and phorbol myristate acetate (PMA). Arachidonic acid was the main product in eicosanoid mixtures released from both cell lines. The prostaglandin pathway was activated in mouse PEC, but not in human THP-1 cells. There were marked differences in the relative potency of different amphiphile preparations for the activation of both cell types. Pretreatment of THP-1 cells with γ-interferon was not required for the activation of arachidonic acid metabolism in the human THP-1 cell line. Treatment with R595 LPS desensitized mouse PEC to subsequent challenge with R595 LPS, but not with either C2-ceramide or PMA. HPLC analysis confirmed that arachidonic acid was the main product released from activated THP-1 cells and suggested that the eicosanoid mixtures released from activated cells contained products other than prostaglandins, leukotrienes, and arachidonic acid.
ACKNOWLEDGMENTS

With my sincerest gratitude and appreciation, I would like to thank my advisor, Dr. George Card, for his guidance, patience, and friendship throughout my studies at the University of Montana. His advice, support and encouragement made the completion of this study possible. He has also seen me through many difficult times in my life and has helped me grow as a student and a person. Also, I would like to thank the members of my Master's committee, Dr. Michael Minnick and Charles Eyer for their invaluable advise and suggestions. Thanks to the Stella Duncan Research Institute and Ribi ImmunoChem Research Inc. for their support. I also wish to give special thanks to Jean Pfau for her continued patience and help that she has provided to me during the time I worked with her in Dr. Card's laboratory.

Finally, but most importantly, I dedicate this work to my family - my parents and grandmother. Their love, encouragement, and silent sacrifices over the years have helped me through thick and thin, and gave me strength throughout the study. With love I would like to thank my dear grandmother, who passed away during the time when I was working on my degree. Her unlimited love, support, understanding and guidance have made me who I am today. I love her dearly.
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Chapter I

Introduction

Activated macrophages play a critical role in host defense against tumors and microbes, in the development of an inflammatory response, in homeostasis and in diseases such as atherogenesis, and carcinogenesis. Macrophages possess numerous physiologic properties that may be modulated during activation. These include alterations in intracellular constituents, plasma membrane components, and secretion of both protein and lipid mediators (1).

As shown in Fig 1, a variety of metabolic activities have been described in activated macrophages. The metabolic activities which are commonly used to measure macrophage activation include the respiratory burst, secretion of proteins and glycoprotein mediators, release of tumor necrosis factor (TNF) and other cytokines, display of altered surface properties, and the release of arachidonic acid metabolites (eicosanoids) (4).

Arachidonic acid is an essential fatty acid which is incorporated into membrane phospholipids. The products of arachidonic acid metabolism (eicosanoids) mediate a number of reactions that have various important biological effects. It has also been suggested that the release of other mediators (e.g. cytokines) may be dependent on the activation of arachidonic acid metabolism. Macrophages are a major source of the eicosanoids and, by consideration of their importance in host defense, it is clear that their
Inflammation
- Interleukin 1
- Prostaglandins, Leukotrienes
- Complement components
- Clotting factors

Bacterial destruction
- Oxygen metabolites
- Lysozyme
- Acid hydrolases
- Cationic proteins

Tissue damage
- Oxygen metabolites
- Acid hydrolases
- C3a

Tissue healing
- Elastases
- Collagenases
- Interleukin 1
- Hyaluronidase

Lymphocyte activation
- Antigen presentation
- Antigen processing
- Interleukin 1

Tumor destruction
- Oxygen metabolites
- Tumor necrosis factor
- C3a
- Proteases

Fig. 2. Secretory products of a macrophage.
release constitutes an important aspect of macrophage function.

The activity of macrophages is regulated by extracellular signals which can either enhance or diminish the ability of macrophages to carry out a certain function. Bacterial amphiphiles, including lipopolysaccharides, lipoteichoic acids, monophosphoryl lipid A, etc., are substances which can enhance the immune response by causing sensitive cells, such as macrophages, neutrophils and lymphocytes, to release a variety of mediators (4). These mediators act as messengers which have many significant biological functions, including inflammation (interleukins, eicosanoids), tumor destruction (tumor necrosis factor), lymphocyte activation (interleukins). This study was focused on the release of eicosanoids (arachidonic acid metabolites) by the macrophages (murine and human THP-1) activated with different bacterial amphiphiles.

**ARACHIDONIC ACID**

Eicosanoids are derivatives of arachidonic acid, a C20 polyunsaturated fatty acid (5-cis-, 8-cis-, 11-cis-, 14-cis-eicosatetraenoic acid) (4) (Fig. 2). After activation of phospholipase A2, arachidonic acid is released from membrane phospholipid, and is then converted to different eicosanoid products. There are three major classes of eicosanoids which are produced by three major eicosanoid biosynthetic pathways: the cyclooxygenase pathway (prostaglandins), the
5'-lipoxygenase pathways (leukotrienes), and the 12'- and 15'-lipoxygenase pathway (lipoxins).

The synthesis of the prostaglandin family begins with the enzyme cyclooxygenase. Phospholipase A2, which liberates arachidonic acid from the phospholipids in cell membranes, is the rate-limiting step in eicosanoids synthesis (Fig. 3, 4).

LEUKOTRIENES

The leukotrienes (LTs) are a family of biologically active lipids derived from arachidonic acid by the action of a 5'-lipoxygenase (26). They are produced from polymorphonuclear cells, neutrophils, eosinophils, monocytes, lymphocytes, macrophages, and mast cells (16). This family includes the dihydroxyeicosanoid LTB4, a potent chemotactic agent, and the peptidolipid leukotrienes LTC4, LTD4 and LTE4, which are responsible for the potent smooth muscle contractile effect of slow-reacting substance of
Fig. 3: Arachidonic acid pathways

Fig. 4: Eicosanoid biosynthetic pathways
anaphylaxis (SRS-A)(26). Leukotrienes C4 and D4 have been shown to be potent myotropic agents, while Leukotriene E4 is somewhat less potent. These and other products of the lipoxygenase-pathway have been implicated in the pathology of asthma and other hypersensitivity reactions (6). In addition, the eukotrienes have potent effects on white cell function, (including a variety of phagocytic cells, as well as lymphocytes), bronchial tone, and several other biological target systems (21).

Because of so many important biological functions and profound biochemical effects, this family has been the subject of intense interest and there has been considerable recent interest in quantifying these compounds in biological samples.

PROSTAGLANDINS

Prostaglandins are cyclopentanoic acids derived from arachidonic acid which exert hormone-like effects in many physiological processes. The primary prostaglandins are divided into three types based on the functionality in the cyclopentane ring - E type (β-hydroxy-ketones), F type (1,3-diols), and A type (α,β-unsaturated ketones). The natural prostaglandins are also grouped into the mono-, bis-, or trisunsaturated classes, according to the number of carbon-carbon double bonds in the parent E-type prostaglandin. All of the prostaglandins have in common the prostanoic acid
skeleton, and typical prostaglandins, like PGE2, have four oxygen-functionalized carbon atoms.

The biosynthesis of prostaglandins is initiated by an enzyme associated with the endoplasmic reticulum, called prostaglandin endoperoxide synthase. The enzyme catalyzes simultaneous oxidation and cyclization of arachidonic acid. The enzyme has two distinct activities, cyclooxygenase and peroxidase (Fig. 5).
Fig. 5: Prostaglandin pathways and species
Prostaglandins are released from many types of tissue in response to injury such as anaphylactic shock, mechanical trauma, and inflammation. Pharmacological properties of prostaglandins include vasodilatation, increased vascular permeability, and leukocytic emigration (10). Prostaglandins cause either vasoconstriction or vasodilatation, depending on the type of prostaglandin, the type of vascular bed and the animal species. Prostaglandins of the A and E series, in most vascular beds, induce vasodilatation of both arterioles and venules. In man, intravenous prostaglandins cause headache and also pain along the veins into which they are infused. When administered intradermally or intramuscularly in concentrations much higher than those expected to occur in inflammation, PGE1 causes a long-lasting overt pain. With PGF2a, there is an initial brief effect followed by a delayed gradual increase. In addition, PGE1 can enhance the granuloma formation by cotton pellets and increase collagen synthesis in chick embryo tibiae. It is also the most powerful pyretic agent known, when injected either into cerebral ventricles or directly into the anterior hypothalmus (10).

Prostaglandins are likely to be important in bone metabolism because they are potent multifunctional regulators of both formation and resorption. They are produced in abundance by bone cells, and by the cells adjacent to the bone in the marrow and the periosteal tissues. Moreover, prostaglandins probably mediate the
effects of mechanical forces on the skeleton. Thus they are likely to play a critical role in skeletal physiology and in the pathologic responses of bone. Recent studies have confirmed that prostaglandins, particularly PGE2, are potent stimulators of resorption that act by increasing both the replication and differentiation of osteoclast precursors. Prostaglandins have biphasic effects on bone formation. An inhibitory effect on collagen synthesis can be demonstrated in cell and organ culture and appears to be transcriptional, acting through a PGF receptor that activates protein kinase C (25). Several recent studies have shown that the lung is one of the major sites of prostaglandin synthesis and inactivation. A variety of physiological and pathological stimuli including hypoxia, anaphylaxis and hyperinflation cause the release of prostaglandins from lung. Thus, it has been suggested that there is a relationship between the ability of the lung to handle prostaglandin synthesis, release and metabolism, and pulmonic diseases. In contrast to the peptido-leukotrienes, inhaled prostaglandin E2 attenuates smooth muscle constriction in airway passages (23).
**BACTERIAL AMPHIPHILES**

The bacterial amphiphiles which will be used in this study include: lipopolysaccharide (R-form LPS and S-form LPS), monophosphoryl lipid A (MLA) and lipoteichoic acids (LTA) from different gram-positive bacteria.

**LIPOPOLYSACCHARIDES**

Lipopolysaccharide (LPS) is the endotoxin which is localized on the surface of bacterial cells, and, together with phospholipids and proteins, forms the outer membrane of Gram-negative bacteria (5). It can produce adverse reactions including circulatory and metabolic failures and often lethal shock in animals. These effects have been attributed largely to the release of several chemical mediators such as interleukins, TNF, and eicosanoids from macrophages. It is well known that LPS is able to activate the complement cascade and prime polymorphonuclear leukocytes or monocytes-macrophages to release these various mediators. The in vitro culture of polymorphonuclear leukocytes or peritoneal macrophages (PM) with LPS promotes an enhanced respiratory burst and release of eicosanoids, events considered important in the defense against bacterial infection as well as in the progress of tissue damage (31). The influence of endotoxin on the biosynthetic processes of eicosanoids remains unclear (8).

LPS is composed of three distinct structural parts:
O-antigenic repeating polysaccharide, core-oligosaccharide, and a hydrophobic component designated lipid A (29) (Fig. 6). There is considerable variation in the amounts of polysaccharide in LPS so that some LPS consists predominantly of lipid A and is called R-form LPS, whereas others have abundant polysaccharide (the O-side chain) and is called S-form LPS. Although the lipid A part of LPS is responsible for the endotoxic activities, the polysaccharide moiety influences the elimination kinetics, mediates specific LPS binding with macrophages and lymphocytes, and is essential for activation of the alternate pathway of the complement system (8). Since R-form and S-form LPS differ in their relative contents of polysaccharide, it is possible that they have differential effects on the release of chemical mediators and subsequent pathophysiological consequences.
Monophosphoryl lipid A (MLA) is an attenuated derivative of LPS that lacks many of the endotoxic properties of the parent molecule and yet retains potent adjuvant and immunostimulating activities (Fig. 7). MLA, marketed as MPL immunostimulant, has been tested in several clinical trials involving a variety of indications, formulations, and routes of administration. Many studies have shown that MLA is safe in humans at doses that are active with respect to a number of immunological markers, including induction of cytokines, activation of cytotoxic T cells, and protection against a subsequent endotoxin challenge (22). Other evidence has shown some relationship between MLA and several mediators: MLA induced rapid accumulation of interferon-gamma (IFN-γ) in mice.

Fig. 7: Structure of monophosphoryl lipid A (MLA)
Tumor necrosis factor-alpha (TNF-α) appeared to be a cofactor for IFN-γ induction by MLA. With low dose of MLA (<5 micrograms), IFN-γ induction was dependent upon exogenous TNF-α administered either in advance of or with MLA (13).

The monophosphoryl lipid A homologs derived from *Salmonella minnesota* strain R595 have been characterized by the combination of FAB-MS (Fast atom bombardment and mass spectrometry) and 2D nuclear magnetic resonance spectroscopy which included a detailed evaluation of the hepta-, hexa-, and pentaacyl MLA (5). This study was interested in determining the effects of this partial structure of the lipid A moiety of gram-negative lipopolysaccharide, monophosphoryl lipid A, on eicosanoid metabolism in macrophages.

**LIPOTEICHOIC ACID**

Lipoteichoic acid (LTA) is a different type of amphiphile which is produced by Gram-positive bacteria (11) (*Fig. 8*). Most LTAs which have been characterized consist of a 1,3, linked polyglycerophosphate moiety in a phosphodiester linkage to a membrane hydrophobic glyceroglycolipid which is anchored at the outer surface of the cytoplasmic membrane, with the glycerophosphate chain extending into the environment (4). This glyceroglycolipid anchor is usually composed of a disaccharide unit linked to a diacylglycerol. The length of the polyglycerophosphate
chain varies from 16 to 40 glycerophosphate units, depending on the bacterial species (11,33).

Fig. 8: Structure of lipoteichoic acid (Staphylococcus aureus)

The number of group substitutions in the glycerol residues also varies among bacterial species. Bacterial LTAs can be divided into four groups based on the chain substitution: group A lacks substituents; group B carries only D-alanine substituents; group C carries only glycosyl substituents; and group D carries both of these substituents (4).

It has been well known that the inflammatory components implicated in gram-positive bacteremia are cell wall components, toxins, and enzymes. The role of LTA in gram-positive bacterium inflammation has not been well documented. Some recent research reported that although it was relatively non-toxic, lipoteichoic acids could activate arachidonic acid metabolism and eicosanoid release from
macrophages (17). There is some evidence to show that there are humoral and cellular factors that recognized both LPS and LTA, and LTA had some immunological and biological properties in common with LPS (2,14).
OBJECTIVES

The major objective of this study was to compare the relative amounts of leukotrienes, prostaglandins, and arachidonic acid released from macrophages activated with different bacterial amphiphiles, including ipopolysaccharide (S-form LPS and R-form LPS) and monophosphoryl lipid A (MLA) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, and phorbol myristic acetate (PMA).

SPECIFIC AIMS

(1). An analytical strategy was developed for the rapid extraction, separation, and quantification of the relative amount of leukotriene, prostaglandin and arachidonic acid released from activated macrophages. Procedures currently available for eicosanoid analysis are expensive (e.g. EIA, RIA) and/or very time consuming (e.g. HPLC). Although these have been useful for some clinical studies and other experiments involving a limited number of samples, they are not suitable for the analysis of a large number of samples.

(2). This procedure was used for an analysis of the relative amount of prostaglandin, leukotriene, and arachidonic acid released from macrophages treated with different bacterial amphiphiles.

The analytical strategy developed in Specific Aim#1 would allow the screening of a large number of samples. The working assumption is that substances
which activate cells to produce markedly different mixtures of prostaglandins and leukotrienes will have different activities in whole animals.

(3). Selected amphiphiles (based on the results obtained in Specific Aim #2) were used to study the relative kinetics of prostaglandin and leukotriene release from activated macrophages.

Some studies suggest that the accumulation of one eicosanoid (e.g. PG-E2) may cause a down-regulation of the synthesis of other eicosanoids.

(4). Samples which showed significant differences of the relative amounts of leukotriene and prostaglandin were selected for complete analysis by HPLC and EIA assay. These procedures provide the quantitative analysis of each of the major prostaglandins and leukotrienes as well as arachidonic acid and other derivatives. Likewise, samples from the kinetic experiments which showed significant changes of eicosanoid families over time were analyzed with HPLC and EIA assay.
Chapter II

Materials and Methods

Materials

Octadecyl (C18) minicolumns were purchased from Amersham International (Amersham Place, Buckinghamshire, England). Labelled fatty acid and eicosanoids were purchased from American Radiolabeled Chemicals Inc (St.Louis, MO). Unlabeled lipids, Cellgro RPMI 1640 medium, and fetal bovine serum (FBS) were obtained from Sigma Chemicals (St.Louis, MO). Six to ten week-old ICR mice were obtained from Jackson laboratory. THP-1 cells, LPS preparations, and vitamin D3 (α-1, 25 dihydro-xyvitamin D) were provided by Ribi ImmunoChem Research Institute (Hamilton, MT). LTA preparations were from Sigma Chemicals (St. Louis, MO), and Ecolume fluor was obtained from ICN Biochemicals (Irvine, CA). Prostaglandin E2 and Leukotriene C4 Enzyme Immunoassay Kits were obtained from Cayman Chemical Company (Ann Arbor, MI).

Fractionation of eicosanoids on C18 minicolumns

Initial studies were focused on the development of one-step column-centrifugation procedures for the rapid separation of leukotrienes, prostaglandins and arachidonic acid. The reverse-phase C18 bonded silica gel has been proven to be very effective for extraction of prostaglandins.
and leukotrienes from biological fluids (27). Known mixtures of prostaglandin E2 (PGE2), leukotriene D4 (LTD4) and arachidonic acid (AA) containing tritium-labelled lipid were used to evaluate the separation procedures. Three mixtures of known lipids were used; each contained a mixture of unlabelled PGE2 (280 pmole), LTD4 (200 pmole), and arachidonic acid (1000 pmole) plus one labelled lipid. Mixture 1 contained the unlabelled lipid mixture plus $^3$H-PGE1 (0.1 μCi), mixture 2 contained the unlabelled lipid mixture plus $^3$H-LTD4 (0.01 μCi), and mixture 3 contained the unlabelled lipid mixture plus $^3$H-arachidonic acid (0.15 μCi).

Solvent systems and elution schemes evaluated in this study were selected from solvent systems which have been used in HPLC and TLC (thin layer chromatography) procedures for separation of these lipids (28,30). The following solvents were selected for the final elution scheme: solvent A (A), acetonitrile-methanol-ddH$_2$O-acetic acid-EDTA (1280:800:1916:4:2 grams); solvent B (B), solvent A-methanol (50:50); solvent C (C), solvent A-ddH$_2$O (75:25); solvent D (D), solvent A-methanol (75:25); and methanol. Samples were applied to the C18 minicolumns in water. Prostaglandins were eluted by successively pushing 1 ml each of solvents C, A, and D through the minicolumn with a 1 ml syringe. Leukotrienes were then eluted with solvent B, and finally arachidonic acid was eluted with methanol. Labelled lipid in
each fraction was counted in a Beckman LS7500 scintillation counter.

Macrophage Cultures

Two types of macrophage cultures were used in this study: mouse peritoneal exudate cells (mouse PEC) and human THP-1 monocytic cells. These two cell lines have been well characterized as excellent models for the study of murine and human macrophages, respectively.

Mouse Peritoneal Exudate Cells

Mice were killed with CO\textsubscript{2} and PECs were recovered by injecting 10 ml of RPMI medium containing 20 \mu g/ml of gentamicin into the peritoneal cavity. Peritoneal fluids were removed, transferred to a centrifuge tube, and centrifuged at 1000 rpm for 10 minutes. The cells were then resuspended in 10 ml of RPMI medium, counted using a hemacytometer, and the cell density was adjusted to 2 - 2.5 \times 10^{6} \text{ cells/ml}. The cell suspension was added (1.0 ml/well) to a 24 well flat-bottom plate. Cells were incubated at 37°C in a chamber with 5% CO\textsubscript{2} for 2 hours to allow macrophages to adhere. Non-adherent cells were then removed and fresh medium was added.

Human THP-1 Cells

Human THP-1 cells were grown in RPMI medium containing
10% FBS serum in 75 cm² sterile tissue culture flasks at 37°C in 5% CO₂. The cell density was adjusted to 1.5 - 2.5 x 10⁵ cells/ml.

**Bacterial Amphiphiles**

Bacterial amphiphiles which were used in this study included lipopolysaccharide (LPS) and lipoteichoic acid (LTA) preparations which have been shown to activate total arachidonic acid metabolism (4). LPS preparations included smooth LPS from *Salmonella abortus* (S-LPS), rough LPS from *Salmonella minnesota* R595 (R-LPS), diphosphoryl lipid A (DPL) and monophosphoryl lipid A (MLA) obtained from R-595 LPS, and deacylated LPS preparations. LTA preparations included LTA from *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *S. faecalis*.

**Labeling Procedure**

[^3]H] arachidonic acid was used to metabolically label the eicosanoids. Adherent mouse peritoneal exudate cells on the 24-well plate were washed with fresh medium, and then 1 ml of medium containing[^3]H]-arachidonic acid (0.5 μCi/well) was added to each well. Cells were then incubated overnight at 37°C in 5% CO₂.

Human THP-1 cells were pretreated with vitamin D3 (1 μM/ml) overnight in a 75 cm² sterile tissue culture flask at 37°C in 5% CO₂. After overnight pretreatment, cells were
harvested, counted, and transferred to a centrifuge tube, centrifuged at 1000 rpm for 10 minutes. Then the cells were resuspended in 24 ml of RPMI medium with 10% FBS serum containing $[^3]H$-arachidonic acid (0.5 μCi/ml). The cell suspension was then added (1.0 ml/well) to a 24 well flat-bottom plate, and incubated overnight at 37°C in 5% CO$_2$. At the end of the overnight incubation, the unincorporated label was removed and the cells were washed three times with fresh medium.

**Macrophage Challenge**

Cells were challenged in medium with serum with an amphiphiles, or ceramide, or phorbol myristate acetate (PMA). Cells were incubated at 37°C with 5% CO$_2$ for different challenging time period, and/or with different concentrations of the bacterial amphiphiles, as indicated. At the end of the incubation period the medium from each well was collected and centrifuged in an Eppendorf 5414 table top centrifuge to remove any cells. Lipids released from cells labeled with $[^3]H$-arachidonic acid was determined in 100 μl from each sample collected in scintillation vials with 4 mls of Ecolume fluor. The remaining 900 μl from each sample was analyzed with the C18 minicolumn separation procedure for analysis of the relative amount of prostaglandins, leukotrienes and arachidonic acid as described above.
Samples which showed significant differences in the relative amounts of leukotrienes and prostaglandins were selected for complete analysis by HPLC.

**HPLC Analysis**

The high-performance liquid chromatograph was performed with a 4.6 x 250 mm Silica C18 column, which was attached to a PERKIN-ELMER Series 410 LC Pump, a FLO-ONE\Beta Series A-100 radioactivity detector, and a PERKIN-ELMER LC-90 UV Spectrophotometric detector. The solvent system was a gradient of solvent A (acetonitrile:methanol:ddH2O:acetic acid:EDTA = 1280:800:1916:4:2 gram) and methanol eluted at the flow rate of 1 ml/min in the following order: 0 min, 100% A; 30 min, 100% A; 31 min, 100% methanol; 51 min, 100% methanol; 52 min, 100% A; 67 min, 100% A. Tritium-labelled PGE1, LTD4, and arachidonic acid were selected as standards which were the representative of different eicosanoid families for the radioactivity detector.
Chapter III

RESULTS

Binding Affinity of Different Eicosanoids on C18 Minicolumns

Initial studies were focused on an analysis of the binding affinity of eicosanoids on C18 minicolumns. Samples consisted of 280 pmole of unlabelled PGE2, 1100 pmole of unlabelled arachidonic acid, and 200 pmole of unlabelled leukotriene D4 plus either 0.1 μCi of $^3$H-PGE1 or 0.01 μCi of $^3$H-LTD4 in 1 ml of RPMI with 10% FBS culture medium. RPMI medium with 10% FBS serum, which was used for culturing both mouse and human THP-1 macrophages, was added to make the final value of each sample to be 1 ml. A 50 μl aliquot from each sample was removed to determine total CPM loaded on the columns. The remaining portion of each sample was loaded onto each of two C18 minicolumns. After each sample passed through the column, a 50 μl aliquot of the eluate was counted to determine the amount of labelled eicosanoid which was not bound on the column. Over 96% of $^3$H-PGE1 and 88% of $^3$H-LTD4 were bound to the C18 minicolumns. The amount bound was not significantly increased by passing the effluent through the columns a second time.
Binding Affinity of Different Eicosanoids on Silicic Acid Columns

The binding of PGE1 and LTD4 to silicic acid was measured in different solvents in order to select the most appropriate solvent for loading and eluting samples. The following solvents were selected for this study: 99% ethanol, ethanol:H2O (4:1), ethanol:H2O (2:1), ethanol:H2O (1:4), hexane, toluene, and methanol. Samples, 100 μl each, consisted of mixed unlabelled eicosanoids as described above, were labelled with either 0.1 μCi [3H]-PGE1 or 0.01 μCi [3H]-LTD4. 400 μl of each solvent listed above was added to make the final value of each sample to be 0.5 ml. A 10 μl aliquot from each sample was removed to determine total CPM loaded. Silicic acid was loaded into 1.5 ml centrifuge tubes, 40 mg each tube. Samples were then loaded into the tubes and mixed with silicic acid. After 15 minutes, all tubes were centrifuged at 14,000 rpm for 30 seconds, and a 10 μl aliquot of the supernatant of each sample was removed to determine the amount of labelled eicosanoids which did not bind to the silicic acid in the centrifuge tubes. Our results indicated that among the different solvents listed above, eicosanoids had high binding affinity with silicic acid in either hexane or toluene. Over 98.7% of 3H-PGE1 and 98.3% of 3H-LTD4 bound with silicic acid in hexane, and over 98.6% of 3H-PGE1 and 98.1% of 3H-LTD4 bound with silicic acid in toluene. Based on these results, toluene was
selected as the first solvent in the elution system.

Separation of Leukotrienes, Prostaglandins, and Arachidonic Acid on C18 Minicolumns

Three samples which consisted of the unlabelled eicosanoid mixture described above were labelled with \(^{3}\text{H}\)-PGE2, \(^{3}\text{H}\)-LTD4, or \(^{3}\text{H}\)-AA. A 50 μl aliquot was taken from each sample and counted to determine total CPM loaded on the columns. The remaining portion of each sample was loaded onto C18 minicolumns, and eluted successively with solvents C, A, and D (fraction #1), solvent B (fraction #2), and finally methanol (fraction #3). The recovery for \(^{3}\text{H}\)-PGE2, \(^{3}\text{H}\)-LTD4, and \(^{3}\text{H}\)-AA was 89%, 54.34% and 96%, respectively (Fig. 9).

A sample containing a mixture of labelled PGE2 (36.6% of total), LTD4 (15.3% of total), and arachidonic acid (48.1% of total) was loaded onto the C18 minicolumn. As shown in (Fig. 10), the amount recovered in each fraction was almost identical to the expected value.
Fig. 9: Separation of standard mixture of prostaglandins, leukotrienes, and arachidonic acid on C18 minicolumn. The standard mixture of unlabeled eicosanoids consisted of 280 pmole of PGE2, 1100 pmole of arachidonic acid, and 200 pmole of leukotriene D4. Mixture #1, 2, and 3 consisted of the standard mixture plus labeled $^3$H-PGE1 (0.1 μCi/sample) (solid bar), $^3$H-LTD4 (0.01 μCi/sample) (spotted bar), and $^3$H-AA (0.12 μCi/sample) (hatched bar), respectively. The lipid was applied to a C18 minicolumn in RPMI medium. Fraction 1 was eluted with solvent C, A and D, successively, fraction 2 with solvent B, and fraction 3 with methanol.
Fig. 10: Fractionation of an eicosanoid mixture on C18 minicolumns.
A known mixture consisting of 0.1 µCi $^3$H-PGE1, 0.01 µCi $^3$H-LTD4, and 0.12 µCi $^3$H-AA was fractionated on C18 minicolumn as described in the legend of Fig. 9. The amount of label recovered in each fraction (solid bar) is compared to the actual amount of each labeled lipid added to the mixture (hatched bar).
Separation of Leukotrienes, Prostaglandins, and Arachidonic Acid on Silicic Acid Columns

Three samples which consisted of the unlabelled eicosanoid mixture as described above were labelled with 0.1 µCi [³H]-PGE₁, 0.01 µCi [³H]-LTD₄, or 0.12 µCi [³H]-AA. A 10 µl aliquot from each sample was removed to determine total CPM loaded on the column. Silicic acid was loaded onto centrifuge columns, 60 mg each column. Samples were then loaded onto each one of three centrifuge columns, and sequentially eluted twice with each of the following solvents: toluene, solvent A₁ (toluene:ethyl acetate:methanol=60:40:0), solvent A₂ (toluene:ethyl acetate:methanol=60:40:2), solvent A₃ (toluene:ethyl acetate:methanol=60:40:20), solvent B₁ (acetonitrile:methanol:ddH₂O:acetic acid:EDTA=1280:800:1916:4:2 grams), solvent B₂ (solvent B₁:methanol=50:50), and methanol. As shown in Fig 11, the silicic acid column procedure could separate LTD₄ from both PGE₁ and arachidonic acid, but it was not effective for separation of prostaglandins and arachidonic acid. In order to confirm this separation result, fraction #3, 4, and 5, which had the highest CPM counts from the [³H]-PGE₁-silicic acid column, were mixed together as sample A. And fraction #9 and 10, which had the highest CPM counts from [³H]-LTD₄-silicic acid column, were also mixed together as sample B. Sample A and B were then dried, resuspended in 100 µl of 99% ethanol each, loaded
onto each of two new silicic acid columns, respectively, and eluted as described above. The results, as shown in Fig 12, confirmed that $^3$H-PGE1 were eluted with toluene, solvent A1, A2 and A3, and $^3$H-LTD4 were eluted with solvent B1 and B2. Based on these findings, we defined the toluene - solvent A1 - A2 - A3 as the prostaglandin fraction, and Solvent B1 - B2 - methanol as the leukotriene fraction.

In order to compare the separation effectiveness of the silicic acid column procedure and the C18 minicolumn procedure, three samples containing a labelled mixture of $^3$H-PGE1 only, $^3$H-LTD4/$^3$H-PGE1 = 38/62, or $^3$H-LTD4 only, were loaded onto each of three silicic acid columns and eluted as described above. The amount of label recovered in each fraction was closer to the applied value for the C18 minicolumns (Fig. 10) than for the silicic acid columns (Fig. 13).
Fig. 11: Separation of the standard mixture of prostaglandins, leukotrienes, and arachidonic acid on silicic acid columns. Mixture #1 (solid bar), #2 (hatched bar), and #3 (open bar) were prepared as described in Fig. 9. The lipid was applied to the silicic acid columns in RPMI medium. Fraction: T (toluene); A1 (toluene:ethyl acetate:methanol=60:40:0); A2 (toluene:ethyl acetate:methanol=60:40:2); A3 (toluene:ethyl acetate:methanol=60:40:20); B1 (acetonitrile:methanol:ddH2O:acetic acid:EDTA=1280:800:1916:4:2 grams); B2: (B1:methanol=50:50); M:methanol.
Fig. 12: Confirmation of the separation efficiency of prostaglandins and leukotrienes on silicic acid column. Fraction #3, 4, and 5 from the $^3\text{H}$-PGE1-silicic acid column as shown in Fig. 11 were mixed together as sample "A:$^3\text{H}$-PGE1", and fraction #9 and 10 from the $^3\text{H}$-LTD4-silicic acid column in Fig. 11 were mixed as sample "B:$^3\text{H}$-LTD4". Samples A and B were then dried, resuspended in 100 μl of 99% ethanol, loaded onto two new silicic acid columns, and eluted as described in the legend of Fig. 11.
Fig. 13: Fractionation of an eicosanoid mixture on silicic acid columns.
Three samples, each containing the standard unlabeled mixture as described in Fig. 9 plus $^3$H-PGE1 only (Sample #1), $^3$H-LTD4/$^3$H-PGE1=38/62 (Sample #2), and $^3$H-LTD4 only (Sample #3), were fractionated on three silicic acid columns as described in the legend of Fig. 11. The amount of label recovered in each fraction (solid bar) is compared to the actual amount of each labeled lipid added to the mixture (hatched bar).
The relative Amount of Label in the Different Eicosanoid Fractions Released from LPS Activated THP-1 Cells Was Similar Throughout the Activation Period

THP-1 cells (5.25 x 10⁵ cells/ml x 20 ml) were pretreated with vitamin D3 (1 μM/ml) and metabolically labelled with [³H] arachidonic acid (0.5 μCi/ml) overnight. The cells were then challenged with smooth-LPS from S. abortus or rough-LPS from S. minnesota (R595) at a concentration of 1000 ng/ml. One ml samples were removed at 0, 15, 30, 60, 90, and 120 minutes after the addition of LPS, and then centrifuged. A 100 μl aliquot of the supernatant was removed from each sample and counted for total label released (Fig. 14). The remaining portion of each sample was subsequently fractionated on C18-minicolumns. The major eicosanoid recovered at each time point was arachidonic acid. Only trace amounts of label were recovered in the prostaglandin or leukotriene fractions in the mixture released from S. abortus LPS treated cells (Fig. 15). Less than 5% of the total bound lipid was recovered in the prostaglandin fraction of the lipid released from the cells challenged with R595 LPS, and both arachidonic acid (40% to 60%) and leukotrienes (20% to 30%) were recovered from C18 minicolumn separation (Fig. 16). In addition, between 30% and 40% of the label released from the cells did not bind to the C18-minicolumns when applied in RPMI medium. These products were apparently quite polar.
Fig. 14: Time course for label released from human THP-1 cells challenged with *S. abortus* or *S. minnesota* (R595) LPS. Human THP-1 cells (8.7 x 10^5 cells/ml x 40 ml) were pretreated with vitamin D3 (1 µM/ml) and metabolically labelled with ^3H-arachidonic acid (0.5 µCi/ml) overnight. The cells were then challenged with either *S. abortus* LPS or *S. minnesota* LPS at a concentration of 1000 ng/ml in RPMI medium with 10% FBS. One ml samples were removed at 0, 15, 30, 60, 90, and 120 minutes after the addition of LPS, and centrifuged. A 100 µl aliquot of each supernatant was counted to determine total label released from the cells.
Fig. 15: Time course for eicosanoid release from activated human THP-1 cells challenged with *S. abortus* LPS. THP-1 cells were pretreated and challenged as described in the legend of Fig. 14. After the 100 μl aliquot from each sample was taken to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: ³H-labelled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
Fig. 16: Time course for eicosanoid release from activated human THP-1 cells challenged with *S. minnesota* LPS. Human THP-1 cells were pretreated and challenged as described in the legend of Fig. 14. After the 100 µl aliquot was removed from each sample to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: $^3$H-labelled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
THP-1 Macrophages Activated with LPS from *S. minnesota* (R595) Released Both PGE$_2$ and LTC$_4$

In order to quantify individual eicosanoids, EIA kits were used to analyse the lipids released from human THP-1 cells activated with *S. minnesota* LPS.

THP-1 cells (7.4 x 10$^5$ cells/ml x 20 ml) were pretreated with vitamin D3 and metabolically labelled with $^3$H-arachidonic acid overnight as described above. *S. minnesota* LPS was then used to challenge the cells at a concentration course of 0, 0.1, 1, 10, 100, 1000 ng/ml following the same experimental procedure as previous. As shown in Fig. 17, *S. minnesota* LPS activated arachidonic acid metabolism in THP-1 cells. The Prostaglandin E2 and Leukotriene C4 Enzyme Immunoassay kits were used to analyse eicosanoids released from the cells. As shown in Fig. 18, both prostaglandin E2 and leukotriene C4 were recovered, and the results also showed that more leukotriene C4 was released than the amount of prostaglandin E2.
Fig. 17: CPM released from human THP-1 cells challenged at various concentrations with *S. minnesota* LPS (R595). Human THP-1 cells (6.9 x 10^5 cells/ml x 20 ml) were pretreated and labeled as described in the legend of Fig. 14. The cells were then challenged with *S. minnesota* LPS at the concentration course of 0, 0.1, 1, 10, 100, 1000 ng/ml in RPMI medium with 5% FBS for two hours. One ml samples were then collected, centrifuged, and a 100 µl aliquot of each supernatant was counted to determine total label released from the cells.
Fig. 18: Enzyme immunoassay analysis of eicosanoids released from human THP-1 cells challenged with *S. minnesota* LPS (R595) at various concentrations. Human THP-1 cells were pretreated, labelled, and challenged as described in the legend of Fig. 17. A 50 μl aliquot of each sample was analysed for prostaglandin E2 and for leukotriene C4 with the specific EIA kits.
THP-1 cells (7.5 x 10^5 cells/ml x 20 ml) were pretreated with vitamin D3 and metabolically labelled with [^3H]-arachidonic acid overnight as described above. The cells were then challenged with LTA at a concentration of 1000 ng/ml following the same experimental procedure as described above. *S. faecalis* LTA activated arachidonic acid metabolism in human THP-1 cells (*Fig. 19*). Samples were collected at intervals and fractionated on C18 minicolumns. Between 20% to 30% of the label released from the cells did not bind to the column. The bound label was recovered primarily in the arachidonic acid fraction (between 40% and 60%) and the leukotriene fraction (between 20% to 25%) (*Fig. 20*).
**Fig. 19:** Time course for label released from human THP-1 cells challenged with *S. faecalis* LTA.

Human THP-1 cells (7.5 x 10⁵ cells/ml x 20 ml) were pretreated and labelled as described in the legend of Fig. 14. The cells were then challenged with *S. faecalis* LTA at a concentration of 1000 ng/ml in RPMI medium with 10% FBS. One ml samples were removed at 0, 15, 30, 60, 90, and 120 minutes after the addition of LTA, and centrifuged. A 100 µl aliquot of each supernatant was counted to determine total label released from the cells.
Fig. 20: Time course for eicosanoid release from activated human THP-1 cells challenged with *S. faecalis* LTA. Human THP-1 cells were pretreated and challenged as described in the legend of Fig. 19. After the 100 μl aliquot was removed from each sample to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: ³H-labelled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
The Relative Amount of Label Recovered in Different Eicosanoid Fractions was Similar for THP-1 Cells Activated with Different LTA preparations

THP-1 cells (3.4 x 10^5 cells/ml x 15 ml) were differentiated with vitamin D3 and labelled with ^3H-arachidonic acid as described above. The cells were then challenged with LTA from *S.pyogenes*, *B.subtilis*, and *S.aureus* in RPMI medium with 5% FBS for two hours. As shown in Fig. 21, *S.pyogenes*, *B.subtilis*, and *S.aureus* LTA activated arachidonic acid metabolism in THP-1 cells, and *S.aureus* LTA appeared to have the most potent effect for this activation. Samples from cells challenged with 1000 ng/ml of each amphiphile were analyzed on C18 minicolumns. The relative amounts of prostaglandins, leukotrienes, and arachidonic acid were similar for each of these LTA preparations (Fig. 22) and the *S.faecalis* preparation (Fig. 20). About 50% of the total label released from the cells was recovered in arachidonic acid fraction, and about 10% of the label was recovered in leukotriene fraction. Less than 2% of the label was found in prostaglandin fraction. Between 35% and 40% of the label did not bind to the C18 minicolumn.

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Fig. 21: Label released from human THP-1 cells challenged at various concentrations with *S.pyogenes*, *B.subtilis*, and *S.aureus* LTA.

Human THP-1 cells (3.3 x 10^5 cells/ml x 15 ml) were pretreated and labelled as described in the legend of Fig. 14. The cells were then challenged with *S.pyogenes*, *B.subtilis*, and *S.aureus* LTA at the concentration course of 0, 0.1, 1, 10, 100, 1000 ng/ml in RPMI medium with 5% FBS for two hours. All the samples were then collected, centrifuged, and a 100 µl aliquot of each supernatant was counted to determine total label released from the cells.
Fig. 22: Eicosanoids released from activated human THP-1 cells challenged with 1000 ng of LTA from *S. pyogenes*, *B. subtilis*, or *S. aureus*. Human THP-1 cells were pretreated and challenged as described in the legend of Fig. 21. After the 100 μl aliquot was removed from each sample to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: \(^3\)H-labelled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
Pretreatment of THP-1 Cells with Human Gamma Interferon did not Alter the Pattern of Arachidonic Acid Metabolism

γ-IFN pretreatment has a marked influence on the response of some cells to LPS. Some macrophage cultures require exposure to γ-IFN in order to respond to LPS. To determine the effect of γ-IFN on arachidonic acid metabolism in human THP-1 cell line, THP-1 cells (4.5 x 10^5 cells x 30 ml) were pretreated with human γ-IFN at 20 ng/ml during the overnight labeling period. Cells without γ-IFN pretreatment were used as control. Cells were then challenged with R595 LPS in RPMI medium with 5% FBS for 2 hours. As shown in Fig. 23, arachidonic acid metabolism was activated in both γ-IFN pretreated and non-pretreated cells, and the activation was almost identical in both cell preparations, indicating that γ-IFN was not required for activating arachidonic acid metabolism in human THP-1 cell line.
Fig. 23: Effect of human γ-interferon on arachidonic acid metabolism in human THP-1 cells.
Human THP-1 cells (4.5 x 10^5 cells/ml x 30 ml) were pretreated and labelled as described in the legend of Fig. 14. Half of the cells were pretreated with human γ-interferon at 20 ng/ml overnight. *S. minnesota* LPS was then used to challenge both γ-IFN pretreated (solid line) and non-pretreated cells (broken line) in RPMI medium with 5% FBS for two hours. One ml samples were then collected, centrifuged, and a 100 μl aliquot of each supernatant was counted to determine total label released from the cells.
Mouse Peritoneal Macrophages (PEC) were More Sensitive to PMA than Human THP-1 Macrophages

Some macrophage cultures (e.g. mouse peritoneal macrophages) are very sensitive to the phorbol myristate acetate (PMA). PMA is an analogue of diglyceride and therefore does not require the amphiphile receptor(s) in the membrane. To compare the effects of PMA, *S. minnesota* LPS, and *S. faecalis* LTA on arachidonic acid metabolism in human THP-1 monocytic cells and mouse PEC, THP-1 cells were distributed into a 12-well flat-bottom plate at a concentration of 4.8 x 10^5 cells/well, and mouse PEC were at a concentration of 1.9 x 10^6 cells/well. *S. minnesota* LPS, *S. faecalis* LTA, and PMA were then used to challenge the cells at a concentration of 1000 ng/ml in RPMI medium with 10% FBS. Three wells of the cells were used as controls for unchallenged cells in each plate. After four hours incubation at 37°C in a chamber with 5% CO₂, one ml samples were removed from each well, and then centrifuged. A 100 µl aliquot of the supernatant was removed from each sample and counted for total label released. The remaining portion of each sample was fractionated on C18 minicolumns.

*S. minnesota* LPS was the most potent activator (2000 ± cpm/10^5 cells), and PMA had the least effect (1000 ± cpm/10^5 cells) in human THP-1 cells. In contrast to the THP-1 cells, PMA had the strongest effect for the activation in mouse PEC macrophages (*Fig. 24*). The composition of the eicosanoid

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mixture released from PMA treated THP-1 cells was similar to LPS and LTA treated cells (Fig. 25). Mouse PEC cultures released more label in the prostaglandin fraction than human THP-1 cells (Fig. 26).
Fig. 24: Comparison of the effects of S. minnesota LPS, S. faecalis LTA, and PMA on arachidonic acid metabolism in human THP-1 cells and mouse peritoneal macrophages (PEC). THP-1 cells (9.6 x 10^5 cells/ml x 40 ml) were pretreated and labelled as described in the legend of Fig. 14. Mouse PEC were collected (1.9 x 10^6 cells/ml x 12 ml), washed with RPMI medium without serum, and metabolically labelled with ^3H-arachidonic acid (0.5 μCi/ml) overnight. Both of the cell lines were then challenged with S. minnesota LPS, S. faecalis LTA, or PMA at a concentration of 1000 ng/ml in RPMI medium with 10% FBS for four hours. One ml samples were then removed, centrifuged, and a 100 μl aliquot was removed from each supernatant to be counted for total label released from the cells.
Fig. 25: Eicosanoids released from activated human THP-1 cells challenged with *S. minnesota* LPS, *S. faecalis* LTA, or PMA.

THP-1 cells were pretreated and challenged as described in the legend of Fig. 24. After the 100 μl aliquot was removed from each sample to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: ^3^H-labelled polar products which did not bind to the C18 minicolumn; PG:prostaglandin family; LT:leukotriene family; AA: arachidonic acid family.

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Fig. 26: Eicosanoids released from activated mouse peritoneal macrophages (PEC) challenged with *S. minnesota* LPS, *S. faecalis* LTA, or PMA.

Mouse PEC were collected, washed, labelled, and challenged as described in the legend of Fig. 24. After the 100 µl aliquot was removed from each sample to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: $^3$H-labelled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
Comparison of the Effects of C2-ceramide, Sphingomyelinase, and Dihydroceramide on Arachidonic Acid Metabolism in Mouse Peritoneal Macrophages (PEC)

To compare the effects of C2-ceramide, sphingomyelinase, and dihydroceramide on arachidonic acid metabolism in mouse PEC, mouse PEC were collected (1.1 x 10^6/ml x 26 ml), washed, and labelled overnight as described in "Materials and Methods". Following the same experimental procedure as described above, C2-ceramide, sphingomyelinase, and dihydroceramide were used to challenge the cells in RPMI medium with 2.5% FBS at the concentrations of 2.5, 5, 10, 20, 40, 80 μM/ml, 0.03, 0.06, 0.125, 0.25, 0.5, 1 unit/ml, and 2.5, 5, 10, 20, 40, 80 μM/ml, respectively. After four hours incubation at 37°C in the chamber with 5% CO₂, all the samples were collected, centrifuged, and transferred to clean centrifuge tubes. A 100 μl aliquot from each sample was removed and counted for total label released. As shown in Fig. 21, both C2-ceramide and sphingomyelinase activated arachidonic acid metabolism in mouse PEC. Dihydroceramide had no apparent effect on this activation.
Fig. 27: Comparison of the effects of C2-ceramide, sphingomyelinase, and dihydroceramide on arachidonic acid metabolism in mouse peritoneal macrophages (PEC). Mouse PEC were collected (1.1 x 10^6 cells/ml x 24 ml), washed, and labelled as described in the legend of Fig. 24. The cells were then challenged with C2-ceramide or dihydroceramide at 2.5, 5, 10, 20, 40, 80 μM/ml, or with sphingomyelinase at 0.03, 0.06, 0.125, 0.25, 0.5, 1 unit/ml in RPMI medium with 2.5% FBS for four hours. One ml samples were then collected, centrifuged, and a 100 μl aliquot was removed from each supernatant to count for total label released from the cells.
Effect of R595 LPS Pretreatment of Macrophage Cultures (Mouse PEC) on Subsequent Challenge with S. minnesota LPS, Ceramide, and Phorbol Myristic Acetate (PMA)

To understand the nature of desensitization by R595 LPS treatment, mouse PEC were collected (2.54 x 10^6 cells/ml x 12 ml), washed, and treated with S. minnesota LPS at a concentration of 100 ng/ml in RPMI medium with 10% FBS on a 24-well flat-bottom plate, 1 ml per well. Nine wells of the cells were used as control (untreated) samples. After three hours incubation at 37°C in the chamber with 5% CO₂, the medium was removed, and RPMI medium containing ^3H-arachidonic acid was used to label the cells (0.5 μCi/ml) overnight as described before.

After overnight incubation, the medium was removed, and the cells were washed three times with RPMI medium without serum. Then S. minnesota LPS, ceramide, and PMA were used to challenge both LPS treated and control (untreated) cells in RPMI medium with 10% FBS at concentrations of 1000 ng/ml, 80 μM/ml, and 1000 ng/ml, respectively. Three wells of each LPS-treated and untreated cells were used as control samples for unchallenged cells. After two hours incubation at 37°C in the chamber with 5% CO₂, one ml samples were removed, centrifuged, and transferred to clean tubes. A 100 μl aliquot of the supernatant was removed from each sample and counted for total label released. The remaining portion of each sample was fractionated on C18 minicolumns.
As shown in Fig. 28, for the untreated cells, *S. minnesota* LPS activated arachidonic acid metabolism, and ceramide was less effective for the activation. In contrast, the pretreated cells showed no response to R595 LPS, but an enhanced response to PMA. No apparent change of activation was observed for the effect of ceramide between LPS-treated and untreated mouse PEC. C18 minicolumn separation showed that there was no apparent difference in the composition of the eicosanoid mixture released from LPS-treated and untreated mouse PEC which were challenged with *S. minnesota* LPS and PMA. $^3$H-labelled polar products, arachidonic acid, leukotrienes, and prostaglandins were all recovered from the separation. Apparent difference was observed from the separation of samples released from LPS-treated and untreated mouse PEC which were challenged with ceramide. All $^3$H-labelled polar products, arachidonic acid, leukotrienes, and prostaglandins were recovered from the samples released from untreated cells. But only $^3$H-labelled polar products and arachidonic acid were recovered from the samples released from LPS-treated cells. No label was recovered in the leukotriene and prostaglandin fractions (Fig. 29).
Fig. 28: Effect of R595 LPS pretreatment of macrophage cultures (mouse PEC) on subsequent challenge with *S. minnesota* LPS, C2-ceramide, and PMA. Mouse PEC were collected (2.54 x 10⁶ cells/ml x 6 ml), washed, and pretreated with R595 LPS at a concentration of 100 ng/ml in RPMI medium with 10% FBS for three hours. The medium was then removed, and the cells were labelled overnight as described in the legend of Fig. 24. The cells were then challenged with *S. minnesota* LPS at 1000 ng/ml, C2-ceramide at 80 μM/ml, or PMA at 1000 ng/ml respectively in RPMI medium with 10% FBS for two hours (hatched bar). The same amount of untreated mouse PEC were labelled and challenged following the same experimental procedure as described above (solid bar). After two hours challenging, all samples (1 ml each) were removed, centrifuged, and a 100 μl aliquot from each supernatant was taken to count for total label released from the cells.
Fig. 29: Eicosanoids released from activated mouse peritoneal macrophages, both R595 LPS pretreated and untreated, challenged with S. minnesota LPS, ceramide, or PMA.

Mouse PEC were collected, pretreated with R595 LPS, labelled, and challenged as described in the legend of Fig. 28. After the 100 μl aliquot from each sample was taken to be counted, the remaining portion of both R595 LPS pretreated and untreated samples were fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: ³H-labelled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
HPLC Analysis

Standard mixture containing unlabelled LTD4 (100 pmole) plus $^3$H-PGE1 (0.1 μCi), $^3$H-LTD4 (0.05 μCi), and $^3$H-AA (0.15 μCi) was analysed through HPLC. The HPLC system was performed as described in "Materials and Methods", and these components were well quantified using the in line radioactivity flow detector (Fig. 30).

Samples released from THP-1 cells challenged with R595 LPS as described above were analysed with HPLC system, and this analysis confirmed the C18 minicolumn separation result that arachidonic acid was the main product in the eicosanoid mixture released from the cells. (Fig. 31).

Samples released from THP-1 cells challenged with R595 LPS as described above were fractionated on C18 minicolumns, and the prostaglandin, leukotriene, and arachidonic acid fractions were then concentrated and analysed with HPLC system. Arachidonic acid was recovered as main product in the arachidonic acid fraction. The HPLC system lacked the sensitivity to detect label in the prostaglandin or leukotriene fractions. When Samples were pooled, concentrated, and then analysed with HPLC system, the leukotriene fraction showed the peak which appeared in the area where the peak of arachidonic acid appeared.
Fig. 30: HPLC analysis of standard mixture of eicosanoids. Standard mixture containing unlabeled LTD4 (100 pmole) plus \(^3\)H-PGE\(_1\) (0.1 μCi), \(^3\)H-LTD4 (0.05 μCi), and \(^3\)H-AA (0.15 μCi) was analysed through HPLC. HPLC was carried out on a 4.6 x 250 mm silica C18 column, which was attached to a PERKIN-ELMER Series 410 LC pump and a FLO-ONE\Beta Series A-100 radioactivity detector. The solvent system was a low pressure gradient of solvent A (acetonitrile:methanol:ddH\(_2\)O :acetic acid:EDTA= 1280:800:1916:4:2 grams) and methanol eluted at the flow rate of 1 ml/min in the following order: 0 min, 100% A; 30 min, 100% A; 31 min, 100% methanol; 51 min, 100% methanol; 52 min, 100% A; 67 min, 100% A.
Fig. 31: HPLC analysis of eicosanoids released from human THP-1 cells challenged with *S. minnesota* LPS. Human THP-1 cells were pretreated, labeled, and challenged as described in the legend of Fig. 17. After counting 100 μl aliquot from each sample and running the EIA assay, all the rest of samples were mixed together and analysed through HPLC. HPLC system was performed as described in the legend of Fig. 30.
Membrane Phospholipid was not Recovered in Eicosanoid Fractions Released from Activated Human THP-1 Cells

When lipid from the prostaglandin and leukotriene fractions was concentrated and subsequently fractionated by HPLC, all of the label eluted in the arachidonic acid fraction. This suggested that these eicosanoid fractions might have contained arachidonate esterified to more complex lipid (e.g. phospholipid) and that the arachidonate was hydrolyzed from the glycerol during concentration step.

In order to quantify the amount of arachidonate which was released from cells as complex phospholipids, THP-1 cells were labeled with either $^3$H-arachidonic acid at 0.5 μCi/ml or $^{32}$P at 5 μCi/ml overnight. Both $^3$H-AA and $^{32}$P labeled cells were then challenged with either *S. minnesota* LPS or *S. faecalis* LTA at 0, 0.1, 1, 10, 100, 1000 ng/ml in RPMI medium with 5% FBS. After incubation at 37°C with 5% CO$_2$ for 2 hours, all samples were removed and then centrifuged. A 200 μl aliquot of the supernatant was removed from each sample and counted for total label released from the cells, and the remaining portion of each sample was fractionated on C18 minicolumns. As shown in Fig. 32, both $^3$H and $^{32}$P was released from cells challenged with *S. minnesota* LPS or *S. faecalis* LTA. C18 minicolumn separation showed different results between $^3$H and $^{32}$P labeled cells. The recovery of $^3$H-label was similar to the results described above, but almost all the $^{32}$P-label was recovered.
in the water phase. The lipid which eluted from the C18 minicolumn in the prostaglandin or leukotriene fractions did not appear to contain phospholipid (Fig. 33).
Fig. 32: Label released from human THP-1 cells challenged at various concentrations with either S.minnesota (R595) LPS or S.faecalis LTA.

Human THP-1 cells (8.6 x 10^5 cells/ml x 15 ml) were pretreated with vitamin D3 (1 μM/ml) and metabolically labeled with either ^3H-arachidonic acid at 0.5 μCi/ml or ^32P at 5 μCi/ml overnight. The cells were then challenged with either S.minnesota LPS or S.faecalis LTA at the concentration course of 0, 0.1, 1, 10, 100, 1000 ng/ml in RPMI medium with 5% FBS for two hours. One ml samples were then collected, centrifuged, and a 200 μl aliquot of each supernatant was counted to determine total label released from the cells.

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Fig. 33: Eicosanoids released from activated human THP-1 cells challenged with *S. minnesota* LPS or *S. faecalis* LTA. Human THP-1 cells were pretreated, labeled, and challenged as described in the legend of Fig. 32. After the 200 µl aliquot was removed from each sample to be counted, the remaining portion of each sample was fractionated on C18 minicolumns as described in the legend of Fig. 9. Unbound: $^3$H-labeled polar products which did not bind to the C18 minicolumn; PG: prostaglandin family; LT: leukotriene family; AA: arachidonic acid family.
Chapter IV
DISCUSSION

Development for Rapid Separation of Different Eicosanoids

Many techniques have been used to separate and extract different eicosanoids. Classical techniques for extraction of prostaglandins or leukotrienes from biological fluids and plasma are liquid-liquid or liquid-solid extractions. In liquid-liquid extractions, many different organic solvents, such as chloroform, diethyl ether or ethyl acetate, have been used (27). However, this technique is time-consuming because multi-step extractions and large volumes of organic solvents may be necessary for an efficient extraction. Liquid-solid extractions have employed solid adsorbents such as silica or octadecylsilane silica materials from which adsorption of a compound from the liquid phase onto the adsorbent material and subsequent desorption may result in different recoveries depending on the type of adsorbent utilized. Silica and C18 (ODS) extraction columns (Sep-Paks) are the most common choice. Another octadecylsilane silica material available in disposable columns of different sizes distributed by J.T Baker has also been found to accomplish the extraction of prostaglandins and lipoxygenase metabolites of arachidonic acid from buffer with good recoveries (27).

Based on previous research results from our laboratory
(4), the major classes of eicosanoids we were interested in were the prostaglandin family, leukotriene family, and arachidonic acid. Several groups have described procedures to extract prostaglandins or leukotrienes from plasma or other biological fluids, however, a technique for simultaneous extraction and separation of all prostaglandins, leukotrienes, and arachidonic acid has not been demonstrated. A major objective of this present study was to develop a procedure for the simultaneous extraction and separation of the three classes of eicosanoids with liquid-solid extraction technique based on the solid adsorbents and using organic solvents which have been used in HPLC or TLC procedures.

Comparison of Eicosanoid Separation Efficiency of Silicic Acid Column Procedure and C18 Minicolumn Procedure

Initial studies for quick separation of different eicosanoids were focused on the evaluation of one-step column-centrifugation procedures. For our initial experiments, small centrifuge columns were developed using silicic acid as absorbant, which was expected to have markedly different affinities for the prostaglandin family and the leukotriene family. It was essential that all of the different eicosanoids have high binding affinity in the initial solvent. Each of the eicosanoids could then be sequentially eluted with different solvents. We tested
the binding affinity of the eicosanoids in different solvents, and toluene was finally selected as the solvent for loading samples on silicic acid columns. For both the silicic acid procedure and C18 minicolumn procedure, the solvent systems evaluated in this study for sequential elution were selected from solvents which have been used in thin-layer chromatography (TLC) and HPLC systems. Fractionation of the different classes of eicosanoids on hydrophobic C18 minicolumns or silicic acid columns is based on the difference in polarity of leukotrienes (LT), prostaglandins (PG), and arachidonic acid (AA) (polarity: PG > LT > AA). The silicic acid column procedure required more complicated solvent systems than the C18 minicolumn procedure, and this procedure was only effective for separation of leukotrienes from prostaglandins and arachidonic acid, but not for separation of prostaglandins and arachidonic acid (Fig. 11). Neither the separation or the recovery of this procedure was as effective as the C18 minicolumn procedure (Fig. 10, 13). Based on these observations, the C18 minicolumn procedure was finally selected for eicosanoid analysis in this study.

LPS and LTA Activate Eicosanoid Metabolism in Human THP-1 Cell Line

The human monocytic leukemic cell line, THP-1, which differentiates toward macrophages in response to phorbol 12-myristate 13-acetate (PMA) or vitamin D3, is an established
model of induced macrophage differentiation (3). After differentiation with 1,25-dihydroxyvitamin D3, previous studies showed that THP-1 cells responded to LPS stimulation with a greatly enhanced secretion of proinflammatory cytokines and mediators including TNF-α and the arachidonic acid metabolites, such as prostaglandin E2 (PGE2), thromboxane B2, and prostaglandin F2α (PGF2α). This increase required the expression of the CD14 antigen, an integral component of a high-affinity LPS receptor (20). Our results indicated that THP-1 cells responded well to activation with LPS from S. minnesota (R595) and S. abortus, and to lipoteichoic acid from S. faecalis, S. pyogenes, B. subtilis, and S. aureus. Activation significantly enhanced release, which was concentration-dependent and time dependent, of arachidonic acid metabolites.

The rate-limiting enzyme in the arachidonic acid cascade is phospholipase A2 (PLA2), which cleaves arachidonic acid residues from membrane phospholipids. This enzyme was reported to be activated by LPS in macrophages, causing it to localize to the plasma membrane and release arachidonic acid (20). It has been documented that relative potency of LPS varied with the proportion of lipid A in the LPS molecule. Previous studies indicated that the ratio of lipid A to polysaccharide in LPS has significant influence on the cellular mechanisms and subsequent toxic effects of endotoxins and the consequence of gram-negative bacterial
infections. Due to the structural differences between 
*S. minnesota* LPS (R-LPS) and *S. abortus* LPS (S-LPS), they 
might have different effects on the activation of 
phospholipase A₂, causing different subsequent 
pathophysiological consequences.

Our results of C18 minicolumn separation showed there 
were some differences of the arachidonic acid metabolites 
released from THP-1 cells challenged with either *S. minnesota* or *S. abortus* LPS. Both arachidonic acid and 
leukotrienes, which are the 5'-lipoxygenase products, were 
recovered from the mixture of eicosanoids released from THP-1 
cells which were challenged with *S. minnesota* LPS, but no 
measurable amount of prostaglandins were found. In contrast, 
only arachidonic acid was recovered from the eicosanoid 
mixture released from THP-1 cells challenged with smooth-LPS 
from *S. abortus*, suggesting that *S. abortus* LPS stimulation 
did not activate the 5'-lipoxygenase pathway in the THP-1 
cell line. Label was recovered, however, in the leukotriene 
fraction from THP-1 cells challenged with *S. minnesota* 
(R595) LPS, indicating that the 5'-lipoxygenase pathway 
was activated in these cells.

Chemically, LTA is different from LPS, but it shares 
some common physical properties with LPS. LTA is localized 
at the outer surface of the cytoplasmic membrane of Gram-
positive bacteria (11). Both LTA and LPS are anionic, 
amphiphilic polymers, which contain carbohydrate and 
phosphorus, and both have a hydrophobic glycolipid moiety.
and a hydrophilic end. LTA induces some of the same biological and immunological responses which are induced by LPS, but compared with LPS, LTA is less toxic. There are differences in the relative potencies of different LTA preparations. In mouse PEC cultures, *S. faecalis* and *S. aureus* LTA are comparable to LPS, whereas *B. subtilis* LTA is less potent (4). In this study, we used LTA from *S. faecalis*, *S. pyogenes*, *B. subtilis*, and *S. aureus* to challenge both THP-1 cells and mouse PEC macrophages. Our results indicated that THP-1 cells responded well to the stimulation from all of these LTA preparations. C18 minicolumn separation of the eicosanoid mixture showed compositions similar to that released by *S. minnesota* LPS (R595) challenged cells. Both arachidonic acid and leukotrienes were recovered from the separation, but no measurable prostaglandins were found. These results suggested that the pathway of cell activation by these LTA preparations was similar to the activation pathway of R595 LPS. They could activate both phospholipase A₂ and 5'-lipoxygenase pathways, causing the release of arachidonic acid and leukotrienes. No label was recovered in the prostaglandin fraction. The key enzymes in prostaglandin synthesis are the constitutive cyclooxygenase, prostaglandin H synthase-1 (PGHS-1), and the mitogen-induced cyclooxygenase (PGHS-2). The C18 minicolumn separation results suggest that cyclooxygenase pathway is not activated in the human THP-1 cell line. The result that LTA had
similar effect as LPS on stimulating arachidonic acid release in human THP-1 cells is quite interesting since LTA is less toxic than LPS, and it has not been implicated in pathological conditions.

HPLC system was applied in this study to analyse each fraction of prostaglandin, leukotriene, or arachidonic acid, which were fractionated on C18 minicolumns. HPLC analysis confirmed the C18 minicolumn separation results that arachidonic acid was the main product in eicosanoid mixture released from THP-1 cells challenged with *S. minnesota* LPS. The HPLC analysis lacked the sensitivity to detect label in the prostaglandin and leukotriene fractions. In order to increase sensitivity, the leukotriene fractions from several samples were pooled, concentrated by evaporation, and then analysed by HPLC. All of the label in these pooled samples was eluted in the arachidonic acid fraction. Since the amount of arachidonic acid in the eicosanoid mixture was much higher than the amount of prostaglandins or leukotrienes, it was possible that when the mixture was fractionated on C18 minicolumn, some arachidonic acid might be eluted with the leukotriene fraction, causing the recovery of arachidonic acid in leukotriene fraction with HPLC analysis. This is unlikely, however, because when the label recovered in the leukotriene fraction was applied to a fresh column and re-fractionated all of the label was again recovered in the leukotriene fraction. The same results were obtained when label recovered in the prostaglandin fraction.
was refractionated. Another possible explanation for this observation could be that some other products, which were eluted in the PG or LT fraction on the column but subsequently chromatographed with AA on the HPLC, were released from the THP-1 cells during the R595 LPS challenge. Since we just used $^3$H-LTD4, $^3$H-PGE1, and $^3$H-AA as standards for HPLC analysis and also for C18 minicolumn procedure, it is very possible that some other products which could be separated from arachidonic acid on the column but not on HPLC.

In contrast, EIA assay results showed that both PGE2 and LTC4 were recovered from the eicosanoid mixture released from THP-1 cells challenged with S. minnesota LPS. But compared with LTC4, the amount of PGE2 was much less. The amount of PG measured by EIA was below the sensitivity of the HPLC. EIA assay could measure prostaglandins at the concentration as low as 5 pg/ml. A very low amount of PGE2 in eicosanoid mixture could not be detected with C18 minicolumn procedure or HPLC assay, but it could be measured with EIA assay.

S. minnesota LPS, S. faecalis LTA and PMA Activate Eicosanoid Metabolism in Mouse Peritoneal Macrophages

The toxicity of lipopolysaccharide is a problem in the practical use of LPS to enhance the immune response. This has led to a search for naturally occurring or modified forms of LPS which have reduced toxicity but retain immune
stimulatory activity. Much evidence has suggested that the endotoxic properties of LPS are largely determined by the lipid A component which interacts with a defined humoral factor (LPS binding protein) and the cellular recognition molecular receptor, CD14. The toxicity levels of different lipid A preparations show marked differences (5). Removal of a phosphate group from lipid A results in a marked reduction in pyrogenicity and toxicity. Likewise, removal or substitution of the normal fatty acids of lipid A causes decreased toxicity (4). Although lipid A is considered to be responsible for virtually all the biological activities of LPS, the polysaccharide moiety determines the elimination kinetics, LPS binding specificity with macrophages, and the activation of the alternate pathway of the complement system. This is at least part of the reason that smooth and rough LPS have differential effects on the release of chemical mediators.

It has been previously demonstrated that smooth LPS from Pasteurella hemolytica caused marked increases in plasma PG, TxB2 and serotonin in sheep (7). Emau and Giri reported (8) that both smooth and rough LPS caused immediate increases in plasma arachidonic acid to its maximal levels at various times during the infusion, and the rise in plasma arachidonic acid metabolites was greater in response to smooth than to rough LPS. They also found although both smooth and rough LPS produced an early transient rise in plasma serotonin, the two LPSs differed in their abilities
to decrease plasma serotonin and histamine during the later part of endotoxemia.

LTA was also reported to stimulate the respiratory burst in human blood monocytes (17), but the immunological implications of the interaction of LTA with cells for the immune response remains unclear. Some previous results suggested that LTA would interact with host immune cells directly. The hydrophobic component of LTA is less complex than lipid A of LPS, and it has been proposed that the reason LTA is less toxic is because LTA is closer in structure to eukaryotic membrane glycolipids than lipid A (34). It has been suggested that the hydrophobic glycolipid region would be the biologically active part in LTA for attachment to unidentified receptor(s) on the surface of macrophages, such as lipid A in LPS is the minimal structure which is responsible for most of the biological properties of LPS (18).

In our laboratory, Rahul Jasuja reported (4) that lipoteichoic acid (LTA) from the Gram-positive bacteria S. aureus, S. faecalis, B. subtilis, and B. stearothermophilus activated eicosanoid release from mouse peritoneal macrophages (mouse PEC). The amount of arachidonic acid released was comparable to that released by mouse PEC which were activated with toxic LPS from S. abortus. B. subtilis LTA stimulated arachidonic acid release, but the amount released was much less than the other LTA preparations. Lipopolysaccharide (LPS) from S. abortus initiated
eicosanoid release and showed a maximum release at lower concentrations than LTA. The total amount of eicosanoids released was about the same for LPS and *S. aureus* and *S. faecalis* LTA. Phorbol myristic acetate (PMA) was also tested, and the result showed it was another activator for arachidonic acid metabolism. It has been known that PMA, which is an analog of diacylglycerol, can permeate the cell membrane and activate protein kinase C, causing the release of arachidonic acid via phospholipase A2 (35). This previous study did not determine the relative amount of the different classes of eicosanoids in the materials released from different cell lines.

My study confirmed that *S. minnesota* LPS (smooth LPS), *S. faecalis* LTA, and PMA activated eicosanoid metabolism in mouse peritoneal macrophages (mouse PEC). Furthermore, C18 minicolumn separation showed that prostaglandins, leukotrienes, and arachidonic acid were recovered in the eicosanoid mixture released from mouse PEC challenged with R595 LPS, LTA, and PMA, indicating that both cyclooxygenase and 5'-lipooxygenase pathways were activated in mouse PEC.

Comparison of the Effect of *S. minnesota* LPS, *S. faecalis* LTA, and PMA on Eicosanoid Metabolism in Human THP-1 cells and Mouse Peritoneal Macrophages

There were marked differences in the relative amount of different eicosanoids recovered from the mixture released from mouse PEC or human THP-1 cells. When both of the cell
lines were challenged with *S. minnesota* LPS, *S. faecalis* LTA, and PMA, C18 minicolumn separation of eicosanoid mixtures from activated mouse PEC showed label in the prostaglandin, leukotriene, and arachidonic acid fractions. Only the leukotriene and arachidonic acid fractions were recovered from human THP-1 cells. This result is quite interesting since the same results were obtained by stimulation with LPS, LTA, or even PMA, which is not a bacterial amphiphile. The type of eicosanoids released did not vary with the type of activation but with the different cell lines. The constitutive cyclooxygenase PGHS-1 and inducible cyclooxygenase PGHS-2 are responsible for prostaglandin synthesis, whereas 5'-lipoxygenase is responsible for leukotriene synthesis. It has been reported that the mitogen-inducible cyclooxygenase, PGHS-2, could be induced upon LPS stimulation in THP-1 monocytes, and is generally thought to be the isoform responsible for the resultant burst of prostaglandin and thromboxane synthesis (24). Recently, Mazzucco and Warr reported (20) that the fungal metabolite trichodimerol (BMS-182123), which has demonstrated inhibition of LPS-stimulated tumor necrosis factor-α (TNF-α) secretion in both human and murine in vitro macrophage models, interfered with LPS-induced eicosanoid secretion through an inhibition in the induction of the inducible cyclooxygenase, PGHS-2, at the level of transcription.
In this study, little prostaglandin was released from THP-1 cells challenged with LPS, LTA, and PMA. On the other hand, the stimulation of the amphiphiles (both LPS and LTA) and PMA could activate the cyclooxygenase pathway in mouse peritoneal macrophages, resulting in the release of prostaglandins. The mechanisms for this preferable activation need further studies.

Another comparison of the effect of LPS, LTA, and PMA on eicosanoid metabolism in human THP-1 cells and mouse PEC shows that LPS, LTA and PMA activate these two cell lines with different stimulative potency. Both of the cell lines could responded well to LPS (R595), LTA (S. faecalis), and PMA stimulation and released eicosanoids. But R595 LPS appeared to have the most potent stimulative effect on THP-1 cells, releasing two times more eicosanoid than PMA. On the other hand, PMA was the most potent activator for eicosanoid metabolism in mouse PEC, and S. faecalis LTA had the least effect for this activation.

PMA acts as an analogue for diglyceride. A possible explanation for this observation is that PMA has different binding affinity for the protein kinase C in these two cell lines. DAG is a source for arachidonic acid by the action of diglyceride lipase, and it also can activate protein kinase C, which plays an important role in leukocyte activation, differentiation of monocytes to macrophages, and arachidonic acid release (Fig. 34). It has been previously reported that PMA could specially activate protein kinase C, causing the
release of arachidonic acid. Based on our observation, we suggest that PMA might have lower binding ability in THP-1 cells compared with in mouse PEC, resulting in lower protein kinase C activation. Another possibility is that in THP-1 cells, protein kinase C may not be linked with the arachidonic acid metabolic pathway as closely as in mouse PEC, so that this enzyme has less of an effect on arachidonic acid release in THP-1 cells.

The reason that LPS and LTA have different effects on eicosanoid release in THP-1 and mouse cells might be related to their different receptors on the cells. Several LPS receptors have been identified. The 53- to 55-kDa cell surface glycoprotein CD14, which is a glycoposphatidylinositol anchored protein, has been identified as the main LPS receptor on leukocytes, enabling them to be stimulated with LPS by binding serum LPS-binding protein (LBP)-LPS complexes at low LPS concentrations. This process is facilitated by the catalytic activity of the serum protein LPS-binding protein (LBP), which accelerates the binding of LPS to CD14 (32). CD18 and LDL-scavenger receptor are also LPS receptors, but they do not appear to be involved in signal transduction. Receptors of LTA have not been identified. LTA has not been found to bind the CD14 receptor, but it has been pointed out that LTA from several Gram-positive bacteria binds a 28 kDa LPS binding protein in mouse serum (19). Previous investigations showed that the macrophage scavenger receptor (SR), a glycosylated
trimeric transmembrane protein, binds directly to many Gram-positive bacteria, possibly via LTA. SR binding to other ligands is dependent upon the spatial characteristics of the repeating negative charge of the ligand. Therefore, Greenberg and Fischer (12) investigated SR recognition of
Fig. 34: Pathway of arachidonic acid release
LTA species with various charge densities and distributions, and they demonstrated that the SR binds directly to many Gram-positive bacteria via negative charges and possibly also recognizes polyanionic hydrophilic chains of LTAs. Their data support the role of surface associated LTA as a major ligand of Gram-positive bacteria for binding to SR. Based on our findings, we suggest that the distribution of LPS and LTA receptors on these two cell lines is different. Both LPS and LTA receptors which could function in signal transductions might be more abundant on THP-1 cells than on mouse PEC. This different distribution of the receptors could lead to the different stimulative potency of LPS and LTA on these two cell lines, causing more eicosanoids released from THP-1 cells than from mouse PEC when challenged with LPS or LTA. The other possibility that could explain our results would be that the LPS and LTA receptor on mouse cells might not be linked to the arachidonic acid metabolic pathway as closely as in THP-1 cells, so LPS or LTA challenging has less effect on activating arachidonic acid metabolism in mouse cells than human THP-1 cells.

The Nature of Desensitization by S. minnesota (R595) LPS Treatment in Mouse Peritoneal Macrophages

We tested the ability of S. minnesota LPS to desensitize mouse peritoneal macrophages, and the data presented in our study showed the treatment with R595 LPS desensitized the macrophage cultures to subsequent response
to LPS challenge. Furthermore, we found that the cell culture pretreated with R595 LPS had good response to PMA challenge, releasing even more eicosanoids than the cells without LPS pretreatment.

Rahul Jasuja reported (4) that *S. faecalis* and *S. aureus* LTA desensitized mouse PEC to LPS challenge, but *B. subtilis* LTA could not. He also found that the desensitization was not specific for pretreatment with LTA. Mouse PEC pretreated with LPS were desensitized to LPS challenge, and cells pretreated with LTA were unresponsive to subsequent LTA challenge. But *S. faecalis* LTA could not desensitize the cells to PMA and calcium ionophore A23187 challenge. My study confirmed that R595 LPS could desensitize mouse PEC to LPS challenge, and the C18 minicolumn separation result showed the main classes of eicosanoids released from either desensitized or non-desensitized cells were similar.

Previous studies from our laboratory (4) indicated that the desensitization was not the result of less $[^3H]$-arachidonic acid uptake during the labeling period, or the result of a general down-regulation of arachidonic acid metabolism. Cells pretreated with LTA during the adherence period had an enhanced rate of uptake of $[^3H]$-arachidonate during the labeling period, and remained responsive to PMA challenge, indicating that there was an increase in the turnover of arachidonic acid in these stimulated cells, resulting in an increase in both uptake and release. There
are several possible mechanisms which may be responsible for the desensitization of cells to LPS challenge, such as alterations of amphiphile receptors, the targets of signal transduction, or the cellular regulators of the signal transduction pathways. The best characterized LPS receptor CD14, as described above, can function by delivering LPS to the signaling receptor. CD18 and the LDL-scavenger receptor have not been reported to function in signal transduction. Previous results suggested that blocking these receptors does not desensitize cells to LPS challenge, and the desensitization is not the result of a down-regulation of any of the known receptors (9). The possible explanations for desensitization could be that it is the result of changes in cellular elements involved in the interaction of the receptor and transduction system or some single step in signal transduction pathways. Both tyrosine kinase and protein kinase C have been implicated in the LPS activation pathway. As shown in Fig. 34, in the pathway of arachidonic acid release, the steps for cells desensitized with LPS could occur at a point before protein kinase C activation.

We also found that R595 LPS pretreatment could not desensitize mouse PEC to PMA challenge, which was similar to Jasuja's (4) observation that S. faecalis LTA could not desensitize mouse PEC to PMA challenge. It has been shown that the mechanism for PMA activating arachidonic acid metabolism is that PMA permeates the cell membrane and
directly activates protein kinase C, causing arachidonic acid release via phospholipase A2. This mechanism could explain the result that R595 LPS and *S. faecalis* LTA pretreatment did not desensitize cells to PMA challenge since the pretreatment caused desensitization step which occurred at a point before protein kinase C activation, so the subsequent treatment of PMA still activated protein kinase C, causing the release of arachidonic acid. Our observation that subsequent PMA challenge of R595 LPS pretreated cells caused even more arachidonic acid release than untreated cells could be due to the three hour pretreatment of R595 LPS. Previous study in our laboratory (4) indicated that mouse PEC pretreated with LTA from *S. pyogenes*, *S. aureus*, and *S. faecalis* during the adherence period had an enhanced rate of uptake of [³H]-arachidonate during the labeling period. Based on this observation, the explanation for our result is that pretreatment with R595 LPS enhanced the uptake of [³H] arachidonate during the labeling period, which would increase the amount of label in the lipid pool, so that more arachidonic acid was released during subsequent PMA challenge.

It has been suggested that ceramide is an intracellular modulator of cell growth and differentiation, and it is also an important second messenger in signal transduction in cells of myeloid lineage (15). Examination of the structures of LPS and ceramide have revealed a strong similarity between the molecules. This present study showed that C2-
ceramide and sphingomyelinase had the effect on activating arachidonic acid metabolism in mouse PEC, and dihydroceramide, which was used as a negative control, had no apparent effect on this activation. R595 LPS could not desensitize the cells to subsequent challenge with C2-ceramide or PMA. Most recently, Jean Pfau (unpublished data) in our laboratory reported that both LPS and ceramide could activate arachidonic acid metabolism in human THP-1 cells, and prior exposure to ceramide did not desensitize the cells to subsequent challenge with either LPS or ceramide, nor could LPS desensitize the cells to challenge with ceramide, which is very similar to our result in mouse PEC.

Macrophage-endotoxin interaction has many destructive pathophysiological consequences, such as endotoxic shock. Desensitization of the responding cells by pretreatment with bacterial amphiphiles could be an effective method for minimizing the harmful effects caused by macrophage-endotoxin interaction.
Summary

1. A C18 minicolumn procedure was developed to separate different eicosanoid mixtures into prostaglandin fraction, leukotriene fraction, and arachidonic acid fraction.

2. The relative amount of label in the different eicosanoid fractions released from LPS activated THP-1 cells was similar throughout the activation period. THP-1 macrophages activated with LPS from S. minnesota (R595) released both PGE₂ and LTC₄.

3. The kinetics of activation of THP-1 cells with lipoteichoic acid were similar to the kinetics of cells activated with LPS. The relative amount of label recovered in different eicosanoid fractions was similar for THP-1 cells activated with different LTA preparations.

4. Pretreatment of THP-1 cells with human gamma interferon did not alter the pattern of arachidonic acid metabolism.

5. Mouse peritoneal macrophages (PEC) were more sensitive to PMA than human THP-1 macrophages.
6. R595 LPS pretreatment desensitized mouse peritoneal macrophages to subsequent challenge with R595 LPS, but this treatment could not desensitize the macrophage cultures to subsequent challenge with either C2-ceramide or PMA.

7. The composition of the eicosanoid mixture released from PMA treated THP-1 cells was similar to LPS and LTA treated cells.

8. Mouse PEC cultures released more label in the prostaglandin fraction than human THP-1 cells.

9. HPLC analysis confirmed the C18 minicolumn separation results that arachidonic acid was the main product released from THP-1 cells challenged with *S. minnesota* LPS, and no measurable amount of prostaglandin was recovered. HPLC result also suggested that some product other than prostaglandins or leukotrienes was released from THP-1 cells during this challenging.
Literature Cited


