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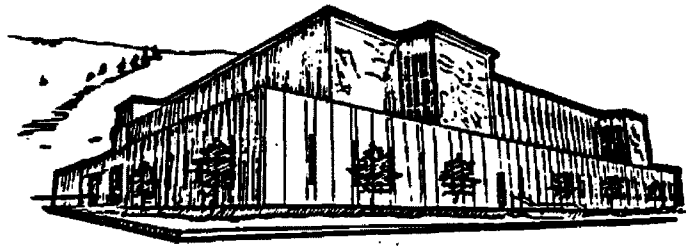
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University of  
**Montana**



**EFFECT OF LIPOTEICHOIC ACIDS ON ARACHIDONIC ACID  
METABOLISM IN MACROPHAGES**

**By**

**Rahul R Jasuja**

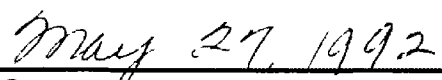
**B.Sc., University Of Bombay, St. Xavier's College, 1987**

**Presented in partial fulfillment of the requirements  
for the degree of  
Master of Science  
University Of Montana  
1992**

**Approved by**

  
**Chairman, Board of Examiners**

  
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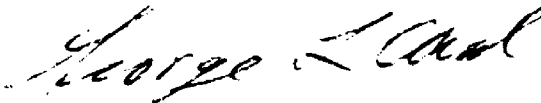
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Effect of lipoteichoic acids on arachidonic acid metabolism in macrophages (87 pp.)

Director: George L. Card



Lipopolysaccharide (LPS) from gram negative bacteria is a potent amphiphile which stimulates sensitive cells (e.g. macrophages, neutrophils) to secrete biologically active molecules that have protective (enhanced immune response, tumor cell destruction) and pathological (hypersensitivity reactions, toxicity) activities. Lipoteichoic acids (LTA) isolated from gram positive bacteria are chemically different but have structural features and physical properties similar to LPS. LTA, unlike LPS, has no reported toxicity. This study involved a comparison of the relative ability of LTA and LPS to activate arachidonic acid metabolism in macrophage cultures. Peritoneal macrophages from mice were labelled with [<sup>3</sup>H] arachidonic acid and stimulated with LTA or LPS. LPS from *Salmonella abortus* or LTA from *Streptococcus faecalis* or *Staphylococcus aureus* or *Bacillus steorothermophilus* activated arachidonic acid metabolism. Pretreatment with LTA from *Streptococcus faecalis* or *Staphylococcus aureus* desensitized macrophages to subsequent challenge with toxic LPS from *Salmonella abortus*. LTA from *Bacillus subtilis* activated arachidonic acid metabolism to a lesser extent, but did not desensitize macrophages to challenge with LPS. Cells desensitized to LPS and LTA responded to phorbol myristate acetate stimulation. This suggests that desensitization is an initial step in the arachidonic acid activation pathway occurring at some point preceding the release of diglyceride. These findings may be important in controlling endotoxic shock. Inhibition of phospholipase C, protein kinase C and phospholipase A<sub>2</sub>, steps in the arachidonic acid release pathway, blocked arachidonic acid release in cells stimulated by LPS or LTA. This suggests that arachidonic acid release in macrophages stimulated by LPS and LTA are similar, at least to some extent, both involving phospholipase C, protein kinase C and phospholipase A<sub>2</sub> in the activation process.

## Acknowledgements

I dedicate this work to my family - "Mummy", "Papa", and "Gudiya". Their unlimited love, support and understanding over the years have helped me through thick and thin. I am thankful to Dr. Card for providing the guidance and expertise that made this study possible. A combination of Dr. Card's enthusiasm and personality made it a pleasure to work in his laboratory. Thanks to Drs. Deborde and Hill, for their invaluable advise and suggestions, and to the Stella Duncan Research Institute for its support. I would also like to express my gratitude to uncles and aunts, especially Rick, Hans, Savitri, and Manori, for making life a little easier for me. Last, but not the least, I wish to acknowledge friends, colleagues, and the splendor, spirit, and unmatched lifestyle of Montana that made my stay in Missoula so special.

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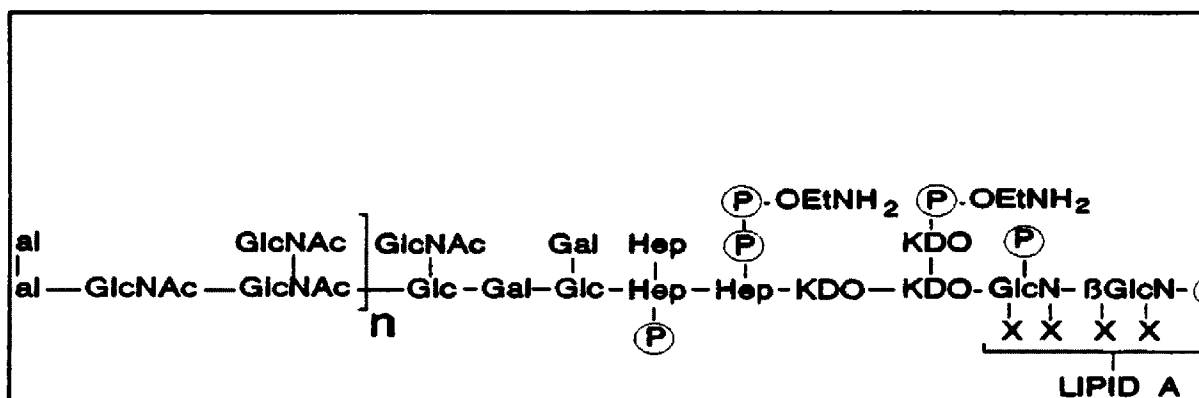
## INTRODUCTION

Biological response modifiers (BRM) are substances which enhance the immune response by causing sensitive cells (macrophages, neutrophils, lymphocytes) to release a variety of mediators. These mediators act as messengers which are released from one cell to activate another cell, or as mediators which promote phagocytosis, activate natural killer cells, enhance antibody production and activate cells involved in specific and non-specific resistance. The mediators include tumor necrosis factor (TNF) (44,52) the interleukins (22,44,52), platelet activating factor (PAF) (22,44), interferons (44) and eicosanoids (22,44) among others. This study is focused on the release of eicosanoids by macrophages. Eicosanoids are derivatives of arachidonic acid (5-*cis*-, 8-*cis*-, 11-*cis*-, 14-*cis*- eicosatetraenoic acid), a C<sub>20</sub> polyunsaturated fatty acid (40). Several bacterial structural components and products are potent biological response modifiers. The lipopolysaccharide (LPS) of gram-negative bacteria is one of the most well characterized BRM. LPS stimulates macrophages to release arachidonic acid which is metabolized to eicosanoids that have protective (e.g. tumor cell destruction, bacterial killing) and pathological (e.g. inflammation, hypersensitivity) properties. Lipoteichoic acids of gram-positive bacteria have a

different chemical structure but some of the same physical properties of LPS. The major objective of this study was to compare the activities of LTA to LPS in macrophage cultures.

### Lipopolysaccharide

Lipopolysaccharide (LPS, also called endotoxin) is an integral component of the outer membrane of gram negative bacteria. LPS consists of three regions having contrasting biological and chemical properties - 'O' specific polysaccharide chain, core polysaccharides, and the lipid A region which is linked to the core polysaccharide by means



**Figure 1. Structure of lipopolysaccharide**

of 2-keto-3-deoxyoctalonic acid (KDO) (Fig. 1) (31). The toxic activity of LPS has been shown to reside in the lipid A region (38,62). The majority of lipid A structures studied contain a  $\beta(1-6)$ -linked D-glucosamine disaccharide that carries two phosphoryl groups- one bound as an ester to the non-reducing glucosamine and the other to the glycosidic hydroxyl group of the reducing glucosamine

(Fig.2) (38). This lipid A backbone has substituted groups (designated R1-R6 in Fig. 2). These groups include nitrogen containing residues, D-arabinose, phosphate (R1, R2, R3) and long chain acyl groups (R5 and R6). The R4 group is the KDO group that links lipid A to the polysaccharide core (38). Evidence for the involvement of the lipid A in binding to macrophages came from experiments that showed that monoclonal antibodies against the lipid A moiety inhibited LPS binding to macrophages (46). Tahri-Jouti *et. al* (46)

have shown that enzymatic or chemical removal of the fatty acids of lipid A reduced binding of LPS to macrophages. LPS can bind directly to a receptor(s) or as a complex

with a plasma

protein - lipopolysaccharide binding protein (LBP), (39).

The LPS-LBP complex binds to the CD14 receptor on the surface of macrophages (60). Studies using monoclonal antibodies to synthetic derivatives of lipid A suggested that more than a single LPS receptor exists on the surface

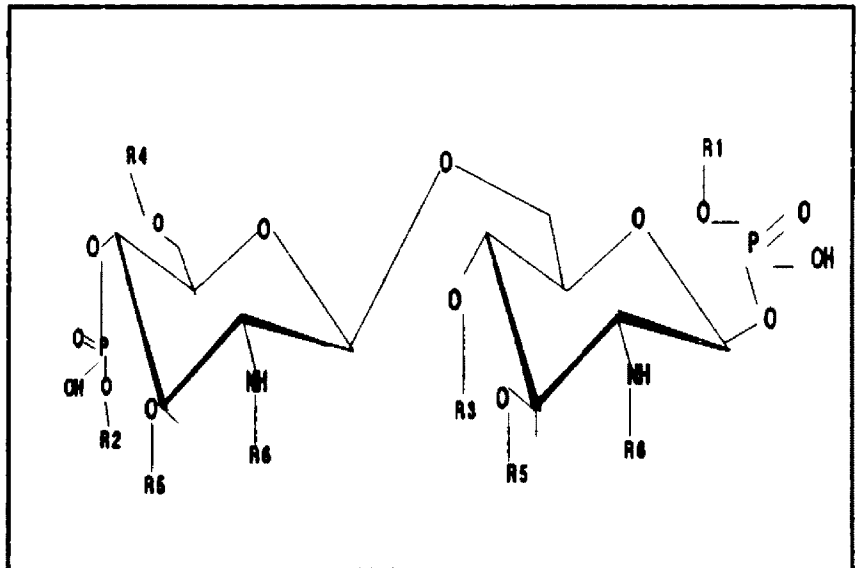


Figure 2. Structure of lipid A

of a macrophage and that different spatial configurations of lipid A are recognized by different receptors (46).

LPS is responsible for many of the harmful manifestations seen in patients with gram negative sepsis or endotoxemia. Among these are pyrogenic activity, hypoglycemia, changes in blood clotting and blood pressure, and organ damage which can result in shock and even death (22,37). Neutrophils, platelets, vascular endothelium cells and macrophages appear to be the major cells involved in endotoxic injury (22). The interaction which initiates endotoxic injury appears to occur between LPS and macrophages, resulting in the release of cytokines and lipid mediators (37).

Tumor necrosis factor (TNF) and interleukin-1 (IL-1), released by macrophages on LPS stimulation, appear to be the primary mediators of endotoxic shock (22,37). Evidence that the macrophage is the critical cell in the host response to LPS comes from studies on LPS resistant mouse strain C3H/HeJ, and LPS sensitive mouse strain C3H/HeN (22). These mice are genetically identical except for the LPS resistance/sensitive gene. In contrast to LPS sensitive mice, the LPS resistant mice do not produce certain cytokines and show decreased prostaglandin (an eicosanoid derived from arachidonic acid) release in response to LPS. Further, the LPS resistant C3H/HeJ mice normally cannot be sensitized to endotoxin by galactosamine, a compound that

markedly increases sensitivity to endotoxin in normal animals. If macrophages from C3H/HeN mice are transferred to LPS resistant C3H/HeJ mice, then they can be sensitized by galactosamine.

### Lipoteichoic Acid

Lipoteichoic acid (LTA), like LPS, is a bacterial amphiphile. All LTAs, which have been characterized, consist of an anionic polymer with a terminal hydrophobic group (14,55). Most LTAs which have been characterized consist of a 1,3, linked polyglycerophosphate moiety in a phosphodiester linkage to a membrane glyceroglycolipid (14,15,56). The hydrophobic glyceroglycolipid is anchored at the outer surface of the cytoplasmic membrane, with the glycerophosphate chain extending into the environment. The glyceroglycolipid anchor of most LTAs

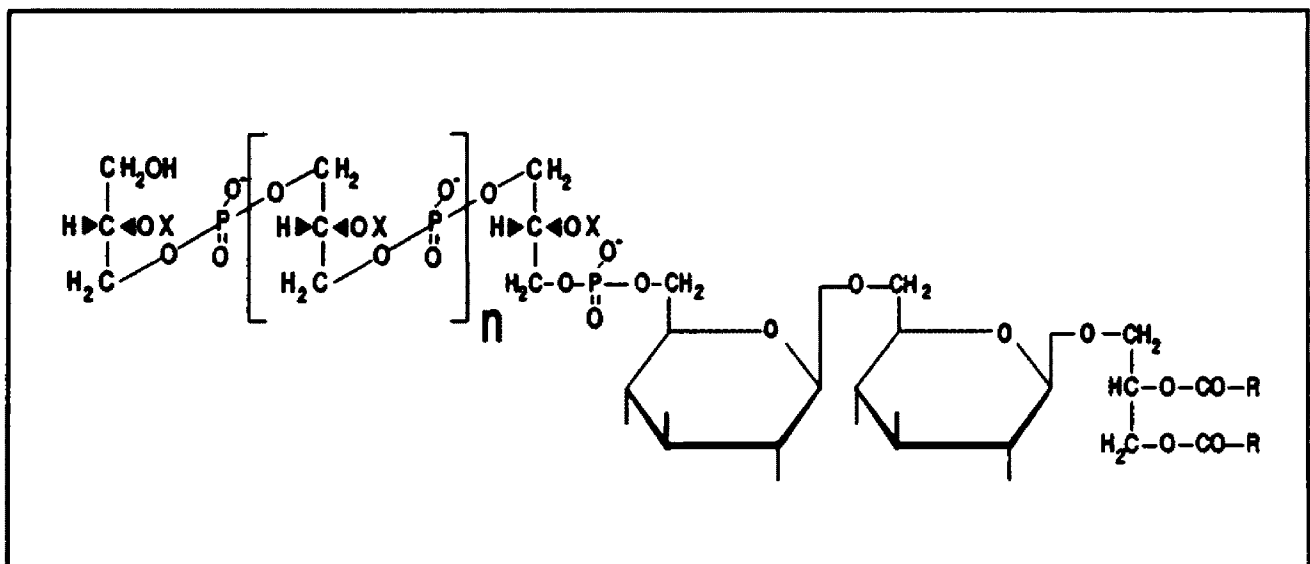


Figure 3. Structure of Lipoteichoic acid (*Staphylococcus aureus*)

consists of a disaccharide unit linked to a diacylglycerol

(dihexosyldiacylglycerols) (Fig. 3) (14,15). The glyceroglycolipids of some LTAs have three fatty acid residues (triacylglycerols). This third fatty acid is bonded to C<sub>6</sub> of the diacylglycerol linked saccharide and anchors the LTA more firmly in the membrane (14). In LTAs that have a third fatty acid the saccharides are linked by a C<sub>1</sub> - C<sub>2</sub> or C<sub>1</sub> - C<sub>4</sub> bond, leaving the C<sub>6</sub> of the diacylglycerol linked saccharide free to bind the fatty acid. The most common fatty acids in LTA are C<sub>16</sub> and C<sub>18</sub> saturated fatty acids (14). LTAs with a non-glycosylated lipid anchor are rare, but have been identified in a few bacterial species. The length of the polyglycerophosphate chain varies from 16 to 40 glycerophosphate units, depending on the bacterial species (14,15,56). The number of group substitutions in the glycerol residues also varies from one bacterial species to another. Based on chain substitution, bacterial LTA can be divided into four groups: group A lacks substituents, group B carries only D-alanine substituents, group C carries only glycosyl substituents, and group D carries both these substituents. No other substituents have been identified (56).

The role of LTA in pathogenesis of gram positive bacteria has received relatively little attention. The inflammatory components implicated in gram positive bacteremia are cell wall components (peptidoglycan, teichoic acids, and protein A), toxins ( $\alpha$ -toxin, enterotoxin), and



enzymes (proteinase, coagulase). No role for LTA in gram-positive bacteremia has been reported. Recently, Brade et al (6) reported that a 28 kD protein of normal mouse serum bound both LTA and LPS. This 28 kd serum protein bound to the lipid A-proximal inner core region of LPS. Initial investigations have shown that the sugar acid KDO is required for binding of this protein. Brade et al. (6) suggest that an unidentified moiety, similar to KDO in conformation, may exist in LTA. It is evident from this that there are humoral factors and cellular factors that recognize LPS and LTA. Several research groups (47,61) have reported that LTA has some immunological and biological properties in common with LPS. For example, Tarsi-Tsuk and Levy (47) recently reported that LTA from *Streptococcus faecalis* stimulates the respiratory burst in blood monocytes.

#### Macrophage Activation

Activated macrophages play a critical role in host defense against tumors and microbes, in the inflammatory response, in homeostasis and in disease such as atherogenesis, and carcinogenesis (Fig. 4) (21). Adams (1) has pointed out that macrophage activation is not restricted to the development of anti-microbial or anti-neoplastic function, but can be broadly defined as an increased competence to perform a variety of functions associated with host defense. The functional ability of macrophages is

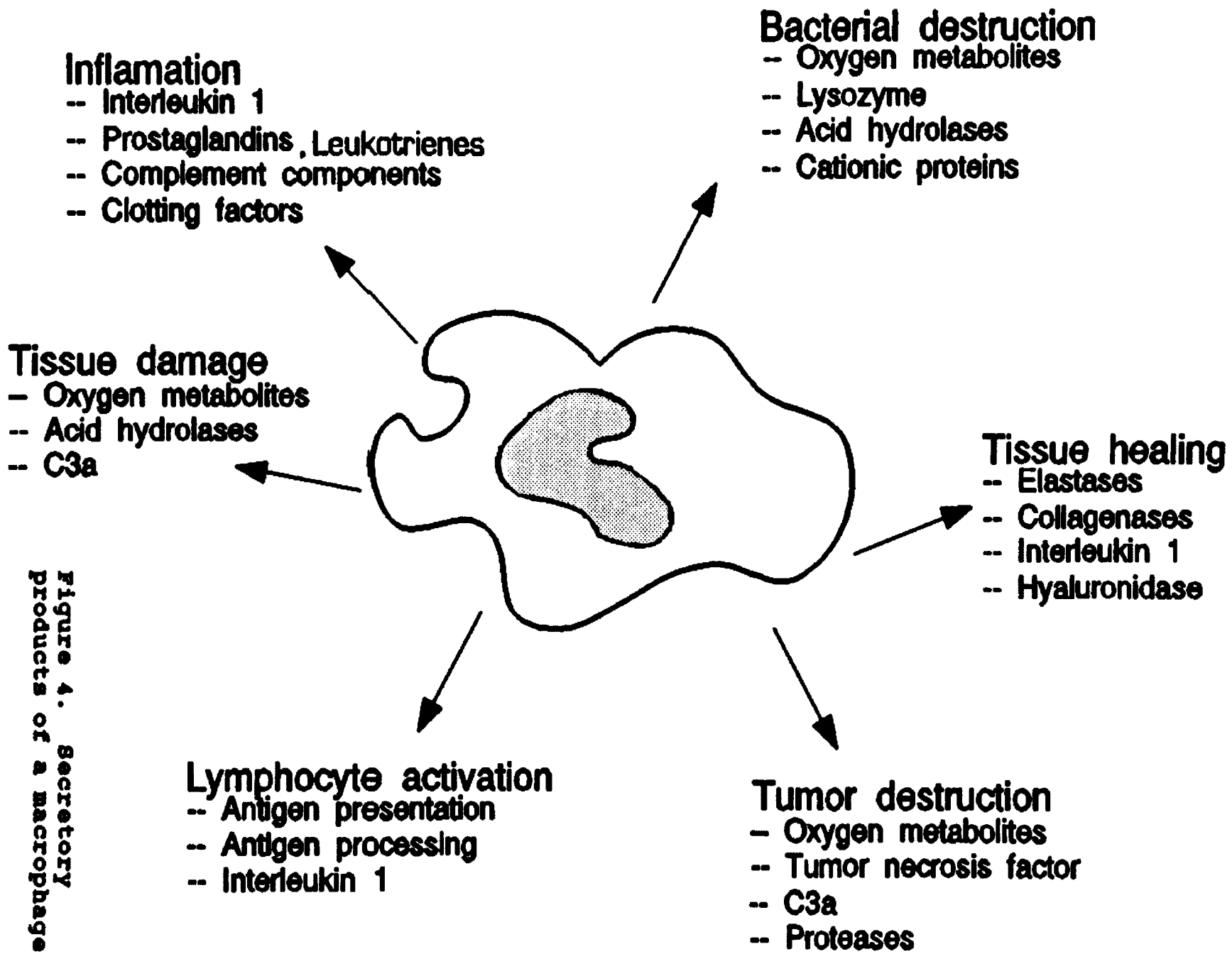
strictly regulated by extracellular signals. These extracellular signals can either enhance or diminish the ability of macrophages to carry out a certain function (21). The precise mechanisms of macrophage activation are diverse, but there is always the involvement of two fundamental steps: (1) initial recognition of the extracellular stimuli; and (2) subsequent physiological changes that result in one or more of the following; secretion of potent mediators (cytokines), changes in surface properties, and metabolic changes (e.g. respiratory burst).

#### **Measure of Macrophage activation**

Activated macrophages show a variety of metabolic activities (1) (Fig. 4). The metabolic activities that are most commonly used to measure macrophage activation are described.

**1) Respiratory burst:** Activated cells show an enhanced oxidative metabolism called the respiratory burst. This results from the activation of the NADPH oxidase system which catalyses the synthesis of metabolites (e.g. superoxide anion, hydrogen peroxide, hydroxyl radical etc.), which have potent anti- microbial activities. This respiratory burst can be measured by cytochrome c reduction (47).

**2) Secretion of proteins and glycoprotein mediators:**

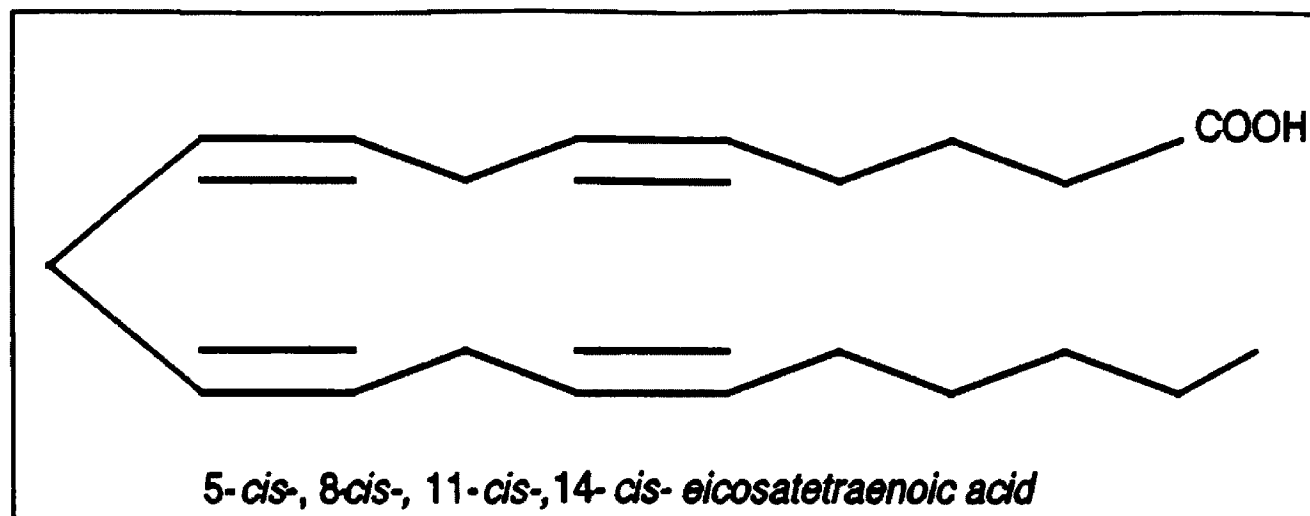


Activated macrophages secrete several different kinds of proteins and glycoproteins that have enzymatic or immunostimulating activity. These include plasminogen activator, lysosomal hydrolases, cytotoxic proteinases, and the interferons (44).

**3) Release of tumor necrosis factor (TNF) and interleukin-1 (IL-1):** TNF and IL-1, which appear to be the primary mediators of endotoxic shock (35) are synthesized de novo and are released as a consequence of microbial stimulation, in particular LPS, or tissue injury (23,37,44).

**4) Display of altered surface properties:** A selective remodelling of the membrane, as a consequence of activation, results in expression of several receptors that enhance macrophage function. Some of the components expressed by activated macrophages are receptors for complement (CR3), receptor for the Fc portion of immunoglobulin (FcR), and the  $\alpha_2$ -macroglobulin receptor (44).

**5) Release of arachidonic acid metabolites (eicosanoids):** Arachidonic acid is released from membrane phospholipids by the action of phospholipase C and/or phospholipase A<sub>2</sub>. It is then metabolized to other eicosanoids either by the cyclooxygenase pathway (prostaglandins, thromboxanes) or the lipoxygenase pathway (leukotrienes, hydroxyeicosatetraenoic acid and lipoxins) (35,40). The products of arachidonic acid metabolism mediate a number of reactions that have various biological effects.



**Figure 5. Structure of Arachidonic Acid**

In this study, cell activation was assayed by the release of eicosanoids from cells labeled with [ $^3\text{H}$ ] arachidonic acid. Arachidonic acid (Fig. 5) is an essential fatty acid which is incorporated into membrane phospholipids (40). There is little, if any, free (non esterified) arachidonic acid in unstimulated cells. In an analysis of the eicosanoid products released from mouse macrophage cultures stimulated with LPS from *S. abortus equi*, Aderem and Cohn (2) recovered over 97% of the total released label as prostaglandins and leukotrienes, and less than 2% as free arachidonic acid.

Several studies have suggested that the release of other mediators may be dependent on some step(s) or products of arachidonic acid metabolism. For example, Tarsi-Tsuk et al (47) reported that inhibition of arachidonic acid

metabolism caused a decrease in the respiratory burst (17). Likewise, release of TNF was suppressed by the inhibition of arachidonic acid metabolism (35).

### **Mechanism of Macrophage Activation**

The mechanism of signal transduction in phagocytic cells (macrophages and neutrophils) has been investigated in some detail with the synthetic peptide N-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) (43) and to a lesser extent with LPS. The most well characterized steps in macrophage activation are summarized below.

**Receptors and G-protein:** G-proteins are a group of plasma membrane components that serve a signal transducing function between a variety of receptors and intracellular components (21,50). Common characteristics of G-proteins include GTPase activity and a heterotrimeric  $\alpha\beta\gamma$  structure (50). A model of the receptor mediated stimulation of phosphatidylinositol ( $\text{PIP}_2$ ) specific phospholipase C (PLC) via G-protein is depicted in Fig. 6A (42,44). Receptor-ligand binding stimulates GTP to substitute for GDP on the  $\alpha$ -subunit of G-protein which promotes dissociation of the  $\alpha$  subunit from the  $\beta$  and  $\gamma$  subunits (44,50). This forms the activated G-protein - GTP complex. The G-protein - GTP complex causes the activation of a phosphatidylinositol-4,5-bisphosphate ( $\text{PIP}_2$ ) specific phospholipase C (PLC) (44,50).

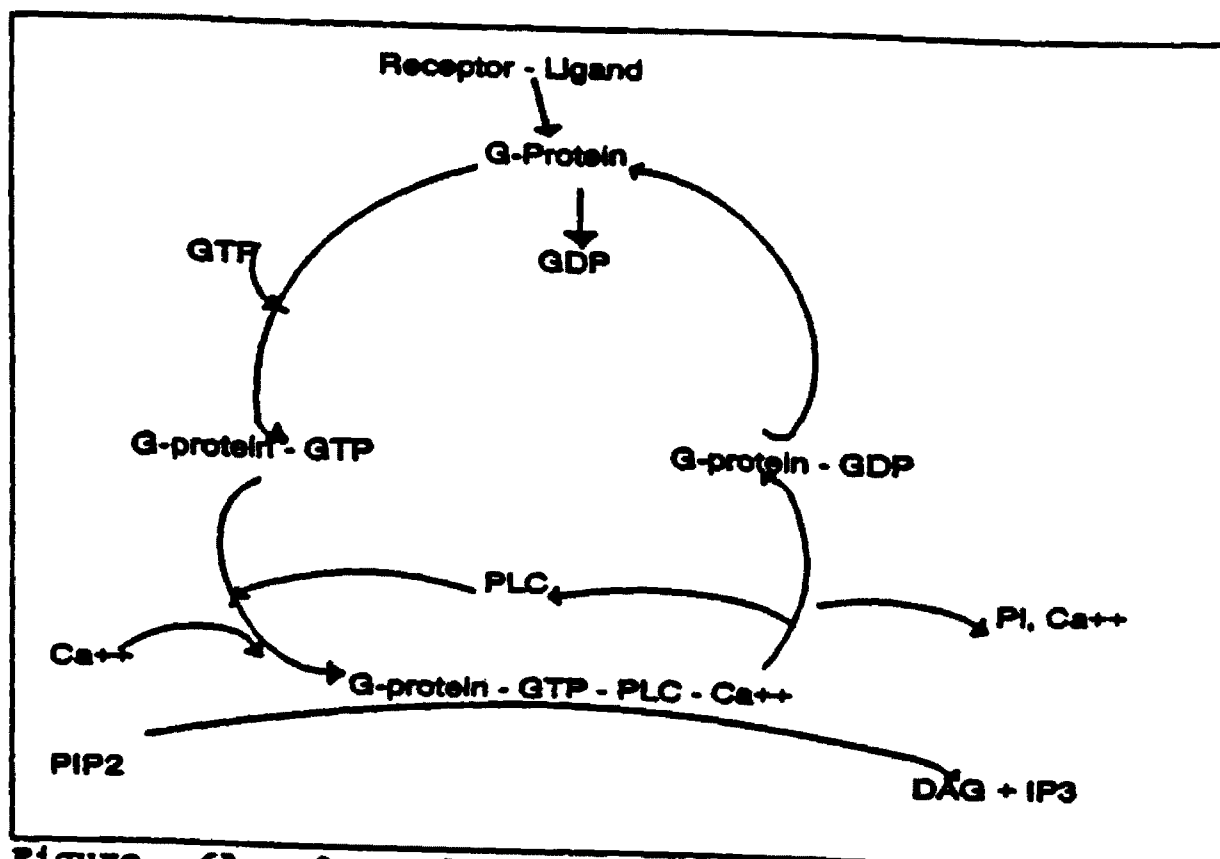


Figure 6A. A model for G-protein activation of phospholipase C

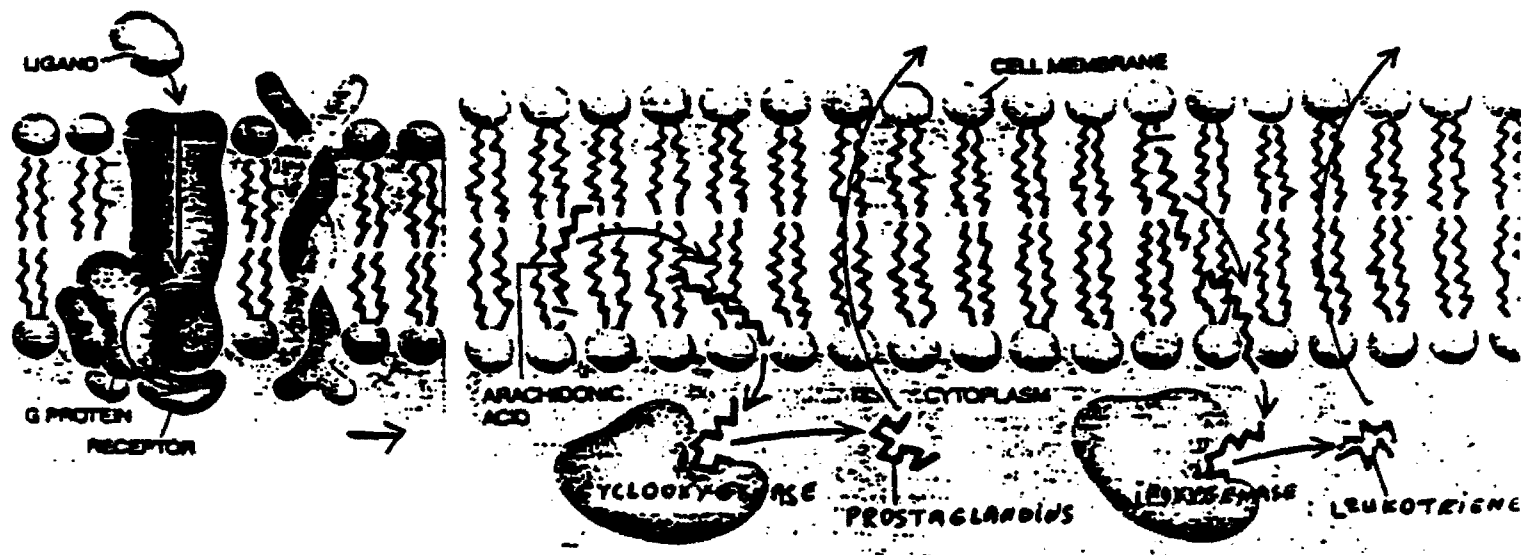
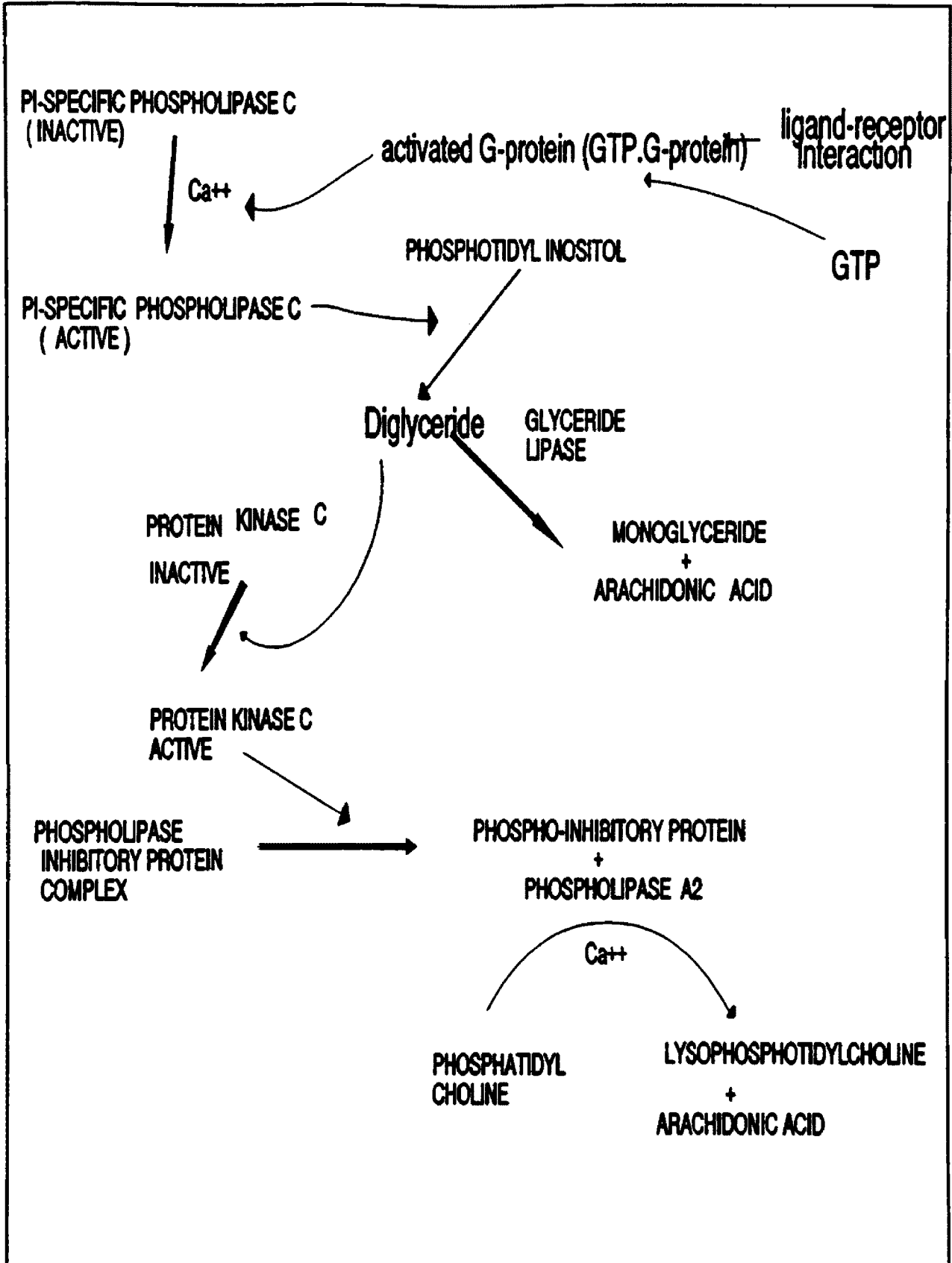


Figure 6B. Ligand-receptor interaction activates a G-protein. The G-protein then activates phospholipases which in turn release arachidonic acid from phospholipids. Arachidonic acid is metabolized by either the cyclooxygenase or lipoxygenase pathway. (Adapted from *Scientific American* vol. 264. (1)).

The activated PLC (i.e. G-protein - GTP - PLC - calcium complex) then hydrolyses  $\text{PIP}_2$  to diacylglycerol (DAG) and inositol triphosphate ( $\text{IP}_3$ ) (42,42,50). Both, DAG and  $\text{IP}_3$  derivatives are mediators.

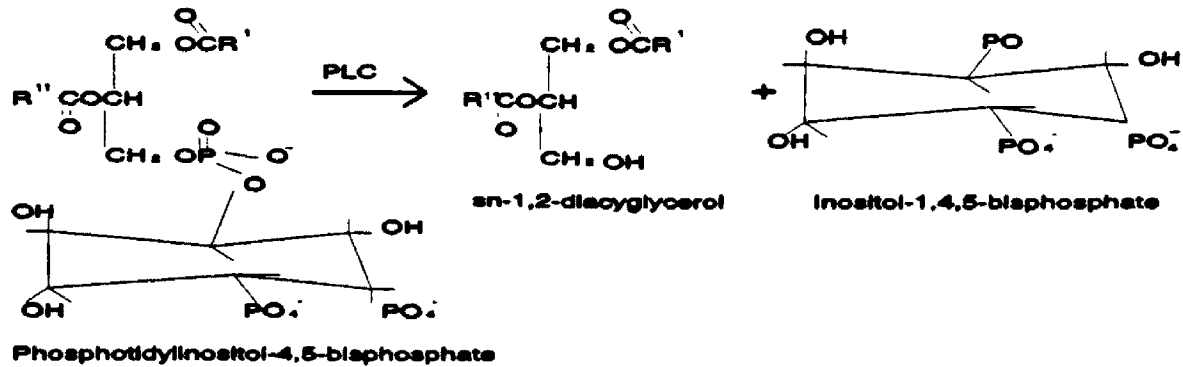
A protein of 60-70 KD, which is coupled to a G-protein, has been identified as the receptor for f-Met-Leu-Phe peptides by covalent cross-linking techniques (44). Subsequent to the addition of agonist, the number of cell surface receptors is initially decreased by endocytosis, and then increased, which suggests that the receptor is recycled (44). A receptor which binds LPS, referred to as CD14, has been identified on the surface of macrophages and monocytes (60). CD14 is a 55 KD glycoprotein that is attached to the membrane via a phosphatidylinositol anchor (60). CD14 appears to be a receptor for a complex of LPS and a 60 KD serum glycoprotein, called lipopolysaccharide binding protein (LBP) (60). LBP forms high affinity complexes with smooth as well as rough LPS. LBP will also bind to the bacterial cell surface. Wright et al (60) exposed whole blood to various concentrations of LPS in the presence or absence of monoclonal antibodies to CD14. LPS at low concentrations induced TNF production. This response was eliminated by blocking antibody to CD14, 3C10 or 60b. It was suggested that CD14 facilitates binding of LPS-LBP complexes to the cell surface in such a way that other proteins or components of the cell surface were activated





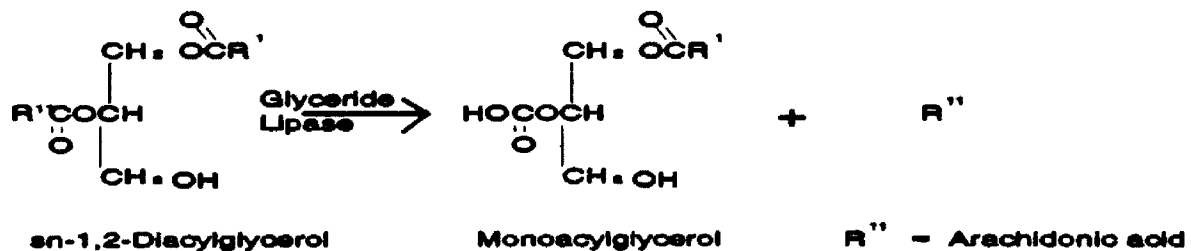
**Figure 7. Pathway of arachidonic acid release**





to a decrease in the  $\text{Ca}^{2+}$  requirement of PLC, such that the enzyme is active at ambient  $\text{Ca}^{2+}$  concentrations (51).

**Diacylglyceride and Protein Kinase C:** The diacylglycerol (DAG) formed from action of PLC is a source for arachidonic acid by the action of a diglyceride lipase. DAG is also an important mediator which can activate protein kinase C.

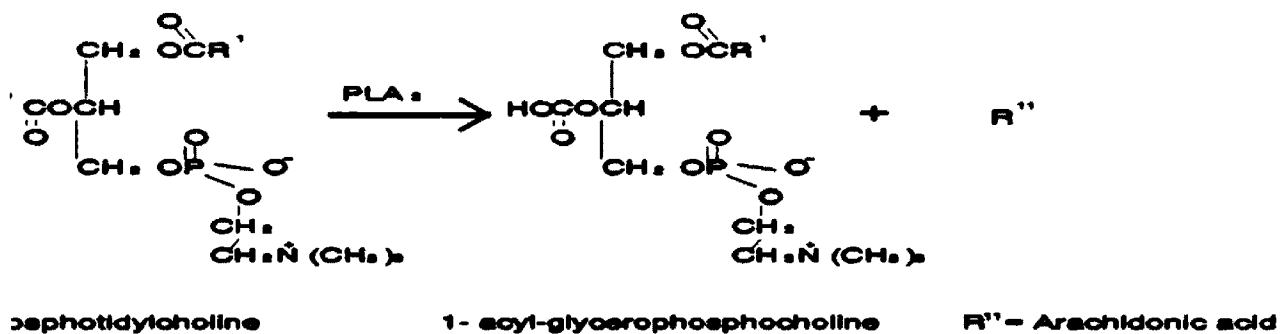


Protein kinase C plays a central role in leukocyte activation and may be involved in differentiation of monocytes to macrophages (59). Studies using phorbol esters (33), which are analogues of DAG for activation of protein kinase C, have shown that activation of protein kinase C involves its redistribution from the cytosolic to the

membrane fraction (36,59). In unstimulated polymorphonuclear leukocytes (PMN) 90% of the protein kinase C activity is in the cytosolic fraction; however, on stimulation by f-met-leu-phe, protein kinase C is translocated to the plasma membrane (33,36). Inhibition of protein kinase C translocation to the plasma membrane by pharmacological agents inhibits the respiratory burst (59). Cell permeable analogues of DAG that activate protein kinase C stimulate different activation pathways (11,16). For example, the DAG analogue sn-1,2-didecanoylglycerol stimulated degranulation without increasing superoxide production by PMNs. In contrast, another DAG analogue, sn-1,2-dioctanoylglycerol stimulated both degranulation and superoxide production in PMNs. Coussens *et al.*, (10) suggested that protein kinase C is present in isozyme forms or is compartmentalized. These observations also suggest that different activation pathways (e.g. respiratory burst, secretion of metabolites) are regulated differently (10).

The source of DAG is not necessarily PI. Snyderman and Uhing (44) found that DAG production stimulated by receptor-ligand interaction occurred from two sources - PI and PC. These workers suggested that DAG production occurred in two phases: first from PI hydrolysis, which was accompanied by an increase in calcium concentration in the cytosol, and second, from the hydrolysis of phosphatidylcholine by calcium stimulated PLC.

**Phospholipase A<sub>2</sub>:** Protein kinase C has several effects including phosphorylation of several proteins that modulate gene expression and activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (21). PLA<sub>2</sub> cleaves arachidonic acid from membrane phosphotidylcholine (PC), which is the second pathway of arachidonic acid release.



Researchers are divided on whether arachidonic acid released in response to a specific receptor-ligand interaction occurs by a PLC dependent pathway or PLA<sub>2</sub> or both. It is likely that different stimuli elicit arachidonic acid release from different sources. Walsh *et al.*, (53) have reported that zymosan and ionophores stimulate arachidonic acid release in neutrophils by PLA<sub>2</sub>. Experiments performed by Walsh *et al.*, (53) demonstrated that neutrophils stimulated with zymosan and ionophores showed no increase in levels of labeled DAG or monoacylglycerol, which are products formed by the action of phospholipase C on phosphatidylinositol (see Fig. 6). Sporn *et al.*, (45) have shown that the biologically active oxidant H<sub>2</sub>O<sub>2</sub>, and the

particulate stimuli zymosan, activated arachidonic acid metabolism in alveolar macrophages by different pathways. In their study, the protein kinase C (PKC) inhibitor, staurosporine, inhibited zymosan stimulated arachidonic acid metabolism, but was relatively ineffective in inhibiting  $H_2O_2$  stimulated arachidonic acid metabolism. Thus, zymosan activates PKC, which then activates phospholipase A<sub>2</sub> mediated arachidonic acid metabolism. In contrast,  $H_2O_2$  activates arachidonic acid metabolism by at least two pathways, a PKC dependent pathway involving PLA<sub>2</sub> activity and a second pathway that is PKC independent. Sporn et al., (45) suggested two possible PKC independent mechanisms of activating arachidonic acid metabolism - release of arachidonic acid from diacylglyceride (DAG) by the action of glyceride lipase (i.e. by activating phospholipase C) and by inhibition of arachidonic acid reacylation. Arachidonic acid turnover rates in macrophages are high (30) and inhibition of its reacylation would result in accumulation of free arachidonic acid which would activate arachidonic acid metabolism. Kroner et al., (29) have demonstrated that sulfhydryl reactive chemicals (e.g. para-chloromercuribenzoate) stimulated arachidonic acid and prostaglandin release by a PLA<sub>2</sub> independent mechanism by inhibiting reacylation of arachidonic acid into phospholipids.

**Role of Calcium:** Calcium is necessary in receptor mediated leukocyte activation. Inositol-1,4,5-trisphosphate (IP<sub>3</sub>), formed by the action of phospholipase C on phosphatidyl inositol, has been shown to cause the release of calcium from intracellular stores (7,37). The calcium ionophore (e.g. A23187) will stimulate leukocytes in the presence of sufficient extracellular calcium. Verghese et al., (50) have shown that the sequence of ligand-receptor interaction, G-protein activation, phosphatidylinositol metabolism, and calcium influx are necessary events in activation of monocytes. They showed that inhibition of G-proteins with *Bordetella pertussis* toxin inhibited subsequent steps of phosphatidylinositol metabolism, calcium mobilization and cell activation. Tarsi-Tsuk et al., (47) have shown that stimulation of the respiratory burst in monocytes with *S. faecalis* lipoteichoic acid (LTA) required an increase in cytosolic calcium levels. Treatment of these monocytes with calcium ionophore, however, caused a negligible respiratory burst, suggesting that an increase in cytosolic calcium levels is not the sole requirement for an LTA mediated respiratory burst. According to Aderem and Cohn (2), stimuli that mobilize large amounts of intracellular calcium, such as zymosan and immune complexes (particulate stimuli), cause secretion of both cyclooxygenase and lipoxygenase products, whereas stimuli that mobilize small amounts of calcium, such as PMA (soluble stimuli) promote

secretion of only the cyclooxygenase products. They demonstrated that PMA, in the presence of calcium ionophore A23187, would activate the 5-lipoxygenase pathway and that this resulted in secretion of both products. According to Aderem and Cohn (2), small amounts of calcium, such as those generated by PMA alone, are sufficient to stimulate phospholipases but not 5-lipoxygenase, i.e. phospholipases have a higher affinity for calcium than 5-lipoxygenase. Their suggestions are consistent with the  $K_m$  values of purified phospholipase C and 5-lipoxygenase.

In contrast to the above observations, Klein et al., (28) reported that an influx of extracellular calcium into the cytosol is not a necessary step in activation of neutrophils by LPS. However, intracellular calcium processes are essential for activation of neutrophils by LPS. They measured expression of C3bi (a receptor for complement that is expressed in activated neutrophils) in LPS stimulated neutrophils and found no increase in expression of C3bi. Inhibition of the influx of extracellular calcium into the cytosol by EGTA and calcium-channel blockers had no effect on expression of C3bi. Quin-2, an agent that binds calcium and interferes with intracellular calcium metabolism, partially inhibited C3bi expression. This inhibition was overcome by A23187, which, by allowing influx of extracellular calcium, reversed the effects of quin-2 (28).



### Desensitization of Macrophages

Upon repeated administration of small amounts of LPS, the response of the host to LPS challenge decreases. This phenomenon is called tolerance or desensitization (18,32). Although the mechanism of LPS induced tolerance is unknown, tolerance in other systems has been shown to involve modification of membrane receptors (14). Haas et. al., (20) reported that mice which were desensitized to LPS were able to activate nuclear factor  $\kappa$ B (NF- $\kappa$ B), a DNA binding protein required for TNF gene transcription, nevertheless, transcription of the TNF gene did not occur. Henricson et. al., (21) observed that pretreatment with either LPS at sublethal doses or monophosphoryl lipid A (MPL), a relatively nontoxic LPS derivative, could induce a state of tolerance in mice when subsequently challenged with lethal doses of LPS. LPS (25  $\mu$ g) and MPL (200  $\mu$ g) induced comparable levels of colony stimulating factor (CSF), a cytokine that stimulates proliferation of bone marrow derived precursor cells, however, the levels of interferon (IFN), TNF, and interleukin-6 (IL-6) were approximately 10-fold lower in MPL pretreated mice. They suggested that the diminished toxicity of MPL may be due to significantly lower levels of toxic products such as TNF, IFN, and IL-6 released, yet enough is produced to permit a low dose

synergy required for induction of tolerance (35). Further, they suggested that chemical differences between LPS and MPL may cause conformational differences in the lipid A moiety such that MPL binds with a lower affinity to the LPS signalling receptor, or that the conformation of MPL allowed it to interact with an alternative lipid A binding protein.

Coffee et al (9) have suggested that altered G-protein responses could be involved in tolerance to endotoxin. Endotoxin tolerant and control macrophage cultures were treated with the stable non-hydrolyzable GTP analogue GTP[ $\gamma$ -S] which stimulates arachidonic acid release. There was significantly decreased thromboxane release in response to GTP[ $\gamma$ -S] in endotoxin tolerant macrophages. Arachidonic acid depletion, loss of phospholipase activity, or loss of cyclooxygenase activity were shown to be unlikely reasons for the decrease because calcium ionophore A23187 caused more thromboxane release by the endotoxin tolerant cells than the control cells. Since tolerance to endotoxic shock is also accompanied by tolerance to other forms of shock, including epinephrine shock, hemorrhagic shock, and myocardial ischemia (9), it is possible that decreased or altered G-protein function may cause resistance to other stimuli by preventing signal transduction beyond the receptor. This cross tolerance cannot be transferred passively, indicating that it is cellular and not humoral (9).

### Inhibitors of the pathway of arachidonic acid release and arachidonic acid metabolism

The pathological effects mediated by the products of arachidonic acid metabolism can be inhibited by agents that interfere with the activation of arachidonic acid release or by agents that block metabolism of arachidonic acid to biologically active prostaglandins, thromboxanes, leukotrienes and lipoxins. Commonly used drugs such as aspirin (17) and indomethacin (17,41) prevent prostaglandin formation by inhibiting cyclooxygenase activity, whereas drugs such as benoxaprofen (17) inhibit the lipoxygenase activity preventing leukotriene formation. Inhibitors that prevent arachidonic acid release, exert their effect by blocking one or more steps in the activation pathway. Some of the commonly used inhibitory agents of arachidonic acid release are listed below.

- a) Neomycin: inhibits phosphoinositide metabolism, and therefore phospholipase C mediated arachidonic acid release (8);
- b) Hydrocortisone: inhibits phospholipase C (17);
- c) Quinacrine: inhibits phospholipase A<sub>2</sub> activity (13);
- d) Staurosporine: inhibits protein kinase C (54);
- e) H-7: inhibitor of protein kinase C (25);
- f) Pertussis toxin: inhibits function of G-proteins and therefore transduction of signal beyond receptor (4).

## OBJECTIVE

The major objective of this study was to compare the effect of lipoteichoic acid (LTA) from gram-positive bacteria to that of lipopolysaccharide (LPS) from gram-negative bacteria on arachidonic acid metabolism in macrophage cultures. Native LPS from *E. coli* and related organisms is highly toxic and has potent immunostimulatory activity. The development of effective biological response modifiers from LPS has focused on the efforts to reduce the toxicity while retaining the immunostimulatory properties of the LPS molecule. Lipoteichoic acid from several gram-positive bacteria has been reported to be non-toxic. If LTA has immunostimulatory activity then, it may have potential as a therapeutic agent.

### **Specific aims:**

1. To determine the relative ability of different LTA preparations to activate arachidonic acid metabolism in mouse peritoneal macrophages. Several studies have suggested a correlation between the immunostimulatory and toxic properties of different LPS preparations and their relative potency as measured by eicosanoid release.
2. To determine the effect of lipoteichoic acid treatment

on the ability of macrophage to respond to LPS. The toxemia which occurs in gram-negative infections results, in part at least, from products produced by activated macrophages. A substance which specifically desensitizes macrophages to LPS could be an important agent for controlling these infections.

3. To determine the pathway of macrophage activation. Specific inhibitors of different steps in eicosanoid metabolism will be used in an attempt to characterize the pathway of arachidonic acid release and subsequent metabolism.

## MATERIALS AND METHODS

### Collection of resident macrophages from mouse peritoneum.

Mice were killed with CO<sub>2</sub> then 10 ml of cold Iscove's or RPMI medium (Sigma Chemicals, St.Louis, MO), pH 7.1, without serum, was injected into the peritoneal cavity. The fluid in the peritoneum was aspirated carefully so that no bleeding occurred. About 7 - 8 ml of medium was retrieved per mouse. The cells were sedimented in a Beckman model J-6B centrifuge at 250 x g for 10 minutes at 4°C, then resuspended in 10 ml medium with 10% fetal bovine serum (FBS, Hyclone laboratories, Logan, UT).

Cells were counted using a hemacytometer and the cell density adjusted to 2 - 2.5 x 10<sup>6</sup> cells/ml. The cell suspension was added (0.5 ml/well) to a 24 well flat-bottom plate. Cells were incubated at 37°C, in a chamber with 5% CO<sub>2</sub> for 2 hours to allow macrophages to adhere. Non-adherent cells were then removed and fresh medium added.

### Treatment of macrophages with LTA and LPS prior to challenge.

Macrophage cultures were treated prior to the labeling and challenge step during the second hour of the adherence period (pretreatment). Cells were treated with 10 µg/ml of LTA or LPS as required. Control cells received medium

without LTA/LPS. After one hour treatment at 37°C the cells were washed and labeled in fresh medium.

#### **Labeling of cells with [<sup>3</sup>H] arachidonic acid.**

Adherent cells (macrophages) were washed once with medium, then 1 ml (or 0.5 ml) of medium containing [<sup>3</sup>H] arachidonic acid (0.5 μCi/well or 0.25 μCi/well) in medium with 10% FBS was added to each well. Cells were incubated overnight at 37°C in a chamber with 5% CO<sub>2</sub>.

#### **Challenge of macrophages with LTA or LPS.**

At the end of the overnight incubation the excess label was removed and the cells washed three times with medium. Cells were then challenged with 0.5 ml LPS from *S.abortus* (Ribi Immunochem, Hamilton, MT) in medium with serum at concentrations of 0.1, 1, 10, 100, and 1000 ng/ml or with 0.5 mls LTA from *S.faecalis*, *S.aureus* or *B.subtilis* (Sigma Chemicals, St.Louis, MO) in medium with serum at concentrations of 1, 10, 100, 1000, and 10000 ng/ml. In some experiments the cells were challenged in medium without serum, as indicated. Cells were incubated at 37°C for 2 hours in a chamber with 5% CO<sub>2</sub>. At the end of the incubation period the medium from each well was collected and centrifuged in an Eppendorf 5414 table top centrifuge to exclude any cells. Released [<sup>3</sup>H] arachidonic acid was determined in 200 μl duplicate samples collected in scintillation vials with 4 mls of flour

(Ecolume, ICN Biochemicals, Irvine, CA) and read in a Beckman LS 7500 scintillation counter. For determination of total counts, adhered cells were lysed with 0.5 ml of 0.5% triton X-100 to release label, then the wells were scraped, and radioactivity read in duplicate samples as described above.

**Treatment with agents that modulate the arachidonic acid pathway:**

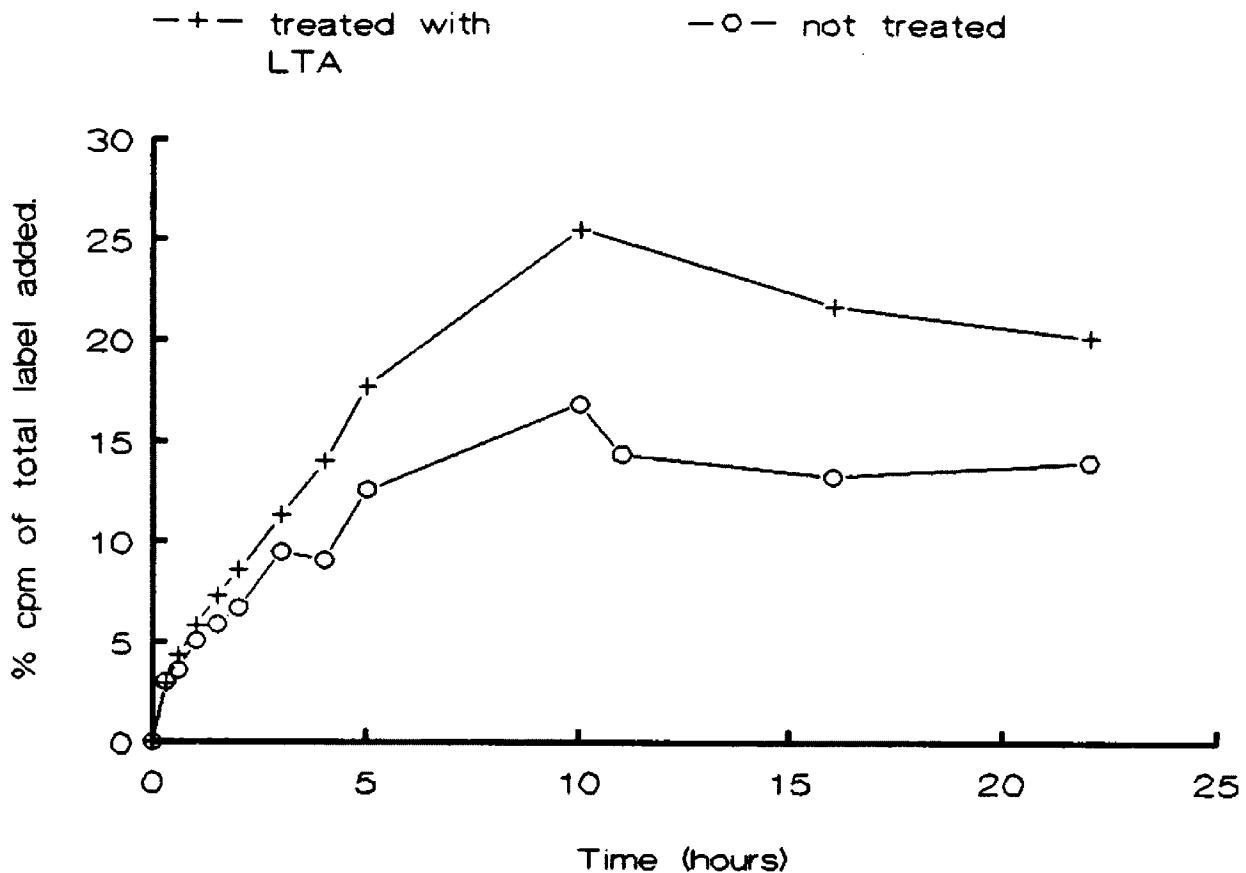
Phorbol myristic acetate (PMA) and calcium ionophore A23187 were used as activators of the arachidonic acid pathway. PMA and calcium ionophore A23187 were used to challenge cells, as described above for LTA and LPS, at concentrations of 1, 10, 100, 1000, and 10000 ng/ml in media with or without serum, as indicated. Indomethacin (41), neomycin (8), quinacrine (13), and staurosporine (54) were used as inhibitors of specific steps of the arachidonic acid activation pathway. Inhibitors were used either in the pretreatment, labeling, or challenge period, as indicated. When used in the challenge step, the inhibitor was added 30 minutes prior to addition of LPS or LTA followed by a 2 hour incubation period. The concentrations of the inhibitors used were: indomethacin  $10^{-6}$ , quinacrine 10 mM and 1 mM, staurosporine 1  $\mu$ g/ml and 10  $\mu$ g/ml, and neomycin 6.2 mg/ml and 1 mg/ml.



## RESULTS

### Effect of LTA and LPS on [<sup>3</sup>H] arachidonic acid uptake

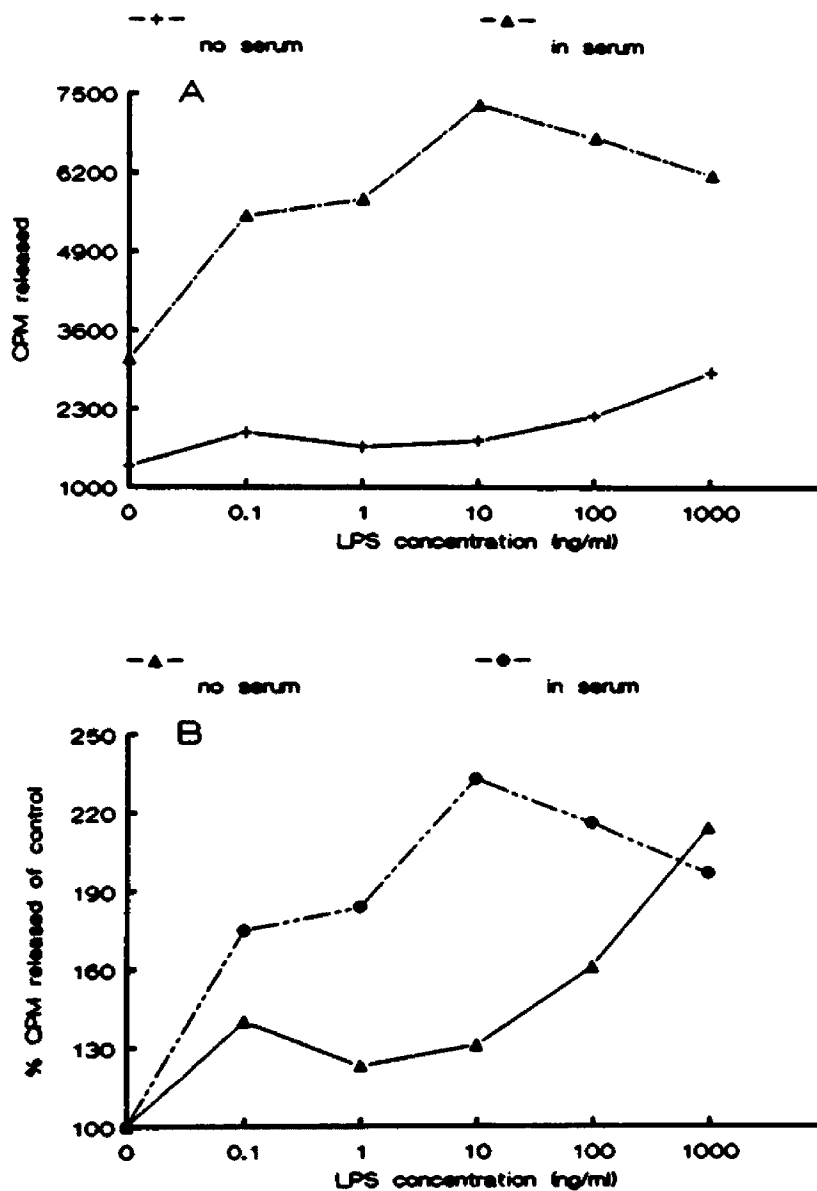
One of the goals of this study was to determine the effect of LTA on the subsequent sensitivity of macrophage cultures to LPS. It was important, therefore, to show that LTA did not block the uptake of (<sup>3</sup>H) arachidonic acid during the labeling period. Cells were treated with 10 µg/ml of *S. faecalis* LTA in RPMI medium for one hour prior to the addition of label. Samples were taken from duplicate wells at intervals for 24 hours. Label not taken up by cells was assayed by measuring label in medium (data not shown). Label taken up by cells was measured by aspirating the remaining medium, then washing the adhered cells three times to remove any remaining label. The cells were then lysed by adding 0.5 ml triton X-100 to each well. The wells were scraped, and label taken up by cells counted. It is clear from the results shown in Fig. 9 that LTA treatment actually increased (<sup>3</sup>H) arachidonic acid uptake. After 10 hrs, LTA treated cells showed an uptake of 26 % of the total label added, whereas untreated cells took up 17 % of the total label. The results suggested that activation results in increased turnover of membrane arachidonic acid.



**Fig. 9: Effect of LTA on [ $^3\text{H}$ ] arachidonic acid uptake.** Resident peritoneal macrophages from mice were pretreated with  $10\mu\text{g/ml}$  of *S. faecalis* LTA in RPMI medium (+) or with RPMI medium alone (o) for one hour. The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid ( $0.25\text{ mCi/well}$ ). At intervals the cells were washed to remove label in medium, lysed with  $0.5\text{ ml/well}$  of  $0.5\%$  triton X-100, the wells scraped, and label taken up by cells measured. Values are the mean of four samples.

**Effect of serum on eicosanoid release.**

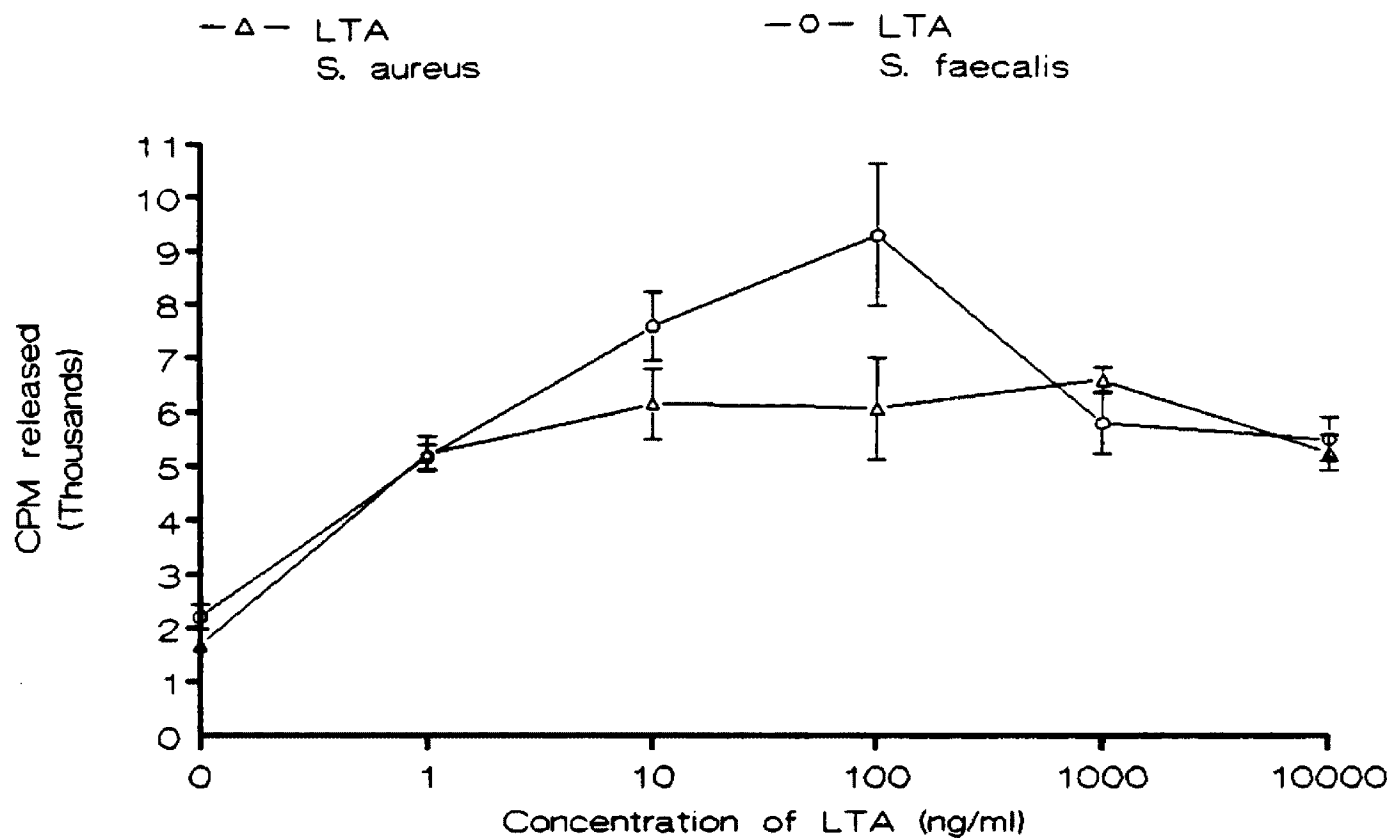
The influence of serum during the challenge period was studied by challenging cells with LPS in RPMI with and without 10% fetal bovine serum (FBS). Cells challenged in RPMI with serum released more label than cells challenged in RPMI alone (Fig. 10A and 10B).



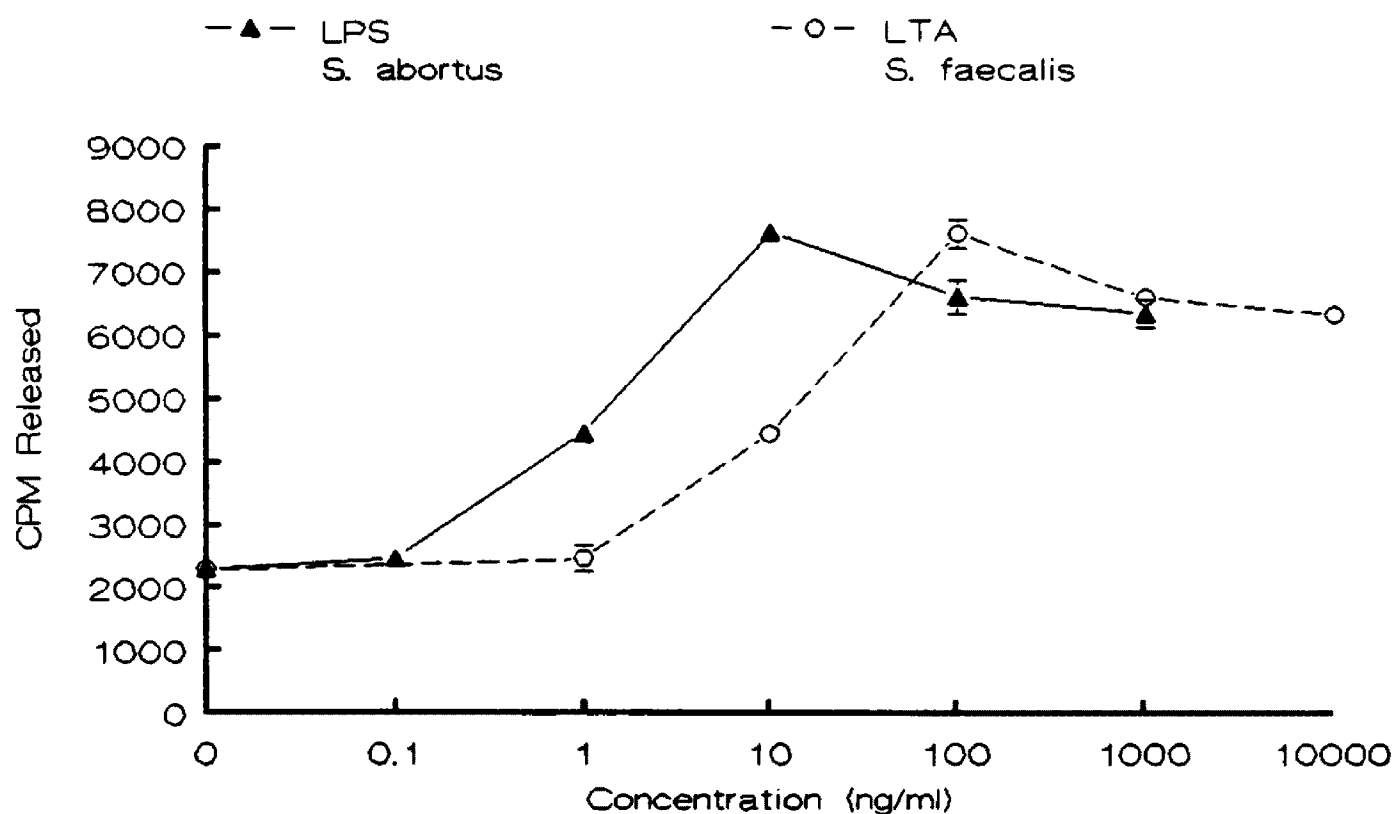
**Fig. 10: Effect of serum during the challenge period.** Resident peritoneal macrophages from mice were labeled with [ $^3\text{H}$ ] arachidonic acid, washed, and challenged with *S. abortus* LPS in RPMI with or without serum (10% FBS). Data shown as CPM released (A) and % CPM released of control (B).

### LTA activated arachidonic acid metabolism

Mouse peritoneal macrophages were labeled with [<sup>3</sup>H] arachidonic acid and challenged with LTA from the Gram-positive bacteria *S. aureus* (Fig. 12), *S. faecalis* (Fig. 12), *B. subtilis* (Fig. 14A), and *B. stearothermophilus* (Fig. 14B). The response to LTA was compared to the response to LPS from *Salmonella abortus* (a smooth strain) (Fig. 13). LPS from *S. abortus* initiated eicosanoid release and showed a maximum release at lower concentrations than LTA. Nevertheless, all of the LTA preparations activated eicosanoid release in the macrophage cultures. The total amount of eicosanoid released was about the same for LPS and *S. aureus* and *S. faecalis* LTA (Fig. 13). *B. subtilis* LTA stimulated arachidonic acid release, but the amount released was much less than the other LTA preparations (Fig. 14A). LTA from *B. stearothermophilus* stimulated release of about three times as much arachidonic acid as LTA from *S. faecalis* or *S. aureus*. This LTA must be further purified before it can be compared to the other preparations.

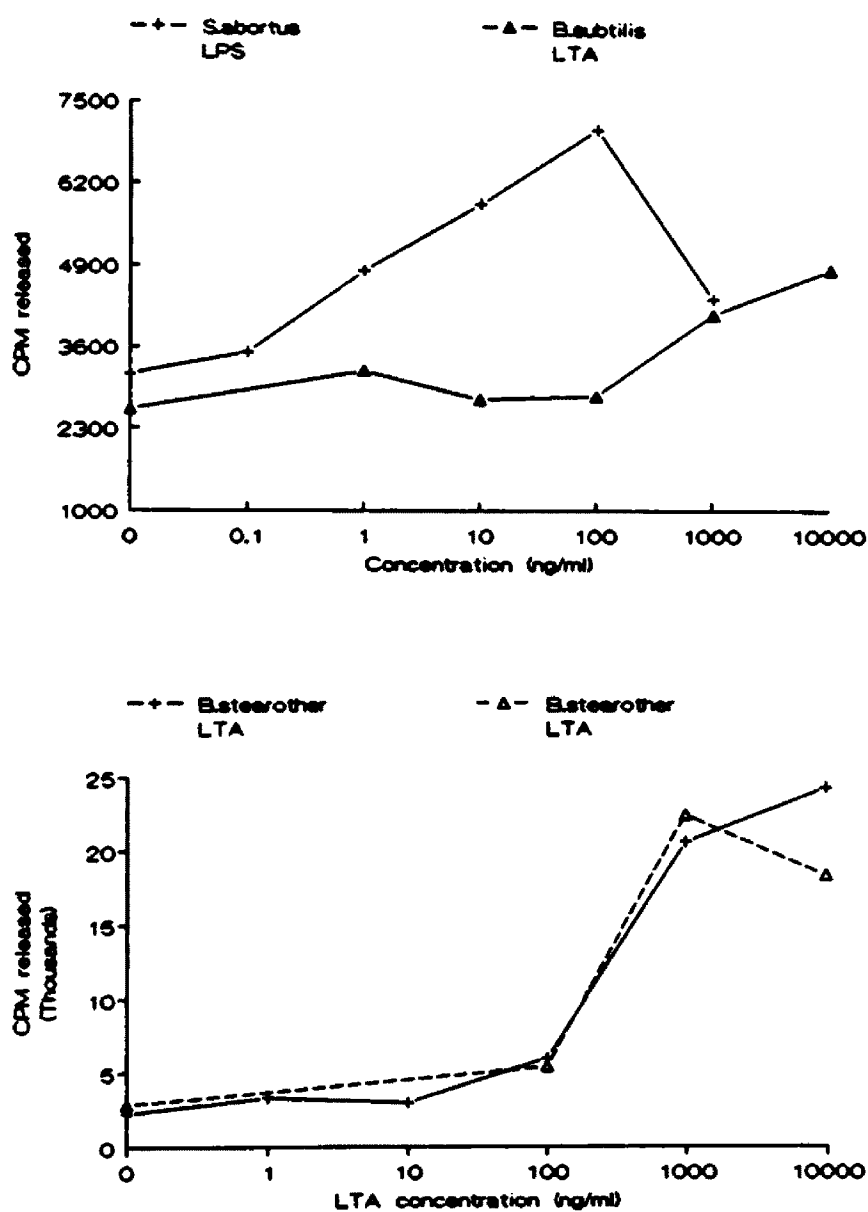


**Fig. 11: LTA from *S. aureus* and *S. faecalis* activated arachidonic acid metabolism in mouse peritoneal macrophages.** Resident peritoneal macrophages from mice were labeled with [ $^3\text{H}$ ] arachidonic acid, washed, and challenged with LTA from *S. aureus* or *S. faecalis*.



**Fig. 12: Release of arachidonic acid by LTA and LPS was comparable.**

Resident peritoneal macrophages from mice were labeled with [ $^3\text{H}$ ] arachidonic acid, washed, and challenged with *S. abortus* LPS or *S. faecalis* LTA.



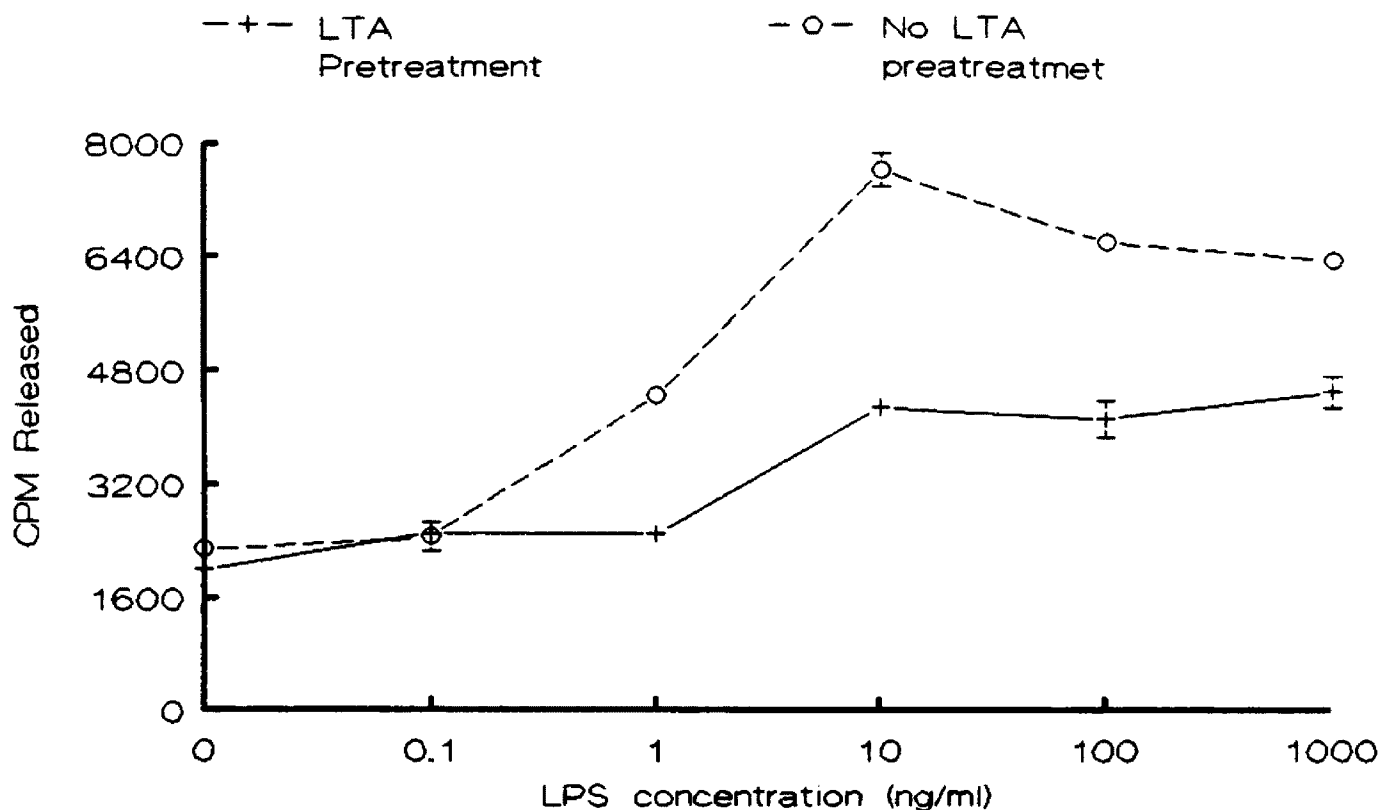
**Fig. 13: Effect of LTA from *Bacillus subtilis* and *Bacillus stearothermophilus* on arachidonic acid release.**

Resident peritoneal macrophages from mice were labeled with [ $^3\text{H}$ ] arachidonic acid, washed, and challenged with LTA from *B. subtilis* (A) or *B. stearothermophilus* (B).



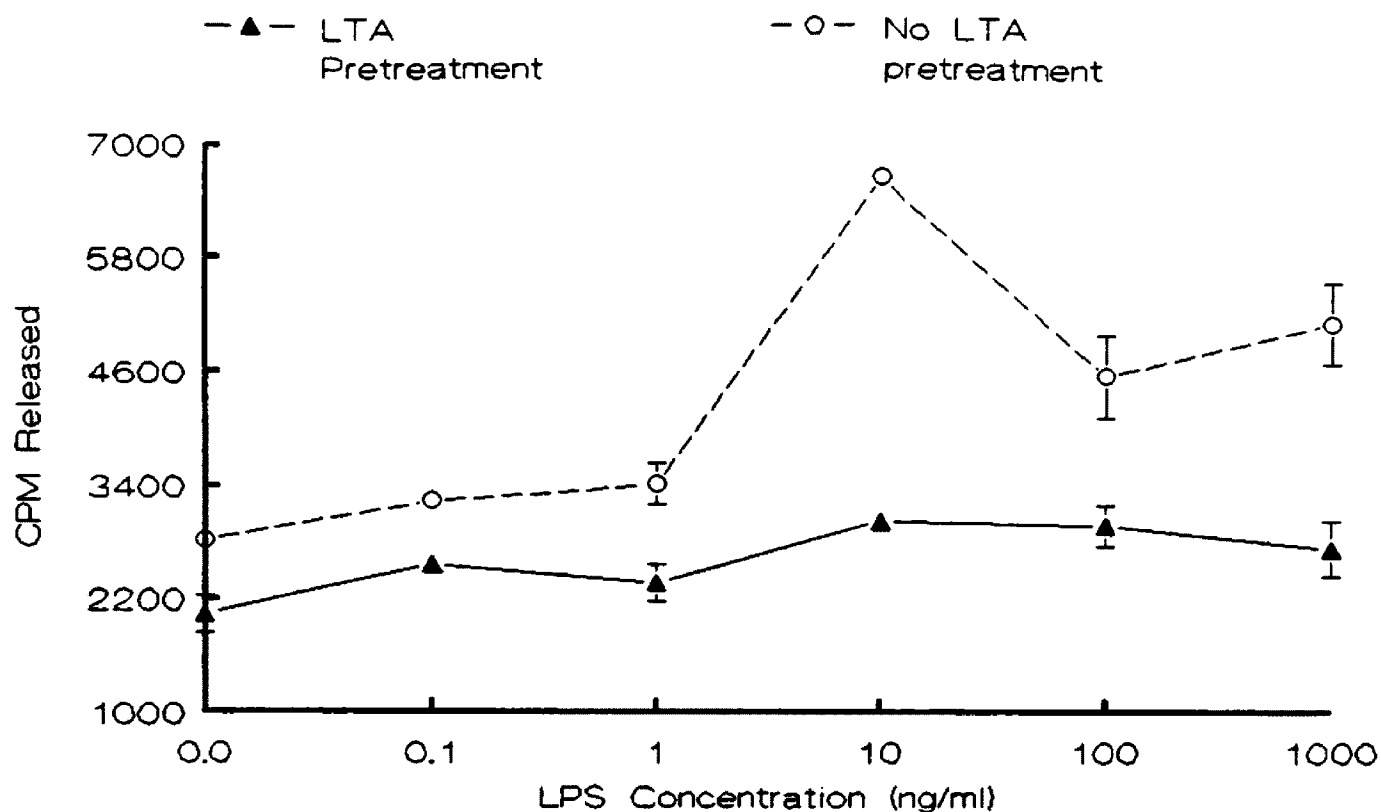
**LTA desensitized macrophages to *S. abortus* LPS challenge**

Macrophage cultures were pretreated for one hour with 10 µg/ml LTA from *S. faecalis*, *S. aureus*, *B. subtilis* or *B. stearothersophilus*. Cells were then labeled with [<sup>3</sup>H]arachidonic acid overnight, then washed and challenged with LPS from *S. abortus*. LTA from *S. faecalis* and *S. aureus* desensitized macrophage cultures to challenge with LPS from *S. abortus* (Fig. 15 and 16) as was evident from a marked decrease in [<sup>3</sup>H]arachidonic acid released. LTA from *B. subtilis* did not desensitize macrophages to LPS challenge (Fig. 17). When *B. stearothersophilus* was used to pretreat cells under the same conditions as above, the desensitization was complete (Fig. 18). As stated above, this was a crude preparation of *B. stearothersophilus* LTA and the results are questionable. Desensitization was not specific for LTA treatment or LPS challenge - cells treated with *S. abortus* LPS and subsequently challenged with the same LPS were desensitized (Fig. 19) and cells treated with *S. faecalis* LTA and subsequently challenge with the same LTA were also desensitized (Fig. 20).



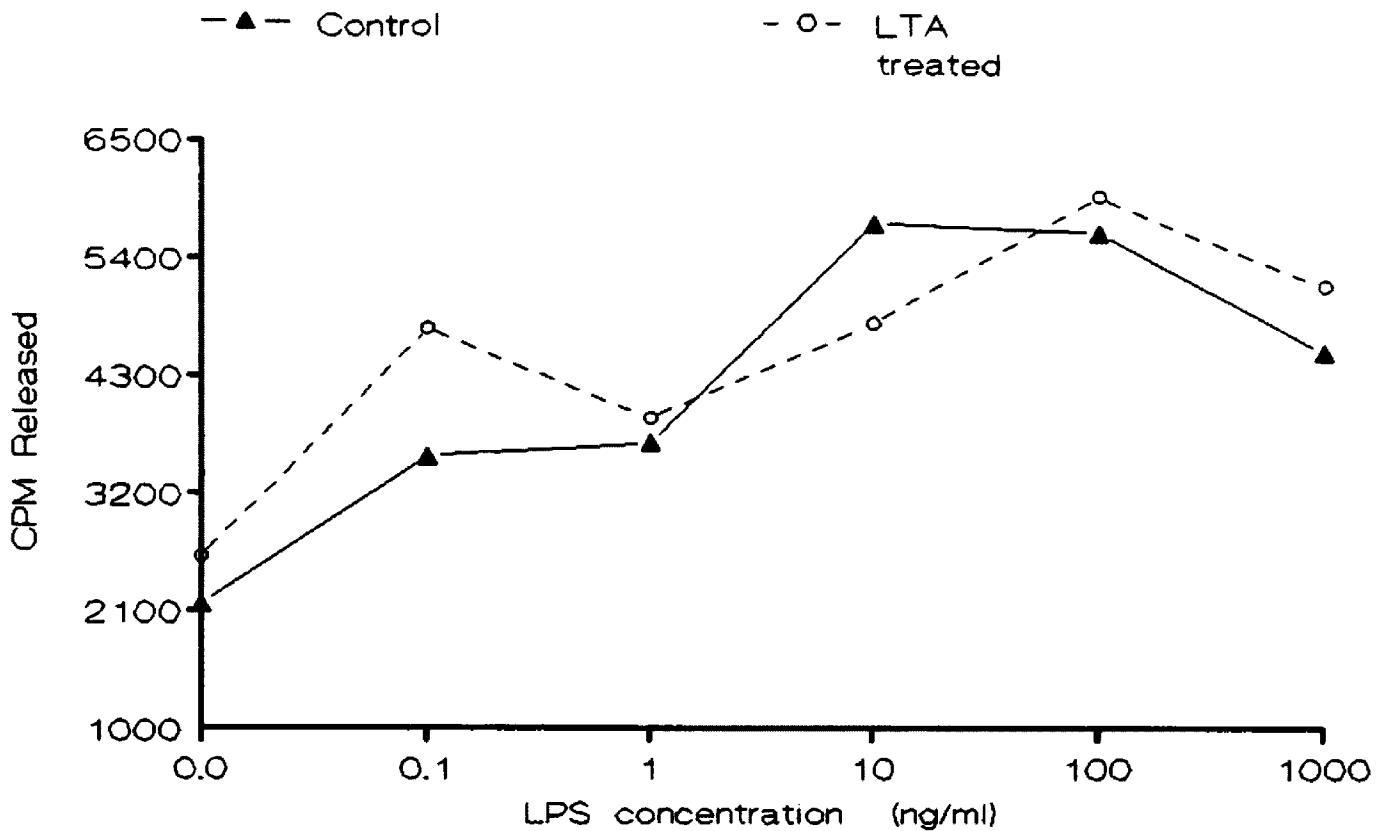
**Fig. 14: *S. faecalis* LTA desensitized macrophages to *S. abortus* LPS challenge.**

Resident peritoneal macrophages from mice were pretreated with either 10  $\mu\text{g/ml}$  of *S. faecalis* LTA in Iscove's medium or with Iscove's medium alone (no pretreat). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS.



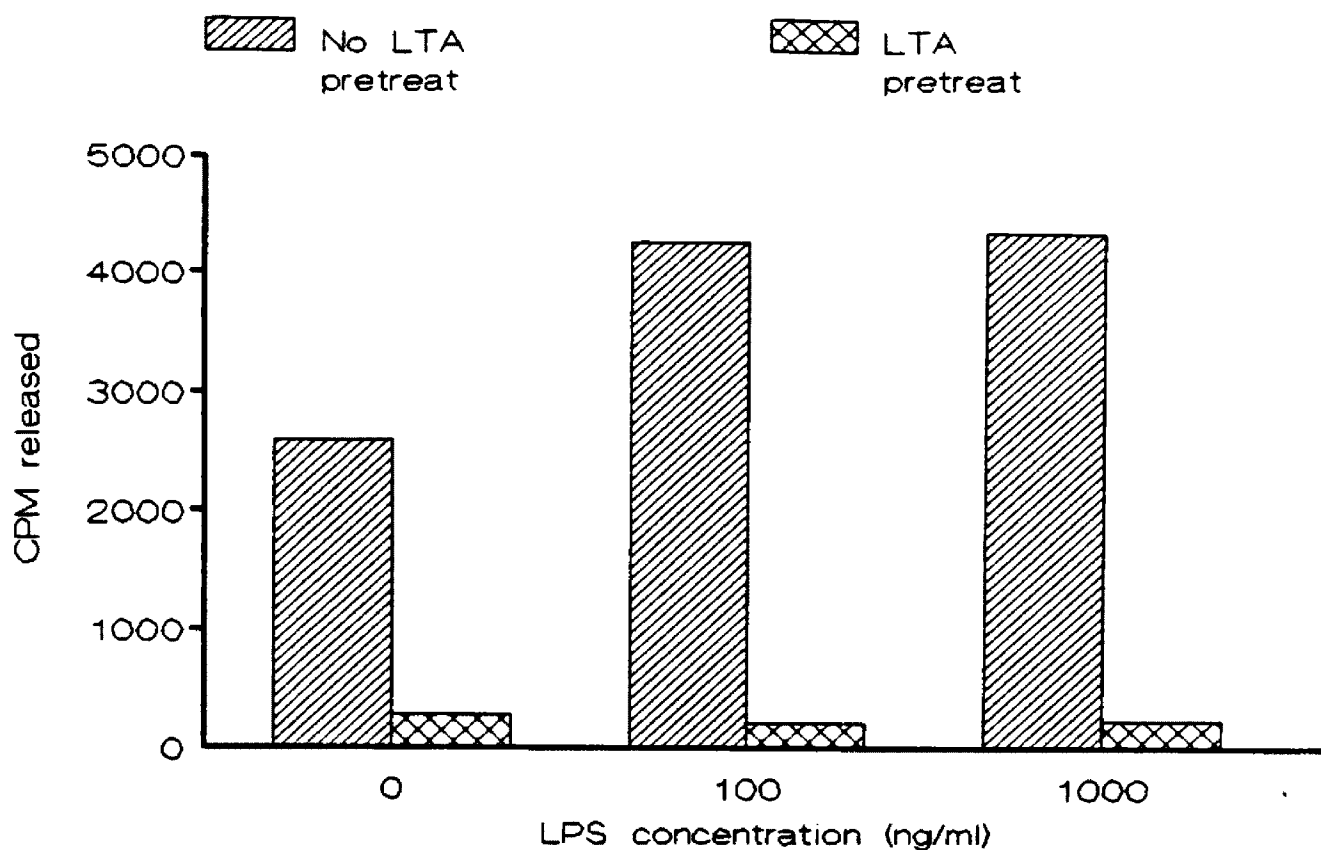
**Fig. 15: *S. aureus* LTA desensitized macrophages to *S. abortus* LPS challenge.**

Resident peritoneal macrophages from mice were pretreated with either 10  $\mu\text{g/ml}$  of *S. aureus* LTA in Iscove's medium or with Iscove's medium alone (no pretreat). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS.



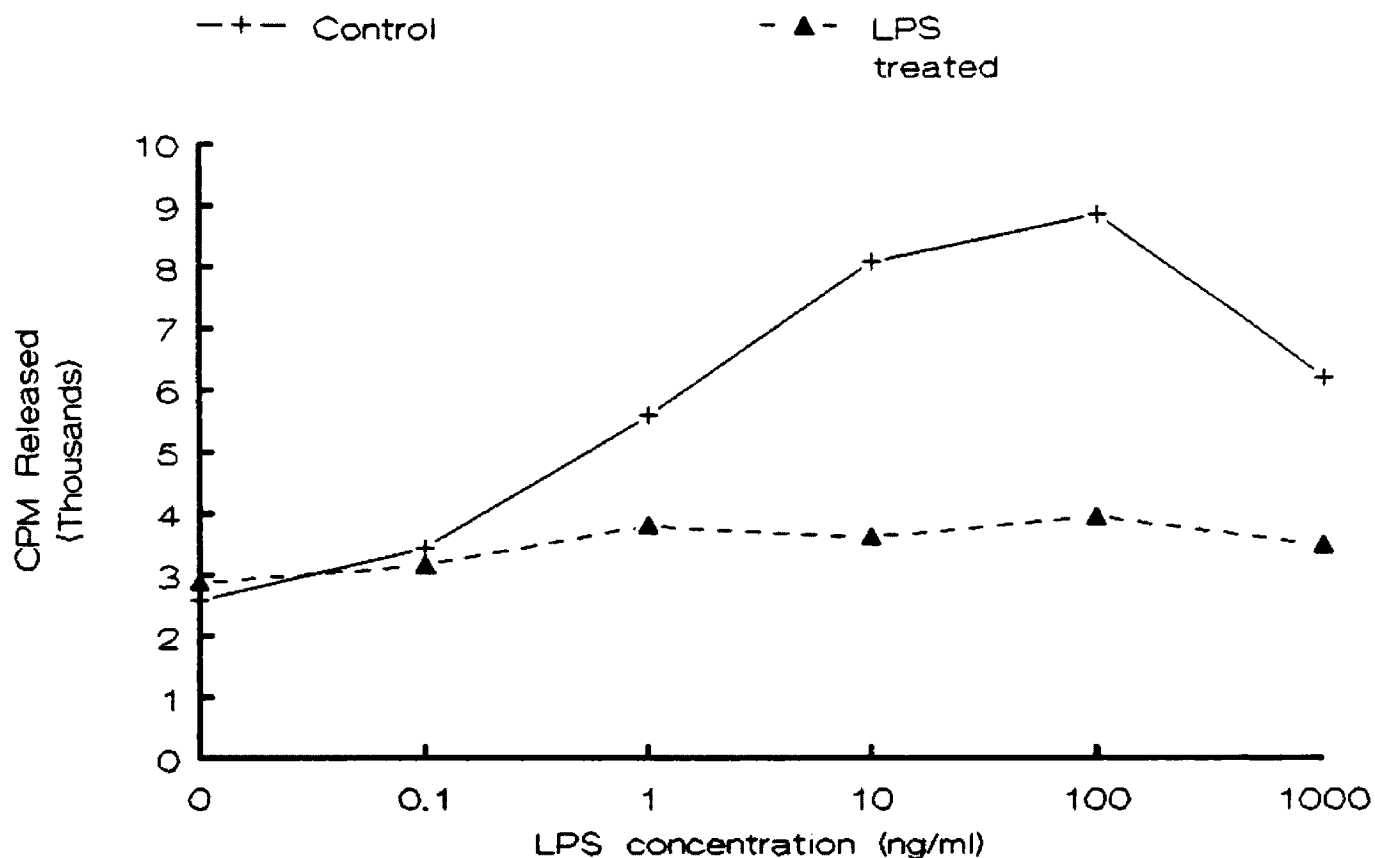
**Fig. 16: *B. subtilis* LTA did not desensitize macrophages to *S. abortus* LPS challenge.**

Resident peritoneal macrophages from mice were treated with either 10  $\mu\text{g/ml}$  of *B. subtilis* LTA in Iscove's medium or with Iscove's medium alone (no pretreat). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS.



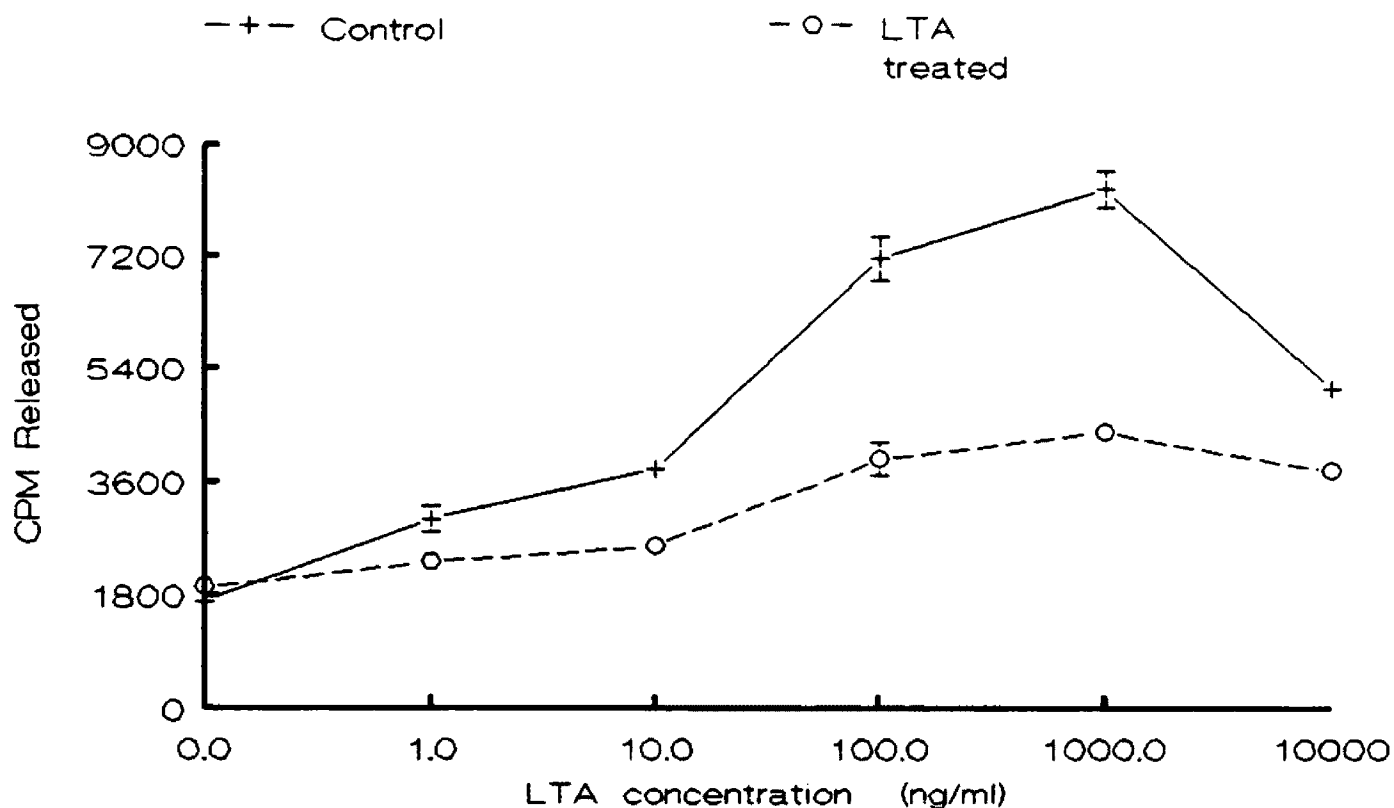
**Fig. 17: *B. steorothermophilus* LTA desensitized macrophages to *S. abortus* LPS challenge.**

Resident peritoneal macrophages from mice were treated with either 10  $\mu\text{g/ml}$  of *B. steorothermophilus* LTA in Iscove's medium or with Iscove's medium alone (no LTA treatment). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS.



**Fig. 18: *S. abortus* LPS desensitized macrophages to *S. abortus* LPS challenge.**

Resident peritoneal macrophages from mice were treated with either 10  $\mu\text{g/ml}$  of *S. abortus* LPS in Iscove's medium or with Iscove's medium alone (no LPS treatment). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS.



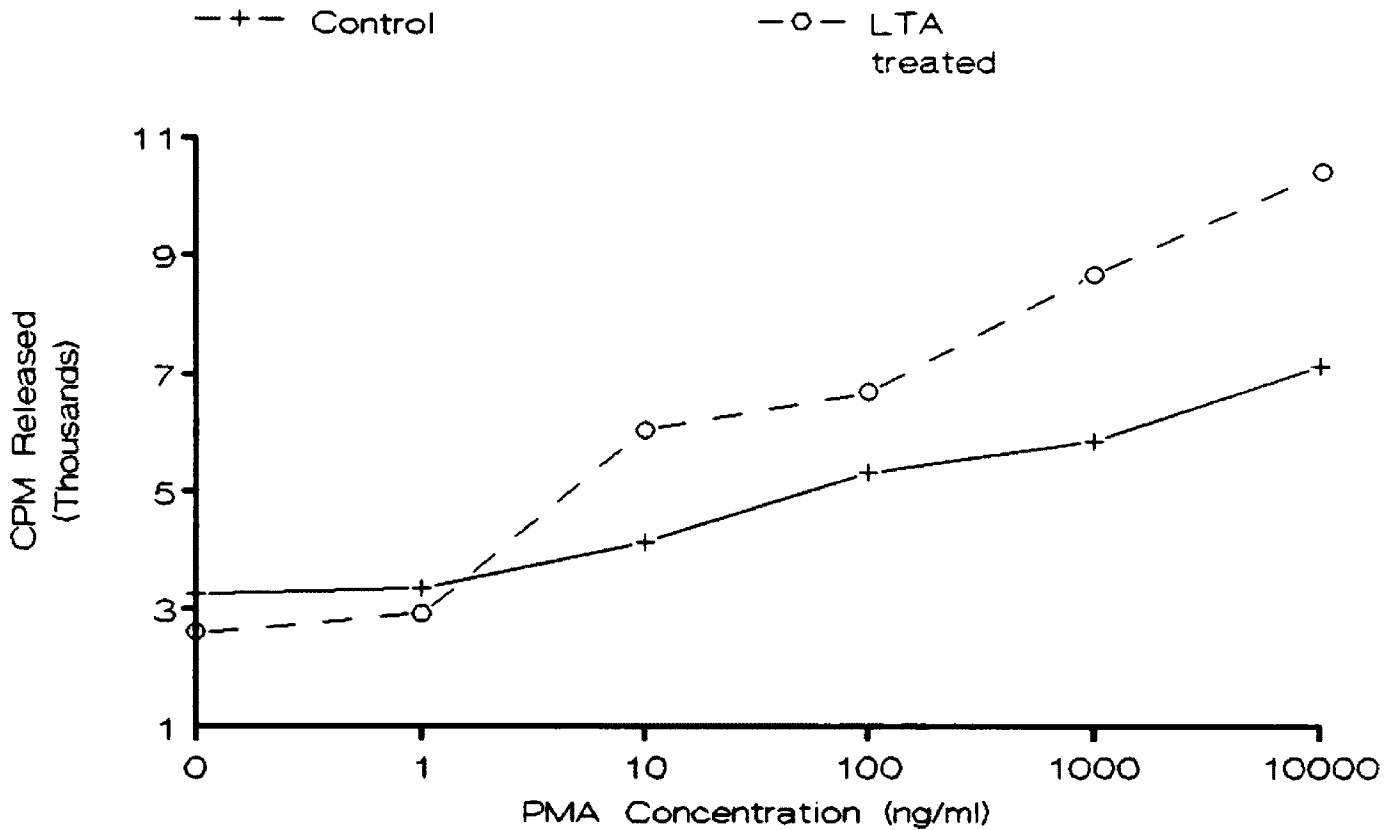
**Fig. 19: *S. faecalis* LTA desensitized macrophages to *S. faecalis* LTA challenge.**

Resident peritoneal macrophages from mice were treated with either 10  $\mu\text{g/ml}$  of *S. faecalis* LTA in Iscove's medium or with Iscove's medium alone (no LTA treatment). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. faecalis*

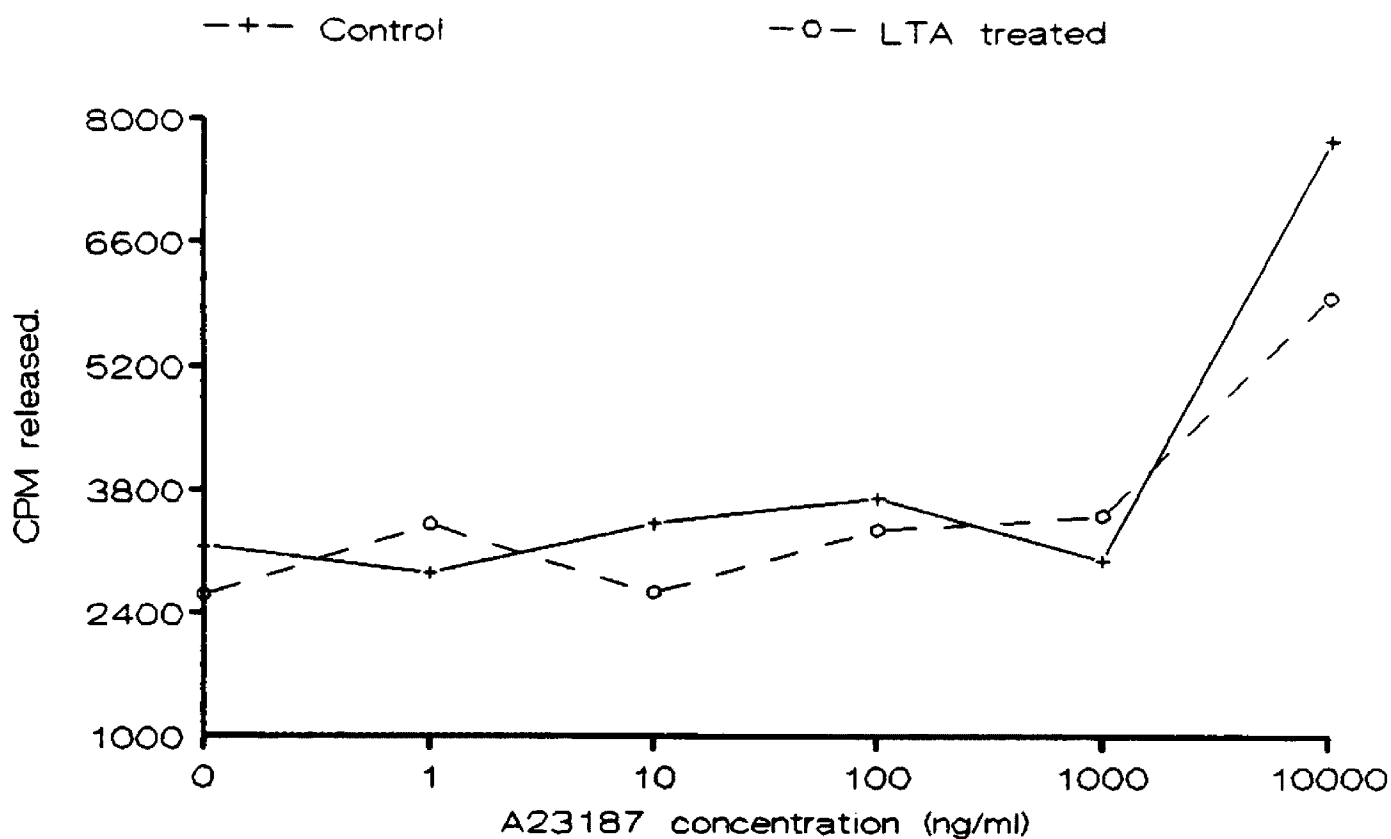
**Effect of LTA treatment of macrophage cultures on subsequent challenge with PMA and the calcium ionophore A23187**

To understand the nature of desensitization by LTA treatment, macrophage cultures were treated with LTA from *S. faecalis* then challenged with phorbol myristic acetate (PMA), a stimulus that bypasses receptor mediated activation of cells. Cells treated with LTA were not desensitized to PMA challenge (Fig. 21). When calcium ionophore A23187, an agent that causes an influx of calcium into the cytosol, was used to challenge LTA treated cells, no desensitization occurred (Fig. 22).





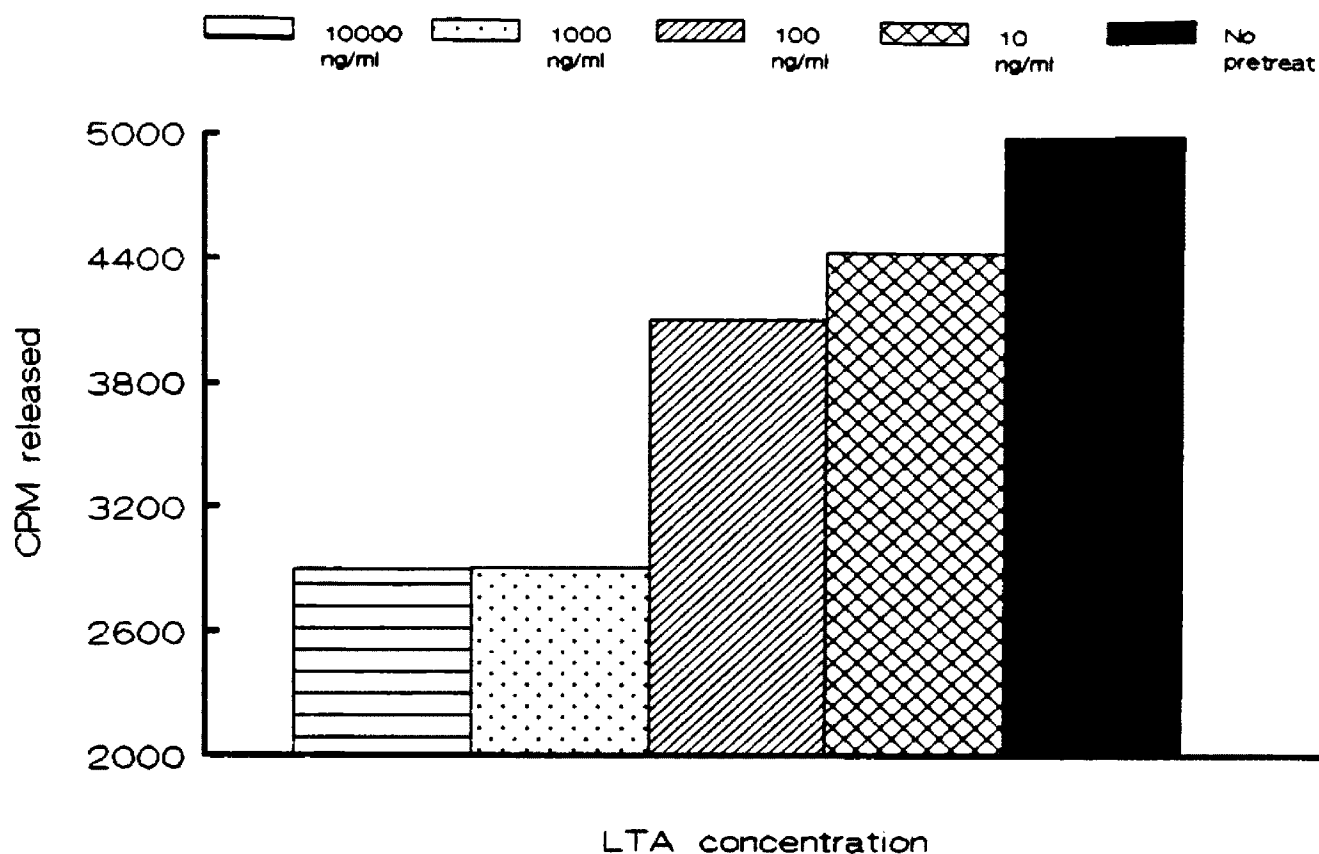
**Fig. 20: *S. faecalis* LTA did not desensitize macrophages to phorbol myristate acetate (PMA) challenge.** Resident peritoneal macrophages from mice were treated with either 10  $\mu\text{g/ml}$  of *S. faecalis* LTA in Iscove's medium or with Iscove's medium alone (no LTA treatment). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with PMA.



**Fig. 21: *S. faecalis* LTA did not desensitize macrophages to calcium ionophore A23187 challenge.** Resident peritoneal macrophages from mice were treated with either 10  $\mu\text{g/ml}$  of *S. faecalis* LTA in Iscove's medium or with Iscove's medium alone (no LTA treatment). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with A23187.

**Effect of LTA concentration on desensitization**

Fig. 23 shows the relative ineffectiveness of *S. faecalis* LTA concentrations of 10 and 100 ng/ml in desensitizing cells to subsequent LPS challenge. Whereas LTA concentrations of 1000 and 10,000 ng/ml were equally effective in desensitizing cells to LPS challenge.

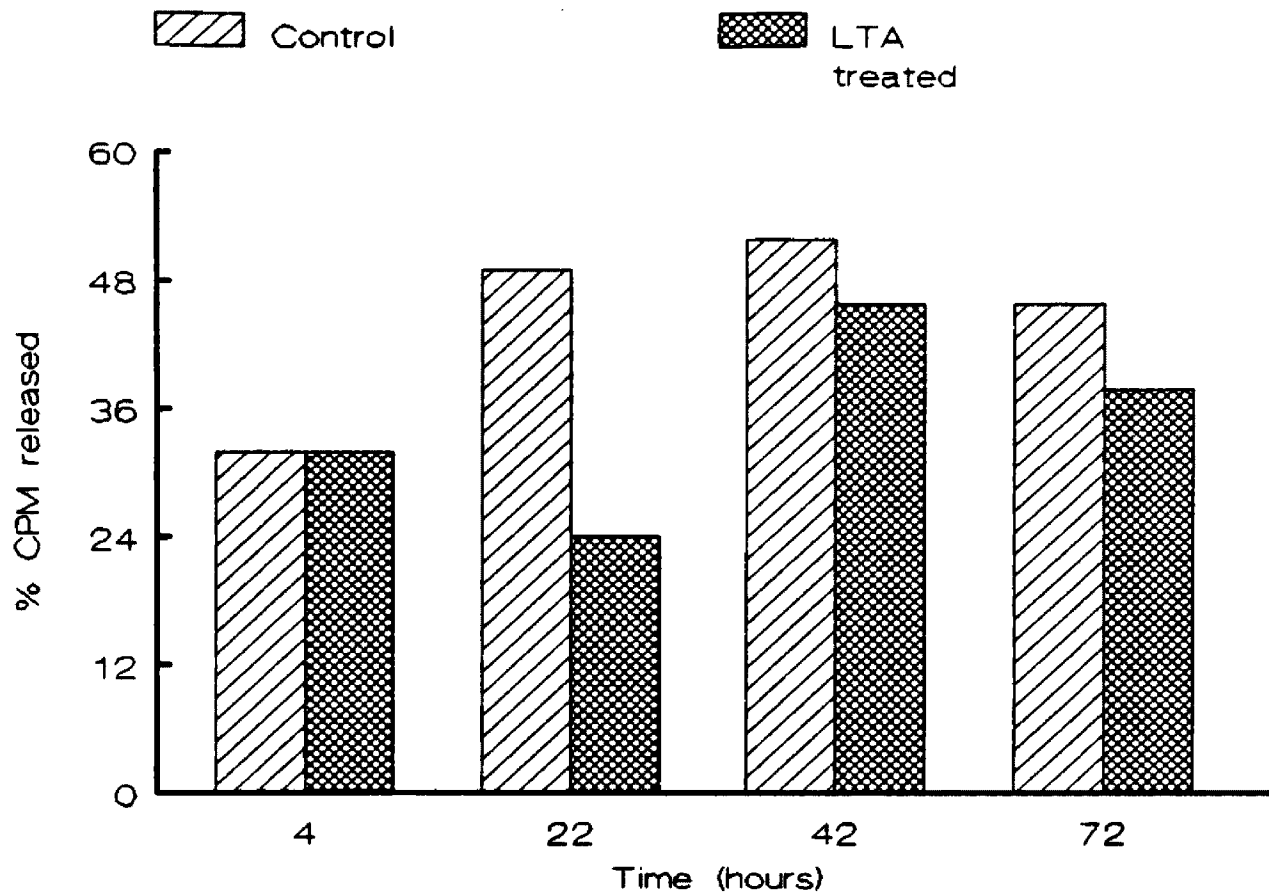


**Fig. 22: Effect of LTA concentrations on desensitization.** Resident peritoneal macrophages from mice were pretreated with either 10ng/ml, 100ng/ml, 1000ng/ml, or 10,000ng/ml *S. faecalis* LTA in Iscove's medium for one hour. The cells were then incubated with [<sup>3</sup>H] arachidonic acid overnight, washed and challenged with 10  $\mu$ g/ml *S. abortus* LPS.

**Effect of time period between pretreat and challenge**

To determine the period of time for which macrophage cultures remain desensitized after LTA treatment cells were treated with 10  $\mu\text{g/ml}$  of LTA, labeled, washed three times to remove any remaining label and subsequently challenged with *S. abortus* LPS at various intervals over a period of 72 hours.

Fig. 24 shows that cells challenged with LPS at 4 hours were not desensitized. At 22 hours cells were desensitized and then gradually became more responsive to LPS challenge at 42 and 72 hours.

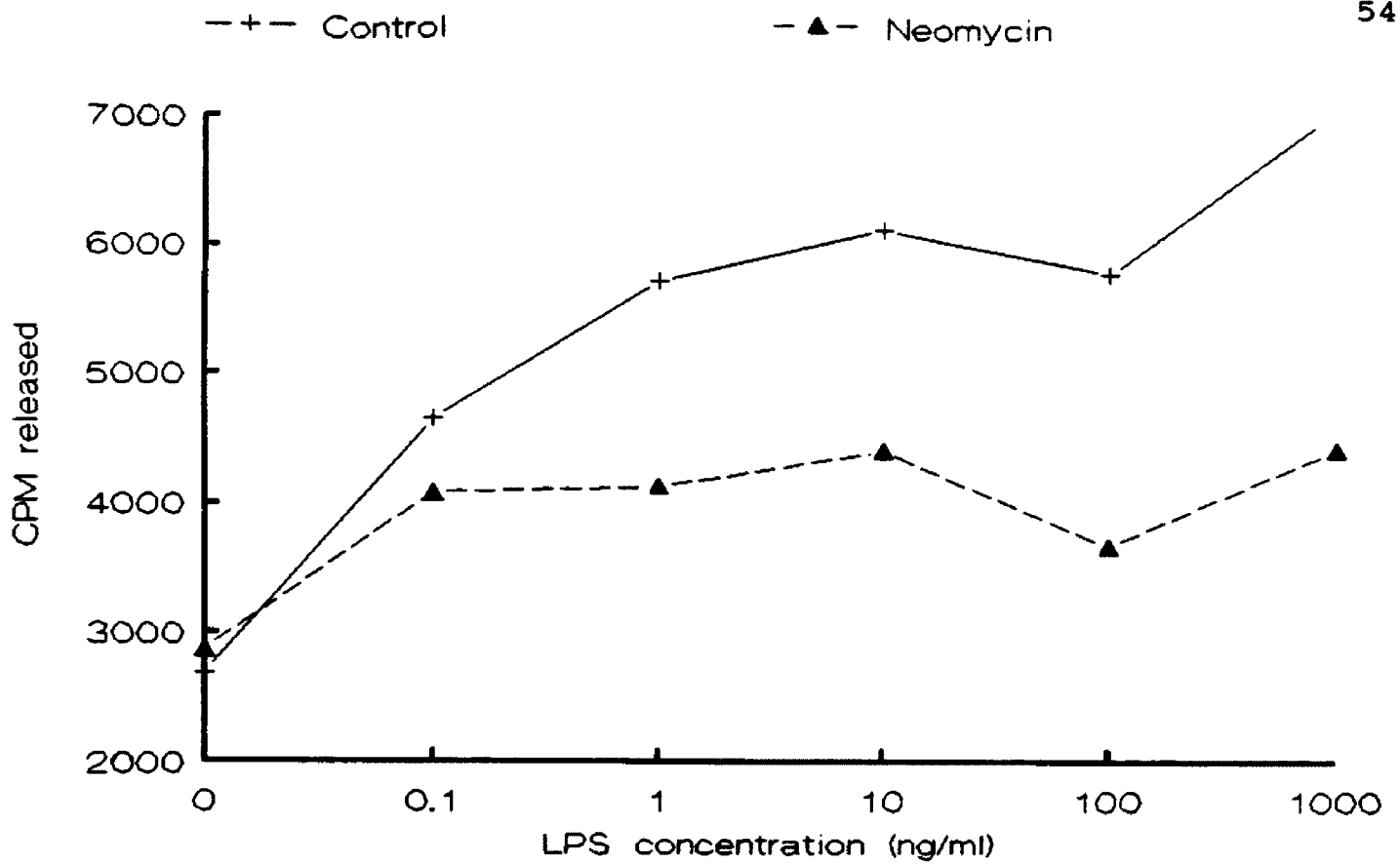


**Fig. 23: Effect of time period between LTA treatment and subsequent LPS challenge.**

Resident peritoneal macrophages from mice were treated with 10  $\mu\text{g/ml}$  of *S. faecalis* LTA in Iscove's medium or with Iscove's medium alone (no LTA treatment). The cells were then incubated with [ $^3\text{H}$ ] arachidonic acid. At various intervals, over a period of 72 hours, the cells were washed to remove label, and challenged with 10  $\mu\text{g/ml}$  *S. abortus* LPS.

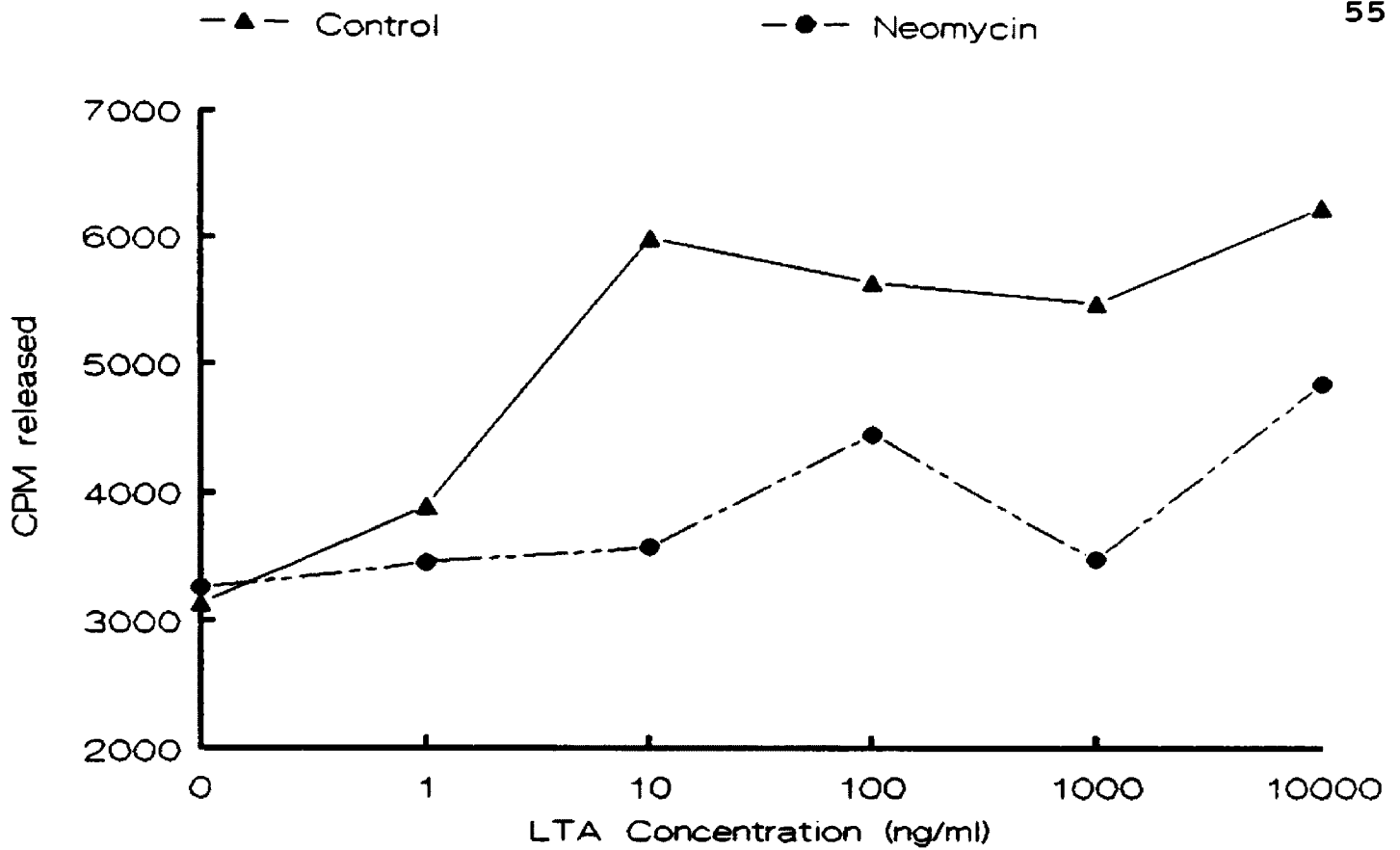
### **Effect of Neomycin on LPS and LTA stimulation of macrophage cultures**

There is evidence for both phospholipase C dependant and phospholipase C independent pathways of arachidonic acid mobilization. The role of phospholipase C in LTA activation of arachidonic acid metabolism was evaluated by treating cells with neomycin, which inhibits phospholipase C. Cells were allowed to adhere, labeled with [<sup>3</sup>H]arachidonic acid overnight, washed and treated with 6.2 mg/ml neomycin for 30 min. The cells were then challenged with LPS. Both, LPS (Fig. 25) and LTA (Fig. 26) stimulated cells, showed a significant decrease in arachidonic acid release as compared to controls. Lower concentrations of neomycin were ineffective in inhibiting arachidonic acid release (data not shown).



**Fig. 24: Neomycin with LPS challenge.**  
Resident peritoneal macrophages from mice were labeled with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS with or without Neomycin.



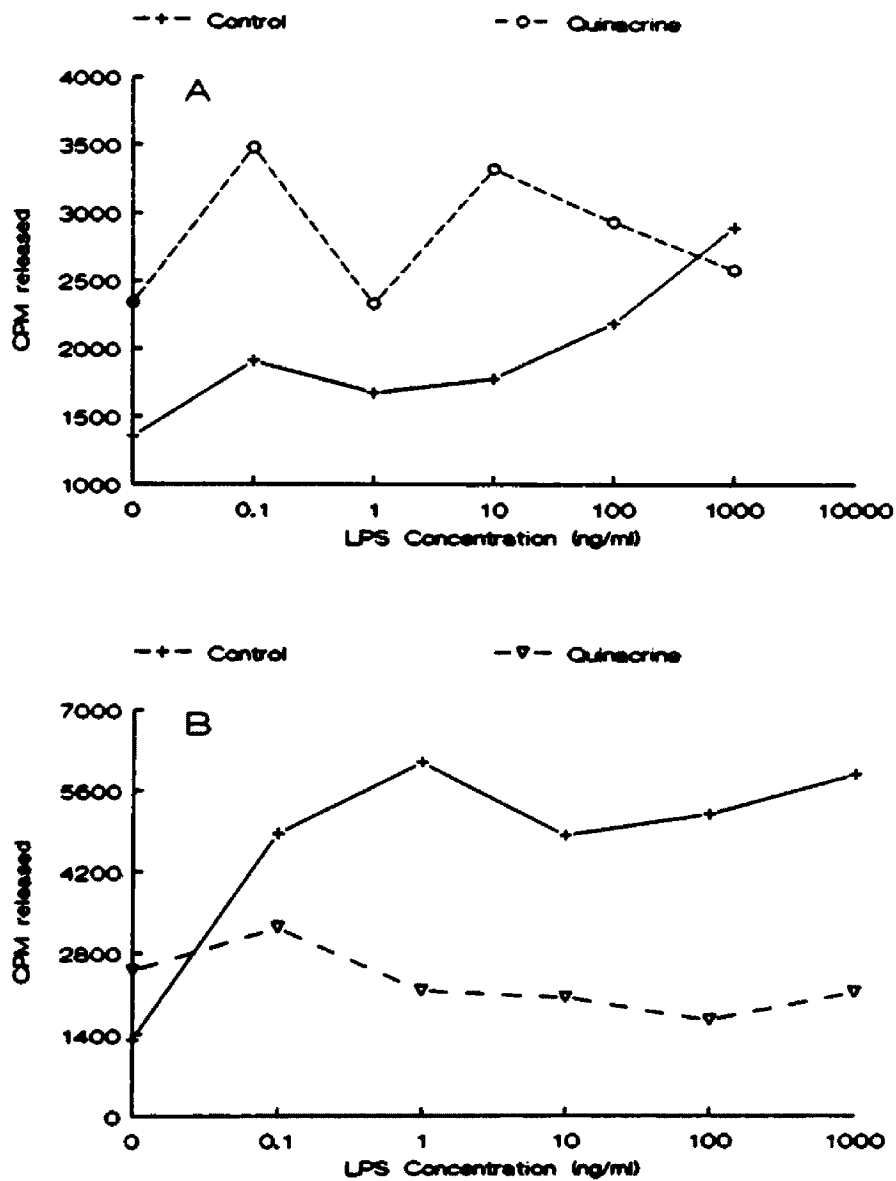


**Fig. 25: Neomycin with LTA challenge.**

Resident peritoneal macrophages from mice were labeled with [ $^3\text{H}$ ] arachidonic acid overnight, washed, and challenged with *S. faecalis* LTA with or without Neomycin.

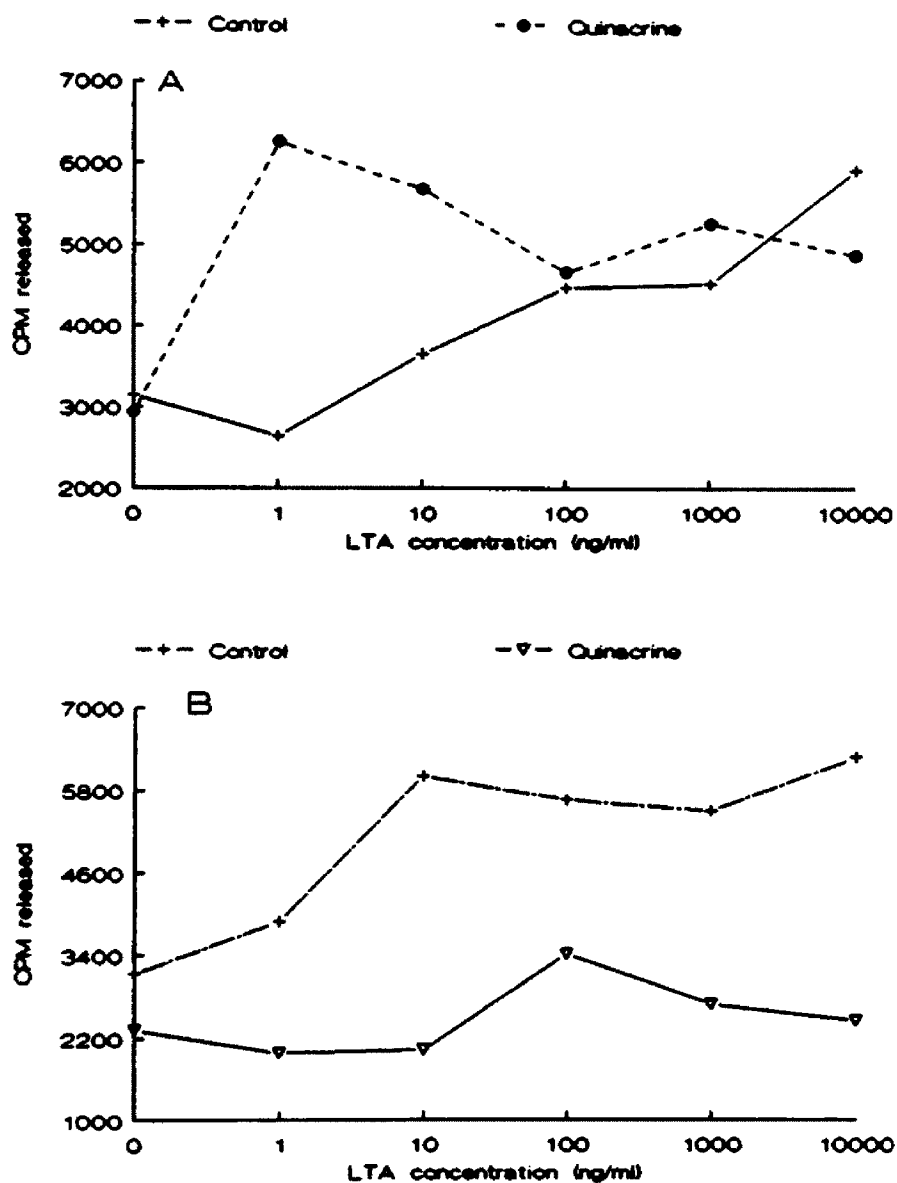
### **Effect of quinacrine on LTA and LPS stimulation of macrophage cultures**

Quinacrine is an inhibitor of phospholipase A<sub>2</sub>. Cells were allowed to adhere, labeled, washed, and then treated with 10 mM or 1 mM quinacrine. Thirty minutes after the addition of quinacrine the cells were challenged with LPS or LTA. Quinacrine did not inhibit arachidonic acid release at 1 mM (Fig. 27A and Fig. 28A). At a ten-fold higher concentration (10 mM), however, quinacrine inhibited arachidonic acid release (Figs. 27B and 28B). At lower concentrations of quinacrine (1 mM), cells released label without LPS stimulation (Fig. 27A). This suggests that quinacrine might stimulate arachidonic acid below inhibitory concentrations. Also, quinacrine at 1 mM appeared to sensitize cells to subsequent challenge with normally non-activating concentrations of LPS and LTA (Fig. 27A and 28A).



**Fig. 26: Quinacrine with LPS challenge.**

Resident peritoneal macrophages from mice were labeled with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS with or without 10<sup>-3</sup>M quinacrine (A) or 10<sup>-2</sup>M quinacrine (B).

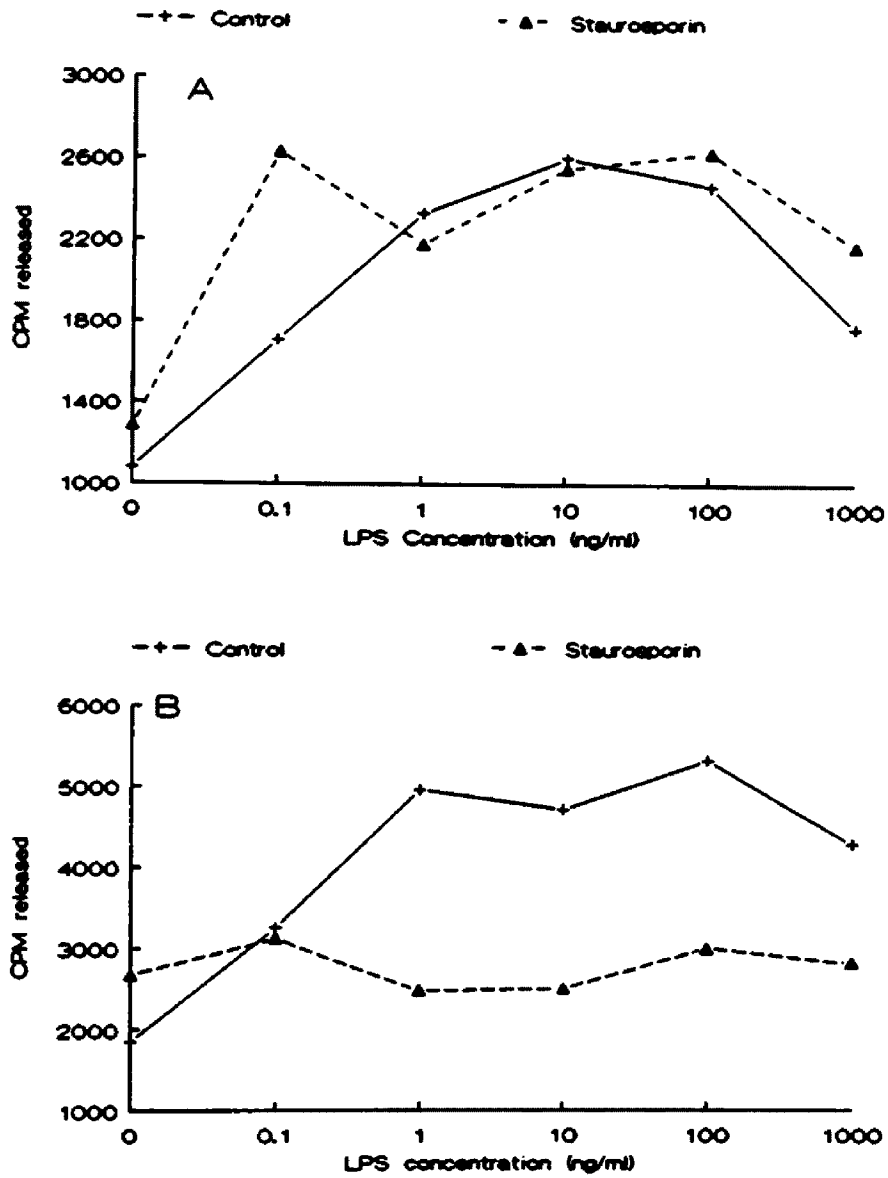


**Fig. 27: Quinacrine with LTA challenge.**

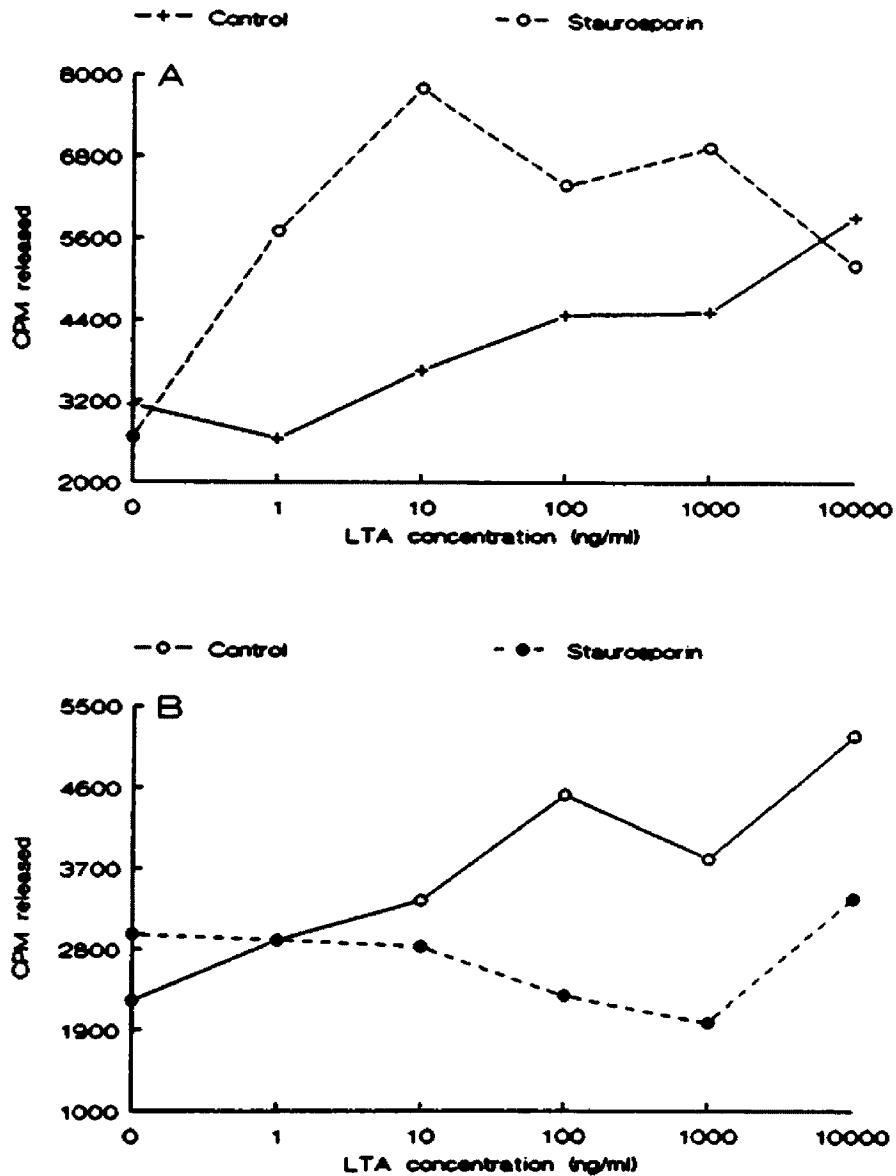
Resident peritoneal macrophages from mice were labeled with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. faecalis* LTA with or without 10<sup>-3</sup>M quinacrine (A) or 10<sup>-2</sup>M quinacrine (B).

### **Effect of staurosporine on LPS and LTA stimulation of macrophage cultures**

The role of protein kinase C in activating arachidonic acid release in macrophage was studied using staurosporine, an inhibitor of protein kinase C. Cells were cultured, labeled, and washed as described above. Staurosporine at concentrations of 1  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$  was added to the cells 30 min before challenge. Staurosporine at 1  $\mu\text{g/ml}$  did not inhibit arachidonic acid release in LPS or LTA stimulated cells (Figs. 29A and 30A). At 10  $\mu\text{g/ml}$ , however, staurosporine was an effective inhibitor of arachidonic acid release in both LPS and LTA challenged cells (Figs. 29B and 30B).



**Fig. 28: Staurosporine with LPS challenge.** Resident peritoneal macrophages from mice were labeled with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS with or without staurosporine at 10<sup>3</sup> ng/ml (A) or 10<sup>4</sup> ng/ml (B).



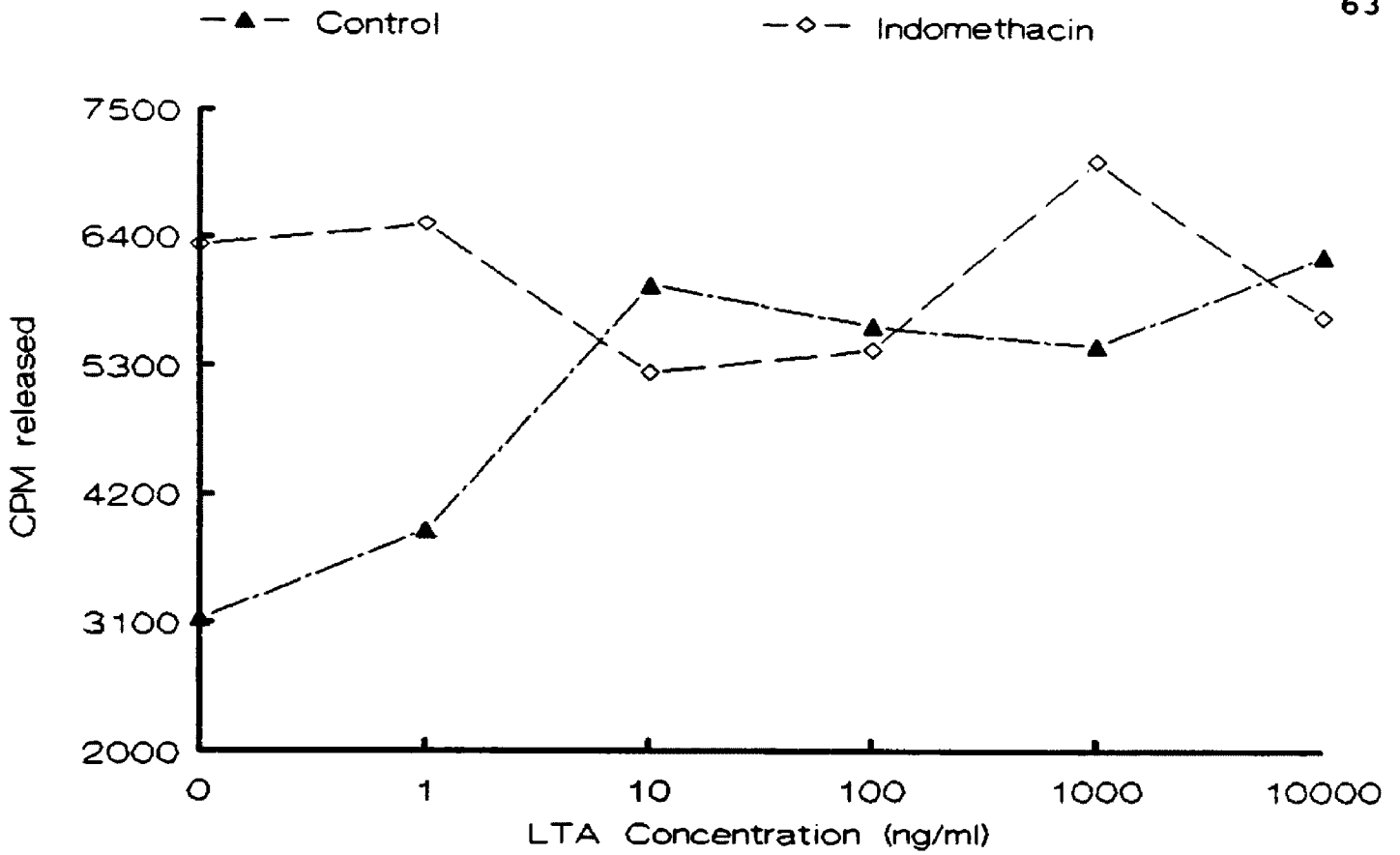
**Fig. 29: Staurosporine with LTA challenge.**

Resident peritoneal macrophages from mice were labeled with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. faecalis* LTA with or without staurosporine at 10<sup>3</sup> ng/ml (A) or 10<sup>4</sup> ng/ml (B).

### **Effect of Indomethacin on arachidonic acid release**

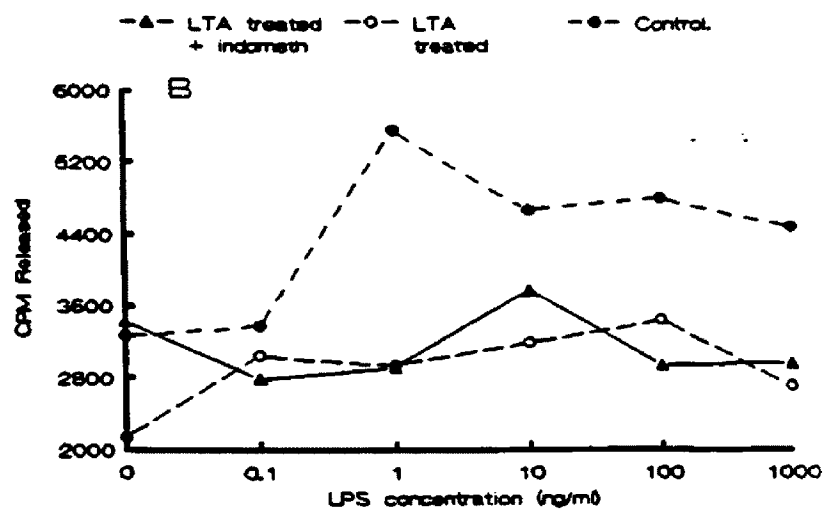
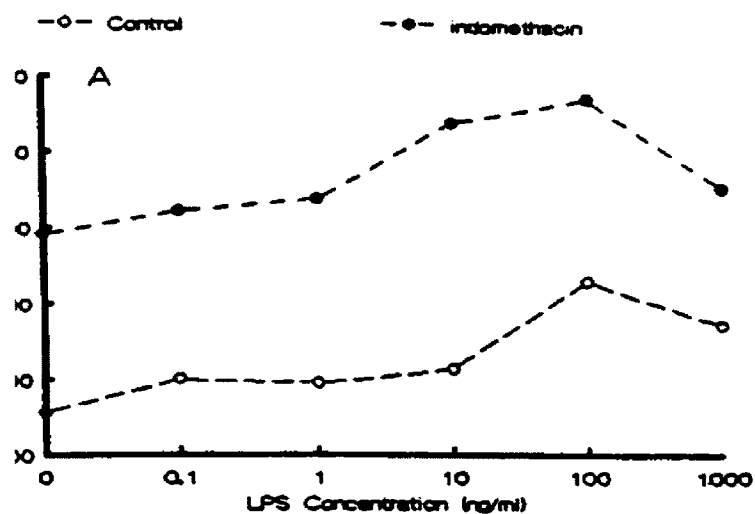
Indomethacin is an inhibitor of cyclooxygenase. Prostaglandin E<sub>2</sub>, a product of the cyclooxygenase pathway, has been implicated in down-regulating the effects of LPS on macrophage. If the accumulation of PGE<sub>2</sub> contributes to desensitization then indomethacin should influence the response. Cells were allowed to adhere to wells, washed, labeled with [<sup>3</sup>H]arachidonic acid overnight with or without 10<sup>-6</sup> M indomethacin, washed again, and challenged with LPS. Cells treated with indomethacin during the labeling period released twice the amount of [<sup>3</sup>H] arachidonic acid in unstimulated cells (control wells with no LPS challenge). The effect of indomethacin on desensitization was studied by pretreating cells with 10 µg/ml LTA in the presence or absence of 10<sup>-6</sup> M indomethacin. The cells were then washed, labeled overnight, and challenged with LPS. Indomethacin (10<sup>-6</sup> M) had no effect on pretreatment (Fig. 32B). To determine the effect on LTA and LPS stimulation of macrophages, indomethacin was added 30 minutes before addition of LPS or LTA. Figs. 31 and 32C show a basal increase in arachidonic acid release (wells without LPS or LTA) by indomethacin treated cells compared to cells without indomethacin. On LPS or LTA stimulation, indomethacin treated cell show little increase in arachidonic acid release as compared to cells without indomethacin.





**Fig. 30: Indomethacin with LTA challenge**  
Resident peritoneal macrophages from mice were labeled with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. faecalis* LTA with or without Indomethacin (10<sup>-6</sup>M).

Fig. 31: Effect of indomethacin on arachidonic acid release



**(A): Indomethacin with label.**

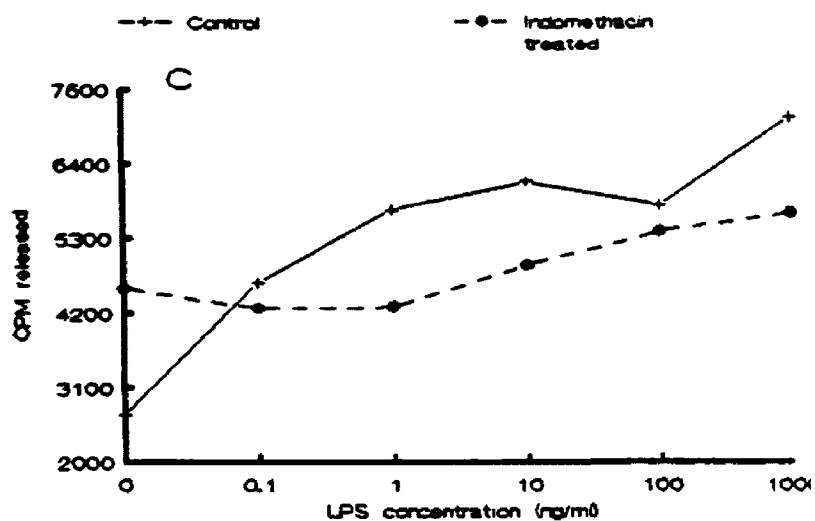
Cells were labeled with [<sup>3</sup>H] arachidonic acid with or without Indomethacin ( $10^{-6}$ M) overnight, washed, and challenged with *S. abortus* LPS.

**(B): Indomethacin with pretreat.**

Cells were treated with either  $10\mu\text{g/ml}$  *S. faecalis* LTA with or without Indomethacin ( $10^{-6}$ M) or with media alone. The cells were then incubated with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS.

**(C): Indomethacin with challenge.**

Cells were labeled with [<sup>3</sup>H] arachidonic acid overnight, washed, and challenged with *S. abortus* LPS with or without Indomethacin ( $10^{-6}$ M).



## DISCUSSION

### **LTA activates arachidonic acid release in macrophages**

LTA triggered arachidonic acid release in mouse peritoneal macrophages (Fig. 11). The amount of arachidonic acid released was comparable to that released by macrophages activated by stimulation with toxic LPS from *S. abortus*. Recently, Tarsi-Tsuk et al. (47) reported that LTA stimulated the respiratory burst in human blood monocytes. The immunological implications of the interaction of LTA with cells involved in the immune response is not clearly understood. LTA is localized at the outer surface of the cell membrane of gram-positive bacteria and is often released from the cell surface (14). It is likely, therefore, that it would interact with host immune cells. The finding that LTA can stimulate arachidonic acid release in mouse peritoneal macrophages is especially interesting considering the reports that LTA is much less toxic than LPS. LTA induces some of the same biological and immunological responses which are induced by LPS (Table 1) but it has not been implicated in any destructive pathological infection.

It is not known if the metabolic events which result in the pyrogenic or toxic activities of LPS are the same or different than the metabolic events which contribute to the

immunostimulatory (protective) activities of LPS. The preparation of a chemically modified LPS with immunostimulatory

**Table 1.** Comparison of some of the properties of LPS and LTA (adapted from ref. 57)

Property	LPS	LTA
Pyrogenicity	+	-
Lethal toxicity	+	-
Immunogenicity	+	-
Mitogenicity	+	-
Eukaryotic membrane binding	+	-
Hypersensitivity	+	-

activity but greatly reduced toxicity (49) suggests, to some extent at least, that these activities can be separated. Chemically, LTA is different from LPS, but it shares common physical properties with LPS. Both LPS and LTA are amphiphiles with a hydrophobic glycolipid moiety and a hydrophilic end. The lipid A region of LPS is responsible for most of the biological properties (31). Removal of a phosphate group from Lipid A (i.e. formation of

monophosphoryl lipid A (MPL)) results in marked reduction in pyrogenicity and toxicity (49). Also, removal of normal fatty acids or substitution of the normal fatty acids in lipid A (e.g., substitution of myristic acid with palmitic acid) results in decreased toxicity (49). The hydrophobic component of LTA is less complex than lipid A of LPS (57). Wicken and Knox (57) have pointed out that LTA is closer in structure to eukaryotic membrane glycolipids than lipid A and have suggested that this may be the reason it is less toxic.

As shown in Figs. 11 and 13 there were differences in the relative potencies of different LTA preparations. *S. faecalis* and *S. aureus* LTA were comparable to LPS, whereas, *B. subtilis* LTA was less potent. Our initial assumption was that the differences in potency between *B. subtilis* and *S. aureus* and *S. faecalis* might be due to differences in the chemical structure of the lipoteichoic acids (Fig. 32). The LTA preparations of *S. aureus* and *S. faecalis* are similar in glycerol/phosphate and alanine/phosphate content. LTA of *B. subtilis* differs in having a lower glycerol/phosphate and alanine/phosphate content. On the other hand, the glycolipid moiety of *B. subtilis* and *S. aureus* is similar, whereas that of *S. faecalis* is different (14,26,56). Table 2 summarizes the differences in the three LTA preparations.

Table 2. (data obtained from Sigma Chemical Co. and ref. 14)

LTA	gly/phos	ala/phos	Glycolipid
<i>S. faecalis</i>	0.8	0.13	Glc( $\alpha$ 1-2)Glc( $\alpha$ 1-3)acyl <sub>2</sub>
<i>S. aureus</i>	0.8	0.1	Glc( $\beta$ 1-6)Glc( $\beta$ 1-3)acyl <sub>2</sub>
<i>B. subtilis</i>	0.6	0.05	Glc( $\beta$ 1-6)Glc( $\beta$ 1-3)acyl <sub>2</sub>

A fourth LTA preparation tested was from *B. stearothermophilus*.

This was a partially purified preparation, therefore, the results must be interpreted cautiously. Figure 14 demonstrates that *B. stearothermophilus* LTA stimulated macrophages to release three to four times more arachidonic acid than LTA preparations from *S. faecalis* or *S. aureus*. The LTA of *B. stearothermophilus* is devoid of sugar residues in its hydrophobic region (Card, unpublished data). The alanine/phosphate and glycerol/phosphate contents have not been determined. It was anticipated that *B. stearothermophilus* LTA would be less potent because it lacked carbohydrate residues in the lipid region. In analogy with LPS, for which lipid A is the minimal structure that possesses most of the biological properties of LPS (31,62), it was expected that the biologically active part in LTA would be the hydrophobic glycolipid region, possibly for attachment to an, as yet, unidentified receptor on the surface of macrophages. The greater amount of arachidonic acid released by *B. stearothermophilus* LTA could have resulted from contamination with other cell wall material such as



*peptidoglycan or protein. B. stearothermophilus* LTA was prepared in our laboratory, and only partially purified.

***S. faecalis* and *S. aureus* LTA desensitize macrophage to LPS challenge.**

Treatment with LTA from *S. faecalis* and *S. aureus* desensitized macrophage cultures to subsequent response to LPS challenge (Figs. 14 and 15). This desensitization was not the result of less [<sup>3</sup>H] arachidonic acid uptake during the labeling period. Macrophage cultures treated with LTA took up more label than those which were not treated (Fig. 9). This suggests that there was an increase in the turnover of arachidonic acid in these stimulated cells, resulting in an increase in both release and uptake.

The possibility of desensitizing the macrophage response, which results in the deleterious effects of endotoxin (and other inflammatory stimuli such as TNF, bacterial exotoxins, etc.) is of importance, considering the many destructive pathophysiological effects that are inflicted as a consequence of macrophage-endotoxin interaction (35). Endotoxic shock is one of the most serious consequences of hospital-acquired infections (62). In recent years strategies to treat endotoxic shock have focused on the development of pharmacological agents that inhibit the formation of potent mediators (22,62). The successful strategies for treating endotoxic shock suggest



that the eicosanoid products of arachidonic acid are important mediators in this condition (5,62). Steroidal (5,62) and non-steroidal (17) pharmacological agents have been used to control the harmful effects mediated by endotoxin. Steroidal inhibitors (e.g. glucocorticoids) act by binding to a specific receptor on the cell and initiating synthesis of proteins (e.g. macrocortin and lipomodulin) that inhibit phospholipase activity, thus preventing arachidonic acid release (5). Non-steroidal anti-inflammatory agents interfere with arachidonic acid metabolism (17). For example, aspirin and indomethacin inhibit cyclooxygenase (17). Both steroidal and non-steroidal agents, inhibit the formation of lipid mediators and other potent cytokines such as tumor necrosis factor (TNF) and platelet activating factor (PAF).

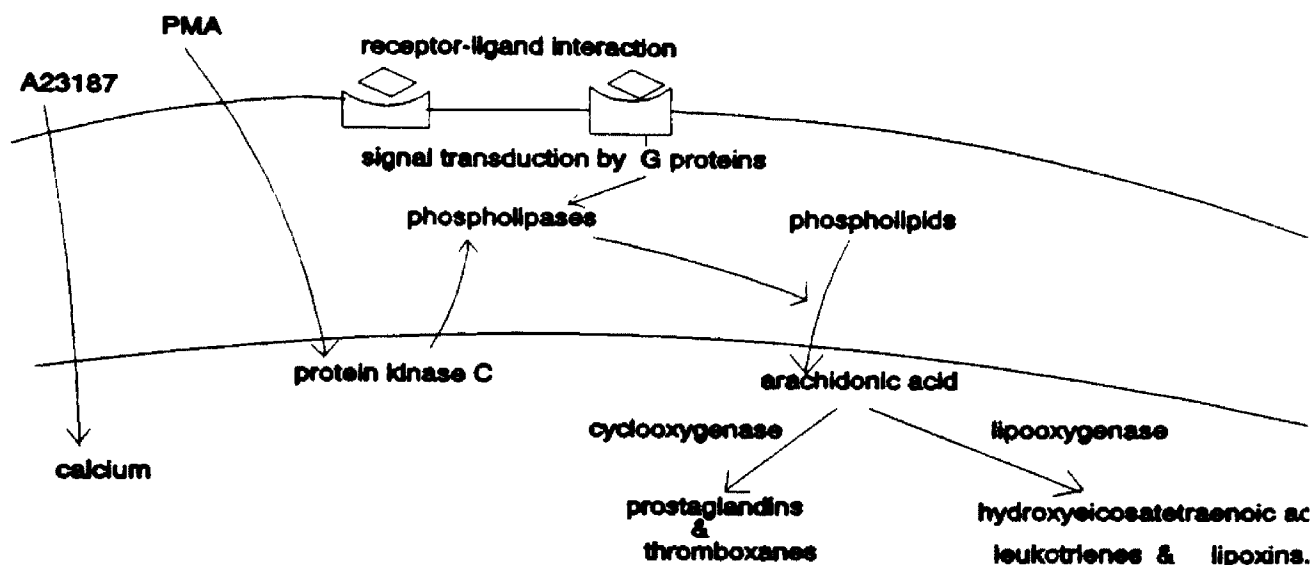
Desensitization of responding cells by pretreatment with biological response modifiers (BRM) such as lipoteichoic acid, polynucleotide complexes (34), muramyl peptides (12) sublethal doses of native endotoxin (19), and derivatives of lipid A (49) may be an effective method of controlling the harmful effects mediated by LPS. MPL, which is the most promising, has greatly reduced toxic properties of LPS, but retains the properties of being a potent adjuvant (49). Our finding that LTA desensitized macrophages to LPS challenge is important in view of the fact that LTA, like MPL, is reported to be non-pyrogenic,

relatively non-toxic, and a potent immunogen.

Lipoteichoic acid from *B. subtilis*, did not desensitize macrophage cultures to LPS challenge (Fig. 16). *B. subtilis* LTA is chemically different from *S. aureus* and *S. faecalis* LTA in its alanine/phosphate and glycerol/phosphate content. It is possible that desensitization requires a certain specificity and stringency in structure that *B. subtilis* LTA lacks. It should be noted that *B. subtilis* LTA, besides being ineffective in desensitizing macrophage cultures, was also relatively ineffective in stimulating arachidonic acid release.

As shown in Fig. 18 desensitization was not specific for pretreatment with LTA. Cells treated with LPS were desensitized to subsequent LPS challenge, and cells treated with LTA were unresponsive to subsequent LTA challenge (Fig. 19).

In an attempt to understand the nature of the desensitization process and the cell components involved, cells were treated with the non-receptor mediated cell agonist phorbol myristic acetate (PMA) and the calcium ionophore A23187. Cells pretreated with LTA from *S. faecalis* were not desensitized to PMA challenge (Fig. 20). PMA, an analog of diacylglycerol, permeates the cell membrane and activates protein kinase C, resulting in arachidonic acid release via phospholipase A<sub>2</sub> (Fig. 33) (59,44). Since pretreatment with LTA did not effect PMA action, it is



**Figure 33. Arachidonic acid release mediated by non-receptor mediated stimuli - phorbol myristic acetate (PMA) and calcium ionophore A23187, and receptor mediated stimuli - LPS and LTA**

likely that the desensitization step occurred at a point before diacylglyceride release. As shown in Fig. 21 pretreatment with LTA did not desensitize cells to calcium ionophore A23187 challenge. Thus, either desensitization did not affect the calcium dependent steps or calcium influx into the cytosol can overcome the desensitization. Recent reports have suggested that down-regulation of protein kinase activity may play a role in desensitization (45). It was reported by Sporn *et al.* (45) that cells incubated in the presence of PMA for 18 hours had reduced protein kinase C levels, which resulted in a diminished response to stimuli (45). The relative effectiveness of LTA for desensitization may be related to the relative potency in stimulating eicosanoid release. LTA from *B. subtilis* stimulated arachidonic

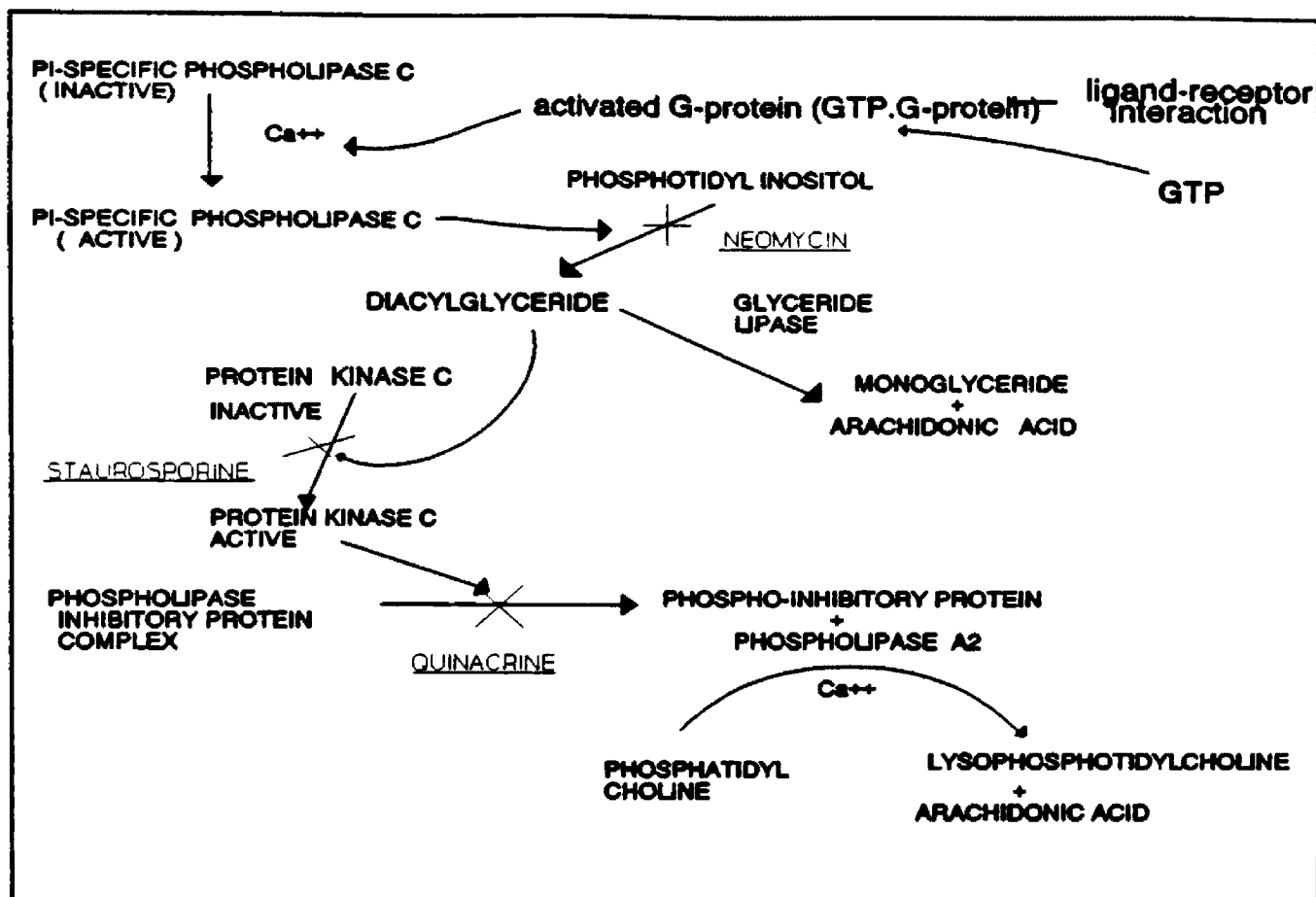
acid release moderately, and was ineffective in desensitizing macrophage cultures to LPS challenge. All other LTA preparations that were tested stimulated arachidonic acid release to a greater degree and were effective in desensitizing macrophage cultures to LPS challenge. Other factors that might play a role in desensitization include modulation of receptors such that the second stimulus binds with a lower affinity (24), saturation of available sites (7); or steric hindrance caused in the microenvironment of the receptor resulting in incomplete signal transduction. Desensitization by some treatments is against a wide range of stimulants which have different receptors (27). This suggests that desensitization may involve many receptors or may involve a step(s) subsequent to receptor-stimuli interaction that is common to many stimuli. Introna et al., (27) suggested that desensitization may be controlled at the transcriptional level. They reported that stimulation of cells with LPS resulted in an increase in expression of *c-fos* mRNA (*c-fos* mRNA codes for a group of "early" proteins that are induced by LPS). The levels of *c-fos* mRNA increased rapidly on LPS stimulation and then decreased. During a period of 2-3 hours following treatment, during which *c-fos* mRNA remained at low levels, cells were desensitized and subsequent challenge with LPS did not induce higher *c-fos* mRNA levels. Full sensitivity to LPS was restored 20 hours

later, when levels of *c-fos* mRNA were elevated to normal levels. Data presented in this study showed that macrophages remain desensitized to LPS at least 20 hours after pretreatment (Fig.24). It is possible that different physiological branches of the activation process are desensitized at different times and for different lengths of time.

### **The Pathway Of Arachidonic Acid Release By LTA and LPS**

The rationale behind using inhibitors in this study was to detect differences in the mode of arachidonic acid release by LPS and LTA. In recent years much research has focused on the specific steps involved in the activation processes of cells stimulated by bacterial stimuli (LPS, peptidoglycan, exotoxins) and physiological stimuli (hormones, interferons, eicosanoids etc.) (1,21). It is known that different stimuli activate macrophages via different molecular mechanisms (1,21). For example, according to Aderem et al., (3), the calcium ionophore A23187 activates macrophages by a pathway that does not involve activation of protein kinase C, whereas PMA activates macrophages by a protein kinase C dependent pathway.

The phospholipase C inhibitor, neomycin inhibited arachidonic acid release in cells stimulated by both LPS and LTA (Fig. 24 and 25). Neomycin binds to



**Figure 34. Pathway of arachidonic acid release showing steps at which the inhibitors acts.**

phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) preventing hydrolysis by phospholipase C (8). Thus, neomycin prevents formation of diacylglyceride (DAG) which is a source of arachidonic acid by the action of diacylglyceride lipase. DAG also activates protein kinase C (PKC), which in turn activates phospholipase A<sub>2</sub> (PLA<sub>2</sub>) to release arachidonic acid from phosphatidylcholine (PC). Inhibition of PI hydrolysis by neomycin also prevents formation of inositol-1,4,5-trisphosphate (IP<sub>3</sub>), which acts as a second messenger in mobilizing intracellular calcium stores. According to the model shown in Fig. 34, inhibition of the PLC pathway

would prevent arachidonic acid release from DAG by diglyceride lipase and prevent the activation of PLA<sub>2</sub> which acts on phosphatidylcholine (PC) to release arachidonic acid.

As shown in Figs. 26 and 27, when the PLA<sub>2</sub> inhibitor quinacrine (47,48), was used there was inhibition of arachidonic acid release with 10<sup>-2</sup> M quinacrine, but 10<sup>-3</sup> M quinacrine appeared to prime macrophages for arachidonic acid release by LPS and LTA. Concentrations of quinacrine lower than 10<sup>-3</sup> M had a priming effect (data not shown). The high basal release of arachidonic acid in the control wells (wells with quinacrine but no LPS challenge) indicated that quinacrine, at concentrations below inhibitory levels, stimulated arachidonic acid release from macrophages, but was inhibitory at higher concentrations. Other research groups (47) have used considerably lower concentrations of quinacrine (10<sup>-6</sup> M) to inhibit arachidonic acid release and the respiratory burst in human blood monocytes.

Data obtained with neomycin and quinacrine suggested that inhibition of either PLA<sub>2</sub> or PLC significantly inhibited arachidonic acid release. As shown in Fig. 34, inhibition of PLC would inhibit PLA<sub>2</sub> mediated arachidonic acid release since PLC generates DAG which is required for activation of PKC, which then activates PLA<sub>2</sub>. However, PLA<sub>2</sub> could be activated by a PLC independent G-protein pathway. This G-protein mediated activation of PLA<sub>2</sub> could release

arachidonic acid from PC and/or phosphatidylethanolamine (PE) (30). Specific inhibition of PLA<sub>2</sub> should not block PLC mediated arachidonic acid release from DAG. Thus, if LTA and/or LPS stimulate release of arachidonic acid by a PLC dependent pathway, then quinacrine should not inhibit arachidonic acid release by LPS and/or LTA. Recent reports suggested that the action of quinacrine may not be specific (48). Tauber and Simons (48) have suggested that quinacrine may inhibit all phospholipases by binding to phospholipids and changing membrane potential which disturbs membrane architecture (48,55). Quinacrine may, therefore, inhibit glyceride lipase, which converts DAG to arachidonic acid and monoacylglycerol, in addition to inhibiting PLA<sub>2</sub> activity. This would prevent arachidonic acid release by either a PLC dependent pathway (from DAG) or a PLA<sub>2</sub> pathway (from PC).

Staurosporine was used to determine the role of protein kinase C in LTA and LPS stimulation of arachidonic acid release. Staurosporine at 10 µg/ml inhibited arachidonic acid release from macrophages challenged with LPS or LTA. Cells stimulated with LPS or LTA in the presence of 1 µg/ml staurosporine were not inhibited, releasing arachidonic acid equal in amount (or more) to cells challenged with LPS or LTA without staurosporine. Therefore, lower concentrations of staurosporine, like quinacrine, enhanced LPS and LTA mediated arachidonic acid release. This is in keeping with the observations of Watanabe et al., (54) that staurosporine



had dual effects: it released arachidonic acid at low concentrations and inhibited arachidonic acid release at higher concentrations. Stimuli have been reported as being PKC dependent or PKC independent in releasing arachidonic acid. For example PMA and the calcium ionophore A23187 are PKC dependent, whereas  $H_2O_2$  and hormonal stimuli are PKC independent (45). LTA and LPS mediated arachidonic acid release appeared to be PKC dependent. This suggests that  $PLA_2$  hydrolysis of PC is a major source of arachidonic acid release from LPS and LTA stimulated cells.

Indomethacin, which is an inhibitor of cyclooxygenase, prevents formation of prostaglandins and thromboxanes (41). One of the prostaglandins, prostaglandin  $E_2$  ( $PGE_2$ ), down-regulates the activated state of the cell (35). According to Wightman and Dallob (58) prostaglandins are synthesized endogenously and accumulate during the period the macrophages are cultured. The addition of indomethacin during the overnight labeling period (about 18 hours) resulted in a significantly higher total amount of arachidonic acid released when cells were subsequently challenged with LPS (Fig. 31A). The addition of indomethacin during the LTA pretreatment step had no effect on desensitization (Fig. 31B). This suggests that  $PGE_2$  does not play a role in the desensitization process.

### Summary

1. The release of  $^3\text{H}$ -arachidonic acid from the lipid pool of peritoneal macrophages activated by treatment with lipoteichoic acid from *Streptococcus faecalis* and *Staphylococcus aureus* was similar to that observed with macrophages treated with toxic lipopolysaccharide from *Salmonella abortus*. Macrophage cultures treated with *Bacillus steorothermophilus* LTA stimulated  $^3\text{H}$ -arachidonic acid release to a significantly higher extent, more than any other LTA or LPS preparation.
2. Pretreatment of macrophages with lipoteichoic acid from *Streptococcus faecalis* and *Staphylococcus aureus* desensitized peritoneal macrophages to challenge with toxic lipopolysaccharide from *Salmonella abortus*.
3. Lipoteichoic acid from *Bacillus subtilis* activated arachidonic acid metabolism to some extent but did not desensitize macrophages.
4. Peritoneal macrophages, desensitized to lipopolysaccharide and lipoteichoic acid challenge, responded to phorbol myristate acid.
5. Inhibitors that blocked arachidonic acid release in macrophage cultures stimulated by LPS also blocked arachidonic acid release in macrophage cultures stimulated by LTA.

### **Future Directions**

The relationship between the chemical nature of potent agonists, the cellular events they induce in cells of the immune response, and the resulting biological response they trigger is not clear. LPS and LTA may stimulate different branches of the arachidonic acid activation pathway, resulting in differences in the types and amounts of prostaglandins and leukotrienes produced. A qualitative and quantitative study of the prostaglandins and leukotrienes produced by HPLC, enzyme immunoassays, or radioimmunoassays may explain differences in the cell response and the subsequent biological effect.

The nature of the desensitized state observed in macrophages treated with LTA and subsequently challenged with LPS appears to be different from that caused by pharmacological agents that inhibit arachidonic acid metabolism (e.g. non-steroidal agents) or those that inhibit protein synthesis (e.g. steroidal agents) and needs to be addressed.

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