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FINAL TECHNICAL REPORT FOR ONR GRANT N00014-89-J-1916

JULY 1, 1989 TO OCTOBER 31, 1993

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The last year of this period covered was a no cost extension, necessitated by initial staffing difficulties for the performance of the work proposed initially. This report will be divided into two parts: Part one, list of publications; part two, thematic grouping of the results obtained and a discussion of their significance.

PART ONE

During the period of support six papers were published in peer reviewed journals. Three manuscripts have been submitted and are in various stages of the peer review process. The ten abstracts refer to either oral or poster presentations at various national meetings and symposia.

PAPERS PUBLISHED

1. Mayer, A.M.S. and J.A. Spitzer. Continuous infusion of E. coli endotoxin in vivo primes in vitro superoxide anion release in rat polymorphonuclear leukocytes and Kupffer cells in a time-dependent manner. Infection and Immunity, 59, 4590-4598, 1991.
2. Dowty, B.J., E. Rodriguez de Turco, A. Hagar and J.A. Spitzer. A nonlethal intravenous dose of endotoxin influences opsonized zymosan-stimulated [³²P]-phospholipid turnover in rat alveolar macrophages. Circ. Shock, 36, 307-311, 1992.
3. Mayer, A.M.S., R.A. Pittner, G.E. Lipscomb and J.A. Spitzer. The effect of *in vivo* tumor necrosis factor administration of superoxide anion production and protein kinase C activity of rat alveolar macrophages. Am. J. Physiol. 264, L43-L52, 1993.
4. Spitzer, J.A. and A.M.S. Mayer. Early neutrophil infiltration into the liver and attendant changes in arachidonic acid metabolism and superoxide generation upon endotoxin infusion. J. Surg. Res. 55, 60-67, 1993.
5. Mayer, A.M.S. and J.A. Spitzer. Modulation of superoxide anion generation by mannoalide arachidonic acid and staurosporine in liver infiltrated neutrophils in a rat model of endotoxemia. J. Pharmacol. Exp. Therap. 267, 400-409, 1993.
6. Mayer, A.M.S. and J.A. Spitzer. Modulation of superoxide generation in *in vivo* lipopolysaccharide-primed Kupffer cells by staurosporine, okadaic acid, mannoalide, arachidonic acid, genistein and sodium orthovanadate. J. Pharmacol. Exp. Therap. 268, Jan. 1994.

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PAPERS SUBMITTED

1. Vindrola, O., Mayer, A.M.S. and J.A. Spitzer. Characterization of prohormone convertase PC1 (PC3) and PC2 in rat neutrophils and macrophages. Effect of LPS and $\text{TNF } \alpha$.
2. Mayer, A.M.S., Zhang, P. and J.A. Spitzer. Effect of *E. coli* lipopolysaccharide and tumor necrosis factor- α on β_2 integrin expression and O_2^- anion generation in rat polymorphonuclear leukocytes.
3. Spitzer, J.A., Zhang, P. and A.M.S. Mayer. Functional characterization of peripheral circulating and liver recruited neutrophils in endotoxic rats.

ABSTRACTS

1. Dowty, B.J., Rodriguez de Turco, E.B. and J.A. Spitzer. Endotoxin attenuates zymosan-induced phospholipid turnover in rat alveolar macrophages. FASEB J. 4, A354, 1990.
2. Dowty, B.J., E. Rodriguez de Turco, S. Nelson, A. Hagar and J.A. Spitzer. Effect of systemic endotoxin on superoxide anion release and signal transduction in rat alveolar macrophages. FASEB J. 5, A1629, 1991.
3. Mayer, A.M.S. and J.A. Spitzer. In vivo tumor necrosis factor (TNF) administration primes PMA-induced superoxide (SO) production by rat alveolar macrophages. FASEB J. 5, A1620, 1991.
4. Mayer, A.M.S., R. Pittner, G. Lipscomb and J.A. Spitzer. The effect of *in vivo* tumor necrosis factor (TNF) administration on superoxide anion (SO) production and protein kinase C activity of rat alveolar macrophages. FASEB J. 6, A1332, 1992.
5. Spitzer, J.A. and A.M.S. Mayer. Effects of inhibitors of cyclooxygenase, lipoxygenase, and PLA_2 on endotoxin-primed superoxide release by sequestered neutrophils and Kupffer cells. J. Cell. Biochem. 816C, 168, 1992.
6. Spitzer, J.A. and A.M.S. Mayer. Nitric oxide (NO) synthesis and superoxide (SO) production by circulating and liver infiltrated neutrophils of endotoxemic rats. FASEB J. 7, A825, 1993.
7. Mayer, A.M.S. and J.A. Spitzer. Modulation of PMA-stimulated O_2^- generation in *in vivo* endotoxin primed rat Kupffer cells by arachidonic acid, phospholipase A_2 , protein kinase C and serine-threonine phosphatase inhibition. FASEB J. 7, A162, 1993.
8. Spitzer, J.A., P. Zhang, A.M. S. Mayer. Expression of β_2 -integrins on circulating and liver recruited neutrophils and Kupffer cells of endotoxic rats. Circ. Shock. S-2, 26, 1993.

9. Mayer, A.M.S., J.A. Spitzer. Arachidonic acid modulates phorbol ester-stimulated O_2^- generation in rat liver sequestered neutrophils and Kupffer cells in endotoxemia. Circ. Shock, S-2, 16, 1993.
10. Mayer, A.M.S., P. Zhang, J.A. Spitzer. Increased expression of CD11b/c and CD18 is dissociated from anti-CD11b/c MAb-stimulated O_2^- generation in rat LPS and TNF-treated neutrophils. Shock, 1994.

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PART TWO - THEMATIC GROUPING OF THE RESULTS AND THEIR SIGNIFICANCE

The overall thrust of these investigations was the study of signal transduction mechanisms in rat alveolar macrophages, Kupffer cells and polymorphonuclear leukocytes (neutrophils or PMN) upon endotoxin (LPS) or tumor necrosis factor (TNF) treatment. These three cell types play prominent roles in host defense mechanisms against microbial invasion. Alveolar macrophages are the major mononuclear phagocytes found in a unique position outside the epithelial lining of the air spaces. In the lung the ingestion of inhaled microorganisms as well as inorganic particles by alveolar macrophages constitutes an important first line of host defense against infection. Kupffer cells, the resident macrophages of the liver in the sinusoidal space, provide an important defense function against microorganisms or endotoxin reaching the liver via the portal vein. The recruitment of neutrophils to the liver is an integral part of the early inflammatory response. When activated, neutrophils contribute significantly to microbicidal activity through chemically mediated measures i.e. the release of granular contents and through changes in redox potential via reactive oxygen and reactive nitrogen intermediates. Neutrophils at the wrong place, at the wrong time, and in excess can also cause serious tissue damage.

Alveolar macrophages were the focus of our investigations published in publications #2 and #3. In publication #2 we reported that infusion for three hours of a nonlethal intravenous dose of endotoxin caused significant changes in opsonized zymosan-stimulated [32 P] phospholipid turnover in alveolar macrophages. The significance of this finding was the demonstration of the ability of a nonlethal dose of endotoxin to perturb phospholipid-dependent signal transduction mechanisms in cells isolated from a compartment other than the one in which endotoxin was administered. It is possible that these cross-compartmental effects on signaling mechanisms may contribute to decreased immunocompetence of alveolar macrophages observed in extrapulmonary infections.

We probed the effect of *in vivo* TNF administration on superoxide production and PKC activity of rat alveolar macrophages in publication #3.

In vivo TNF administration led to enhanced phorbol myristate acetate [PMA]-stimulated superoxide anion secretion. Intratracheal injection of TNF did not result in enhancement of spontaneous or agonist-stimulated superoxide release. In these studies also cross compartmental effects of TNF were detected on respiratory burst activity. The changes we have seen in both cytosolic and membrane PKC were similar between the saline control and TNF- α injected groups, suggesting that the enhancement of PMA-stimulated superoxide release seen in TNF- α injected rats is not due to the state of activation of PKC. Thus, other as yet unidentified transduction pathways are likely to be involved in the priming of PMA-stimulated superoxide production resulting from *in vivo* TNF treatment. Our results supported the suggestion made by other investigators that a PKC-independent pathway involving protein kinases other than PKC as well as serine-threonine phosphatases may have a role in the activation of NADPH-oxidase and superoxide production. Our results contribute to a better understanding of the signal transduction pathways involved in enhanced respiratory burst activity of alveolar macrophages of TNF treated animals. Such understanding of the mediation of host defense mechanisms of potential injury in excess is necessary to identify possible targets for prevention and/or rational therapeutic intervention in lung injury during sepsis and trauma.

In publications #1 and 6 we have investigated the priming of hepatic Kupffer cells by *in vivo* endotoxin treatment for *in vitro* superoxide release and the details of the mechanisms of the signal transduction pathways that are operational in this priming. Based upon the data presented in publication #1 we concluded that after a 3 hour endotoxin infusion Kupffer cells are primed for agonist-stimulated release of superoxide, but to a lesser extent than the neutrophils that have been recruited to the liver by that time. After a 30 hour infusion of a nonlethal dose of endotoxin infiltrating mononuclear phagocytes are found in the liver in increased numbers and are primed for agonist-induced superoxide release while the priming of neutrophils have diminished. A significant corollary of this study is that *in vivo* priming by endotoxin infusion for 3 or 30 hours is not reversed by the experimental protocol utilized for cell recovery (approximately 3 hours), thus suggesting that *in vivo* LPS priming of superoxide release may ultimately lead to severe impairment of liver function and metabolism observed during endotoxemia and sepsis, if not therapeutically blocked at an early time point.

In publication #6 we explored the modulation of superoxide generation in *in vivo* LPS-primed Kupffer cells by staurosporine, okadaic acid, mannoalide, arachidonic acid, genistein, and sodium orthovanadate. The results of these studies revealed that likely signaling pathways include protein kinase C, protein serine-threonine phosphatases, phospholipase A₂ and arachidonic acid, but not protein tyrosine kinases, phosphatases, and cyclooxygenase and 5 lipoxygenase products of arachidonic acid metabolism. These findings could have implications on the design of novel therapeutic approaches for the modulation of enhanced superoxide release by Kupffer cells in endotoxemia.

In publications# 1, 4, and 5 we studied hepatic recruited neutrophils in terms of superoxide formation and arachidonic acid metabolism in a rat model of endotoxemia. One of the hallmarks of the early inflammatory response is the recruitment of neutrophils from the circulation into sites of inflammation, especially into the liver and lung. In these studies we discovered that infusion of endotoxin in a nonlethal dose for 90 min accentuated neutrophil infiltration into the liver and resulted in significant modulation of the eicosanoid profile, consisting of a major shift in PGD₂/PGE₂ to PGE₂, and LTD₄ and 12-HETE. The significant shifts in eicosanoid profile of the mixed Kupffer cell and neutrophil fraction coincided with an enhanced respiratory burst by these cells, suggesting that signals originating from cyclooxygenase and lipoxygenase activity or arachidonic acid itself may participate in the modulation of superoxide release. The different time courses of neutrophil influx and alterations of arachidonic acid metabolism and respiratory burst activity suggest further that the mere influx of neutrophils is not sufficient and adequate time for intercellular communication is necessary for the expression of endotoxin-induced changes in paracrine or autocrine regulation of arachidonate metabolism and proximal host defense mechanisms. The increase in respiratory burst activity of Kupffer cells and neutrophils may in part increase their effectiveness in host defense mechanisms. However, priming by endotoxin may also enhance the capacity of these cells to cause tissue injury which might happen in conditions associated with endotoxic shock or infection-induced adult respiratory distress syndrome.

Generation of superoxide anion is part of the microbicidal killing mechanism of leukocytes. When generated in excess, superoxide may be also be harmful and locally toxic to a particular organ where it may accumulate, for example in the lungs and liver during endotoxemia. Superoxide can by spontaneous dismutation change into hydrogen peroxide and

it can react with hydrogen peroxide to form more toxic radicals such as singlet oxygen and hydroxyl radicals and can induce the formation of chemotactic lipid, that may induce the influx of more neutrophils to the liver. All of these products of oxygen reduction may account for the induction of tissue injury observed in early endotoxemia and may participate in the killing of hepatocytes.

The early accumulation of neutrophils in the liver after endotoxin exposure raises the possibility that these cells may contribute to endotoxin-induced liver injury. In support of this hypothesis is the finding that neutrophil depletion attenuates liver injury associated with endotoxin exposure. Since the extracellular release of reactive oxygen metabolites, including superoxide anion and hydrogen peroxide may be instrumental in causing liver injury, an important issue to be addressed for the prevention or treatment of endotoxin-induced liver injury is the modulation of the mechanism of superoxide release in liver infiltrated neutrophils. Our studies demonstrated the participation of phospholipase A₂, arachidonic acid and protein kinase C in PMA-stimulated superoxide generation in LPS-induced liver sequestered as well as circulating blood neutrophils. The findings should have implications on the design of novel therapeutic approaches for the modulation of superoxide release in the pathogenesis of endotoxin hepatotoxicity.

Continuing our work towards the elucidation of some of the mechanisms that are instrumental in host defense against microbial invasion- whether blood borne or through inhalation- we turned our attention to the kinetics of diapedesis and subsequent accumulation of neutrophils in the liver. Key elements in these processes are the expression or configuration of adhesion proteins on the phagocyte or the endothelium, the two main factors that coordinate the targeting of cellular defenses. Oxygen free radicals, of which superoxide is an example as well as reactive nitrogen intermediates, of which nitric oxide is an example constitute part of the phagocytes' microbicidal armament. These two species of free radicals i.e. superoxide and nitric oxide (NO) function in a major way in mounting initial host defense strategies by virtue of their bacterial killing activities, regulation of vascular control (NO), chronic influence on neutrophil migration and platelet aggregation (NO) and modulation of hepatocyte function (NO). Superoxide and nitric oxide generation, however, may lead to serious cytotoxicity and tissue damage due to excessive formation of these free radicals. By better understanding the regulation and mediation of these mechanisms, sites for beneficial therapeutic interventions can be and are being identified. For example, scavenging of free radicals in ischemia-reperfusion injury and the administration of an inhibitor of nitric oxide synthase for improvement of hemodynamic status in septic shock patients have been found useful.

The sequence of events leading to neutrophil emigration in response to extravascular inflammatory stimuli can be summarized as follows: Signals are generated at the inflammatory site that activate the circulating phagocyte and adjacent endothelium. As a consequence of activation one or both cell types become adhesive. The circulating phagocytes then adhere to the endothelium, migrate along the endothelial surface, diapedese between the endothelial junctions and finally emigrate through subendothelial matrix to participate in the inflammatory reaction in the tissue.

Aggregation of phagocytes in the vessel lumen can lead to microvascular occlusion and produce ischemia. In the process of activation, adherence and transendothelial migration

phagocytes may release products (e.g. oxidants, proteases, phospholipase products of cytokines) that injure or alter endothelial function. Finally, once sequestered in the tissue phagocytes may mediate damage by the release of inflammatory mediators. Phagocyte-mediated vascular and tissue injury has been implicated in the pathogenesis of a variety of clinical entities including adult respiratory distress syndrome (ARDS), ischemia-reperfusion injury following myocardial infarction, shock, sepsis, organ failure and many others.

In the light of these considerations we have undertaken a functional characterization of peripheral circulating and liver recruited neutrophils in endotoxic rats in terms of NO and superoxide generation, β_2 integrin expression, phagocytosis and eicosanoid profile. The results of these studies have been submitted and are listed as #3 of the submitted manuscripts. Liver neutrophils produced significantly more NO both in the absence and in the presence of an *in vitro* endotoxin challenge than did circulating blood PMNs. No significant difference was observed in PMA-stimulated superoxide generation. Endotoxin infusion significantly upregulated the expression of CD11b/c in circulating and even more so in liver PMN. Phagocytosis was also significantly enhanced by *in vivo* endotoxin treatment in blood PMN and liver PMN showed even greater phagocytic activity than blood PMN or Kupffer cells. The percent distribution of prostaglandins D₂ and E₂ of total eicosanoids was significantly higher and that of thromboxane B₂ and 5,12 and 15 HETEs was significantly lower in liver than in blood PMN. The implications of altered neutrophil function may extend to mechanisms of host defense and hepatotoxicity associated with sepsis and endotoxemia.

In submitted manuscript #2 we demonstrated that increased surface expression of CD11b/c and CD18 is dissociated from anti-CD11b/c monoclonal antibody stimulated superoxide release in *in vivo* LPS and TNF-treated rat neutrophils.

Taken together our results obtained at the cellular and molecular level, pertaining to host defense and potential tissue toxic mechanisms are likely to contribute to the transition of results obtained in animal models to applicability to therapeutic interventions and improved care of severely impaired patients in combat casualty situations.

Further relevance to the mission of the combat casualty care program is provided by the fact that increased expression of CD11/CD18, the adhesion molecule necessary for emigration of circulating leukocyte is present in several inflammatory disorders associated with neutrophil activation, including patients with burns, trauma and sepsis. This increased expression is often mediated by inflammatory mediators generated *in vivo* and may contribute through enhanced cell adhesion to neutropenia and/or the microvascular injury observed under these conditions.