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Effects of endotoxin on neonatal pig Kupffer cells

by

Jacqueline Kinyamu Akunda

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Toxicology

Major Professor: Franklin Ahrens

Iowa State University

Ames, Iowa

1998

UMI Number: 9911578

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ABSTRACT

Kupffer cells (KC) were isolated by sequential perfusion of neonatal pig livers with buffers and collagenase and purified by density gradient separation using arabinogalactan. Pulmonary Alveolar Macrophages (PAM) were recovered by broncho-alveolar lavage from the same pigs for comparison and to serve as control macrophages for the assays performed. Activity of the two cell types was determined on an equal number of cells using in-vitro methods to evaluate phagocytosis, bactericidal activity, production of superoxide anion (O_2^-), tumor necrosis factor alpha (TNF-α) and nitric oxide (NO). Both KC and PAM demonstrated similar phagocytic activity of 125-iodoxyuridine–labeled *S. aureus*. PAM produced more O_2^- as measured by their ability to reduce cytochrome-C and killed more *S. aureus* as measured by reduction of MTT. Both PAM and KC produced TNF-α when incubated for a period of four hours with lipopolysaccharide (LPS) or opsonized zymosan. Neither KC nor PAM produced nitric oxide when incubated in vitro with LPS or zymosan. These results suggest that KC from neonatal pigs are immunologically active, and that there are inherent differences in the two macrophage populations, probably enhancing their roles in the different organs.

In an additional study, the effects of endotoxin pretreatment on the activity of KC and PAM, and hepatic microsomal enzyme activity was investigated. Neonatal pigs were injected with LPS intraperitoneally at a dose of 50 μg/kg body weight. Rectal temperature taken 5 hours post-administration of LPS was significantly higher ($p<0.05$) than that of the control animals. KC from LPS treated pigs had higher bactericidal and phagocytic activity ($p<0.05$, $p< 0.01$ respectively) and a higher production of O_2^- ($p< 0.05$) than KC from control animals. PAM from both groups had similar bactericidal and phagocytic activity, and O_2^-

production. Hepatic microsomal enzyme activity of aniline hydroxylase was not affected by pretreatment of neonatal pigs with LPS.

Tetracyclines (TC) have been shown to have anti-inflammatory effects in addition to the antimicrobial action. The effects of in-vivo administration of chlortetracycline (CTC) on ex-vivo perfused pig livers was investigated. The retention and clearance of *Salmonella choleraesuis* (SCS), and production of C-reactive protein (CRP), and haptoglobin (HPG) by whole livers were studied. In addition, CTC modulation of production of TNF-α by pig Kupffer cells was studied. Pigs were dosed orally with CTC for three days, and given LPS 24 hours before removal of the liver. *Salmonella* retention and clearance by the livers of pigs fed CTC was lower ($p < 0.01$ and $p < 0.05$ respectively) than the control livers. An increase in CRP and HPG by the liver after a three-hour perfusion was observed. Further, CTC decreased the production TNF-α by cultured Kupffer cells incubated in-vitro with LPS. CTC may thus assume more subtle roles than just killing bacteria. It's modulation of production of TNF-α suggests a potential for attenuating the acute phase response.

GENERAL INTRODUCTION

Introduction

Kupffer cells (KC) are macrophages found in the liver sinusoids. They make up about 29 % of all the non-parenchymal cells in the liver (Laskin, 1996). Because of this large pool, the liver is a major immunologic organ. It is however much less studied than most other immunologic organs. Previous studies on KC have been restricted to lower mammals especially rats and mice (Fahmi et al., 1995; Bautista et al., 1990; Bellezzo et al., 1996). The pig was chosen for this study because of the high economic loss associated with bacterial pathogenesis and septicemia in pigs, and the need to increase our understanding of the mechanisms involved in the response of KC to endotoxin and subsequently, the defensive role the liver plays in endotoximea.

Although septic diseases of pigs are numerous, and the liver and especially KC play a major role in scavenging of lipopolysaccharide (LPS) during endotoximea in other species like rats (Decker, 1990), no work has been done on their role in septicemic diseases of pigs. To our knowledge, this is the first study that evaluates KC function in pigs.

Tetracyclines, a group of broad spectrum antibiotics are reported to have anti-inflammatory effects. Chortetracyline (CTC), one of the members of this group is widely used as a feed additive in pig production, for promotion of growth. The effect of tetracycline on acute phase response and inflammation in pigs is not known.

The objective of this research was to add to the understanding of pig KC and their involvement in the inflammatory response; to compare the activities of KC with the more widely studied pulmonary alveolar macrophages (PAM) and to evaluate the potential value of pig KC for immuno-toxicological studies. In addition, the acute phase response in the liver and it's modulation by chlortetracycline was evaluated.

Thesis Organization

This thesis has five main sections. The thesis begins with a general introduction and literature review, followed by three manuscripts prepared for submission to Veterinary Immunology and Immunopathology. The general introduction and literature review lays the groundwork on which the study is based and summarizes previous work in the research area, methods used, and the rationale for the assays utilized in this study. The first manuscript deals with comparisons of the immunological activity of KC and PAM. The bactericidal activity, phagocytic activity, and the production of superoxide anion (O_2^-) of the two cell types are compared. The production of nitric oxide (NO) and TNF-α is evaluated. The second paper of the thesis deals with the effects of pretreatment of neonatal pigs with lipopolysaccharide (LPS) on KC and PAM function. The bactericidal and phagocytic activity, and production of O_2^- by cells from control and LPS treated animals are compared. The last paper reports an evaluation of the effects of chlortetracycline (CTC) on the production of TNF-α by KC, and the effect of pre-treatment of neonatal pigs with CTC on the clearance and retention of *Salmonella choleraesuis* (SCS). In addition, the effect of CTC on production of acute phase proteins

by the liver is evaluated. Each of these sections has it's own bibliography. The last major division of the thesis is a general summary and conclusions. This outlines the conclusions drawn from the three distinct studies that make up the three manuscripts. There is a short appendix with extra figures and tables that are not included in the text which provide more detail to the reader.

All this work was supervised by Dr. Franklin Ahrens and Dr. Theodore Kramer. The immunological assays and Kupffer cell isolation was performed in Dr. James Roth's laboratory.

Literature Review

Kupffer cells

Kupffer cells (KC) are macrophages residing in the liver sinusoids. They are found within the sinus adhering to the endothelial lining, at times with projections into the space of Disse. They are stellate in shape, and are preferentially located in periportal sinusoids. Most have an abundant cytoplasm containing lysosomes or dense bodies of varying diameters (Wisse et al., 1996). Because of their position in the hepatic sinusoid, they are the first macrophages to come into contact with any noxious material arising from the hepatic portal vein. KC in the rat comprise the largest pool of macrophages in the body, constituting 80-90% of all body macrophages (Laskin, 1996; Bellezo et al., 1996) and therefore perform an important function of clearing the portal blood of substances like LPS (Decker, 1990). They also endocytose old and foreign cells, parasites, bacteria, viruses and tumor cells (Wisse et al., 1996; Laskin, 1996).

KC posses a high pinocytic, phagocytic and digestive capacity. These processes are accompanied by the release of specific molecules and secretory products that include reactive oxygen species, cytokines, and arachidonate metabolites (Decker, 1990). A large number of soluble substances are able to activate KC. These include: endotoxin, which is one of the most potent activators, zymosan, Bacille Calmette-Guerin (BCG), interferon gamma (IFN γ), colony stimulating factors (CSF), macrophage activating factors (MAF), platelet aggregating factor (PAF) and phorbol myristate acetate (PMA). When activated, KC secrete important molecules such as O_2^-, and NO, which are cytotoxic (Wisse et al., 1996) eicosanoids, platelet activating factor, leukotrienes, cytokines and hydrolytic enzymes that aid in the destruction of the antigen. KC secrete cytokines with immuno-regulatory functions and inflammatory activity including interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), PAF, transforming growth factor beta (TGF-β) and IFN γ.

KC posses both Fc and C3b receptors and therefore can phagocytose opsonized and non-opsonized particles. They have the capacity to act as antigen presenting cells for induction of T-lymphocyte responses.

KC from normal animals are thought to be in a low grade state of activation due to small quantities of endotoxin that permeate the intact gastro-intestinal mucosal barrier. However, during sepsis and acute inflammation, KC are highly activated (Callery et al., 1992). Such activated KC appear larger, more stellate, are highly vacuolated, and display an increased cytoplasmic to nuclear ratio (Laskin and Pendino, 1995). They also adhere to, and spread on culture dishes more rapidly than KC from normal animals. They

exhibit enhanced phagocytic, chemotactic, and cytotoxic activity against tumor cells for example; increased metabolic activity and increased release of O_2^-, hydrogen peroxide, NO, proteolytic enzymes, and cytokines (Laskin et al., 1996; Callery et al., 1992).

In fetal and neonatal liver, KC form the center of erythroblastic islands, controlling the development of red blood cells. KC can also be sub-fractionated into classes of different diameter, which show functional differences and are found in different locations within the liver lobule.

Pulmonary alveolar macrophages

Pulmonary Alveolar Macrophages (PAM) are resident macrophages in the lungs of mammalian species and are found in the alveolar spaces of the lungs (Chitko-McKown et al., 1991). PAM are considered to be the major cells responsible for immune surveillance of the lung (Chitko-McKown et al., 1992). They are the predominant immunocytes found in the lung lavage fluid of pigs after 48 hours of age , and may be the first line of defense against organisms invading the lower respiratory tract. Studies performed on PAM indicate that these cells are capable of protecting against invasion by foreign organisms by the production of cytokines, phagocytosis and cytotoxicity (Chitko-McKown et al., 1991). On stimulation with biological response modifiers, PAM, like KC, become activated with subsequent enhanced function. PAM are easily recovered with broncho-alveolar lavage as these are the majority of cells in the lavage fluid (Pabst, 1994). Like KC, PAM from different species vary in their ability to produce immune mediators. Ovine, bovine, and rat PAM have been shown to produce NO (Bogdan et al.,

1997; Chitko-Mckown et al., 1992), while human PAM do not produce NO with a variety of stimulants (Michaliszn et al., 1995,). Bogdan et al. (1997), investigated the induction of NO in ovine PAM and found that PAM cultured with no stimulation for 24, 48 or 72 hours produced low concentrations of NO that was not increased following co-culture with LPS and other additives.

Studies on pig PAM have included a demonstration of their phagocytic and bactericidal activity (Chitko-Mckown et al., 1991; Thanawongnuwech et al., 1997), their cytotoxicity, tumoricidal activity, and production of cytokines (Chitko-McKown et al., 1991; Turek et al., 1994). A single study reported on the production of NO by pig PAM. The study examined the effects of poly-unsaturated fatty acids on some functions of porcine lung immune cells. The investigators found that LPS did not increase PAM nitrite production over basal levels (Turek et al., 1994).

Lipoplysaccharide

Lipoplysaccharide (LPS) is a major component of the cell wall of gram negative bacteria. It is also commonly referred to as endotoxin. The structure of purified LPS is similar among species and strains of gram negative bacteria. The general structure consists of polysaccharide core and lipid domains (Raetz, 1990). The polysaccharide region is a repeating oligosaccharide termed O-antigen polysaccharide which extends outward from the cell wall. This region determines the different serotypes of bacteria and varies between the phyla and the genera of these bacteria. A core octulonate links the O-polysaccharide with the lipid region. The lipid region termed 'lipid A' is a major

component of the outer leaflet of the cell wall lipid bilayer. The lipid A region is similar in the different bacterial species. Many of the biological effects of LPS are attributed to this region of the molecule.

LPS is an integral part of the gram negative bacterial cell wall, therefore exposure to this toxin occurs during cell division of the organisms or after death of bacteria, for example after bactericidal antibiotic therapy. LPS may also be absorbed from the gut especially the colon. Such LPS is derived from indigenous gram negative bacteria which are part of the normal gut flora (Ditter et al., 1983). Any disruption of the intestinal mucosa altering it's permeability leads to LPS absorption. The absorption of LPS from the gut wall in normal animals is controlled by bile salts.

In experimental studies, LPS is often administered intravenously. Intravenously administered LPS is taken up by many tissues including liver, lungs, kidney, spleen and adrenal glands. In rodents, circulating LPS accumulates predominantly in the liver (Warner et al., 1988). In contrast to rodents, the lungs appear to play a major role in the clearance of circulating LPS in other animal species such as sheep (De Camp et al., 1992). Resident intravascular macrophages such as KC and pulmonary intravascular macrophages (PIM) play an important role in the removal of LPS and this may explain the differences in the body handling of LPS since not all animals have PIM. Polymorphonuclear (PMN) cells also play a role in neutralizing LPS by a protein, which specifically binds to the lipid A region of LPS. This protein, referred to as bacterial permeability increasing protein (BPI) can lyse and kill gram negative bacteria by binding to LPS on the bacterial surface (Hewett and Roth, 1993).

LPS is important since infection by gram-negative bacteria may lead to death resulting from shock and multiple organ failure. In humans, a large percentage of deaths of hospitalized patients can be attributed to gram negative bacterial sepsis (Haven et al., 1989), and LPS is a major contributing factor of this high mortality. LPS is implicated in tissue injury in bacterial infections and usually causes inflammation, necrosis and the appearance of large numbers of PMNs in the tissues (Hewett and Roth, 1993).

Several surface receptors bind LPS. The type of receptor binding is dependent on the nature of the LPS. LPS may be cell free, cell associated or bound to serum lipid binding protein (SLBP). Serum CD18 binds LPS coated particles like intact bacteria to phagocytic cells. CD14 or CD-like antigens on cells bind LPS bound to SLPB. Cell free LPS binds to plasma membrane proteins of certain cells or to plasma membrane lipids. Interaction of LPS with these receptors initiates transmembrane signals and signal transduction pathways and the subsequent response (Hewett and Roth, 1993).

In vivo, LPS causes circulatory shock associated with reduced cardiac output and decreased venous return. LPS activates the coagulation system leading to disseminated intravascular coagulopathy (DIC) which is an important contributing factor to the pathogenesis of circulatory shock and mortality associated with severe endotoximea. LPS also causes detrimental effects to other organs including the kidney (gastro-intestinal tract (GIT) and liver.

Although endotoxin is considered to be toxic to cells in vivo, it is important to note that it is not toxic to cells in-vitro. Endotoxin becomes toxic to cells and tissues because of the products released by KC and other inflammatory cells (Wisse et al., 1996).

LPS has an anti-tumor and anti-metastatic effect and activates natural killer cells (Takahashi et al., 1996). More importantly, LPS is a B-cell mitogen and can trigger T-independent immunoglobulin M (IgM) antibody response. Under appropriate conditions, LPS pretreatment in vitro can also prime macrophages for enhanced production of reactive oxygen intermediates as well as TNF-α and NO. Macrophages can also be deactivated by small amounts of LPS (Fahmi et al., 1995).

Many studies have been carried out on the effect of LPS on KC both in vivo and in-vitro (Fahmi et al., 1995; West et al., 1988; Limuro et al., 1994; Meszaros et al., 1991). However, these studies have been done mainly on KC of rats and mice.

Fujita et al. (1995) investigated the mechanism responsible for endotoxin induced liver injury. *E. coli* LPS administered intravenously increased the capacity of hepatic macrophages (KC) to produce chemical mediators including O_2^-, IL-1 and TNF-α in male rats. By using gadolinium chloride, the investigators also demonstrated that hepatic macrophages played a crucial role in liver injury and that TNF-α was the most likely factor implicated in the development of endotoxin induced liver injury. Gadolinium chloride is a rare earth metal that is reported to block phagocytosis by liver macrophages selectively in their surface attachment and engulfment phases (Limuro et al., 1994). Both activation of hepatic macrophages and factors in the plasma were essential in the occurrence and development of endotoxin induced liver injury (Fujita et al., 1995).

When animals are injected with endotoxin, the major portion is engulfed by hepatic macrophages leading to secretion of inflammatory monokines, particularly TNF-α from the activated KC. TNF-α may injure hepatocytes and sinusoidal endothelial cells

(Fujita et al., 1995). LPS has also been found to increase the glucose uptake by KC. The enhanced glucose metabolism of these immunologically active cells is part of the hepatic immune system response and subserves the antibacterial defense of the body (Meszaros et al., 1991).

Callery et al. (1992) demonstrated that human KC produced IL-6 in response to bacterial LPS stimulation. In a recent study, Yoshioka et al. (1997) demonstrated that bovine cultured KC expressed mRNAs of TNF-α, IL-1 α and β and IL-6, when stimulated for 3 hours with endotoxin. Dieter et al. (1995) reported that in-vitro treatment of KC with LPS leads to release of TNF-α, nitric oxide and eicosanoids but not the generation of O_2^-. Billiar et al. (1989) found that the addition of LPS or killed *E-coli* to cocultures of KC and hepatocytes induced a profound decrease in hepatocytes (HC) protein synthesis, with an increase in citrulline which was mediated via an L-arginine dependent mechanism. The increase in citrulline was not a result of the conversion of the other amino acids by hepatocytes to substrate for citrulline or NO_2/NO_3 production by the KC.

LPS in vivo stimulates the release of super oxide anion. Bautista et al (1990) demonstrated that KC from normal rats did not produce super O_2^-. In contrast, those obtained from LPS treated rats, produced O_2^- with or without macrophage activators such as PMA, opsonized zymosan or calcium ionophore.

Acute phase response

The acute phase response is a systemic inflammatory response to infection and injury. This response is commonly triggered by LPS release resulting from gram negative bacterial infections. The response consists of a series of cascading events, starting with the release of TNF-α, IL-1 and IL-6 from macrophages, and includes the induction of fever, increased synthesis of hormones such as ACTH and hydrocortisone, leukocytosis, and ending with secretion of hepatocyte derived acute phase proteins (APP) such as C-reactive protein (CRP) serum amyloid A (SAA), haptoglobin (HPG), $\alpha 2$ macroglobulin, anti-proteinases, and fibrinogen. Serum concentrations of APP increase rapidly during infection and remain elevated throughout the infection. APP are important in infection because they act as opsonins and also activate the complement system (CRP) (Roitt et al., 1989). The anti-proteinases are believed to protect against tissue injury.

The profile of acute phase proteins differs among species and is dependent on the inflammatory stimulus. In man and dog, CRP is a major acute phase protein but it is not an APP in cattle (Eckersall et al., 1996). In the pig, several individual proteins have been associated with infection or pathological lesions including acid soluble glycoprotein, ceruloplasmin, alpha 2-globulin, HPG, and CRP. CRP and HPG have been identified as the best markers for the identification of inflammatory lesions in pigs (Eckershall et al., 1996), while alpha 2-globulin is named as the pig major acute phase protein (pig-MAP) (Lampreave et al., 1994). In a study where turpentine was used to stimulate a sterile inflammatory response, serum concentration of CRP and HPG peaked within the second day of treatment. Other acute phase proteins studied such as ceruloplasmin increased by

the fourth day of treatment, α1-acid glycoprotein fluctuated during the period of infection

and acid soluble glycoprotein showed a two fold increase (Eckersall et al., 1996).

Chortetracycline

Tetracyclines (TC) are a family of antibiotics active against a wide range of gram-

negative and gram-positive bacteria. They are also reported to have anti-inflammatory

properties that are independent of their antibacterial activity (Shapira et al., 1997).

Chlortetracycline was the first tetracycline introduced in 1948. It is elaborated by

Streptomyces aureofaciens. Tetracycline the compound, is a derivative of

chlortetracycline while other members of this group of antibacterial agents are

semisynthetic derivatives. Tetracyclines are effective against both gram-negative and

gram-positive bacteria and other organisms such as rickettsia, mycoplasma, chlamydia,

some mycobacteria and amebae. Tetracyclines have been used extensively for the

treatment of infectious diseases, localized inflammatory diseases like chronic acne and

periodontal disease (Shapira et al., 1997). TCs are also widely used as an additive to

animal feeds to facilitate growth and control infections (Hathaway et al., 1996;

Henderson et al., 1997; Bousquet et al., 1997).

Recent studies have demonstrated that TCs have anti-inflammatory properties not

related to their antimicrobial activity, but rather to their ability to inhibit the activity of

protein kinase C, mammalian collagenases and other related matrix metalloproteases

(Milano et al., 1997). Inhibition of cytokine secretion is a possible pathway by which TC

may function in some clinical situations especially those involving inflammatory

reactions. It has been reported that TC protects mice against LPS-induced septic shock and inflammatory lesions. Tetracyclines also reduce LPS-induced TNF-α levels in serum and inhibit LPS induced TNF-α and IL-β secretion in human monocytes in-vitro, suggesting that they are potent drugs for preventing LPS induced pathology. The mechanism of action involves blockade of post-transcriptional events of cytokine production rather than early intracellular events since the TC do not inhibit mRNA accumulation (Shapira et al., 1996). LPS induced secretion of cytokines from monocytes involves specific intracellular signal transduction events (Shapira et al., 1996) consisting of proteolytic cleavage of the membrane anchored TNF-α and the release of the soluble cytokine. Tetracyclines have been shown to inhibit both protein kinase C (PKC) and metaloproteinase activities (Golub et al., 1991; Webster et al., 1994).

Milano et al. (1997) found that TC treated mice were protected from a lethal dose of intraperitoneal injection of LPS. In their study, the authors demonstrated that TC acted in early events triggered in response to LPS and was no longer protective if injected more than 1 hour after the injection of endotoxin. Tetracycline also inhibited TNF-α, IL-1 and nitrate accumulation in the blood. Unlike other studies that have shown that TCs decrease the ability of the macrophages to synthesize IL-1α and TNF-α, these investigators found the contrary. They concluded that TC may modulate other pathways that could in turn be responsible for inhibition of the cytokine synthesis. They also found that TCs reduce plasma nitrate levels in-vivo and production of NO from peritoneal macrophages in-vitro (Milano et al., 1997). In a more recent study, D'Agostino et al. (1998) have demonstrated that the reduction of NO by tetracycline was a post-

transcriptional event since the TCs reduced NO synthase but did not inhibit formation of inducible NO synthase mRNA.

Shapira et al. (1997) have shown that daily systemic administration of TC for four days following LPS challenge reduced the size of the LPS-caused lesions and blocked LPS-stimulated TNF-α secretion. They found that TC causes the retention of membrane associated TNF- α on monocyte membranes, thereby preventing the release of TNF-α into the culture media. In another study, Shapira et al. (1996), found that TC was a potent inhibitor of TNF-α secretion by cementum-stimulated monocytes, suggesting a novel mechanism for TC in periodontal therapy, where TC is widely used.

Kloppenburg et al. (1995) found that minocycline, a tetracycline derivative, reduced production of IL-2, IFN-γ and TNF-α by T-cells activated via the T-cell receptor, thus demonstrating that TCs have immunomodulating effects on human T-cells.

Lin et al. (1993), have demonstrated that intrapleural injection of TC induces the release of cytokines like TNF-α and IL-6, which may be the mechanism of the sclerosing effect of TC. However, the subjects of this study were patients with malignant pleural effusion.

Hepatic microsomes

Microsomes are microvesicles that are formed following homogenization of the liver and subsequent differential centrifugation. They are obtained by high centrifugation speeds of over 100, 000 x g. Microsomes have enzymes that catalyze the phase one

biotransformation reactions. These enzyme systems include the cytochrome P450 system and the mixed function amine oxidase.

Microsomal enzymes are of importance because they are involved in biotransformation of compounds, making these compounds more polar and therefore readily excreted. Decrease of the enzymes activity may lead to toxicity of a compound that would ordinarily not be toxic. Such decrease in enzyme activity has been associated with acute phase response (APR) following inflammatory stimuli, such as infection, inflammation or tissue damage (Monshouwer et al., 1996). In acute phase response, there is increased production of acute phase proteins such as serum amyloid A, C-reactive protein and haptoglobin, and a reduction in production of albumin and other proteins that are not involved in the APR. This may result in reduced synthesis of biotransformation enzymes and the enzymes necessary for production of cofactors.

In a study to evaluate the effects of *E-coli* LPS on oxidative biotransformation in pigs, Monshouwer et al. (1996) found that the total cytochrome P450 (CYP450) content and microsomal cytochrome P450 activities were significantly decreased after 24 hours. Studies have been reported in other species where microsomal enzymes are depressed following infection with live organisms (Elsheikh et al., 1997). Monshouwer et al. (1996) suggest that using LPS to induce acute phase response is a better model for studying the effects of APR on biotransformation enzymes since it is simpler than live bacterial infection and mimics very well the bacterial infection model. The authors further suggest that it is likely that the depression of CYP450 after LPS is mediated by

pro-inflammatory cytokines such as TNF-α, IL-1α and IL-6. Other mechanisms have also been proposed for this depression of the enzyme activity.

Studies on pig Kupffer cells

Caperna et al. (1985) isolated neonatal porcine KC by centrifugal elutriation and demonstrated their phagocytic ability on 5 μm latex particles. This is the only documented study on phagocytic ability of neonatal pig KC. In another report, Peterson (1987) studied the drug metabolizing enzymes in rat, mouse, pig and human macrophages and the effect of phagocytic activation. Aryl hydroxylase activity (AHH) was detectable in both KC and hepatocytes isolated from pig liver biopsy material. The macrophage AHH activity was depressed following phagocytic activation in vitro by latex beads.

Isolation procedures for Kupffer cells

Two common methods have been employed for isolation of Kupffer cells. Most investigators use centrifugal elutriation (Caperna et al., 1986; Knook and Wisse, 1982) while others use density gradient separation (Olynk et al., 1994). The procedures involve enzymatic digestion of the liver with collagenase followed by pronase (to destroy hepatocytes) or digestion with collagenase alone. Calcium-free perfusion at the beginning of the procedure without the dissociating enzymes causes detachment of desmosomes between parenchymal cells which start to drift apart. Addition of EDTA to the perfusion, makes the desmosomes detach more rapidly. This step is followed by addition of calcium which is required for collagenase activity. Incubation of liver

preparations with pronase after collagenase treatment can be used to destroy the parenchymal cells but this treatment has disadvantages since the KC may take up substances from destroyed parenchymal cells. Also there is formation of parenchymal cell derived particles (blebs) with the same density as the KC, which makes subsequent separation very difficult (Knook and Wisse 1982).

Cells that contaminate KC cell preparations vary with the type of perfusion and incubation used. After collagenase treatment, hepatocytes are the main contaminants, but white and red blood cells and cellular debris are also found in the preparations. Parenchymal cells can be removed by low centrifugation at no more than 50 x g for a few minutes. Knook and Wisse (1982) recommend two 30 second centrifugations in the rat, while Caperna et al. (1985) and Peterson (1987) both employed 50 x g centrifugations for two minutes for pig KC.

To purify the KC, three common methods are utilized, selective attachment (Peterson et al., 1987), density gradient separation (Olynk et al., 1994) or centrifugal elutriation (Caperna et al., 1985). Selective attachment is based on the observation that endothelial cells do not attach well on plastic and thus KC can be purified by culturing on plastic dishes and washing the non-adherent cells a few hours later. The success of this procedure depends of the enzymes used for digestion. Pronase digestion gives better purity cells. Endothelial cells obtained from livers digested with only collagenase may also attach to plastic (Knook and Wisse, 1982). Density gradient separation has been utilized by investigators with reported success. Different reagents are used for gradient preparations. These include metrizamide, percoll, arabinogalactan, nycondez or ficoll.

Centrifugal elutriation is most often employed by investigators and leads to relatively pure cell preparations (Knook and Wisse, 1982).

Rationale for the macrophage activity assays

Because the immunological activity of neonatal pig KC is not documented, we selected assays that have been previously performed on other macrophages and other phagocytic cells to assess their function.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay that measures the reduction of MTT by live cells. The assay is based on reduction of MTT to purple formazan crystals. The crystals are solubilized and the resulting color (optical density) (OD) of the solution measured at a wavelength of 550 nm with a spectrophotometer. This assay has been used widely to determine the number of live versus dead cells or organisms (Stevens et al., 1991; Thanawongnuwech et al., 1997). In our study, the MTT assay was utilized to evaluate the percent bacteria killed by the KC and PAM. To evaluate the killing of *Salmonella choleraesuis* (SCS) by KC and PAM we used the traditional method of colony counts since the MTT assay gave inconsistent results.

Ingestion of *Staphylococcus aureus* for evaluation of macrophage phagocytosis has been documented for PAM (Chitko-McKown et al., 1991; Thanawongnuwech et al., 1997) and other phagocytic cells especially neutrophils (Roof et al., 1989). It is a widely used reliable assay for phagocytic activity. The assay utilizes *S. aureus* bacteria labeled

with radioactive iodine. The bacteria taken up by the cells can be quantified by determining the cell associated ^{125}I in a gamma counter.

The cytochrome-C reduction assay measures the production of O_2^- by phagocytic cells. Production of O_2^- has been documented for pig PAM (Thanawongnuwech et al., 1997), rat KC and alveolar macrophages (Bautista et al., 1991, Badger, 1986), and porcine neutrophils (Roof et al., 1989).

Production of NO on stimulation of cells with LPS is documented for macrophages including KC and PAM (Billiar et al., 1989; Bogdan et al., 1997). NO can be quantified in the supernatant collected from cultured cells activated by a variety of stimuli. Analysis of NO can be achieved in different ways. The most commonly used is the Griess reaction (Billiar et al., 1989; Bogdan et al., 1997; Roland et al., 1994). The Sievers NO analyzer® was used in this study, because of low detection limits and rapid analysis. The Sievers NO analyzer measures NO based on a gas-phase chemiluminescence between NO and ozone. The nitrates and nitrites in the sample are reduced using vanadium (III) and an inert gas is used to purge NO from solution, resulting in a peak of NO from subsequent chemiluminescence. The response of the NO analyzer is proportional to the NO, NO_2^- and NO_3^- in the sample and the injection volume. The detection limit is approximately 1 picomole of nitrate, nitrite or NO injected into the purge vessel (Sievers® NO Analyzer, Operation and Service Manual, 1997).

Production of TNF-α or it's mRNA is documented for porcine PAM (Baarsh et al., 1991), rat KC (Fujita et al., 1995), and bovine KC (Yoshioka et al., 1997) in response to LPS and other stimuli. The L929 cell line used for the TNF-α bio-assay has been

previously used in pig TNF-α studies (Baarsh et al., 1991). The human recombinant

TNF-α is used as a standard since there is cross-reaction between pig and human TNF-α.

The pig TNF-α ELISA which is more specific for the species was used to confirm our

bioassay.

Objectives

Since there is lack of knowledge on the functional role of pig KC in gram

negative sepsis in this species, we designed our experiments to study:

1. The immunological activity of neonatal pig KC. PAM were used as controls to

   validate the assays and also for comparison of functional activity,

2. The role of KC in pig endotoximea,

3. The effect of CTC on the acute phase response in the liver.

Based on previous studies in KC obtained from other species we hypothesized

that:

a) KC from neonatal pigs are capable of phagocytosis, bactericidal activity and

   production of O_2^-,

b) LPS can stimulate in-vitro production of TNF-α and NO from neonatal pig KC,

c) LPS injected intraperitoneally simulates endotoxin from the gastro intestinal tract into

   the liver and that KC would be activated by this LPS,

d) LPS induces an acute phase response that can depress hepatic drug metabolizing

   enzymes,

e) Chlortetracycline would modulate immune mechanisms during acute phase response.

By evaluating the function of KC from controls and animals exposed to LPS it can be determined whether LPS activates KC, and if so, the characteristics of the activation. In addition, comparison of KC and PAM will provide information on the relative role of each cell type in the clearance of gut derived endotoxin.

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IMMUNOLOGICAL ACTIVITY OF NEONATAL PIG KUPFFER CELLS - COMPARISON WITH PULMONARY ALVEOLAR MACROPHAGES

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Abstract

Kupffer cells (KC) were isolated by sequential perfusion of neonatal pig livers with buffers and collagenase and purified by density gradient separation using arabinogalactan. Pulmonary Alveolar Macrophages (PAM) were recovered by broncho-alveolar lavage from the same pigs for comparison and to serve as control macrophages for the assays performed. Activity of the two cell types was determined on an equal number of cells using in-vitro methods to evaluate phagocytosis, bactericidal activity, production of superoxide anion (SOA), tumor necrosis factor alpha (TNF-α) and nitric oxide (NO). Both KC and PAM demonstrated similar phagocytic activity of 125-iodoxyuridine-labeled *Staphylococcus aureus* (*S. aureus*). PAM produced more superoxide anion (SOA) than KC as measured by the ability of the SOA to reduce cytochrome-C, and killed more *S. aureus* as measured by reduction of MTT. Both PAM and KC killed *Salmonella choleraesuis* (SCS). Although numerically PAM killed more SCS than KC, the difference was not significant. Both PAM and KC produced TNF-α when incubated for a period of four hours with lipopolysaccharide (LPS) or opsonized

zymosan (OZ). Neither KC nor PAM produced NO when incubated in vitro with LPS or

OZ. These results suggest that KC from neonatal cells are immunologically active, and

that there are inherent differences in the two macrophage populations, possibly due to

their distinct roles in the different organs. The evaluation of the activity of pig Kupffer

cells can be a useful tool in the study of mechanisms of endotoxic liver injury.

Introduction

Kupffer cells (KC) are fixed macrophages residing in the liver sinusoids. Because

of their position in the hepatic sinusoids, they are the first macrophages to come into

contact with any noxious material arising from the hepatic portal vein. Pulmonary

alveolar macrophages (PAM) are resident macrophages in the lungs of mammalian

species and are found in the alveolar spaces of the lungs. They are the predominant

immunocytes found in the lung lavage fluid of pigs after 48 hours of age (Chitko-

McKown et al., 1991). Both KC and PAM are capable of protecting the host against

invasion by foreign organisms by mechanisms of phagocytosis and killing, production of

cytokines, cytotoxicity and cytostasis. Studies performed on rat and mouse KC indicate

that they are phagocytic, secrete arachidonic acid metabolites, cytokines and reactive

oxygen species (Decker, 1990). However investigations on the immunological activity of

pig KC have not been reported. Our objective was to isolate KC from neonatal pigs

using methods previously described for other species and evaluate their immunological

activity. Activity of PAM isolated from the same animal were evaluated with KC to

serve as controls and for comparison of the two cell types. Assays documented for PAM

and KC from other species were used. These assays included, an evaluation of phagocytic ability, bactericidal activity, and production of TNF-α, nitric oxide and superoxide anion. The information obtained will aid in understanding the role of KC in the immune response of neonatal pigs and form a basis for further studies.

Materials and Methods

Animals

Neonatal pigs seven to ten days old were obtained from a commercial pig breeder. Animal procedures conformed to the guidelines set by the Committee for Animal Care at Iowa State University. The animals were anaesthetized using halothane, washed in soapy water and placed on dorsal recumbency on an angled surgical board. The abdominal area was swabbed with betadine and a surgical drape was applied. The abdominal cavity was opened and the hepatic portal vein isolated and cannulated just before its entry into the liver. The liver was perfused in situ via the hepatic portal vein with cold heparinized physiological saline to remove the blood. The abdominal vena cava was isolated, tied off and severed just above the kidneys. The thoracic cavity was opened and the thoracic vena cava was transected. The liver was then excised and mounted onto a re-circulating perfusion apparatus (MX International, Aurora, CO). The liver was perfused sequentially with a) Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium and with EDTA (2 mM), single pass; b) DPBS without calcium or magnesium but with glucose (15 mM), sodium bicarbonate (25 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml), re-circulated for 15 minutes; c) DPBS with calcium and

magnesium, single pass; d) Williams medium E (Gibco BRL Products, Grand Island
NY) supplemented with sodium bicarbonate (25 mM), penicillin (50 units/ml),
streptomycin (50 μg/ml), deoxyribonuclease (40 units/ml), collagenase (0.1 %) (Sigma
Chemical Co. St. Louis, MO) for 15 minutes (Gerlach et al., 1994). The liver capsule
was ruptured, cut into small pieces and incubated for an additional 15 minutes in the
collagenase solution (d) at 37° C. The cell suspension was filtered through sterile gauze
to remove the large tissue particles and then filtered through a sterile 80 μm nylon mesh.

Isolation and culture of Kupffer cells

Liver cells were isolated according to a method described by Olynk et al., (1994),
with slight modifications of the specific gravities used for preparation of the gradients,
and the reagents used for the washes. The procedures were as follows. Hepatocytes were
separated from the non-parenchymal cells (NPC) by differential centrifugation at 50 x g
for 2 minutes and the supernatants collected and pooled. The supernatant obtained was
centrifuged at 500 x g for 3 minutes and washed three times with phosphate buffered
saline (PBS) pH 7.2. The cell suspension was layered on a sterile step-gradient of
purified arabinogalactan with densities of 1.055, 1.065, 1.070 and 1.080. Centrifugation
was performed at 50 000 x g for 30 minutes at room temperature in a Beckman SW41
rotor. KC were recovered at the interface of 1.070 and 1.080 density layers and washed
three times in PBS by centrifugation at 500 x g for 3 minutes. The cells were re-
suspended at a concentration of 2.5×10^6/ml in Williams medium E supplemented with
penicillin 100 units/ml, streptomycin 100 μg/ml, 10% fetal calf serum, L-glutamine 2

mM, Hepes buffer and sodium bicarbonate. Cells were plated in 96 or 24 well plates at a density of 3-5 x 10^5 or $1x10^6$ cells per well, respectively. The cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2-95% air. After 24 hours, the medium was removed and the adhered cells washed with sterile PBS. Fresh medium was then added. The isolation procedure yielded a total of 6.4 x 10^7- 1.4 x 10^8 cells per liver. Viability was assessed by trypan blue dye exclusion. The purity was assessed by ingestion of latex beads, morphology and non specific esterase stain using the α- naphthylacetate esterase assay (Sigma Chemical, St. Louis MO). Two hundred cells were scored for viability and purity.

Isolation and culture of pulmonary alveolar macrophages

Pulmonary alveolar macrophages were recovered from the same pigs as used for recovery of KC using a method described by Baarsh et al. (1991). The lungs were removed after removal of the liver and PAM were collected by lung lavage using PBS. The lavage fluid was dispensed and aspirated several times. A total volume of 50 ml of lavage fluid was used. The lavage collected was filtered through a 50 μm nylon mesh, washed three times by centrifugation at 500 x g. The cells were re-suspended in culture medium as used for KC, counted and cultured in a humidified atmosphere of 5 % CO_2 at 37°C for 1 hour and then washed to remove the non-adherent cells. Fresh medium was added and the plates incubated for 24 hours, at which time the medium was changed again. Cells were cultured for 48 hours before performing the assays. The viability of

the cells was assessed using trypan blue dye exclusion, and the purity by non-specific

esterase stain as used for KC.

Bactericidal assays

Evaluation of *Staphylococcus aureus* killing by MTT assay: Bactericidal

activity of macrophages on *S. aureus* was evaluated by colorimetric assay according to a

method described by Stevens et al., (1991), with slight modifications as described. On

the third day after isolation, cultured cells were washed with PBS and the medium

replaced with 0.05 ml of Williams medium E with 5% fetal calf serum for the bactericidal

assay. The cells (5×10^5) were incubated with antibody-opsonized *S. aureus* (5×10^6) for

one hour at a ratio of 10 bacteria per cell. Following this incubation, cells were lysed by

addition of saponin. Standard wells were set up by adding bacteria onto wells in which

the cells had been lysed with saponin. The viable bacteria were determined by addition

of 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT). The live *S.*

aureus reduces MTT to purple formazan crystals. The formazan crystals were solubilized

by adding isopropanol and the amount of formazan quantified by measuring the optical

density (OD) at 595 nm. Absorption of the formazan is directly related to viable bacteria

and was used to determine the percent of *S. aureus* killed by KC and PAM according to

the formula:

$$\% \text{ killing} = 1 - \left(\frac{\text{OD sample - OD of cells only}}{\text{OD of standard - OD of cells only}} \right) \times 100$$

Evaluation of *Salmonella choleraesuis* killing by colony counts: *Salmonella choleraesuis*, (SCS) strain TK 38 was streaked onto MacConkey medium and one colony isolated and inoculated into tryptose soy broth (TSB) for two hours, at which time the bacteria were washed in PBS and standardized to an optical density (OD) of 0.2. (approximately 2×10^8 bacteria/ml). For the killing assay, the bacteria were diluted 10 fold in minimum essential medium (MEM) and used at this concentration. The bacteria were opsonized by incubating with immune serum for 30 minutes at room temperature.

KC and PAM were isolated and cultured as previously described. After 48 hours, media was removed and MEM with 5 % fetal calf serum was added to each well and to three extra wells as standards for the assay. The wells were incubated with bacteria (bacteria to macrophage ratio 10:1), for one hour, after which the cells were lysed with saponin, and aliquots from each well were taken for colony counts. The counts of bacteria only and bacteria with cells were determined on serial dilutions of the aliqouts. 10 µl of the dilutions were plated in sixtuplicates and the results recorded. Percent killing was evaluated according to the formula.

$$\% \text{ killing} = \left(\frac{(\text{CFU bacteria only}) - (\text{CFU of cells} + \text{bacteria})}{\text{CFU of bacteria only}} \right) \times 100$$

Phagocytic assay

The ability of the KC and PAM to ingest bacteria was evaluated according to a method previously described (Roof and Kramer, 1989) with slight modifications as described. Heat killed *S. aureus* labeled with ^{125}I-iodo-deoxyuridine (Udr) (Amersham, Arlington Heights, IL) was used to evaluate ingestion by macrophages. The test was conducted in duplicate and the average of the duplicate values used for calculation. Cells (1×10^6) in a 24 well plate were washed with warm PBS. This was followed by the addition of 0.3 ml of Earle's balanced salt solution (EBSS), 0.05 ml of 1:6 dilution of porcine anti *S. aureus*, and 0.05 ml of [125]I -Udr-labeled *S. aureus*, (bacteria to cell ratio of 75:1). A standard with *S. aureus* but no macrophages, and a background with all reactants except cells were also set up. The reagents were added and the plate incubated for one hour at 37° C with gentle agitation. After 1 hour, 0.5 ml of a PBS solution containing 0.5 units of lysostaphin (Sigma Chemical, St. Louis, MO) was added to the test wells and the blank but not the standard wells, and the plates incubated for an additional 30 minutes at 37° C. Cold PBS (1.0 ml) was added to each well to stop the reaction. The PBS was aspirated and the cells washed and then detached by incubating for 30 minutes with trypsin-EDTA. Detached cells were placed in clean tubes and placed in a gamma counter to determine the counts per minute (cpm) of radioactivity present. The percent ingestion of *S. aureus* was calculated according to the formula:

$$\% \text{ ingestion} = \left(\frac{(\text{cpm in reaction tube}) - (\text{cpm in background tube})}{(\text{cpm in standard tube}) - (\text{cpm in background tube})} \right) \times 100$$

Cytochrome C reduction assay

The cytochrome C reduction assay measures the amount of superoxide anion (O_2^-) produced by the phagocytic cells during the oxidative metabolic burst by measuring the change in optical density due to reduction of cytochrome C by the O_2^-. The assay was carried out according to a method described by Roof and Kramer, (1989). The assay was conducted in triplicate and the average taken for all three readings. The standard reaction mixture contained 0.1 ml of cytochrome C solution in Hanks balanced salt solution (HBSS), 1×10^6 KC or PAM in 0.05 ml of HBSS, and 0.05 ml of pre-opsonized porcine zymosan (10 mg/ml) as the stimulant. The O_2^- production by resting porcine KC and PAM was measured by replacement of zymosan with HBSS. After incubation for 1 hour at 37° C with agitation, the mixture was centrifuged and the supernatant collected into a fresh 96 well plate for the reading of the O.D at 550 nm. The data is presented as OD x 1000

Nitric oxide and TNF-α assays

Macrophages were plated at a density of 1×10^6 /well in a 24 well plate and incubated in a humidified atmosphere of 5% carbon dioxide in air. After 24 hours incubation, the macrophages were washed with PBS and fresh medium was added. After 48 hours incubation, the medium was removed and fresh medium added with or without LPS at different concentrations (1, 5, 10 or 50 μg/ml), or opsonized zymosan (5 mg/ml) as stimulants. The plates were incubated for a further 4 or 24 hours and the media were collected and centrifuged at 750 x g for 5 minutes to pellet any cellular debris and the

opsonized zymosan. The supernatant obtained was used as test sample for TNF-α and NO.

NO Assay: NO in the medium was determined by a Sievers® NO analyzer (Sievers Instruments, Inc. Boulder, CO).

TNF-α Assay: Procedures for TNF-α bioassay were as previously described by Baarsh et al. (1991) with slight modifications. The L929 fibroblast cell line was cultured in modified eagles medium (MEM) containing 10 % fetal calf serum (Gibco Laboratories, Grand Island, NY) supplemented with L-glutamine (2 mM), Hepes, sodium bicarbonate, penicillin (100 units/ml) and streptomycin (100 µg/ml). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 C. The L929 cells were grown in 75 cm^2 tissue culture flasks to a monolayer and sub-cultured by scraping to dislodge the adherent cells. Cells were centrifuged at 500 x g and re-suspended in culture medium. A 0.1 ml suspension with 2.5 x 10^5 cells/ml was added to each well in a 96 well culture plate for use in the bioassay. Blanks without cells were set up with each plate. The plates were incubated overnight at 37° C before addition of test supernatants. Human recombinant TNF-α and rabbit anti human TNF-α polyclonal antibody (Genzyme, Cambridge, MA) were used as positive and negative controls. A few of the test supernatants were tested with a pig TNF-α ELISA (Endogen, Woburn MA), to confirm the presence of the cytokine, since the ELISA is specific for the pig.

Data analysis

Comparisons between the different groups were made using the Student's t-test, where two groups were compared. For the evaluation of TNF-α production using the bioassay, analysis of variance (ANOVA), SAS general linear models procedure (PROC. GLM) was utilized. For all the data, a p-value of 0.05 or less was considered significant.

Results

Macrophage isolation

The isolation procedures yielded an average of 9.8 x 10^7 KC and 3.14 x 10^7 PAM from each pig . There were sufficient numbers from most animals to perform all the assays. The number of KC obtained were comparable to those obtained from neonatal pigs by others although the procedures for isolation were different (Caperna et al., 1985).

The purity of the KC after the collagenase treatment was greater than 85 %. The contaminating cells were mainly hepatocytes and endothelial cells. After culture, the purity of the cells improved to greater than 90 % since the hepatocytes and endothelial cells did not attach well on the plastic culture plates and could be washed off. The viability as measured by trypan blue was greater than 90 % for KC. After one hour in culture, the KC spread out to become more stellate. This morphology remained as long as the cells were in culture. Cells kept for up to two weeks displayed no change in morphology.

The numbers of PAM obtained were fewer than those previously reported from the porcine lung. This is probably because of the size of the lungs in the young pigs used

in the present study compared with those of older pigs used in the previous report (Thanawongnuwech et al., 1997). The purity of PAM was greater than 90 %. The contaminating cells were monocytes, some lymphocytes and a few neutrophils. The viability was greater than 95%. PAM in culture retained their spherical structure.

Bactericidal and phagocytic activity

Killing and ingestion of *S. aureus*: Both KC and PAM exhibited bactericidal and phagocytic activity. KC ingested an average of 24 % while PAM ingested 21.2 % of the iodinated *S. aureus*. Although numerically, KC ingested more bacteria than PAM, the differences were not significant. PAM exhibited a significantly higher ($p<0.01$) bactericidal activity (69.1 %) as compared to KC (55.9 %). The bactericidal activity was higher than the phagocytic ability as evaluated by our assays (Table 1.1).

Killing of *Salmonella choleraesuis*: Killing of SCS by KC and PAM was evaluated by counting the colony forming units of SCS incubated with or without macrophages. The results are presented in Table 1.2. KC killed a mean of 33.9 % SCS while PAM killed a mean of 53.5% bacteria. Although numerically % killing was higher for PAM than KC, there was no significant difference between the two. The lack of significance may be due to the few number of trials in this assay.

Production of superoxide anion

The ability of PAM and KC to produce O_2^- in response to opsonized zymosan (OZ) was evaluated by measuring the optical density (OD) change associated with reduction of ferricytochrome C. The production of O_2^- calculated as the OD x 1000 is presented in Table 1.3. When KC were stimulated in-vitro with opsonized zymosan, the change in OD was slightly higher than the blank, while for un-stimulated cells, only one trial had a change in OD higher than the blank. Thus, the mean OD for unstimulated KC is from seven trials, six of wich were no different from the blank. Stimulated KC had a significantly higher production of O_2^- (p< 0.05) than the non-stimulated cells. PAM produced more superoxide anion than KC, with or without stimulation with OZ. Both resting and stimulated PAM induced a change in OD higher than the blank from all trials. The ability of PAM to produce O_2^- when stimulated with OZ was much higher than that of KC (p<0.01). PAM that were un-stimulated also produced more O_2^- than un-stimulated KC (p<0.05).

Nitric oxide production

Stimulation of both KC and PAM with LPS and zymosan did not elicit any nitric oxide production. The levels in the medium, unstimulated and stimulated cells were similar in the six trials carried out.

TNF-α production

In-vitro stimulation of KC and PAM with LPS led to production of TNF-α as evidenced by the cytotoxicity of the supernatants obtained on the L929 fibroblast cell line. Figure 1.1 shows the percent cytotoxicity of supernatants produced by KC and PAM when different doses of LPS were used. There was a statistically significant linear relationship between the dose of LPS and the production of TNF-α by PAM. With KC, there was an initial increase of TNF-α with increasing doses of LPS followed by a decline at doses of 10 μg/ml or higher (Figure 1.1). To confirm that the macrophages produced TNF-α, a pig TNF-α ELISA was used. Cells were incubated with 5 μg/ml LPS and supernatants collected 4 hours later. TNF-α activity was measured with a commercial ELISA system (Pig TNF-α ELISA), using a biotinylated sandwich system as described by the manufacturer (Endogen Inc, Wilburn MA). The results of this analysis are presented in Table 1.4. Both KC and PAM produced significantly higher levels of TNF-α when incubated with LPS as compared to cells only. KC incubated with medium only produced 58 pg/ml of TNF-α, while those incubated with LPS produced 409 pg/ml. PAM produced 136.7 pg/ml of TNF-α with no stimulation and 2278 pg/ml upon stimulation with LPS.

Discussion

KC play a major role in the clearance of noxious material from hepatic blood. Although KC from other species have been studied, only two studies report on neonatal

pig KC function. Caperna et al. (1985) demonstrated the phagocytosis of latex beads by neonatal pig KC thus confirming their role as phagocytic cells. Peterson, 1987, demonstrated a decrease in hemeoxygenase activity when KC from neonatal pigs phagocytosed latex beads. Phagocytosis by KC from other species has also been demonstrated. Olynk et al. (1994) demonstrated the phagocytic ability of rat KC on both yeast and latex beads. We established that KC were as active as PAM in uptake of opsonized *S. aureus*.

Studies on porcine PAM are more widely documented (Chitko–McKown et al., 1991; Thanawongnuwech et al., 1997). One study documented a phagocytic ability similar to that of the present study (Chitko-McKown et al., 1991) while the other reported a higher phagocytic ability (Thanawongnuwech et al., 1997). In our study, the bactericidal activity of 69.1 % for PAM was similar to that reported by Thanawongnuwech et al. (1997), but higher than previously reported (Chitko-McKown et al., 1991). The variability could be due to several factors including the age of the pig and the differences in methodologies. Neonatal pig neutrophils were found to be less active than those of older pigs and it is a well established fact that young animals have a less active immune system as evidenced by studies documenting decreased aspects of immune function in cells from neonates (Shi et al., 1994). The ability of KC to kill SCS, a gram negative bacterium, was also demonstrated. Although percent killing by PAM was numerically greater than the killing by KC, the difference was not statistically significant.

We expected the phagocytic ability of the cells to be higher than the bactericidal activity, since phagocytosis precedes killing. The greater bactericidal activity as compared to the phagocytic ability has been observed before by other investigators (Chitko-McKown et al., 1992). This higher bactericidal activity could be explained by the higher ratio of bacteria to cells used in the phagocytosis assay (75:1) compared to that used in the bactericidal assay (10:1). In addition, in the phagocytosis assay, the cells and bacteria were incubated for one hour. This time was sufficient to allow regurgitation of bacteria, with subsequent loss of the radioactivity, and consequently lower counts per minute and lower phagocytosis. Previous investigators who have made the same observation have attributed the phenomenon to the heat inactivation step during preparation of bacteria, and altered antigenic determinants on the surface of *S. aureus* causing reduction in opsonization by serum anti bodies (Chitko-McKown et al., 1992). This could not be the case in our study, because the serum used in opsonizing the bacteria was obtained using heat killed *S. aureus* bacteria.

Macrophages brought into contact with phagocytosable material like zymosan particles show the phenomenon of oxidative burst. Superoxide anion is produced by membrane associated oxidases of the macrophages activated by the inflammatory stimulus. The superoxide radical rapidly dismutates to hydrogen peroxide. In the presence of divalent cations, hydrogen peroxide and O_2^- form hydroxyl radicals and molecular oxygen. Such reactive oxygen intermediates have been linked to membrane, protein and DNA damage, lipid peroxidation and cytotoxicity (Laskin and Pendino, 1995). In rat KC, the superoxide anion radical (O_2^-) is the major product. Superoxide

anion produced by KC helps macrophages to inactivate and destroy phagocytosed organisms or particles. This is one of the major killing mechanisms for phagocytic cells.

Superoxide anion production measured by reduction of cytochrome C is documented in pig PAM and rat KC that were stimulated in-vitro with LPS (Thanawongnuwech et al., 1997; Fujita et al., 1995). In our study we demonstrated that while PAM produced O_2^- consistently when stimulated with zymosan, such was not the case with KC. In some cases, we did not detect any O_2^- activity from the KC. Furthermore, we have demonstrated that KC produce more O_2^- if the pig is pre-treated with LPS. Thanawongnuwech et al. (1997) found that PAM produced more O_2^- than pulmonary intravascular macrophages (PIM). Since reactive oxygen species could lead to tissue injury, it is possible that regulation of these destructive oxidation products may be more controlled in KC to reduce tissue injury. KC may thus rely on other mechanisms of killing which are oxygen independent.

Tumor necrosis factor-α (TNF-α) is a very important component of a group of signal molecules produced by macrophages after inflammatory stimulation. It has profound effects on the immune system of the host under conditions of stress and disease. TNF-α generates a multitude of effects including cachexia, inflammation and septic shock (Baarsch et al., 1991). It has been described as a mediator for tumor necrosis seen after tumor implants in mice and as an agent responsible for anorexia in chronic neoplastic or inflammatory disease (Decker, 1990). TNF-α is also recognized as a mediator of cytotoxicity and lethal vascular coagulation. The cytotoxic potential is thought to involve the production of oxygen-derived radicals such as superoxide anion,

hydrogen peroxide, singlet oxygen and hydroxyl radicals. TNF-α may also contribute to increased resistance against infection by priming macrophages to generate large amounts of oxygen derived radicals when these cells are subsequently challenged by soluble particulate stimuli. Such radicals, although beneficial, may induce injury that can lead to organ failure if excessively produced (Bautista et al., 1991).

LPS is among the most potent inducers of TNF-α secretion. Secretion may also be stimulated after exposure to some viruses like Sendai and Newcastle disease virus. In the rat, KC seem to be the most abundant source of this cytokine. The effects of TNF-α are manifold. In the rat KC, TNF-α triggers production of superoxide anion by KC and mediates most of the effects of LPS. TNF-α in-vivo primes the KC to release more superoxide anion during short term non-lethal TNF infusion, leading to the release of toxic oxygen metabolites that may contribute to organ failure (Bautista et al., 1991).

Production of TNF-α has been documented in porcine PAM (Baarsh et al., 1991; Chitko-McKown et al., 1991) and rat (Dieter 1995; Lichtman et al., 1996; Olynk et al., 1994) and mouse KC on stimulation with LPS Fahmi et al., 1995). The present study documents the production of TNF-α from neonatal pig KC and PAM as occurs in other species.

Nitric oxide (NO) is an organic signal molecule secreted by many cells including hepatocytes (Billiar et al., 1989; Curran et al., 1990), KC (Habrecht et al., 1995), pulmonary alveolar macrophages (Nicholson et al., 1996) and endothelial cells (Moncada et al., 1991). Activated macrophages produce NO in relatively large amounts via the NADPH dependent nitric oxide synthase enzyme (Laskin and Pendino, 1995). It is an

endothelium derived relaxing factor and a powerful agent in adjusting blood pressure in small vessels. NO is rapidly converted to nitrite and finally nitrate. LPS activated rat KC are able to synthesize NO from L-arginine. In the liver, NO may play a role in the regulation of hepatic blood flow (Decker, 1990). NO has also been implicated in macrophage mediated cytotoxicity in the intracellular destruction of pathogens and in the regulation of cellular proliferation. NO and it's oxidation products, nitrite and nitrate, are produced by activated hepatic macrophages and are thought to be involved in altered hepatic function following sepsis or trauma. NO may also contribute to tissue injury induced by xenobiotics and is involved in the regulation of hepatic DNA synthesis and apoptosis. It reacts with superoxide anion forming peroxynitrite, a relatively long lived cytotoxic oxidant that has been implicated in stroke, heart disease and immune complex-mediated pulmonary edema. Peroxynitrite may also initiate lipid peroxidation and can directly react with sulfhydryl groups in cell membranes (Laskin, 1996).

Although KC and PAM of the rat and mouse have been found to release nitric oxide when stimulated with LPS, release of NO or inducible nitric oxide synthase enzyme (iNOS) activity has not been documented in pig KC or PAM on stimulation with LPS only. Consequently, we followed protocols previously used to stimulate rat and mouse KC to produce NO. In our study, the NO levels were identical in supernatants from stimulated cells, non stimulated cells and in the media. Thus, we concluded there was no detectable nitric oxide produced by KC and PAM stimulated with LPS or zymosan during a 24 hour period. This lack of NO production may be due to a requirement of more than one stimulus to elicit such a response (Roland et al., 1994), or

simply that neonatal pig KC and PAM do not produce NO. This latter conclusion is supported by a recent report by Pampush and coworkers (1998). In their study, pig peripheral blood mononuclear cells (PBMC), splenocytes, or alveolar macrophages failed to produce inducible nitric oxide synthase when stimulated with Concanavalin A (ConA) and LPS or recombinant porcine interferon γ (IFN–γ) and LPS. In vitro engulfment of *Hemophilus parasuis* also failed to induce iNOS or nitrite formation. There are many species differences between cell responses to stimuli. Neonatal pig KC may behave like human, ovine or murine PAM which do not produce any detectable levels of NO when stimulated with LPS or LPS in combination with IFN-γ (Michaliszyn et al., 1995; Bogdan et al., 1997). Similarly, human KC did not produce any NO when stimulated with LPS alone or together with IFN-γ (Roland et al., 1994). Turek et al. (1994) reported that LPS did not increase macrophage nitrite production over basal levels in pigs. Harbrecht et al., (1995) found that exposure of isolated rat KC to LPS alone resulted in an increase in both total nitrate and nitrite as well as NO at 24 and 48 hours. Roland et al. (1994) reported that, similar to rat KC, human KC released IL-1, IL-6, TNF-α, transforming growth factor β (TGF-β) and prostaglandin E_2 (PGE$_2$). However, unlike rat KC NO could not be detected regardless of whether the human KC were exposed to LPS, INF-γ or a combination of the two.

Nitric oxide is produced by activated macrophages as an intermediate metabolite of the oxidation of L-arginine and plays an important role in macrophage mediated antimicrobial and antitumor responses (Bilyk et al., 1995). Nitric oxide is an important antimicrobial mechanism of phagocytes from mice and rats (Michaliszyn et al., 1995).

This may not be the case in species whose macrophages do not produce NO, even when activated.

Nitric oxide is also important in the pathogenesis of liver diseases. For example, during endotoxemia, inhibition of NO synthesis enhances the degree of hepatic injury, implicating a protective effect of NO. This may be due to stabilization of the hepatic micro-circulation which modulates the effects of toxins on the liver. Nitric oxide is a known scavenger of toxic oxygen-derived radicals and has been shown to have a protective effect against tissue injury in endotoxemia (Bautista and Spitzer, 1994).

In our study, nitric oxide concentrations were identical whether wells contained LPS-exposed KC or PAM, un-stimulated cells, or medium only. We recognize that induction of nitric oxide may require more than one signal but we did not test the hypothesis in this study. In a few trials on hepatocytes, we found that the production of nitric oxide was up-regulated when supernatants from KC treated with LPS were added to the hepatocytes (data not shown). In addition, we found that isolated, perfused whole livers treated with different agents including LPS, or salmonella for upto three hours had no change in the nitric oxide level in the perfusates (data not shown). Our results are consistent with other investigators who have reported that pig KC and PAM do not produce nitric oxide on stimulation with LPS (Turek et al., 1994).

In summary, we found that neonatal pig KC are immunologically active, they have a phagocytic ability comparable to PAM, and a lower bactericidal activity. The ability of PAM and KC to kill SCS, a gram negative bacterium that is a specific pathogen for pig was demonstrated, further emphasizing the role both cell types play in defense of

the pig against invasion by bacteria. While PAM produce significant amounts of superoxide anion spontaneously and even more on stimulation by opsonized zymosan, KC produce insignificant amounts in comparison. The ability of both cell types to produce TNF-α on stimulation with LPS emphasizes their role as possible scavengers for LPS during endotoxemia and their importance in immune function. KC and PAM from neonatal pigs do not produce nitric oxide when stimulated with LPS in-vitro.

Acknowledgements

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Table 1.1: Bactericidal and phagocytic activity of Kupffer cells (KC) and pulmonary alveolar macrophages (PAM) (Mean % activity, n = 6/group)

	KC (Mean ± S.E.M)	PAM (Mean ± S.E.M)
% killing of *S. aureus*	55.9 ± 1.6*	69.1 ± 1.5
% phagocytosis of *S. aureus*	24.0 ± 2.7	21.2 ± 3.3

*Significant difference between the two means, p<0.01
S.E.M - standard error of the mean

Table 1.2: Killing of *Salmonella choleraesuis* by Kupffer cells and pulmonary alveolar macrophages (PAM) (n=4/group).

| | Colony forming units/10 µl Mean ± S. E. M | | % killing |
	Cells + SCS only	SCS only	Mean ± S. E. M
KC	27.5 ± 2.1	43.2 ± 5.7	33.9 ± 7.5
PAM	14.8 ± 1.6	33.9 ± 3.7	53.5 ± 9.5

SCS was incubated with KC and PAM for one hour. 10 µl of serial dilutions of aliquots from wells were drop plated in sixtuplicates and the colony forming units counted. Values shown are means of the counts averaged for the four trials carried out. The % killing of SCS was calculated from the colony counts.
S.E.M - standard error of the mean

Table 1.3: Production of Super oxide anion by Kupffer cells (KC) and pulmonary alveolar macrophages (PAM) with or without stimulation by opsonized zymosan (OD x 1000), (n=6/group)

	Cells + opsonized zymosan Mean ± S.E.M	Cells only Mean ± S.E.M
KC	427 ± 9.2**	407 ± 7.7*
PAM	644 ± 28.3	445 ± 17.1

* - Means of KC and PAM groups are significantly different, $p < 0.01$
**- Means of KC and PAM groups are significantly different, $p < 0.05$
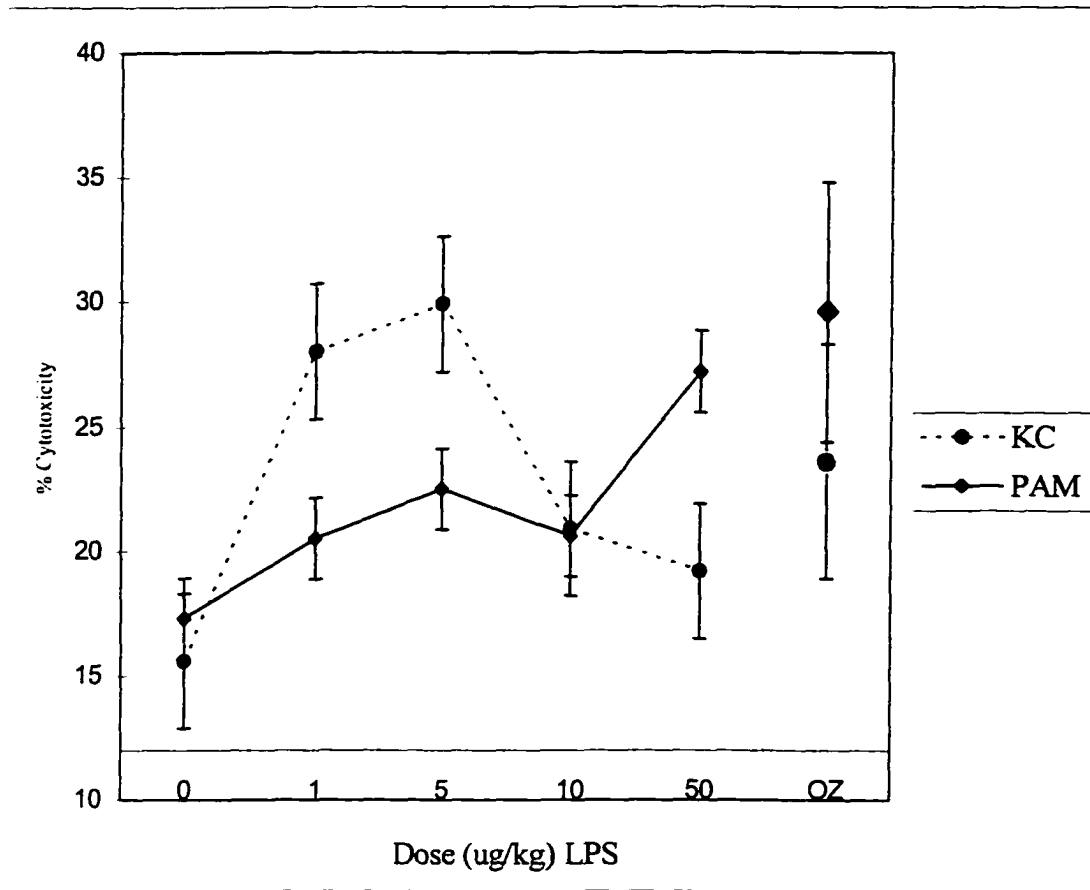S.E.M - standard error of the mean

Figure 1.1: **Production of TNF-α by Kupffer cells (KC) and pulmonary alveolar macrophages (PAM) incubated with different concentrations of LPS and opsonized zymosan (OZ), (% cytotoxicity against L929 cells) (n=7/group)**

Table 1.4: Production of TNF-α (pg/ml) by Kupffer cells (KC) and pulmonary alveolar macrophages (PAM). Cells were incubated with 5 μg/ml LPS for four hours. TNF-α was measured by a commercial TNF-α ELISA system. Mean ± S.E.M are indicated (n=4/group)

	Control (cells only)	LPS (cells + LPS)
KC	58 ± 17.8	409 ± 158*
PAM	136.7 ± 35.8	2278 ± 543**

* -Mean is significantly higher than control, p<0.05,
** -Mean is significantly higher than control, p<0.01

ENDOTOXIN IN-VIVO ENHANCES THE IN-VITRO ACTIVITY OF NEONATAL PIG KUPFFER CELLS, BUT NOT PULMONARY ALVEOLAR MACROPHAGES

J. Kinyamu Akunda, Franklin A. Ahrens, Theodore T. Kramer

Abstract

Neonatal pigs were injected with endotoxin (*E.coli* lipopolysaccharide) intraperitoneally at a dose of 50 μg/kg body weight. The temperature recorded 5 hours post-administration of LPS was higher than that of the control animals. Kupffer cells (KC) were isolated by sequential perfusion of neonatal pig livers with buffers and collagenase and purified by centrifugal elutriation. Pulmonary alveolar macrophages (PAM) were recovered by broncho-alveolar lavage from the same pigs for comparison and to serve as control macrophages. KC from LPS treated pigs had higher bactericidal and phagocytic activity, and a higher production of superoxide anion (O_2^-) than KC from control animals. PAM from control and LPS treated pigs had similar bactericidal and phagocytic activity, and similar rates of production of O_2^-. The hepatic microsomal enzyme activity of aniline hydroxylase was not affected by LPS pretreatment. The response of neonatal pig KC to endotoxin makes them a valuable tool in the study of mechanisms of endotoxic liver injury.

Introduction

Kupffer cells (KC) are sessile macrophages found in the hepatic sinusoids. In this position, they function to clear the bloodstream of circulating bacteria, viruses and other particulate matter (West et al., 1988). Endotoxins enter the liver via the hepatic portal vein. When liver function is normal, endotoxins are bound to LPS binding protein. The protein bound LPS is internalized by liver macrophages and does not spill over into the systemic circulation (Limuro et al., 1994). During infection by gram negative bacteria, however, the uptake of LPS may lead to excess production of peptide mediators and reactive oxygen species which may injure the tissues. The liver is a major immunological organ and KC play an important role in the clearance of endotoxin. There is, however, a great species variation in the type of cells that are involved in clearance of LPS. In the rat, KC are major scavengers of LPS but in sheep, Decamp et al., (1992) demonstrated that while bacteria, gold colloid and iron oxide particles injected into the mesenteric vein were taken up mainly by KC, nearly 50 % of endotoxin eluded hepatic clearance and was subsequently removed in the lungs.

Endotoxemia leads to the acute phase response characterized by a series of events including fever, increased lassitude, loss of appetite and an increased synthesis of acute phase proteins (Van Miert, 1995). During the acute phase response, several processes involved in drug disposition are altered, including a decrease in hepatic biotransformation. Studies in 3 month old pigs have shown that *E.coli* LPS administered intravenously induces an acute phase response associated with a significant decrease of microsomal cytochrome P450 (CYP450) dependent activities. The decrease in CYP450

activities was accompanied by losses of some apoproteins (Monshouwer et al., 1996).
The depressed CYP450 activity by LPS is thought to be mediated by pro-inflammatory
cytokines such as TNF-α, IL-1 and IL-6 since administration of these cytokines
reproduces the LPS-induced depression (Chen et al., 1992; Wright and Morgan, 1991).

Since there is a lack of knowledge about the mechanisms of endotoxemia in the
pig, we designed this experiment to study the role of KC in gut-derived endotoxemia in
neonatal pigs. KC from control animals and animals exposed to LPS were isolated in
order to characterize LPS induced changes. In addition, PAM were isolated from the
same animals to provide information on the relative role of each cell type in clearance of
gut-derived endotoxin. By evaluating the effect of LPS administration, changes
associated with the acute phase response initiated by LPS may be determined. Since
microsomal enzyme activity during the acute phase response is decreased in some
species, hepatic microsomal metabolism was also evaluated in neonatal pigs, using a gut-
derived endotoxin model.

Materials and Methods

Animals

Pathogen free neonatal pigs were obtained from the Veterinary Clinical Sciences
Department at Iowa State University (ISU). The animals were treated according to the
guidelines set by the Committee for Animal Care at ISU. Two neonatal pigs were
obtained from the same litter. One pig was pretreated with LPS at 50 μg/kg
intraperitoneally and the other served as the control. A total of 14 pigs were utilized in

this study. There were seven pigs in each group (LPS treatment and controls). After administration of LPS, rectal temperature was recorded, and the hematology performed to determine the response to endotoxin. After 24 hours, the pigs were sacrificed and KC and PAM were isolated as previously described (Caperna et al., 1985; Baarsh et al., 1991).

Isolation of cells

The liver was perfused with collagenase and the parenchymal cells separated as previously described (Caperna et al., 1985). KC for this study were isolated by centrifugal elutriation (JE 6B elutriator rotor) according to a method described by Caperna et al., (1985). The elutriation buffer consisted of PBS with 1 % bovine serum albumin (Sigma Chemical CO. St. Louis MO). The cell suspension was introduced at a flow rate of 10 ml/min at a centrifuge speed of 2500 rpm. The flow rate was gradually raised to 31 ml/minute and the red blood cells, endothelial cells, and small KC were run off. The centrifuge speed was reduced to 1000 rpm and the next fraction that contained KC was collected in 200 ml buffer. The cells were washed in PBS, counted, scored for viability and purity as described previously (Thanawongnuwech et al., 1997). The purity of the cells was over 95 % and the viability greater than 80 % as evaluated by trypan blue dye exclusion.

PAM were isolated by lung lavage from the same pigs. They were washed twice in PBS and re-suspended in the same medium as the KC. Equal numbers of PAM and KC were plated on either 24 or 96 well plates for the assays.

Bactericidal assay

Bactericidal activity was evaluated by a colorimetric assay according to a method described by Stevens et al. (1991), with slight modifications as described. On the third day after isolation, cells were washed with PBS and the medium replaced with 0.05 ml of Williams medium E, with 5% fetal calf serum for the bactericidal assay. The cells (5 x 10^5) were incubated with antibody-opsonized *S. aureus* (5 x 10^6) for one hour at a ratio of 10 bacteria per cell. Following this incubation, cells were lysed by addition of saponin. Standard wells were set up by adding bacteria onto wells in which the cells had been previously lysed with saponin. The viable bacteria were determined by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT). The live *S. aureus* reduces MTT to purple formazan crystals. The formazan crystals were solubilized by adding isopropanol and the amount of formazan quantified by measuring the optical density (OD) at 595 nm. Absorption of the formazan is directly related to viable bacteria and was used to determine the percent of *S. aureus* killed by KC and PAM according to the formula:

$$\% \text{ killing} = 1 - \left(\frac{\text{OD sample - OD of cells only}}{\text{OD of standard - OD of cells only}} \right) \times 100$$

Phagocytic assay

The ability of the KC and PAM to ingest bacteria was evaluated according to a method previously described by Roof and Kramer, (1989) with slight modifications. Briefly heat killed *S. aureus* labeled with ^{125}I-iodo-deoxyuridine (Udr) (Amersham, Arlington Heights, IL) was used to evaluate ingestion by macrophages. The test was conducted in duplicate and the average of the duplicate values used for calculation. Cells (1×10^6) in a 24 well plate were washed with warm PBS. This was followed by the addition of 0.3 ml of Earle's balanced salt solution, 0.05 ml of 1:6 dilution of porcine anti *S. aureus* serum and 0.05 ml of ^{125}I -Udr-labeled *S. aureus*, (bacteria to cell ratio of 75:1). A standard with *S. aureus* but no macrophages, and a background with all reactants except cells were also included. The reagents were added and the plate incubated for one hour at 37° C with gentle agitation. After 1 hour, 0.5 ml of a PBS solution containing 0.5 units of lysostaphin (Sigma Chemical, St. Louis, MO) was added to the test wells and the blank but not the standard wells, and the plate incubated for an additional 30 minutes at 37° C. Cold PBS (1.0 ml) was added to each well to stop the reaction. The PBS was aspirated, the cells washed, and then detached by incubating for 30 minutes with trypsin-EDTA. Detached cells were transferred to clean tubes and placed in a gamma counter to determine the counts per minute (cpm) of radioactivity present. The percent ingestion of *S. aureus* was calculated according to the formula:

$$\% \text{ ingestion } = \left[\frac{(\text{cpm in reaction tube}) - (\text{cpm in background tube})}{(\text{cpm in standard tube}) - (\text{cpm in background tube})} \right] \times 100$$

Cytochrome C reduction assay

The cytochrome C reduction assay measures the amount of O_2^- produced by the phagocytic cells during the oxidative metabolic burst by measuring the change in optical density due to reduction of cytochrome C by the O_2^-. The assay was carried out according to a method described by Roof and Kramer, (1989). The assay was conducted in triplicate and the average taken for all the three readings. The standard reaction mixture contained 0.1 ml of cytochrome C solution (2.5 mg/ml) in HBSS, 1 x 10^6 KC or PAM in 0.05 ml of HBSS, and 0.05 ml of pre-opsonized porcine zymosan (10 mg/ml) as the stimulant. The O_2^- production for resting porcine KC and PAM was measured by replacement of zymosan with HBSS. After incubation for 1 hour at $37°$ C with agitation, the mixture was centrifuged and the supernatant collected into a fresh 96 well plate for the reading of the OD at 550 nm. The data is presented as the OD x 1000.

Hepatic microsomal metabolism

Hepatic microsomes were isolated by differential centrifugation (Chhabra et al., 1972). Briefly, liver homogenate obtained from collagenase perfusion was centrifuged at 900 x g for 20 minutes. The supernatant obtained was centrifuged for 25 minutes at 192,000 x g for 15 minutes. The supernatant was discarded and the microsomal pellet suspended in KCl (1.15 %) - Hepes (1.25 mM) so that 1 ml contained microsomes from 0.5 g of wet liver. An aliquot of the microsomal suspension was removed and assayed for protein concentration by the method of Lowry et al. (1951).

Hepatic microsomal metabolism was evaluated by measuring the aniline hydroxylase activity. Aniline hydroxylase activity was measured by the formation of p-aminophenol from aniline by a spectophotometric method (Imai et al., 1966). The reaction mixture contained 8 mM aniline, 0.32 mM NADP, 3 mM glucose-6-phosphate, 2.5 mm $MgCl_2$, 1.3 units of glucose-6-phosphate dehydrogenase, 8 mM nicotinaminde, 100 mM Tris-acetate buffer, pH 8.0, and 0.5 ml of microsome suspension in a final volume of 1 ml. The reaction was carried out for 20 minutes at 37° C aerobically, with moderate shaking and stopped by addition of 0.5 ml of 20 % of trichloroacetic acid. After centrifugation, 1 ml aliquot of the supernatant was removed into a clean tube and 0.5 ml of 10 % Na_2CO_3 was added, followed by 1 ml of 2 % phenol in 0.2 N NaOH. The resulting blue color was measured at 630 nm after standing for 30 minutes. The enzyme activity was determined as nanomoles of p-aminophenol formed per mg protein per 20 minutes.

Data analysis

Comparisons of data obtained from control and LPS treated animals were made using the Student's t-test. A p-value of 0.05 or less was considered significant.

Results

Rectal temperature was recorded in control pigs and LPS-treated pigs. The mean temperature was 102.7° F in the control pigs and 103.4° F in LPS treated animals five

hours post treatment. The LPS treated animals had a significantly higher temperature ($p<$ 0.01) compared to the control animals (Table 2.1).

Effect of LPS pretreatment on microsomal enzyme activity

Microsomal enzyme activity was evaluated by determining the aniline hydroxylase enzyme activity (AHA). There was no difference between the AHA activity in the control and LPS treated pigs (54.4 and 59.4 nanomoles of p-aminophenol/mg protein/20 minutes respectively (Table 2.1).

Effect of LPS pretreatment on phagocytic and bactericidal activity

The effect of LPS on the ability of KC and PAM from control and LPS pretreated pigs to ingest and kill *S. aureus*, was evaluated. The results are presented in table 2.2. KC from LPS treated animals had a significantly higher ($p<0.05$) bactericidal activity (77.8 %) as compared to KC from control animals (66.6 %). The ability to ingest iodinated *S. aureus* was higher ($p< 0.01$) for LPS derived KC as compared to those derived from control animals (24.3% and 15.6 % respectively). The ingestion and killing of *S. aureus* by PAM was not altered by pre-treatment with LPS. PAM from control animals had similar percent phagocytic activity with PAM from LPS pretreated pigs (20.4 and 22.6 % respectively). Similarly, PAM from control and LPS pre treated animals killed 70.1 and 76.2 % of *S. aureus* bacteria respectively. Thus, KC activity was enhanced when pigs were pretreated in-vivo with LPS but the activity of PAM was not significantly altered.

Effect of LPS pretreatment on superoxide anion production

The O_2^- production was enhanced in KC from LPS treated animals when compared to KC from control animals (p< 0.05) (Table 2.3). PAM from LPS treated animals had a slightly higher production of O_2^- when stimulated with OZ than PAM from control animals but the difference was not statistically significant (Table 2.3).

Discussion

We induced an acute phase response as evidenced by the rise in body temperature 5 hours after LPS administration. The animals were not clinically sick. The rise in temperature after administration of LPS is a response similar to that observed in pigs three months of age that were given LPS intravenously, intermittently over a four hour period (Monshouwer et al., 1996).

The present study demonstrates the bactericidal and phagocytic activity of neonatal pig Kupffer cells and their enhanced function when LPS is administered prior to isolation of the cells. The enhanced production of superoxide anion in KC has previously been documented for rat KC. KC from normal rats did not produce superoxide anion, while those KC obtained from LPS treated rats produced O_2^- with or without macrophage activators (Bautista et al., 1990). The present study extends these observations to pigs. However, in pigs the enhanced O_2^- production was only observed when KC were stimulated in-vitro with opsonized zymosan. There was no significant difference in PAM from control and LPS treated animals. This lack of difference between PAM isolated from control animals and those isolated from LPS treated animals, and the enhanced

activity of KC, can be explained by the part each of these cell types play in gut-derived endotoxemia. KC may play a larger role in the clearance and thus LPS does not reach the lungs in significant concentration.

We analyzed the microsomal enzyme activity by evaluating aniline hydroxylase activity. Although studies in pigs have reported the depression of microsomal enzyme activity (Monshouwer et al., 1996), we did not observe this phenomenon in neonatal pigs. We speculate that this inconsistency could be explained by the age of the pigs. The microsomal enzyme activity in neonates is not well developed as evidenced by the low activity in control pigs, therefore the response to LPS was not measurable. The inconsistency could also be due to the difference in the dose of LPS and the route of administration. We used a dose of 50 $\mu g/ml$ intraperitoneally while the investigators in the mentioned study, infused a total of 85 $\mu g/ml$, intravenously, intermittently over a four hour period.

In summary, we found that low doses of endotoxin administered intraperitoneally led to an elevation of body temperature of the piglets five hours after exposure, an indication of a systemic reaction to the LPS. The administration of LPS in-vivo enhanced the bactericidal activity, phagocytic activity and the production of O_2^- by KC, but had no significant effect on isolated PAM. The microsomal enyme activity in neonatal pigs was not affected. The activation of KC by low doses of LPS without serious clinical ailments may be useful in pig production. It is possible that low doses of LPS could be utilized to ameliorate the effects of gut-derived endotoxins from enteric infections which are particularly common in neonates.

Acknowledgements

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Table 2.1: Mean (± S.EM) of temperature, and microsomal enzyme activity (aniline hydroxylase activity) in LPS treated and control pigs (n=6/group).

	Control Mean ± S.EM	LPS Mean ± S.EM
Temperature (°F)	102.7 ± 0.1**	103.4 ± 0.1
AHA Activity (nmoles p-aminophenol/mg/20 min)	54.4 ± 18.1	59.4 ± 24.2

**significant difference between the two means, p<0.01
S.E.M - standard error of the mean.

Table 2.2: **Bactericidal and phagocytic activity of Kupffer cells (KC) and pulmonary alveolar macrophages (PAM) isolated from control and LPS treated pigs (n=7/group)**

	Bactericidal Activity (*S. aureus*) % Mean ± S.E.M		Phagocytic Activity % Mean ± S.E.M	
	Control	LPS	Control	LPS
KC	66.6 ± 3.0	77.8 ± 3.4*	15.6 ± 4.0	24.3 ± 3.4**
PAM	70.1 ± 7.0	76.2 ± 5.7	20.4 ± 4.7	22.6 ± 5.1

*Significant difference between controls and LPS treated pigs, p<0.05.
* *Significant difference between controls and LPS treated pigs, p<0.01.
S.E.M - standard error of the mean.

Table 2.3: **Production of superoxide anion (O_2^-) by Kupffer cells (KC) and pulmonary alveolar macrophages (PAM) isolated from control and LPS treated pigs with or without stimulation by opsonized zymosan (OZ) (OD x 1000) (n=7/ group)**

	Control Pig		LPS treated	
	Cells + OZ Mean ± S.E.M	Cells only Mean ± S.E.M	Cells + OZ Mean ± S.E.M	Cells only Mean ± S.E.M
KC	443.2* ± 8.5	432.8 ± 17.6	512.6 * ± 14.9	417.2 ± 7.2
PAM	627.4 ± 85.0	431.8 ± 14.4	695.0 ± 40.2	463.4 ± 24.8

*Significant difference between control and LPS treated animals, p<0.05
S.E.M - Standard error of the mean

CHLORTETRACYCLINE MEDIATES ACUTE PHASE RESPONSE IN *EX-VIVO* PERFUSED PIG LIVERS, AND INHIBITS PRODUCTION OF TNF-α BY ISOLATED KUPFFER CELLS

J. Kinyamu Akunda, Ervin Johnson, Franklin A. Ahrens, Theodore T. Kramer

Abstract

Tetracyclines (TC) have been shown to have anti-inflammatory effects in addition to their antimicrobial action. We investigated the effects of in-vivo administration of chlortetracycline (CTC) on ex-vivo perfused pig livers. The retention and clearance of *Salmonella choleraesuis* (SCS), production of C-reactive protein (CRP), and haptoglobin (HPG) by whole livers were studied. In addition, we studied the in-vitro modulation of production of TNF-α by pig Kupffer cells. Pigs were dosed orally with CTC for three days, and given *Salmonella* LPS 24 hours before removal of the liver. *Salmonella* retention and clearance by livers of pigs given CTC was lower than by control livers ($p < 0.01$ and $p < 0.05$ respectively). We demonstrated an increase in CRP and HPG by liver from control pigs after a three-hour perfusion. Further, CTC decreased the production TNF-α by cultured Kupffer cells incubated in-vitro with LPS. Thus, CTC may assume more subtle roles than just killing bacteria. Its modulation of production of TNF-α suggests a potential for attenuating the acute phase response. However, this possible

beneficial action of CTC was accompanied by a marked reduction of SCS uptake by the liver and a significant decline in the antimicrobial effect of the liver.

Introduction

The acute phase response is a systemic inflammatory response to infection or injury. This response is commonly triggered by LPS release resulting from gram negative bacterial infections. The response consists of a series of cascading events, starting with the release of TNF-α from macrophages, and ending with the hypersecretion of acute phase proteins. Tetracyclines (TC) are a group of broad spectrum antibiotics that have been shown to have anti-inflammatory properties which are independent of their antibacterial activity (Shapira et al., 1997). Studies have demonstrated that TC protect mice from a lethal dose of LPS and inhibit TNF-α, IL-1 and nitrate secretion into the blood (Milano et al., 1997). TC have also been shown to reduce LPS-caused lesions and block LPS-stimulated TNF-α secretion by monocytes (Shapira et al., 1997; Kloppenburg et al., 1996). Other studies have found an increase in production of TNF-α and IL-6 by TC in human patients with malignant pleural effusions (Lin et al., 1993). Mice were significantly protected from LPS endotoxemia by TC if given prior to LPS, but TC had no direct effect on TNF-α synthesis by peritoneal macrophages in-vitro. However, the authors proposed that it may modulate other inhibitory pathways (Milano et al., 1997).

Previous studies conducted in this laboratory have demonstrated the presence of TNF-α and IL-6 in perfusion fluid of livers perfused with LPS or *Salmonella*

choleraesuis (SCS) (Frank et al., 1996; Kramer et al., 1993). In addition, the authors

have shown earlier that *ex-vivo* perfused pig livers are capable of retaining and clearing

infused SCS (Kramer et al., 1993). When liver perfusates from prior LPS or *Salmonella*

perfused livers were added to the perfusion system, *Salmonella* clearance was abolished

(Frank et al., 1996; Kramer et al., 1993). This effect was attributed to passively induced

acute phase reaction.

The present investigation was undertaken to assess the effect of chlortetracycline

(CTC) on liver retention and clearance of SCS, and on the production of TNF-α and two

acute phase proteins, C-reactive protein (CRP) and haptoglobin (HPG). In addition, the

production of TNF-α by Kupffer cells (KC) and its modulation by chlortetracycline were

investigated.

Liver perfusion fluids at time 0 and 180 min of perfusion with SCS were

compared. Livers in 2 groups of pigs were perfused with approximately 10^8 colony

forming units (CFU) SCS, and perfusate levels of TNF-α, CRP and HPG were compared.

Cultured KC from pigs pretreated with LPS were incubated with or without CTC and

LPS and the production of TNF-α production measured.

Materials and Methods

Pigs and liver perfusion

Pigs, 7-14 days old were obtained from a specific pathogen-free herd. The liver

perfusion procedure was conducted under general anesthesia, as described earlier

(Chalstrey et al., 1971; Kramer et al., 1993).

Two groups, 4 perfused livers in each group were studied for the following criteria: SCS retention and clearance; CRP and HPG production at 0 min and 180 min of perfusion.

Group I livers were from pigs given an intraperitoneal injection of 10 µg/kg LPS 24 hours before liver perfusion; Group II livers were from pigs dosed orally with CTC (4.4 mg/kg) every 12 hours for a total of five doses, and LPS (10 µg/kg) intraperitoneally 24 hours before liver perfusion. A third group of pigs were pretreated with LPS 50 µg/kg intraperitoneally 24 hours prior to perfusion. KC were isolated, cultured and used for studies of effects of CTC added in vitro on TNF-α production.

Perfusion fluid processing

Perfusion fluids were collected at 0 minutes and 180 minutes of perfusion. The fluids were sterile filtered and concentrated in a CH2, Amicon spiral cartridge concentrator to a standard protein concentration.

Isolation and testing of C-reactive protein and haptoglobin

CRP was isolated from sterile, concentrated perfusates by the immobilized *p*-aminophenyl phosphoryl choline affinity chromatography method of Volanakis et al. (1978), as modified for the removal of non-specifically absorbed amyloid-P (Christener & Mortensen, 1995). Briefly, the perfusate was dialysed into borate buffered saline, pH

8.0, with 5mM Ca^{++}. The dialysed perfusate was applied to a commercially available phosphoryl choline agarose column (Pierce®). CRP was eluted from the column with 10 mM EDTA and purified on a DEAE sephracel column (Pharmacia®).

HPG was purified from perfusates by the DEAE anion exchange chromatography method of Connell and Shaw, (1961). Briefly, perfusate and DEAE resin were dialyzed against 0.005 M Na-acetate buffer, pH 4.7. The perfusate was applied to the column, and proteins were eluted from the column with 0.005 M Na-acetate buffer, pH 4.7, containing 0.01 M NaCl. HPG eluted from the column after elution of all other proteins, and was collected as a protein peak at 280L. The HPG rich fraction was further purified and concentrated by precipitation at 55 % saturation with ammonium sulfate. Finally, presumptive HPG was poured onto a Sephadex G-200 (Pharmacia™) column, equilibrated with Tris-NaCl buffer, pH 4.7, and the HPG collected with the first (exclusion) bed volume. Levels of CRP and HPG in eluates were determined by SDS-PAGE.

Evaluation of effects of chlortetracycline on production of TNF-α by Kupffer cells

Isolation and culture of Kupffer cells: The liver was perfused with collagenase and the parenchymal cells separated as described previously (Caperna et al., 1985). Briefly, KC were isolated by centrifugal elutriation (JE 6B elutriator rotor). The elutriation buffer consisted of PBS with 1 % bovine serum albumin (Sigma Chemical CO., St. Louis MO). The cell suspension was introduced at a flow rate of 10 ml/min and a centrifuge speed of 2500 rpm. The flow rate was gradually raised to 31 ml/minute and

the red blood cells, endothelial cells, and small KC were run off. The centrifuge speed was reduced to 1000 rpm and the next fraction which contained KC was collected in 200 ml buffer. The cells were washed in PBS, counted, scored for viability and purity as described previously (Thanawongnuwech et al., 1997). The purity of the cells was over 95 % and the viability greater than 80 % as evaluated by trypan blue dye exclusion. The cells were re-suspended at a concentration of 2.5 x 10^6/ml in Williams medium E supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, 10% fetal calf serum, L-glutamine 2 mM, hepes buffer (2 mM) and sodium bicarbonate. Cells were plated in 24 well plate at a density of or 1x10^6 cells per well. The cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2-95% air. After 24 hours, the medium was removed and the adhered cells washed with sterile PBS. Fresh medium was then added.

TNF-α production and the effect of CTC: 48 hours after isolation and culture of KC, the medium was aspirated and replaced with fresh medium, or medium containing 0, 0.1, 0.25, 0.5, and 1.0 mg/ml of CTC (Figure 2). After 30 minutes, LPS (5 μg/ml) was added to each well, and the cells incubated for an additional 4 hours. Media was collected, centrifuged to remove debris and stored at -70 C until analysis for TNF-α was carried out.

ELISA Detection of TNF-α: TNF-α was measured with a commercial ELISA system (Endogen®, Pig TNF-α ELISA, Woburn MA), using a biotinylated antibody sandwich system.

Data analysis

Data from the study of whole livers was analyzed by Students' t- test, while that from KC was analyzed by analysis of variance (ANOVA) using SAS general linear model procedure (PROC. GLM).

Results

Salmonella retention and clearance by whole livers

Pig livers from LPS pre-treated pigs in group I retained 64.2 ± 5.7 percent SCS, at time 0, and cleared 88.3 ± 2.6 percent of the retained SCS during the 180 min perfusion (Table 3.1A). Livers from CTC dosed and LPS pre-treated pigs in group II retained 7.4 ± 1.9 percent SCS at time 0, and cleared 22.0 ± 18.5 percent of the retained SCS during the 180 min perfusion (Table 3. 1B).

Effects of chlortetracycline on acute phase protein production

Perfusion fluids from livers of pigs injected with LPS (10 μg/kg) and perfused with SCS had demonstrable levels of HPG and CRP. The HPG and CRP bands intensified after the 180 minute perfusion, indicating an acute phase response to SCS

(Figure 3.1A). The HPG and CRP response to SCS perfusion was more variable in pigs that were given CTC for 3 days prior to perfusion (Figure 3.1B). Some pig livers from CTC treated pigs responded with an intensified HPG and CRP band after 180 minutes perfusion (Nos. 163 and 164); another responded with an intensified CRP, but not HPG band (No. 161); in liver No 162, the HPG and CRP concentrations declined during the 3-hour perfusion. These observations indicate that pig livers perfused with *Salmonella* for 3 hours can respond with acute phase protein synthesis. CTC feeding prior to perfusion did not have a consistent effect on acute phase protein synthesis.

Effects of chlortetracycline on production TNF-α by Kupffer cells

Cultured Kupffer cells were incubated with or without LPS or with LPS and different concentrations of CTC. The results are presented in figure 3.2. KC without LPS produced a mean of 58.8 pg/ml of TNF-α. KC incubated with LPS for four hours produced a mean 734.3 pg/ml of TNF-α. KC incubated with LPS produced significantly higher amounts of TNF-α than the controls ($p< 0.05$). TNF-α production of KC incubated with CTC and LPS was significantly reduced regardless of the concentration of CTC ($p<0.05$) (Figure 3.2).

Discussion

Our results on production of TNF-α by KC pretreated with CTC indicate that CTC inhibited the production of TNF-α by Kupffer cells stimulated with LPS. There was an inhibition of TNF-α production regardless of the dose of CTC used. These results

are consistent with other investigations that have demonstrated an inhibition of production of TNF-α by antimicrobial agents of the tetracycline group, with subsequent anti-inflammatory effects. Administration of TC for four days following LPS challenge, reduced the size of LPS-caused lesions and blocked LPS-stimulated TNF-α secretion by human monocytes (Shapira et al., 1997). Tetracyclines blocked and reversed both spontaneous and interleukin 1β-induced nitric oxide activity in ex-vivo conditions (Amin et al., 1996). LPS-treated mice pretreated with tetracyclines showed a significant inhibition of TNF-α, IL-1α, and nitrate secretion in the blood, events that were directly related with their survival. In mice treated with tetracyclines, a significant decrease of inducible nitric oxide synthase activity was observed in spleen and peritoneal cells compared with that detected in mice treated with LPS alone (Milano et al., 1997).

In this study, we show that livers from control pigs produced both HPG and CRP, the major pig acute phase proteins, within three hours of infusion of *Salmonella*. Studies in pigs have shown that both HPG and CRP in serum reach a peak on the second day after stimulation of an acute phase response (Eckersall et al., 1996). These two acute phase proteins are likely to be the best markers for the identification of inflammatory lesions in pigs (Eckersall et al., 1996). The elevated levels of CRP and HPG in pig livers after only three hours of stimulation demonstrated here attest to the rapidity of the acute phase response. The results of CTC pre-treated pigs were equivocal since while two of the pigs had diminished production of both CRP and HPG, the other two maintained substantial production in the three hours of perfusion. Thus, it cannot be concluded that CTC pretreatment blocked the acute phase response. However, livers from control pigs

cleared over 80 % of the SCS infused into the liver, while livers of pigs treated with CTC had diminished clearance. The reduced uptake of SCS by the livers from CTC treated animals may be a reflection of the effect of TC on the phagocytic function in the liver. Studies have shown that TC inhibits bacteria uptake by phagocytic cells (Forsgen et al., 1982).

Clearance of SCS by the liver was diminished in livers from CTC fed pigs compared to controls. TC have been postulated to inhibit neutrophil antibacterial activity (Paape et al., 1991) but other studies have suggested that this effect is observed only at sub-therapeutic doses of TC (Myers et al., 1995). The inhibition of bactericidal activity by TC may explain the reduced clearance of SCS by the liver of CTC fed pigs and warrants further investigation.

The therapeutic effect of CTC may thus assume more subtle roles than just killing bacteria. The principal secondary effect seems to be modulation of TNF-α and of the resulting inflammatory reaction. Since an excessive inflammatory reaction may lead to pathological consequence, including the often fatal acute phase reaction, the modulating role of CTC may be more important than its antimicrobial role. In the present instance, we have shown that CTC inhibited production of TNF-α by KC, suggesting a potential for attenuating the acute phase inflammatory reaction. However, this possible beneficial action of CTC was accompanied by markedly reduced SCS uptake by the liver and significant decline in the antimicrobial effect of the liver.

Acknowledgements

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Table 3.1: Effects of LPS or LPS plus chlortetracycline pretreatment on retention and clearance of *Salmonella choleraesuis* in the perfused pig liver

Liver no. and inocculum	Percent SCS retained at 0 min	Percent SCS cleared at 180 min
A. LPS treatment		
AK 157 2.2X10^8 CFU	50.9	89.0
AK 158 2.2X10^8 CFU	58.7	95.2
AK 159 8.4X 10^7 CFU	75.5	84.0
AK 160 8.4X 10^7	71.5	84.9
Group mean ± S.E.M.	64.2 ± 5.7	88.3 ± 2.6
B. CTC and LPS treatment		
AK 161 1.0X10^8 CFU	10.1	76.9
AK 162 1.0X10^8 CFU	9.7	11.0
AK 163 5.2X10^8 CFU	8.1	0.0
AK 164 5.2X 10^8 CFU	1.7	0.0
Group mean ± S.E.M.	7.4 ± 1.9*	22.0 ± 18.5**

*Significantly different from group A retention, $p<0.01$

**Significantly different from group A clearance, $p<0.05$.
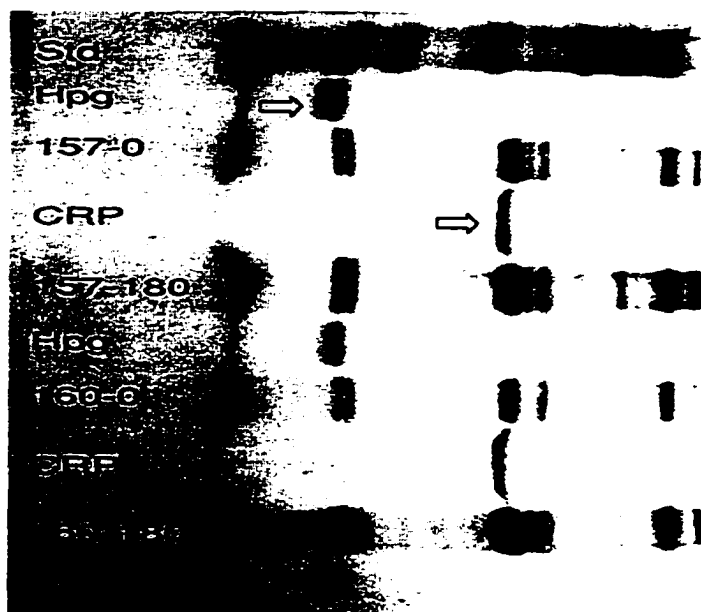
Figure 3.1A: SDS-PAGE identification of C-reactive protein (CRP) and haptoglobin (HPG) (Arrows) from liver perfusate at time zero, and three hours after infusion of *Salmonella choleraesuis*. Liver numbers 157 and 160 were from control pigs.
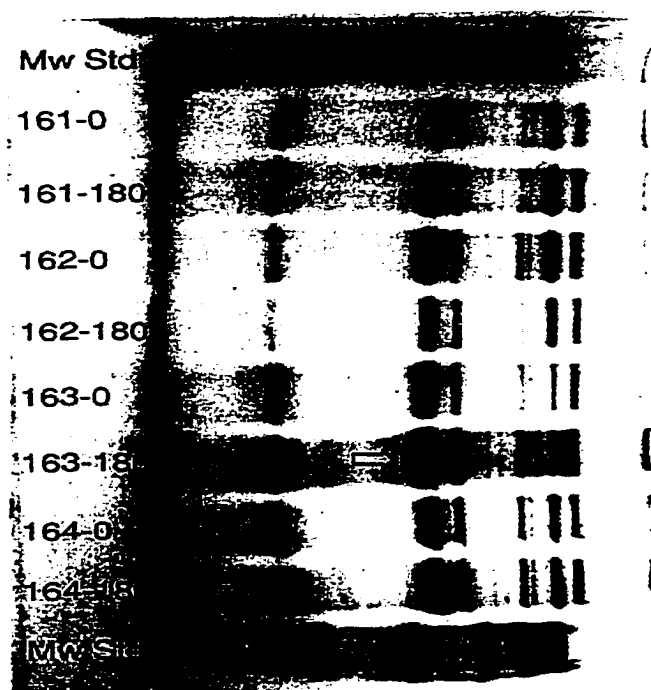
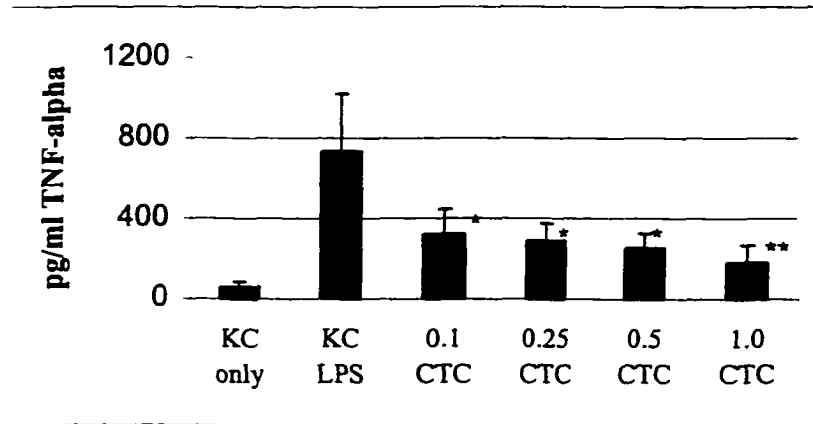**Figure 3.1B: SDS-PAGE identification of C-reactive protein (CRP) and haptoglobin (HPG) (arrows) from liver perfusate at time zero, and three hours after infusion of *Salmonella choleraesuis*. Liver numbers 161 to 164 were from chlortetracycline pretreated pigs.**

Figure 3.2: Levels of TNF-α in culture supernatants of Kupffer cells (KC). KC
were incubated without LPS (KC only), with LPS 5 μg/ml, or with
different concentrations of CTC (mg/ml). Levels of TNF-α (pg/ml) in
the culture supernatants were measured by TNF-ELISA. Bars are
mean and the standard error of the mean. *-Means are significantly
different from LPS *p< 0.05, and ** p< 0.01

GENERAL CONCLUSIONS

The immunological activity of neonatal pig Kupffer cells (KC) was studied and comparisons made with the activity of pulmonary alveolar macrophages (PAM). Neonatal pig KC are immunologically active although their activity is lower than that of PAM. The KC are able to ingest and kill both gram negative *(Salmonella choleraesuis)* and gram positive *(Staphylococcus aureus)* bacteria. KC were found to produce insignificant amounts of superoxide anion (SOA) even when stimulated with opsonized zymosan. PAM on the other hand produced significant amounts of SOA both spontaneously and on stimulation. The inability of KC to produce SOA implies that these cells must use other mechanisms for killing. Both KC and PAM produced TNF-α when incubated in-vitro with LPS thus emphasizing their role in the immune response. Neither KC nor PAM produced nitric oxide on incubation with LPS or opsonized zymosan.

When LPS was administered parenterally, the pigs had an acute phase response as evidenced by the rise in temperature of the treated pigs as compared to the controls. KC isolated from the LPS-pretreated animals were more metabolically active. These cells phagocytosed and killed more bacteria and also produced more superoxide anion. The activity of PAM from both LPS treated animals and the controls was similar. This observation may explain the relative role of each of these cell types in endotoxemia. LPS administered in-vivo did not affect the hepatic microsomal enzyme activity (aniline hydroxylase activity).

The modulation of acute phase response by chlortetracycline (CTC) was also studied. Livers were obtained from CTC dosed pigs or controls. Livers from CTC pretreated pigs had a diminished ability to retain and clear SCS from the liver, implying a diminished phagocytic and bactericidal activity of the liver. The neonatal porcine liver was found capable of producing acute phase proteins (C-reactive protein and haptoglobin), within a period of three hours. In addition, CTC was found to inhibit the production of TNF-α by KC in vitro. Thus, CTC like other tetracyclines has anti-inflammatory effects in addition to its antimicrobial effect.

APPENDIX

The number, viability and purity of Kupffer cells (KC) and pulmonary alveolar macrophages (PAM), obtained from neonatal pig liver and lung

	KC	PAM
Number of cells	9.8×10^7	3.1×10^7
Viability	>90 %	> 95 %
Purity	>85 %	> 90 %
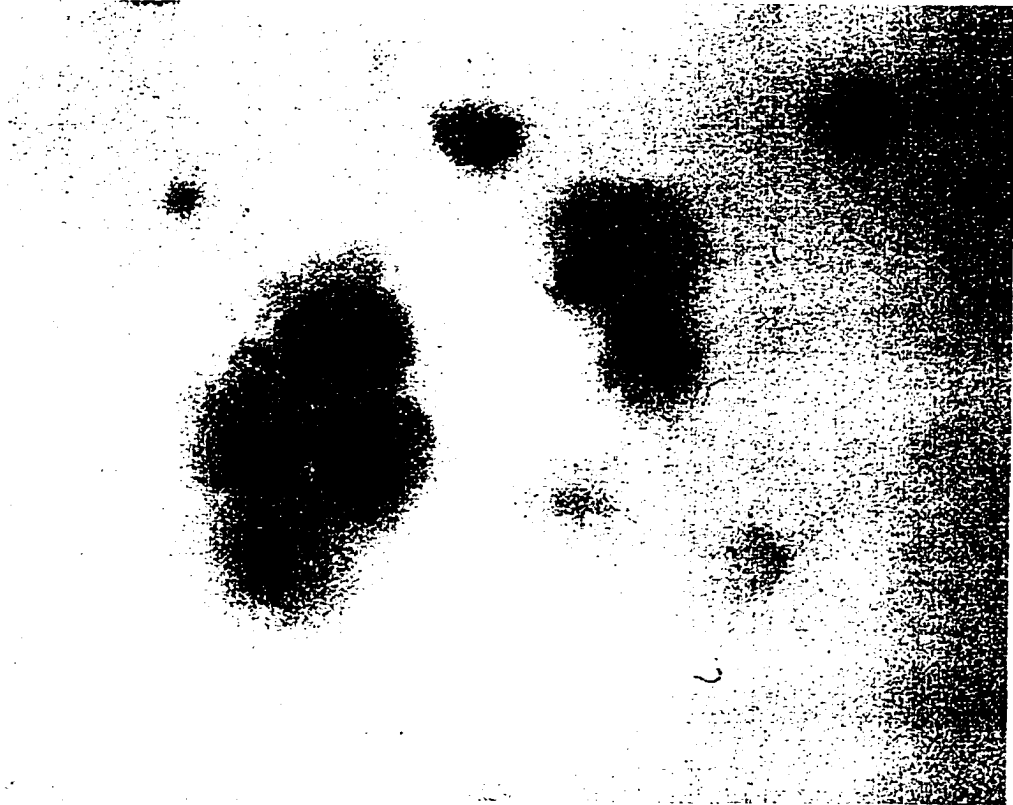
Cytospin of KC isolated by collagenase perfusion and purified by step gradient in arabinogalactan (Magnification 40X)

KC after 24 hours in culture, showing the ingestion of latex beads (arrow). Notice the spreading nature in culture, the Kupffer cells acquire a stellate form. (Magnification 100X)
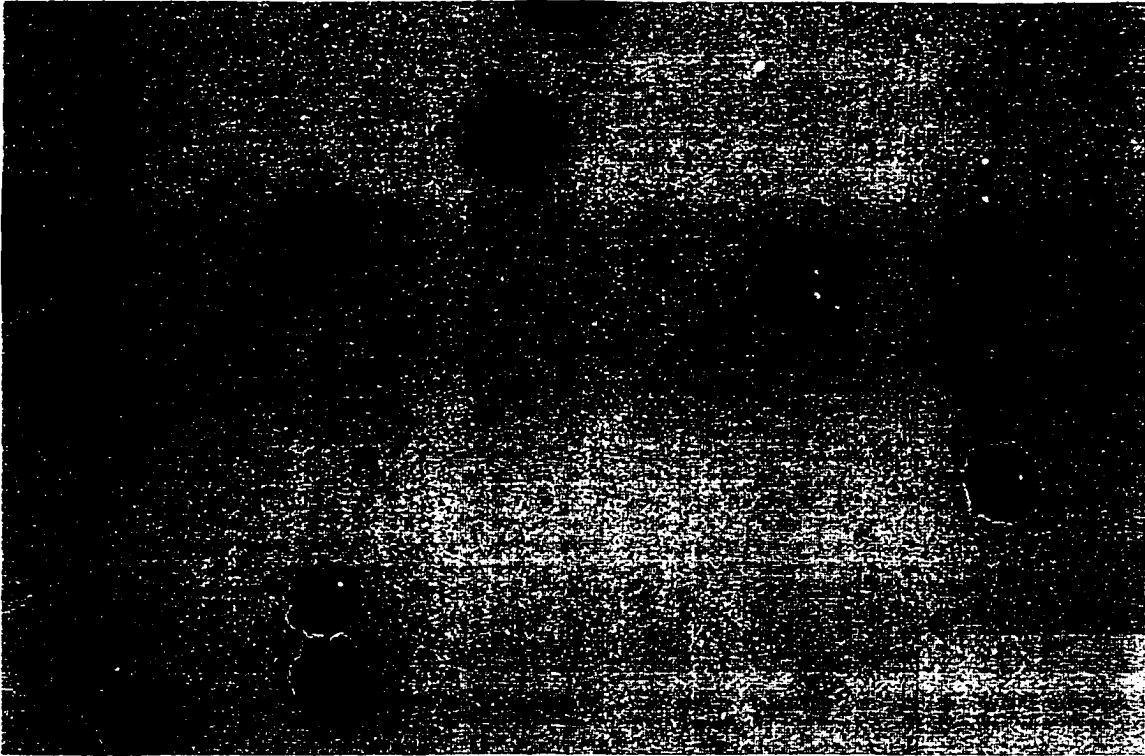
Pulmonary alveolar macrophages (PAM), isolated by broncho-alveolar lavage from neonatal pig lungs.

Production of TNF-α by Kupffer cells (KC) and (PAM) pulmonary alveolar macrophages (PAM), measured by % cytotoxicity on L929 cells (n=6/group).

	0 μg LPS (control)	1 μg LPS	5 μg LPS	10 μg LPS	50 μg LPS	Opsonized Zymosan
KC	15.9 ± 2.2	28 ± 2.6*	29.9 ± 2.2*	20.9 ± 2	19.2 ±3.6	23.6 ± 4.7*
PAM	17.3 ± 3.8	20.5 ± 3.3	22.5 ± 2.2	20.6 ± 4.6	27.2 ± 5.6	29.6 ± 5.2

Values are Mean ± S.E.M
*Significantly higher than control p<0.05

ACKNOWLEDGEMENTS

Completing a Ph.D program and writing a thesis requires hard work, patience and help. The completion of this work could not have been possible without Gods' grace and the help, support and encouragement of so many people. I list only a few of them here.

I'd like to express my sincere gratitude to my major Professor Dr. Franklin Ahrens for his guidance and support throughout my graduate studies. His assistance in the technical work, his patience and his encouragement throughout has been instrumental in the achievement of my goal. Thank you Dr. Ahrens for your time, and for seeing me through my Ph.D.

I wish to thank Dr. Theodore Kramer whom I worked with closely, and from I whom I learned a lot of immunology. My work benefited from his valuable suggestions and contributions. His financial support for the last year of my study is also greatly acknowledged and his kindness is appreciated.

I'm grateful to my other committee members Dr. James Roth who also availed his laboratory for my use, Dr. Donald Dyer, and Dr. Monica Howard for giving their time and their contributions to the of success of this study and my future career. Their interest in my work and my progress was very encouraging to me.

I thank the staff in the Biomedical Sciences Department (BMS) for their support through the years: Vern Hoyt and Linda Erickson for having answers to so many of my questions. All the friends and colleagues in the college of Veterinary Medicine I cannot list here, my gratitude goes to you.
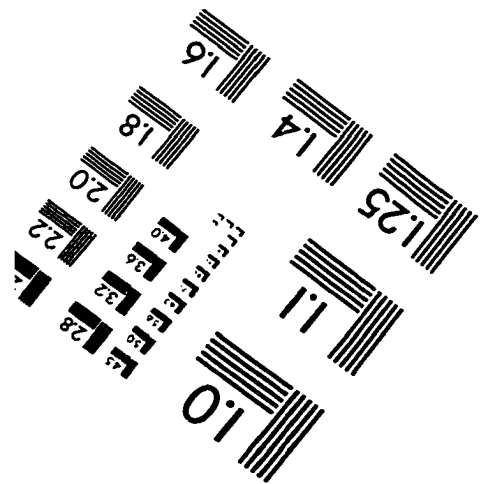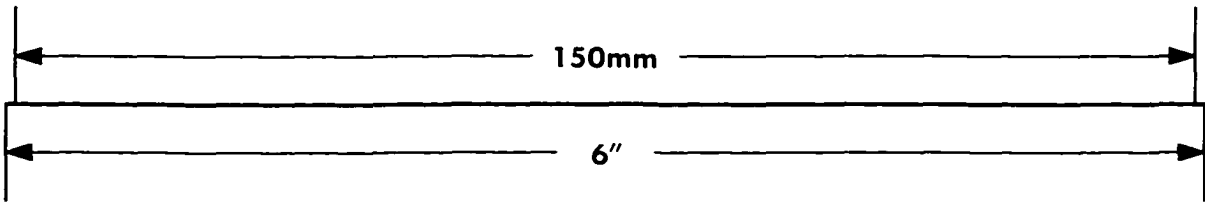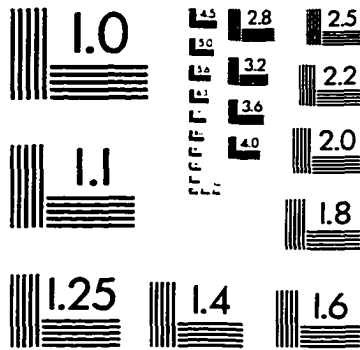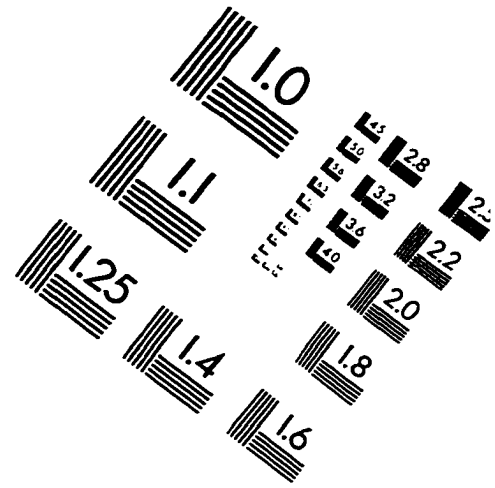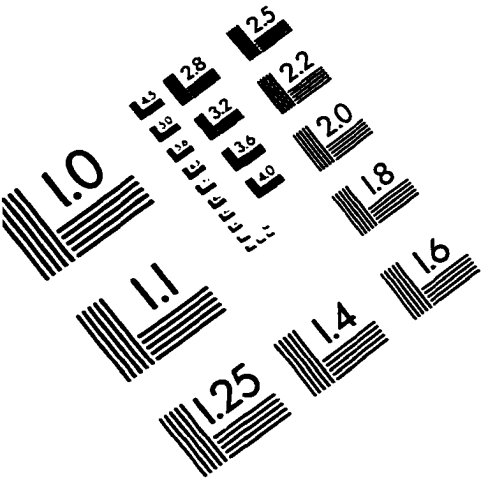
My appreciation goes to Dr. Dagmar Frank and Laura Hamilton for their generosity, kindness and support. Your Friendship is very special to me. The members of Friends of International Women, were a constant source of encouragement. I'm grateful that they shared their time and friendship with me. The Kenyan women at Iowa State were also a source of inspiration and support.

My education at Iowa State University was supported from a number of sources. I acknowledge the contributions of the Department of Residence and thank Dr. Don Whalen my mentor; BMS Department, and the research grants under Dr. Ahrens and Dr. Kramer. I also thank the P.E.O organization for granting me the International Peace Scholarship and for the kindness of their members, especially the Boone Chapters.

I could not have accomplished this without support of close relatives and family in the United States. Notably I wish to thank my sister Dr. Harriet Kinyamu, my niece Denise Karimi, Dr. Susan Kaaria, Wanja and Mugure Kebaara for their support and comfort. My life was more full because they were a part of it.

I wish to thank my parents Mr. and Mrs. Kinyamu for their upbringing and teaching me the value of education in my early years. I will forever be grateful for their love and support. Finally my sincerest gratitude to my husband Eric Akunda, for being my best critic and friend and giving me support in many ways. My son, Chris Akunda is a source of inspiration and his smile at the end of the day rewards all efforts. I dedicate this dissertation to them and to the memory of my mother-in-law Mrs. Christabel Akunda. I know you would have been proud of us.

IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"