COPPER SUPPLEMENTATION AND MONOCYTE FUNCTION IN GROWING BEEF CALVES

by

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Abstract

The effect of dietary copper (Cu) supplementation with Cu-Sulfate ($CuSO_4$) or Cu-Lysine (CuLy) on Cu status and bovine monocyte function was evaluated through a series of experiments. Initially, two *in vitro* techniques, immunomagnetic (IM) and culture flask adherence (CF), were compared for isolation of a viable, homogeneous monocyte population. The CF technique for monocyte isolation resulted in both a greater number of cells exhibiting phagocytic activity, as well as, an increased phagocytic capacity compared to monocytes recovered by the IM technique. Culture flask adherence appears to be an efficient technique for isolation of a viable, homogeneous population of bovine monocytes.

Copper status and monocyte function were evaluated in beef calves supplemented with Cu over a 2 year study period. Fifty-four weaned calves were allotted to one of three Cu treatment groups in a 150 d feeding trial. Plasma Cu concentration was increased in CuLy-supplemented calves

over controls and $CuSO_4$-supplemented calves on d 42, 84, and 126. Calves supplemented with Cu had increased ceruloplasmin activity on d 84, 126, and 150 as compared to controls. Hepatic Cu measured on d 150 was decreased in controls compared to Cu-supplemented calves. Monocyte cell number and function from CuLy-supplemented calves showed increased phagocytosis on d 84 and 126 and increased oxidative burst on d 42 and 126 compared to controls.

Dietary Cu supplementation was repeated using 45 calves in a 120 d study. CuLy-supplemented heifers had increased major histocompatability complex (MHC) class II expression on d 68, 82 and 110 compared to $CuSO_4$-supplemented and control group heifers. Heifers supplemented with Cu had increased plasma Cu concentrations on d 82 and 110 compared to controls.

The effect of vaccination on monocyte function was evaluated in Cu-supplemented beef heifers. Vaccination with *B. abortus* Strain 19 increased monocyte oxidative burst, phagocytic activity, and MHC class II expression in heifers. Copper supplementation and source of Cu supplement influenced monocyte response to vaccination. Monocyte response appeared to be higher in CuLy-supplemented heifers after vaccination compared to $CuSO_4$-supplemented and control heifers.

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Dedication

This work is dedicated to the memory of my father, whose "Reverence for Life" has infiltrated my being and illuminated my world.

WE NEED ANOTHER AND A WISER AND PERHAPS A MORE MYSTICAL CONCEPT OF ANIMALS.

Remote from universal nature, and living by complicated artifice, man in civilization surveys the creature through the glass of his knowledge and sees thereby a feather magnified and the whole image in distortion. We patronize them for their incompleteness, for their tragic fate of having taken form so far below ourselves. And therein we err, and greatly err. For the animal shall not be measured by man. In a world older and more complete than ours they move finished and complete, gifted with extensions of the senses we have lost or never attained, living by voices we shall never hear. They are not brethren, they are not underlings; they are other nations, caught with ourselves in the net of life and time, fellow prisoners of the splendour and travail of the earth.

Henry Beston

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Chapter I
Introduction

Historically, infectious diseases have caused significant economic losses to the beef producer. In recent years animal nutritionists and producers have begun to realize potential benefits from dietary trace mineral supplementation. Although the mechanisms are not completely understood, specific trace minerals have been shown to play an important role in disease resistance and improved animal performance. Copper is a component of cells and enzyme systems necessary to help protect against disease challenge, and therefore has been targeted for its influence on the normal function of the immune system in animals (Graham, 1991).

For centuries, the reddish-brown metallic element identified as copper (Cu) was utilized by by various craftsman for everything from tools to coins. The identification of Cu in plant and animal tissues was thought to be solely a consequence of soil contamination. In the early 1900's Hart and associates showed that Cu is a component in living systems. Their discovery opened Pandora' box, for the essential role of Cu in growth and in the prevention of a multitude of clinical and pathological disorders which have since been revealed. In 1931, the ruminants' requirement for Cu was realized, indirectly, by association of commonly seen conditions such as ovine enzootic ataxia (swayback); bovine falling disease, hair and wool depigmentation and bone abnormalities linked with inadequate dietary Cu intake (Becker et al., 1965; McDowell, 1992). After Cu was proven essential for growth and hemoglobin formation (Hart et al., 1928), numerous other Cu-dependent enzymes were identified. Ceruloplasmin and superoxide dismutase are two Cu-containing enzymes specifically targeted for their role in immune function.

Altered immune cell function as a consequence of dietary Cu deficiency was first reported in laboratory species. Prohaska and others have demonstrated suppressed T and B cell function, impaired neutrophil oxidative metabolism and candidacidal activity, and altered secretory capacity of leukocytes in Cu-deficient rats and mice (Prohaska &

Lukasewycz, 1981, 1989; Prohaska et al., 1983; Lukasewycz & Prohaska, 1982, 1983, 1985; Vyas & Chandra, 1983; Davis et al., 1987, Failla et al., 1988; Flynn et al., 1984; Flynn & Yen, 1981; Prohaska et al., 1988a). Recently, Babu and Failla (1990) reported a positive correlation between monocyte Cu concentration and cellular oxidative capacity and killing ability.

Ruminant studies have focused mainly on induced Cu deficiencies in sheep and cattle that resulted in impaired cell-mediated immunity and neutrophil dysfunction (Boyne & Arthur, 1986; Jones & Suttle, 1981; Zin et al., 1991). The influence of dietary Cu alterations on monocyte immunoresponsiveness has received little consideration. In light of previous murine (Babu and Failla, 1990; Jain and Williams, 1988; Duwe et al., 1981; Lawrence and Jenkinson, 1987) studies, it is likely that the bovine monocyte system would be responsive to dietary Cu supplementation and that immunocompetence could be enhanced in the growing ruminant.

Cattle production has advanced from the small backyard farm to corporate run operations. Increased economic and environmental constraints require efficient cattle production systems in order to meet market demands; optimal animal health is an essential aspect of this type of production. Macro- and micronutrient deficiencies can result in compromised immune function and, ultimately, production losses. Marginal Cu deficiencies often go undetected but have been shown to affect cattle growth and performance (Bremner & Mills, 1981; Herd et al., 1990; Clark et al., 1994). Supplementation with Cu sources of high biological availability and monitoring physiological parameters of Cu status that are sensitive to marginal Cu deficiencies are two avenues by which to enhance optimal cattle health and production.

The present set of experiments were designed to address these aforementioned areas of cattle production and optimal health by (i) determining the effects of various Cu supplements on immune cell function and Cu status in growing beef calves; (ii) characterizing the effects of vaccination stress and dietary Cu on immune response in beef calves; and (iii) evaluating monocyte activity as an indicator of Cu status in the beef calf.

Chapter II

Literature Review

This review of the literature will begin with the animal's copper requirement, briefly discuss sources to meet those requirements, review the mechanism (s) by which those sources are utilized by the animal, assess copper utilization by the animal and, indirectly, re-evaluate Cu requirements.

Copper Requirements and Dietary Sources

Trace mineral requirements are not well characterized, particularly in regard to the effect of nutrition on immune function. The National Research Council (NRC) and the Agricultural Research Council (ARC) have established minimal requirements (Table 1) for trace mineral nutriture. These recommendations have been formulated presumably for healthy, disease-free animals. The complicated interactions of environmental (climate, soil, plant) and physiological (growth, pregnancy, lactation, illness, production) stressors can alter nutrient requirements. It has been proposed that during high milk production, rapid growth, or stress, NRC and ARC recommendations are probably inadequate and should be exceeded. Specific recommendations for increasing dietary Cu have not been established for beef cattle but suggested levels range from 4 to 8 times current NRC recommendations, DM basis (Larson et al., 1995; Herd, 1990).

Concentrations of Cu in crops and forages vary geographically and are dependent on soil factors, rainfall and drainage patterns, pH, plant species management, and harvesting techniques (Graham, 1991). Native pasture, the main nutrient source for beef cattle, can be an inadequate source of many trace minerals including copper. Tall fescue (*Festuca arundinacea* Schreb.) and various clovers (*Trifolium species*) are commonly utilized forages in Virginia that have a reduced Cu availability in ruminants (Puls, 1988). In a comprehensive review of bovine Cu status, Smart et al. (1992) summarized Cu concentrations in feeds utilized in beef production systems. On a dry

Table 1. Current Recommendations for Dietary Copper for Beef Cattle

	National Research Council	Agricultural Research Council
Copper	4 - 10 mg/kg DM	
Growth	--------	8 - 15 mg/kg DM
Pregnancy	--------	13 - 20 mg/kg DM
Lactation	--------	8 - 14 mg/kg DM

Adapted from Graham, 1991.

matter (DM) basis the average Cu concentration was below NRC recommendations beef cattle of 8 to 10 mg Cu/kg DM, with grass hays ranging from 4.4 to 6.2 mg Cu/kg; legume hays from 7.0 to 11.0 mg/kg; cereal silage 4.1 to 6.5 mg/kg; and corn silage 5.2 to 7.5 mg/kg. Oats, barley and corn were below 6.5 mg Cu/kg and pasture was approximately 2.7 mg Cu/kg. These types of feedstuffs also had a low (0.050) absorption coefficient for copper (Suttle, 1986).

Several known dietary interactions predictably decrease Cu availability in feeds, most notably the interaction between copper and molybdenum. Soil and plant Mo concentrations markedly alter Cu bioavailability. Copper uptake in plants is optimal in poorly drained, clay soils with an acid pH. Increasing soil pH reduces Cu concentrations of plants but favors Mo uptake. Copper:Molybdenum ratios of less than 2:1 in feedstuffs result in conditioned Cu deficiencies in beef cattle. Molybdenum, along with sulfur (S), forms thiomolybdates in the rumen. These Mo-S complexes bind to available dietary Cu and thereby decrease its absorption and utilization (McDowell, 1992).

Additional sources of Cu include parenteral and oral supplements. Copper glycinate and Cu-edetate have been used in subcutaneous injection (Boila 1984a;1984b). Oral supplements available in the United States include Cu boluses, Cu-oxide wires, and inorganic or organic Cu salts (Graham, 1991). Parenteral administration of Cu has been more problematic than oral supplementation with reports of irritation and abcessation at the injection site, acute toxicosis and death. Oral toxicosis, however, has been reported more commonly in young livestock (SMTA, 1980). Copper in the form of oxide, sulfate and amino acid complexes were supplemented to beef cattle rations to determine bioavailability of the three Cu sources (Clark et al., 1993a, Kegley & Spears, 1994). Bioavailability of Cu was lower with the oxide form compared to the sulfate and amino acid complexed forms of Cu supplement.

Metal amino acid chelates are thought to be more efficiently absorbed and potentially more efficiently transported to target tissues (Kratzer & Vohar, 1981). Enhanced bioavailability of the chelates over simple salts is dependent upon the stability of

the chelates in the intestine and their solubility in water or lipids (Shah, 1981). Copper complexed by amino acids, peptides and certain proteins increases Cu retention, particularly Cu complexed with L-amino acids (Kirchgesner & Grassman, 1978). Copper absorption and retention were increased in stressed beef calves that were supplemented with Cu-Lysine (organic form) compared to calves supplemented with Cu-Sulfate (inorganic form). Cu-Lysine supplemented calves also gained more weight during the feeding trials than calves supplemented with Cu-Sulfate (Kincaid & Blauwiekel, 1986; Wittenburg, 1991; Nockels et al., 1993). Concurrent studies indicated no difference in bioavailability between the two Cu sources (Kegley & Spears, 1994, Clark et al, 1993b).

Copper Disposition: Absorption through Excretion

Ingested Cu is poorly absorbed especially by ruminant species. Mature ruminants absorb only 1 to 3%, whereas younger animals absorb 15 to 30%. Suttle (1975) showed the apparent availability of Cu in pre-weaned lambs was 47% compared to 10% after weaning.

Copper is absorbed in all segments of the digestive tract, but the majority is absorbed in the abomasum and small intestine either as free Cu or more likely as ligand-bound copper. The exact nature of these ligands is not completely understood but it is agreed that numerous factors affect ligand binding and thus Cu uptake from the gut. The acidic environment of the stomach appears to enhance Cu solubility and transport across gastric mucosa, at the same time though, these gastric secretions enhance solubility of other dietary metals resulting in direct competition for gastric absorption (Cousins, 1985).

Copper that by-passes absorption in the stomach to reach the small intestine, is influenced by a variety of dietary components, including fiber, phytate, ascorbic acid, thiomolybdate, and amino acids. The extent of exchange of Cu with intestinal binding ligands is not well known, but competition for various ligands could also be a factor that

influences the extent of uptake. Copper absorption is enhanced when complexed with amino acids as compared to inorganic complexes such as $CuSO_4$ (Kirchgesner & Grassman, 1978; Kincaid & Blauwiekel, 1986; Wittenburg, 1991; Nockels et al., 1993) The L-isomer form of amino acids has been shown to be more efficiently absorbed as Cu-amino complexes than the D-isomer form (Kirchgesner & Grassman, 1978). Conversely, absorption studies utilizing the amino acid histidine (Marceau et al., 1970; Gollan & Deller, 1973) did not show improved rates of copper absorption over inorganic Cu complexes. In general, high protein diets enhance Cu uptake (Davis et al., 1962; Engel et al., 1967; Greger & Snedeker, 1980; Lonnerdal et al., 1982, Turnland et al., 1982), but the digestibility of dietary protein can have a marked effect on the formation of Cu-amino acid and peptide complexes. Citrate, gluconate, and phosphate complexes can enhance Cu absorption (Cousins, 1985). Likewise, Cu in the forms of Cu_2CO_3, $Cu_2(NO_3)_2$, Cu-oxalate and Cu-EDTA are more bioavailable sources of Cu than $CuSO_4$(Chapman & Bell, 1963; Nichols et al., 1993). Dietary components such as phytate, fiber , fructose and ascorbic acid (Cousins, 1985) when complexed with Cu, decrease Cu uptake. Copper thiomolybdates ($CuMoS_3$ and $CuMoS_4$) are known antagonists for Cu absorption in ruminants. While stable complexes of Cu thiomolybdates are insoluble and impair ruminal uptake, absorbed oxythiomolybdates are thought to be associated with infertility and growth failure seen with secondary hypocuprosis (Bremner et al., 1987). Additionally, dietary zinc (Zn) and iron (Fe) interfere with copper absorption (Graham, 1991). Other endogenous substrates can also influence Cu absorption. For instance, bile (Gollan & Deller, 1973) and pancreatic secretions (Cousins, 1985) have a negative influence on resorption of secreted copper. Biliary secretion of Cu is promoted by glucocorticoids (Benson, 1979; Henkin, 1974). The extent of control that endogenous secretions may have on Cu metabolism during trauma and stress has not been established.

Once absorbed into the intestinal cell, Cu is compartmentalized and its fate in the cell may, in part, be regulated by cellular concentrations of Zn and its influence on metallothionein (Figure 1). Transfer of Cu out of the intestinal cell to the portal circulation

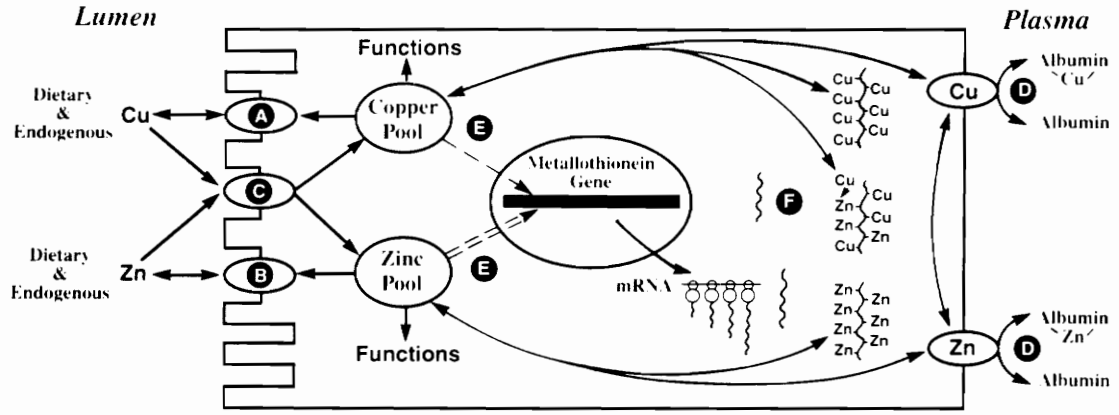

Figure 1. Schematic representation of copper uptake at the brush border membrane and transport through the intestinal cell. (**A**) Lumenal Cu of dietary and / or endogenous origin is transported across brush border membrane surface. (**B**) Similar transport of zinc. (**C**) Potential interaction between high lumenal concentrations of Cu and Zn for a common transport system, endogenous binding factors, or receptors. Once in the intestinal cell, nutrient metals interact with intracellular pools. (**D**) Copper and Zn within the enterocyte are transferred to albumin for plasma transport. (**E**) High intracellular concentrations of Cu or Zn activate metallothionein promoter gene. (**F**) Thionein polypeptides have high binding affinity for Cu which can prevent Cu transport across the basolateral membrane to plasma, thus reducing Cu absorption.
Adapted from Cousins, 1985.

is generally assumed to occur with albumin (Sarkar et al., 1978; Cousins, 1985), although investigators (Evans, 1973; Weiner & Cousins, 1980) have shown that plasma Cu accumulated in hepatic cells whether presented to the liver as amino acid complexes, namely histidine, or albumin bound .

Copper is contained within two plasma pools in transport to the liver. Ninety-five percent of the total Cu is tightly bound to ceruloplasmin. The remaining 5% is loosely bound to albumin and amino acids and these two pools remain separate with no exchange of Cu during transport to the liver (Cousins, 1985). Once in the hepatocyte, Cu is transferred to metallothionein-like protein and other metalloenzymes. Liver is the central organ of Cu metabolism and, therefore, closely reflects overall Cu status. Copper concentration of hepatocytes is influenced by dietary Cu supply as well as by specific disease factors, therefore a fuller understanding of hepatic metabolism is warranted.

Hepatic Metabolism of Copper

Copper uptake by the liver follows first-order kinetics (Cousins, 1985). Investigators have suggested that the uptake mechanism at specific sites on or in the hepatocyte cell wall is responsible for the accumulation-binding phenomenon observed in kinetics studies. Investigators have also demonstrated, through liver cell culture studies, that Cu uptake is regulated by factors such as culture media concentrations of amino acids and temperature fluctuations. Surprisingly though, it was found that the presence of metals such as zinc, nickel, magnesium, cobalt and iron did not influence hepatic uptake kinetics of copper (Harris & Sass-Kortsak, 1967; Weiner & Cousins, 1980; Cousins, 1985). The data collectively suggest that Cu uptake follows a passive carrier-mediated mechanism.

Regulation of Cu accumulation within the hepatocyte is thought to be under hormonal control. Epinephrine and glucagon stimulate Cu accumulation, whereas estradiol-17ß and testosterone (Cousins, 1985) lower hepatic Cu accumulation (Figure 2). Interestingly, the hormonal regulation of Cu metabolism has inputs that are similar to mechanisms that influence amino acid transport (Kilberg et al., 1980), in which case,

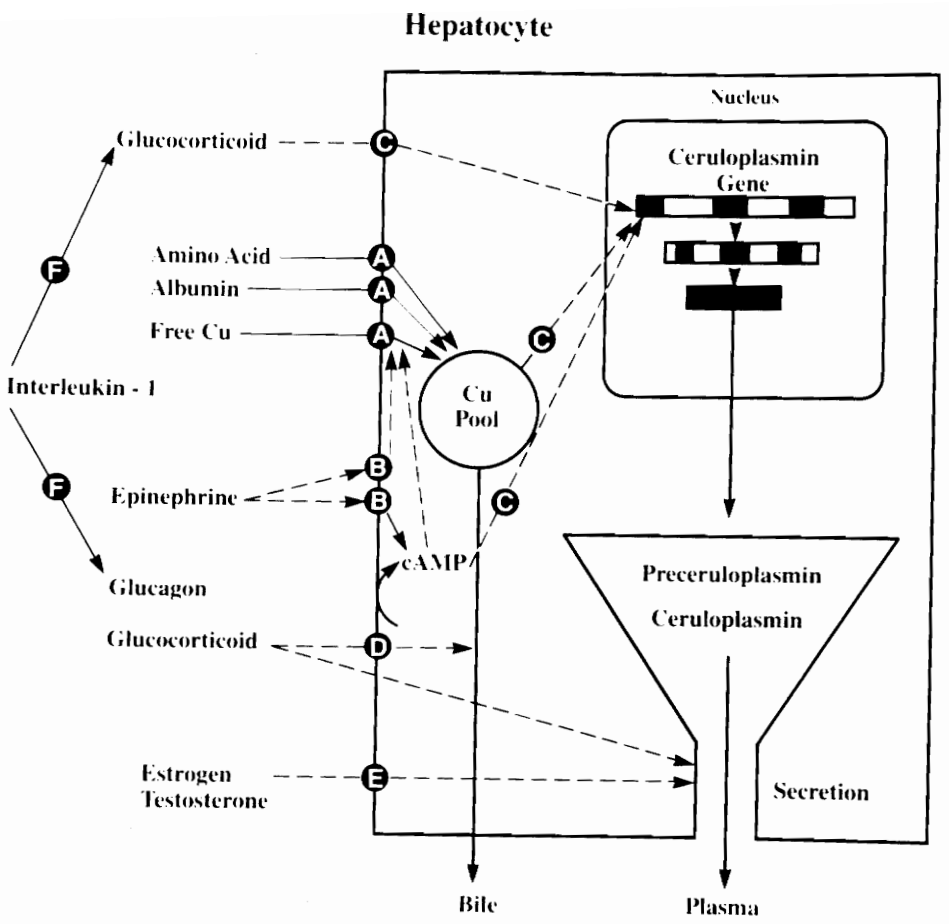

Figure 2. Schematic depicting hepatic metabolism of copper. Key components of regulation including uptake, redistribution to intracellular copper pools, and ceruloplasmin synthesis and secretion. (**A**) Copper uptake from portal circulation involves amino acid-bound, albumin-bound, or free Cu; then Cu is distributed among compartments represented as a Cu pool. Hormonal regulation includes: (**B**) Epinephrine, a major stimulus for intracellular Cu accumulation; (**C**) Transcription of the ceruloplasmin gene is, regulated by glucocorticoids, Cu and / or intracellular cAMP which is regulated by epinephrine and glucagon; (**D**) Glucocorticoids increase biliary excretion of Cu and ceruloplasmin secretion; (**E**) Estrogen and testosterone increase ceruloplasmin secretion; (**F**) Interleukin -1 increases plasma ceruloplasmin directly or indirectly via hormones.
Adapted from Cousins, 1985.

enhanced membrane transport of the amino acids that chelate Cu may result in increased hepatic uptake of the mineral.

Copper is distributed throughout various subcellular compartments of liver cells. The nuclear fraction contains approximately 27% of hepatic Cu, mitochondria 7%, rough endoplasmic reticulum (ER) microsomes 7% and smooth ER microsomes 3%. The largest proportion (54%) of hepatic Cu is found in the supernatant (cytosol) fraction (Smeyers-Verbeke et al., 1977). Lysosomes also accumulate appreciable amounts of copper. In Cousins' review of murine radioisotope studies for Cu absorption, Cu is first associated with lower molecular weight proteins (metallothionein) and, with time, is transferred to higher molecular weight proteins such as superoxide dismutase (SOD) and cytochrome oxidase, important cuproenzymes involved in immune function. These Cu-containing enzymes and others will be discussed in relation to physiologic functions of copper.

Excretion of hepatic Cu depends on many factors, specifically intracellular factors that favor retention of Cu, and the availability of circulating ligands that transfer Cu from hepatocytes. Darwish and co-workers (1983) demonstrated a steady state between Cu influx and efflux, with imbalances resulting mainly from an altered efflux or prolonged hepatic retention. The etiology of these Cu accumulating disease states is not known, but protein synthesis may be a central factor in regulating hepatic Cu, because mineral levels increase during an inhibition of either protein transcription or translation. (Cousins, 1985; Weiner & Cousins, 1980).

Hepatic secretion of Cu is principally in the form of ceruloplasmin and extrahepatic uptake is presumably mediated by this metalloenzyme (Cousins, 1985; Graham, 1991). Copper bound to low molecular weight moieties, however, can also be donated to tissues. For example, bioflavonoids (DiSilvestro & Harris, 1983) and glycylhistidyllysine (Pickart & Thaler, 1980) chelated to plasma Cu can donate Cu to tissues. Once transported to target organs, Cu participates mainly as a cuproenzyme in numerous cell functions.

Fecal excretion accounts for the highest proportion of ingested Cu, most of which is unabsorbed. Absorbed Cu is actively excreted via bile and to a lesser extent through

urine, milk, intestine and perspiration (Underwood, 1977)

Physiological Functions of Copper

Approximately 10 proteins are accepted as true cuproenzymes and are responsible for the variety of specialized copper functions, however, only a few of these proteins have been a focus of Cu metabolism studies through the years.

Iron Metabolism

Copper is an indirect catalyst in hemoglobin function via iron oxidation. Iron, stored as ferritin, must be converted to the ferrous form for mobilization and / or incorporation into hemoglobin or myoglobin. Ceruloplasmin (ferroxidase) catalyzes the oxidation of Fe, permitting it to bind with the Fe-transport protein, transferrin (McDowell, 1992).

Chronic anemia often observed in mineral deficient animals, is poorly characterized but may be induced by either a Cu or Fe deficiency. Increased red blood cell fragility and viscosity and altered membrane lipid peroxidation are reported characteristics which can be associated, directly or indirectly, with impaired intracellular Fe mobilization and heme synthesis (Williams et al., 1985; Jain & Williams, 1988; Suttle et al., 1987).

Connective Tissue Integrity, Bone Formation, and Cardiac Function

Copper deficiency can result in a failure of collagen to undergo cross-linking and maturation. Lysyl oxidase is the Cu-containing enzyme which is necessary to add hydroxyl groups to lysine residues in collagen to allow cross-linking between collagen fibers. These cross-links give the proteins structural rigidity and elasticity. Abnormal bone formation is a clinical sign associated with Cu-deficient animals. Lameness is evident, long bones fracture easily, and some animals show joint swelling or enlargement. A failure in elastin cross-linking results in aortic aneurysms and ruptures or sometimes myocardial fibrosis,

evidenced as sudden death or "falling disease" in cattle (Graham, 1991; McDowell, 1992).

Pigmentation and Keratinization Abnormalities

A principal manifestation of Cu deficiency in animals is alteration of hair color (pigmentation) or achromotrichia. Hair and wool color changes can be attributed to a lack of tyrosinase activity. Tyrosinase is the cuproenzyme responsible for conversion of tyrosine to melanin, and a breakdown in this pathway is the probable cause of alteration in hair color (Graham, 1991).

Changes in the character of wool and hair, such as crimp and coarseness can also be associated with Cu deficiency. The crimp in wool is dependent upon formation of disulfide groups and orientation of long-chain keratin fibrils in the fiber. Abnormal sulfhydryl cross-linkage in keratin into disulfide groups is a characteristic of Cu deficiency that results in weak and coarse hair fibers (McDowell, 1992).

Central Nervous System - Cellular Respiration

Cytochrome oxidase is a Cu-dependent enzyme involved in both cellular respiration and central nervous system (CNS) function. It is the terminal enzyme in the electron transport chain which is required for ATP production. Degeneration and necrosis of neuronal cells appear to be a associated with decreased cytochrome oxidase which results in defects of mitochondrial enzyme function. Whether cell degeneration and necrosis is a consequence of deficient cytochrome oxidase or visa versa is controversial (Miller & O'Dell, 1987). The link between Cu deficiency and enzootic ataxia (swayback) in young ruminants has been described as a manifestation of incomplete myelin formation (McDowell, 1992). Phospholipid is the major component of myelin and decreased cytochrome oxidase in Cu deficiency leads to depressed phospholipid synthesis by liver mitochondria. The alteration in myelin synthesis results in neurological disturbances associated with swayback (O'Dell, 1984). Dopamine-ß-hydroxylase, a Cu-containing enzyme, catalyzes the oxidation of the neurotransmitter, dopamine, to norepinephrine.

Central nervous system function can likewise be altered by changes in these neurotransmitters.

Reproduction

Calving problems, retained placenta, and skeletal abnormalities in newborns have been attributed to Cu deficiency (Graham, 1991). Fetal death and resorption have resulted from Cu deficiency in laboratory species (McDowell, 1992). Abnormalities detected in the embryos of Cu deficient mammals have been categorized as defects in red blood cell and connective tissue formation during early embryonic development. Reproductive problems including delayed puberty, altered estrous cycle length, cystic ovarian disease, impaired ovulation, and reduced conception rates were linked to Cu deficiency induced by interactions with molybdenum (Mo) and iron (Fe) (Phillippo et al., 1982; 1987; Graham, 1991). Although Fe and/or Mo treatment decreased Cu concentration in beef cows, parenteral or oral Cu supplementation did not appear to correct the mineral-induced infertility or improve reproduction performance in general.

The role of copper and molybdenum in fertility is inconclusive at this time, but disturbances in luteinizing-hormone (LH) release appear to be the common denominator in many of the observed reproductive problems. The association is based on several studies indicating that Cu alters release of gonadotropin-releasing hormone from the median eminence (Barnea et al., 1988a). Thymic gonadal regulation depends on hormone interactions among the hypothalamus, pituitary, gonads, and thymus (the HPG-thymic axis). Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates LH release from the pituitary, and LH stimulates sex steroid (estrogen or testosterone) production by the gonads. A negative feedback mechanism causes GnRH release from hypothalamus to be depressed and LH release from the pituitary to be inhibited (Grossman, 1985). Copper most likely influences reproductive regulation by way of a receptor mechanism involving Cu and facilitory ligands such as histadine, histamine and glycine, to stimulate release of LHRH from the median eminence (Barnea et al., 1988b).

Copper and Immune Function

Cuproenzymes

Altered immunity associated with Cu deficiency involves various cuproenzymes, some of which have previously been mentioned as participating in other physiological functions. Investigations into the relationship of Cu and immune function have centered around cuproenzymes 1) acting in an antioxidant role; 2) involved in energy metabolism; and 3) acting in the control of neuroendocrine function. Specific enzymes include ceruloplasmin and Cu, Zn-superoxide dismutase (SOD) as antioxidants; cytochrome oxidase (CO) in energy metabolism and ; dopamine-ß-hydroxylase, as well as peptidyl glycine alpha-amidating monooxygenase (PAM), in neuroendocrine control.

Copper deficiency has been associated with brain catecholamine changes. Prohaska and co-workers (1992; 1993) have reported several studies that measured an increase in dopamine concentration, while norepinephrine concentration and dopamine-ß-hydroxylase (DBH) activity have decreased. The synthesis of norepinephrine from dopamine is catalyzed by the Cu enzyme complex, DBH (Harper, 1979). Enzyme activity appears to be correlated with the Cu-to-enzyme ratio. Maximal dopamine hydroxylation occurs at a stoichiometry of 2 mol of Cu / mole of enzyme subunit (Ash et al., 1983). Klinman and coworkers (1984) showed that DBH catalytic activity is dependent on Cu levels. Low brain norepinephrine, resulting from decreased DBH activity, may indirectly alter immune response in animals. Reduction of alpha and beta adrenergic receptor site binding and subsequent altered central catecholamine metabolism may influence immunohumoral messenger release and cyclic-AMP (cAMP) action in a Cu-deprived immune system (Besedovsky et al., 1983; Feller et al., 1981; Keller et al., 1982; Govoni et al., 1977; Prohaska & Lukasewycz, 1989; Ray & Wallis, 1980).

The enzyme PAM requires Cu and ascorbate as cofactors to modify numerous neuroactive peptides (Eipper et al., 1983), the most common being vasoactive intestinal peptide (VIP). Vasoactive intestinal peptide couples with a lymphocyte receptor to activate

cyclase, which leads to a cAMP-dependent cascade. Indirectly, through peptides such as VIP, PAM can influence the immune system.

The relationship between Cu and energy metabolism is through the alteration of ATP production by cytochrome oxidase. As previously mentioned, phospholipid synthesis is ,in part, regulated by cytochrome oxidase. The phospholipid content of immune system cells can also be altered by reduced cytochrome oxidase. The biosynthesis of leukocyte plasma membranes requires energy. It is possible that decreased CO may limit ATP flux and ultimately alter immune system cell membrane protein and phospholipid content (Prohaska & Lukasewycz, 1990). Altered cell membranes can impair the immunoreactivity of leukocytes.

Ceruloplasmin is an acute phase glycoprotein that tightly binds approximately 6 Cu atoms per molecule. The catalytic activity and potential functions of this enzyme have been categorized as: 1) oxidation of Fe^{2+}- to Fe^{3+}-transferrin in the regulation of hepatic Fe mobilization; 2) oxidase activity for aromatic amines; 3) transport of Cu to tissue sites; 4) serum antioxidation; and 5) endogenous modulation of the inflammatory response (Frieden, 1980). These last three categories are most closely linked to host defense mechanisms. Ceruloplasmin is considered the primary source of Cu for extrahepatic tissues either through a membrane receptor mechanism or by direct endocytosis. The receptor mechanism involves ceruloplasmin Cu^{2+} reduction at the membrane receptor, and subsequent transfer of Cu^{1+} to an unidentified intracellular acceptor. An alternative to this mechanism involves endocytosis of intact ceruloplasmin molecules and then release of Cu^{1+} by proteolysis and recycling of the glycoprotein portion back to the cell membrane (Frieden, 1980; Owen, 1965; Linder & Moor, 1977). Intracellular Cu can then be oxidatively transferred to apoenzymes such as lysyl oxidase.

The antioxidant function of ceruloplasmin was proposed based on the observations that increased ceruloplasmin activity was associated with the generation of oxidation

products (O_2^- and H_2O_2). Superoxide ion (O_2^-) is generated from the oxidation of ferrous iron. Ceruloplasmin through its ferroxidase activity can catalyze the oxidation of Fe^{2+} with production of H_2O_2 rather than superoxide generation (Baldwin et al. , 1993). Ceruloplasmin also can serve as a scavenger of superoxide radicals (Goldstein et al., 1979), inhibit metal ion-induced lipid peroxidation (Cousins, 1985), and have a protective effect on Fe-stimulated erythrocyte lysis (Lovstad, 1981). The radical-scavenging potential of ceruloplasmin is lower than that of SOD but, because ceruloplasmin is extracellular as well as intracellular, it has antioxidant potential in the plasma. More recently, ceruloplasmin has been shown to enhance phagocytosis of neutrophils and monocytes by binding to the plasma membrane and triggering oxidative metabolism within the cell (Saenko et al., 1994). Figure 3 shows how the regulation of Cu metabolism can affect physiologic functions and specifically relate to overall host-defense processes.

Acquired and Innate Immunity

The relationship of Cu status and leukocyte function has been explored using the murine model. Copper-deficient rats were shown to have higher mortality rates when infected with *Salmonella typhinurium* compared to Cu adequate counterparts (Newberne et al., 1968). Multiple studies over the past 15 years have reported suppressed T and B cell function in Cu-deficient rats and mice based upon reduced T cell proliferation to mitogen stimulation, altered lymphocyte subset populations in spleen, decreased splenic T lymphocyte and antibody-producing cells, decreased B cell mitogen response, and impaired cell-mediated immunity to leukemia cells (Lukasewycz & Prohaska, 1982; 1983; 1985; Prohaska & Lukasewycz, 1981; 1989; Prohaska et al., 1983 ; Vyas & Chandra, 1983; Davis et al., 1987, Failla et al., 1988). Likewise, Cu-deficient mice have altered phagocytic cell activity with impaired respiratory burst and candidacidal activity, as well as reduced cytokine (IL-1, IL-2, IL-5) secretory capacity (Babu & Failla, 1990a; 1990b; Flynn et al., 1984; Flynn & Yen, 1981; Prohaska et al., 1988a).

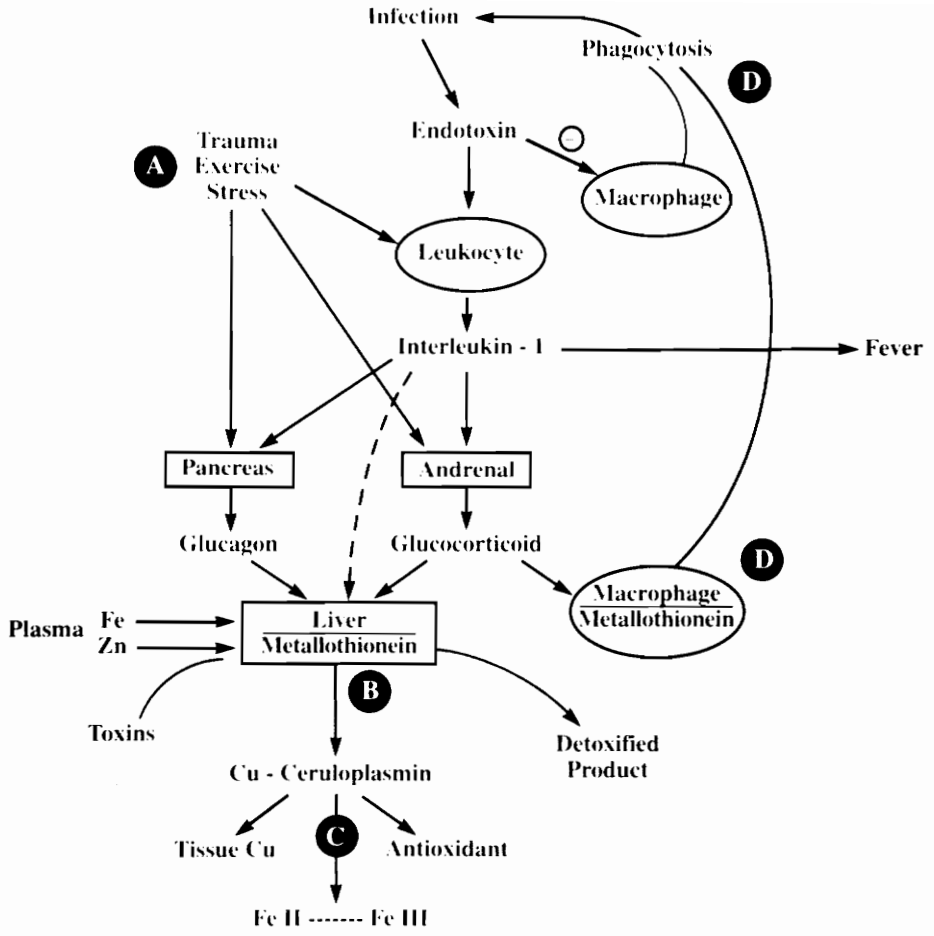

Figure 3. Hypothesized relational aspects between physiological stressors, immune response, and copper function. (**A**) Stress and trauma increase plasma glucagon, glucocorticoids, and interleukin-1, (**B**) which promotes hepatic secretion of ceruloplasmin via increased metallothionein levels. (**C**) Elevated plasma ceruloplasmin relates to protection from oxidative damage. (**D**) Macrophages synthesize metallothionein in response to glucocorticoids, which in turn increases phagocytic capacity of monocytes, possibly through intracellular Cu mechanisms.
Adapted from Cousins, 1985.

Immune cell function studies designed to evaluate the effects of Cu on immune response in ruminants are limited in comparison to laboratory animals. Neutrophils from genetically Cu-deficient sheep (Woolliams et al., 1986) and cattle fed Cu-deficient diets (Jones & Suttle, 1981; Boyne & Arthur, 1981; 1986; Xin et al., 1991) exhibited decreased killing ability of ingested *Candida albicans* and *Staphylococcus aureus* , but phagocytic activity of the cells was not impaired. Gestating beef cows fed diets supplemented with $CuSO_4$ and high levels of Fe showed a decrease in neutrophil phagocytosis compared to cows supplemented with either $CuCO_3$ and/or no Fe supplement. Humoral response to chicken gamma-globulin was not different among the Cu- and/or Fe-supplemented treatment groups in that study (Niederman et al., 1994). Lambs genetically selected for low Cu status were more susceptible to bacterial infection and showed lower mixed lymphocyte responses to pokeweed mitogen, phytohemmagglutinin and concanavalin A than did high Cu genotype lambs (Woolliams et al., 1986). Yong et al.(1985) showed no effects on blood lymphocyte responses when lambs were Cu depleted by administration of tetrathiomolybdate. Lilley et al. (1985) reported that alveolar macrophages from Cu-deficient cattle had no *in vitro* bacteriostatic or bacteriocidal activity compared to a 50% killing activity by macrophages from Cu-adequate cattle. Increased incidence of abomasal ulceration, intramammary infections and parasitic infestation have been indirectly related to altered leukocyte function in ruminants fed Cu-deplete rations (Mills et al., 1976; Lilley et al., 1985; NMC, 1994; Hucker & Yong, 1986).

Assessment of Copper Status

Techniques for detecting and controlling trace element and nutrient imbalances in livestock differ widely in character and effectiveness. Analytical measurements are a more specific, accurate, and precise assessment of trace mineral status compared to subjective determinants such as body condition and hair coat changes. Criteria commonly used for

status assessment include: dietary intake versus requirement, balances studies, elemental analysis of liver, blood, urine, and hair and, more recently, measurement of trace element-specific biochemical and physiological functions (Mertz, 1985). A variety of conditions influence Cu availability in soil, therefore, soil Cu content is not an accurate indication of plant Cu concentrations. Evaluation of Cu status, by monitoring dietary Cu, has limited value unless the concentrations of other elements that interact with Cu, such as Mo, S, Cd, and Zn, are also monitored. Trace mineral requirements have been routinely derived from balance studies or tissue composition.

Blood and Hepatic Copper Concentrations

Plasma Cu concentrations reflect the Cu status of animals but the normal range is wide and, in some references, the values for adequate, marginal and deficient may overlap. A reference range for cattle plasma and liver Cu is depicted in Table 2. In cattle, the normal range for plasma Cu is 0.6 to 1.5 ug/mL; plasma Cu concentrations below 0.6 μg/mL indicate deficiency, but concentrations above 0.6 μg/mL do not necessarily imply adequacy (Claypool et al., 1975). Assessment of Cu status via plasma Cu is losing favor (Suttle, 1986; Mills, 1987) but it can be useful diagnostic tool when used in conjunction with other measures of Cu status.

Liver Cu content reflects Cu status because the liver stores Cu and the majority of Cu metabolism occurs in hepatocytes. Low intakes of available Cu by ruminants are usually accompanied by marked declines in hepatic Cu reserves. In severe depletion, total liver Cu declines by 50 % every 25-30 days. The rate of release of liver Cu is usually sufficient to maintain plasma Cu within the normal range until liver Cu falls below 30 mg Cu/kg DM. Hepatic stores and endogenous losses of Cu have been positively correlated (Bremner & Mills, 1981) implying that hepatic marginality is an adequate indicator of Cu deficiency. The value of blood and liver mineral concentrations is in assessing long-term changes in trace element body content, not for interpreting acute physiological responses. Assessment of physiological alterations involving trace elements is necessary to identify the

Table 2. Evaluation of copper status of cattle.

Tissue	Adequate	Marginal	Deficient
Liver, µg/g (wet basis)	25-100	5-25	0.5-10
Plasma, µg/ml	0.8 -1.5	0.6-1.2	0.2-1.0

Adapted from Puls, 1990.

pathological consequences of sub- or supra-optimal trace mineral intake before productivity declines or diagnostically characteristic clinical signs of severe deficiency become evident (Mills, 1987).

Biochemical (Enzyme) Activity

Cu-dependent enzymes have been suggested for determination of Cu status. Ceruloplasmin (Blakley & Hamilton, 1985; Wikse et al., 1992) and superoxide dismutase (Suttle & McMurray, 1983) have been investigated because determination of enzyme activity is more easily accomplished with them than with other cuproenzymes. Although ceruloplasmin activity declines with established hepatic deficiencies, and low ceruloplasmin can be detected before clinical signs of Cu deficiency develop (Mills, 1976), it has also been shown to increase during the acute phase of many infections. Synthesis of this enzyme is altered by inflammation, hormones and copper. A multitude of factors, both physiological and experimental, increase plasma ceruloplasmin (Cousins, 1985). Acute-phase proteins such as ceruloplasmin increase after tissue injury, localized acute inflammation, and chronic inflammatory disease. The enzyme changes related to physiologic stresses are thought to be due to altered rates of synthesis (Chang et al., 1976; Holtzman & Gaumnitz, 1970; Weiner & Cousins, 1983). Infection is a physiologic stress that increases ceruloplasmin synthesis. *Salmonella* -infected chickens showed a marked increase in ceruloplasmin, as have chicks subjected to *E. coli* endotoxin (Starcher & Hill, 1965; Curtis & Butler, 1980). The immediate increase in ceruloplasmin observed in these studies after infection was thought to be attributable to hepatic release of ceruloplasmin and / or hormonal induction of ceruloplasmin synthesis. A biphasic response for both serum Cu and ceruloplasmin was observed when hamsters were injected with *E. coli* endotoxin, suggesting an initial immediate release of hepatic ceruloplasmin followed by a slower onset of de-novo synthesis (Etzel et al., 1982). Ceruloplasmin response to infection with intracellular organisms such as *B. abortus* has not been investigated.

Cytokines secreted in response to stress, particularly immune challenge, can

increase serum ceruloplasmin, and the increase in ceruloplasmin after exercise (Cousins, 1985) is possibly related to interleukin-1 (IL-1) release. Many factors such as these can alter ceruloplasmin concentrations and interfere with accurate interpretation of Cu status.

SOD is a Cu, Zn erythrocyte enzyme that is principally controlled by Cu status at the time of erythrocyte synthesis. It converts potentially toxic superoxide free radicals to H_2O_2. Copper depletion and subsequent repletion studies with both cattle and sheep indicate that erythrocyte SOD declines and recovers more slowly than plasma Cu. These studies further suggest that differences in the rate of decline of both SOD and plasma Cu during depletion directly reflect severity of the deficiency (Suttle & McMurray, 1983). Trace element deficiencies can predispose the animal to accumulate lipid hydroperoxides, which cause tissue damage if not disposed of properly. This can lead to 'oxidative stress' affecting many organ systems. Quantitating the Cu, Zn SOD status can be a useful tool in monitoring Cu status, but the time of measurement relative to immune challenge, acute or chronic, may influence diagnostic interpretation. Monitoring nutrient insufficiencies through detection of physiological criteria allows early intervention.

Leukocyte Function / Activity

One of the earliest functional defects in Cu-deficient cattle is failure of microbial defense mechanisms (Boyne &.Arthur, 1986). It was suspected initially that this failure was perhaps one manifestation of a generalized decline in SOD activity because microbicidal activity is contingent upon a free-radical process. The loss of microbicidal function, however, precedes SOD loss and appears more closely related to a failure to produce superoxide within the phagosome which serves to destroy or neutralize foreign antigens (Arthur & Boyne, 1985). Thus, measuring killing activity, in conjunction with alterations in other Cu-dependent functions, could be a means for the early detection of Cu-related physiological changes resulting in altered immune response and ultimately poor animal performance.

The neutrophil has traditionally been singled out as the indicator of

immunocompetence in the animal model although the macrophage shares similar cell functions. Although these two cells are similar, macrophages are distinguished from neutrophils by: their capacity to differentiate locally, their capacity for mRNA transcription and protein synthesis, their ability to modify their cellular repertoire in response to external stimuli, the diversity of their secretory products which affect both macrophages and other cell types, their ability to process antigens, and their long life in tissues (Ho, 1989). A direct relationship between dietary Cu adequacy and monocyte cell function has been reported in rats (Babu & Failla, 1990b) indicating that the ability of the cell to phagocytize organisms was contingent upon adequate macrophage Cu concentrations.

Like other cells in the body, phagocytes interact with their environment through membrane receptors. Phagocytes possess receptors for many growth factors and cytokines. They also are known to express receptors for major histocompatability (MHC) class II antigens and for the constant region (Fc) of immunoglobulin G. Engagement of IgG FcR by immune complexes or IgG-opsonized particles leads to phagocytosis. It is believed that the Fc receptor (FcR) expressed on the monocyte/macrophage mediates phagocytosis by being a cation channel and regulating protein kinase C activation which, in turn, phosphorylates important proteins necessary for the final cell response. Adenylate cyclase activation results in generation of cAMP which regulates activation of protein kinases. Adenylate cyclase activation is induced by the neurotransmitter epinephrine. Synthesis of this neurotransmitter is indirectly controlled by activity of a Cu-dependent enzyme, dopamine-ß-hydroxylase (Ho, 1989).

Efficient activation of the immune system requires the cooperation of many different types of cells. Originally T and B lymphocytes were felt to mediate most immune responses. Important sub-populations of these classes of lymphocytes must interact in unique and highly regulated ways to affect proper immune responses. Another group of cells, which includes the monocyte, are referred to as accessory, or antigen presenting, cells and have been shown to contribute to immune response. Monocytes have multiple functions in host defense that include antigen presentation and T cell activation.

Monocytes phagocytize and process foreign protein into peptides. These peptides are presented to CD4+ T cells through surface expression of class II immune associated glycoproteins (Ia) also known as MHC molecules. These Ia molecules are the products of immune response genes and thus exert control over specific immune responses. The mechanism involves Ia and antigen interaction along with a specific T-cell receptor. Each tissue has been described as expressing varying percentages of Ia determinants. For example, peritoneal cavity and lung have a limited expression compared to thymus and spleen where expression is high (Unanue et al., 1984). The basal expression of Ia on monocytes is characterized by: (i) cell age, in that Ia develops mainly in young macrophages (monocytes), (ii) transient nature, and (iii) independence from mature T cell and overt antigen stimulation. Membrane integrity and cellular metabolism are vital components in Ia expression of monocytes. Altered fatty acid, protein and lipid composition of cell membranes has been demonstrated in Cu-deficient rats (Korte &Prohaska, 1987 ; Johnson & Kramer, 1987; Jain & Williams, 1988). Copper-deficient rats exhibit hypercholesterolemia, which is believed to influence membrane cholesterol levels and, subsequently, membrane integrity (Duwe et al., 1981). Researchers have disputed the notion of increased lipid peroxidation of macrophage cell membranes as a consequence of altered cuproenzyme activity (Lawrence and Jenkinson, 1987; Korte & Prohaska, 1987).

The second function of the macrophage involves the ability of this cell to produce soluble factors with immunoregulatory potential. A very important one of these being IL-1, which is involved in the antigen-specific activation of lymphocytes (Rosenwasser & Gurka, 1989). Flynn and co-workers (1984) have shown, through *in vitro* procedures, that mixed leukocyte populations in Cu-deplete cultures exhibit an impaired production of IL-1.

In summary, Cu alters immune response through regulation of immune cell functions. With a better understanding of the cellular relationships involved in activation of the immune response, it becomes evident that alterations in phagocyte cell (monocyte)

activity because of dietary deficiencies can result in a cascade of immunological dysfunctions. Early detection of decreased immune response related to dietary trace mineral changes could circumvent future production losses.

Chapter III

Comparison of Immunomagnetic and Flask Adherence Cell Techniques for Isolation of Bovine Monocytes

Abstract

This study was designed to compare two techniques, immunomagnetic (IM) and culture flask adherence (CF), for isolation of a homogenous monocyte population from cattle. Whole blood samples were collected from six beef calves (mean weight 250 kg) whose total white blood cell counts and differentials were within the normal range for the bovine species. Monocyte separation was initiated with a Ficoll-Hypaque density gradient to isolate the mixed monocyte / lymphocyte population from whole blood samples. Further purification of monocytes by the IM and CF treatment techniques showed an increased ($P < 0.001$) number of cells recovered by CF compared to IM treatment but, monocytes recovered from the IM treatment were 90% homogenous compared to 65% homogenity of monocytes from the CF treatment. All recovered cell populations were 95% viable based on trypan blue stain. Monocytes recovered from the CF treatment had increased ($P < 0.01$) phagocytic ability at 37 C (measured by flourescent emission) compared to IM recovered monocytes. The percentage of monocytes able to phagocytize beads was increased ($P < 0.001$) in the CF treatment group at 4 C and 37 C. Hydrogen peroxide (H_2O_2) release from monocytes recovered by the CF treatment showed a mean channel flourescence of 100 with approximately 20% of the cells responding to stimuation with cytochlasin B. Monocyte numbers were inadequate from the IM treatment to run H_2O_2 release. The results indicate that CF technique yielded an adequate number of functional monocytes that could be used in bovine monocyte studies. Although the IM technique yielded a more homogenous monocyte population, the number and function of those monocytes were unacceptable for bovine monocyte studies. Culture flask adherence appears to be a reliable and reproducible technique for bovine monocyte isolation.

Introduction

Infectious diseases cause significant economic losses in animal production systems. In the United States alone, estimated annual losses of over $2 billion from mastitis and respiratory disease are reported in the dairy and beef cattle industries (Graham,1991). Research has focused on aspects of neutrophil and lymphocyte function in regards to health and disease resistance in the bovine (Arthur & Boyne, 1985; Boyne & Arthur, 1986; Jones & Suttle, 1981; Xin et al., 1991). Monocytes also play an important role in the cascade of humoral and cellular immune responses elicited by infectious agents or invading organisms (Babu & Failla, 1990). These cells not only have phagocytic activity but also immunoregulate immune responses by secreting various monokines such as IL-1 and TNF.

Although monocytes account for only 2 to 7 % of the leukocyte population in the bovine species, they influence lymphocyte activation, chemotaxin release, and intracellular killing of bacteria in the diseased animal (Ho, 1989; Rosenwasser & Gurka, 1989; Politis et al., 1991; Francey et al., 1992; Nash et al., 1992).

Alterations of the immune system can influence animal health and promote optimal production. Optimal nutrition helps to assure optimum performance by the animal and optimum health at the cellular level. An immune system deficient in micronutrients necessary for cellular activation, regulation, and growth will be unable to respond to disease challenges. Therefore, dietary nutrient deficiencies can help target immunodeficiency problems before animal health and performance are irreversibly damaged. Changes in mononuclear phagocyte activity may be a reliable barometer of immune system alterations in the animal. Numerous investigations on mononuclear phagocyte biology using peritoneal-derived mononuclear cells have been reported in murine models. Monocyte isolations by flask adherence have yielded very homogeneous cell populations in the rat and mouse. Although isolation and recovery of murine monocytes is well documented and easily performed, monocytes from other species exhibit dissimilar

properties when collected via murine monocyte isolation protocols. Therefore, the isolation and culture techniques commonly used for laboratory animals may not be transferable to other species, especially the bovine. A simple, timely and reproducible technique for isolation of bovine monocytes has not been determined.

The objective of this study was to compare a newly-introduced immunomagnetic cell separation technique to a traditional technique, flask adherence, for the isolation of bovine monocytes. Percent yield, viability, and homogeneity of the isolated monocyte populations were determined. Functional assays utilized to ascertain recovered cell activity included hydrogen peroxide (H_2O_2) release and phagocytic activity.

Materials and Methods

Animals and Facilities - Weaned crossbred beef calves (mean initial weight 250 kg) were used in the experiment. Calves were housed at the Virginia Tech Beef Center in an outside lot and fed a corn silage plus soybean meal ration. The calves were weighed on platform scales and blood samples were obtained at the time of weighing.

Experimental Design & Methodologies - Six calves were randomly chosen from the herd for this experiment. Whole blood was collected by jugular venapuncture from all calves into 30 ml syringes that contained 15 ml of Alsevars solution for cell separation techniques and also into EDTA-coated vacutainer tubes for total leukocyte counts. Both immunomagnetic and flask adherence separation techniques were run in triplicate on blood from all six calves. The experiment included a stepwise procedure of monocyte isolation and recovery from whole blood utilizing both immunomagnetic and flask adherence cell separation techniques. Recovered monocytes were then tested for viability and activity via two *in vitro* systems.

(i) Bovine monocyte isolation and recovery.

Complete blood cell counts were determined on blood collected in EDTA-coated

Vacutainer® tubes for each calf. Monocyte cell isolation was initiated with a continuous Ficoll-Hypaque density gradient separation of blood collected in Alsevars solution. This technique (Appendix A) was modified from a density gradient technique reported by Goddeeris et al., 1985. This type of separation resulted in a multilayered cell population based on cell density with red blood cells on the bottom of the tube, covered by a thick layer of granulocytes, then a very thin layer of mononuclear cells and finally a top layer of plasma (Figure 1). The monocyte / lymphocyte layer was aspirated by Pasteur pipette into 15 ml tubes. Cells were counted on a hemocytometer and stained with trypan blue to determine viability prior to treatment allocation. An equal number of cells ($1x10^6$) were allocated to the immunomagnetic and flask adherence treatments. Both techniques are described in detail in Appendices B and C. Immunomagnetic separation entails either a positive or negative selection process. An indirect, negative selection was chosen for the immunomagnetic technique. In this procedure, the lymphocytes in the mixed monocyte / lymphocyte population were bound to beads coated with anti-lymphocyte monoclonal antibodies (Moab). These lymphocyte-Moab-bead complexes were then magnetically pulled out of solution. The remaining cell fraction consisted of a fairly homogeneous monocyte population (Figure 2).

Cells were counted after monocyte isolation by hemocytometer to determine percent cell recovery. Viability of cells was determined by trypan blue stain. Cytospins were prepared and stained with alpha-napthyl acetate esterase for accurate monocyte identification. The purified monocyte populations were then used for flow cell cytometry analysis of H_2O_2 release and phagocytic activity.

(ii) Testing of recovered monocytes for immune response from the two separation techniques.

Measurement of H_2O_2 Release using DCFH-DA

Cells obtained from the multi-step monocyte isolation procedure (Appendices A, B,

and C) were used to measure monocyte cell activation and oxidative metabolism. The procedure to measure H_2O_2 release from monocytes (Appendix D) is a modification of that reported by Bass et al; (1983) which measures fluorescence emitted from activated monocytes generating hydrogen peroxide (H_2O_2). Dichlorofluorescin diacetate (DCFH-DA) is a non-fluorescent probe which diffuses into cells and, in the presence of H_2O_2, is oxidized to the fluorescent analog, 2',7'-dichlorofluorescein (DCF).

Briefly, cells were diluted to a concentration of 1 x 10^6 cells/ml in Hanks balanced salt solution (HBSS) and 1 µl / ml of 5 mM DCFH-DA was added to all samples (final concentration of 5 µM DCFH-DA). Cells were incubated in a 37 C waterbath for 15 minutes. Samples were divided into two tubes that contained 1 x 10^6 cells/ml each. One tube received 12.5 µl of the stimulant, cytochalasin B (1/100 dilution of stock solution); the other tube received 12.5 µl of HBSS, which was used as the negative control for each sample (calf). All samples were incubated for 10 minutes in the 37 C waterbath. A shift in fluorescence intensity was measured using a laser flow cell cytometer [a] emitting at 525 nm.

Phagocytosis of fluorescent beads

Cells obtained from the multi-procedure monocyte isolation (Appendices A, B, and C) were used to measure phagocytic uptake of fluorescent beads by a procedure (Appendix E) similar to that reported by Steinkamp et al., 1982.

Isolated monocytes were diluted in Krebs ringer bicarbonate-gelatin solution (KRH; 0.76% NaCl, 18.5 mM Hepes-Tea, 4.8 mM KCl, 2.4 mM $MgSO_4$, 0.1 gm gelatin) to a concentration of 1 x 10^6 cells/ml. A mixture of 0.5 ml of cells in KRH, 50 µl homologous sera and 10 µl of polystyrene beads[b] was diluted 1/10 in HBSS and then

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[a]Coulter Epics 752

[b]Fluoresbrite® beads, 1 micron, Polysciences, Warrington, PA

incubated for 1 hr in 50 ml round bottom tubes at 4 C and 37 C. The reaction was stopped with 5 ml phosphate buffer solution (PBS)-gelatin-EDTA (0.1 gm gelatin, 0.1 gm EDTA/100 ml PBS). Fluorescence was measured on a laser flow cell cytometer emitting at 525 nm. Phagocytic activity was correlated with mean channel fluorescent emission using the cold incubation (4 C) as the control for each sample (calf).

Statistical Analyses - All data were tested for normality and homogeneity of variance. Recovered monocyte cell populations were tested in triplicate. A mean value was derived resulting in one value per animal per treatment. Percent yield, cell viability, and phagocytic activity were compared between the two techniques by a paired t-test [c]. Total cell recovery from the immunomagnetic technique was too low to run both monocyte function tests, therefore H_2O_2 release was not determined on cells from the immunomagnetic technique.

Results

Total leukocyte counts for the six calves ranged from 3,221/µl to 6,983/µl (Table 1). The mixed monocyte/lymphocyte populations obtained, via the Ficoll-Hypaque density gradient separation phase of monocyte isolation were within a range of 3.7×10^7 to 9.4×10^7 cells. The mean number of cells recovered from the flask adherence technique was increased ($P < 0.001$) compared to the immunomagnetic technique. Percent recoveries from the flask adherence and immunomagnetic isolation techniques were 14.0 and 0.8%, respectively (Figure 3). Ninety-five percent of the recovered cells from both treatments were determined to be viable by trypan blue stain. Assessment of recovered cells, stained with monocyte-specific alpha-napthyl acetate, showed that the cell population consisted of

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[c]SAS Institutes Inc., Cary, NC, 1985

90% monocytes when recovered by way of immunomagnetic technique, and 65% monocytes when recovered by the flask adherence technique. These populations of cells were used to determine monocyte activity. Information on specific monocyte activity was collected using a flow cytometric gating technique. This technique allowed visualization of specific cell populations based on relative differences in the size and granularity of the cells as measured by forward scatter and side scatter, respectively (Figure 4).

Hydrogen peroxide (H_2O_2) release from monocytes recovered by the CF treatment showed a mean channel fluorescence range from 50 to 146 and percent of cells reacting to stimulation with cytochalasin B ranged from 10 to 30%. There was not a sufficient number of monocytes recovered from the immunomagnetic technique to run H_2O_2 release for this treatment group.

Phagocytic activity, measured as mean channel fluorescent emission, of monocytes stimulated at 37 C was different ($P < 0.01$) between treatment groups. An increase in fluorescence was observed from monocytes obtained via the flask adherence technique compared to the immunomagnetic technique. A difference between treatment groups was not observed when cells were incubated at 4 C. Treatment group means were not equal ($P < 0.001$) for percent of cells that phagocytosed fluorescent beads at both 4 C and 37 C (Figures 5 and 6). The mean percent of cells separated by the immunomagnetic technique that phagocytosed beads at 4 and 37 C was 12.5 and 14.3, respectively; the percent of cells that phagocytosed beads from the flask adherence at 4 and 37 C was 25.0 and 39.1, respectively. An increased number of monocytes were isolated from the flask adherence versus the immunomagnetic technique.

Discussion

It is necessary to have reliable methods for obtaining highly enriched populations of monocytes in order to study cellular interactions in the immune responses of cattle. The

methods developed for depletion and enrichment of human and mouse mononuclear cells (Mosier, 1967) have not been reproducible for monocyte isolation in the bovine. The isolation of mononuclear leukocytes from larger species is technically more limiting and cells appear to exhibit dissimilar properties in response to isolation protocols. Studies of macrophage populations from large animal species have been restricted to cells derived from bronchoalveolar lavages and mammary gland tissue.(Politis et al.,1991; Francey et al., 1992; Nash et al., 1992). The common isolation technique to obtain a homologous population of monocytes has been gradient separation and tissue culture flask incubation. A reproducible, inexpensive technique for isolation of a functional bovine monocyte population has yet to be reported.

Cells from laboratory and small animal species, have been separated by several techniques including gravity sedimentation, continuous and discontinuous density-gradient centrifugation, flotation, centrifugal elutriation, flow cytometry and, adherence to glass or plastic (Weiss et al, 1989). Commonly used density gradients include Percoll and Ficoll-Hypaque. Percoll is a preparation of colloidal silica particles coated with polyvinylpyrrolidone. Ficoll-Hypaque is a combination of a hydrophilic polymer of sucrose and a radioopaque contrast medium. Weiss et al; (1989) adapted a simple continuous Percoll density-gradient technique for isolation of granulocytes and mononuclear cells from dogs, cats, horses, and cattle. Cell purity was 100%, cell viability exceeded 95 %, and percent recovery of mononuclear cells approximated 70%. Cell function was not tested in this report. Separation with Ficoll-Hypaque solution (Dooley et al., 1982) has likewise resulted in highly purified mononuclear cell populations, but neutrophil viability and function were reported to be adversely affected by some aspect of this separation technique. Goddeeris et al; (1986) compared several different techniques for both enrichment of bovine monocytes and also their depletion from peripheral blood. Adherence to plasma-coated gelatin was the most efficient and reproducible method for enrichment of monocytes, whereas depletion of peripheral blood monocytes was best achieved by defibrination. Purity of the cell population was improved by fluorescence

activated cell sorter (FACS) with a monocyte-specific monoclonal antibody as well as by adherence to polystyrene flasks, but recovered monocytes did not respond to concanavalin A and phytohaemagglutinin unless gelatin/plasma monocytes were used. Monocyte recovery from the polystyrene flask adherence was greater than cell recovery from the Percoll method (64% and 33 %, respectively).

In the present study, initial mononuclear cell isolation was accomplished by density-gradient separation with Histopaque 1077®, a Ficoll-Hypaque preparation. This density gradient was chosen over Percoll because it was less labor intensive, and more conducive to processing multiple samples at one time. The mononuclear cell layer was easily visualized and aspirated from surrounding cell layers. Cell viability (95%) of aspirated monocytes was equal to that reported in the literature for Percoll density-gradient and various other monocyte separation techniques.

Purity of the cell population was further attempted by (i) immunomagnetic cell separation using the Biomag® preparation and (ii) by adherence to polystyrene culture flasks. Previous studies suggest that the combination of gradient separation and flask adherence has inherent disadvantages that restrict their use and hamper their routine application in the clinical laboratory. Low recovery rate of the desired cell population from the gradient interface, variable intervals for flask incubation, large margin of error associated with cell typing, recovery techniques that alter cell activity, and the time required for these procedures would decrease the reproducibility for isolation of monocytes from large animal species (Chinsakchai & Molitor, 1992; Yam et al., 1971; Hammer & Weber, 1974; Li et al., 1973). The immunomagnetic technique is based on cell selection of a desired population using magnetic beads that are coated with specific monoclonal antibody, to either positively or negatively select out monocytes from a mixed cell suspension. Theoretically, this technique could overcome many of the proposed disadvantages of flask adherence separation. A negative selection was employed in this study, which left monocytes suspended in solution while the lymphocytes were magnetically removed from

the cell suspension. Although the purity of the recovered monocyte population from the Biomag® technique was 90%; total monocyte recovery using this technique was significantly lower compared to the total monocytes recovered from flask adherence. These results differ from those reported previously (Goddeeris et al., 1986). Monocytes trapped between Moab-coated beads and pulled out of solution may have accounted for the low cell recovery. The cell suspension agitation and strong magnetic force may have disrupted monocyte cell structure , thereby decreasing the number of functional cells recovered.

Cell function of the recovered monocytes appeared to be dependent upon the separation technique employed. In general, monocytes recovered by flask adherence exhibited increased cell function compared to those recovered by the immunomagnetic technique. Although cold-shocking cells and then scrapping the flask surface has been thought to decrease cell function, that did not appear to be the case in this study. In contrast, magnetic bead separation appeared to be more traumatic to the desired cell population. Premature activation of monocytes by substrates used in the immunomagnetic technique may have affected the subsequent testing of monocyte cell function.

The time required to complete monocyte separation using the Biomag® technique was approximately 4 hr. In contrast, overnight cell culture was required to obtain monocytes from the flask adherence technique. Although a time-saving technique, the monoclonal antibodies necessary for immunomagnetic cell separation method are expensive. Considering cell yield, cell function and cost, Ficoll-Hypaque density-gradient in conjunction with flask adherence purification appears to be a reproducible, inexpensive method for isolation of bovine monocytes.

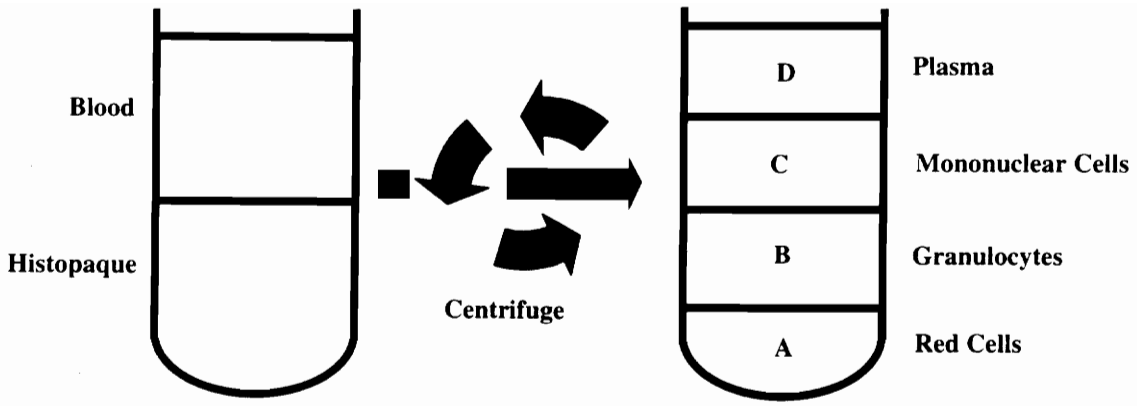

Figure 1. Schematic of Ficoll-Hypaque density gradient. After centrifugation cells are layered according to density, (**A**) red cells, (**B**) granulocytes, (**C**) mononuclear cells, (**D**) plasma. Mononuclear cell layer is gently aspirated off with a Pastuer pipette.

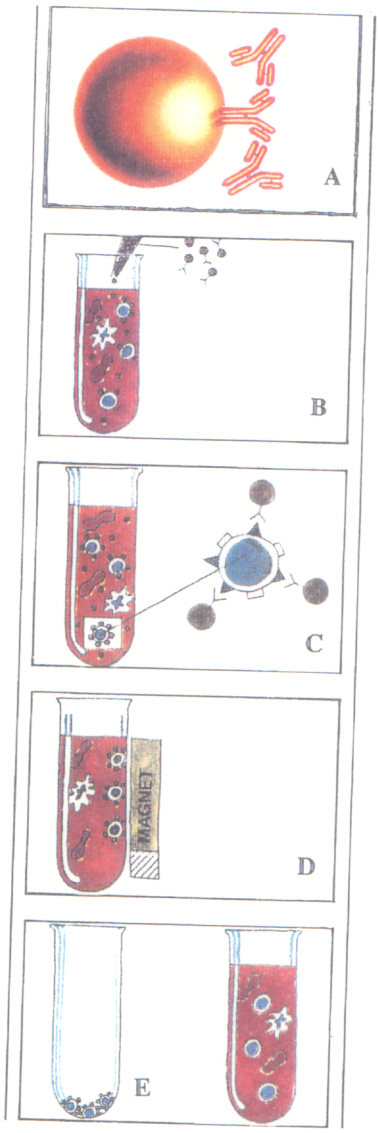

Figure 2. Schematic of immunomagnetic cell separation technique. (**A**) Magnetic beads coated wihtlymphocyte-specific monoclonal antibodies. (**B**) and (**C**) Bead-monoclonal antibody complex cultured with mixed leukocyte cell population to allow specific cell binding of lymphocytes to monoclonal antibodies. (**D**) Magnet affixed to culture vessel to magnetically remove lymphocytes from the cell suspension. (**E**) Target cells (monocytes) remain in suspension for easy retrieval.

Table 1. White blood cell[1] and monocyte counts[2] of study calves

Calf	WBC	Monocytes	
	cells/μl	cells/μl	%
B215	3,221	97	3.0
2B52	3,487	129	3.7
B223	4,021	169	4.2
B220	4,330	190	4.4
2B55	5,576	318	5.7
242	6,983	461	6.6

[1]Mean of triplicate Coulter counter readings.

[2]Derived from differential counts made on blood smears stained with Wright-Giemsa stain.

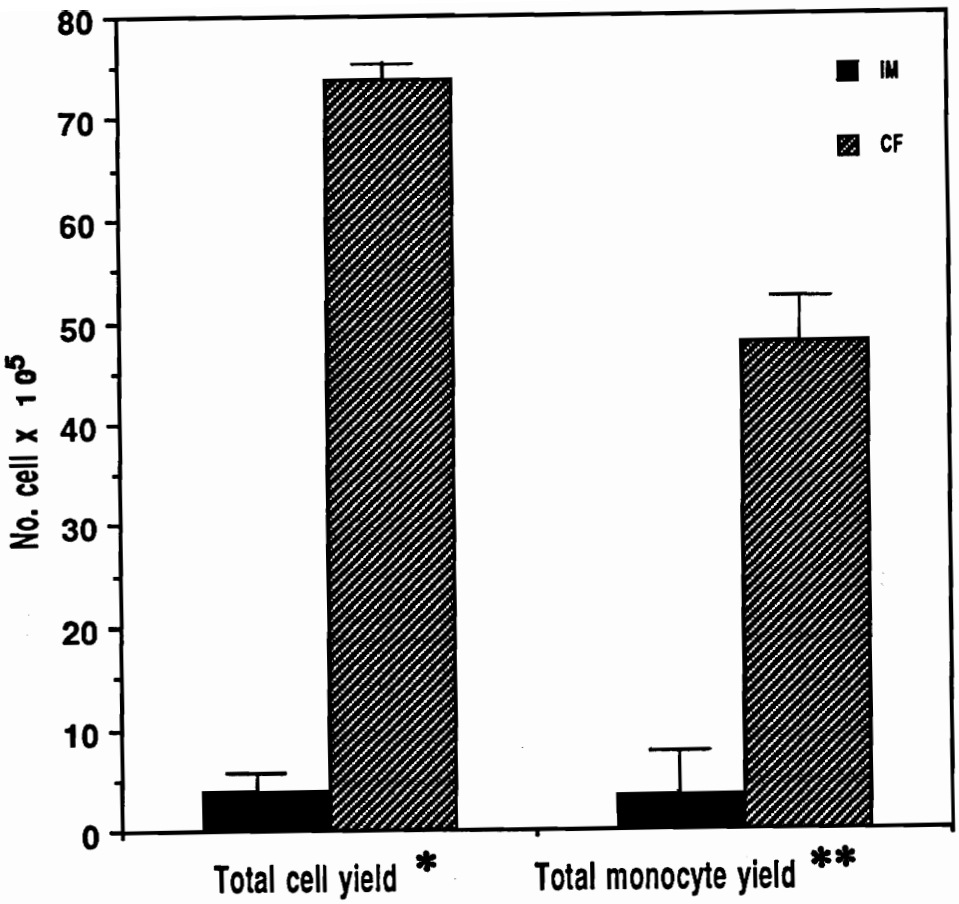

Figure 3. Cell yield from immunomagnetic (IM) and culture flask adherence (CF) techniques for bovine monocyte isolation Mononuclear cells were initially separated from whole blood via a Ficoll-Hypaque gradient density procedure; and further purified by immunomagnetic (IM) and culture flask adherence (CF) techniques (treatments). Total cell yield includes non-homogenous monocyte populations recovered from the IM and CF treatments. Monocyte yield is based on 90% homogenity of the recovered cell population for IM and 65% homogeneity for CF treatments as determined by alpha-napthyl acetate esterase staining. *Indicates treatment means are not equal ($P < 0.001$) based on paired t-test. **Treatment means not equal ($P < 0.01$).

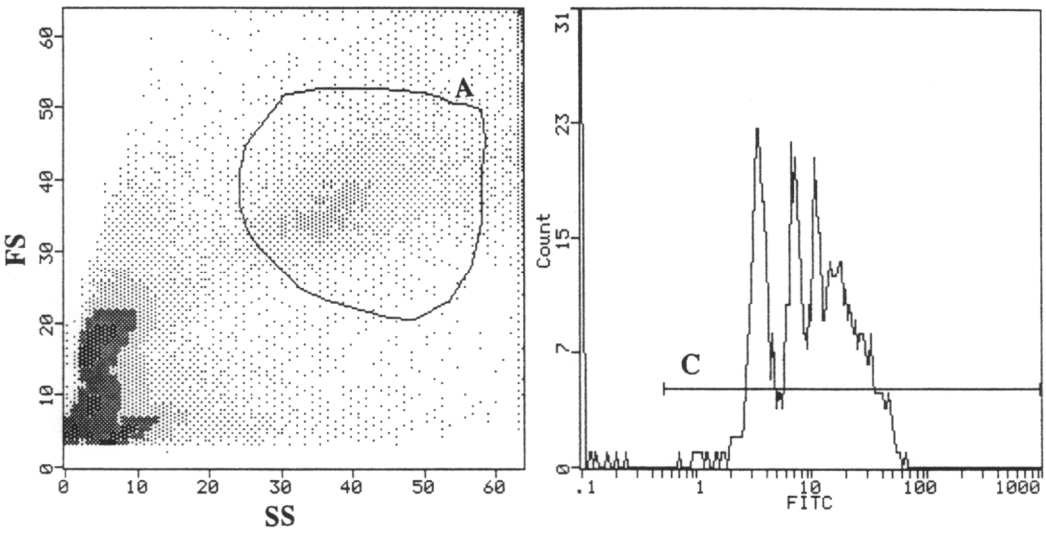

Figure 4. Histogram depicting the gating technique used to identify the monocyte cell population. The technique is based on forward and side scatter characteristics of the target cell population. (**F S**) Forward scatter measures cell size. (**S S**) Side scatter measures cell granularity. (**A**) Monocyte cell population. (C) Fluorescence measured on the gated monocyte population.

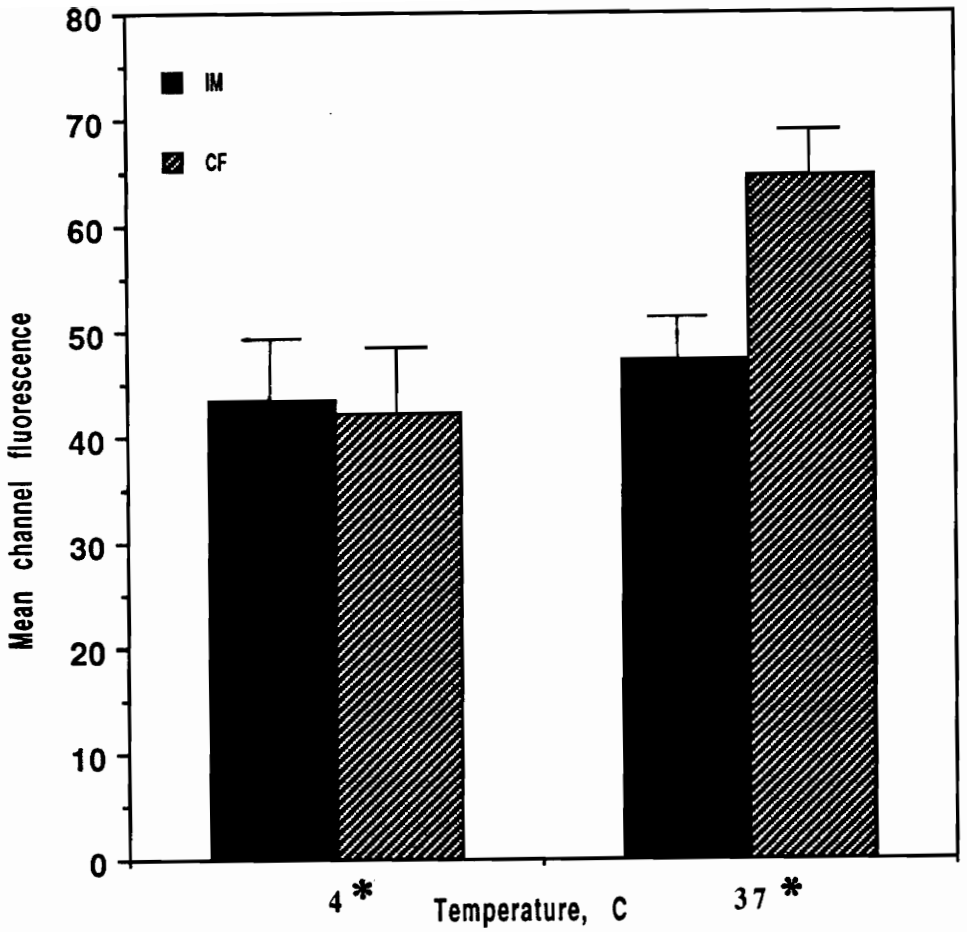

Figure 5. Phagoctyic activity of bovine monocytes recovered from immunomagnetic (IM) and culture flask adherence (CF) techniques. Mononuclear cells were initially separated from whole blood via a Ficoll-Hypaque gradient density procedure. Phagocytic activity is measured by mean channel fluorescent emission, where fluorescence is defined as channel number, channel 0-256. Columns depict the mean fluorescence (±SE) for monocytes incubated at 4 C and 37 C based on treatment (technique). *Treatment means are not equal $P < 0.01$.

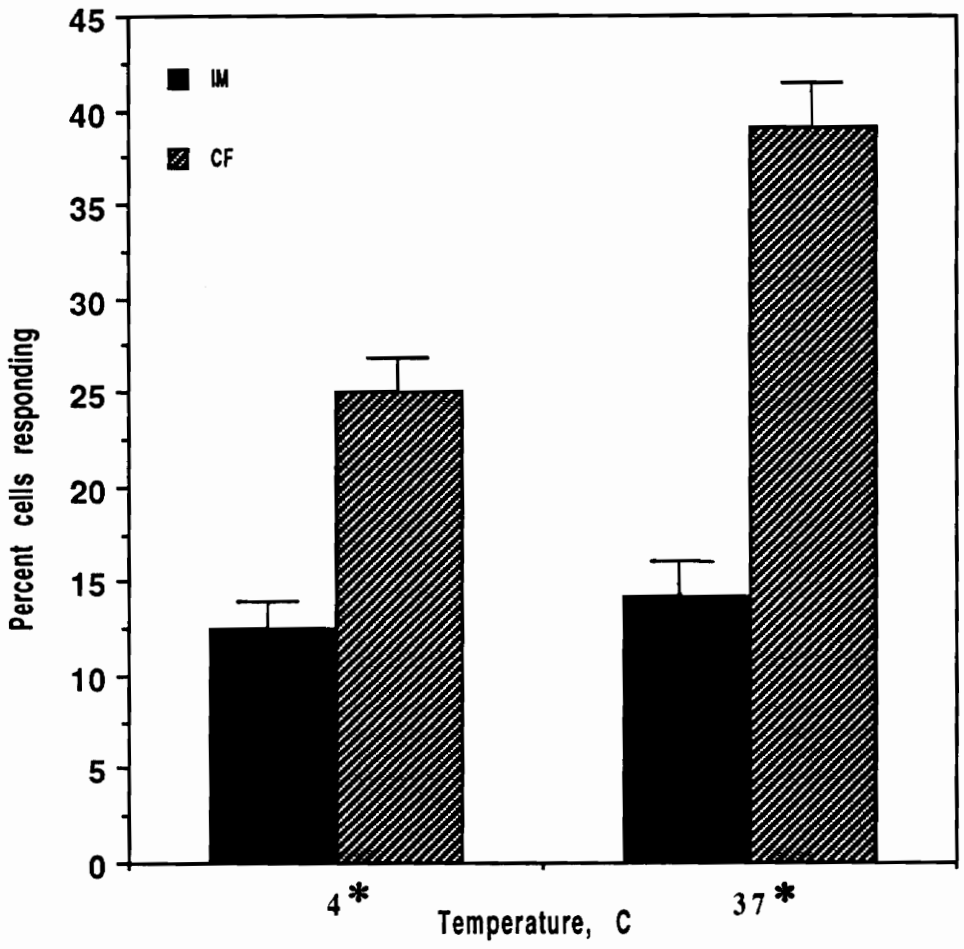

Figure 6. Percent of bovine monocytes recovered from immunomagnetic (IM) and culture flask adherence (CF) techniques that phagocytized fluorescent beads at 4 C and 37 C. Mononuclear cells were initially separated from whole blood via a Ficoll-Hypaque gradient density procedure; and further purified by the IM and CF techniques (treatments). Columns depict the mean percentage of cells (±SE) that phagocytized. *Treatment means are not equal $P < 0.001$.

Chapter IV

Copper Supplementation and Vaccination Effects on Copper Status and Immune Function in Beef Calves.

Part 1. Effect of Copper Supplementation on Copper Status and Selected Monocyte Functions in Beef Calves

Abstract

Effects of dietary copper (Cu) supplementation and vaccination on Cu status and monocyte function in growing beef calves were evaluated. Fifty-four weaned calves (mean initial weight 275 kg) were allotted by weight, breed, and gender to 1 of 3 treatment groups: no supplemental Cu (Control); Cu-Lysine (CuLy), and Cu-Sulfate ($CuSO_4$). Average daily intake of Cu for treatment groups was 31, 98 and 99 mg/hd/d, respectively, based on DM intake of 6.2 kg/hd/d. Nine heifers from each treatment group were vaccinated with *Brucella abortus* Strain 19 on d 37. Monocyte peroxide release and phagocytosis were assessed 3-5 d post-vaccination. Calves supplemented with CuLy had increased ($P < 0.05$) plasma Cu concentrations on d 84 and d 126 over control and $CuSO_4$-supplemented calves. Ceruloplasmin activity ($P < 0.05$) and hepatic Cu ($P < 0.001$) were lower in controls compared to Cu-supplemented calves. Cellular release of H_2O_2 by monocytes was greater ($P < 0.05$) in $CuSO_4$-supplemented calves as compared to controls on d 0. Hydrogen peroxide release measured in monocytes from CuLy supplemented calves was increased ($P < 0.10$) compared to the control group on d 42 and d 126. Monocytes from vaccinated calves had a higher ($P < 0.05$) H_2O_2 release and phagocytic response to stimulation compared to non-vaccinated calves. Body weights tended ($P < 0.10$) to be greater in the Cu-supplemented groups compared to controls during the 150 d study, with evidence of a breed effect ($P < 0.05$). These results indicate that Cu

supplementation, especially as a mineral amino chelate (CuLy) enhances monocyte activity, and may therefore enhance the non-specific defense mechanisms and the general immune response in growing beef calves.

Introduction

Adequate copper nutriture is required for normal function of the immune system. This is well recognized, especially with regards to the branch of the immune system referred to as acquired immunity. For example: impaired antibody production (Vyas & Chandra, 1983), T and B cell blastogenesis (Lukasewycz & Prohaska, 1983; Babu & Failla, 1989), and neutrophil killing ability (Niederman et al., 1994; Boyne & Arthur, 1981) have been associated with primary and conditioned Cu deficiency. In comparison, innate immunity, another branch of the immune system that refers to cell types and secretory products that provide protection against tumors and invading organisms, has received little attention with regards to Cu nutriture. The monocyte plays a key role in innate immunity as a phagocytic cell that presents antigens and secretes monokines essential for lymphocyte activation.

Copper in Monocyte Function

Copper has three specific roles in the function of the monocyte. The first would be as a component of superoxide dismutase (SOD), an inhibitor of superoxide anions generated from the oxidative burst that accompanies phagocytosis of an organism. Secondly, Cu is involved in the Haber-Weiss reaction that converts intracellular hydrogen peroxide to hydroxyl radicals. The third aspect of macrophage function involves the Cu-dependent enzyme, ceruloplasmin (ferroxidase), and its association with intracellular iron (Fe) in an antioxidant capacity. The oxidative state of intracellular Fe, which is dependent upon presence of ferroxidase, influences the fate of cellular metabolites such as hydrogen peroxide and the ability of monocytes to participate in perpetuating the cellular immune response (Baldwin et al., 1993). Any one of these aspects of monocyte function, and thus immune response, can be interrupted with alterations in Cu status of the animal.

Monitoring copper status

Accurate assessment of marginal Cu deficiency by use of conventional determinants such as blood Cu has been difficult. No clear delineation of laboratory values exists between the adequate, marginal and deficient ranges for blood and hepatic Cu. Physiological indicators, such as sub-optimal immune function measured as cell function impairment, could possibly assess Cu status more accurately and earlier as compared to conventional analysis of blood and hepatic Cu concentrations (Mills, 1981a). Early intervention of Cu deficiency in beef cattle can enhance health and reduce production costs associated with lowered immunocompetence.

Objectives

The purpose of this study was to evaluate the effects of dietary Cu changes on immune response / status in growing beef calves and compare organic versus inorganic forms of Cu supplementation. This was accomplished by:

(i) designing a Cu supplementation feeding trial for growing beef calves that compared non-supplemented, inorganic Cu, and organic Cu supplementation.

(ii) determining monocyte cell immune response at specific intervals in the Cu supplementation feeding trial.

(iii) assessing Cu status via conventional methods including plasma Cu, hepatic Cu, and Cu-dependent enzyme activity.

(iv) evaluating a stress component, that being routine vaccination with *B. abortus* Strain 19, in terms of dietary Cu supplementation.

(v) determining the effect of Cu supplementation on body weight and average daily gain.

Materials and Method

Animals, Facilities and Feed - Weaned Angus, Polled Hereford, and Limousin steer and heifer calves (mean body weight 275 kg) were housed in adjacent outside lots containing a fence-line feed bunk, automatic waterer, and free-choice white salt. Calves were fed once daily a Cu-deficient diet of corn silage and soybean meal (5 mg Cu / kg DM) for 4 wk preceding and during the 150 d study. Nutrient analysis of the ration fed to calves is presented in Table 1. Copper was incorporated into the mineral supplement [a] according to treatment protocol and was top-dressed daily in the ration at the rate of 113 g/hd/d. Composition of the mineral supplement is listed in Table 2.

Experimental Design and Methodologies - Fifty-four weaned beef calves were allotted by weight, breed and gender to 1 of 3 Cu-treatment groups for 150 d. Dietary treatment groups consisted of: 1) Control - no Cu supplementation , 2) Cu- Lysine (CuLy), and (3) Cu-Sulfate ($CuSO_4$). Mineral with added Cu was supplemented for treatment groups 2 and 3 to supply approximately 20 mg Cu/kg in the total diet. Copper content of the mineral supplement for each treatment group was: control - 0.35 mg/kg, $CuSO_4$ - 600 mg/kg, and Cu-Ly - 600 mg/kg. Composition of the mineral supplement is listed in Table 2. Blood was collected for plasma Cu and serum ceruloplasmin activity every 28 d.

The adjusted sample size (n=27) for monocyte testing was determined by way of a power calculation. Sample size to determine significance at the 0.05 level was calculated using data derived from bleeding on d 0 and relationships between standard deviation of the sample mean, and sample size. Hydrogen peroxide release of monocytes showed the greatest variance, therefore, sample size was adjusted based on the power calculation using data for H_2O_2 release.

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[a]Southern States Feed Co-op, Richmond, VA.

Heifers (age 9-11 mo) from each treatment group were vaccinated on d 37 with the standard dose of *B. abortus* Strain 19 to assess effects of vaccination stress. At the end of the 150 d study, liver was biopsied on a sub-sample (n=5) from each treatment group.

Measurement of blood Cu concentration - Blood was collected via venipuncture into heparinized Vacutainer® tubes and plasma was separated for Cu determination. Plasma samples were diluted three-fold with deionized water and analyzed with appropriate external standards by atomic absorption spectrophotometry.

Ceruloplasmin oxidase activity - The procedure used was a modification of the micro-method technique described by Smith et al, 1983. Briefly, p-phenylenediamine (ppd) substrate was prepared in 0.8 M sodium acetate buffer (32.8 g/L sodium acetate adjusted with acetic acid to pH 8.7). Reaction-inhibiting agent was subsequently prepared with the ppd substrate-buffer (45 mg sodium azide (NaN_3) / ml buffer). Standards were prepared using bovine ceruloplasmin[b]. Oxidase activity of the standard was 2600 units/ml and there were 102 units/mg of ceruloplasmin protein. This activity was used to calculate the amount required for preparation of the 2.00 mg/ml standard. All other standards (1.00 mg/ml, 0.50 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml) were made by diluting the 2.00 mg/ml concentration. Ninety-six well flat-bottom plates[c] were loaded with 20 µl of serum sample and standards in triplicate. The inhibiting agent (280 µl) was then added to blank wells and ppd substrate buffer (280 µl) to sample wells. Plates were incubated for 30 minutes at 37 C. Standard curves and ceruloplasmin oxidase values were calculated from a software program[d] integrated with a UV kinetic microplate reader set at 550 nm.

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[b]Sigma Chemical, St. Louis , MO

[c]ICN Bio Medicals, Costa Mesa, CA

[d]Softmax®Molecular Devices, Merlo Park, CA

Hydrogen peroxide release from stimulated monocytes using DCFH-DA - Peripheral monocytes were isolated from whole blood samples by Ficoll gradient separation (Goddeeris et al., 1986) and culture flask adherence techniques (Appendices A and C). Briefly, whole blood was layered over the Ficoll solution[e] and centrifuged at 2200 x g for 35 minutes. The white blood cell layer was removed, allocated to 75 cm^2 culture flasks[f], and cultured for 16 hr at 37 C, 5% CO_2 in complete media (RPMI 1640, 10 % fetal bovine serum, 1 mM Na pyruvate, 100 U penicillin, 100 µg streptomycin and 2 mM L-glutamine) for monocyte isolation. Adherent cells were recovered by washing flasks with ice-cold (4 C) Hanks balanced salt solution (HBSS) with 10 mM EDTA and gentle scraping. Recovered monocytes were tested for H_2O_2 release via a procedure (Appendix D) modified from Bass et al., 1983. Briefly, cells were diluted to a concentration of 1 x 10^6 cells/ml in HBSS and 1 µl/ml of 5 mM dichlorofluorescin diacetate (DCFH-DA) was added to all samples (final concentration of 5 mM DCFH-DA). Cells were incubated in a 37 C waterbath for 15 minutes. Samples were divided into two tubes containing 1 x 10^6 cells/ml. One tube received 12.5 µl of the stimulant, cytochalasin B (1/100 dilution of stock solution); the other tube received 12.5 µl of HBSS, which was used as the negative control for each sample (calf). All samples were incubated for 1minutes in the 37 C waterbath. A shift in fluorescence intensity was measured using a laser flow cell cytometer.[g]

Phagocytosis of fluorescent beads - Isolated monocytes were diluted to concentrations of 1 x 10^6 cells/ml in Krebs ringer bicarbonate-gelatin solution (KRH;

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[e]Histopaque 1077, Sigma Chemical, St. Louis , MO

[f]Falcon® Vented Tissue Culture Flask, Baxter Scientific Products, Charlotte, NC

[g]Coulter Epics 752

0.76% NaCl, 18.5 mM Hepes-Tea, 4.8 mM KCl, 2.4 mM $MgSO_4$, 0.1 gm gelatin) to test phagocytic activity. The procedure was modified from that reported by Steinkamp et al; (1982) and is outlined in detail in Appendix E. Briefly, a mixture of 0.5 ml of cells in KRH, 50 µl homologous sera and 10 µl of polystyrene beads[h] was diluted 1/10 in HBSS and then was incubated for 1 hr in 50 ml round bottom tubes at 4 C and 37 C. The reaction was stopped with 5 ml phosphate buffer solution (PBS)-gelatin-EDTA (0.1 gm gelatin, 0.1 gm EDTA/100 ml PBS). Fluorescence was measured using a laser flow cell cytometer set at 525 nm. Phagocytic activity was correlated with mean channel fluorescent emission using the cold incubation (4 C) as the control for each sample (calf).

Liver biopsies - Liver from 5 calves/group was biopsied at the end of the 150 d study (Pearson & Craig, 1980). Hepatic specimens averaged 1 g in weight and were stored frozen at -20 C until digested with nitric and perchloric acids. Samples were analyzed (wet basis) for Cu, S, Zn, Fe, and Mo by indirectly coupled plasma spectrometry.

Statistical Analysis - The general linear model procedure[i] was used to analyze the data. Animal was used as the experimental unit. The model included dietary treatment, vaccination status, breed, and all two-way interactions. Differences among treatment means ($P<0.05$) were separated by a Tukey test.

Results

Plasma Cu concentrations in calves (n=54) did not differ between treatment groups at the initiation of the study. At the end of the 5 month study, plasma Cu concentrations

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[h]Fluoresbrite® Beads, 1 micron, Polysciences, Warrington, PA

[i]Proc GLM, SAS Institutes Inc., Cary, NC, 1985

were increased ($P < 0.05$) in Cu-supplemented groups compared to the control group (Table 3). Within the subset of calves (n=27) chosen for immune response testing, plasma Cu concentration differed among treatment groups on days 42, 84 and 126. Plasma Cu was decreased ($P < 0.05$) on d 42 in the the non-supplemented calves compared to CuLy-treated calves. CuLy-supplemented calves had higher plasma Cu concentrations (Table 3) than both $CuSO_4$ and non-supplemented calves on days 84 ($P < 0.001$) and 126 ($P < 0.05$). Vaccinated calves (Figure 1) had increased plasma Cu concentrations compared to non-vaccinated calves on days 84 ($P < 0.05$) and 126 ($P < 0.01$). Vaccinated calves supplemented with CuLy tended to have increased plasma Cu concentrations compared to both $CuSO_4$ and non-supplemented calves (Figure 1).

Ceruloplasmin oxidase activity of calves (n=54) was higher ($P < 0.05$) in control calves versus Cu-supplemented calves at initiation of the study. On d 150, enzyme activity of Cu-supplemented calves was increased ($P < 0.05$) over controls. Enzyme activity for the subset (n=27) of calves differed ($P < 0.05$) on d 84 and 126 between control and Cu-supplemented calves (Table 3).

Hepatic Cu concentrations for control calves were decreased($P < 0.001$) compared to both CuLy and $CuSO_4$-supplemented calves (3.3, 38.3, 26.5 μg Cu/g tissue, wet basis, respectively).

Cellular release of H_2O_2 after stimulation with cytochalasin B was greater ($P < 0.05$) in Cu-supplemented calves compared to non-supplemented calves (Table 5). Hydrogen peroxide release from monocytes of CuLy-supplemented calves was increased ($P < 0.10$) on d 42 and 126 over that of non-supplemented calves. An interaction ($P < 0.10$) between dietary treatment and vaccination was found on d 42 and 126 (Figure 2). Effect of vaccination alone ($P < 0.05$) for H_2O_2 release from stimulated monocytes was evident on d 42 and 84 post-vaccination. Phagocytic activity of monocytes in the CuLy-supplemented calves was lower ($P < 0.05$) than both $CuSO_4$ and non-supplemented calves at the start of the study (Table 6). By d 84, CuLy-supplemented calves showed increased ($P < 0.05$) monocyte phagocytosis compared to control and

$CuSO_4$-supplemented calves. A vaccination effect was demonstrated by d 42 post-vaccination. Monocytes from vaccinated calves showed increased ($P < 0.05$) phagocytic activity over monocytes from non-vaccinated calves (Figure 3). Vaccinated calves fed CuLy supplement had greater ($P < 0.10$) monocyte peroxide release and phagocytic activity than non-supplemented calves. Monocyte phagocytic activity of vaccinated calves supplemented with$CuSO_4$ was variable. An interaction between dietary treatment and vaccination was evident by d 84 ($P < 0.10$) and significant ($P < 0.05$) by d 126. The total number of monocytes responding to *in vitro* stimulation was increased ($P < 0.05$) in CuLy-supplemented calves by d 42 after vaccination stress compared to control and $CuSO_4$-supplemented calves (Table 7). The number of monocytes responding to stimulation tended to be higher in the Cu-supplemented groups compared to controls on d 84 and 126.

Body weights did not differ among dietary treatment groups by d 150 of the study, however, a breed effect ($P < 0.05$) was observed with Angus and crossbred calves consistently weighing heavier than controls (Tables 8&9).

Discussion

Earlier studies support a relationship between Cu status and disease susceptibility, indicating that Cu deficiency may compromise host defense systems (Orr et al., 1990; Eng, 1993). Copper deficiency impairs ability of the monocyte to generate a normal respiratory burst and alters phagocytic ability of the cell (Babu & Failla, 1990; Lilley et al., 1985). However, the effect of Cu-supplementation on monocyte function in response to vaccination stress in cattle has not been evaluated.

In the present study, plasma Cu concentrations of control calves were lower than that of Cu-supplemented calves 5, 47, and 113 d after vaccination with *B. abortus* S19. Calves supplemented with CuLy maintained higher plasma Cu concentrations than $CuSO_4$-

supplemented and control calves 47 and 89 d after vaccination stress. Nockels and co-workers (1993) reported similar results from a mineral balance trial comparing CuLy and $CuSO_4$ absorption and retention post ACTH stress. Negative Cu retention during ACTH-induced stress was reversed by dietary Cu repletion. In their study, calves fed CuLy had 53% greater apparent Cu absorption and increased Cu retention during repletion compared with calves fed $CuSO_4$. Increased plasma Cu after vaccination in CuLy-supplemented calves suggested differences in bioavailability between the two Cu sources fed in this study. In lactating beef cattle, supplemental Cu from Cu proteinate resulted in the highest liver Cu concentrations, intermediate concentrations were detected with $CuSO_4$ and the lowest concentrations were found with supplemental Cu oxide (Clark et al., 1993). A study comparing relative Cu bioavailability from different Cu sources for growing cattle showed $CuSO_4$ and CuLy were similar in bioavailability, but Cu oxide was essentially unavailable (Kegley & Spears, 1994). A severe snow storm on d 82-90 of my study, would be considered an environmental stress, and might account for decreased plasma Cu concentrations on d 84.

Klasing et al; (1993) reported that stress increases blood Cu and decreases liver Cu, while absorption and excretion remain constant. After stress, Cu requirements of the animal are increased. Acute phase proteins, such as ceruloplasmin, change mineral metabolism by increasing the demand for minerals during early phases of the immune response. Ceruloplasmin activity for control calves was higher than both Cu-supplemented groups at the start of the study but dropped below supplemented calves after vaccination. This would suggest that either control caves absorbed less Cu during the study period or Cu stores were depleted in vaccinated control calves during immune system challenge and unavailable for incorporation into the acute phase protein, ceruloplasmin. All calves were handled identically throughout the study, therefore, the initial difference between control and Cu-supplemented groups on d 0 would most likely be attributed to individual animal variation. Stress associated with vaccination and ambient temperature changes can account

for the lowered ceruloplasmin activity on d 42 and 84 while, cessation of these stressors may have accounted for the gradual increase of enzyme activity at bleeding intervals thereafter.

As previously stated, Cu plays an indirect role in monocyte function by way of the Cu-dependent enzyme, ceruloplasmin. One of the many proposed functions of ceruloplasmin includes serum antioxidation, in which it acts as a scavenger of free radicals and superoxide ion. The oxidation of ferrous iron leads to superoxide ion formation within the monocyte. This in turn can lead to peroxidative damage. Ceruloplasmin, through its ferroxidase activity, can catalyze the oxidation of Fe^{2+} with concomitant production of water and oxygen, lowering the likelihood of superoxide generation by Fe^{2+} (Cousins, 1985). Phagocytosis of organisms by the monocyte results in cell activation and the cascade of oxidation products. Calves in this study were vaccinated with *B. abortus* Strain 19, a gram-negative, intracellular bacterium that can be phagocytosed and replicate within the monocyte. The decrease in ceruloplasmin activity after vaccination may be a consequence of increased utilization as an antioxidant by monocytes.

The primary focus of Cu deficiency on immune response has been associated with acquired immunity, that is, decreased antibody production and impaired blastogenic response to T and B cell mitogens (Vyas & Chandra, 1983; Prohaska & Lukasewycz, 1981; Koller et al., 1987). Monocytes mainly function in innate immunity, an alternate branch of the immune system, as phagocytic cells, antigen presentors, and secretory cells. Fully activated macrophages have tumoricidal properties, whereas macrophages that are partially activated possess a greater ability to ingest particles and generate superoxide radicals than resident cells (Cohn, 1978). The Cu content of the macrophage reflects the Cu status of the diet (Carpenter et al., 1986). Decreased cellular concentrations of Cu affect monocyte function (Babu & Failla, 1990; Carville & Strain, 1988). When exposed to a stimulus, monocytes are activated and produce reactive oxygen species referred to as the respiratory burst. The burst can be measured as H_2O_2 release. In our study, Cu-

supplemented calves had increased H_2O_2 release from stimulated monocytes compared to non-supplemented calves. Stress from vaccination additionally increased monocyte response, with CuLy-supplemented calves showing an increased H_2O_2 release from monocytes over control calves.

An indirect influence of supplemental Cu on oxidative capacity of the monocyte is associated with intracellular relationships of iron and *B. abortus* vaccination. Iron is needed by bacteria for intracellular growth (Graham, 1991). Brucella, by using their siderophores to chelate Fe, can deplete the intracytoplasmic Fe pool of macrophagesto a level below that needed for catalysing the generation of hydroxyl radicals (Baldwin et al., 1993). In addition, gamma interferon, a cytokine involved in monocyte activation, has been reported to decrease transferrin receptors on the monocyte and thus lower intracellular Fe concentrations (Byrd & Horwitz, 1989). The decrease in cell number and mean channel fluorescence of monocytes from calves in the control group, especially after vaccination, may be a consequence of intracelluar Fe depletion leading to decreased monocyte oxidative function. Dietary Cu supplementation, particularly in the CuLy form, potentially increased the Cu available for cellular functions. Subsequently Cu could have been utilized as a catylst in the Haber-Weiss reaction in lieu of Fe, and thus potentiate the monocyte oxidative burst in response to *B. abortus* vaccination.

Decreased phagocytosis and candidacidal activity both have been observed in bovine neutrophils (Boyne & Arthur, 1981; 1986). However, macrophages obtained from Cu-deficient rats demonstrated adequate phagocytosis of foreign organisms but a decreased intracellular killing ability compared to macrophages from Cu-adequate control rats (Babu & Failla, 1990). Microbicidal activity depends upon a free radical-mediated process; H_2O_2 release from cells will decline prior to their phagocytic activity. In the present study, phagocytosis of fluorescent beads by monocytes from control calves was decreased compared to supplemented calves. Monocytes from CuLy-supplemented calves phagocytized more beads than the $CuSO_4$ group suggesting that Cu was more available for cellular functions when fed in the proteinate form. The data suggest that cellular Cu was

depleted in non-supplemented calves and resulted in diminished monocyte function.

Copper deficiency, associated with a low level of plasma ceruloplasmin, leads to decreased antimicrobial activity of phagocytes (Boyne & Arthur, 1981) and decreased activity of natural killer cells (Koller et al., 1987). Conversely, Cu deficiency can also be associated with increased acute and delayed inflammatory responses. These observations suggest that ceruloplasmin has immunomodulating activity and is able to normalize immune response of target cells. This regulatory mechanism of cerulpolasmin is thought to be associated with membrane binding. Receptors for ceruloplasmin have been identified on the surface of red blood cells (Barnes & Frieden, 1984) and white blood cells, including monocytes (Kataoka & Tavassoli, 1985). Ceruloplasmin enhances monocyte phagocytosis and oxidative metabolism by binding to the cell membrane and, at the same time may protect cells and tissues against harmful oxygen radicals released during cell activation (Saenko et al., 1994). The decrease in phagocytic activity exhibited by monocytes in the control calves may have been, in part, a consequence of altered Cu status as indicated by both lowered ceruloplasmin and plasma Cu concentrations. Decreased ceruloplasmin activity would have resulted in decreased membrane binding and a dampened phagocytic ability of the monocyte Whether plasma Cu concentration can directly influence the number and/or function of ceruloplasmin membrane receptors has not be investigated.

Plasma Cu, although significantly lower in non-supplemented calves in our study was still considered within the marginal reference range (0.4 to 0.7 μg/ml). Hepatic Cu concentrations of 3.3 μg Cu/g tissue (wet basis) in the control calves is considered adequate by some investigators (Smith & Coup, 1973) yet deficient by others (Puls, 1990). Thus, the delineation between marginal and deficient is still ill-defined in the literature. In this study, low hepatic Cu stores, along with dietary Cu intake below NRC recommendations for beef cattle (8-10 mg/kg), suggests a marginal deficiency state for calves in the control group (NRC, 1984). Assessment of Cu status by use of conventional measurements such as blood Cu is often inaccurate. Hepatic samples for Cu determination are considered invasive and more cumbersome to obtain, although they are very sensitive

measures of Cu status. In rats fed a 55 % restricted Cu diet, hepatic and blood Cu concentrations were adequate to marginal yet, monocyte activity was significantly lower than that of controls (Babu & Failla, 1990). Results of our study are in agreement with these findings and both studies support Mills' proposal that phagocytic cell function may provide a sensitive indicator of Cu status (Mills, 1987).

The present study demonstrated that H_2O_2 release and phagocytic activity of monocytes obtained from calves stressed by vaccination are enhanced by supplementing Cu at twice that recommended by NRC for growing beef calves. Copper may be more available for celluar functions when supplemented as CuLy than as $CuSO_4$. Dietary Cu supplementation optimizes immune cell function and, therefore, may enhance disease resistance in growing beef calves.

The differences observed between vaccinated and non-vaccinated calves, as well as the interaction between diet and vaccination (Figures 1, 2 & 3) at specific bleeding intervals, suggests a possible protective role for Cu during periods of production-related stress. Differences observed between vaccinated and non-vaccinated groups may be masked by gender, as studies report a sexual dimorphism in regard to immune response (Grossman, 1985). These findings will be covered in further detail in the discussion section of Chapter IV, Part 2. To substantiate these findings further, a gender-specific vaccination study was designed and described in Part 2.

Table 1. Nutrient composition of basal diet[1] fed to beef calves

Item	Nutrient concentration (DM basis)
	%
Crude protein	9.3
Acid detergent fiber	22.2
Neutral detergent fiber	37.5
Calcium	0.18
Phosphorus	0.25
Potassium	1.08
Magnesium	0.15
NE_m	1.61 Mcal/kg
NE_g	1.08 Mcal/kg
	mg/kg
Copper	5.0
Molybdenum	1.2
Zinc	26.0
Manganese	39.0
Iron	170.0

[1]Diet consisted of corn silage fed ad libitum plus 0.7 kg soybean meal/hd/d.

Table 2. Composition of mineral supplement fed to beef calves[1]

Mineral	Concentration
	%
Calcium	21.2
Phosphorus	6.0
Sodium	6.6
Chloride	10.3
Potassium	1.0
Magnesium	1.5
Sulfur	1.5
Iron	0.139
	mg/kg
Copper[2]	0.35
Iodine	35.00
Cobalt	16.00
Selenium	52.00
Zinc	160.00
Manganese	151.00

[1]Mineral top-dressed daily at 113 g/hd/d.

[2]$CuSO_4$ and CuLy treatments contained 600 mg Cu/kg.

Table 3. Effect of Cu treatment on plasma Cu concentration of growing beef calves[1].

	Plasma Cu (μg/ml)				
Treatment	Day 0	Day 42	Day 84	Day 126	Day 150
Control[2]	0.80	0.62[a]	0.43[c]	0.59[a]	0.69[a]
$CuSO_4$[3]	0.78	0.69[ab]	0.54[d]	0.59[a]	0.78[b]
CuLy[4]	0.77	0.73[b]	0.64[e]	0.68[b]	0.78[b]
SEM	0.03	0.02	0.02	0.02	0.02

[1]Values are treatment means for n=18 calves/treatment on d 0 and150; n=9 calves/treatment on d 42, 84 and 126. Heifers were vaccinated on d 37.

[2]Mineral mix with no Cu added.

[3]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[4]Cu supplied as CuLy (600 mg Cu/kg) in mineral mix.

[a,b]Concentrations within each column with different superscripts differ ($P < 0.05$).

[c,d,e]Concentrations within each column with different superscripts differ ($P < 0.001$).

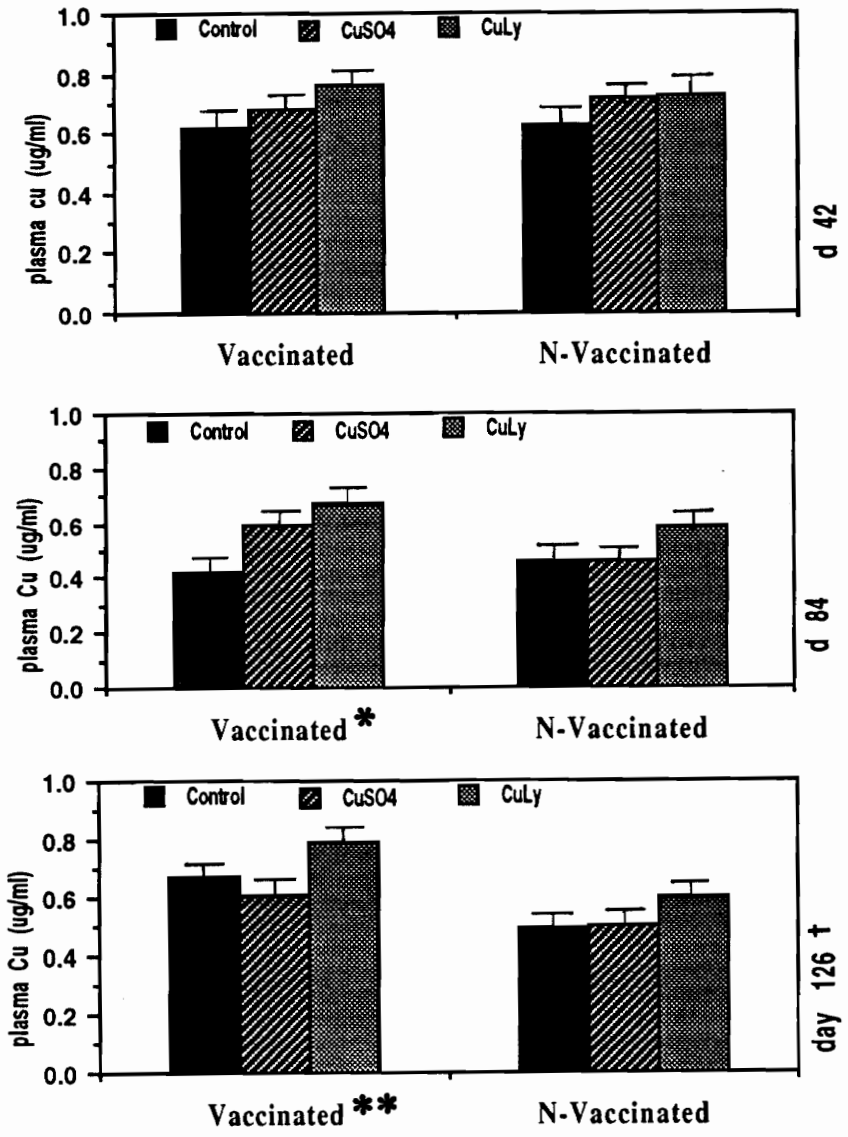

Figure 1. Plasma Cu concentrations for *B. abortus* Strain 19 Vaccinated and (non) N-Vaccinated control, $CuSO_4$-supplemented and CuLy-supplemented beef calves on d 42, 84 and 126. Vaccination was administered on d 37 of 150-d study. Columns depict the mean value for treatment groups based on supplement and vaccination. Significant difference *($P < 0.05$), **($P < 0.01$) between Vaccinated and N-Vaccinated within day. †Represents an interaction ($P < 0.10$) between supplement and vaccination.

Table 4. Effect of Cu treatment on serum ceruloplasmin activity of growing beef calves[1]

	Ceruloplasmin oxidase activity (mg/ml)[2]				
Treatment	Day 0	Day 42	Day 84	Day 126	Day 150
None[3]	0.57[a]	0.24	0.24[a]	0.26[a]	0.27[a]
$CuSO_4$ [4]	0.40[b]	0.36	0.36[b]	0.35[b]	0.37[b]
CuLy[5]	0.44[b]	0.34	0.35[b]	0.36[b]	0.40[b]
SEM	0.02	0.03	0.02	0.02	0.02

[1]Values are least square means of n=18 calves/treatment on d 0 and 150; n=9 calves/treatment on d 42, 84 and 126. Heifers were vaccinated on d 37.

[2] 1 mg of ceruloplasmin protein = 102 units of ceruloplasmin oxidase activity.

[3]Mineral mix with no Cu added.

[4]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[5]Cu supplied as CuLy (600 mg Cu/kg) in mineral mix.

[a,b]Concentrations within each column with different superscripts differ ($P < 0.05$).

Table 5. Effect of Cu treatment on monocyte hydrogen peroxide release in growing beef calves.[1]

	Mean channel fluorescent emission [2]			
Treatment	Day 0	Day 42	Day 84	Day 126
None[3]	87[a]	66[c]	108	105[c]
$CuSO_4$ [4]	124[b]	81[cd]	104	110[cd]
CuLy[5]	102[ab]	94[d]	112	126[d]
SEM	8.2	8.8	4.1	6.1

[1]Values are treatment means of n=9 calves/treatment. Heifers were vaccinated on d 37.

[2]Mean fluorescence of the responding cells, where fluorescence is defined as channel number; channel range 0 to 256.

[3]Mineral mix with no Cu added.

[4]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[5]Cu supplied a CuLy (600 mg Cu/kg) in mineral mix.

[a,b]Concentrations within each column with different superscripts differ ($P < 0.05$).

[c,d]Concentrations within each column with different superscripts differ ($P < 0.10$).

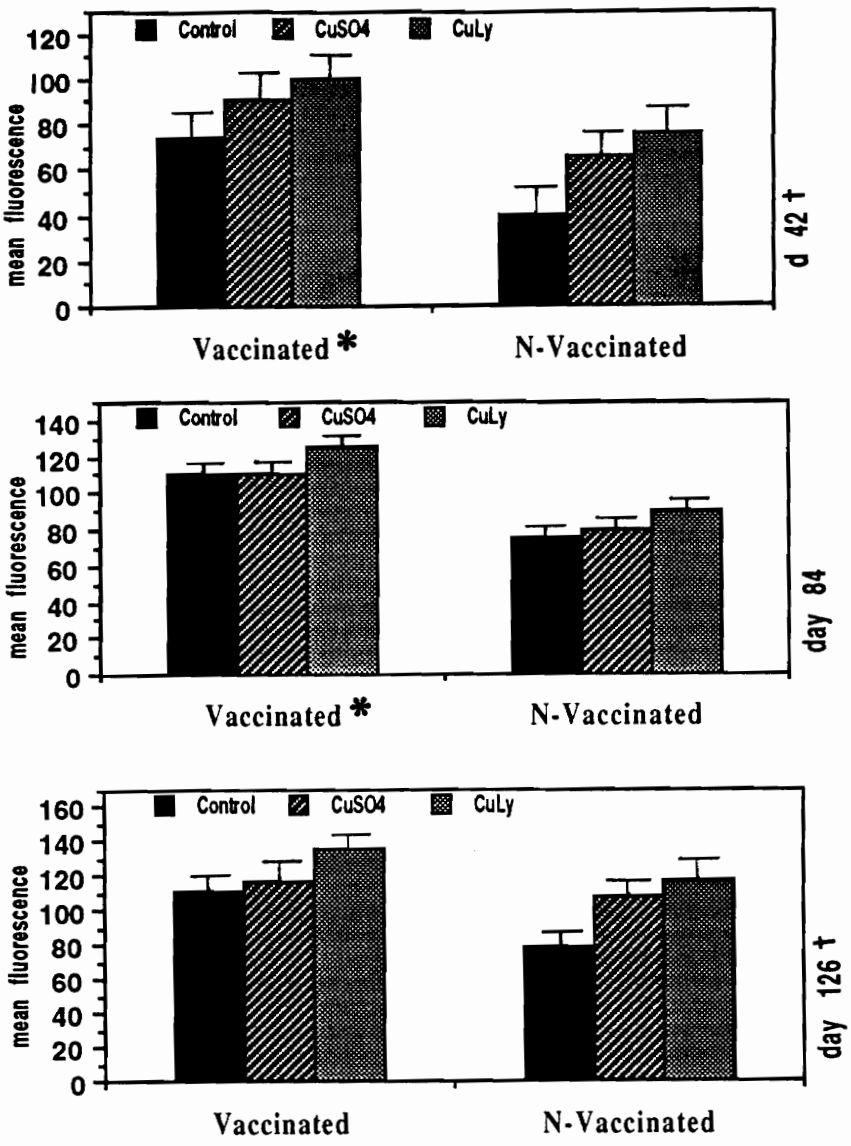

Figure 2. Hydrogen peroxide release for *B. abortus* Strain 19 Vaccinated and (non) N-Vaccinated control, $CuSO_4$-supplemented and CuLy-supplemented beef calves on d 42, 84 and 126. Vaccination was administered on d 37 of 150 d study. Columns depict the mean fluorescence for treatment groups based on supplement and vaccination, where fluorescence is defined as channel number; channel range 0-256. *Significant difference ($P < 0.05$) between Vaccinated and N-Vaccinated within day. †Represents an interaction ($P < 0.10$) between supplement and vaccination.

Table 6. Effect of Cu treatment on phagocytic activity of monocytes in beef calves[1]

	Mean channel fluorescent emission[2]			
Treatment	Day 0	Day 42	Day 84	Day 126
Control[3]	35[a]	16	6[a]	20[c]
$CuSO_4$[4]	27[a]	10	14[ab]	27[cd]
CuLy[5]	9[b]	18	20[b]	32[d]
SEM	3.8	6.0	2.4	5.5

[1]Values are treatment means for n=9 calves/treatment. Heifers were vaccinated on d 37

[2]Mean fluorescence of the responding cells, where fluorescence is defined as channel number; channel range 0 to 256.

[3]Mineral mix with no Cu added.

[4]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[5]Cu supplied as CuLy (600 mg Cu/kg) in mineral mix.

[a,b]Concentrations within each column with different superscripts differ ($P < 0.05$).

[c,d]Concentrations within each column with different superscripts differ ($P < 0.10$).

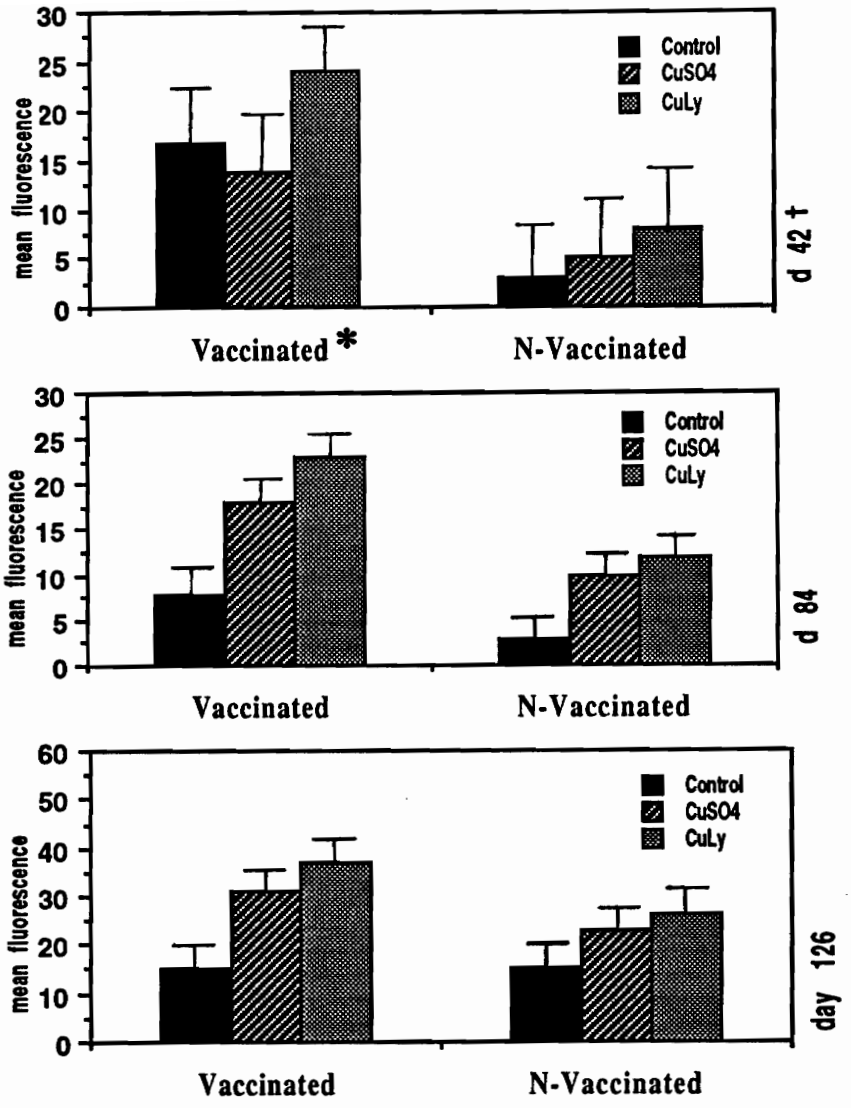

Figure 3. Phagocytosis of fluorescent beads for *B. abortus* Strain 19 Vaccinated and (non) N-Vaccinated control, $CuSO_4$-supplemented and CuLy-supplemented beef calves on d 42, 84 and 126. Vaccination was administered on d 37 of 150 d study. Columns depict the mean fluorescence for treatment groups based on supplement and vaccination, where fluorescence is defined as channel number; channel range 0-256. *Significant difference ($P < 0.05$) between Vaccinated and N-Vaccinated within day. †Represents an interaction ($P < 0.10$) between supplement and vaccination.

Table 7. Effect of Cu treatment on number of monocytes responding to *in vitro* stimulation in beef calves[1]

	Total monocytes[2]			
Treatment	Day 0	Day 42	Day 84	Day 126
None[3]	11.6	5.5[a]	4.8[a]	7.9[c]
$CuSO_4$[4]	8.0	6.4[a]	9.5[b]	9.2[cd]
CuLy[5]	9.5	12.3[b]	11.3[b]	12.0[d]
SEM	1.09	0.88	0.85	1.15

[1]Values are means of n=9 calves/treatment. Heifers were vaccinated on d 37.

[2]Number of monocytes responding to *in vitro* stimulation via cytochalasin B and fluorescent beads in 100 cells.

[3]Mineral mix with no Cu added.

[4]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[5]Cu supplied a CuLy (600 mg Cu/kg) in mineral mix.

[a,b]Numbers within each column with different superscripts differ ($P < 0.05$).

[c,d]Numbers within each column with different superscripts differ ($P < 0.10$).

Table 8. Mean body weight[1] by breed of calves fed Cu-supplemented and non-supplemented diets for 5 months

	Body weight (kg) ± SEM		
Breed	Day 1	Day 42	Day 150
Angus[a]	284± 5.7	319± 6.3	384± 7.3
Crossbred[a]	284± 7.2	314± 7.9	379± 9.2
Limousin[b]	226±11.3	259±12.6	320±14.5
P. Hereford[b]	249± 9.3	277±10.3	341±11.9

[1]Values are least square means.

[a,b]Weights within each column with different superscripts differ ($P < 0.05$).

Table 9. Body weights[1] of various beef breeds fed three copper treatments[2]

Breed		Angus	Crossbred	Limousin	Polled Hereford
	Treatment		Body weight (kg)		
DAY1					
	Control[3]	283	290	220	246
	$CuSO_4$[4]	284	276	233	255
	CuLy[5]	284	287	225	247
DAY 42					
	Control	312	313	250	271
	$CuSO_4$	316	308	270	280
	CuLy	329	321	258	281
DAY 150					
	Control	374	379	303	335
	$CuSO_4$	384	369	339	338
	CuLy	395	391	319	352

[1]Values are least square means.

[2]Treatments consisted of mineral mix supplemented to basal ration of corn silage and soybean meal.

[3]Mineral mix with no Cu added.

[4]Cu supplied as $CuSO_4$ in mineral mix.

[5]Cu supplied as CuLy in mineral mix.

Chapter IV

Copper Supplementation and Vaccination Effects on Copper Status and Immune Function in Beef Calves.

Part 2. *Brucella abortus*-Stimulated Monocyte Response in Vaccination-Stressed Heifer Beef Calves Supplemented with Dietary Copper

Abstract

This 60-d study was designed to evaluate the effect of vaccination on monocyte response in Cu-supplemented heifers. Fourteen weaned beef heifers (mean weight 250 kg) were supplemented with either Cu-Lysine (CuLy) or Cu-Sulfate ($CuSO_4$) and vaccinated with *B. abortus* Strain 19 on d 30 or d 60. Monocytes were collected on d 0, 35 and 65 and tested for oxidative burst (H_2O_2 release) and phagocytic activity using immunofluorescent techniques. Time of vaccination influenced ($P < 0.001$) monocyte phagocytosis and H_2O_2 release. Heifers vaccinated on d 30 had increased ($P < 0.05$) H_2O_2 release by monocytes compared to non-vaccinated heifers at that time period; monocytes from heifers vaccinated on d 60 had increased ($P < 0.05$) H_2O_2 release compared to the non-vaccinated group on d 60. Monocyte activity on d 30 was increased over d 0 activity for both dietary treatment groups. Heifers vaccinated on d 30 showed a decrease in monocyte H_2O_2 release from d 30 to d 60. Phagocytic activity of monocytes increased from d 30 to d 60 in all groups except the CuLy-supplemented heifers vaccinated on d 30. A decrease in monocyte H_2O_2 release on d 60 was observed in both CuLy-supplemented groups. A time-by-vaccination interaction was evident for monocyte H_2O_2 release ($P < 0.001$) and phagocytosis ($P < 0.10$). The results of this study: (i) indicated that vaccination with *B. abortus* Strain 19 stimulates monocyte activation and phagocytosis in beef heifers and (ii) helped to substantiate the vaccination effects reported in the previous study where gender differences may have masked monocyte response to vaccination.

Introduction

Researchers observed that interactions between immune response and reproduction existed as early as 1898 and that these interactions were hormonally regulated. Grossman (1985) has summarized the supportive evidence derived from clinical and experimental studies regarding gonadal steroid regulation of immune function. Immune studies indicated that (i) a sexual dimorphism exists in the immune response (IR), (ii) IR is altered by gonadectomy and sex steroid hormone replacement, (iii) IR is altered during pregnancy, and (iv) organs responsible for the IR contain receptors for gonadal steroids.

Sex Steroids and Immunity

Circulating levels of estrogen, androgens, and progesterone can affect immune system function and vice versa. These interactions are mediated through the hypothalmic-pituitary-gonadal-thymic axis, and depend on pituitary luteinizing hormone. Numerous studies indicate humoral and cell-mediated immunity are affected by sex steroid hormones. Females showed higher titers of IgG, IgM and IgA to various antigen challenges compared to males. Estradiol depressed suppressor and cytolytic T cell activity in humans and laboratory animals, often resulting in increased mortality. Castrated animals did not exhibit this immune dysfunction suggesting that the immunodepressive effects of circulating estrogens are negated by castrations and immune response is enhanced (Kittas & Henry, 1980; Wira & Sandoe, 1980; Grossman, 1985).

Cultured lymphocytes from castrated and sex hormone-treated rats showed similar responses for T lymphocyte mitogen response and DNA synthesis. However, serum from castrated rats, when added to T lymphocyte cultures, showed a marked stimulatory effect over estradiol (Grossman, 1985). An increase in the mass of peripheral lymphocytes and thymus tissue has been associated with castration (Chiode, 1940). Castrated animals undergo structural alterations of thymic tissue that appear to be regulated by sex hormone receptors located both in thymic tissue and, controversially, in thymic lymphocytes

(Grossman, 1985; Pearce et al., 1981; Danel et al., 1983).

Phagocytic cell function is likewise regulated by sex hormones. Activated macrophages from pregnant mice were shown to be less bacteriocidal for *Toxoplasma gondii* (Luft & Remington, 1984). Macrophage-regulated resistance to infection and / or vaccination with *B. abortus* is altered in pregnant cattle (Crawford et al., 1988;1991; Winter et al., 1986; Nicoletti, 1977; Berman et al., 1954; Alton et al., 1975). Certain pathogens, particularly intracellular organisms, are known to induce more severe disease in pregnant females because a non-specific depression of the IR occurs during pregnancy and this most likely contributes to diminished resistance to antigen challenge (Weinberg, 1984).

Objectives

In the previous 5 month study, selected immune cell functions were evaluated in beef calves vaccinated with *B. abortus* Strain 19 in conjunction with dietary Cu supplementation. Differences in monocyte activity were observed between vaccinated and non-vaccinated calves and, within these groups a diet-by-vaccination effect was evident. The vaccinated group consisted of heifer calves and the non-vaccinated group was exclusively steers, therefore the magnitude of immune response to vaccination, with respect to dietary treatment, may not have been accurately interpreted. The objective of this study was to verify the effect of vaccination on monocyte activity in cattle of the same sex. This was accomplished by designing a study using weaned beef heifers and measuring their monocyte response after vaccination.

Materials and Methods

Animals, Facilities and Design - Fourteen weaned heifer beef calves (Angus, Polled Hereford, Crossbred) weighing approximately 250 kg were used in this experiment. Calves were housed at the Virginia Tech Beef Center and were allotted by weight and breed to either 1) Cu-Lysine (CuLy) or 2) Cu-Sulfate ($CuSO_4$) dietary treatments. These two Cu

supplements were identical to those used in the previous 5 month study. The mineral[a] was top-dressed to the daily ration of ground corn at a rate of 99 g/hd/d. The total Cu concentration of the diets approximated 16 ppm DM basis.

Experimental Design & Methodologies - The experimental design was a 2 x 2 factorial with diet and vaccination as treatment factors. Animals were fed one of two Cu supplements and vaccinated with *B. abortus* Strain 19 at two time periods. All calves were bled on d 0 for monocyte testing. On d 30, 3-4 calves from each treatment group were vaccinated with the standard dose of *B. abortus* Strain 19. Five days post-vaccination, blood was collected from all calves for monocyte testing. On day 60, the remaining calves from each treatment group were given the *Brucella* vaccine. Five days following this second vaccination series, blood samples were taken from all calves for monocyte testing. Hydrogen peroxide release and phagocytosis tests were carried out with isolated monocytes. Body weights were recorded at the start of the study.

Monocyte Preparation - Peripheral monocytes were isolated from whole blood samples by Ficoll gradient separation (Goddeeris et al., 1986) and culture flask adherence techniques as described in Appendices A and C. Briefly, whole blood was layered over the Ficoll solution[b] and centrifuged at 2200 x g for 35 minutes. The white blood cell layer was removed, allocated to 75 cm^2 culture flasks[c], and cultured for 16 hr at 37 C, 5% CO_2 in complete media (RPMI 1640, 10% fetal bovine serum, 1 mM Na pyruvate, 100 U penicillin; 100 µg streptomycin, 2 mM L-glutamine) for monocyte isolation. Adherent cells were recovered by washing flasks with Hanks balanced salt solution (HBSS) with 10 mM EDTA at 4C and gentle scraping.

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[a]Southern States Feed Co-op, Richmond, VA

[b]Histopaque 1077®, Sigma Chemical, St. Louis, MO

[c]Falcon® Vented Tissue Culture Flask, Baxter Scientific Products, Charlotte, NC

Hydrogen peroxide release from stimulated monocytes using DCFH-DA - Recovered monocytes were tested for H_2O_2 release via a procedure modified (Appendix D) from Bass et al.,1983. Briefly, cells were diluted to a concentration of 1 x 10^6 cells/ml in HBSS and 1 µl/ml of 5 mM dichlorofluorescin diacetate (DCFH-DA) was added to all samples. Cells were incubated in a 37 C waterbath for 15 minutes. Samples were divided into two tubes containing 1 x 10^6 cells/ml. One tube received 12.5 µl of the stimulant, cytochalasin B (1/100 dilution of stock solution); the other tube received 12.5 µl of HBSS, which was used as the negative control for each sample (calf). All samples were incubated for 10 minutes in the 37 C waterbath. A shift in fluorescence intensity was measured using a laser flow cell cytometer[d] emitting at 525 nm.

Phagocytosis of fluorescent beads - Isolated monocytes were diluted to concentrations of 1 x 10^6 cells/ml in Krebs ringer bicarbonate-gelatin solution (KRH; 0.76% NaCl, 18.5 mM Hepes-Tea, 4.8 mM KCl, 2.4 mM $MgSO_4$, 0.1 gm gelatin) to test phagocytic activity. The procedure was modified from that reported by Steinkamp et al; (1982). and is outlined in detail in Appendix E. Briefly, a mixture of 0.5 ml of cells in KRH, 50 µl homologous sera and 10 µl of polystyrene beads[e] was diluted 1/10 in HBSS and then was incubated for 1 hr in 50 ml round bottom tubes at 4 C and 37 C. The reaction was stopped with 5 ml phosphate buffer solution (PBS)-gelatin-EDTA (0.1 gm gelatin, 0.1 gm EDTA/100 ml PBS). Fluorescence was measured using a laser flow cell cytometer set at 525 nm. Phagocytic activity was correlated with mean channel fluorescent emission using the cold incubation (4 C) as the control for each sample (calf).

Statistical Analysis - All data were tested for normality and homogeneity of variance. Repeated measures were taken on the same animals over time, therefore, the

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[d]Coulter Epics 752

[e]Fluoresbrite® Beads, 1 micron, Polysciences, Warrington, PA

GLM procedure[f] for Repeated Measures Analysis of Variance was performed on the data. The Huynh-Feldt Epsilon Univariate Hypothesis Test was used for analysis of within-subject effects. Interactions with time were included in the model to determine whether the treatment (vaccination with *B. abortus* and/or Cu supplementation) effect changed over time. Analysis of Contrasts was used to compare average response changes between time periods. Vaccination treatment means were separated by Tukey's HSD ($P < 0.05$).

Results

Vaccination with *B. abortus* Strain 19 on d 30 and d 60 influenced ($P < 0.001$) monocyte response in terms of oxidative burst, measured as H_2O_2 release, and phagocytosis of fluorescent beads (Table 1 and 2). Heifers vaccinated on d 30 had increased ($P < 0.05$) monocyte H_2O_2 release compared to the non-vaccinated heifers at that time period; monocytes from heifers vaccinated on d 60 had increased ($P < 0.05$) H_2O_2 release compared to the non-vaccinated group on d 60 (Figure 1). An increase in monocyte activity was observed on d 30 for both dietary treatment groups compared to d 0. Phagocytic activity continued to increase for all groups by d 60 except for the CuLy-supplemented calves vaccinated on d 30 (CuLy I) (Figure 2) . A time-by-vaccination interaction was evident for H_2O_2 release of monocytes ($P < 0.01$) and tended towards significance ($P = 0.10$) for phagocytic activity (Table 1 and 2).

Discussion

In the preceding part of this chapter, differences were observed for monocyte activity and plasma Cu concentration in vaccinated (heifers) and non-vaccinated (steers)

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[f]Proc GLM, SAS Institutes Inc., Cary, NC, 1985

calves after vaccination with *B. abortus* Strain 19. Interactions between dietary treatment and vaccination were also observed at various days post-vaccination throughout the study period. Characterizing the nature of these observed differences may be difficult because of gender influences on immune response. Grossman (1985) described the relationship between sex steroid hormones and immune response, in particular, the role of circulating estrogen levels on leukocyte function. He reported numerous studies that compared leukocyte function in castrated mice and rats and in their estrogen-supplemented counterparts. Serum from castrated animals had a profound stimulatory effect on T cell activity compared to estradiol. In my previous study, heifers (vaccinated calves) showed no increase in monocyte response over steers (non-vaccinated) on d 84 and 112 (Figures 4 & 5, Part 1). It is possible that monocytes from the vaccinated heifers elicited a strong response to stimulation, but that response was masked by an enhanced immune response from steers. In the present study, the effect of gender was eliminated by utilizing calves of the same sex ,therefore, the effect of vaccination on monocyte function could be clearly evaluated.

Vaccination with *B. abortus* Strain 19 influenced monocyte function in that H_2O_2 release and phagocytic activity of the group vaccinated on d 30 were increased over the non-vaccinated group for d 30. A similar response was observed between vaccinated and non-vaccinated animals on d 60. This stimulatory effect on monocytes was expected based on the normal cascade of events after the immune system is challenged. The challenge in this study was the Brucella vaccine at two separate time periods. Unanue and co-workers (1984) described the response of monocytes to invading organisms, beginning with phagocytosis of the organism. Uptake by the cell then stimulates an intracellular oxidative response. The duration of monocyte response is dependent upon several factors including: cell age, persistence of a stimulant, intercellular communications, and cell environment. Oxidative burst of monocytes from calves vaccinated on d 30 decreased by d 60 (Figure 1). During that time period, a second vaccination was not encountered, thus, the immune system was not stimulated in that manner and monocyte activation most likely diminished.

Monocyte phagocytic activity measured on d 30 and d 60 appeared to be influenced by dietary interactions and, thus, immune response over time was more difficult to interpret.

Monocyte response of calves vaccinated on d 60 was increased over their monocyte response measured on d 30. This suggests that *B. abortus* vaccination stimulated monocyte activation in heifers. The increased monocyte response between d 0 and d 30 for calves vaccinated on d 60 suggests a dietary effect from Cu supplementation. Babu and Failla (1990a) reported similar results for murine-derived monocytes. Oxidative burst and killing activity of monocytes, as well as monocyte Cu concentration, were increased in Lewis rats fed Cu-adequate diets compared to Cu-deplete controls. An impaired respiratory (oxidative) burst of neutrophils from Cu-deficient cattle (Boyne & Arthur, 1981; 1986) and rats (Babu & Failla, 1990b) has also been reported.

Diet tended to influence monocyte H_2O_2 release over time in that the CuLy-supplemented calves showed greater increases from d 0 to 30, and from d 30 to 60 for the calves vaccinated on d 60 only. Dietary effect on phagocytic activity was not so clearly defined, suggesting that oxidative burst of monocytes is more sensitive to Cu availability. These results agree with the previous year's study, described in Chapter 4, Part 1, that showed monocyte oxidative capacity was altered prior to phagocytic activity. Macrophages obtained from Cu-deficient rats exhibited adequate phagocytosis of foreign organisms but a decrease in respiratory burst and candidacidal activity (Babu and Failla, 1990a). Suttle and Jones (1989) showed a similar response in neutrophils from Cu-depleted ruminants. These studies support the belief that microbicidal activity depends upon a free radical-mediated process; H_2O_2 release from cells will decline prior to their phagocytic activity.

The present study verified the effect of vaccination on monocyte stimulation within a cattle of the same sex. Phagocytosis and monocyte activation, measured as H_2O_2 release, were increased in all heifers in response to vaccination with *B. abortus* Strain 19. These results suggest that, in the previous study, the magnitude of vaccination effect at various time periods for the heifers was most likely muted by gender-related effects on immune response. Copper supplementation of heifers in this study appeared to enhance monocyte

response to vaccination. Differences between Cu source were not as clearly delineated in this study compared to the previous Cu-supplementation study, which ran for 120 d. This study lasted only 60 d which may not have been sufficient time for dietary effects to be recognized. Additionally, the small sample number may have lessened the probability of detecting diet effects.

Table 1. Repeated Measures Analysis of Variance[1] for H_2O_2 release of cytochalasin B-stimulated monocytes from Cu-supplemented heifers.

Source	Df[2]	Adj Pr>F Huynh-Feldt Epsilon
Time	2	0.0001
Time * Diet[3]	2	0.1228
Time * Group[4]	2	0.0024
Time * Diet * Group	2	0.5373

[1]GLM procedure for Repeated Measures of Variance, UnivariateTests of Hypotheses for Within-Subject Effects. Monocytes were collected at three separate 30 d intervals.

[2]Degrees of freedoom

[3]Diet refers to Cu supplement: a) Cu as CuLy (600 mg Cu/kg); b) Cu as $CuSO_4$ (600 mg Cu/kg).

[4]Group refers to heifers vaccinated on d 30 or d 60.

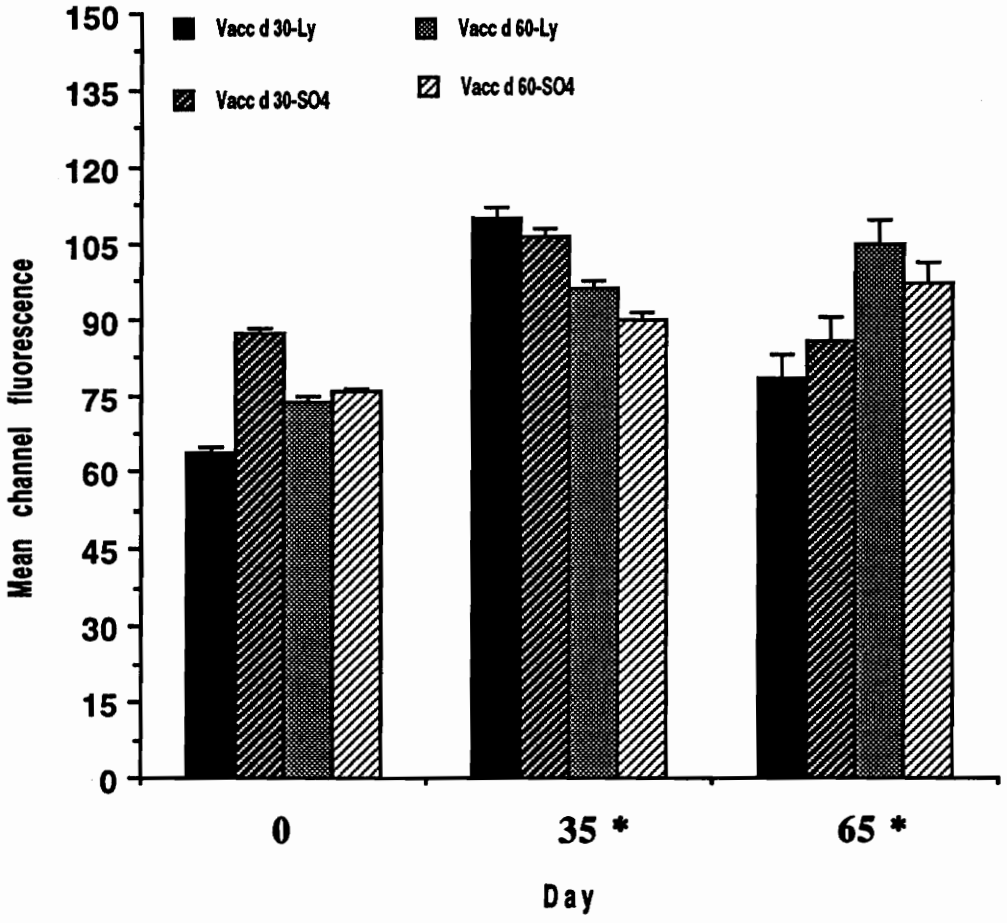

Figure 1. Hydrogen peroxide release of monocytes from Cu-supplemented heifer beef calves vaccinated with *Brucella abortus* Strain 19. Calves were supplemented for 60 d with Cu-Sulfate (SO_4) or Cu-Lysine (Ly) and vaccinated with *Brucella* on either d 30 or d 60. Blood samples were collected from all calves on d 0, 35, and 65. Monocytes were isolated and tested for oxidative burst (H_2O_2 release) in response to stimulation with cytochalasin B. Values are mean fluorescence of responding cells, where fluorescence is defined as channel number; channel range 0 to 256, +/- SEM for the treatment groups. *Treatment means for **vaccinated** calves differ from **not vaccinated** on specified days ($P < 0.05$).

Table 2. Repeated Measures Analysis of Variance[1] for phagocytosis of fluorescent beads by monocytes from Cu-supplemented heifers.

Source	Df[2]	Adj Pr>F Huynh-Feldt Epsilon
Time	2	0.0009
Time * Diet[3]	2	0.4600
Time * Group[4]	2	0.1472
Time * Diet * Group	2	0.7143

[1]GLM procedure for Repeated Measures of Variance, UnivariateTests of Hypotheses for Within-Subject Effects. Monocytes were collected at three separate 30 d intervals.

[2]Degrees of freedom

[3]Diet refers to Cu supplement: a) Cu as CuLy (600 mg Cu/kg); b) Cu as $CuSO_4$ (600 mg Cu/kg).

[4]Group refers to heifers vaccinated on d 30 or d 60.

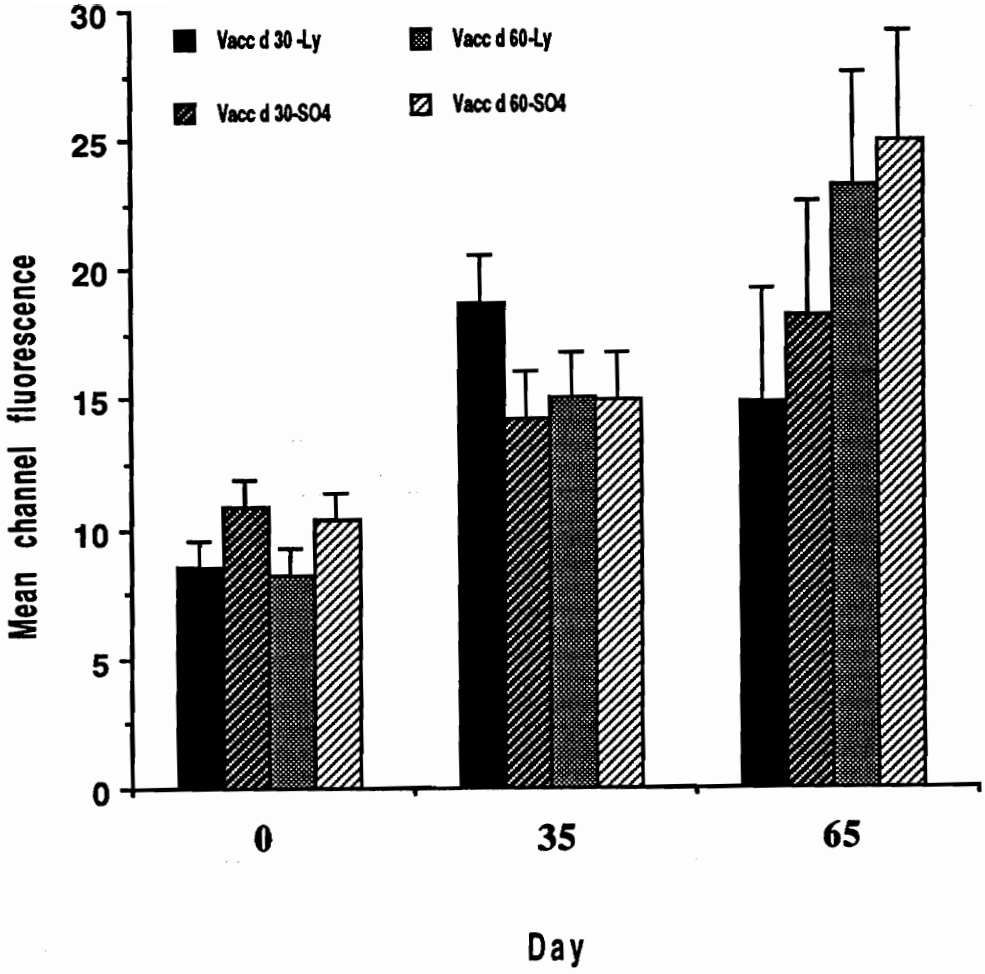

Figure 2. Phagocytic activity of monocytes from Cu-supplemented heifer beef calves vaccinated with *Brucella abortus* Strain 19. Calves were supplemented for 60 d with Cu-Sulfate (SO_4) or Cu-Lysine (Ly) and vaccinated with *Brucella* on either d 30 or d 60. Blood samples were collected from all calves on d 0, 35, and 65. Monocytes were isolated and tested for phagocytosis of fluorescent beads. Values are mean fluorescence of responding cells, where fluorescence is defined as channel number; channel range 0 to 256, +/- SEM for the treatment groups.

Chapter V

Effect of Copper Supplementation on the Expression of Major Histocompatability Complex Class II Antigens in Bovine Monocytes

Part 1. Comparison of RB51 and 2308 *B. abortus* Lipopolysaccharide Antigens as Stimulants for MHC Class II Expression on Bovine Monocytes

Abstract

This study was designed to compare the ability of lipopolysaccharide (LPS), extracted from rough and smooth variants of *Brucella abortus*, to stimulate monocyte expression of major histocompatability complex (MHC) class II molecules *in vitro*. Forty-five weaned beef calves (mean weight 277 kg), were randomly assigned to one of three treatments: 1) Control - fed a corn silage plus soybean meal diet (basal) with no Cu supplementation; 2) Cu-Sulfate ($CuSO_4$) + basal and; 3) Cu-Lysine (CuLy) + basal. Blood monocytes were isolated at specified times and co-cultured with one of three stimulants: a) complete media supplemented with 10 µg/mL of crude *B. abortus* Strain RB51 lipopolysaccharide; b) complete media supplemented with 10 µg/mL of *B. abortus* Strain 2308 lipopolysaccharide and c) complete media, acting as a control lipopolysaccharide. The MHC class II antigen expression was determined using immunofluorescent techniques. Monocytes stimulated with Strain RB51 LPS exhibited an increased MHC class II expression compared to monocytes stimulated with S2308 over all time periods ($P < 0.001$). A diet-by-LPS treatment interaction was evident ($P < 0.05$). The MHC class II antigen expression at various times was altered by diet ($P < 0.05$). These data provide evidence that crude *B. abortus* Strain RB51 lipopolysaccharide has the ability to stimulate bovine monocytes to express MHC class II molecules *in vitro*. Strain RB51 LPS appears to promote MHC class II expression to a greater degree than the more virulent Strain 2308 and, thereby, is useful for *in vitro* bovine monocyte studies.

Introduction

Brucella abortus is a facultative intracellular bacterium that causes abortions and reduced fertility in cattle (Enright, 1990) and chronic zoonotic infections in humans (Young, 1983). The bacterium contains an O-chain component as part of its lipopolysaccharide (LPS). This O-chain appears to be the immunodominant antigen to which a major portion of the antibody response is directed in naturally infected animals and animals immunized with the smooth *Brucella* species (Schurig et al., 1991). Currently, *B. abortus*-induced abortions in cattle are prevented by immunizing heifers with the *B. abortus* Strain 19 vaccine,. although protective, the Strain 19 vaccine induces production of antibodies to the LPS O side chain of *B. abortus*. This humoral immune response can complicate interpretation of diagnostic serology used to detect cattle that have been naturally infected with *B. abortus*. The use of a stable, rough variant of smooth *B. abortus* that lacks the O-chain could potentially overcome serologic misinterpretations.

Characteristics of rough Strain RB51

Protective immunity to the *Brucella* bacterium results mainly from activated macrophages, antigen specific lymphocytes and other cell-mediated immune responses (Stevens et al., 1994d). Therefore evaluation of cellular immune responses in cattle has been the focus in trying to identify *B. abortus* antigens that might replace the attenuated Strain 19 vaccine. Rough strains of *B. abortus* have been described including 45/20 (McEwen, 1940) and RB51(Schurig et al.,1991), but RB51 appears to be the most satisfactory. Strain RB51 is a rough mutant derived from the virulent *B. abortus* Strain 2308 and has an outer membrane similar to Strain 19 and Strain 2308, as well as, all the Strain 2308 proteins that stimulate cell-mediated immune responses in mice (Stevens et al., 1995) and cattle (Stevens et al., 1994a). Strain RB51 differs from these smooth strains in that it lacks the LPS O antigens. Mice and cattle vaccinated with Strain RB51 have cell-mediated immune responses to Strain 2308 bacteria and are protected from abortions

associated with Strain 2308 challenge, however, animals do not produce antibodies to the O antigens, which are detected by serologic tests for brucellosis (Schurig et al., 1991). Comparative studies of immune responses in mice vaccinated with *B. abortus* Strain 19 or Strain RB51 and subsequently challenged with Strain 2308 showed that the mice vaccinated with RB51 have enhanced resistance to infection with 2308 (Schurig et al., 1991). Immune and pathologic responses in mice infected with strains 2308, 19 and RB51 suggest that, although RB51 gives a less persistent immune response, germinal centers in the spleen were larger and the lymphocyte depletion commonly seen with Strain 19 and Strain 2308 was not observed (Stevens et al., 1994c). Cattle vaccination studies have likewise indicated similar immune responses with Strain RB51 compared to Strain 19 (Stevens et al., 1994a).

Factors influencing MHC Class II Expression on Monocytes

As mentioned earlier, monocytes play an integral role in protection against intracellular invaders such as *B. abortus* . The monocyte protects initially with antigen engulfment, followed by antigen processing and presentation to lymphocytes via MHC class II molecules. Bovine macrophages can present *B. abortus* smooth Strain 19 in the context of MHC class II molecules to T cells (Splitter and Everlith, 1986). The influence of Strain RB51 in bovine MHC class II antigen presentation activity has not been investigated.

Because expression of MHC-encoded molecules is absolutely required for antigen presentation and development of helper and cytotoxic T cells, it follows that any process that modulates their expression may significantly influence immune responses. In which case, the regulation of Ia antigen (MHC class II molecules) on antigen presenting cells may well be a critical step in the control of immune responsiveness. Macrophages can be divided into two subpopulations (Unanue et al., 1984). The Ia positive subset expresses cell surface Ia molecules and can mediate antigen presentation. The second subset, Ia negative, lacks both membrane Ia and functional activity for T cell activation. Eventually,

all Ia positive macrophages will become Ia negative, and new stem cells give rise to Ia positive progeny. Therefore, Ia glycoprotein subsets are transient and expression at any point in time is influenced by the numerous factors affecting macrophage activation. The extent of Ia expression can be regulated by: interaction of macrophages with some agent that triggers phagocytosis, products of antigen-stimulated T cells preferentially inducing Ia expression in young macrophages, and 'inhibitory' or 'stimulatory' products that alter Ia expression.

Agents such as mineral oil, peptone, and thioglycollate have been shown to increase macrophage numbers *in vivo* but have not increased the proportion of Ia positive macrophages. In contrast, live bacteria induced an increase in both number of macrophages and percentage of Ia-bearing macrophages. Induction of Ia expression has also been demonstrated *in vitro* with primary cell cultures of peritoneal macrophages (Beller & Ho, 1982; Steinman et al., 1980), bone marrow-derived macrophage colonies (Calamai et al., 1982), and macrophage-like cell lines (McNicholas et al., 1983). Gamma interferon (King & Jones, 1983) and indomethacin (Snyder et al., 1982) have been shown to induce Ia macrophages *in vitro.* Lipopolysaccharide, which has profound effects on a wide variety of macrophage functions (Morrison & Ryan, 1979), has also been shown to alter Ia expression.

LPS Regulation of Monocyte Activation and MHC Class II Expression

Lipopolysaccharide acts at picomolar concentrations as a very potent monocyte agonist. Beller & Unanue (1980) reported that intraperitoneal injection of mice with LPS caused little to no change in Ia expression, whereas Steeg and co-workers (1982) demonstrated through *in vitro* studies that LPS inhibited expression of Ia induced by gamma-interferon, and actually decreased Ia expression on human monocytes (Yem & Parmely, 1981). Ziegler et al. (1984) later showed that the regulatory effects of LPS on Ia expression was dose dependent. In recent years, researchers have detailed the chemical structure of LPS, specifically its lipid A domain that is recognized by the monocyte, and

thus initiates signal transduction events leading to cell activation via receptor dependent mechanisms (Ulevitch and Tobias, 1994; Hara-Kuge et al., 1991; Raetz et al., 1991). This receptor mechanism, which involves a membrane bound receptor comprised of LPS complexed to a plasma protein (LBP) and membrane-bound or soluble CD14 glycoprotein, lends to a fuller understanding of how LPS stimulates cells. Mathison and co-workers (1992) have shown that LPS, isolated from the rough or smooth form of gram-negative bacteria, has similar stimulatory effects on monocytes through this protein-bound receptor mechanism. The ability of LPS extract of the gram-negative, rough Strain RB51 *B. abortus*, to stimulate monocyte expression of MHC class II molecules (Ia antigens) has not been evaluated.

Objectives

This study was designed to compare *B. abortus* smooth Strain 2308 and rough Strain RB51 LPS extracts as *in vitro* stimulants for MHC class II expression on bovine monocytes. This was accomplished by co-culturing bovine monocytes with Strain RB51 and Strain 2308 lipopolysaccharide and measuring MHC class II molecule expression via flow cytometric analysis.

Materials and Methods

Animals and Facilities - Weaned Angus, Polled Hereford and Crossbred heifers, 6-8 mo of age (mean weight 250 kg) were used for blood sources in this experiment. All animals were housed at the Virginia Tech Beef Center in outside pastures or lots containing a fence-line feed bunk, automatic waterer and free-choice white salt. Calves were fed once daily a ration consisting of corn silage and soybean meal, top-dressed with a mineral mix. Mixed grass hay in round bales was available in each lot.

Experimental Design and Methodologies - Fifteen ml of blood were collected via jugular venipuncture from each calf into an equal amount of Alsevars solution (Sigma Chemical, Co., St. Louis, MO) for a total volume of 30 ml. Calves were bled on d 68 ,82 and 96 of the study. On d 68 all calves were vaccinated with 2 ml of *Brucella abortus* Strain 19.

Monocyte Isolation and MHC Class II Molecule Stimulation

Monocytes were isolated and purified according to procedures outlined in Appendices A and C. Briefly, whole blood collected in Alsevars solution was layered over 15 ml of Histopaque 1077® (Sigma Chemical, Co., St. Louis, MO) and centrifuged for 35 minutes at 900 x g. The mononuclear layer (monocytes and lymphocytes) was removed and washed 3 times in Hanks balanced salt solution (HBSS) at 1000 x g for 10 minutes. Recovered cells were diluted to 1 x 10^6 cells per ml in RPMI 1640 media (Sigma Chemical Co., St.Louis, Mo.) supplemented with 100 U of penicillin per ml (Sigma Chemical Co., St. Louis Mo.), 100 µg streptomycin per ml (Sigma Chemical Co., St.Louis Mo.), 200 mM L-glutamine (Sigma Chemical Co., St. Louis Mo.), 100 mM Na pyruvate and 10 % fetal bovine serum (FBS) (Gibco, BRL Life Technologies, Grand Island,N.Y.), referred to as complete media, and incubated in cell culture flasks in a 37 C, 5% CO_2, humid environment for 4 hours. After the four hour incubation, media was poured off and an equal amount of fresh media was added to each culture flask. Cells were introduced to one of three challenges at this time: a) complete media, acting as a control; b) complete media supplemented with 10 µg/ml of crude *B. abortus* Strain RB51 lipopolysaccharide; and c) complete media supplemented with 10 µg/ml of *B. abortus* Strain 2308 lipopolysaccharide. Cells were then returned to the incubator for an additional 16 hours.

The monocyte fraction of the cell suspension was recovered by pouring off media, and adding 5 ml of HBSS supplemented with 10 mM ethylenediaminetetraacetic acid (EDTA), (Sigma Chemical Co., St. Louis, Mo.) at 4 C. The HBSS©EDTA was allowed to sit for 12 minutes on the monocyte layer. Each flask was then gently scraped to facilitate

cell removal, and rinsed with 10 ml of HBSS to insure recovery of all cells. Each sample was washed 3 times with HBSS and centrifuged at 1000 x g for 10 minutes. Samples were counted using a hemocytometer and cell volume was adjusted to 1 x 10^6 cells per ml.

B. abortus Strain RB51 and Strain 2308 LPS Extract Preparation

Crude RB51, equivalent to RB51 from the aqueous extract phase, was prepared in the following manner. Twenty trypticase soy agar (TSA) plates were streaked with strain RB51 and incubated at 37 C in 5% CO_2 for 72 hr. Organisms were harvested from plates by washing the surface several times with sterile distilled water and killed by adding an equal volume of acetone to the bacterial suspension for 3 hr at room temperature with stirring. Cells were centrifuged at 10,000 x g for 10 minutes. The cell pellet was washed twice with sterile water, resuspended in 10 ml 10% NaCl, 4M urea, 0.001% ß-mercaptoethanol (ME), and incubated overnight at 40 C with shaking. The suspension was centrifuged at 10,000 x g for 10 minutes and the supernatant removed. The recovered supernatant was dialyzed in 12,000-14,000 mwco dialysis tubing at 4 C overnight, lyophilized and called crude RB51 LPS (Wise, 1995).

Smooth Strain 2308 LPS from the phenol phase was extracted from bacterial cells using a procedure described previously (Schurig et al., 1991). Both LPS extracts were reconstituted in PBS to a stock solution 10x greater than working solution and subsequently diluted to working solution (1µg/µl) at the time of cell culture.

Major Histocompatibility Complex (MHC) Class II Measurement

MHC Class II expression was measured by a fluorescence labelling technique. Monocytes were cold-incubated (4 C) with anti-MHC Class II, (TH14B), monoclonal antibody (Moab), (VMRD, Inc., Pullman, Wa.) diluted 1:2500, for 1 hr. Cells were washed 3 times in HBSS and centrifuged at 1000 x g for 10 minutes. Bound Moab was then tagged with the fluorescent marker, fluorescein isothiocyanate (FITC)-conjugated AffiniPure Rabbit Anti©Mouse IgG (H+L), (Jackson Immuno Research Laboratories, West Grove, Pa.). The FITC was used at a final concentration of 7.5 µg/0.5 ml of sample,

(1:250 dilution of product) and cold-incubated (4C) with Moab-tagged monocytes for 1 hr. Cells were washed 3 times in HBSS and centrifuged at 1000 x g for 10 minutes. Each sample was then diluted to a final concentration of 1 x 10^6 cells/ml in HBSS. Two tubes were prepared for each experimental sample. One tube was incubated with the FITC only and acted as the non-specific fluorescent control. The other was incubated with both FITC and MHC Class II moab. Fluorescence was measured using a Coulter Epics 752 Flow Cytometer, equipped with an argon laser, exciting at 488 nm. Emission was read at 525 nm. The difference in mean channel fluorescence between the two samples (FITC vs FITC & MHC Class II moab) was used to measure the expression of MHC Class II in response to the different *in vitro B. abortus* lipopolysaccharide challenges.

Statistical Analysis - Since repeated measures were taken on the same animals over time, the GLM procedure for Repeated Measures Analysis of Variance was performed on all data. The Huynh-Feldt Epsilon Univariate Hypothesis Test was used for analysis of within-subject effects. Analysis of Variance of Contrasts Variables was used to compare average response changes from treatments between time periods. Differences between LPS treatment means were separated by Tukey's HSD analysis ($P < 0.05$).

Results

The time period at which monocytes were challenged with rough and smooth strains of *Brucella abortus* LPS affected ($P < 0.001$) surface expression of MHC class II molecules (Table 1). The response of monocytes over time was dynamic ($P < 0.05$), with the CuLy-supplemented group showing an increased response at time periods 2 and 3 compared to control and CuSO4-supplemented groups (Figure 1). The diet-by-LPS treatment interaction affected ($P < 0.05$) monocyte expression of MHC class II molecules over time. This interaction was strongly influenced by the response of monocytes in the

CuLy/RB51 group. The relationship between rough Strain RB51 and smooth Strain 2308 did not change over time ($P = 0.87$) with monocytes challenged with RB51 consistently exhibiting increased MHC class II molecule expression. Contrasts (Table 2) between time periods indicated that the average response changed between time periods 1 and 2 ($P < 0.05$) and between time periods 2 and 3 ($P < 0.001$).

Discussion

Monocyte stimulation *in vitro* with crude *B. abortus* Strain RB51 lipopolysaccharide (LPS), extracted from the aqueous phase of LPS preparation, and with Strain 2308 LPS extracted from the phenol phase repeatedly resulted in increased MHC class II expression from the Strain RB51 group. The data from this comparative study help to detail further the characteristics of rough Strain RB51.

Lipopolysaccharide has been shown to be a potent stimulant of macrophage cell activation, although reports specific to the regulation of Ia-positive antigens (MHC class II molecules) have been controversial. Our study suggests that *B. abortus* LPS, whether as rough or smooth strains, induces Ia-positive cells. In contrast to these findings, Mathison and co-workers (1992) found that lipopolysaccharide binding protein (LBP) lowered the stimulatory dose of LPS, and the rate of cellular responses to LPS was similar when LPS with different core and /or O-antigen structures were compared. Their determination for cell activation was TNF cytolytic activity from elicited macrophages, and the macrophage activators included LPS isolated from a number of gram negative bacteria including *Salmonella, Escherichia coli, and Klebsiella species*. These differing results could be attributed to the bacterial species utilized in each study, such that *Brucella abortus* LPS may have different stimulatory capacities. Previous studies have shown similar induction of Ia expression from peritoneal-elicited macrophages (Steinman et al., 1980), as well as, peripheral and bone marrow-derived macrophages (Calamai et al., 1982). Monocyte origin, therefore, should not have been a factor in study differences.

The process of MHC Class II molecule expression has been outlined in detail (Englehard, 1994). More recently, a novel mechanism whereby monocytes recognize LPS has been proposed (Mathison et al., 1992), thereby expanding the areas for speculative interpretation of our results. Interference with Ia antigen expression on the monocyte could be associated with a defect in the binding of antigen. This could cause a cessation of expression or delay in expression until after the time that surface molecule determination was made. A defect in antigen processing, after internalization by the monocyte, could likewise result in interruption of presentation via the Ia antigen. Another aspect of MHC class II expression to consider is modulation of Ia by anti-Ia antibodies. Unanue et al; (1984) reported that anti-Ia antibodies can selectively cap Ia from the membrane. The difference in structure of Strain 2308 LPS and Strain RB51 LPS could have potentially altered expression of Ia by interrupting the steps associated with MHC-antigen binding and resulted in delayed Ia expression. The decreased response at time period 2 may have been a manifestation of Ia expression interference by one or a combination of the above reasons.

Since Ia-positive macrophages are not a stable subset, but rather dependent upon cell age and activation state, the difference in Strain 2308 and Strain RB51 LPS stimulation of Ia expression may be a consequence of various factors. Strain 2308 LPS, coming from a more virulent strain, may have possibly eliminated that proportion of the monocyte population which was Ia positive, stimulation of secretory products by the monocyte that would be inhibitory to Ia expression could possibly have been induced by the 2308 strain of LPS. Ziegler and co-workers (1984) reported that the differences between *Listeria monocytogenes'* LPS-induced Ia populations during early and late phases of stimulation most likely were related to prostaglandins. Prostaglandins, as well as other secretory substances, are known to inhibit MHC class II expression (Unanue et al., 1984). The stimulatory effects of LPS are dose-dependent (Ziegler et al., 1984) and, although in our study, LPS strains were added to monocyte cell cultures at the same dry weight dose, the actual *in vitro* LPS dose may have been greater compared to the crude Strain RB51 LPS resulting in inhibition of Ia expression in the Strain 2308 group.

The kinetics of Ia induction may also be involved in strain differences observed in our study. Prior to the start of the study, monocytes were challenged with LPS for 2 to 72 hours *in vitro* (data not shown) and MHC class II expression was compared. Based on this preliminary work, a 16 hour co-culture period with LPS was chosen. Because of individual animal variation, this 16 hour culture with LPS may not have allowed an optimal stimulatory effect by Strain 2308 LPS. Beller and Ho (1982) have shown induction of Ia antigens within 3 days after stimulation with gram negative *L. monocytogenes*, either *in vivo* or *in vitro*. Viability of isolated bovine monocytes in our cell cultures appeared to diminish considerably by day 2, suggesting optimal LPS stimulation of bovine monocytes *in vitro* occurs prior to 48 hours. The amplification loop involved in monocyte activation, MHC class II induction and optimal expression would appear to vary within T-cell dependent or independent environments and possibly among gram negative bacterial species.

LPS is found in the outer membrane of gram-negative bacteria such as *Brucella abortus*. Recognition of LPS at the cell surface is required for LPS uptake or initiation of transmembrane signaling leading to cell activation. Westphal and associates (1986) established that the adjuvant effects of bacterial LPS are mediated by the lipid A portion of the LPS macromolecule. Ziegler et al. (1984) showed Ia-inducing activity was lipid A dose-dependent, with Ia induction greater with Re595 LPS (80% lipid A) compared to the wild-type LPS (5% lipid A). Lipid A was not measured in the LPS extracts used in this study, but differences in the lipid A content of Strain 2308 and Strain RB51 LPS may have accounted for the variation in MHC class II expression in monocytes. Although much attention has been given to the immunomodulatory effects elicited by the lipid A fraction of LPS, the polysaccharide (PS) portion of some, but not all, preparations of LPS have been reported to have immunostimulatory properties (Friedman et al., 1987). The stimulatory aspects of PS fractions include: induction of various cytokines, stimulation of B-cell mitogens, activation of alternative complement pathways, and facilitation of LPS binding to macrophages. Therefore, the possibility exists that PS fractions of Strain RB51 and Strain

2308 LPS differ enough to affect monocyte MHC class II expression.

An initial step in LPS-induced Ia antigen expression of monocytes is membrane binding and then subsequent transmission of a signal or of LPS itself across the cell membrane occurs. Several proteins have been suggested to mediate LPS recognition at the cell surface. Recently, the LPS-LBP-CD14 complex has been recognized as the basis for a membrane receptor mechanism in LPS cell activation. Lipopolysaccharide binding protein, a unique serum protein, appears to bind initially to LPS and facilitate LPS attachment to CD14, a glycoprotein either membrane-bound or soluble, intimately involved in LPS uptake by the cell. Lipopolysaccharide binding protein binds with high affinity to the most chemically conserved region of LPS lipid A. Therefore, differences in lipid A content among *B. abortus* strains 2308 and RB51 may ultimately effect LPS binding and recognition. Likewise, other serum proteins have been recognized to modify physical, chemical and biological properties of LPS. In which case, the effect of fetal bovine serum (FBS) incorporated into the culture media on LPS stimulatory capacity can not be overlooked as a factor in study differences. Also, characteristics inherent in each of the strains of *B. abortus* LPS used in our study may influence LPS internalization and its subsequent intracellular signal transduction via tyrosinase phosphorylation, and indirectly affect Ia antigen expression.

The dietary effect on increased MHC class II expression was observed to be most significant in monocytes isolated from the CuLy-supplemented calves. Whether this enhancement of antigen expression was the result of increased dietary copper, dietary lysine or both is not clear. The lysine supplied by the CuLy mineral was less than 1% of the calves' daily lysine requirement. Theoretically, that micro-measure of lysine should not significantly affect immune response, however, studies the previous year indicated that CuLy-supplementation enhanced monocyte oxidative burst and phagocytic activity. Dietary, as well as environmental requirements for optimal immunostimulatory effects of Strain RB51 needs to be investigated further.

This comparative study has provided additional information on the potential immunoprotective characteristics of *B. abortus* Strain RB51. Because the LPS fragment of this rough mutant can stimulate MHC class II expression of bovine monocytes and stimulate MHC class II antigens to a greater degree than its parent strain, 2308, Strain RB51 LPS is a suitable alternative stimulant for monocyte cell culture studies.

Table 1. Repeated Measures Analysis of Variance for MHC class II expression of monocytes stimulated with *B. abortus* RB51 and 2308 Lipopolysaccharide.[1]

Source	Df[2]	Adj Pr>F Huynh-Feldt Epsilon
Time	2	0.0001
Time * Diet[3]	4	0.03
Time * Trt[4]	2	0.87
Time * Diet * Trt	4	0.04

[1]GLM procedure for Repeated Measures of Variance, Univariate Tests of Hypotheses for Within-Subject Effects. Monocytes were collected at three separate 14 d intervals.

[2]Degrees of freedom.

[3]Diets consisted of a basal ration (corn silage and soybean meal) supplemented with a mineral mix containing a) no copper (Cu); b) Cu as $CuSO_4$; c) Cu as CuLy.

[4]Treatments consisted of 10 μg/ml of: crude *B. abortus* Strain RB51 and Strain 2308 LPS *in vitro*.

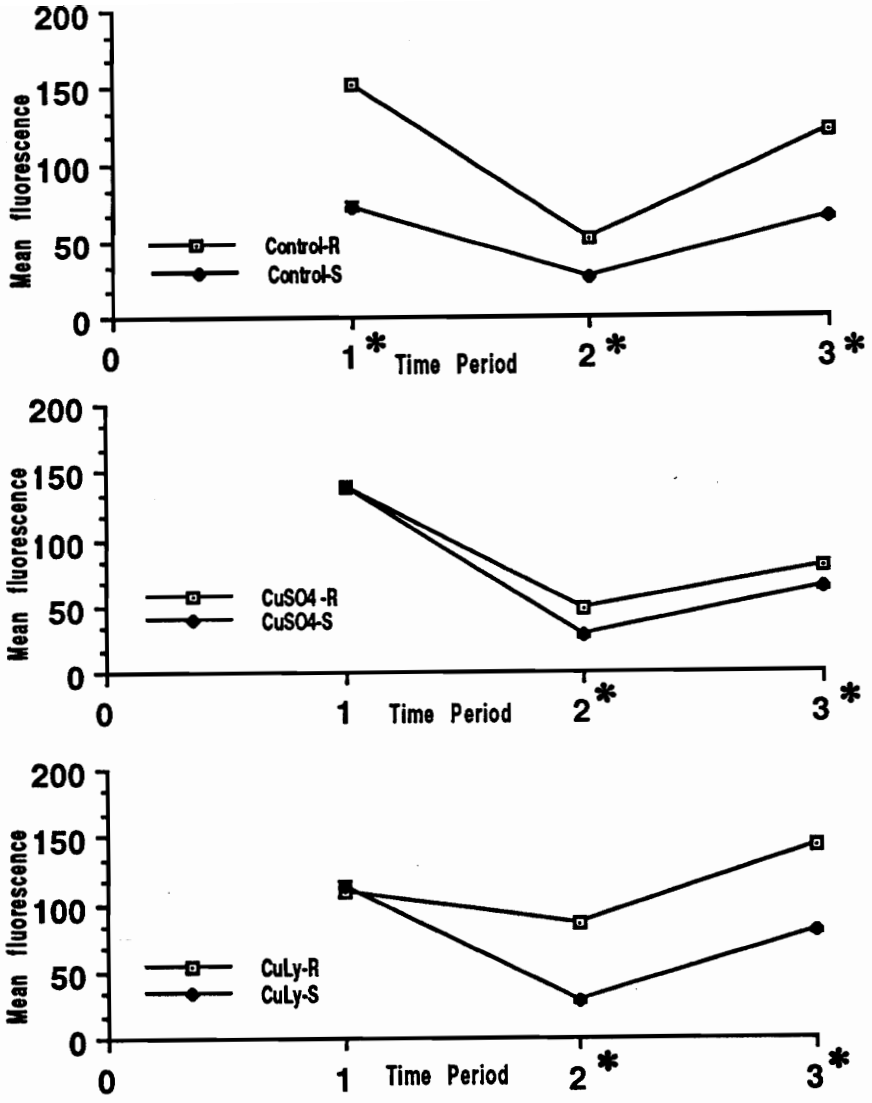

Figure 1. Bovine monocyte MHC class II expression in response to *in vitro* stimulation with *Brucella abortus* lipopolysaccharide (LPS). Monocytes were collected at time period 1 (d 68), time period 2 (d 82), and time period 3 (d 96) from beef calves fed one of three Cu supplements : Control (Cu deplete), Cu-Sulfate ($CuSO_4$), or Cu-Lysine (CuLy) for 120 d. Isolated monocytes were stimulated *in vitro* with rough Strain RB51 (R) and smooth Strain 2308 (S) *B. abortus* LPS and MHC class II expression was measured via immunofluorescent markers. Values are mean fluorescence of responding cells, where fluorescence is defined as channel number; channel range 0 to 256, +/- SEM for each treatment group. * LPS treatment means differ by Tukey's ($P < 0.05$).

Table 2. Repeated Measures Analysis of Variance Contrasts for MHC class II expression of monocytes stimulated with *B. abortus* Strain RB51 and Strain 2308 Lipopolysaccharide.[1]

Source	Contrast: Time 1 vs 2		Contrast: Time 2 vs 3	
	Df[2]	Pr>F	Df[2]	Pr>F
Mean	1	0.0032	1	0.001
Diet[3]	2	0.0430	2	0.0047
Animal * Diet	21	0.0004	21	0.0279
Treatment[4]	1	0.7435	1	0.4434
Diet * Treatment	2	0.0582	2	0.1571

[1]GLM procedure for Repeated Measures of Variance, Analysis of Variance of Contrast Variables. Monocytes were collected at three separate 14 d intervals.

[2]Degrees of freedom.

[3]Diets consisted of a basal ration (corn silage and soybean meal) supplemented with a mineral mix containing a) no copper (Cu); b) Cu as $CuSO_4$; c) Cu as CuLy.

[4]Treatments consisted of 10 µg/ml of: crude *B. abortus* Strain RB51 and Strain 2308 LPS *in vitro*.

Chapter V

Effect of Copper Supplementation on the Expression of Major Histocompatability Complex Class II Antigens in Bovine Monocytes

Part 2. Strain RB51 Stimulated Monocyte Response in Brucella Vaccinated Heifer Beef Calves Supplemented with Dietary Copper

Abstract

Forty-five weaned beef calves (mean weight 277 kg) were used to determine the effects of dietary copper (Cu) supplementation and routine vaccination on bovine monocyte activity and Cu status. Calves were randomly assigned to one of three treatments: 1) Control group - fed a basal diet (corn silage plus soybean meal) without Cu supplementation; 2) Cu-Lysine (CuLy) + basal; 3) Cu-Sulfate ($CuSO_4$) + basal. Average daily Cu intake for treatment groups was 44, 111 and 112 mg/hd/d, respectively. On d 68 of the 120 d study, calves were vaccinated with *Brucella abortus* Strain 19. Blood monocytes were isolated and co-cultured with *B. abortus* Strain RB51 crude lipopolysaccharide to determine major histocompatibility complex (MHC) Class II expression and phagocytic activity 14 and 28 d following vaccination. Calves supplemented with $CuSO_4$ had increased ($P < 0.05$) plasma Cu on d 82 compared to control calves. Treatment differences were observed post-vaccination for monocyte expression of MHC class II antigens. CuLy-supplemented calves had increased ($P < 0.05$) MHC class II antigen expression as compared to $CuSO_4$-supplemented and control groups on d 82 and 110. Phagocytic activity of monocytes from CuLy-supplemented heifers was increased ($P < 0.05$) over $CuSO_4$ and control groups on d 82. Weight gains recorded on days 82 and 110 were greater ($P < 0.05$) for Cu-supplemented calves compared to controls. These results suggest Cu supplementation, especially in the organic form

(CuLy), enhances immunocompetence at the monocyte level in beef heifers stressed by vaccination. Also, supplemental dietary Cu in a basal diet with 7 mg Cu/kg increased weight gains in beef heifers.

Introduction

Macrophages of cattle serve as host cells for *Brucella abortus*, a facultative intracellular bacterium, whose infectivity depends upon the ability of the bacterium to survive within the monocyte. Host resistance to disease caused by *B. abortus,* and other intracellular organisms, depends on appropriate interaction between specifically sensitized T cells and macrophages (Splitter & Everlith, 1989). Specific T-helper cells are activated by monocytes that express the class II gylcoproteins encoded by genes of the MHC, in association with the antigens derived from the bacteria. Regulation of expression of class II glycoprotein molecules on the monocyte is, therefore, a critical step in control of immune responsiveness.

Dietary Cu deficiency alters neutrophil function and lymphocyte blastogenic activity in various species (Davis et al., 1987; Jones & Suttle, 1981). Likewise, decreased monocyte oxidative burst, microbicidal activity, and Cu concentration of monocytes have been associated with decreased dietary Cu in the murine model (Babu & Failla, 1990). Intracellular environment (Lin & Ficht, 1995), secretory function (Page et al., 1978), and surface molecule expression (Unanue et al., 1984) of the monocyte are influenced by numerous factors that include nutrient availability.

In previous chapters, the author has suggested that monocyte activation, oxidative burst, and phagocytic activity in beef calves depend upon dietary Cu concentration and stress associated with routine vaccination. Other functions of the monocyte would potentially be altered by dietary Cu intake. The role of dietary Cu on MHC class II expression of bovine monocytes has not yet been investigated.

Objectives

The purpose of this study was to determine the effect of Cu supplementation on MHC class II molecule expression in beef calves vaccinated with *Brucella abortus* Strain 19. This was accomplished by supplementing beef heifers with Cu from two sources,

collecting monocytes, and measuring MHC class II expression on the cell surface in response to vaccination.

Materials and Methods

Animals, Facilities and Feed - Weaned Angus, Polled Hereford and Crossbred heifer calves approximately 8 mo of age (mean weight 275 kg) were housed in adjacent outside lots that contained a fence-line feed bunk, automatic waterer, and free-choice salt feeder. Calves were fed daily a corn silage and soybean meal ration (7 mg Cu/kg DM) for 3 weeks preceding and during the 120 d study. The nutrient composition of the basal diet was analyzed (Table 1). Copper was incorporated into the mineral supplement [a] according to treatment protocol. Mineral was top-dressed daily to the ration at the rate of 113 g/hd/d. Free-choice white salt was available throughout the study period.

Experimental Design and Methodologies - Forty-five weaned beef calves were allotted by weight and breed to 1 of 3 groups for 120 days. Treatment groups consisted of: 1) Control - without Cu supplementation, 2) Cu-Lysine (CuLy) and 3) Cu-Sulfate ($CuSO_4$). Mineral with added Cu was supplemented to treatment groups 2 and 3 to supply approximately 20 mg Cu/kg in the total diet. Composition of the mineral supplement is listed in Table 2. Total daily Cu intake for treatment groups was 44, 111, and 112 mg/hd/d, respectively.

Calf weights were recorded every 28 days. Calves were vaccinated with *B. abortus* Strain 19 on d 68 of the study. Blood was collected for determination of plasma Cu and monocyte testing on d 0, 68, 82 and 110.

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[a] Southern States Feed Co-op, Richmond, VA.

Measurement of blood Cu concentration - Blood was collected via venipuncture into heparinized Vacutainer® tubes and plasma was separated for Cu determination. Plasma samples were diluted three-fold with deionized water and analyzed with appropriate external standards by atomic absorption spectrophotometry.

MHC Class II expression on bovine monocytes in response to vaccination - Monocytes were isolated from whole blood samples by Ficoll gradient separation (Goddeeris et al., 1986) and adherence techniques in culture flasks. The procedures are detailed in Appendices A and C. Briefly, whole blood was layered over the Ficoll solution[b] in 50 ml tubes and centrifuged at 2200 x g for 35 minutes. The mixed monocyte/lymphocyte layer was removed by pipette aspiration; diluted to a concentration of $1x10^6$ cells/ml and cultured at 37 C, 5% CO_2 in vented tissue culture flasks[c] for 4 hr in RPMI 1640 media (Sigma Chemical Co., St.Louis, Mo.) supplemented with 100 U of penicillin per ml (Sigma Chemical Co. St. Louis Mo.), 100 µg streptomycin per ml (Sigma Chemical Co., St.Louis Mo.), 2 mM L-glutamine (Sigma Chemical Co., St. Louis Mo.), 1 mM sodiun pyruvate and 10 % fetal bovine serum (FBS) (Gibco, BRL Life Technologies, Grand Island,N.Y.), referred to as complete media. Media and non-adherent cells were removed and fresh media was added with 10 µl (1µg/ml) of a crude, phenol water LPS extract of *B. abortus* rough Strain RB51[d] for an additional 16 hr incubation. Media was removed and adherent monocytes were recovered by washing flasks with Hanks balanced salt solution (HBSS) with 10 mM EDTA at 4 C and gentle scraping. Recovered cells were diluted to a concentration of 1 x 10^6 cells/ml in HBSS.

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[b]Histopaque 1077®, Sigma Chemical, St. Louis , MO

[c]Falcon®, Baxter Scientific Products, Charlotte, NC

[d]VMRCVM Research Center, Blacksburg, VA.

Monocytes were tested for expression of MHC class II molecules using a modified procedure (Appendix F) from Splitter and Everlith (1989). Briefly, $1x10^6$ cells/ml were exposed to MHC Class II monoclonal antibody (THI4B) (VMRD, Inc., Pullman, WA) diluted 1:2500. A paired cell sample (control) with no monoclonal was prepared for each animal. All samples were incubated for 1 hr at 4 C and washed twice in phosphate buffer solution (PBS). Fluorescein isothiocyanate (FITC) conjugated AffiniPure Rabbit Anti©Mouse IgG (H+L), (Jackson Immuno Research Laboratories, West Grove, Pa.) was added to all samples. A 1 hr incubation at 4 C was followed by two washings in PBS buffer. Cells were then analyzed by flow cytometry. Mean channel fluorescent emission was measured on a laser flow cell cytometer emitting at 525 nm. The MHC class II expression was correlated with fluorescent emission.

Phagocytosis of fluorescent beads - Isolated monocytes were diluted to concentrations of 1 x 10^6 cells/ml in Krebs ringer bicarbonate-gelatin solution (KRH) to test phagocytic activity. The procedure followed was modified (Appendix E) from that reported by Steinkamp et al; (1982). A mixture of 0.5 ml of cells in KRH, 50 µl homologous sera and 10 µl of polystyrene beads[e] diluted 1/10 in HBSS, was then incubated for 1 hr in 50 ml Nalgene tubes at 4 C or 37 C. The reaction was stopped with 5 ml PBS-gelatin-EDTA. Fluorescence was measured using a laser flow cell cytometer emitting at 525 nm. Phagocytic activity was correlated with mean channel fluorescent emission using the cold incubation (4 C) as the control for each sample (calf).

Determination of Brucella titers - Standard tube agglutination was used to determine agglutinin titer to *B. abortus* following a described procedure (Alton et al., 1975).

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[e]Fluoresbrite® Beads, 1 micron, Polysciences, Warrington, PA

Statistical Analysis - The general linear model[f] was used to analyze the data. Animal was used as the experimental unit. The model included dietary treatment, breed, and all two-way interactions. Differences among means ($P < 0.05$) were separated by a Tukey test.

Results

Plasma Cu concentration of controls was higher ($P < 0.05$) than Cu-supplemented heifers on d 0 and 68, but lower than the $CuSO_4$ and CuLy-supplemented heifers after vaccination with *B. abortus* Strain 19 on d 82 and 110 (Table 3).

Monocyte cell function, as measured by phagocytic activity and MHC class II antigen expression, was used as a partial indicator of immune response. Phagocytic activity did not differ among treatment groups prior to vaccination with *B. abortus* but, within 14 d after vaccination, monocytes from CuLy-supplemented heifers showed increased ($P < 0.05$) bead uptake (mean channel fluorescence) and tended to have a greater number of cells phagocytizing fluorescent beads compared to controls (Table 4). Expression of MHC class II molecules on monocytes from CuLy-supplemented heifers was increased ($P < 0.05$) over controls on d 68, and over both controls and $CuSO_4$-supplemented heifers on d 82 and 110. Monocytes from CuSO4-supplemented heifers exhibited increased ($P < 0.05$) MHC class II expression compared to controls on d 82 and 110 (Table 5). The number of monocytes expressing MHC class II molecules 14 d after vaccination was increased ($P < 0.05$) in the CuLy-supplemented heifers compared to control and $CuSO_4$-supplemented calves.

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[f]Proc GLM, SAS Institutes Inc., Cary, NC, 1985

On d 0, 89% of the calves had no titer to *B. abortus* antigen. The remaining animals had low titers due to vaccination at a previous time. At 2 weeks after vaccination with *B. abortus* Strain 19, all calves had a titer of 1:25 or more. At 6 weeks after vaccination, 16% of the calves became negative. Calves supplemented with CuLy tended to have numerically higher titers than both $CuSO_4$-supplemented and control calves (Figure 1).

Heifers supplemented with CuLy and $CuSO_4$ gained ($P < 0.05$) more weight during the 120 d study (Figure 2) compared to non-supplemented calves (356 and 344 vs 319 kg, respectively). CuLy-supplemented heifers had increased ($P < 0.05$) body weights on d 82 and 110 compared to controls. Heifers in the $CuSO_4$-supplemented groups showed increased ($P < 0.05$) body weights over controls on d 110 only.

Discussion

Plasma Cu concentrations were higher in control heifers, at the start of the study, compared to heifers in the Cu-supplemented groups. All calves were maintained on the same diet prior to the study, and initial treatment allocation was not based on plasma Cu concentration, therefore, differences among treatment groups on d 0 were random. Dietary Cu supplementation to heifers, as either the sulfate or lysine form, maintained or slightly increased plasma Cu concentrations of heifers by d 68. Conversely, plasma Cu concentrations of non-supplemented heifers decreased throughout the study. Blood Cu increases in response to stress, most likely, because of increases in Cu-containing acute phase proteins in the plasma (Klasing, 1993; Nockels et al., 1993). Mobilization of hepatic Cu stores and increased gastrointestinal absorption could likewise elevate plasma Cu concentrations during stress. In this study, plasma Cu continued to decrease in the control and $CuSO_4$-supplemented group up to 6 weeks after vaccination stress. This may reflect a decreased bioavailability of the sulfate form of Cu supplement or low hepatic Cu

concentrations in these heifers at initiation of the study. Interactions with dietary Fe, in the gastrointestinal tract, may have influenced the availability of Cu in the control and $CuSO_4$-supplemented groups. High dietary concentrations of Fe, S, and Mo can disrupt intestinal absorption of Cu (Graham, 1991) and iron-induced Cu deficiency has been reported (Boyne & Arthur, 1986). Niederman et al; (1994) reported decreased serum Cu and immune cell function in gestating beef cattle that were supplemented with 1,600 mg of Cu ($CuSO_4$) and 3,000 mg of Fe/kg of trace mineral salt compared to Cu supplemented as $CuCO_3$. They suggested that ruminal reduction of $CuSO_4$ to the insoluble sulfide may result in altered bioavailability of Cu, and that Fe in high concentrations may compete with Cu for metallothionein to decrease Cu absorption from the rumen. Iron concentration of the basal diet (342 mg/kg) in this study exceeded NRC recommendations for beef cattle (1984) of 50 to 100 mg/kg. Iron could have interfered with Cu absorption and $CuSO_4$ reduction in the rumen may have altered availability.

Dietary Cu deficiency influences both Cu concentration and function of monocytes in the Lewis rat (Babu & Failla, 1990). Respiratory burst and killing ability of macrophages decreased in Cu-deficient animals, but phagocytic activity of the cell was not different from the Cu-adequate control animals. Because H_2O_2 release from cells will decline prior to their phagocytic activity, and killing ability is contingent upon this free radical-mediated process (Ho, 1989), this would suggest that respiratory burst and microbicidal activity may be more sensitive indicators of cellular Cu alterations than phagocytic activity. In the present study, phagocytosis of fluorescent beads by activated monocytes from control calves was lower than that from CuLy-supplemented calves in response to vaccination stress. Monocytes from the $CuSO_4$-supplemented group had an intermediate response, suggesting that Cu was most available for cellular functions when fed in the proteinate (CuLy) form, and that control heifers were Cu-deficient at the cellular level. Potentially, this Cu deficiency could have led to decreased phagocytic activity of monocytes. The specific components of cellular activity in which Cu deficiency influences

monocyte function (i.e. SOD, Haber-Weiss reaction, and with transferrin metabolism) have been discussed previously.

An additional physiologic function of activated monocytes includes the capacity to present antigens to T-helper cells for T cell activation (Ho, 1989). This aspect of the immune response, directed by the monocyte, may also be affected by Cu status of the animal. Antigen presentation is a multi-step process initiated by phagocytosis of antigens, followed by processing and, ultimately, recycling of antigens to the cell surface in association with MHC class II molecules (Unanue et al., 1984). Expression of MHC class II molecules (Ia Antigens) was determined in this study but the presentation of processed antigens was not evaluated. One would assume, by the nature of antigen presenting cells, that Strain RB51 LPS antigen binds to Ia and is presented on the cell surface in context with the MHC class II molecule. The study conducted by Splitter and Everlith (1986) supports this assumption in that they have shown bovine macrophages can present *B. abortus* antigens in the context of MHC class II molecules to lymphocytes.

On d 68, expression of class II molecules, stimulated with crude LPS preparation obtained from *B. abortus* rough Strain RB51, was not different between Cu-supplemented groups, but a difference was apparent between controls and CuLy-supplemented heifers. MHC class II molecule expression would be expected to increase after *in vivo* vaccination / *in vitro* stimulation based on the normal response of monocytes exposed to LPS. Monocytes from CuLy-supplemented heifers also exhibited this type of response with increased MHC expression 14 and 42 d after vaccination. The decreased fluorescent emission and number of cells responding to LPS stimulation on d 82 and 110 for the control and $CuSO_4$-supplemented calves compared to the CuLy group, suggests fewer monocytes were available for stimulation and those that were available exhibited decreased function. Profound lymphoid depletion and small germinal centers occurred in spleens of mice for up to 4 weeks post-infection with *B. abortus* Strain 19 (Stevens et al., 1994c). Repletion of the lymphocyte population was not observed until 6 weeks after the exposure

to Strain 19. Based on this observation, the decrease in monocyte number and response reported in our study could be a manifestation of vaccination with Strain 19. Potentially, Cu in the organic form, may protect monocytes, directly or indirectly, from the stress of vaccination.

Monocyte Cu concentrations were not determined in this study, but some speculation can be made as to the possible protective role of CuLy supplementation on the cellular level. Cell deactivation is a natural course of events, since macrophages do not remain activated indefinitely. It has been suggested that deactivation is accelerated by infection with intracellular organisms, e.g. *Brucella* species. Cytokines, such as gamma-interferon (IFN-¥), enhance the ability of macrophages to control growth of brucella (Jiang & Baldwin, 1993; Splitter & Everlith, 1989). Chronic infection of macrophages with *B. abortus*, however, inhibits the ability of IFN-¥ to activate macrophages and activated macrophages are rapidly deactivated. The calves in our study were vaccinated with *B. abortus* which, theoretically, should be phagocytosed and processed by monocytes. *Brucella* is known to persist intracellularly by several mechanisms (Jones & Winter, 1992; Halling et al., 1991), therefore, could act as a chronic infection and deactivation would be accelerated in those cells. The potential exists for CuLy to act in a protective role by slowing cellular deactivation and thereby enhancing monocyte activity.

Prostaglandin, a macrophage-deactivating factor, has been shown to decrease Ia-antigen expression in monocytes. Supplemental Cu may alter prostaglandin secretion by the macrophage, ultimately slowing cell deactivation. Interleukin 1 (IL-1), which also is secreted by the macrophage and directs macrophage recruitment, may be influenced by Cu concentrations and, thus, alter monocyte responses to immune challenge.

Ia (MHC class II molecule) develops mainly in young macrophages (Beller & Unanue, 1981). These newly recruited, young cells, in contrast to older macrophages, have a greater myeloperoxidase activity (Zhan et al., 1991) and, thus, have an increased Ia expression and potential for generation of hypohalides via oxidative burst. Impaired monocyte activity in control and $CuSO_4$-supplemented calves may be related to alterations

in cell membrane properties because of inadequate cellular Cu, since phagocytosis and MHC class II expression are intimately involved with the surface membrane. Hepatocyte membranes from Cu-deficient rats show depressed fluidity (Lei et al., 1988). Investigators have reported protein and lipid composition of erythrocyte membranes from Cu-deficient rats to be altered, resulting in increased erythrocyte viscosity (Johnson & Kramer, 1987; Jain & Williams, 1988). Altered membrane cholesterol levels impair the immune response (Duwe et al., 1981), and rats deficient in Cu exhibit hypercholesterolemia. Cholesterol levels were not monitored in the control or Cu-supplemented groups during our study, but this is an area for further investigation.

Under normal conditions (d 68), LPS stimulation enhances phagocytosis and MHC class II expression. Vaccine stress, however, (i) decreased phagocytic capacity in control and $CuSO_4$-supplemented groups and (ii) decreased the class II expression in response to *in vitro* LPS stimulation in all but the CuLy-supplemented group. Cu-Lysine supplementation appears to make monocytes more responsive to a stimulant like LPS, therefore, able to exhibit better non-specific defense mechanisms for a stronger immune response.

Physiological determinants, such as immune cell dysfunction, are theorized to be sensitive, early indicators of nutrient deficiency (Babu & Failla, 1990; Mills, 1987). More traditionally, however, nutritional adequacy is determined by clinical signs and production changes. Nutrient supplementation in beef cattle is commonly dictated by gain potential which, in turn, affects the profit margin of the producer. This study indicated that dietary Cu supplementation at levels approximately twice that of current beef NRC (1984) recommendations (8-10 mg Cu daily), resulted in significant body weight gains for beef calves. Trace mineral supplementation has been shown to optimize production in beef animals (Chirase et al., 1991; Hermel, 1992). This suggests that certain micronutrient requirements may be understated by current NRC recommendations, especially for optimal immune response in the growing animal. The potential profit margin, based solely on weight gain and dietary Cu supplementation for this experiment, was calculated to be

approximately $20/calf. Enhanced immune responsiveness associated with dietary Cu supplementation, especially during stress-related management practices such as vaccination, likewise, has the potential to decrease costs associated with feeder calf production.

Table 1. Nutrient and energy composition of basal diet fed to heifer beef calves[1]

Item	Nutrient concentration (DM basis)
	%
Crude protein	11.5
Acid detergent fiber	28.7
Neutral detergent fiber	46.7
Calcium	0.29
Phosphorus	0.29
Potassium	1.50
Magnesium	0.22
NE_m	1.61 Mcal/kg
NE_g	1.08 Mcal/kg
	mg/kg
Copper	7.0
Molybdenum	1.7
Zinc	34.0
Manganese	51.0
Iron	342.0

[1]Diet consisted of corn silage fed ad libitum plus 0.7 kg soybean meal/hd/d.

Table 2. Composition of mineral supplement fed to heifer beef calves[1]

Mineral	Concentration
	%
Calcium	21.2
Phosphorus	6.0
Sodium	6.6
Chloride	10.3
Potassium	1.0
Magnesium	1.5
Sulfur	1.5
Iron	0.044
	mg/kg
Copper[2]	0.35
Iodine	35.00
Cobalt	16.00
Selenium	52.00
Zinc	160.00
Manganese	151.00

[1]Mineral top-dressed daily at 113 g/hd/d.

[2]$CuSO_4$ and CuLy treatments contained 600 mg Cu/kg.

Table 3. Effect of Cu treatment on plasma Cu concentrations of heifer beef calves[1]

	Plasma Cu (μg/ml)			
Treatment	Day 0	Day 68[2]	Day 82	Day 110
Control[3]	0.73[a]	0.71[a]	0.53[a]	0.49[a]
$CuSO_4$[4]	0.66[b]	0.67[b]	0.63[b]	0.59[b]
CuLy[5]	0.64[b]	0.65[b]	0.59[ab]	0.62[b]
SEM	0.03	0.03	0.03	0.03

[1]Values are means for n=15 calves/treatment.

[2]Vaccination with *B. abortus* Strain 19.

[3]Mineral mix with no Cu added.

[4]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[5]Cu supplied as CuLy (600 mg Cu/kg)in mineral mix.

[a,b]Concentrations within each column with different superscripts differ ($P < 0.05$).

Table 4. Effect of Cu treatment on phagocytic activity of monocytes in heifer beef calves[1]

	Mean channel fluorescent emission [2]			Total monocytes [3]		
Day	68[4]	82	110	68	82	110
Treatment						
Control[5]	20.7	4.9[a]	26.4	37.2	1.7[a]	7.5
$CuSO_4$[6]	22.2	6.6[a]	28.2	34.3	2.1[a]	10.4
CuLy[7]	26.0	12.4[b]	31.0	27.4	3.1[b]	17.7
SEM	3.6	1.4	2.0	5.17	0.21	2.31

[1]Values are means for n=15 calves/treatment.

[2]Mean fluorescence of the responding cells, where fluorescence is defined as channel number, channel range 1 to 256.

[3]Number of cells phagocytizing fluorescent beads in 100 cells.

[4]Vaccination with *B. abortus* Strain 19.

[5]Mineral mix with no Cu added.

[6]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[7]Cu supplied as CuLy (600 mg Cu/kg) in mineral mix.

[a,b]Values within each column with different superscripts differ ($P < 0.05$).

Table 5. Effect of Cu treatment on monocyte MHC class II expression in heifer beef calves[1]

	Mean channel fluorescent emission [2]			Total monocytes[3]		
Day	68[4]	82	110	68	82	110
Treatment						
Control[5]	81.7[a]	69.4[a]	100.9[a]	44.5	12.8[a]	45.5
$CuSO_4$[6]	85.5[ab]	75.9[b]	120.9[b]	47.2	9.8[a]	46.8
CuLy[7]	92.1[b]	96.6[c]	133.6[c]	47.5	20.9[b]	53.2
SEM	3.3	2.9	5.2	3.77	1.14	1.62

[1]Values are means for n=15 calves/treatment.

[2]Mean fluorescence of the responding cells, where fluorescence is defined as channel number, channel range 1 to 256.

[3]Number of cells expressing MHC class II molecules in 100 cells.

[4]Vaccination with *B. abortus* Strain 19.

[5]Mineral mix with no Cu added.

[6]Cu supplied as $CuSO_4$ (600 mg Cu/kg) in mineral mix.

[7]Cu supplied as CuLy (600 mg Cu/kg) in mineral mix.

[a,b,c]Values within each column with different superscripts differ ($P < 0.05$).

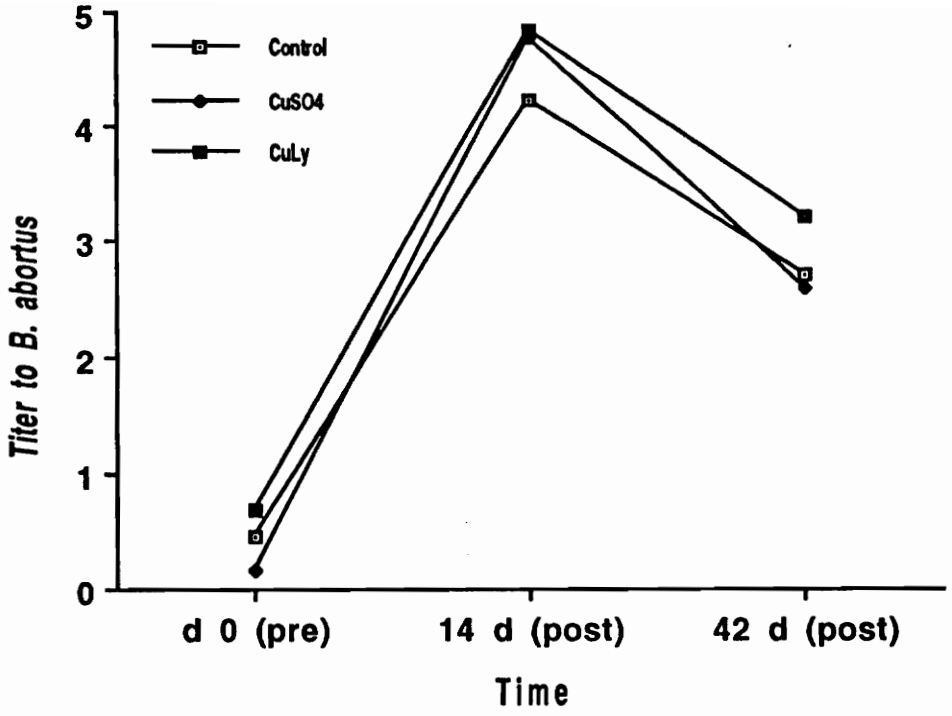

Figure 1. Agglutinin titers to *B. abortus* diagnostic antigen of control, $CuSO_4$-supplemented and CuLy-supplemented beef calves pre- and post-vaccination with *B. abortus* Strain 19. Serial two-fold dilutions (1:25 to 1:800) were converted to a linear scale 0 to 5. Points depict the mean dilution which appeared positive for each treatment group.

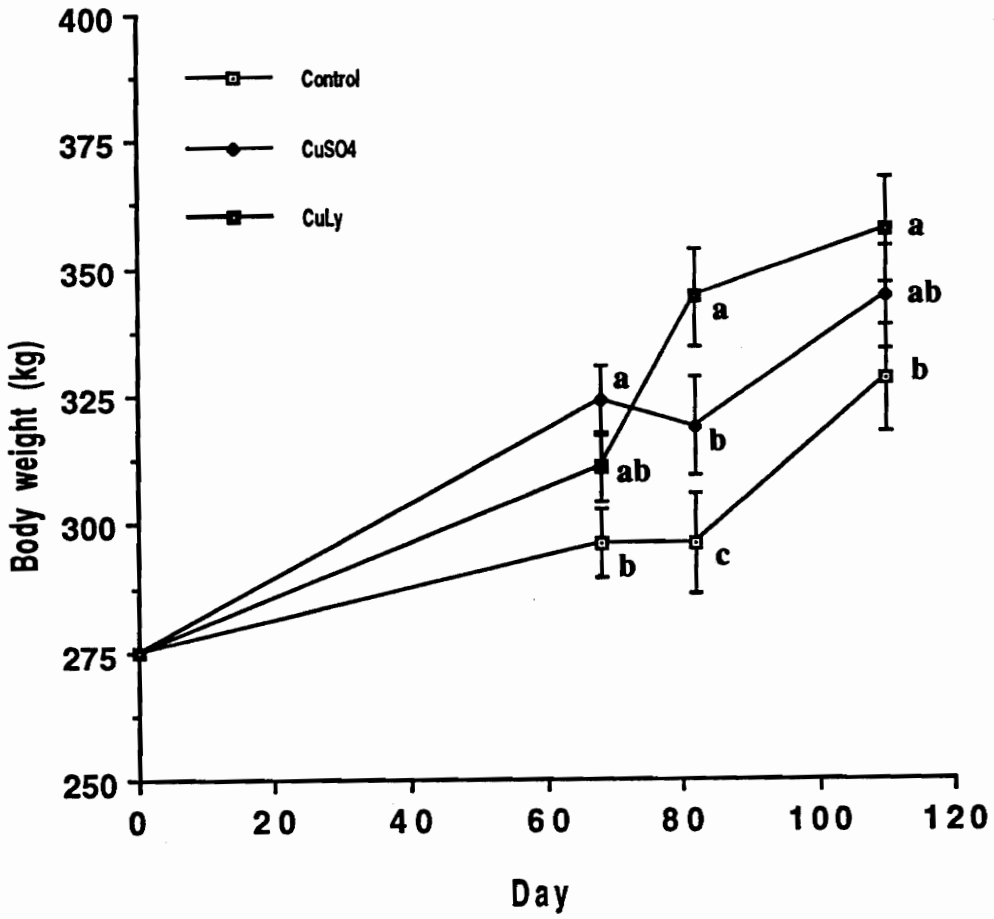

Figure 2. Body weights of control, $CuSO_4$-supplemented and CuLy-supplemented heifer beef calves on d 0, 68, 82, and 110. Diet consisted of corn silage fed ad libitum plus 0.7 kg soybean meal/hd/day. Copper content of the mineral supplement was: Control group - 0.35 mg Cu/kg; $CuSO_4$ and CuLy groups - 600 mg Cu/kg. Points depict the mean body weight +/- SEM for treatment groups. ^{a,b,c}Significant difference ($P < 0.05$) among treatment groups within day.

Summary

Evaluation of selected immune responses in beef calves, supplemented with dietary Cu, was initiated following the development of a reliable *in vitro* system designed to study bovine monocyte function. Density gradient separation of leukocytes, followed by culture flask adherence of monocytes, is the *in vitro* system that consistently resulted in isolation of a viable bovine monocyte population. Though technologically more advanced techniques such as immunomagnetic cell separation are continually being introduced for cell culture study, they are cost prohibitive and inhibit cell function.

Dietary Cu supplementation above current NRC recommendations for beef cattle stimulated monocyte function in growing beef calves. Copper supplementation in the form of an amino acid complex (Cu-Lysine) enhanced monocyte response, in 6-9 month old beef calves, to both *in vivo* and *in vitro* immune challenge, as compared to the inorganic salt (Cu-Sulfate), during three separate study periods. Vaccination with *Brucella abortus* Strain 19 constituted the *in vivo* challenge. Monocyte oxidative burst, phagocytic activity and MHC class II expression were increased post-vaccination stress in calves supplemented with dietary Cu compared to controls. Copper status (plasma Cu, hepatic Cu, and ceruloplasmin activity) was maintained in Cu-supplemented calves in response to vaccination stress, whereas, Cu status declined in non-supplemented calves during each study period. Existing guidelines for dietary Cu may need to be re-evaluated, particularly in regards to cattle undergoing increased physiological stress. The authors' work suggests that increasing the dietary Cu recommendations to 20 ppm , dry matter basis, may optimize the health and production of growing beef calves.

The *in vitro* challenge was directed at the isolated monocytes obtained from Cu-supplemented and non-supplemented calves both pre- and post-vaccination with *B. abortus* Strain 19. Lipopolysaccharide (LPS) extracts from *B. abortus* rough Strain RB51 and smooth Strain 2308 were compared as *in vitro* stimulants of bovine monocyte function. The rough Strain RB51 LPS promoted MHC class II expression on the cell surface to a

greater degree than smooth Strain 2308, and was determined to be useful for *in vitro* bovine studies.

Bovine monocyte function has been shown to be altered by dietary Cu supplementation, therefore, one would suspect that a positive correlation exists between Cu and monocyte activity as measured by oxidative burst, phagocytosis, or surface molecule (MHC class II antigen) expression. Simple linear regression analysis revealed R^2 values ranging from 0.03 to 0.83 for correlations between Cu status and select monocyte function. The weakest correlative values were seen between plasma Cu vs phagocytosis (0.03 to 0.06) and between plasma Cu vs ceruloplasmin (0.03 to 0.40), but correlation coefficients varied depending on time of measurement. Ceruloplasmin activity has been reported,by numerous investigators, as an indicator of Cu status. The weak to moderate correlations between plasma Cu vs ceruloplasmin, calculated from data obtained in these studies, may be reflective of enzymatic assay inaccuracies. Samples obtained at the start of the study were initially stored at -20 C then transferred to the -70 C freezer, and this may have altered enzymatic activity. As with many enzyme assays, the micro-method technique used by the author for determination of ceruloplasmin activity, was a temperature-dependent kinetic assay, therefore, errors were likely to have occurred which may have lead to inaccuracies in the results. Further investigation regarding the concept of ceruloplasmin receptors is needed, because if they exist, this may be a more stable indicator of ceruloplasmin status compared to plasma or serum enzyme activity.

Although the correlation coefficient for liver Cu vs phagocytosis was 0.21, that for liver Cu vs monocyte H_2O_2 release was stronger at 0.52. The linear regression analyses of plasma Cu vs H_2O_2 release (0.33 to 0.41) and Cu vs MHC class II expression (0.59 to 0.83) showed the strongest correlations. This suggests that further investigations to determine a reliable indicator of Cu status in beef cattle should include relationships between variables that show the strongest correlations, such as, monocyte MHC class II expression, H_2O_2 release, and Cu concentrations in liver and blood. The numerical

significance of these relationships, however, should be interpreted in light of the apparent variability inherent in biological systems. An expanded data base for Cu-dependent physiological parameters, such as immune cell function, would strengthen the interpretive value of immunoresponsiveness as an indicator of Cu status. More standardized criteria could possibly be developed through the use of multiple regression analyses, thereby utilizing both physiological and clinical parameters related to Cu status.

In closing, the author believes that marginal Cu deficiency is manifested as subtle production losses and often goes undetected. Optimizing resistance to immune challenge through adequate trace mineral nutrition is paramount to enhancing production. Dietary Cu supplementation is one avenue by which to optimize the immunocompetence of growing beef calves.

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Appendix A

Ficoll-Hypaque Density Gradient Separation

Purpose: Removal of a mixed leukocyte population from whole blood.

Reference: Goddeeris BM, Baldwin CL, ole-Moi Yoi O, Morrison WI. Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. *J. Immumol. Methods* 1986;89:165-173.

Materials:

1. Alsevars® solution - (Sigma Chemical Co.)
2. Histopaque 1077® - (Sigma Chemical Co.)
3. Hanks balanced salt solution (HBSS) - (Gibco / Brl)
4. Trypan blue stain - (Sigma Chemical Co.)
5. 50 ml polystyrene conical tubes - (Fisher Scientific)
6. Hemocytometer

Method:

1. In a 30 cc syringe, collect 15 ml of whole blood into 15 ml of Alsevars® solution from each animal.
2. Place 15 ml Histopaque 1077® into a 50 ml conical tube. Using a 20 gauge needle attached to the 30 cc syringe, gently layer the whole blood/Alsevars® sample over the Histopaque.
3. Centrifuge tubes at 2200 x g, for 35 min. at 27 C. Gently remove from centrifuge.
4. Transfer the mixed monocyte / lymphocyte layer to a clean 50 ml conical tube using a glass pasteur pipette.
5. Add 25 ml HBSS to the transferred cells and centrifuge at 1000 x g, for 10

min. Pour off supernatant.

6. Resuspend the pelleted cells in 25 ml HBSS and centrifuge at 1000 x g, for 10 min. Pour off supernatant.
7. Resuspend the cells in 10 ml HBSS.
8. Remove a small sample of suspended cells from each sample and determine viability with trypan blue stain and a hemocytometer.
9. Count the remaining cells in each sample with a hemocytometer and bring cells to a final concentration of 1 x 10^6 cells/ml.

Appendix B

Immunomagnetic Separation Technique

Purpose: The indirect, "negative selection" method for immunomagnetic cell separation involves attaching monoclonal antibodies to magnetic beads. The monoclonal coated beads are then incubated with a mixed cell population. The cell type which is not desired is bound to the monoclonal antibody and then magnetically removed from solution leaving the desired cell type free for easy retrieval.

In this technique, the mixed monocyte / lymphocyte cell layer removed from the Ficoll-Hypaque density gradient separation was incubated with Biomag® magnetic beads (Advanced Magnetics Inc., Cambridge MA), which were activated with a cocktail of CD_4 and CD_8 monoclonal antibodies. Protocol for negative selection of monocytes involved 4 steps.

Reference: Biomag® and Magnetic Separations. Advanced Magnetics Inc., Cambridge, MA. 1989.

Method:

Step 1: Activation

Add 100 ml Coupling Buffer to 24 ml BIOMAG® in a 25 cm^3 flask. Shake the flask vigorously and magnetically separate by placing BioMag magnet on a flat surface and lay the flask on top of the magnet. Leave this unit in place for 10 minutes. Repeat this process 3 times. Add 48 ml of 5% glutaraldehyde to the flask and rotate on table-top automatic rotator for 3 hrs. Place the flask back on top of the magnet and wait 10 minutes for separation to occur. Aspirate the solution, rinse activated beads with 100 ml coupling buffer and re-separate using the magnet. Repeat 3 times.

Step 2 : Coupling

A) Assume the interface contains on average 4.0×10^7 lymphocytes

$4.0 \times 10^7 \times 0.9 = 3.6 \times 10^7$ cells x 50 particles per cell (for negative selection) = 1.8×10^9 particles required.

1.8×10^9 particles / 5×10^8 particles per ml = 3.6 ml of washed BIOMAG® (1 mg/ml Biomag® contains 5×10^8 particles per ml).

B) Add MoAb with bovine serum albumin (BSA) (using a concentration of 1.5 mg MoAb / 10 mg BSA) to 50 mg BIOMAG® for a final mixture of 3.0 mg MoAb with 20 mg BSA in 15 ml coupling buffer into the activated beads. Add MoAb-BSA to all the activated beads (2 bottles or 1,000 mg) from above activation step and rotate for 24 hrs at room temperature.

C) In a hood, magnetically separate and save supernatant for post-coupling. Add 50 ml of glycine quenching solution to activated beads, shake vigorously then rotate for 30 minutes at room temperature.

Step 3 : Washing and Diluting Coupled BIOMAG® beads

Magnetically separate and aspirate the supernatant from step 2C. Add 50 ml wash buffer, shake vigorously and magnetically separate off supernatant wash. Repeat 3 times. Store Coupled BIOMAG® at 4 C as a suspension in wash buffer (pH 4 - 10).

Step 4 : Procedure for Monocyte Isolation

Wash coupled BIOMAG® beads 2 - 3 times with sterile media and place in a 25 cm^3 flask. Add mixed monocyte / lymphocyte cell suspension, incubate for 15 - 30 minutes at 4 C. Gently invert the flask every 10 - 15 minutes. Magnetically remove lymphocyte population. Gently aspirate supernatant (monocytes) and wash in HBSS twice. Pellet monocytes, dilute in 1 ml buffer and count recovered monocytes. Proceed with flow cytometry measure of H_2O_2 release and phagocytic activity.

Appendix C

Culture Flask Adherence Technique

Purpose: To obtain a purified monocyte population from a mixed cell suspension of lymphocytes and monocytes.

Materials:

1. Complete media - RPMI 1640; 10% fetal bovine serum; 100 U penicillin per ml; 100 μg streptomycin per ml; 2 mM L-glutamine; 1 mM Na-pyruvate (Sigma Chemical)
2. HBSS-EDTA - Hanks balanced salt solution (HBSS) (Gibco / Brl) plus 10 mM Ethylenediaminetetracetic acid (EDTA) (Sigma Chemical Co.)
3. 75 cm^2 cell culture flasks - (Fisher Scientific)
4. Cell scraper - (Fisher Scientific)
5. 50 ml polystyrene, conical tubes - (Fisher Scientific)
6. 37 C, humid incubator ; 5% CO_2

Method:

1. Prepare cells according to the procedure outlined in Appendix A.
2. Dilute cells to a concentration of 1 x 10^6 cells/ml in complete media.
3. Add 1 ml of each cell suspension to a 75 cm^2 flask, then add an additional 9 ml of complete media to each flask.
4. Incubate flasks for 4 hr at 37 C, 5% CO_2.
5. Gently pour off the media in all flasks and replenish with 10 ml of fresh complete media.
6. Incubate flasks for 16 hr at 37 C, 5% CO_2.
7. Gently pour off the media in each flask; add 5 ml of HBSS-EDTA at 4 C.

Let the flasks stand undisturbed for 12 min.

8. Gently scrape the cell layer with a cell scraper and transfer the cells into a 50 ml conical tube.
9. Rinse the flask with 10 ml HBSS-EDTA at 4 C and add wash to the conical tube.
10. Centrifuge cells at 1000 x g, for 10 min. at room temperature. Pour off the supernatant.
11. Resuspend the cell pellet in 10 ml HBSS.
12. Adjust the cell concentration to 1 x 10^6 cell/ml in appropriate buffer for monocyte testing.

Appendix D

Measurement of Hydrogen Peroxide Production using DCFH-DA

Purpose: Measurement of cellular activation and oxidative metabolism. Dichlorofluorescin diacetate (DCFH-DA) is a non-fluorescent probe except in the presence of hydrogen peroxide which is produced by cells undergoing oxidative burst. DCFH-DA diffuses into cells and is hydrolyzed by intracellular esterases to a non-fluorescent analog, 2', 7' - dichlorofluoresin (DCFH), which is trapped within the cell. As hydrogen peroxide (H_2O_2) is produced during the oxidative respiratory burst, DCFH is oxidized to the highly fluorescent 2', 7' - dichlorofluorescein (DCF).

Reference: Bass DA, Parce JW, Dechatelet LR, et al. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J Immunol* 1983;130:1910-1917.

Materials:

1. Hanks balanced salt solution (HBSS) - used as a suspension media for cells
2. 5 mM, 2', 7' - dichlorofluorescin diacetate (M.W. = 487.29) in ETOH.
3. Water bath, 37 C
4. Stimulant of choice - for Chapter 3 and 4, used cytochalasin B (1/100 dilution of stock solution) in HBSS
5. 12 x 75 mm polypropylene tubes

Method:

1. Obtain monocytes from whole blood as described in Appendix A and C.
2. Resuspend cells to a final concentration of 1-1.5 x 10^6 cells/ml in HBSS.

3. Add 1 µl/ml of 5 mM DCF-DA for a final concentration of 5 µM DCFH-DA.
4. Incubate the cells in a 37 C waterbath for 15 min.
5. Aliquot 1 x 10^6 cells/ml into two tubes.
6. Place 12.5 µl of cytochalasin B into one tube. Place 12.5 µl HBSS into the other tube (as a negative control).
7. Incubate sample tubes in 37 C waterbath for 10 min.
8. Analyze cells on a flow cytometer set to emit at 525 nm and measure the shift in fluorescence intensity.

Determination of H_2O_2 release: Release of H_2O_2 is expressed as the mean channel fluorescence and / or the percent cells resulting from the subtraction of the negative control sample from the cytochalasin B-stimulated sample. Fluoresence is defined as the channel number, channel range 0 to 256.

Appendix E

Phagocytosis Assay

Purpose: Phagocytic capacity of monocytes is determined by their ability to phagocytize fluorescent polystyrene beads.

Reference: Steinkamp JA, Wilson JS, Saunders GC, Stewart CC. Phagocytosis: Flow cytometric quantification and fluorescent microspheres. *Science* 1982;215:64-66.

Materials:

1. Krebs Ringer bicarbonate-gelatin solution (KRH) - Mix 97 ml H_2O; 10 ml 9% NaCl; 616 µl 1 M KCl; 600 µl 0.5 M MgSO4; 20 ml 0.1M Hepes-TEA (pH 7.4); 0.108 g Knox unflavored gelatin
2. Homologous sera
3. Polystyrene beads - Fluoresbrite® Beads, 1 micron / FITC conjugated (Polysciences), diluted 1:10 in phosphate buffer solution (PBS)
4. PBS-gelatin-EDTA - Mix 100 ml PBS; 0.1 g Knox unflavored gelatin; 0.1 g EDTA, disodium salt
5. 50 ml round bottom Nalgene tubes

Method:

1. Obtain monocytes from whole blood as described in Appendix A and C.
2. Dilute cells to 1 x 10^6 cells/ml in KRH-gelatin.
3. Place 0.5 ml of cells from each sample into a round bottom tube labelled cold, and 0.5 ml of cells into a tube labelled warm.
4. To each tube add 50 µl homologous sera and 10 µl of beads.

5. Incubate cold-labelled tubes at 4 C and warm-labelled tubes at 37 C for 60 min., no agitating.
6. Place tubes at room temperatue and add 5 ml PBS-gelatin-EDTA to each tube.
7. Centrifuge tubes at 500 x g, for 5 min at room temperature and discard the supernatant.
8. Resuspend cell pellet in 1 ml HBSS.
9. Analyze cells on a flow cytometer set to emit at 525 nm and measure the shift in fluorescence intensity.

Determination of phagocytic activity: Phagocytic activity is expressed as the mean channel fluorescence and / or the percent cells resulting from the subtraction of the 4 C (cold-incubated) control sample from the 37 C (warm-incubated) sample. Fluoresence is defined as the channel number, channel range 0 to 256.

Appendix F

Measurement of Bovine Monocyte MHC Class II Expression

Purpose: To evaluate the immune responsiveness of bovine monocytes by measuring the class II glycoproteins encoded genes of the major histocompatability complex (MHC). Monocytes phagocytize, and process antigens, which are presented on the cell surface via MHC class II molecules, to activate T cells.

Reference: Splitter GA, Everlith KM. *Brucella abortus* regulates bovine macrophage-T-cell interaction by major histocompatibility complex class II and interleukin-1 expression. *Infect Immun* 1989;57:1151-1157.

Materials:

1. Hanks balanced salt solution (HBSS) - (Gibco / Brl)
2. Monoclonal antibody - anti-MHC class II for bovine (TH14B), diluted 1:10 in HBSS. (VMRD, Inc.)
3. Fluorescein (FITC) - conjugated Affinipure Rabbit Anti-Mouse IgG (H+L). (Jackson ImmunoResearch Laboratories, Inc.)
4. 12 x 75 mm polypropylene tubes

Method:

1. Obtain monocytes from whole blood as described in Appendix A and C.
2. Dilute cells to 1 x 10^6 cells/ml in HBSS for a total of 2 ml.
3. Place 1 ml of cells/sample in each of two polypropylene tubes labelled FITC (-) and MHC-FITC (+).
4. Add 4 µl of monoclonal antibody to the (+) tube, and 4 µl of HBSS to the (-) tube. Incubate all tubes on ice for 1 hr.

5. Centrifuge tubes at 1000 x g, for 10 min at room temperature. Pour off supernatant.
6. Resuspend pelleted cells in 1 ml HBSS.
8. Add 4 µl of FITC to all tubes. Incubate all tubes on ice for 1 hr.
9. Centrifuge tubes at 1000 x g for 10 min. at room temperature. Pour off supernatant.
10. Resuspend pelleted cells in 1 ml HBSS and analyze on flow cytometer set to emit at 525 nm.

Determination of MHC class II expression: Expression of MHC class II molecules is measured as the mean channel fluorescence and / or the percent cells resulting from the subtraction of the FITC only (-) sample from the MHC-FITC (+) sample. Fluoresence is defined as the channel number, channel range 0 to 256.

Vita

Korinn Edna Saker was born on July 10, 1958 in Freehold, New Jersey, the daughter of Gretchen E. Saker and the late Raymond J. Saker. She graduated from Freehold Township High School in June, 1976. She received her Bachelor of Science degree in Animal Science from Purdue University in May, 1980. In August of 1983, the author received her Master of Science degree in Animal Nutrition at Clemson University. She received her Doctor of Veterinary Medicine from the University of Georgia, College of Veterinary Medicine in June, 1987 and moved to the New England area to practice veterinary medicine until beginning her Doctor of Philosophy / Clinical Residency program in 1992 at the Virginia-Maryland Regional College of Veterinary Medicine. She completed the Residency program in Clinical Nutrition in May, 1995.

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