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THE EFFECTS OF GLUTAMINE SUPPLEMENTATION IN HEALTHY AND INFECTED EARLY WEANED PIGS

By

Susan Semie Yoo 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

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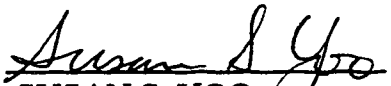

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE EFFECTS OF GLUTAMINE SUPPLEMENTATION IN HEALTHY AND INFECTED EARLY WEANED PIGS in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN NUTRITION AND METABOLISM

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This manuscript is dedicated to my loving parents and sister.

ABSTRACT

The effects of glutamine supplementation in healthy and infected weaned pigs were investigated. Pigs (21d old) were fed either an elemental diets supplemented with glutamine (+GLN) or an equal amount of non-essential amino acids (-GLN). At 26 days of age, animals were intraperitoneally injected with *E.coli* (+Ecoli; 0.5 x 10^8 CFU/kg BW) or buffered saline (-Ecoli) and sacrificed at 28 days of age.

Infection decreased ($P<0.05$) plasma and intramuscular glutamine concentrations but animals that received +GLN diets had higher intramuscular glutamine levels than those that received -GLN diets. Infected animals had elevated ($P<0.05$) total leukocyte counts and blood lymphocyte responses to PMA+IONO were reduced ($P<0.05$). Glutamine supplemented pigs had increased ($P<0.05$) WBC, RBC, HGB, HCT and MCH hemological values, and blood lymphocyte responses to ConA were greater ($P<0.05$) than -GLN animals. The peak response of +Ecoli+GLN animals were greater ($P<0.05$) than +Ecoli-GLN and similar to those of healthy animals.

Hence, glutamine supplementation maintained muscular glutamine concentrations and normalize lymphocyte function in the infected pigs.

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LIST OF ABBREVIATIONS

ANOVA - analysis of variance

BCAAs - branched-chain amino acids

BW - body weight

CD2 - T-cells

CD4 - T-helper cells

CD8 - T-cytotoxic/suppressor cells

CFU - colony forming units

Con A - concanavalin A

DPM - disintegrations per minute

E.coli - *Escherichia coli*

+E.coli - animals intraperitoneally injected with *Ecoli* at 0.5 x 10^8 CFU / kg BW

-E.coli - animals intraperitoneally injected with phosphate buffered saline

+GLN - animals receiving the glutamine (4%, w/w) supplemented diet

-GLN - animals receiving diet without glutamine supplementation

HCT - hematocrit

HGB - hemoglobin

HPLC - high-performance liquid chromatography

ICH_20 - intracellular water

IIP - Illinois ideal amino acid pattern

IL - interleukin

IP injection - injection into intraperitoneal cavity

LD - longissimus dorsi

mAB - monoclonal antibodies

MCH - mean corpuscular hemoglobin

MCHC - mean corpuscular hemoglobin concentration

MCV - mean corpuscular volume

MHC - major histocompatibility complex

MLN - mesenteric lymph nodes

PBS/2 % BSA - phosphate buffer saline with 2% (w/v) bovine serum albumin

PLATE - platelets

PMA + IONO - phorbol myristate acetate with ionomycin

RBC - red blood cells

RDW - red cell distribution width

SEM - standard error of the mean

SI - stimulation index

TCR:CD3 complex - T-cell receptor:CD3 complex

TNF - tumor necrosis factor

TPN - total parenteral nutrition

TSB - tryptic soy broth

WBC - white blood cells

1. INTRODUCTION

Central to the interrelationship between nutritional status and infection in mammals is the immune system. Numerous epidemiological and clinical studies have shown nutritional deficiency is associated with impaired host resistance to infectious diseases. The development of infection leads to hypermetabolic state which further affects nutrient intake and metabolism, and subsequently alters immune function (Figure 1). The examination of nutrition-immunity-infection interactions has provided the bases for immunoregulation via nutrition. The immune system can be modulated by the use of specific nutrients to reduce the morbidity and mortality associated with severe malnutrition. Hence in the management of infectious diseases, nutritional interventions that improve the immune system are of interest.

1.1. HOST DEFENSIVE MECHANISMS - IMMUNITY

From the time of birth to death, animals and humans are subjected to the constant threat of invasion by arrays of infectious and damaging agents. The body has two types of immunity that uniquely recognise and resist foreign attacks. The innate or natural immunity is composed of general inborn defence mechanisms that retain and destroy invading pathogens within the periphery. These protective mechanisms do not require previous exposure or processing of the foreign agents. In contrast, specific or acquired immunity is an adaptive process that provides protection from previously sensitized extracellular and intracellular pathogens. The response involves

specific recognition and an immune memory system that mediates rapid augmented reactions to subsequent confrontation of foreign stimulants known as antigens.

1.1.1. INNATE IMMUNITY

The first line of defence is the relatively impenetrable barriers, the skin and sero-mucous surfaces (Roitt, 1994). Their secretory products, such as lactic acid, mucous and gastric juices, have antimicrobial properties that create an unfavourable environment to most microorganisms. Also, the continual flushing action of these secretory products expels any trapped microbial and foreign particles and prevents stagnation and overgrowth of these invaders. The microbial antagonism associated with the body's normal flora and constant renewal of the epithelial cells provide additional protection.

If the infectious agents manage to penetrate the defenses of the epithelial barrier, they elicit the release of chemical mediators of the inflammatory response and the arrival of phagocytes. These chemical factors consist mainly of proteins of the kinin system, coagulation, complement cascades and vasoactive amines. The general functions of these inflammatory mediators involve limiting the spread of infection, attracting phagocytes to the infected site (chemotaxis), enhancing lysis or phagocytosis of microbes by opsonization, and facilitating specific immune responses (Kinoshita, 1991; Benhaim and Hunt, 1992; Johnston, 1993)

Central to defence against bacteria is phagocytosis. This process involves recognition and attachment to the invader, engulfment and destruction of the microbe by degranulation and oxygen radical production (Benhaim and Hunt, 1992). Macrophages and neutrophils are responsible for this specialized process. When an inflammatory response is stimulated by extracellular bacteria and their toxins, monocytes and neutrophils rapidly infiltrate the infected area in response to chemotaxtic substances. The monocytes quickly develop into activated tissue macrophages and with the neutrophils, they destroy the invading pathogens (Guyton, 1991).

Besides being effective phagocytes, stimulated macrophages are highly secretory cells (Unanue and Allen, 1987). The secretory products include proteases, complement proteins, arachidonate derivatives and cytokines. Cytokines are a group of proteins that mediate and regulate the cells of the immune and inflammatory response as well as promote growth and differentiation of hematopoietic cells. Tumour necrosis factor (TNF) and interleukin-1 (IL-1) are two of the major cytokines produced by macrophages in response to endotoxin from gram-negative bacteria (Lowry, 1993). At low concentrations, these cytokines activate and facilitate the functions of phagocytes and endothelial cells, as well, they act as costimulators for lymphocytes (Abbas et al., 1991). They also stimulate the cascade of cytokine products (IL-1, IL-2, IL-6 and IL-8) by macrophages and other cell types. However, if the stimulus for TNF and IL-1 is strong, they are synthesized in large quantities and released into the bloodstream (Abbas et al., 1991). At high concentrations, they act as

endogenous pyrogens which activate acute phase responses (Saez-Llorens and Lagrutta, 1993).

1.1.2. SPECIFIC IMMUNITY

Central to the operation of specific immunity are the lymphocytes. Infection is associated with increased total lymphocyte counts (Morath et al., 1981). Lymphocytes originate from the common hematopoietic stem cells present in the bone marrow where they can remain to mature into B lymphocytes or migrate to the thymus to develop into T lymphocytes. As these cells mature, they acquire surface receptors that are involved in the development of self tolerance, antigen-specificity and other biological functions. The expressions of these receptors change with the state of maturation and functional activities of the lymphocytes. These receptors have been characterised by monoclonal antibodies and serve as phenotypic markers known as clusters of differentiation (CD) which are used to identify subsets of lymphocyte populations (Bellanti et al, 1994). For example, CD4 and CD8 represent the T lymphocyte subsets, T-helper and T-cytotoxic/suppressor cells, respectively. These function-specific lymphocytes circulate throughout the body's peripheral lymphoid tissues (lymph nodes, spleen and mucosa-associated lymphoid tissues) via afferent blood and efferent lymph to continually survey for antigenic insults (Duijvestijin and Hamann, 1989).

Specific immunity is based on the special properties of lymphocytes which are responsible for two types of immunologic reactions - humoral and cell-mediated immunity. These responses interact synergistically which are co-ordinated by the actions of cytokines, and they are further enhanced by the co-operation of the innate immunity. This interaction is illustrated by specific immune responses to extracellular bacteria.

Humoral immunity is initiated by the binding of the bacterial antigens to the surface immunoglobulins (sIg) of the B lymphocytes and the presentation of the antigen by macrophages or other antigen-presenting cells to T-helper lymphocytes. Activated T-helper cells facilitate the humoral response by secreting IL-2, IL-4 and IL-5 which are important for antibody synthesis, phagocytic and microbicidal functions of phagocytes. Stimulated B lymphocytes proliferate and differentiate into plasma cells. Plasma cells are responsible for the effector response of humoral immunity since they produce and secrete antibodies against the same antigen that initiated the stimulation. These antibodies are released as membrane-associated and secreted forms and have isotype-specific functions (Bellanti et al, 1994). Membrane immunoglobulins present on the surface of a B lymphocyte act as receptors for specific antigens and initiate the humoral response as described above. The secreted antibodies are involved in the effector functions of the immune system. These antibodies can directly inactivate the invading agent by neutralising toxins or agglutinating and precipitating the antigens (Guyton, 1991). As well these antibodies opsonize bacteria to enhance phagocytosis and precoat target cells for antibody dependent cell-mediated cytotoxicity by activating the complement system.

The cell-mediated immune response involves the actions of T lymphocyte subsets. T-cytotoxic/suppressor and T-helper cells have specific roles that regulate the destruction and removal of foreign microbes (Abbas et al., 1991). T-cytotoxic/suppressor cells can lyse infected target cells, provide negative feedback to inhibit antibody reactions, and down-regulate inflammatory responses. Through the production of lymphokines, T- helper cells mediate the action of other immune cells such as phagocytes, B-cells and cytolytic T-cells. Hence, cell-mediated immunity involves direct cellular killing of pathogens by cytotoxic cells or indirect killing by the activities of innate immunity under the regulation of specific immunity.

Essential to T-cell activation and function is the specific receptor system. Unlike B cells that recognise soluble intact antigens, T-cells have receptors that only recognise foreign antigens if displayed as peptide fragments bound to specialised molecules called major histocompatibility complex (MHC) (Bellanti et al, 1994). There are two types of MHC gene products. Class I MHC molecules are found on the surface of nucleated cells in the body, whereas class II MHC molecules are mainly found on antigen-presenting cells such as macrophages, B-cells, and Langerhans-dendritic cells of the skin and lymphoid organs (Unanue and Allen, 1987). The two classes of MHC molecules allow presentation of antigens derived from intracellular pathogens. Thus, viruses, certain bacteria and parasites which can enter cells and multiple safely from humoral immunity and phagocytosis are recognized and destroyed by the T-cells.

The immune system is effective in protecting and eliminating various infectious and damaging agents. However, these active defensive mechanisms require continuous supply of nutrients. For this reason, nutritional deficiency or imbalance can lead to functional impairment of immunity. Hence, this dependency on nutrient supply is an important determinant of host resistance to infection.

1.2. NUTRITION AND IMMUNITY

1.2.1. MALNUTRITION AND IMMUNE RESPONSE

Nutritional deficiencies are the most common causes of secondary immune dysfunction in humans and animals. Studies in developing countries and hospitals have shown that malnutrition affects cell-mediated immunity, lymphocyte subsets, complement system, phagocyte function, secretory antibody response, and antibody affinity (Chandra and Sarchielli, 1993). Because of the close interactive relationship between nutrition and immunity, immunological tests are important functional indices of nutritional status. These immunologic measurements are useful parameters in identifying populations at risk for whom nutritional support and immunomodulation may be beneficial, as well, to help monitor or predict the outcome of dietary treatments (Harvey et al., 1981; Chandra, 1988).

Cell-mediated immunity seems to be most affected by nutritional deficiency. Delayed cutaneous hypersensitivity and mitogen-induced lymphoproliferation tests are often used to assess cellular immune function in vivo and in vitro, respectively. In

moderate to extreme malnourished patients, responses to skin testing of both recall and new antigens involved in delayed cutaneous hypersensitivity test are depressed (Harland, 1965; Law et al., 1973). Similarily, people on energy-reduced diets displayed reduced [^{3}H]-thymidine uptake by lymphocytes stimulated by phytomitogens (Holm and Palmblad, 1976; Field et al, 1990). These mitogens such as concanavalin-A and phorbol myristate acetate with ionomycin activate in vitro blast transformation, DNA synthesis and cell division of T lymphocytes in man and animals (Janossy and Greaves, 1972; Davis and Lipsky, 1985).

Impaired cell-mediated immunity may also be related to alterations in lymphocyte subpopulations. Hence, changes in peripheral lymphocyte counts and T-cell subsets ratio are used to assess immunocompetence. Decreased lymphocyte counts, especially the T-cells, are displayed both in protein-energy malnutrition and in many single nutrient deficiencies (Chandra, 1983; Ozkan et al., 1993). Also, a fall in the ratio of T-helper and T-cytotoxic/suppressor cells has been associated with immunosuppression (Hansbrough et al., 1984).

Whether malnutrition is a consequence of limited access of one or more nutrients, immunological disturbances identified with nutritional deficiency increase susceptibility to infection. This is particularly evident in young children of underdeveloped nations in whom nutritional status is an important determinant of both mortality and morbidity (Chandra, 1972; Dossetor et al., 1977; Hughes et al.,1974; Tomkins, 1981; Ozkan et al., 1993). The risk of death due to infection increase from ~0.1% in the well-nourished to 18% in severely malnourished infants

(Scrimshaw et al., 1968). These children commonly experience deficiencies of protein, iron, zinc, folate, pyridoxine and vitamins A, C and E. The absence of these nutrients are associated with immunological disturbances such as involution of the thymus and other lymphoid tissues, alterations in lymphocyte subpopulations, decreased cell-mediated responses, lowered levels of secretory IgA, impaired activity of phagocytes and decreased production of cytokines (Chandra, 1979 and 1992; Ozkan et al., 1993).

Weaning, the transition from the ingestion of maternal milk to solid food, seems to be a particularly vulnerable period for malnutrition and infection in mammals. The prevalence of infectious disease is highest in children between 6 to 24 months of age in many developing countries (Scrimshaw, 1977). Mata et al. (1977) reported that children of these ages experienced high rates of infection with intestinal disorders being the most common. Many of the children had diarrhoea during the ages of 18 to 23 months, the height of their protracted weaning period. This had been attributed to the replacement of breast milk with poor nutritional weaning diets and continual exposure to infectious agents present in the environment. With the loss of maternal antibodies, the gastrointestinal tract becomes vulnerable to enteropathogenic bacteria and protozoan intestinal infections (Prindull and Ahmad, 1993). Continous exposure to infectious agents may cause intestinal mucosal damage leading to chronic diarrhoea and malabsorption. Therefore, the act of weaning may predispose children to the onset of malnutrition-infection interaction leading to impaired growth and immunosuppression (Mata, 1977; Beisel, 1985).

Similarly, early weaning in piglets has been associated with increased susceptibility to intestinal disturbances, reduced voluntary food intake, poor growth rate and immunosuppression (Miller et al., 1983; Wilson et al., 1989; Rivera et al., 1978; Blecha et al., 1983). If the condition persists without complete recovery, malnutrition may develop and increase the susceptibility to diseases (Walker-Smith, 1993). Post-weaning diarrhoea and wasting pig syndrome are some of the problems arising from weaning (Sarmiento et al., 1988; Morrow-Tesch and Andersson, 1994). Although most pigs rapidly adapt to stresses of weaning and resume their normal growth rate, those pigs that have adjustment difficulties may experience prolonged catch-up growth. As a result, weaning can greatly affect the overall rate of performance and profitability of the market pig (Patience and Thacker, 1989)

Hypermetabolic states also affect immune function. In severe illness or stress conditions the body is in a catabolic state where food intakes are reduced, dietary requirements are increased, and nutrient metabolism is altered (Law et al., 1973; Bistrian et al., 1975). Protein-calorie malnutrition in various degrees is frequently found among post-surgical stress or major trauma patients. In addition, post-surgical stress and major trauma is associated with impaired antibacterial defences which involve cell-mediated immunity, antigen presentation, neutrophil and macrophage function, complement activation, and bacterial opsonization (Pietsch et al., 1977; Lundy and Ford, 1983; O'Mahony et al., 1984; Faist et al., 1986; Polk et al., 1986; White-Owen et al., 1992). Although the pathophysiologic mechanisms involved in immunologic suppression are unclear, stress hormones, cytokines, and lipid-derived inflammatory mediators contribute to the changes in the immune function (Fong and

Lowry, 1990; Baue, 1991). If the catabolic state persists, malnutrition can progress which can further compromise host immunity and predispose the patient to infectious diseases (Saunders et al., 1993; Mainous and Deitch, 1994).

1.2.2. IMMUNOENHANCING NUTRIENTS

Conversely, dietary supplementation restores immunity. The potential role of nutrients in modulating immune function is illustrated in the recovery of cell-mediated responses of malnourished subjects after nutritional therapy (Chandra, 1974; Koster et al., 1981). Repletion and supplementation of specific nutrient deficiency normalize immune response. Of particular importance are the trace elements zinc, copper, and iron, and the vitamins A, E, C, folic acid and pyridoxine (Woodward and Filteau 1990; Sherman, 1992; Bendich, 1992). Although controversy exists over the optimal nutrient mix, investigations focused on defining "optimal nutritional support" now realize that specific nutrients have the potential to enhance immune function when given in excess to the amount found in normal diets. Among the nutrients that have been shown to support immune function are omega-3 fatty acids, nucleotides, arginine and glutamine (Kinsella et al., 1990; Fanslow et al., 1988; Kirk and Barbul, 1990; Newsholme et al., 1985; Alverdy et al., 1992).

The benefits of these specific nutrients for immunocompromised host have been demonstrated by various studies. For example, omega-3 fatty acids produce eicosanoids that have less inhibitory effects on cell-mediated responses than the

eicosanoids produced from omega-6 fatty acids (Kinsella et al., 1990). Also, omega-3 fatty acids are metabolized into less inflammatory metabolites that may alleviate inflammatory damages often experienced during septic shock and endotoxemia (Lee et al., 1985; Lokesh et al., 1986). In animal studies, enteral and parenteral feeding of purified fish oil has improved survival and reduced the inflammatory response to endotoxins (Mascioli et al., 1988 and 1989). Thus, the amount and type of dietary lipids are important in the response and resistance to infection.

The importance of nucleotides, which are absent from most standard parenteral and enteral formulas, has been currently recognized. Dietary nucleotide restriction influence host immune response. Rapidly proliferating immune cells require nucleotides for DNA and RNA synthesis, and animals given nucleotide-free diet have shown immune suppression (Kulkarni et al., 1988). When compared to nucleotide supplemented diets, nucleotide-free diets are associated with low interleukin-2 production, decreased cell-mediated reactions, and low resistance to infection (Fanslow et al., 1988). In contrast, enteral diets supplemented with RNA have been shown to stimulate the maturation and phenotypic expression of T-cells, improve natural killer cell activity and increase resistance to infection (Van Buren et al., 1983 and 1990).

Arginine has been shown to improve wound healing and T-cell functions (Kirk et al., 1993). Enteral diets supplemented with arginine are associated with improved T-cell function, decreased infection rates and mortality in critically-ill patients (Daly et al., 1988; Alexander and Gottschlich, 1989). Arginine also has

powerful secretory effects on the endocrine system and is a precursor for cytoplasmic and nuclear protein synthesis, and production of nitrates, nitrites and nitric oxide (Kirk and Barbul, 1990). Nitric oxide is an important component of cytotoxic reactions and macrophage functions. Hence, these effects suggest arginine to be helpful in immunocompromised situations.

The potential benefits of glutamine are multifactorial. Like arginine, glutamine is an important nutrient for immune response but it is also supports other physiological functions (Figure 2). Glutamine is a vital energy source for the rapidly proliferating cells such as intestinal mucosa cells and lymphocytes (Ardawi and Newsholme, 1982: Souba et al., 1990b; Burke et al., 1989) and supports normal phagocytic activities of macrophages and neutrophils (Wallace and Keast, 1992; Ogle et al., 1994). Glutamine is also an essential precursor for the production of glutathione, neurotransmitters, amino sugars, nucleotides and other amino acids, and it is involved in protein synthesis, acid-base balance and nitrogen transport (Dudrick and Souba, 1991; Souba, 1992). However in critical states, the body's requirements for glutamine seem to exceed its endogenous synthesis. Thus during catabolic situations, provision of additional glutamine in the diet may be essential to optimize physiological functions.

1.3. NUTRITION AND INFECTION

1.3.1. METABOLIC ALTERATIONS ASSOCIATED WITH INFECTION

The prominent effect of infection is its influence on nutrient requirements (Scrimshaw, 1977; Bistrian, 1977; Beisel, 1985). Whether infection develops in response to post-traumatic/surgical stress or is the primary process, the body is subjected to numerous physiological alterations. Systemic reactions associated with infection involve fever, increased production of hepatic acute phase proteins, changes in blood leukocyte patterns, hypermetabolism, negative nitrogen balance and weight loss (Willmore and Kinney, 1981; Beisel, 1987). The complex processes associated with infection require higher intakes of energy and protein nutrients than post-absorptive states. Septic patients experience basal metabolic rates 20 to 50 percent above normal and may lose as much as 40 g nitrogen a day depending on the severity of their infection (Long et al., 1977; Shaw and Wolfe, 1987; Douglas and Shaw, 1989). However, the paradox of infection is the reduction of nutrient intakes due to anorexia, withdrawal of solid foods and factors that limit absorption. Hence, the body depends on endogenous glycogen, fat and protein sources (Beisel, 1984; Douglas and Shaw, 1989).

Associated with the utilization of body nutrient stores during the acute phase response, is the change in the pattern of carbohydrate, fat and protein metabolism. The clinical impact of these metabolic alterations is manifested by hyperglycemia, fluctuations of plasma free fatty acid and triglyceride concentrations, and negative nitrogen balance (Blackburn, 1977; Clowes et al., 1980; Wilmore and Kinney 1981;

Saez-Llorens and Lagrutta, 1993). Mediation of these metabolic and physiological events have been attributed to the complex interactions of immune, endocrine and neurologic systems (Beisel, 1984; Douglas and Shaw, 1989). The extent of these effects on the host depends on the severity and duration of infection, immunologic vulnerability, nutritional status and the timing of therapeutic interventions (Saez-Llorens and Lagrutta, 1993).

Negative nitrogen balance and loss of protein mass are marked effects of infection. During the early stages of infection, catabolism of skeletal muscle may be advantagous since mobilization of nitrogenous substrates provides oxidative fuel as well as substrates for synthesis of proteins important in immune, coagulation and wound healing. Nevertheless, sustained loss of skeletal muscle have negative impact on the outcome of the host since catabolism of myofibrillar proteins are required for locomotion and respiration.

Although the protein balance indicates increased rates of protein catabolism, protein synthesis rates continue simultaneously (Long et al, 1977; Douglas and Shaw, 1989). Infection simultaneously increases anabolic reactions, along with catabolic processes (Beisel, 1985). Redistribution and reutilization of nutrients provide resources for functional and structural needs involved in combating and recovering from infection. Since rapid immune response is dependent on the proliferation and differentiation of immune cells and their secretory products, the host defence system demands continual nutrients to support its actions. Additionally, the liver increases its rate of synthesis to generate proteins required in immune defence and wound

repair. These include acute-phase proteins, various plasma proteins and proteins involved in kinin, complement and coagulation systems (Beisel, 1984). More importantly, anabolic processes must continue after the elimination of the infectious agents for complete recovery since prevention of re-occurrence of infection depends on rapid wound healing, tissue repair and restoration of body nutrient stores.

Catabolic and anabolic processes maintain vital functions during the removal of infectious agents and restoration of homeostasis. These mechanisms require continuous supply of nutrients, and without nutritional support endogenous energy and protein reserves are rapidly utilized. If nutritional deficiency or imbalance develops, functional impairment of various organ systems including the host immune system will occur. In catabolic state, glutamine demand is significantly greater than its supply. Thus, the increased requirement and utilization of glutamine during the time of stress suggest that glutamine may be a conditionally essential amino acid.

1.3.2. CONDITIONAL ESSENTIALITY OF GLUTAMINE

Glutamine is the most abundant amino acid in the mammalian body. Glutamine far exceeds the concentration of other amino acids in the plasma (0.5-0.8mmol/L) and in the skeletal muscle in humans (20mmol/L intracellular water; Souba, 1992; Bergstrom et al., 1974). Glutamine is a neutral glucogenic amino acid with two amine moieties: an α-amine group and an amide group (Figure 2). This unique structure allows glutamine to account for 30 - 35% of all amino acid nitrogen

transport in the blood and makes it an important carrier of ammonia from the peripheral tissues to visceral organs (Souba, 1987). Additionally, the carbon skeleton of glutamine is often used for energy metabolism where 1 mole of glutamine results in the release of approximately 30 moles of ATP after complete oxidation (Souba et al., 1985). Hence, glutamine's abundance and structure contribute to its utilization for numerous physiological functions (Bulus et al., 1989; Dudrick and Souba, 1991).

1.3.2.a. Skeletal Muscle

Skeletal muscle is the key producer and supplier of glutamine. In humans, glutamine constitutes 61% of the free amino acid intracellular pool (excluding taurine; Bergstrom et al., 1974). Daily the muscle supplies approximately 3 times the amount of glutamine ingested in diets to support glutamine uptake by the body, especially, the portal-drained viscera (Souba, 1992). The prime source of glutamine for the intracellular pool is generated by biosynthesis via glutamine synthetase. Glutamine synthetase produces glutamine from glutamate and ammonia in the presence of ATP (Rowe, 1985; Figure 2). Glutamate is synthesized from α-ketoglutarate which can be formed indirectly from TCA cycle intermediates or directly by transamination with amino acids. The main amino acids that are oxidized for glutamine synthesis are alanine, aspartate, glutamate and the branched-chain amino acids (BCAAs - valine, leucine and isoleucine; Newsholme and Leech, 1985). Since skeletal muscle protein is mainly comprised of branched-chain amino acids, these amino acids can serve as the primary amino donors for α-ketoglutarate

(Newsholme and Leech, 1985). As for ammonia, it is a by-product of glutamate dehydrogenase and deamination of AMP to IMP (Moskovitz et al., 1994).

The rate of glutamine mobilization from muscle is regulated by membrane transporters (Newsholme and Parry-Billings, 1990). The uptake and release of glutamine transport occur simultaneously and they are mediated by sodium-dependent, hormone sensitive carriers that are shared by asparagine and histidine (Hundal et al., 1987; Ahmed et al, 1993). Kinetic parameters of glutamine transport provide a mechanism for feedback regulation of plasma glutamine concentrations by being sensitive to changes in the plasma glutamine level (Newsholme and Parry-Billings, 1990). The overall effect of this relationship allows maintenance of constant plasma glutamine concentration.

In the normal, postabsorptive state, intramuscular glutamine concentration is about 30 times greater than the concentration of glutamine in the bloodstream (Lacey and Wilmore, 1990). This large concentration gradient facilitates the net efflux of this amino acid from muscle. However during sepsis, the glutamine gradient is altered due to changes in interorgan glutamine metabolism. Studies investigating the abnormal patterns of amino acids in skeletal muscle of septic patients reveal marked reduction in intracellular glutamine and rises in phenylalanine and BCAA concentrations. (Askanazi et al., 1980; Clowes et al., 1980; Milewski et al., 1982; Petersson et al., 1992). Depending on the seriousness of the infection, the changes can be dramatic and often be of significant duration (Souba and Austgen, 1990).

The profound alterations in intracellular glutamine concentrations are accentuated by increased glutamine demand by the body. During infection, glutamine is highly consumed by splanchnic tissues and immune cells. Since plasma glutamine concentration decreases with increased visceral consumption, the skeletal muscle compensates to maintain constant glutamine concentration in the blood. In an attempt to sustain normal intracellular and/or plasma glutamine concentrations, de novo glutamine synthesis and endogenous protein breakdown of muscle change. Falling intramuscular glutamine levels have been associated with increased muscle glutamine synthetase activity and mRNA abundance (Smith et al, 1984). Also, low glutamine intramuscular concentrations seem to be indicative of whole-body protein catabolism, and are paralleled by low rates of muscle protein synthesis (Millward et al., 1989; Newsholme and Parry-Billings, 1990; Rennie et al, 1994). These metabolic changes seem to respond to intramuscular glutamine concentration. Thus, the size of the intramuscular glutamine pool may influence protein turnover in skeletal muscle in both human and animals (Ahmed et al, 1993).

Despite the effort to maintain constant plasma glutamine concentration, if the duration of the infection is prolonged or the disease becomes more severe, the body's glutamine demand may exceed skeletal muscle synthetic capacity. Consequently, glutamine plasma concentration may eventually become rate-limiting and result in inadequate supply of glutamine for intestinal, hepatic and immune cells. This may explain the correlation between survival in septic patients and their marked depletion of intramuscular glutamine concentration (Roth et al, 1982). To prevent this fatal outcome, several investigators have evaluated the impact of glutamine-enriched diets

on muscle glutamine concentrations and protein synthesis. MacLennan et al. (1987) found that a rise in intramuscular glutamine concentration stimulated protein synthesis in rats. Hammarqvist et al. (1989) have shown that patients receiving postoperative glutamine supplementation experienced smaller decreased in intracellular glutamine concentrations without changes in synthesis rate of skeletal muscle polyribosomes. Thus, these patients had less nitrogen loss than the control group without glutamine. Similarly, Peterson et al. (1994) have demonstrated that postoperative TPN with glutamine (20g) maintains free glutamine levels in skeletal muscle. However when the treatment is discontinued on postoperative day 3 and normal enteral diets (5 g of glutamine) are introduced, intramuscular glutamine concentration drops. These studies suggest exogenous glutamine is needed to sustain free glutamine concentrations in skeletal muscle and to meet glutamine requirements during hypermetaboli states.

1.3.2.b. Splanchnic organs - gut and liver

The small intestine of humans and animals is the principal organ of glutamine consumption (McAnena et al, 1991; Pinkus and Windmueller, 1977). During post-absorptive period, glutamine extraction by the portal-drained viscera is approximately 12-13% in healthy surgical patients and 18-20% in rats (Souba et al, 1990b; Windmueller and Spaeth, 1975). This translates into a net consumption rate of about 1200 nmol/kg BW/min or an uptake of 18-20 g/ day of circulating glutamine by an average-sized adult. However, the average amount of glutamine ingested in the diet is about 5-8 g/d which suggests the high uptake of endogenous glutamine (Souba,

1992). The vast majority of glutamine extracted by the gut is consumed primarily by the mucosal cells, which exhibit a high rate of proliferation and turnover, and contain high glutaminease activity (about 20-30 times greater than glutaminase in skeletal muscle; Pinkus and Windmueller, 1977; Lacey and Wilmore, 1990).

Glutamine is an important nutrient for the maintenance of intestinal metabolism, structure, and function. Glutamine is metabolized to provide oxidative fuel and biosynthetic intermediates for the rapid proliferative mucosal cells, as well provides precursors for hepatic gluconeogensis and ureagenesis (Souba, 1993; Marsman and McBurney, 1995). The ability of mucosal cells to metabolize glutamine may be more important during catabolic states when glutamine depletion may be severe and oral intake of nutrients may be limited. For example, luminal and arterial glutamine delivery to the small intestine of septic patients and endotoxemic rats are diminished (Souba and Austgen, 1990; Austgen, et al., 1991). The low glutamine uptake may be associated with anorexia, impaired transport of glutamine across the basolateral membrane of the enterocyte, and depressed glutaminase activity (Souba et al, 1990a; Ardawi et al, 1991; Salloum et al, 1991). As a consequence of decreased supply of luminal and arterial glutamine, intestinal metabolism, structure and function may be jeopardized. A lack of glutamine availability has been suggested to impair gut barrier function by changes in mucosal permeability, and subsequently translocation of enteric bacteria and their endotoxin may occur (Deitch et al., 1987). This appears to have important therapeutic implications since standard total parenteral nutrition (TPN) solutions are glutamine free and many enteral diets may contain inadequate amounts of glutamine.

Supplementation of oral and parenteral diets with glutamine is beneficial for the small intestine. Glutamine-enriched diets increase mucosal weight and DNA content, decrease villous atrophy associated with prolonged usage of TPN, and increase gut glutaminase activity and glutamine transport (Dudrick and Souba, 1991; Souba, 1993). Lower incidences of sepsis and mortality also have been associated with glutamine supplemented nutrition (Fox et al., 1988). Improved survival has been proposed to be related to diminished bacterial translocation due to intact mucosal structure and barrier function. Luminal perfusion with glutamine has been shown to prevent endotoxin-related increases in ileal permeability in pigs (Dugan and McBurney, 1995).

In contrast to the small intestine, the liver does not readily consume glutamine as a respiratory fuel (Haussinger, 1989). The liver has the capability to synthesize or degrade glutamine depending on its own requirements and on the needs of other organs. During sepsis, the liver becomes the major glutamine consumer in the body, exceeding gut glutamine consumption three-fold (Souba and Austgen, 1990). Hepatic glutamine uptake can increase ten fold following intraperitoneal injection of *E.coli* endotoxin (Austgen et al., 1991). Factors that may contribute to the accelerated uptake of glutamine by the liver include an increase in hepatic blood flow, an increase in circulating concentrations of glutamine and activation of intracellular metabolism and an increased activity of the liver transport system in response to endotoxin (Inoue et al., 1993). This augmented hepatic consumption of glutamine supports increase activity of gluconeogenesis, glutathione production and acute phase protein synthesis associated with infection.

1.3.2.c. Immune cells

Lymphocytes, macrophages and neutrophils are important immunologic cells that require glutamine for normal functions (Ardawi and Newsholme, 1985; Wallace and Keast, 1992; Ogle et al., 1994). Accordingly, these immune cells possess high amounts of glutaminase enzyme, an important regulator of intracellular glutamine metabolism (Ardawi and Newsholme, 1982). Following immunologic challenge or mitogen stimulation, lymphocytes transform into highly active states where rapid cell division and production of chemical mediators occurs (Ardawi and Newsholme, 1985). Compared to resting lymphocytes, mitogen stimulation significantly enhances the rate of glutamine utilization by 10 times (Brand et al, 1986) and causes a threefold increase in glutamine transport of human peripheral lymphocytes (Schroder et al., 1990). Similarly, lymphocytes obtained from rats receiving *Ecoli* endotoxin have greater glutaminase mRNA expression and activity than those from uninfected animals (Sarantos et al., 1993). Glutamine is rapidly metabolized during this activation stage to provide nitrogen precursors for biosynthetic activities and to generate energy and intermediates for cellular reactions (Brand et al, 1986). Thus, stimulated lymphocytes have higher demands for glutamine and their responses may depend on glutamine availability.

Resting and proliferative lymphocytes metabolize glutamine by incomplete oxidation ("glutaminolysis") (Ardawi and Newsholme, 1982 and 1983; Brand et al., 1989). Glutaminase hydrolyses glutamine to glutamate and ammonia. Glutamate undergoes transamination via aspartate aminotransferase with the subsequent

incomplete oxidation by truncated tricarboxylic acid cycle (i.e. from α-ketoglutarate to pyruvate via malate). This metabolic pathway generates adenosine triphosphate (ATP) and supplies aspartate, asparagine and ammonia required for synthesis of nucleotides, glucosamines, guanosine triphosphates and nicotinamide adenine dinucleotides (Tate and Meister, 1973; Lacey and Wilmore, 1990). Therefore, glutaminolysis provides energy and adequate production of metabolized intermediates required for cellular biochemical pathways (Newsholme et al, 1985; Wu et al., 1991).

Incomplete oxidation of glutamine supports lymphocyte responses. Glutamine must be continually used at a high rate by these immune cells, even when they are non-stimulated. Since the response of the immune system to a microbial challenge must be quick, the rate of glutamine utilization needs to be high to provide optimal conditions for rapid response at any time. Glutaminolysis prevents generation of large amount of ATP which would feed-back and diminish the rate of glutamine metabolism (Newsholme et al, 1985) and consequently limit the availability of precursors for purine and pyrimidine nucleotide synthesis. Thus, any factors causing a decrease in the rate of glutamine utilization by lymphocytes can potentially decrease the proliferation rate of these cells (Newsholme and Parry-Billings, 1990). For example, DNA polymerase activity of stimulated cells is depressed in glutamine-deficient medium (Taudou et al., 1983) and [^{3}H]-thymidine incorporation of activated lymphocytes is increased in a dose-dependent manner as the concentration of glutamine increases over the range of 0.01-1mM (Szondy and Newsholme, 1989). If these results can be extrapolated to in vivo conditions, reduced

glutamine levels may limit glutamine utilization by the immune cells and immunosuppression may occur. Hence, the maintenance of adequate supply of plasma glutamine seems important for proper immune response.

It has been hypothesized that immune depression could be reversed in vivo by correction of the blood glutamine concentrations. Inoue et al. (1993) have reported that rats receiving glutamine supplemented TPN had significantly better survival rates (92.1%) than animals on control diets (44.7%) when challenged with *E.coli* induced peritonitis. Also, a reduction in the incidence of clinical infections in patients undergoing bone marrow transplantation has been shown in those who received TPN with glutamine (Ziegler et al., 1992). These studies suggest that glutamine supplementation may reduce the incidence of clinical infection by enhancing immune function. An increase in T-cell DNA synthesis has been observed in patients who received TPN with glutamine after undergoing colorectal resection (O'Riordain et al. 1994). Similarly, investigations examining gut immunology of rats found the addition of glutamine to standard TPN solution maintained both B and T cell populations and improved secretory IgA level (Burke et al., 1989; Alverdy et al., 1992).

Glutamine has numerous physiological functions and its availability is essential in the maintenance of metabolism, structure, and function in several organs. During sepsis both glutamine consumption and production increase, but with time glutamine demand outstrips the body's ability to produce glutamine, and its tissue and plasma levels fall. The provision of exogenous glutamine has been shown to benefit humans and rats in these stressful circumstances.

1.4. GLUTAMINE AND WEANING

Maternal milk supports the nutrient demands of the neonate. For all young mammals, maternal milk provides easily digestible and absorbable carbohydrates, lipids, proteins and micronutrients for growth and development. However, the composition of milk can vary among different species (Jenness and Sloan, 1970). The differences among the species may be contributed to specific requirement needs of their young due to differences in postnatal growth rate, stage of maturity at birth, body composition at birth and environmental peculiarities (Davis et al., 1994). Also, factors such as litter size, lactation load, maternal diet and nursing schedule can influence milk composition.

Despite the differences in milk composition among species, there is a commonality in the overall amino acid pattern of milk. Davis et al (1994) found the amino acids in greatest abundance in milks of several species (including humans and pigs) to be glutamate and glutamine (20% of total amino acids or 182-229 mg of glutamate and glutamine/ g total amino acid). Similarly, Wu and Knabe (1994) have shown glutamine to be the primary amino acid in sow's milk between days 22 and 29 of lactation (1.0 and 3.4 mmol/L defatted milk, respectively). Wu and Knabe concluded that the high concentrations of glutamine present in sow's milk are consistent with the importance of glutamine for maturity of the gastrointestinal tract (Wu et al., 1995). Also at 29 days, elevated rates of glutaminolysis are detected in intraepithelial lymphocytes from pigs (Dugan et al., 1994). Thus, the predominance

of glutamine in milk suggests its potential significance in young mammals and a deficiency of glutamine in their diet may affect their overall growth and development.

1.5. RATIONALE

The importance of glutamine has been recognized in patients that receive TPN. TPN is an important nutritional support for many critically ill and surgical patients but prolonged use of TPN has been associated with atrophy of the gut and immunosuppression. (Boekman and Krill, 1970; Levine et al., 1974; Wilmore et al., 1988). Glutamine is not currently present in standard TPN solutions because of solubility and stability problems (Mainous and Deitch, 1994). However, when glutamine (2% v/v) supplemented TPN is given to surgical patients, improved nitrogen balance, decreased incidence of infection and shortened hospital stay are observed (Zeigler et al., 1992). The beneficial effects of glutamine are attributed to its effect on intestinal barrier function and immunity. Thus, glutamine supplementation has important implications in patient care.

The transition from ingesting maternal milk to solid food is a common and required process of mammalian development. The withdrawal of mother's milk is associated with environmental changes and increased susceptibility of the weanling to infection. Many investigations of humans and rats have shown glutamine supplementation during stressful conditions to have favourable outcomes. However, the requirements for glutamine during weaning are unknown.

The early weaned pig is a practical and suitable animal model to examine the effects of glutamine during weaning. The weaning process in swine is a vulnerable period where a high glutamine source, sow's milk, is replaced with a weanling diet. Numerous studies have endeavoured to define the ideal amino acid profile to optimize

nitrogen utilization and growth in weanling pigs but the role of glutamine has not been examined. The ideal amino acid pattern (IPPA) for 10-kg pigs described by Chung and Baker (1992) does not contain glutamine, nor have the effects of environmental and pathogenic factors on glutamine requirements at weaning been considered. Since hydrolysis of dietary proteins converts glutamine to glutamate, glutamine content of dietary proteins can only be established by amino acid sequencing. However, the elemental diet used by Chung and Baker (1992) provides a dietary regimen where digestibility concerns are not an issue and glutamine level can be controlled. This diet has been reported to support normal growth and efficient nitrogen utilization of weanling pigs.

1.6. HYPOTHESIS

It is hypothesized that addition of glutamine to weanling diets is required during weaning, particularly in the face of infection. This will be evident by:

1. decreased plasma and intramuscular glutamine concentrations and suppressed peripheral lymphocyte responses to mitogens from pigs fed non-glutamine supplemented diets

2. normal plasma and intramuscular glutamine concentrations and improved peripheral lymphocyte responses to mitogens from infected pigs receiving glutamine supplemented diets

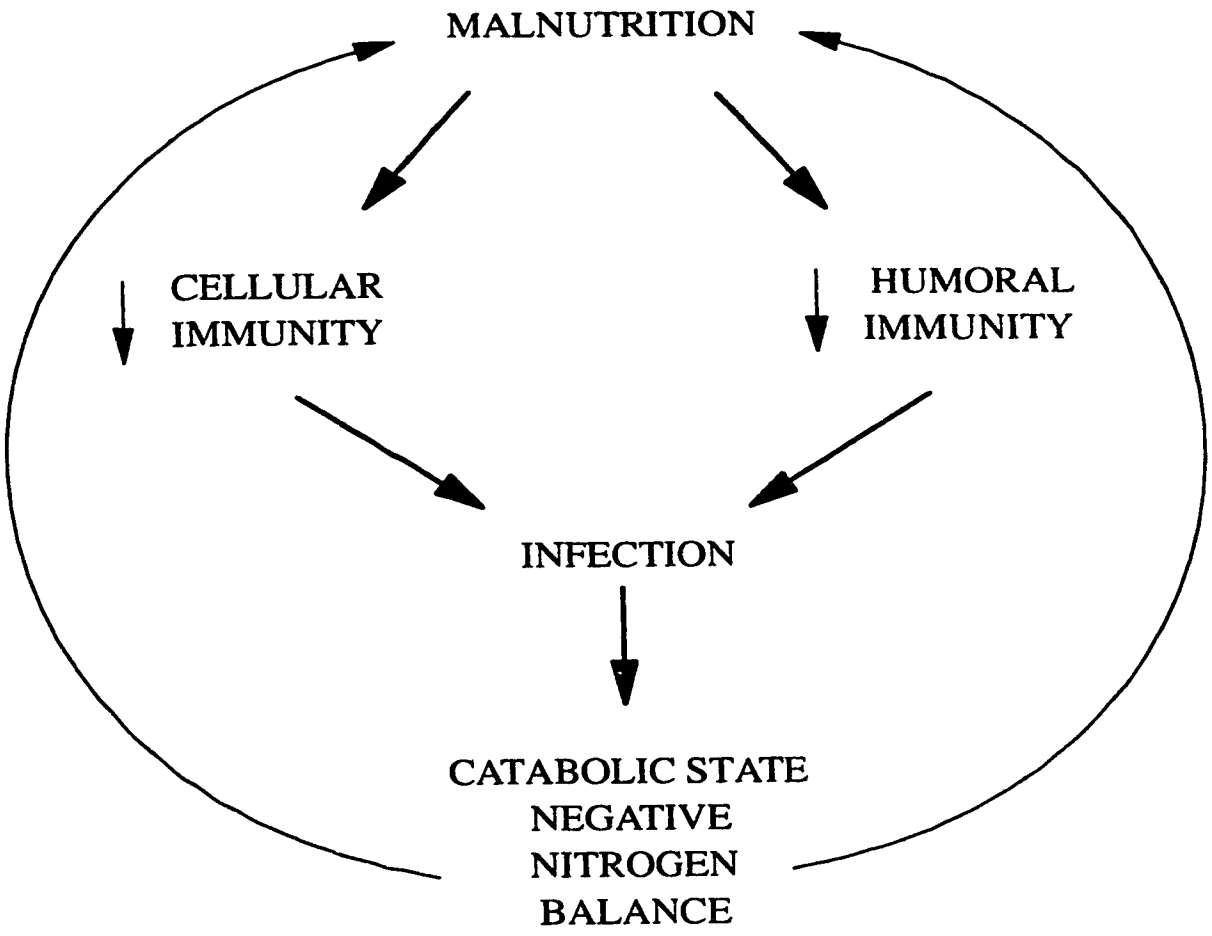


Figure 1. Nutrition-immunity-infection interactions (Mainous and Deitch, 1994).

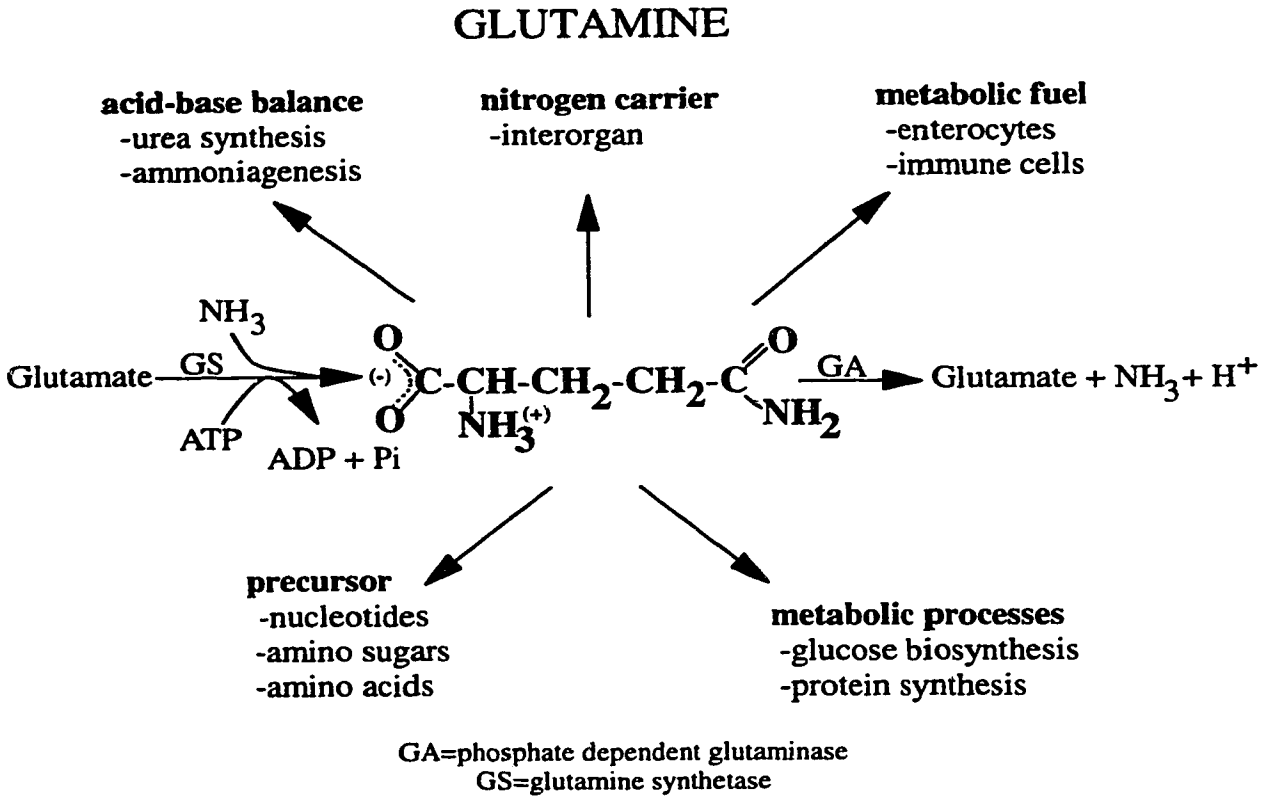


Figure 2. Structure and physiological roles of glutamine (modified from Souba,1992).

2. METHODOLOGY

2.1. ANIMALS

From the University of Alberta Swine Unit, total of fifty-seven Camborough x Canabrid (PIC Ltd) piglets from thirteen litters weighing between 6 kg and 8 kg were obtained for experiments 1 and 2. These pigs were raised with their sows without creep feeding. At 21 days of age, the animals were removed from the sow and their littermates and individually housed in metabolic crates placed in an environmentally-controlled room (28 ± 2 °C; 24-hour light schedule) at the Metabolic Unit of the University of Alberta Edmonoton Research Station. All aspects of the experiment were approved by the University of Alberta Animal Policy and Welfare Committee.

2.2. EXPERIMENTAL DESIGN

2.2.1. EXPERIMENT 1

Twelve pigs (4 / litter) were weaned at 21 days of age. The pigs were given free access to standard grower diet (Table 1) meeting NRC requirements (1988) and water. At 25 days of age, one of three levels of *Escherichia coli* (*E. coli*; $1.0x10^7$, $0.5x10^8$, $1.0x10^8$ colony forming units /kg body weight (CFU/kg BW)) was injected intraperitoneally into these pigs. The animals were monitored over 24 hours for clinical signs of shock (i.e. retching and vomiting, diarrhea, lethargy, prolonged elevated fever, respiratory difficulties, shivering and discoloration of skin; Schrauwen

et al., 1988). At 28 days of age, the animals were anesthetized using halothane (2 - 5 %) with oxygen (1 L / min).

Blood samples (10 mL) were taken by cardiac puncture using 10 mL sterile heparinized vacutainers (143 USP units of sodium heparin; Becton Dickinson, Rutherford, New Jersey). Biopsies of longissimus dorsi (LD) muscle (2 - 3 g) were taken and immediately frozen in liquid nitrogen and later stored in a -50 °C freezer for amino acid analysis. The animals were sacrificed by injection (1 - 3 mL) of commercial euthanasia cocktail (T-61; Hoerchst Canada Inc., Regina, Sask). Daily feed intakes, body weight changes and rectal temperatures were recorded.

2.2 2. EXPERIMENT 2

This experiment involved 45 PIC pigs (5 / litter) where one piglet from each litter was sacrificed at 21 days of age to serve as a control (Figure 3). Two of the remaining four piglets from each litter were assigned to weaning diets (Tables 2 and 3) supplemented with glutamine (+GLN) or an equal amount of nonessential amino acid nitrogen (proline, glycine, taurine, serine, alanine, aspartate) (-GLN). At 26 days of age, one piglet from each diet group was randomly selected and injected intraperitoneally with 0.5×10^8 CFU/kg BW *Escherichia coli* (+Ecoli) or phosphate buffered saline (-Ecoli). Rectal temperature and clinical signs of shock (as described in experiment 1) were monitored for the next 30 hours.

At 28 days of age, the animals were transferred to surgery and anesthetized using 2 - 5 % halothane with oxygen (1 L / min). Blood samples (10 mL) were collected by cardiac puncture using 10 mL sterile heparinized vacutainers. Muscle biopsies (~ 2 - 3 g) of longissimus dorsi were taken and immediately placed in liquid nitrogen and subsequently stored in -50 °C freezer. A mid-line laparotomy was performed and approximately 7 - 10 mesenteric lymph nodes adjacent to the ileum along the mesenteric arteries were removed and placed in sterile phosphate buffer saline with 2 % bovine serum albumin (v/v) (PBS/2%BSA; Sigma Chemicals, St Louis, MO). The animals were sacrificed by injection (1 - 3 mL) of a commercial euthanasia cocktail (T-61).

2.3. DIET COMPOSITION

2.3.1. EXPERIMENT 1

The standard grower diet was designed to meet or exceed requirements for all nutrients for 5 - 10 kg piglets as indicated by the NRC (1988). Composition of the diet is given in Table 1.

2.3.2. EXPERIMENT 2

The chemically defined elemental diets (Table 2 and 3) were formulated to meet or exceed NRC 1988 nutrient requirements for 5 - 10 kg pigs. The weaning

diets had total crude protein content of 17 %. The diets contained either 4 % (w/w) glutamine (+GLN) or an equal amount of non-essential amino acids (-GLN). The essential amino acid (EAA) profiles of both diets (defined as grams of amino acids/100 g lysine) were based on Chung and Baker's (1992) Illinois ideal amino acid pattern (IIP). Diets were isonitrogenous and isocaloric.

2.4. *E. coli* INFECTION

2.4.1. PREPARATION OF *E.coli* INOCULA

The stock of *E.coli* was provided by D. Onderka from Animal Health Division, Alberta Agriculture. The single strained *Escherichia coli* (*E.coli*), serotype O78, had been isolated from coliform septicemia in turkey poults and stored in sheep blood at -50 °C. *E. coli* inocula was cultured under sterile conditions two days prior to infection of pigs as described by Samuels and Baracos (1992).

A small sample of *E.coli* was obtained by dipping a sterile toothpick into the slightly thawed stock of *E. coli*. The sample was smeared onto pre-warmed tryptic soy agar plate (DIFCO Laboratories, Detroit, Michigan) and incubated overnight (minimum of 15 hours) in the incubator (Fisher Isotemp Incubator 300 series, model 3500, Fisher Scientific, Edmonton, Alberta) at 37°C .

Next day, two samples of live *E. coli* were scraped using sterile toothpicks and placed into sterile culture test tubes containing ~12 - 15 mL of pre-warmed tryptic soy broth (TSB, soybean-casein digest; DIFCO Laboratories, Detroit,

Michigan). The tubes were placed into an incubator shaker at 200 RPM (model G25, New Brunswick Scientific Co., INC., Edison, New Jersey) at 37 °C, for 1 hour. The contents of the tubes were poured into a single sterile 500 mL volumetric flask containing ~300 mL of pre-warmed TSB. The flask was further incubated for 4 hours in the shaking incubator. The contents of the flask were poured into six 50 mL sterile centrifuge tubes (Fisher Scientific, Edmonton, Alberta) and centrifuged for 15 minutes at 200 x g in a Jouan CR 4.11 centrifuge (Jouan, France) at room temperature. The pellets were washed and centrifuged three times with sterile phosphate buffered saline (PBS, pH 7.2 composed of 1.01 mM NaH_2PO_4, 8.98 mM Na_2HPO_4, and 145.1 mM NaCl; Sigma Chemicals, St Louis, MO). In the final wash the supernatants were removed by pipette and the pellets of *E.coli* were combined and suspended in sterile PBS. Suspended *E.coli* was stored at 4 °C until next day.

2.4.2. *Ecoli* INOCULATION

The concentration of *E.coli* was determined by surface plating bacterial suspension of 10^{-5} and 10^{-6} dilutions on tryptic soy agar plates Fifteen microliter aliquots from each dilution were plated in duplicate and incubated at 37 °C for 8 to 16 hours. The colonies of duplicated samples were counted and the concentration of suspended *E.coli* was determined and expressed as colony forming units (CFU) / mL as follows

Equation 1: CFU/mL = (number of colonies x dilution factor x 1000) / 15

The live *E coli* solution was diluted to desired dose concentration (i.e. $0.5x10^8$ CFU), and the *E.coli* dose to be administered (CFU/kg body weight) was calculated by the following formula:

$$\textit{Equation 2:}\quad X = \frac{\text{pig weight (kg)} \times N\ (\text{CFU/kg})}{\text{CFU/mL}} \times \frac{14\ \text{mL}}{10\ \text{mL}}$$

where N = the desired dose concentration

X = the volume (mL) of *E.coli* solution

For the inoculum, X mL of desired dose was sampled and further diluted with saline to obtain a total volume of 14 mL. This solution (10 mL) was withdrawn using 10 mL syringe and intraperitoneally injected into the pigs. The non-infected pigs were intraperitoneally injected with 10 mL of sterile PBS.

2.5. LYMPHOCYTE ASSAYS

The following procedures were performed under sterile conditions.

2.5.1. LYMPHOCYTE ISOLATION

2.5.1.1. BLOOD

Heparinised blood was centrifuged for 10 min at 200 x g in a Jouan CR 4.11 centrifuge (Jouan, France) at 24 °C. Plasma was removed and stored at -50 °C for amino acid analysis. The remaining blood was diluted 1:1 with sterile phosphate buffered saline with 2 % bovine serum albumin (PBS/2%BSA, pH 7.4) which

consisted of NaCl (0.580 mM), KCl (0.074 mM), Na_2HPO_4 (0.138 mM), KH_2PO_4 (0.136 mM) and 2% BSA (w/v) (Sigma Chemicals, St. Louis, MO). The diluted blood was carefully placed on top of two-layer gradients of Histopaque 1077 and 1119 (Sigma Diagnostic, St Louis, MO). The sample was centrifuged at room temperature, without brakes, for 30 min at 700 x g. Lymphocytes were pipetted from the PBS/2%BSA- Histopaque1077 interface and washed with PBS/2% BSA. The residual erythrocytes were lysed by suspending the pellet with 4 - 5 mL of water for 45 sec. The reaction was stopped by adding an equal volume of double concentrated PBS. Purified lymphocytes were suspended with PBS/2%BSA, followed by centrifugation at room temperature for 10 min at 200 x g. Lymphocytes were washed and centrifuged twice. In the final washing, lymphocytes were suspended in RPMI-1640 medium (Cellgro, VA, AK) supplemented with 4 % fetal calf serum (v/v), antibiotics (100 IU/ml penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin B), 25 mM Hepes Buffer (Sigma Chemicals, St Louis, MO), 2.5 mM 2-mercaptoethanol, and 4 mM glutamine (ICN Biochemicals, Cleveland, Ohio). Trypan blue exclusion test estimated 98% cell viability in all treatment groups. The lymphocytes were manually counted using hemocytometer and diluted with supplemented culture medium to obtain a cell concentration of $1.5x10^6$ cells/mL for mitogen response assay.

2.5.1.2. MESENTERIC LYMPH NODES (MLNs)

Excess surrounding connective tissue of collected MLNs were removed with forceps. The MLNs were pushed through polypropylene filter mesh (297 mm, Spectrum, Houston, Texas). The cells were washed with 12 mL of PBS/2%BSA and centrifuged for 10 min at 200 x g at room temperature. The pellet was re-suspended with 6 mL buffer solution and placed on top of Histopaque 1077. The cells were centrifuged without brakes at 700 x g for 30 min at room temperature. The top layer of lymphocytes was removed and re-suspended with PBS/2%BSA solution. The cells were again centrifuged for 10 min at 200 x g. Residual erythrocytes were removed by flash lysis (i.e. described in blood lymphocyte isolation procedure 2.5.1.1). Lymphocytes were washed twice with PBS/2%BSA solution. In the final washing, lymphocytes were suspended with supplemented RPMI-1640 culture medium. Trypan blue exclusion test estimated 98% cell viability in all treatment groups. As described above, cells were manually counted by using hemocytometer and concentration of $1.5x10^6$ cells/mL was used for mitogen response assay.

2.5.2. MITOGEN RESPONSE ASSAY

The cell, mitogen and [^{3}H]-thymidine concentrations used in the mitogen assay are described in Appendix 1.

The assay was carried out in triplicate on microtitre plates (round bottom 96-wells plate, Corning, New York) using 200 µL of suspended lymphocytes, giving

a final concentration of $3x10^5$ cells/well. The cells were incubated up to 96 hours at 37 °C in humid air with 5 % carbon dioxide without mitogens or with each of the following mitogens: Concanavalin A (Con A) (5 mg/L; ICN, Irving CA) or Phorbol Myristate Acetate (PMA) (40 µg/L; ICN, Irving CA) with Ionomycin (Iono) (400 µg/L; Sigma Chemicals, St. Louis). The cultured wells were pulsed with 0.05 µCi/well of [^{3}H]-thymidine and 18 hours later thymidine incorporation was determined by liquid scintillation spectrometry in a β-counter.

The mitogen response was recorded as mean decays per minute (DPM) or as stimulation index (SI) as follows:

$$SI = \frac{\text{mean DPM of stimulated cells - mean DPM of unstimulated cells}}{\text{mean DPM of unstimulated cells}}$$

2.5.3. LYMPHOCYTE PHENOTYPE DETERMINATION

For lymphocyte subset identification, T cells were defined as those cells expressing the CD2 (MSA4) antigen and B cells were defined as those cells expressing IgM (Pig45A). Subsets of T cells were identified by the antigens CD4 (PT90A) for helper T cells and CD8 (PT35B) for cytotoxic T cells. These monoclonal antibodies (VMRD Inc., Pullman, WA) were obtained from filtered ascites fluid from mice.

Using an indirect immunofluorescence technique, lymphocytes ($5x10^5$cells/well) were incubated at 4 °C for 30 minutes with one of four mouse anti-swine monoclonal antibodies (mAbs). The excess, unattached mAbs were removed by centrifugation (700 x g for 1 minute) and the cells were washed with PBS with 4 % (v/v) fetal calf serum (FCS) three times. Then a second incubation with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (IgG + IgM; CALTAG Lab, San Francisco, CA) was performed at 4 °C for 30 minutes. After the incubation, lymphocytes were washed and centrifuged (700 x g for 1 minute) 3 times. Lymphocytes were preserved with 1 % paraformaldehyde and preparations were analyzed by flow cytometry (FACScan, Becton Dickinson, Rutherford, New Jersey).

2.6. AMINO ACID ANALYSIS

Blood and muscle samples were evaluated for amino acid concentrations by reversed-phase high-performance liquid chromatography (HPLC) using pre-column derivatization with o-phthalaldehyde (OPA; Jones and Gilligan, 1983). All chromatographic procedures were performed at room temperature, and the samples and standards were analyzed in duplicates as outlined by Sedgwick et al. (1991).

2.6.1. AMINO ACID STANDARD SOLUTION

The amino acid concentrations of the prepared samples were determined by comparing the sample supernatants to protein-free standard solutions. The amino acid

standard solution consisted of protein hydrolysate standard solution supplemented with asparagine, glutamine, citrulline, taurine, and tryptophan (Sigma Chemical Co., St. Louis, MO). In addition to the standard amino acid solution, an internal standard solution composed of ethanolamine (EA) and β-amino-butyric acid (BABA) were also prepared. The final concentrations of EA and BABA in the internal standard solution were 25 mM. The amino acid standard solution was treated in all manner identical to the samples.

2.6.2. PLASMA

To thawed plasma samples or amino acid standards (100 uL), EA/BABA (200 µL) internal standard solution was added and than vortexed. The samples were deproteinized with 400 µL 5 % (v/v) perchloric acid (PCA) and centrifuged at 2800 x g in a Sorval TC centrifuge (Dupont Co. Newtown, CT) for 15 min at room temperature. The supernatant from the PCA treatment was removed and mixed with 300 mL saturated $K_2B_4O_7$ (pH ~ 9.5). The samples were centrifuged again at 2800 x g for 15 min, and the supernatants were used for the HPLC analysis.

2.6.3. LONGISSIMUS DORSI (LD) MUSCLE

The frozen LD muscle samples were placed in liquid nitrogen. Each sample was individually pulverized with a hammer. Approximately 1 g of muscle tissue was

homogenized with ice cold 5 % PCA (5 mL) using glass mortar and pestle in an ice bath. The homogenized mixture was centrifuged at 700 x g in a Jouan, CR.11 centrifuge (Jouan, France) for 15 min at 4 °C and the supernatant was collected and frozen (-30 °C) until free amino acid analysis was performed.

To 100 µL of muscle samples or amino acid standards, 100 µL of EA/BABA internal standard solution was added. The sample was neutralized with saturated $K_2B_4O_7$ (pH ~ 9.5) and precipitants were removed by centrifugation (2800 x g in a Sorvall TC centrifuge, Dupont CO, Newtown CT; for 15 min) The supernatant was used for HPLC analysis.

2.7. HEMATOLOGY

Blood samples (5 mL) obtained by cardiac puncture using EDTA vacutainer (0.05 mL of 15% EDTA (K_3) solution (7.5 mg), Becton Dickinson, Rutherford, New Jersey) were taken to University of Alberta Hospital.

Routine hematology was performed on the blood samples. White and red cells, hemoglobin, hematocrit, red cell distribution width, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelets values were determined by Coulter machine at the University of Alberta Hospital. Differential counts of white blood cells were determined by manual counting.

2.8. STATISTICAL ANALYSIS

A randomized block design was used to compare the different treatment means. The effects of diet and infection were analyzed by two-way analysis of variance with the litter blocked using the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Duncan's multiple range test was used to compare means. All parameters were analyzed by this statistical model. However, additional statistical analysis was performed on mitogen response assays. Within a treatment group, the blood lymphocyte responses to PMA+IONO at 24h, 48h, 72h and 96h were analyzed by one-way ANOVA and Duncan's multiple range test. The responses at 48h and 72h were not significantly different, and the means of these time points were averaged to give the peak mitogen response. To determine treatment differences, the peak mitogen responses from each treatment groups were compared by one-way ANOVA and Duncan's multiple range test. The same statistical model was used to analyzed blood lymphocyte response to Con A. Probability values of $P \leq 0.05$ indicated statistical significance. Results are shown as the mean ± standard error of the mean.

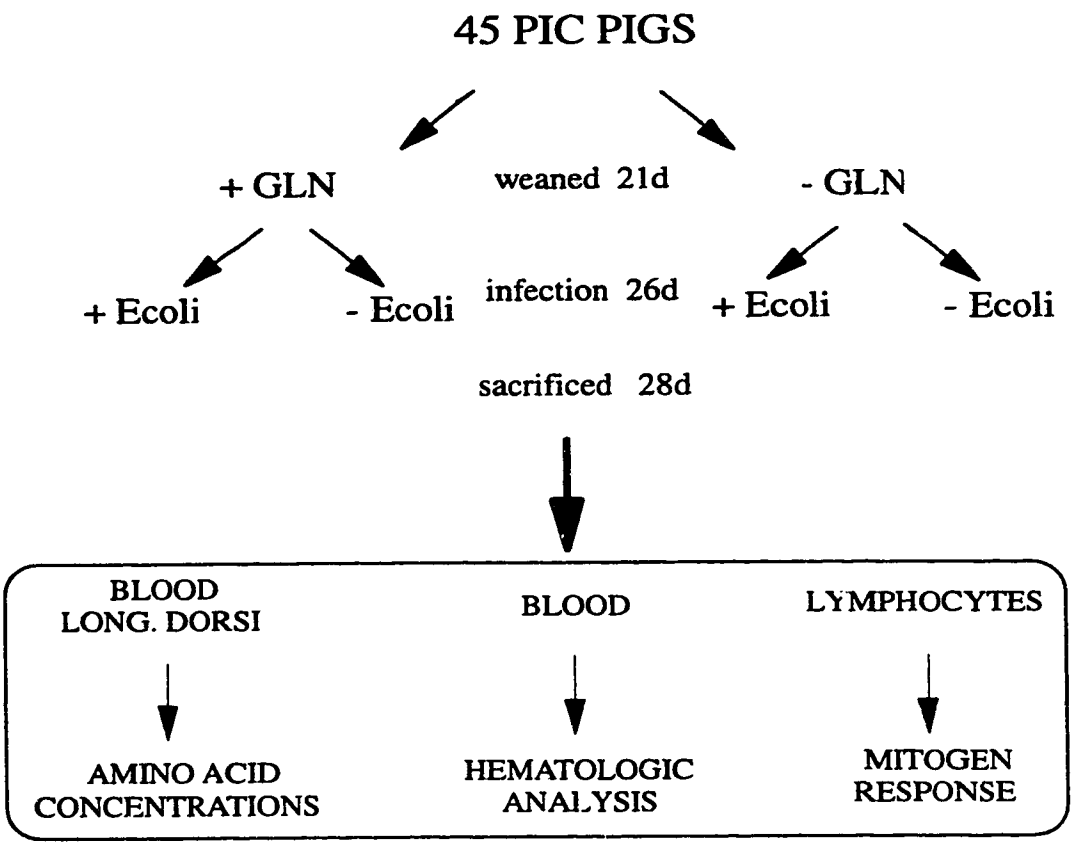


Figure 3. Experimental design of experiment 2.

Table 1.

COMPOSITION OF STANDARD GROWER DIET

	Grower Diet
Ingredients	--g/100kg diet--
Wheat	5.0
Oat grain	20.0
Sugar	10.0
Soybean meal	20.0
Fishmeal	6.5
Whey	14.0
Skim milk powder	20.0
Tallow	2.0
Limestone	0.7
Dicalcium phosphate	0.4
Iodinized sodium chloride	0.4
Mineral mixture*	0.5
Vitamin mixture**	0.5
Crude Protein (% w/w)	24.0

*Mineral mixture contains the following per 100 kg:
300 g $CaCO_3$, 2800 g $Ca_2(PO_4)_3$, 900 g K_2HPO_4, 880 g NaCl, 450 g $MgSO_4.7H_2O$, 65 g $MnSO_4.H_2O$, 50 g Ferric citrate, 10 g $ZnCO_3$, 2 g $CuSO_4.5H_2O$, 900 mg H_3BO_3, 900 mg $Na_2MoO_4.2H_2O$, 4.06 g KI, 100 mg $CoSO_4.7H_2O$, 62.2 mg Na_2SeO_3.

**Vitamin mixture contains the following per 100 kg:
2 g Thiamin-HCl, 5 g Niacin, 1 g Riboflavin, 3 g D-Ca-pantothenate, 4 mg Vitamin B_{12}, 600 mg Pyridoxine-HCl, 60 mg D-Biotin, 400 mg Folic acid, 25 g Ascorbic acid, 200 mg Menadione (Vitamin K), 520000 IU Retinyl acetate (Vitamin A)), 60000 IU Cholecalciferol (Vitamin D_3).

Table 2.

COMPOSITION OF THE CHEMICALLY DEFINED AMINO ACID BASAL DIET

(Modified IIP Diet, Chung and Baker, 1992)

	+GLN Diet	-GLN Diet
Ingredients	--kg/100kg diet--	--kg/100kg diet--
Cornstarch	36.0	34.6
Amino acid mixture	18.9	20.4
Lactose	20.0	20.0
Sucrose	10.0	10.0
Soft Beef Tallow	2.3	2.3
Corn oil	2.8	2.8
Solka floc	3.0	3.0
Mineral mixture*	5.5	5.5
Sodium bicarbonate	1.2	1.2
Vitamin mixture**	0.2	0.2
Choline chloride	0.2	0.2
DL-α-tocopheryl acetate (20mg/kg)	++	++
Ethoxyquin (125mg/kg)	++	++
Crude Protein (% w/w)	17.0	17.0

*Mineral mixture contains the following per 100 kg:
300 g $CaCO_3$, 2800 g $Ca_2(PO_4)_3$, 900 g K_2HPO_4, 880 g NaCl, 450 g $MgSO_4.7H_2O$, 65 g $MnSO_4.H_2O$, 50 g Ferric citrate, 10 g $ZnCO_3$, 2 g $CuSO_4.5H_2O$, 900 mg H_3BO_3, 900 mg $Na_2MoO_4.2H_2O$, 4.06 g KI, 100 mg $CoSO_4.7H_2O$, 62.2 mg Na_2SeO_3.

**Vitamin mixture contains the following per 100 kg:
2 g Thiamin-HCl, 5 g Niacin, 1 g Riboflavin, 3 g D-Ca-pantothenate, 4 mg Vitamin B_{12}, 600 mg Pyridoxine-HCl, 60 mg D-Biotin, 400 mg Folic acid, 25 g Ascorbic acid, 200 mg Menadione (Vitamin K), 520000 IU Retinyl acetate (Vitamin A)), 6000IU Cholecalciferol (Vitamin D_3).

Table 3.

MODIFIED IIP AMINO ACID PATTERN

	+GLN Diet	-GLN Diet
Amino Acids	--kg/100 kg diet--	--kg/100kg diet--
NON-ESSENTIAL		
Glutamine	4.0	-
Proline	0.5	0.9
Glycine	1.2	2.4
Taurine	-	1.0
Serine	-	0.6
Glutamate	4.4	4.4
Alanine	-	1.2
Aspartate	-	1.0
Asparagine	-	-
NEAA-N content	1.5	1.5
ESSENTIAL		
Lysine	1.4	1.4
Arginine	0.6	0.6
Histidine	0.4	0.4
Tryptophan	0.3	0.3
Isoleucine	0.8	0.8
Leucine	1.4	1.4
Valine	0.9	0.9
Threonine	0.9	0.9
Methionine	0.4	0.4
Cystine	0.4	0.4
Phenylalanine	0.7	0.7
Tyrosine	0.6	0.6
EAA-N content	1.3	1.3
NEAA-N : EAA-N ratio	1.2	1.2

3. RESULTS

3.1. EXPERIMENT 1

Daily dry matter (DM) feed intakes of animals did not differ among treatment groups during the first 4 days (Figure 4A). Animals infected with less than $1x10^8$ CFU/kg BW continued to increase feed consumption throughout the experiment whereas those injected with $1x10^8$ CFU/kg BW ate significantly less ($P < 0.05$) after infection. Thus, total and daily average feed consumption of animals receiving $1x10^8$ CFU/kg BW were less ($P < 0.05$) than those animals of receiving the two lowest *E. coli* dosages (Table 4).

All animals lost weight at the beginning of the experiment (Figure 4B). Although body weights and percentage total body weight change (Table 4) did not differ with degree of infection, the most severely infected animals did not gain weight during the experiment whereas animals on the other two treatments did.

Rectal temperatures (Figure 4C) were normal until the day of infection. All animals experienced a small rise ($P < 0.05$) in rectal temperature 6 hours after IP injection. Rectal temperatures returned to normal within 12 hours after infection, except for pigs receiving $1x10^8$ CFU/kg BW that remained elevated ($P < 0.05$).

Plasma and longissimus dorsi (LD) intracellular glutamine concentrations were not significantly different among the treatments. However, plasma arginine, serine and citrulline concentrations (Table 5) decreased ($P < 0.05$) with increasing infection. LD intracellular concentrations of alanine and glycine (Table 6) decreased

($P < 0.05$) at the highest infection rate. Concentrations of other amino acids were not significantly affected by *E. coli* administration.

3.2. EXPERIMENT 2

Daily dry matter feed intakes did not differ among treatments except for day 6 (Figure 5A) where infected animals ate less ($P < 0.05$) than control animals. Total and average daily DM feed intakes did not differ among treatments (Table 7). Total and daily body weights and percentage body weight changes (Table 7) did not differ among the treatments (Figure 5B).

Rectal temperatures were similar before infection (Figure 5C). Control animals continued to have normal rectal temperatures after day 5 whereas rectal temperatures of the infected pigs were significantly ($P < 0.05$) elevated from days 5 to 7.

Non-glutamine diets increased ($P < 0.05$) plasma serine, glycine and taurine concentrations for both -Ecoli and +Ecoli animals (Table 8) but plasma concentrations of other amino acids were not significantly affected by the diet. Infection, regardless of diet, resulted in lower ($P < 0.05$) plasma concentrations of glutamine, histidine, citrulline, ornithine, tyrosine, tryptophan, alanine, glycine, threonine, and methionine. Phenylalanine was the only plasma amino acid to increase ($P < 0.05$) in concentration with infection.

Intracellular glutamine and citrulline concentrations of LD muscle were greater ($P < 0.05$) in the glutamine supplemented animals (Table 9) and glutamine concentrations of the infected pigs were less ($P<0.05$) than the controls. Infected animals also had lower ($P < 0.05$) intracellular histidine and threonine concentrations. Concentrations of branched-chained amino acids and other amino acids did not differ among the treatments. Intracellular asparagine concentrations were significantly ($P < 0.05$) increased with infection.

Glutamine supplementation increased ($P < 0.05$) white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin (HGB) and hematocrit (HCT) (Table 10). However, when the hematological values were adjusted for hematocrit changes, glutamine supplementation significantly ($P<0.05$) increased only total white blood cell counts. Infection significantly elevated ($P < 0.05$) WBC and mean corpuscular hemoglobin (MCH).

The rise in total leukocyte count associated with infection was attributable to an increase ($P < 0.05$) in the absolute number of monocytes, neutrophils and band cells (Table 11). When differential WBC count was expressed as percentage WBC (Table 11), infected animals had greater ($P < 0.05$) percentage of band cells than the healthy animals but the proportion of lymphocytes was decreased ($P < 0.05$). Lymphocyte phenotypes were not significantly different among the treatments (Table 12).

Spontaneous (unstimulated) [^{3}H]-thymidine uptake was not significantly different among the treatments at any time point (Figure 6). Blood lymphocytes of

infected animals had reduced ($P < 0.05$) responses to PMA+IONO at 24h, 48h and 72h timepoints (Figures 7A, 7B, 7C, 8A and 8B). However, when the values of 48 hours and 72 hours were pooled (as described in Section 2.8) to give the peak mitogenic responses there were no significant differences among the treatments.

Glutamine supplemented animals had greater ($P < 0.05$) responses to Con A than -GLN animals (Figures 9A, 9B, 10A and 10B). At the peak mitogen response (Figure 9C and 10C), the stimulation index to Con A of lymphocytes from +Ecoli-GLN animals was significantly reduced ($P < 0.05$) whereas those from +Ecoli+GLN animals did not differ from controls (Figure 10C).

Mesenteric lymphocyte responses to PMA+IONO and Con A were not significantly different among the treatment groups (Figure 11).

The treatment means of 21-day suckled animals represent the initial conditions of the 28-day animals. Plasma glutamine concentrations were not significantly different (Table 13). Twenty-one day old suckled animals had higher ($P<0.05$) plasma concentrations of asparagine, arginine, citrulline ornithine, and tyrosine than the 28-day animals. Plasma serine concentrations of 21-day animals were also higher ($P<0.05$) than 28-day animals but not significantly different from the -Ecoli-GLN group. Plasma isoleucine concentrations were lower in 21-day versus 28-day animals. Similarly, plasma threonine concentrations of 21-day animals were less ($P<0.05$) than those found in 28-days animals but not significantly different from the -Ecoli+GLN animals.

Intracellular muscle glutamine concentrations were not influenced by age (Table 14). Muscle citrulline concentrations of 21-day animals were greater ($P<0.05$) than the 28-day animals but arginine, threonine and isoleucine concentrations were greater ($P<0.05$) in the 28-day animals. Similarly, valine concentrations were greater at 28-day than 21-day ($P<0.05$). Taurine concentrations were highest in the 28-day pigs fed the control diet ($P<0.05$).

The 28-day pigs had significantly higher ($P<0.05$) WBC and RDW values than those of 21-day pigs (Table 15). RBC was lower in 28-day animals fed glutamine-free diets ($P<0.05$) than 21-day or 28-day pigs supplemented with glutamine. MCV and MCH of the 28-day animals were less ($P<0.05$) than the 21-day animals. PLATE was significantly higher ($P<0.05$) in the 21-day pigs than the 28-day animals.

Similar to WBC values, 28-day pigs had greater ($P<0.05$) absolute lymphocyte count than the 21-day animals (Table 16). The absolute counts of other WBC subpopulation groups and leukocyte values expressed as percentage WBC (Table 16) were not significantly influenced by age or glutamine supplementation.

Table 4.

FEED INTAKES AND BODY WEIGHT CHANGES[1]
Experiment 1

	E.coli Dosage (CFU/ kg BW)			
	$1.0x10^7$	$0.5x10^8$	$1.0x10^8$	SEM[2]
Total Feed Intake[3] (g)	1841[a]	1579[a]	812[b]	203
Average Daily Feed Intake (g/d)	263[a]	226[a]	116[b]	29
Initial Body Weight (g)	6913	7215	7120	354
Final Body Weight (g)	7813	8108	7065	435
Average Daily Body Weight Change (g/d)	128	128	-8	45
Total Body Weight Change (g)	900	893	-55	309
% Total Body Weight Change	12.9	12.6	-0.4	4.5

[1] Values are means of different concentrations of *E.coli* for n=4.
[2] Pooled standard error of the mean.
[3] Means with different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

Table 5.

PLASMA AMINO ACID CONCENTRATIONS[1]
Experiment 1

	E.coli Dosage (CFU/kg BW)			
	$1.0x10^7$	$0.5x10^8$	$1.0x10^8$	SEM[2]
Amino Acids		µmol/L		
Alanine	452	496	318	73
Arginine[3]	143[a]	106[ab]	66[b]	14
Asparagine	62	63	47	8
Aspartate	17	18	13	2
Citrulline	83[a]	52[b]	39[b]	6
Glutamate	185	192	175	25
Glutamine	392	386	259	48
Glycine	776	537	417	100
Histidine	71	73	73	13
Isoleucine	162	151	151	17
Leucine	138	143	143	18
Lysine	130	139	131	32
Methionine	30	31	24	4
Ornithine	104	62	51	16
Phenylalanine	57	68	70	5
Serine	112[a]	93[ab]	81[b]	8
Taurine	59	57	58	6
Threonine	72	68	59	6
Tyrosine	68	64	45	7
Tryptophan	38	34	30	5
Valine	189	190	204	26

[1] Values are means of different concentrations of *E.coli* for n=4.
[2] Pooled standard error of the mean.
[3] Means with different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

Table 6.

LONGISSIMUS DORSI INTRACELLULAR AMINO ACID CONCENTRATIONS[1]

Experiment 1

	E.coli Dosage (CFU/ kg BW)			
	$1.0x10^7$	$0.5x10^8$	$1.0x10^8$	SEM[2]
Amino Acids	nmol/g wet muscle tissue			
Alanine[3]	1422[a]	1490[a]	1116[b]	89
Arginine	12766	15050	12352	2009
Asparagine[4]	84	91	102	14
Aspartate	310	256	340	56
Glutamate	1595	1366	1670	229
Glutamine	1582	1510	1067	208
Glycine	2730[a]	2428[a]	1528[b]	235
Isoleucine	157	149	172	25
Leucine	135	154	189	30
Lysine	102	142	141	28
Ornithine[4]	324	278	252	62
Phenylalanine	126	132	123	23
Serine	334	330	350	39
Taurine	3716	3141	3113	43
Threonine	142	112	169	43
Tryptophan[4]	119	108	90	18
Tyrosine[4]	665	589	455	193
Valine	194	213	240	37

[1] Values are means of different concentrations of *E.coli* for n=4

[2] Pooled standard error of the mean.

[3] Means with different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

[4] Values are means of different concentrations of *E.coli* for n=3.

Table 7.

FEED INTAKES AND BODY WEIGHT CHANGES[1]
Experiment 2

	Treatments				
	-Ecoli-GLN	-Ecoli+GLN	+Ecoli-GLN	+Ecoli+GLN	SEM[2]
Total Feed Intake (g)	680	835	703	503	109
Average Daily Feed Intake (g/d)	97	119	100	72	16
Initial Body Weight (g)	7220	7406	7157	7007	139
Final Body Weight (g)	7174	7284	6972	6710	192
Average Daily Body Weight Change (g/d)	-7	-17	-26	-42	21
Total Body Weight Change (g)	-46	-121	-184	-297	146
% Body Weight Change	-1	-2	-3	-4	2

[1] Values are means of different treatment animals for n=9.
[2] Pooled standard error of the mean.

Table 8.

PLASMA AMINO ACID CONCENTRATIONS[1]
Experiment 2

	Treatments				
	-Ecoli-GLN	-Ecoli+GLN	+Ecoli-GLN	+Ecoli+GLN	SEM[2]
Amino Acids			µmol/L		
Alanine[3]**	536	450	397	310	58
Arginine	81	85	76	72	5
Asparagine	48	52	46	44	3
Aspartate	52	24	34	23	10
Citrulline**	62	68	47	44	6
Glutamate	333	285	286	259	37
Glutamine **	388	466	267	302	50
Glycine*/**	1226	917	916	436	184
Histidine**	92	103	70	66	7
Isoleucine	174	179	169	146	15
Leucine	173	196	182	149	18
Lysine	260	209	217	151	31
Methionine**	56	91	46	36	12
Ornithine**	80	72	69	53	8
Phenylalanine**	83	79	106	95	7
Serine*	156	124	156	88	22
Taurine*	323	193	303	128	53
Threonine**	409	336	264	156	65
Tryptophan**	43	40	36	28	4
Tyrosine**	81	83	60	55	11
Valine***	286[ab]	319[b]	297[b]	220[c]	27
BCAAs	633	648	695	514	56
Total*	4477	3625	4039	2635	428

[1] Values are means of different treatment animals for n=9.
[2] Pooled standard error of the mean.
[3] *Treatment mean differences are significant (P<0.05) due to diet.
**Treatment mean differences are significant (P<0.05) due to infection.
***Treatment mean differences are significant (P<0.05) due to diet x infection interaction

Table 9.

LONGISSIMUS DORSI INTRACELLULAR AMINO ACID CONCENTRATIONS[1]

Experiment 2

	Treatments				
	-Ecoli-GLN	-Ecoli+GLN	+Ecoli-GLN	+Ecoli+GLN	SEM[2]
Amino Acids	nmol/g wet muscle tissue				
Alanine	1412	1311	1194	1228	136
Arginine	11257	10110	10260	10602	403
Asparagine	102	117	138	174	17
Aspartate	401	394	409	473	48
Citrulline*	89	102	56	105	14
Glutamate	2197	2316	2155	2182	151
Glutamine */**	1768	2678	1137	1619	314
Glycine	3484	3241	3086	1471	672
Histidine**	190	192	146	146	22
Isoleucine	155	184	167	176	20
Leucine	182	211	202	215	28
Lysine	260	282	252	288	44
Methionine	40	49	40	46	5
Ornithine	321	363	342	313	88
Phenylalanine	127	142	150	130	27
Serine	293	281	332	276	30
Taurine *	4372	3611	4104	3155	273
Threonine **	437	431	331	254	68
Tryptophan	54	51	51	56	6
Tyrosine	294	260	246	271	29
Valine	197	212	220	213	24
BCAAs	533	606	589	604	66
Total **	22849	22460	20519	19819	1119

[1] Values are means of different treatment animals for n=9.

[2] Pooled standard error of the mean.

[3] *Treatment mean differences are significant ($P<0.05$) due to diet.

**Treatment mean differences are significant ($P<0.05$) due to infection.

Table 10.

HEMATOLOGY RESULTS OF WEANED PIGS[1]
Experiment 2

	Treatments				
	-Ecoli-GLN	-Ecoli+GLN	+Ecoli-GLN	+Ecoli+GLN	SEM[2]
	Hematological Parameters (units)				
WBC[3] */** (10^9 /L)	10.79	12.77	15.11	17.53	1.02
RBC * (10^{12} /L)	4.87	5.47	4.98	5.48	0.14
HGB * (G/L)	88.56	98.78	92.22	103.67	2.88
HCT * (L/L)	0.26	0.29	0.27	0.31	0.01
RDW	28.38	26.32	26.20	24.49	1.05
MCV (10^{-15}L)	53.67	53.22	55.33	56.22	1.10
MCH ** (10^{-12}G)	18.20	18.00	18.72	18.97	0.30
MCHC (g/dL)	340.00	340.00	338.00	339.00	23.01
PLATE[4] (10^9/L)	354.00	293.00	427.00	324.00	47.01

Abbreviations:
WBC-white blood cells; RBC-red blood cells; HGB-hemoglobin; HCT-hematocrit; RDW-red cell distribution width; MCV-mean corpuscular volume; MCH-mean corpusular hemoglobin; MCHC-mean corpusular hemoglobin concentration; PLATE-platelets

[1] Values are means of different treatment animals for n=9.
[2] Pooled standard error of the mean.
[3] *Treatment mean differences are significant ($P<0.05$) due to diet.
**Treatment mean differences are significant ($P<0.05$) due to infection.
[4] Values are means of different treatment animals for n=7.

Table 11.

HEMATOLOGY RESULTS OF WEANED PIGS[1]
Experiment 2
(Leukocytes)

	Treatments				
	-Ecoli-GLN	-Ecoli+GLN	+Ecoli-GLN	+Ecoli+GLN	SEM[2]
Leukocytes		Absolute count (x 10^9 cells/L)			
Lymphocyte	7.29	9.46	7.02	7.52	0.75
Monocyte[3] **	0.28	0.27	0.47	0.69	0.15
Neutrophil[4] **	2.54	2.41	4.33	3.52	0.71
Band cells[5] **	0.90	0.48	2.95	4.81	0.91
Eosinophil, Basophil	0.06	0.05	0.21	0.88	0.35
		% of WBC			
Lymphocyte**	69	76	49	45	4
Monocyte[3]	3	2	3	4	1
Neutrophil	21	18	24	18	3
Band cells[5] **	12	4	21	27	6
Eosinophil, Basophil	1	1	2	5	2

[1] Values are means of different treatment animals for n=9.
[2] Pooled standard error of the mean.
[3] Values are means of different treatment animals for n=7
[4] *Treatment mean differences are significant (P<0.05) due to diet.
**Treatment mean differences are significant (P<0.05) due to infection.
[5] Values are means of different treatment animals for n=6

Table 12.

BLOOD LYMPHOCYTE PHENOTYPES[1]
Experiment 2

	Treatments				
	-Ecoli-GLN	-Ecoli+GLN	+Ecoli-GLN	+Ecoli+GLN	SEM[2]
Phenotype	% Lymphocytes[3]				
T cells (CD2)	53	51	60	60	6
T helper cells (CD4)	19	18	17	22	2
T cytotoxic cells (CD8)	25	26	35	30	6
B cells (IgM)	24	30	18	23	5
CD4:CD8 Ratio	0.8	0.7	0.5	0.7	0.1

[1] Values are means of different treatment animals for n=3.
[2] Pooled standard error of the means
[3] 10-20% of lymphocytes were not identified by the mitogens

Table 13.

PLASMA AMINO ACID CONCENTRATIONS[1]

Experiment 2

(Non-infected 28day old pigs vs 21day old pigs)

	28-days		21-days	
	-Ecoli-GLN	-Ecoli+GLN	Suckled	SEM[2]
Amino Acids		µmol/L		
Alanine	536	450	531	46
Arginine[3]	81[a]	85[a]	154[b]	13
Asparagine	48[a]	52[a]	88[b]	7
Aspartate	52	24	28	11
Citrulline	62[a]	68[a]	119[a]	6
Glutamate	333	285	283	34
Glutamine	388	466	368	60
Glycine	1226	917	757	163
Histidine	92	103	111	8
Isoleucine	174[a]	179[a]	123[b]	15
Leucine	173	196	167	19
Lysine	260	209	231	25
Methionine	56	91	91	18
Ornithine	80[a]	72[a]	123[b]	9
Phenylalanine	83	79	77	6
Serine	156[ab]	124[a]	201[b]	17
Taurine	323	193	173	52
Threonine	409[a]	336[ab]	201[b]	54
Tryptophan	43	40	51	5
Tyrosine	81[a]	83[a]	148[b]	14
Valine	286	319	249	25

[1] Values are means of different treatment animals for n=9.

[2] Pooled standard error of the mean.

[3] Means of different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

Table 14.

LONGISSIMUS DORSI INTRACELLULAR AMINO ACID CONCENTRATIONS[1]

Experiment 2

(Non-infected 28day old pigs vs 21day old pigs)

	28-days		21-days	
	-Ecoli-GLN	-Ecoli+GLN	Suckled	SEM[2]
Amino Acids	nmol/g wet muscle tissue			
Alanine	1412	1311	1455	109
Arginine[3]	11257[a]	10110[a]	7122[b]	892
Aspartate	401	394	331	54
Asparagine	102	117	103	16
Citrulline	89[a]	102[a]	163[b]	10
Glutamate	2197	2316	2072	148
Glutamine	1768	2678	2041	304
Glycine	3484	3241	2852	425
Histidine	190	192	119	25
Isoleucine	155[a]	184[a]	72[b]	21
Leucine	182	211	109	35
Lysine	260	282	228	38
Methionine	40	49	52	10
Ornithine	321	363	489	63
Phenylalanine	127	142	69	33
Serine	293	281	344	23
Taurine	4372[a]	3611[b]	3061[b]	254
Threonine	437[a]	431[a]	193[b]	55
Tryptophan	54	51	62	6
Tyrosine	294	260	190	31
Valine	197[ab]	212[a]	139[b]	20

[1] Values are means of different treatment animals for n=9.

[2] Pooled standard error of the mean.

[3] Means of different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

Table 15.

HEMATOLOGY RESULTS OF WEANED PIGS[1]

Experiment 2

(Non-infected 28day old pigs vs 21day old pigs)

	28-days		21-days	
	-Ecoli-GLN	-Ecoli+GLN	Suckled	SEM[2]
	Hematological Parameters (units)			
WBC[3] (10^9/L)	10.79[ab]	12.77[a]	9.31[b]	0.82
RBC (10^{12}/L)	4.87[a]	5.47[b]	5.03[ab]	0.16
HGB (G/L)	88.56	98.78	102.33	3.92
HCT (L/L)	0.26	0.29	0.31	0.01
RDW	28.38[a]	26.32[a]	23.11[b]	1.01
MCV (10^{-15}L)	53.67[a]	53.22[a]	60.67[b]	1.34
MCH (10^{-12}G)	18.20[a]	18.00[a]	20.29[b]	0.31
MCHC (g/dL)	340.00	340.00	338.00	4.00
PLATE (10^9/L)	354.00[ab]	294.00[a]	483.00[b]	45.00

Abbreviations:
WBC-white blood cells; RBC-red blood cells; HGB-hemoglobin; HCT-hematocrit; RDW-red cell distribution width; MCV-mean corpuscular volume; MCH-mean corpusular hemoglobin; MCHC-mean corpusular hemoglobin concentration; PLATE-platelets

[1] Values are means of different treatment animals for n=9.
[2] Pooled standard error of the mean.
[3] Means of different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

Table 16.

HEMATOLOGY RESULTS OF WEANED PIGS[1]

Experiment 2

(Leukocytes)

(Non-infected 28day old pigs Vs 21day old pigs)

	28-days		21-days	
	-Ecoli-GLN	-Ecoli+GLN	Suckled	SEM[2]
Leukocytes	Absolute count (x 10^9 cells/L)			
Lymphocyte[3]	7.29^{ab}	9.46^{a}	6.51^{b}	0.76
Monocyte[4]	0.28	0.27	0.32	0.06
Neutrophil	2.54	2.41	2.09	0.14
Band cells[5]	0.91	0.48	0.31	0.33
Eosinophil, Basophil	0.06	0.05	0.01	0.03
	% of WBC			
Lymphocyte	69	76	67	4
Monocyte[4]	3	2	3	1
Neutrophil	21	18	24	3
Band cells[5]	11	4	4	4
Eosinophil, Basophil	0.7	0.5	0.1	0.3

[1] Values are means of different treatment animals for n=9.

[2] Pooled standard error of the mean.

[3] Means of different letters in the same row are significantly different ($P < 0.05$) from each other as analyzed by one-way ANOVA followed by Duncan's multiple range test.

[4] Values are means of different treatment animals for n=7.

[5] Values are means of different treatment animals for n=6.

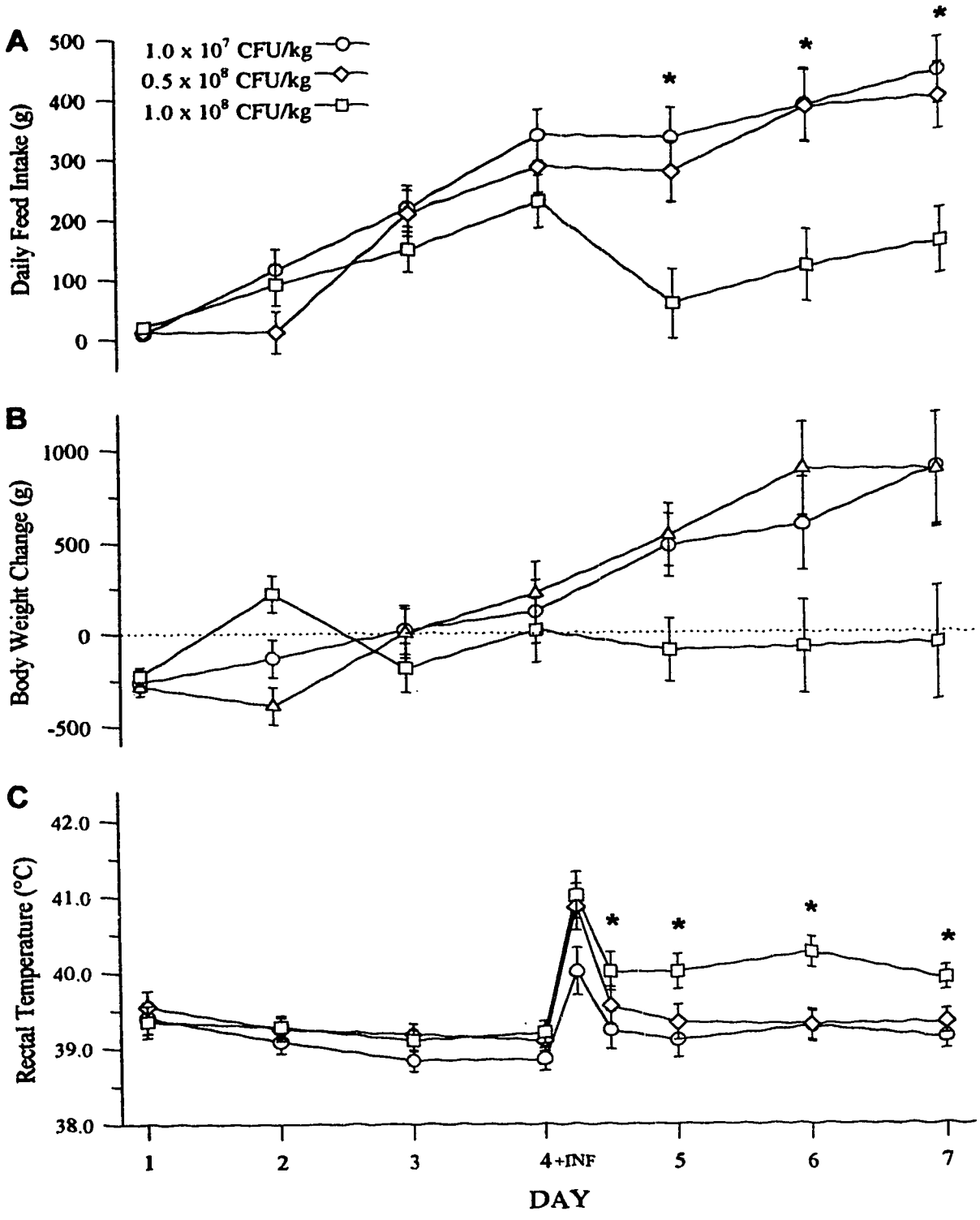


Figure 4: **Experiment 1 - Daily feed intakes, body weight changes from day 0, and rectal temperatures of animals treated with *E.coli*.** Weanling pigs are infected on day 4 as indicated by "+INF". Points represent group means ± SEM of n=4. The asterisks denote significant differences ($P<0.05$) among the group means.

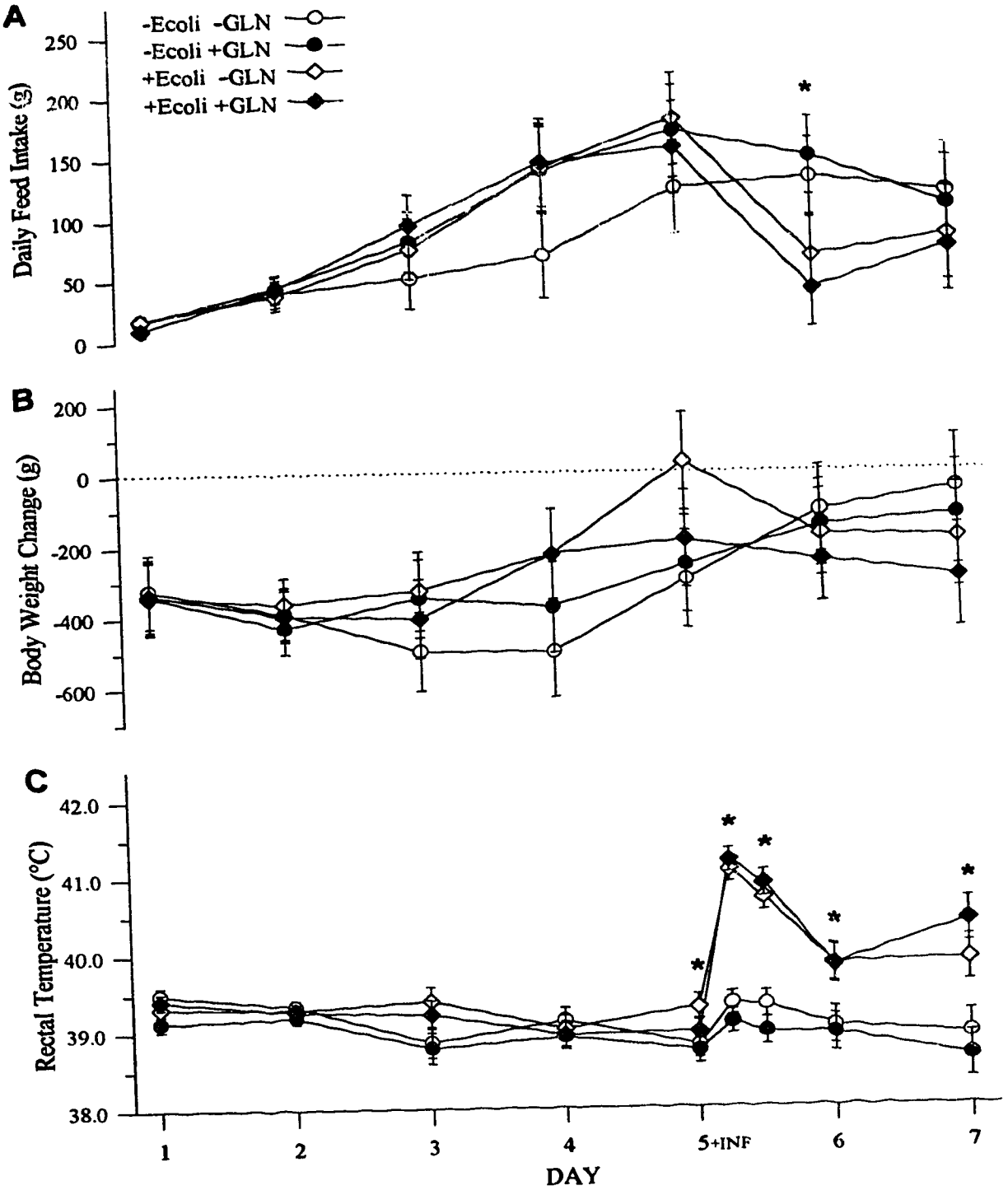


Figure 5: **Experiment 2 - Daily feed intakes, body weight changes from day 0 and rectal temperatures of weaned animals.** Weanling pigs are infected at 0900h on day 5 as indicated by "+INF". Points are group means ± SEM of n=9. The asterisks denote significant differences ($P<0.05$) among the group means due to infection.

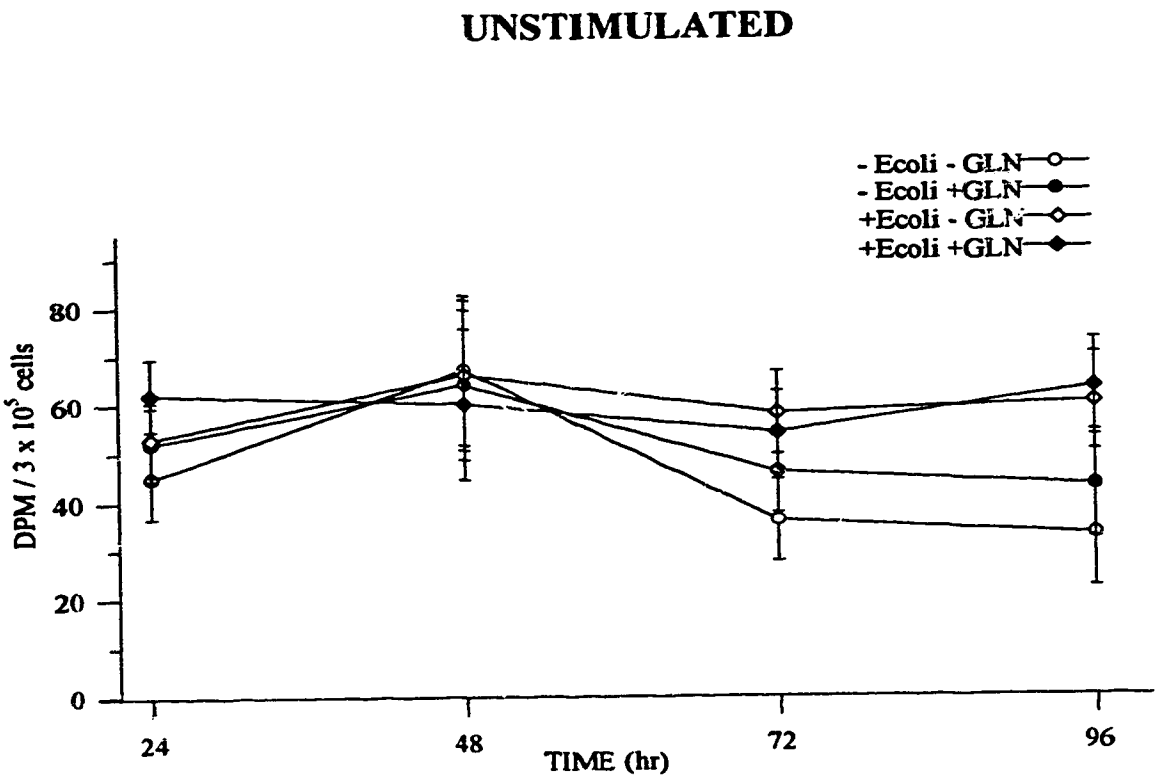
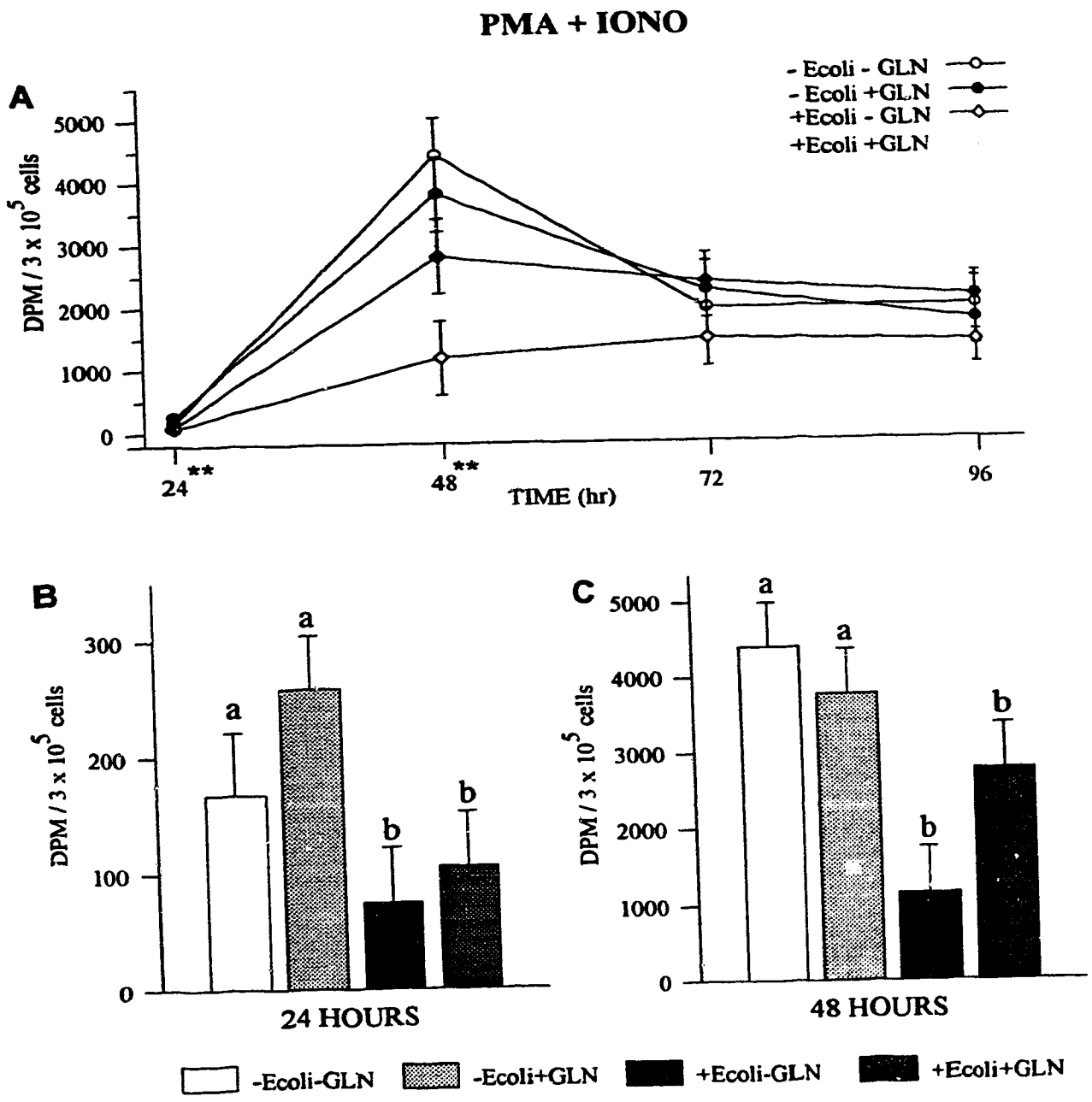
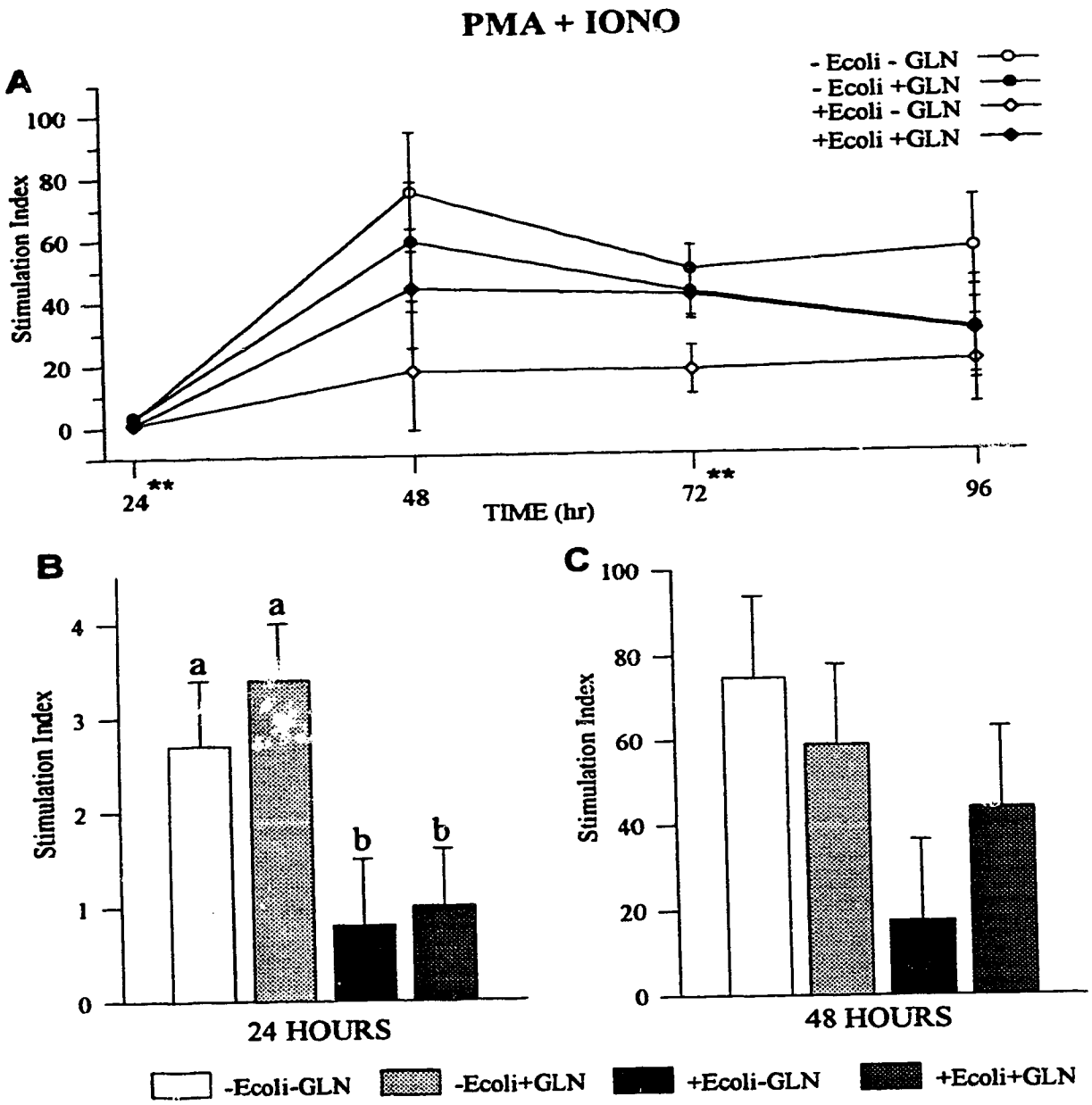


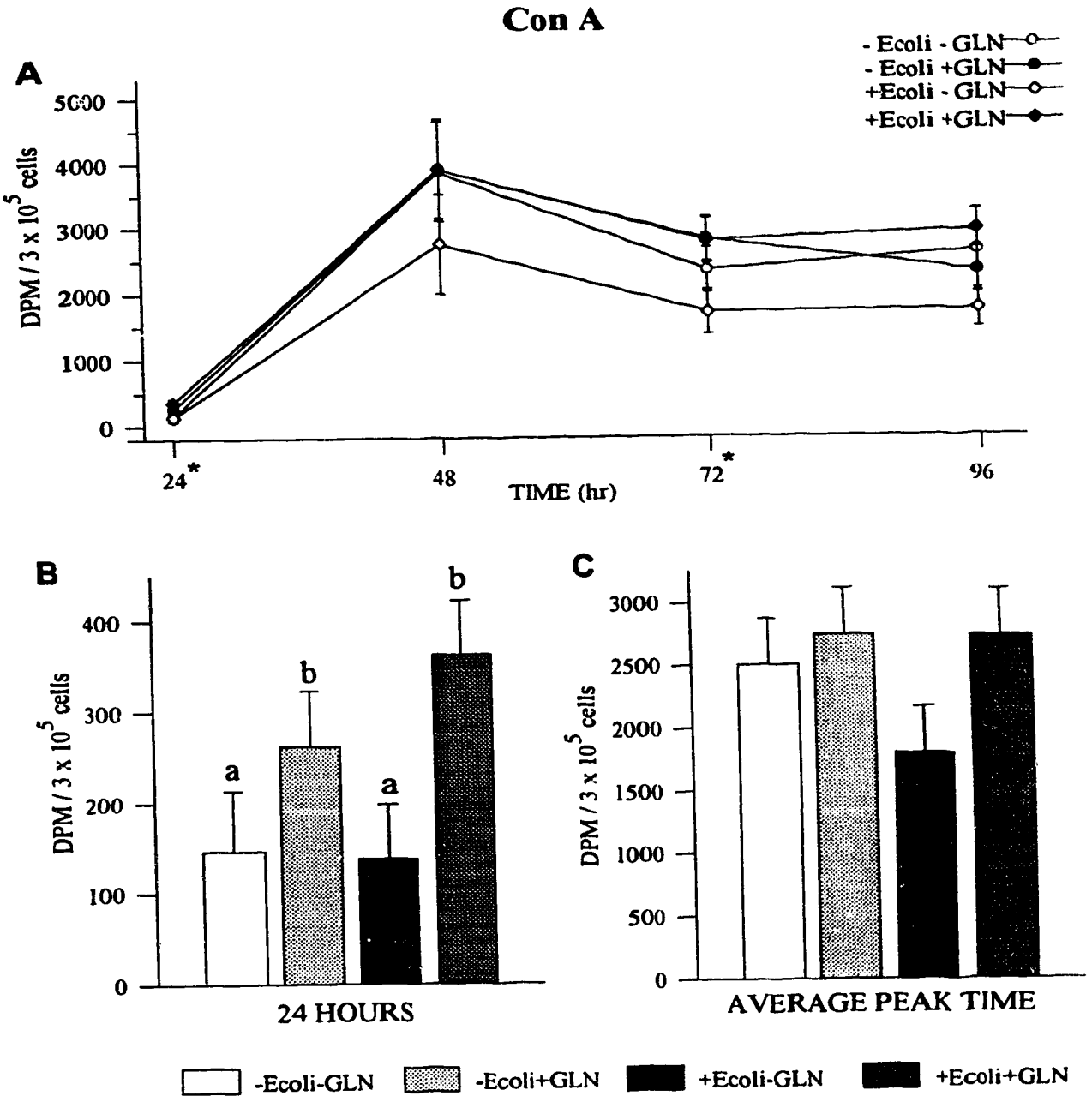
Figure 6: **The rate of [^{3}H]-thymidine uptake by unstimulated blood lymphocytes at 24h, 48h, 72h and 96h.** Responses are expressed as DPM / 3 x 10^5 cells. Values are means ± SEM (n=6) for all time points except for 48h (n=4). The treatment means are not significantly different.



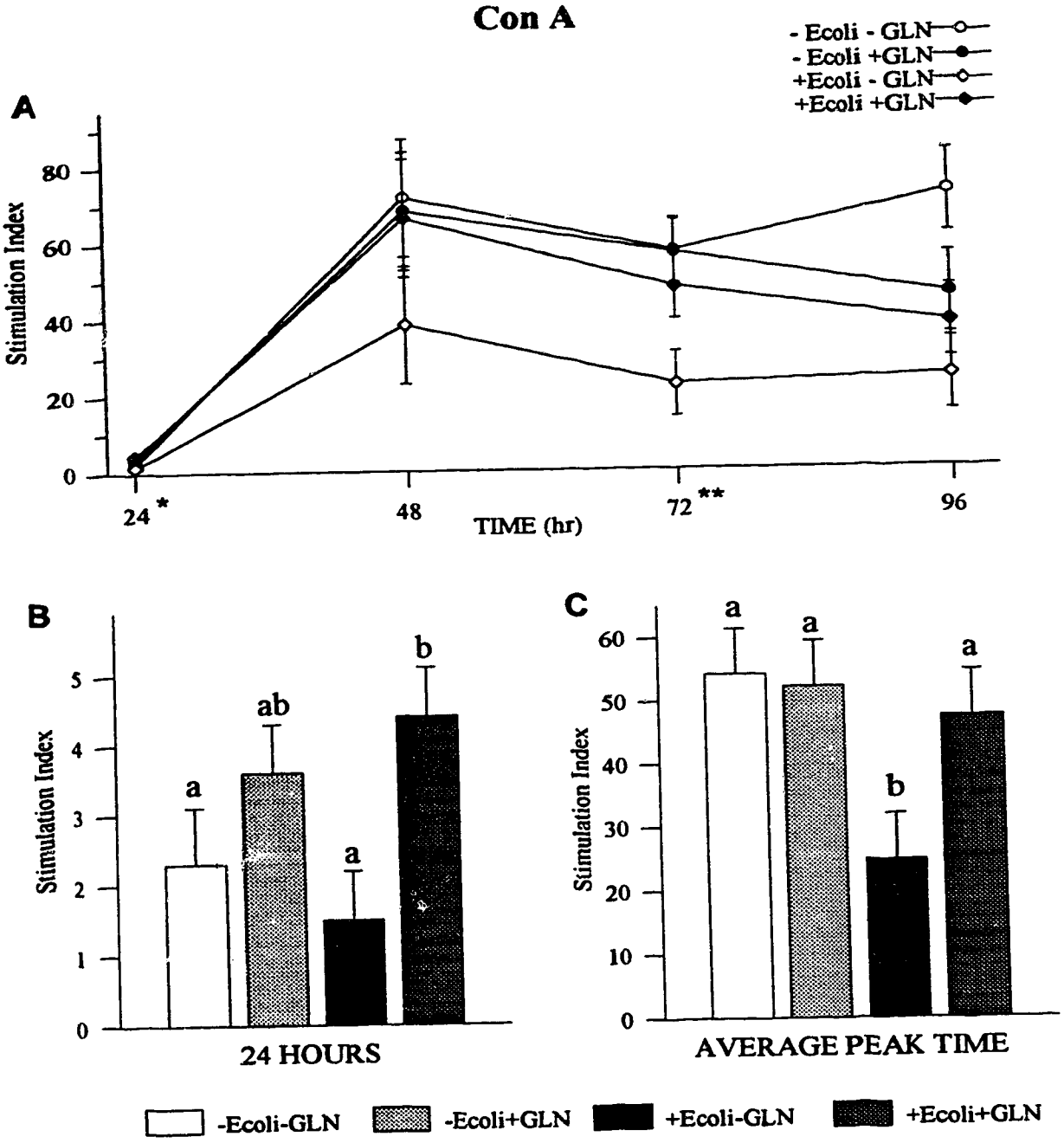
Figures 7A, B and C: **Mitogenic responses of blood lymphocytes to PMA+IONO at 24h, 48h, 72h , 96h.** The rate of [^{3}H]-thymidine uptake is expressed as DPM / 3 x 10^5 cells. Values are means ± SEM (n=5) for all time points except for 48h (n=3). As indicated by asterisks, " ** ", the treatment means at 24h and 48h are statistically different ($P<0.05$) due to infection (Figures A, B, and C). Values with different letters are statistically different ($P<0.05$) as identified by one-way ANOVA and Duncan's multiple-range test (Figures B and C).



Figures 8A, B and C: **Mitogenic responses of blood lymphocytes to PMA+IONO at 24h, 48h, 72h , 96h.** The rate of [^{3}H]-thymidine uptake is expressed as stimulation index ((mean DPM of stimulated cells - mean DPM of unstimulated cells)/ mean DPM of unstimulated cells)). Values are means ± SEM (n=5) for all time points except for 48h (n=3). As indicated by asterisks, " ** ", the treatment means at 24h and 72h are statistically different ($P<0.05$) due to infection (Figures A and B). Values with different letters are statistically different ($P<0.05$) as identified by one-way ANOVA and Duncan's multiple-range test (Figure B). The treatment means are not significantly different from each other at 48h (Figure C).



Figures 9A, B and C: **Mitogenic responses of blood lymphocytes to Con A at 24h, 48h, 72h , 96h and average peak time.** The rate of [^{3}H]-thymidine uptake is expressed as DPM / 3 x 10^5 cells. Values are means ± SEM (n=6) for all time points except for 48h (n=4). As indicated by asterisk, " * ", the treatment means at 24h and 72h are statistically different ($P<0.05$) due to diet (Figures A and B). Values with different letters are statistically different ($P<0.05$) as identified by one-way ANOVA and Duncan's multiple-range test (Figure B). The treatment means are not significantly different from each other at average peak time (Figure C).



Figures 10A, B and C: **Mitogenic responses of blood lymphocytes to Con A at 24h, 48h, 72h , 96h and average peak time.** The rate of [^{3}H]-thymidine uptake is expressed as stimulation index ((mean DPM of stimulated cells - mean DPM of unstimulated cells)/ mean DPM of unstimulated cells)). Values are means ± SEM (n=6) for all time points except for 48h (n=3). As indicated by asterisks, "*" and "**", the treatment means are statistically different ($P<0.05$) due to diet (24h) and infection (72h), respectively (Figures A and B). Values with different letters are statistically different ($P<0.05$) as identified by one-way ANOVA and Duncan's multiple-range test (Figures B and C).

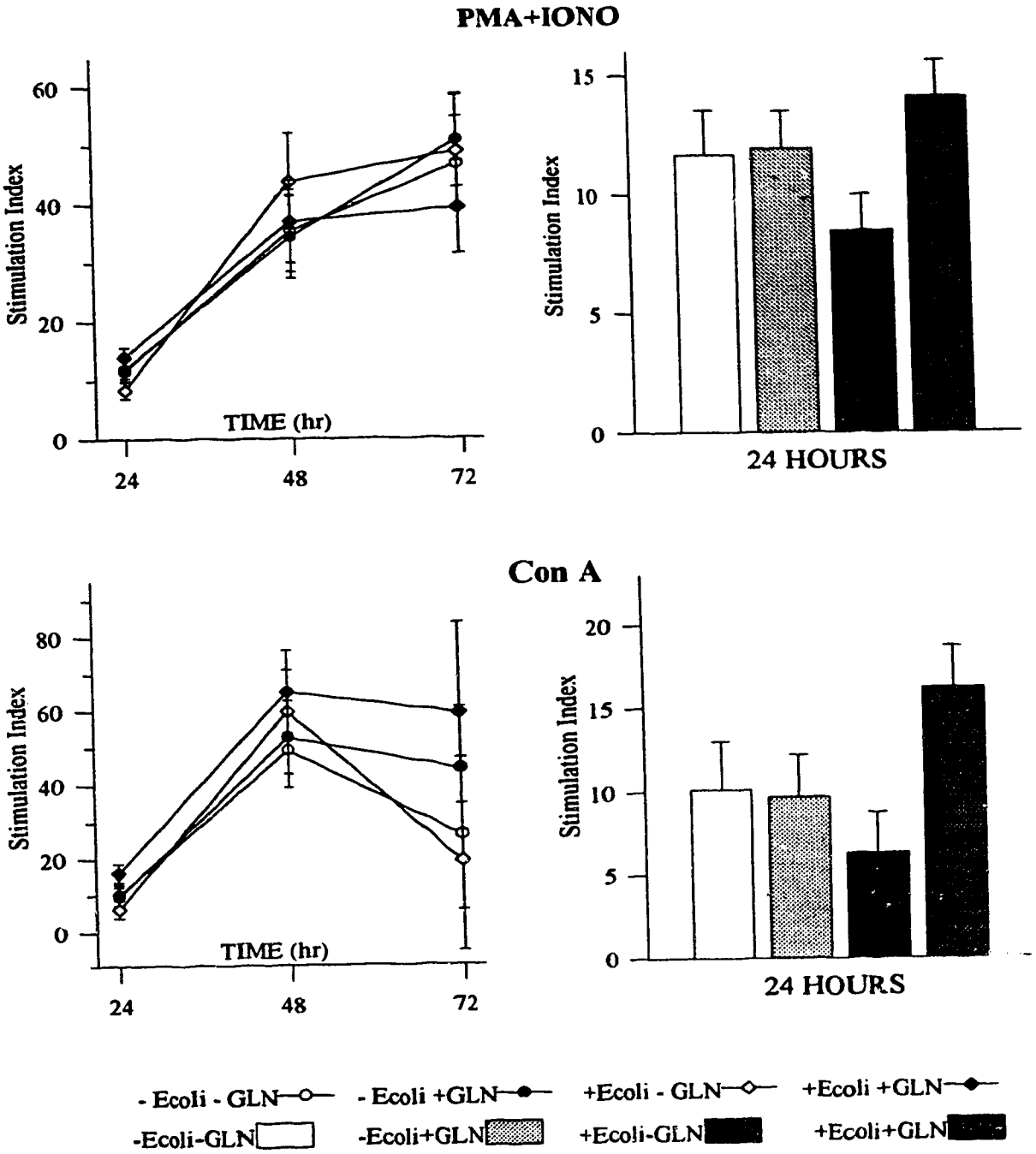


Figure 11: **Effects of treatments on the mitogenic responses of mesenteric lymphocytes at 24h, 48h, and 72h.** Responses to PMA+IONO (n=5) and Con A (n=6) are expressed as stimulation index. Values are means ± SEM. The four treatment means are not significantly different.

4. DISCUSSION

Post-weaning diarrhea is commonly associated with enterotoxigenic *E.coli* (Sarmiento et al., 1988) so an *E. coli* infection model was developed. *E. coli* peritonitis is an effective mean to initiate acute phase reactions. As well, peritonitis reduces cross-contamination since the bacteria are contained within the intraperitoneal cavity. This model also allows control over the dosage and strain of bacteria, and it is very reproducible (Samuels and Baracos, 1992; Inoue et al., 1993).

To establish a reproducible infection model that would allow animals to continue feeding, the effects of three *E. coli* dosages were examined in experiment 1. The results suggest that animals infected with less than $1x10^8$ CFU/kg BW experienced a mild to moderate infection. Feed intakes and body weight gains continued to increase after infection (Table 4; Figures 4A, 4B), rectal temperatures reverted to normal within 24h (Figure 4C), and plasma and intracellular glutamine concentrations did not decrease (Tables 5, 6). Therefore, an infection rate of $0.5x10^8$ CFU/kg BW was chosen for subsequent experiments.

Feed intakes and body weights did not differ among the treatment groups in experiment 2 (Table 7; Figures 5A, 5B). However, it is important to note, all animals were observed to have poor intakes of these elemental diets and consequently, all animals lost body weight (maximum 4 % of initial body weight). Feed intakes of 675 g/day and body weight gains of 450 g/day are performance targets for weanling pigs but various factors can affect feed consumption and growth rates (i.e. age of weaning, environmental changes, removal from sow and littermates, and no access to creep

feeding; Patience and Thacker, 1989). Although Chung and Baker (1992) reported normal feed intakes and body weight gains with elemental diets, these pigs were fed for 3 weeks, and feed intakes were variable and poor during the first 7 days postweaning (Baker, D.H., personal communication). Hence, in this study the palatability of the elemental diet and short feeding schedule may explain these low feed intakes and weight gains.

Despite the selection of a moderate infection level ($0.5x10^8$ CFU/kg BW), the infected pigs in experiment 2 continued to have elevated rectal temperatures 24 hours after *E. coli* injection (Figure 5C). The increased persistence of infection may have been attributed to their low feed intake since nutritional status negatively affects the acute phase response (Saez-Llorens & Lagrutta, 1992).

Infection alters plasma and intracellular amino acid distributions (Askanazi et al., 1980; Clowes et al., 1980; Milewski et al., 1982; Petersson et al., 1992). Plasma amino acid concentrations of septic patients are generally characterized by increased concentrations of aromatic amino acids (phenylalanine and tyrosine) and sulfur containing amino acids (taurine, methionine and cystine), and sometimes decreased concentrations of glutamine. Similarly, skeletal muscles exhibit increased intracellular concentrations of aromatic amino acids, as well as branched-chain amino acids. However, the most notable change in skeletal muscle is the marked decrease of intracellular glutamine level, with up to 50% reductions in muscle glutamine content (Askanazi et al., 1980). Similar changes in plasma and LD intracellular amino acid concentrations were also observed in this study (Tables 8 and 9).

Although a comparison of plasma and muscle intracellular glutamine concentrations of 28-day healthy pigs with 21-day suckled pigs suggests that healthy weaned pigs can synthesize adequate amounts of glutamine (Tables 13 and 14), 28-day infected pigs had significantly lower glutamine concentrations (Tables 8 and 9). The decline in plasma glutamine concentration has been attributed to the changes in glutamine interorgan flow among splanchnic tissues, immune cells and skeletal muscle (Souba and Austgen, 1990; Sarantos et al., 1993). Since alanine, citrulline and ornithine are metabolites of glutamine metabolism by enterocytes (Wu et al., 1994), reduced plasma concentrations of these amino acids displayed by the infected pigs may reflect decreased glutamine metabolism by the small intestine (Table 8). The low glutamine uptake by the small intestine of infected animals has been reported to be due to anorexia, impaired transport of glutamine across the basolateral membrane, and depressed glutaminase activity (Souba et al., 1990a; Ardawi et al., 1991; Salloum et al., 1991). In contrast, hepatic consumption of glutamine increases to support elevated rates of gluconeogenesis, glutathione production and acute phase protein synthesis during infection (Austgen et al., 1991). Also, glutamine uptake is increased in stimulated lymphocytes as a source of biosynthetic precursors and energy for lymphocyte responses (Brand et al., 1986). The increased glutamine consumption by the body is primarily supported by accelerated glutamine release from the skeletal muscle (Newsholme and Parry-Billlings, 1990; Souba, 1992). Thus, the decrease in muscle intracellular glutamine concentration of the infected pigs may be associated with increased glutamine efflux. These results are consistent with other studies that

have examined the effects of infection on glutamine concentrations (Askanazi et al., 1980; Parry-Billings et al., 1989; Ardawi and Majzoub, 1991).

Changes in plasma and muscle concentrations of aromatic and branched-chain amino acids (BCAAs) were also observed in the infected animals. Plasma amino acid profile is influenced by factors that affect the rate of release of amino acids from skeletal muscle and functional status of splanchnic tissues, particularly the liver. Hence, changes in plasma amino acid concentrations may provide an early indications of metabolic alterations of these tissues. Investigators have observed that progressive liver dysfunction is characterized by increased levels of aromatic and sulfur containing amino acids in the blood (Askanazi et al., 1980; Beisel, 1985; Ardawi and Majzoub, 1991). However in this study, the infected pigs displayed decreased plasma tryptophan and tyrosine concentrations and increased phenylalanine concentration (Table 8). Also, plasma concentrations of other amino acids metabolized by the liver such as methionine, threonine, alanine and histidine did not increase, indicating liver function was not impaired in the Ecoli-treated pigs.

Plasma and intracellular branched-chain amino acids concentrations increase during infection (Askanazi et al., 1980). BCAA concentrations are thought to be catabolized in muscle to yield metabolizable energy (Beisel, 1985). BCAAs are important for glutamine synthesis in the muscle since they provide the carbon skeleton for glutamine (Newsholme and Leech, 1985). However in the present study, the infected animals did not display high BCAA concentrations in the muscle or the

plasma (Tables 8 and 9). Nevertheless, Ecoli-treated pigs receiving glutamine supplementation had significantly lower plasma valine levels.

Plasma and intramuscular amino acid profiles can also be altered by diet. Increased concentrations of plasma serine, glycine and taurine reflect the addition of these amino acids in the -GLN diet (Table 8). However, glutamine supplementation did not increase plasma glutamine level. Perhaps, this is attributed to low voluntary feed intake and/or increased utilization of supplemental glutamine by enterocytes, hepatocytes and immune cells. Similarly, Petersson et al. (1994) showed no significant changes in plasma glutamine concentration in post-surgical patients that received glutamine supplemented TPN.

In contrast to plasma glutamine levels, pigs receiving glutamine supplementation had higher intramuscular glutamine concentrations than those animals fed -GLN diets (Table 9). This result is consistent with other studies that have shown glutamine supplementation increases muscle intracellular glutamine concentrations (Hammarqvist et al., 1989; Petersson et al., 1994). Increased muscle glutamine levels may be beneficial to these pigs since low intramuscular glutamine concentrations have been associated with low rates of muscle protein synthesis (Millward et al., 1989; Newsholme and Parry-Billings, 1990; Rennie et al., 1994). MacLeannan et al. (1987) found that a rise in intramuscular glutamine concentration stimulated protein synthesis in rats. Similarly, Hickson et al. (1995) have shown provision of additional glutamine counteracted the effects of glucocorticoid-mediated muscle atrophy. The prevention of atrophy was associated with maintenance of

intramuscular glutamine concentration and a partial reversal of the declines in myosin heavy chain and total protein synthesis.

The plasma and intracellular amino acid analysis reveal intriguing differences in amino acid patterns among species. In humans and rats, glutamine is the most abundant amino acid in plasma and intracellular pools, excluding taurine (i.e. 0.6 mmol/L and 19.5 mmol/L ICH_20, respectively in humans; 1.1 mmol/L and 9 mmol/L ICH_20, respectively in rats; Bergstrom et al., 1976; Turinsky and Long, 1990). However in this study, plasma and intracellular glutamine are not present as the highest amino acid concentrations (Table 5 and 6). The results agree with data of Deutz et al., (1992) who reported plasma glutamine concentrations in piglets to be 0.335 mmol/L , and plasma glycine as the most abundant amino acid (0.650 mmol/L). There are also differences in the pattern of intracellular free amino acids of swine. Unlike humans and rats (~1-2 %; Bergstrom et al., 1974; James et al., 1993), intramuscular arginine concentration accounts for approximately 53-45% of total free amino acids in pigs (Table 9). The high arginine levels may reflect the type of muscle studied. Turinsky and Long (1990) reported muscle-fiber type dependent changes in free amino acid profiles in rats. Indeed, arginine and glutamine intramuscular concentrations of gastrocnemius muscle from weaned pigs are different than that in LD, at 4888 ± 1518 nmol/g wet muscle tissue and 3772 ± 1505 nmol/g wet muscle tissues, respectively (Yoo and McBurney, 1994, unpublished data). These results suggest the effects of muscle type on amino acid profiles need to be further studied.

Hematological data were also analyzed to examine the effects of glutamine supplementation and infection during weaning. The hematological values of all treatment animals were within normal range for 28-day weaned pigs (Table 10; Friendship et al., 1984). Also, a comparison of hematological values of 28-day healthy pigs with 21-day suckled pigs (Tables 15 and 16) displayed expected age-related changes in leukocyte populations and other blood parameters in healthy young pigs (Veterinary Clinical Pathology Laboratory, University of Saskatchewan, personal communication). Although glutamine supplementation increased WBC, RBC, HGB and HCT, when the hematological values were adjusted for differences in hematocrit, glutamine supplementation significantly increased ($P<0.05$) only total WBC.

An increase in total leukocyte count is a hallmark of infection. Activation of the immune system elicits rapid proliferation and differentiation of immune cells (Abbas et al., 1991). The rise in WBC counts (Table 10) of the infected animals was attributed to a significant increase in monocytes, neutrophils and band cells but not lymphocytes (Table 11). Increased populations of neutrophils, monocytes, and tissue macrophages are often detected in bacterial infections (Zimmerman and Ringer, 1992). Phagocytosis is one of the key mechanisms used to rapidly destroy and remove extracellular microbes. Associated with rapid escalation of phagoctyes is an increased release of immature neutrophils from the bone marrow, these cells are referred to as band cells (Bishop et al., 1968).

In contrast to the increase in phagocytic cells, absolute lymphocyte counts did not increase. Although lymphocytes expressed as percentage of WBC, indicated a reduction in lymphocytes in the *Ecoli*-treated animals, absolute lymphocyte counts were within normal ranges (McCauley and Hartmann, 1984), and the relative percentage of B and T cells did not differ with diet or infection (Table 12). It is noteworthy however, that in pigs the proportion of T-cytotoxic/suppressor (CD8) cells is greater than T-helper (CD4) cells (Bala et al., 1992; VMRD Inc, personal communication). This differs from human blood where the proportion of CD4 is greater than CD8 (Erkeller-Yuksel et al., 1992).

It is well established that nutrition has profound effects on host immune defenses. The effect of glutamine supplementation on immune functions has not been studied in the early weaned pigs although early weaning in pigs has been associated with immunosuppression, reduced voluntary food intake and increased susceptibility to intestinal disturbances (Miller et al., 1983; Wilson et al., 1989; Rivera et al., 1987). During this transition period, the protective immunoglobulins from maternal milk is no longer provided (Porter, 1976) and their cell-mediated immunity may become suppressed. Blecha et al., (1983) have demonstrated that lymphocytes isolated from pigs weaned at 2 and 3 weeks of age have depressed responses to phytohemagglutinin (T-cell mitogen) and pokeweed mitogen (B-cell dependent T-cell mitogen).

Similarly, blood lymphocytes of infected animals displayed reduced responses in vitro to PMA+IONO (Figures 7, 8). Glutamine supplementation increased [^{3}H]-thymidine incorporation in response to Con A stimulation (Figures 9, 10).

At the peak response, the stimulation index to Con A of lymphocytes from +Ecoli-GLN pigs was depressed but lymphocytes isolated from +Ecoli+GLN pigs responded similarly to those from the uninfected animals (Figure 10C). These results agree with studies that have shown glutamine supplementation supports immune function during critical states (Burke et al., 1989; Alverdy et al., 1992; Ziegler et al., 1992; Inoue et al., 1993). For example, O'Riordain et al., (1994) have shown enhanced DNA synthesis of T-lymphocytes isolated from surgical patients that received glutamine supplemented TPN.

The differential response of lymphocytes to PMA+IONO and Con A stimulation may reflect different processes involved in lymphocyte activation by these mitogens. PMA and IONO are non-specific mitogens that stimulate all mononuclear cell populations (Field et al, 1990). PMA and IONO work simultaneously to stimulate cells without activating the membrane-binding step that requires antigen-presenting cells and lymphokines. Generally, PMA activates protein kinase C, whereas IONO increases intracellular calcium concentration to mediate protein phosphorylation (Abbas et al., 1991). Unlike PMA+IONO, Con A specifically activates T-cells via binding to a specific membrane receptor (i.e. T-cell receptor:CD3 complex or TCR:CD3) and subsequently phosphorylating membrane-bound proteins (Abbas et al., 1991). In its activation of T-cells, Con A requires assistance of accessory cells and their soluble mediators.

The different response of lymphocytes from infected animals not receiving glutamine to Con A suggests that T-cells are sensitive to glutamine. Horig et al.,

(1993) examined the activation steps of TRC:CD3 complex in lymphocytes (i.e. phytohemagglutinin activation) and demonstrated that activated T-cells first proceed through a phase which is independent of exogenous glutamine followed by glutamine-dependent lymphocyte activation stages. Hence, cell cycle analysis showed that the proportion of cycling cells correlated with glutamine concentration, and at least 0.5 mM exogenous glutamine was required for activation of human lymphocytes. Similarly, Ardawi and Newsholme (1983) have shown rat lymphocyte proliferation upon Con A stimulation is dependent on exogenous glutamine concentration where maximum rates of [^{3}H]-thymidine incorporation is observed at 0.3mM glutamine.

These studies indicate lymphocyte activation via TCR:CD3 complex requires glutamine. Thus, the effects of glutamine supplementation were detected in Con A stimulated lymphocytes and not in PMA+IONO activated cells. Although glutamine supplementation did not significantly increase plasma glutamine concentrations of the infected animals (Table 8), +Ecoli+GLN animals experienced better glutamine status than +Ecoli-GLN animals as indicated by intramuscular glutamine concentrations (Table 9).

In addition, other factors such as changes in the production or recognition of cell membrane receptors, modification in the function or relative numbers of antigen-presenting cells and T cells, and altered production of lymphokines may also influence the results of the mitogen response assay (Field et al., 1990; Bala et al., 1992). For example, macrophages were not measured in this study but monocyte

concentrations (macrophages precursors) tended to be higher in the +Ecoli+GLN animals than +Ecoli-GLN pigs (Table 11). Glutamine has been shown to affect phagocytic activities and IL-1 production of macrophages (Wallace and Keast, 1992) but further studies are required to understand the potential impact of glutamine on antigen-processing and presentation.

MLN lymphocytes responded similarly to glutamine supplementation (Figure 10). Although the values among treatments were not statistically different, glutamine-supplemented, infected pigs tended to have increased [^{3}H]-thymidine incorporation at 24 hours after both PMA+IONO and Con A stimulation. This trend was maintained at 48h and 72h in Con A stimulated cells but not in PMA+IONO activated cells. Like peripheral lymphocytes, MLN lymphocytes have increased rates of glutamine uptake during stimulation (Dudrick et al., 1993). MLN lymphocytes obtained from infected rats with *E.coli* peritonitis have greater glutaminase mRNA expression and activity than those obtained from uninfected animals (Sarantos et al., 1993). It also has been demonstrated that glutamine supplemented TPN solution maintains both B and T populations of lymphocytes isolated from lamina propria as well improved secretory IgA levels (Alverdy et al., 1992). Since gut associated lymphoid tissues constitute about 25% of mucosal cell mass (Jankowski et al., 1994), the maintenance of their immune function may help in the preservation of gut integrity.

4.3. CONCLUSION

The results of this study with piglets were consistent with studies involving glutamine supplementation in humans and rats (Souba et al., 1990; Lacey and Wilmore, 1990; Inoue et al., 1993; Petersson et al., 1992; Moskovitz et al., 1994). Healthy, weaned pigs eating less than normal nutrient requirements did not seem to benefit from exogenous glutamine. However, when the pigs were exposed to a moderate infection, glutamine supplementation maintained normal muscle intracellular glutamine concentrations, leukocyte populations and lymphocyte functions. The provision of glutamine in diets, formulated to have an ideal amino acid profile, was beneficial in moderately infected pigs. It remains to be determined if the addition of glutamine to standard diets would minimize morbidity and weight loss associated with weaning in practical environments.

4.4. FUTURE STUDIES

The effects of starvation on protein metabolism and immune responses are well documented. Since the pigs in this study had low feed intakes, malnutrition may have influenced the results. Thus the effect of glutamine supplementation needs to be studied in pigs with typical feed intakes of 300-500g/d. Changes in receptor function and number, lymphokine production, and phenotypic profile need to be examined in more detail to assess the role of glutamine on lymphocyte function, antigen presentation and processing.

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6. APPENDIX

Determination of cell, mitogen and [^{3}H]-thymidine concentrations

A.1. METHODS

Approximately 20 mL of blood were collected from 28-day old pigs (n=2). Lymphocytes were isolated as stated in the above method 2.5.1.1.

A.1.1. CELL CONCENTRATION:

Different cell concentrations were analyzed at 48h and 72h time periods. ConA and PMA+IONO concentrations were same as methods involved in lymphocyte mitogen response assay (Section 2.5.2.). Stimulation of lymphocytes was measured by [^{3}H]-thymidine incorporation (0.05 mCi/well), as indicated in method section 2.5.2. The cell concentrations used were $3x10^6$ cells/mL, $1.5x10^6$ cells/mL, $1.25x10^6$ cells/mL and $0.75x10^6$ cells/mL.

A.1.2. MITOGEN CONCENTRATION:

Different mitogen concentrations were examined using the procedure described under 2.5.2 with the following modifications. The effects of different mitogen concentrations were examined at 24h, 48h and 72h incubation period using a cell concentration of $1.5x10^6$ cells/mL. [^{3}H]-thymidine incorporation (0.05 mCi/well) was investigated for each of the following mitogens at different concentrations: ConA, 50 mg/L, 5 mg/L and 0.5 mg/L; and either PMA alone at 40 µg/L, or PMA at 400 µg/L, 40 µg/L and 4 µg/L with IONO, 400 µg/L.

A.1.3. [^{1}H]-THYMIDINE CONCENTRATION:

Lymphocytes ($3x10^5$ cells/well) were stimulated with Con-A (5 mg/L) or PMA (40 μg/L) with IONO (400 μg/L). The cultured cells in each well were pulsed with either 1 μCi, 0.5 μCi, 0.05 μCi or 0.025 μCi of [^{3}H]-thymidine, 18 hours prior to harvest time. Thymidine incorporation was determined by liquid scintillation spectrometry in a β-counter as outlined in section 2.5.2.

A.2. RESULTS

Peak mitogen responses seemed similar among all four cell concentrations when expressed as DPM/ 3 x 10^5 cells (Figure A-1). When mitogen responses were expressed as stimulation index, different cell concentrations did not seem to affect mitogen responses.

Unstimulated cell responses for different levels of PMA, PMA+IONO and ConA were expressed as stimulation index (Figure A-2). Blood lymphocyte responses peaked by 48 hours for all levels of PMA+IONO and remained elevated at 72 hours. PMA concentration of 40 μg/L with 400 μg/L of IONO seemed to cause the highest stimulation response.

Con A concentrations influenced lymphocyte [^{3}H]-thymidine incorporations (Figure A-2). ConA level of 50 mg/L did not stimulate lymphocytes, indicating the concentration was too high. However, 5 mgConA/L caused a peak response by 48

hours which was maintained at 72 hours. Although 0.5 mg/L of Con A did not cause a peak response by 48 or 72 hours, lymphocyte proliferation continued to increase with time.

The in vitro proliferative response to mitogens did not increase linearly with increasing [^{3}H]-thymidine concentrations (Figures A-3 and A-4). However, as expected [^{3}H]-thymidine concentration of 0.025 uCi/well concentrations resulted in lowest [^{3}H]-thymidine incorporation rates whereas the highest incorporation rate was observed with 1 uCi/well.

Unstimulated cells responded similarly to each thymidine concentrations at all time points. At the two lowest [^{3}H]-thymidine concentrations, peak responses to PMA+IONO and Con A were observed at 48 hours (Figures A-3 and A-4, respectively). Similarily at the two highest concentrations, peak responses to PMA+IONO and Con A were at 48 hours.

Based on these studies, cell concentrations of 1.5 x10^6 cells/mL with mitogen concentrations of PMA (40 µg/L) with IONO (400 µg/L) and Con A (5 mg/L) in conjunction with [^{3}H]-thymidine concentration of 0.05 uCi/well were used in experiment 2.

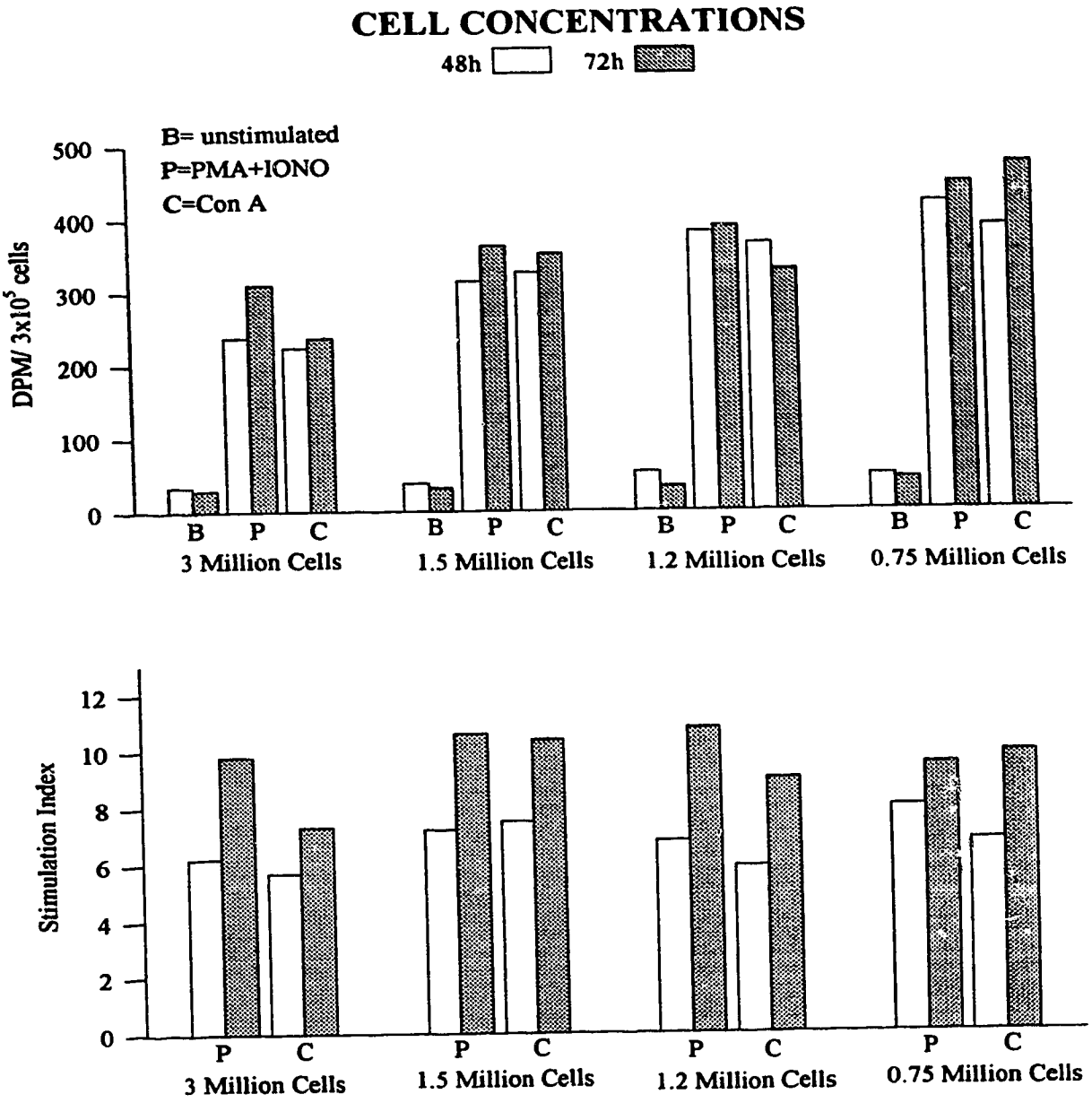

Figure A1: **The effects of different cell concentrations on mitogen response at 48h and 72h.** The responses without mitogen (B = unstimulated) and with PMA+IONO (P) and Con A (C) to four different concentrations of cells are expressed as DPM/ $3x10^5$ cells and stimulation index. The bars represents means of n=2.

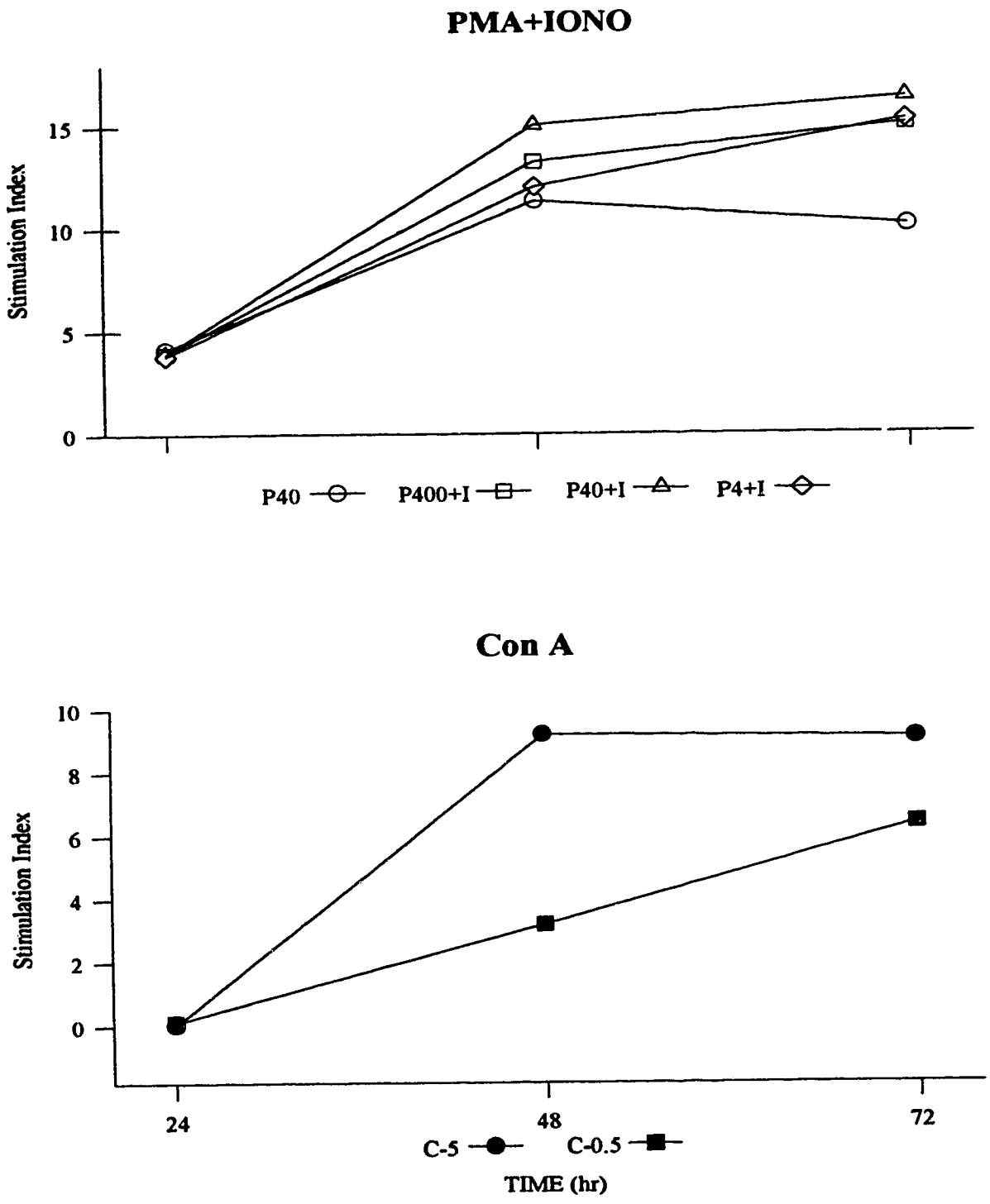

Figure A2: **The effects of different mitogen concentrations at 24h, 48h and 72h.** Responses to four different combination of PMA+IONO and two concentrations of Con A are expressed as stimulation index. Con A concentration of 50 mg/L remained unstimulated, the response is not shown. Each points represents means of n=2.

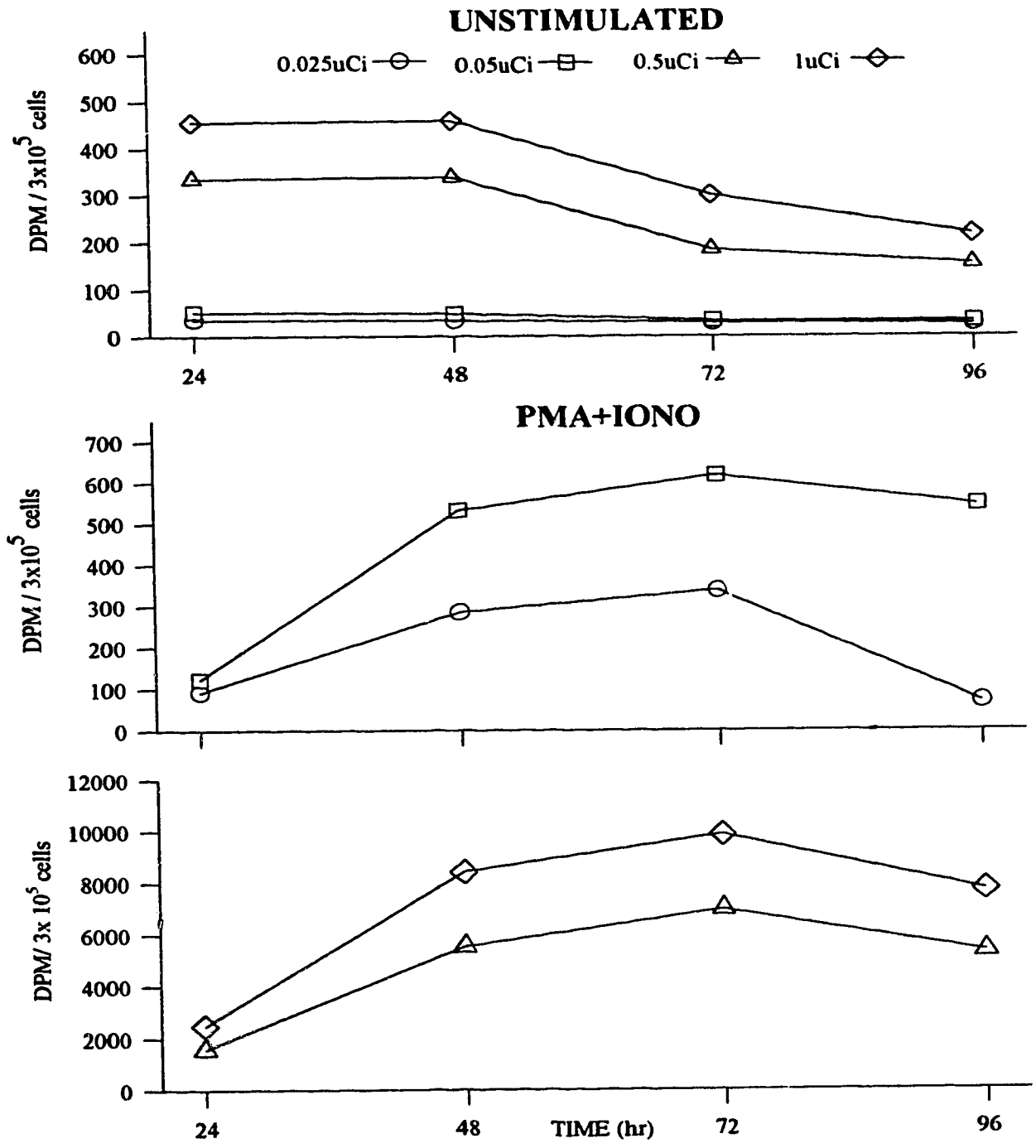

Figure A3: **The effects of different [^{3}H]-thymidine concentrations on PMA+IONO stimulated peripheral lymphocytes at 24h, 48h, 72h and 96h.** The responses in the absence of mitogen (unstimulated) and the presence of PMA+IONO at different [3H]-thymidine concentrations are expressed as DPM/ $3x10^5$ cells. Each point represents a mean of n=2.

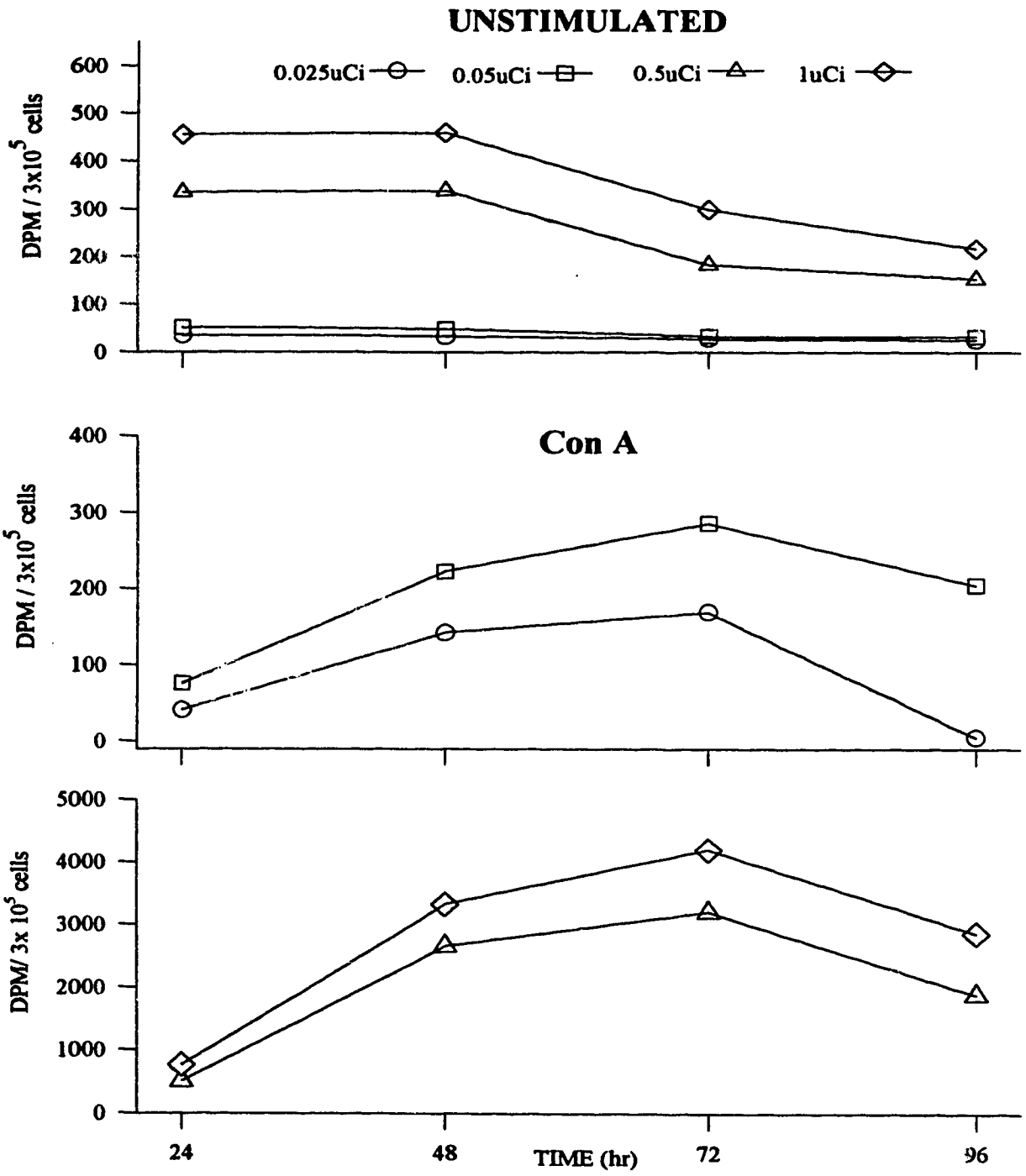

Figure A4: **The effects of different [^{3}H]-thymidine concentrations on Con A stimulated peripheral lymphocytes at 24h, 48h, 72h and 96h.** The responses in the absence of mitogen (unstimulated) and the presence of Con A at different [^{3}H]-thymidine concentrations are expressed as DPM/ $3x10^5$ cells. Each point represents a mean of n=2.