Effect of synthetic and bovine milk conjugated linoleic acid (CLA) on immune function

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Nutrition Science at Massey University, New Zealand

HUI ZHAO

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ABSTRACT

CLA is a collective name for a mixture of positional and geometrical isomers of linoleic acid (*c*-9, *c*-12-octadecadioenic acid) which possess conjugated double bonds. CLA occurs in a variety of foods, but is present at higher concentrations in products from ruminants. Milk fat is the richest natural source of CLA. The objective of this research was to examine the immunomodulatory properties of CLA (both synthetic and natural CLA derived from bovine milk fat). Two experiments were conducted at the Milk and Health Research Centre, Massey University, Palmerston North, New Zealand.

The aim of the first experiment was to investigate the dose effect of different concentrations of synthetic CLA (Tonalin) on immune function. Mice were fed either skim milk powder based diet or the same diet supplemented with 0.1, 0.25, 0.5, 1.0 or 2.0% synthetic CLA (Tonalin) by weight. Animals were immunised orally with a mixture of polio vaccine in sodium bicarbonate (25 μl) and subcutaneously with Fluvax and Tetanus toxoid vaccine on days 7 and 21. After 4 weeks feeding, mice were euthanased by isoflurane overdose. Various immune parameters were measured and the results showed that synthetic CLA (Tonalin) enhanced a range of immune functions. Synthetic CLA stimulated PHA induced T lymphocyte proliferation at 0.25, 0.5 and 1.0% as compared with the control group ($p < 0.05$). Synthetic CLA enhanced macrophage phagocytosis in a dose dependent manner. Synthetic CLA enhanced antibody responses (mucosal and systemic) to vaccines (polio vaccine, Fluvax and Tetanus toxoid). Natural killer cell activity was significantly enhanced in mice fed 0.25 and 0.5% CLA. In general, 0.25% CLA was regarded as the best CLA level which achieved optimal immunoregulating effects.

The aim of the second experiment was to examine the effect of natural CLA derived from milk fat on immune responses in mice. Mice were fed a skim milk powder (SMP) based diet. The control diet was skim milk powder only, without any CLA or milk fat supplementation. The dietary treatments were: ordinary milk fat, fractionated milk fat (1st stage), 0.2% synthetic CLA (Tonalin) and CLA enriched milk fat. Animals were fed these

diets for 28 days. Mice were immunised orally with a mixture of polio vaccine/ovalbumin/cholera toxin in sodium bicarbonate on days 7, 14 and 21 and subcutaneously with Fluvax and ADT (Diptheria and Tetanus toxoid vaccine) on days 7 and 21. Natural CLA was found to stimulate PHA and Con A induced T lymphocyte blastogenesis. Supplementation with natural CLA also led to increased antibody responses to vaccines and increased $CD25^+$ populations in peripheral blood in mice. Natural CLA also enhanced macrophage phagocytosis. Synthetic CLA enhanced a range of immune functions which is consistent with the results in the first experiment.

It is noted that although the CLA content is low in milk fat, the natural CLA derived from milk fat expressed potent effects in enhancing the growth of immune cells and promoting a range of immune functions in mice.

Key words: conjugated linoleic acid (CLA), lymphocyte, macrophage, immunity, milk fat

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

AA	Arachidonic acid
CD	Cluster differentiation
CLA	Conjugated linoleic acid
Con A	Concanavalin A
CT	Cholera toxin
Ig A	Immunoglobulin A
Ig G	Immunoglobulin G
IFN-γ	Interferon-γ
IL-4	Interleukin-4
LPS	Lipopolysaccharide
NK cell	Natural killer cell
OV	Ovalbumin
Polio	Poliomyelitis
PHA	Phytohemagglutinin
PUFA	Polyunsaturated fatty acid
Tet Tox	Tetanus toxoid
Th cell	T helper cell
Ts cell	T suppresser cell
TNF	Tumour necrosis factor

CHAPTER ONE General Introduction

The fact that there is an explosive growth in the market for fresh and fermented food containing *probiotic microbes* indicates that the consumer is ready for the concept of functional foods (Clydesdale, 1997). The demand for functional food products that are able to provide specific health benefits, in addition to basic nutrition, is growing rapidly (Clydesdale, 1997).

Conjugated linoleic acid (CLA), is a newly recognised nutrient which has captured the attention of the nutrition and scientific communities. CLA is a collective name for a mixture of positional and geometrical isomers of linoleic acid (*c*-9, *c*-12- octadecadioenic acid) which possess conjugated double bonds. The *c*-9, *t*-11 and *t*-10, *c*-12 isomers are regarded as the biologically active forms of CLA (i.e. a *cis* double bond between the 9^{th} and 10^{th} carbons and a *trans* double bond between the 11^{th} and 12^{th} carbon atoms) (Ha *et al.*, 1990; Ip *et al.*, 1991; Belury, 1995). CLA is known to occur in a variety of foods, but is present at higher concentrations in ruminant products, with milk fat being the richest natural dietary source of CLA (Ha *et al.*, 1987; Parodi, 1994).

The concentration of CLA in milk fat varies substantially from 0.24% to 2.8% (Riel, 1963). The fluctuation is seasonal, with the highest values during the times in the season when the cows are fully pasture fed (Dhiman *et al.*, 1995). Milk from pasture-fed cows has a substantially higher CLA level than milk from cows fed grain.

CLA has been shown to exhibit a range of health benefits, including inhibition of carcinogenesis (mammary, stomach, skin and colorectal cancers) (Belury, 1995; Ha *et al.*, 1990; Ip *et al.*, 1991, 1994), antiatherogenic (Lee *et al.*, 1994; Nicolosi *et al.*, 1993), immunomodulatory (Miller *et al.*, 1994; Cook *et al.*, 1993), regulation of body fat (Pariza *et al.*, 1996) and growth promoting effects (Chin *et al.*, 1994). The present study is focused on the immunomodulatory properties of CLA. CLA has been found to enhance mitogen-induced lymphocyte blastogenesis, lymphocyte cytotoxic activity, and macrophage

phagocytic activity in experimental animals (such as rodents and pigs) (Chew *et al.*, 1997; Wong *et al.*, 1997). CLA was also observed to reduce catabolic effects induced by immune stimulation and thus inhibit weight loss (Cook *et al.*, 1993). However, systemic studies of the effect of dietary CLA on immune function are not available. The effect of CLA on antibody-mediated (mucosal and systemic) and other aspects of immunity remains to be determined.

An important point to be mentioned is that almost all of the studies that have been carried out to date have used the synthetic form of CLA, therefore the effect of natural CLA derived from milk fat on the immune system is still unknown.

The immune system is crucial for the human body to protect against infectious diseases and cancers (Sell, 1987). Deficiency or hyper-activation of any component of the immune system can predispose an individual to a greater risk of infection or may enhance the severity of a disease. Thus the production of specialised milk fats containing elevated levels of CLA that are able to enhance disease resistance may be a way for the dairy industry to regain the confidence of the consumer and promote the health benefits of milk fat. This, along with the development of a new market niche, would result in increased dietary intakes of CLA, without compromising total fat and cholesterol intake.

The dairy industry in New Zealand makes a key contribution to the national economy, accounting for approximately 19% of annual export earnings, or around 4.3 percent of the GDP. New Zealand's temperate climate makes year round pasture grazing possible, which is beneficial in elevating the amount of CLA in milk fat. This creates an ideal opportunity for the New Zealand dairy industry to utilise and market milk fat which already has naturally higher levels of CLA than most other countries.

There were two objectives in this study, one was to investigate the dose effect of synthetic CLA (Tonalin) on immune function in mice. The other was to investigate the effect of natural CLA derived from milk fat on immune function in mice.

A review of the literature on the effect of dietary fatty acids and CLA on human health and immune function is presented in Chapter Two. Chapters Three and Four each describe an individual experiment and include materials and methods, results and a preliminary discussion. A general discussion which brings all results together and the general conclusion are presented in the final chapter.

CHAPTER TWO Literature Review

2.1 Introduction

People usually take for granted that fat in the diet, (cholesterol and saturated fatty acids in particular), is harmful and may contribute to certain so called 'diseases of affluence' or 'western' diseases, such as cardiovascular disease and diabetes. These ideas have damaged the image of milk and dairy products as popular, basic and almost obligatory food items. Recent studies have shown that certain biologically active components present in milk fat and dairy products may actually act as chemopreventive agents for certain diseases. Of these agents, conjugated linoleic acid (CLA) is the most important. Some people have even described CLA as a "magic bullet" (Khosla, 1997).

This review will first discuss the general effects of dietary fatty acids on human health. The chemopreventive role of certain milk fat components will then be explained, followed by a focus on the newly recognised nutrient CLA, where I will discuss its physiological effects and outline recent research in this field.

2.2 Dietary fatty acids and health effects

As a class, the fatty acids are the simplest of the lipids. Dietary fatty acids are components of the more complex lipids. They are of great importance as a source of energy, providing most of the calories from dietary fat (Groff *et al.*, 1995).

Fatty acids are grouped into three classes, namely saturated (SFA) (such as palmitic acid), monounsaturated (MUFA) (such as oleic acid) and polyunsaturated (PUFA) fatty acids. PUFAs consist of two families of fatty acids, omega-6 and omega-3, designated by the location of the first double bond counting from the methyl end of the fatty acid molecule (Figure 2-1). Linoleic acid (LA) is the parent fatty acid of the omega-6 family and alpha-linolenic acid (LNA) is the parent fatty acid of the omega-3 family (Padmavati *et al.*, 1997).

These unsaturated fatty acids can not be synthesized in animal cells and are therefore called essential fatty acids (EFA). LA and LNA can be incorporated into body lipids and can also be metabolized to long chain n-6 (arachidonic acid, AA) and long chain n-3 PUFA (Eicosapentaenoic, EPA; and docosahexaenoic acids, DHA) (Figure 2-2). AA is found predominantly in the phospholipids of grain-fed animals, whereas EPA and DHA are found in the oils of fish. These long chain fatty acids have a variety of important biological activities and can be converted into eicosanoids which influence blood clotting, smooth muscle contraction and mediate inflammatory reactions (Padmavati *et al.*, 1997).

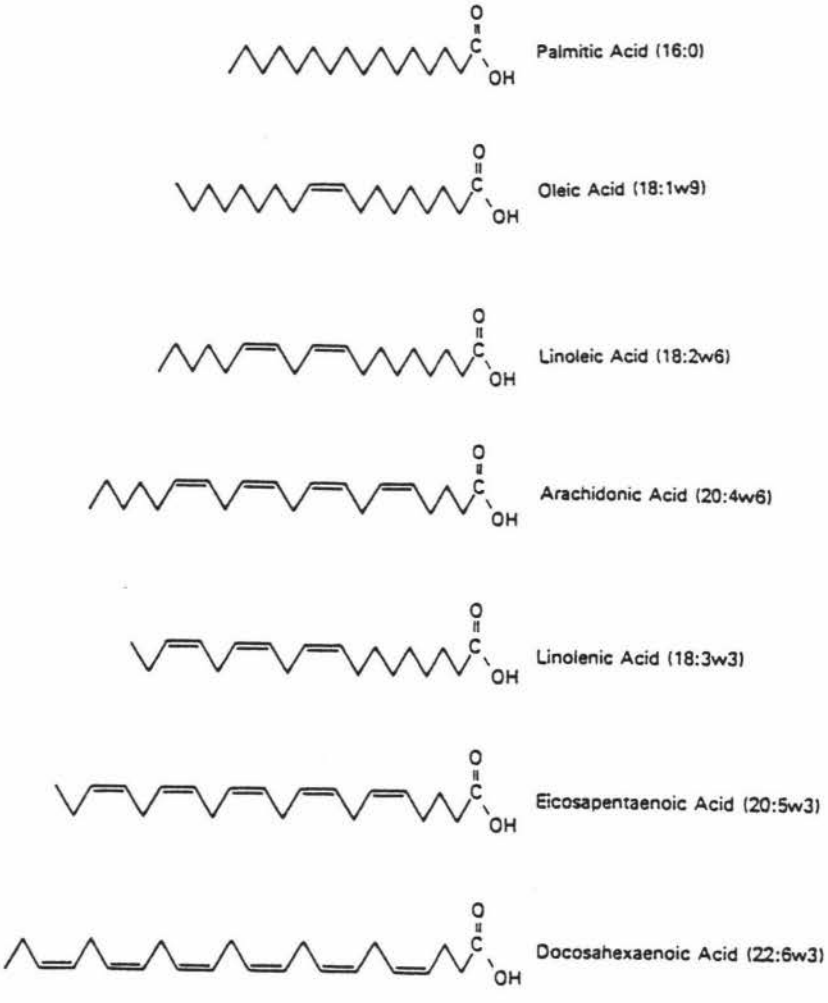

Figure 2-1 Structural formulas for omega-6 (linoleic acid, 18:2 n-6) and omega-3 (alpha-linolenic acid, 18:3 n-3) fatty acids (Padmavati *et al.*, 1997).

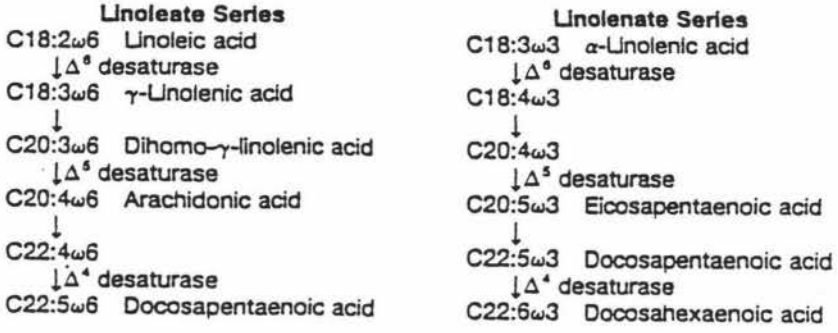

Figure 2-2 Essential fatty acid metabolism desaturation and elongation of ω6 and ω3 (Padmavati *et al.*, 1997).

2.2.1 Dietary fatty acids and cardiovascular diseases

Cardiovascular disease (CVD) is the leading cause of death and disability in many developed and developing countries. It is well documented that dietary factors are major components of the modifiable risk factors (British Nutrition Foundation, 1992). There is a strong connection between diet and blood cholesterol levels, with elevated blood cholesterol levels being a well-recognized risk factor for CVD (Lichtenstein *et al.*,1998).

The major lipoprotein classes are chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Chylomicrons primarily shuttle dietary triglycerides and other fat-soluble components from the gut to peripheral tissues. After a period of time in the circulation, triglyceride-depleted chylomicron remnants are taken up by the liver, which then secretes VLDL particles. The components for these particles come from both the chylomicron remnants and *de novo* hepatic synthesis. Once in circulation, the VLDL particles lose triglyceride and other components, and become enriched in cholesterol. These cholesterol-enriched particles are now called low-density lipoprotein (LDL). LDLs are the major cholesterol-carrying particles in circulation. A high level of LDLs seems to promote the development of cardiovascular diseases (CVD). Hence LDL levels are one of the best predictive factors for the risk of CVD (Lichtenstein *et al.*, 1998). The last major class of lipoprotein particles, HDL, tends to promote cholesterol flux from peripheral tissues and has positive effects in reducing the risk of CVD.

Early studies on the cholesterol-altering impact of dietary fats concluded that saturated fatty acids raised blood cholesterol levels, while polyunsaturated fatty acids reduced serum cholesterol levels and monounsaturated fatty acids were neutral (Sommerfeld, 1998). Dietary *trans* fatty acids, similar to saturated fatty acids, have been demonstrated to raise blood cholesterol levels (Judd *et al.*,1994). There is a consensus in the current scientific community that a diet rich in saturated fat and cholesterol raises LDL levels and contributes to an increased risk of cardiovascular disease (Schaefer, 1997). Recommendations suggest that saturated fat should be restricted to < 10% of energy and cholesterol be restricted to < 300mg/day (Expert Panel, 1990). It has been assumed that either a diet in which unsaturated fatty acids replace saturated fatty acids, or a low fat, high carbohydrate diet would lower the risk of CVD by lowering blood cholersterol levels (Grundy *et al.*, 1982). Indeed, diets that replace saturated fatty acids with unsaturated fatty acids do result in lower LDL levels. In addition to blood LDL-cholesterol pattern, the atherogenic lipoprotein phenotype pattern also increases the risk for CVD (Katan *et al.*,1997). Raised triglycerides, small LDL particles, and HDL-cholesterol levels, are three abnormalities commonly associated with one another and are known as the lipid triad. Low-fat, high-carbohydrate diets not only reduce blood LDL levels, but also reduce HDL levels and raise fasting triglyceride concentrations. Thus, one disadvantage of high-carbohydrate diets appears to be the aggravation of the lipid triad (Katan *et al.*, 1997).

The American National Cholesterol Education program have recommended that total fat, saturated fat and cholesterol be decreased to less than 30% of energy, 10% of energy, and 300% mg/day respectively, to reduce heart disease risk in the general population. A diet that is low in saturated fatty acids, *trans* fatty acids and cholesterol, and relatively high in unsaturated fatty acids gives the best lipoprotein pattern (Katan *et al.*, 1997). It keeps the LDL-cholesterol relatively low, while at the same time, mitigating the lipid triad. Results from a recent study by Hu *et al* (1997) strongly support these recommendations. Women who consumed diets low in saturated and *trans* fatty acids, and relatively high in unsaturated fatty acids were at lowest risk for developing CVD in this study. A recent study reported that in men, a reduction of total fat and saturated fatty acids from 36% and 12% of energy to 27% and 8% of energy, resulted in a significant decrease in total- and LDL-cholesterol levels

(Knopp *et al.*, 1997). Further reducing total fat and saturated fatty acid intake to 22% and 6% of energy, conferred no additional benefit.

2.2.2 Dietary fatty acids and cancer

There is some epidemiologic evidence that demonstrates an association between dietary fat intake and breast, prostate, colon and lung cancer in humans. However, there is no direct evidence of cause and effect relationships, with the data being highly controversial (World Cancer Research Fund, 1997). Some of the controversy derives from the limited ability to accurately assess total energy and fat consumption, and in the difficulty in assessing effects of dietary fat independent of total energy or micronutrient intake and other environmental factors, such as physical activity (Greenwald *et al.*, 1997).

Cancer may be regarded as developing in three stages. The first stage is "initiation", in which some agent, for example, a chemical carcinogen, causes permanent alteration in the DNA of a particular cell. The initiator might come from the diet or be produced during metabolism in the body. In the next stage, "promotion", the promoter increases the chance of the misinformation, inserted into the genetic code by the initiator, being expressed. Finally, the "propagation" stage involves factors that stimulate the uncontrolled growth that is characteristic of the cancer cell. Cancer is not a homogeneous disease with a single cause and cancers affecting different tissues may be quite different in their natural histories. Environmental factors, such as diet, can have totally different effects on various types of cancer (Gurr, 1992).

There is little experimental evidence that demonstrates that fats normally encountered in the human diet can act as initiators, a possible exception being a role for peroxidized fat in gastrointestinal cancer. It is generally believed that dietary fats may act in the promotion stage, although the mechanism is not clear. Many animal experiments have shown that a high level of dietary fat promotes the development of mammary tumors initiated by 7, 12-dimethylbenz[a]athracine (DMBA).

Of those cancers with a putative link to dietary fat intake, breast cancer has been the most extensively studied. Analysis of international data has found that there is a strong positive correlation between per capital fat intake and age-adjusted incidence and mortality from breast cancer (Wynder *et al.*,1986). Potential mechanisms supporting a relationship between dietary fat and breast cancer can be classified as either direct or indirect. Direct mechanisms include: 1) conversion of essential fatty acids to eicosanoids, 2) reactions between oxygen and conjugated double bonds of polyunsaturated fatty acids resulting in the production of reactive oxygen species with the potential to induce DNA damage, and 3) interaction between fatty acids and genomic DNA leading to alterations in gene expression. Potential indirect mechanisms are: 1) alterations in the hypothalamus-pituitary axis that in turn alters hormone levels, 2) effects on membrane-bound enzymes such as mixed-function oxidases that can lead to alterations in estrogen catabolism; 3) structural and functional changes in cell membranes that can alter the hormone activity and growth factor receptors; and 4) effects on immune function (Wynder *et al.*,1997).

While the focus of observational studies has been on the relationship between total fat intake and breast cancer risk, there are some data suggesting that certain fatty acids may predispose the consumer to some cancers more than others. A number of case-controlled studies showed a 50% increase in the relative risk of developing breast cancer among women ingesting high intakes of saturated fat (Howe *et al.*,1990). Animal studies indicated that the fatty acids of the n-6 series enhance tumor development, whereas fatty acids of the n-3 series delay or reduce tumor development (Cave, 1996).

Results from some cohort studies suggested that there is a positive relationship between prostate cancer and fat intake (Pienta and Esper, 1993). This association appears to be strongest for animal (saturated) fat. A potential mechanism for this relationship between prostate cancer and fat intake is that a high fat intake might modify levels of sex hormones (Pienta and Esper, 1993).

In case-controlled studies, dietary fat appears to be consistently associated with colon cancer (Carroll, 1994). Specific factors include a particularly high intake of fat, especially animal

fat and low intake of dietary fibre and calcium, both of which are important in its etiology (Reddy, 1995). The possible mechanism of the relationship between colon cancer and fat intake may be that dietary fat stimulates the secretion of bile acids that are important for the digestion of fat. Not all bile acids are absorbed and unabsorbed bile acids are carried down to the colon where they may be metabolised by many of the species of microorganisms present there. Some of the microbial metabolites of bile acids may be carcinogenic (Guar, 1992). It is reported that individuals consuming high calorie, energy-dense diets, high in fiber and calcium, had less risk of developing colon cancer relative to similar low-fiber, low-calcium diets (Slattery *et al.*,1997). Other factors affecting development of colon cancer include family history (especially at a young age of diagnosis), sex, and site of the tumor within the colon (Lichtenstein *et al.*,1998).

Experimental studies with animals have demonstrated that there is a requirement for n-6 polyunsaturated fat for the development of mammary tumors (FAO, 1994). Tumor yields increase with addition of linoleic acid to the diet up to a threshold of about 4-5 percent of total calories. When this threshold is reached, increasing total fat causes a further increase in the incidence and yield of tumor, apparently independent of the type of fat added (Ip, 1987). Colon cancer also seems to have a requirement for linoleic acid.

Fish oils, containing mainly n-3 polyunsaturated fatty acids, do not appear to promote mammary cancer when fed at high levels, although they may have a stimulating effect at low levels (Carroll, 1989). Studies with mixtures of n-3 and n-6 fatty acids indicate that the promotional effect of n-6 fatty acids may be neutralized by a high ratio of n-3 to n-6 fatty acids in both mammary and colon cancer (Reddy, 1992).

There are abundant data showing that animals fed high fat diets develop tumors of the mammary gland, intestine, skin and pancreas more readily than animal fed low-fat diets. These data are consistent with the epidemiologic evidence linking dietary fat with cancer of the breast, colon, pancreas and prostate.

2.2.3 Dietary fatty acids and the immune system

Interest in the effects of fatty acids and dietary lipids on the immune system dates back many years. The first review of the literature surfaced as long ago as 1978 (Meade and Mertin, 1978). Interest has intensified with the elucidation of the roles of eicosanoids derived from arachidonic acid (AA) in modulating inflammation and immunity (Goodwin and Ceuppens, 1983; Roper and Phipps, 1994; Hwang, 1989; Kinsella *et al.*,1990), and with the knowledge that the metabolism of AA to yield these mediators can be inhibited by the long chain n-3 polyunsaturated fatty acids (PUFA) (Hwang, 1989; Kinsella *et al.*,1990; Calder, 1996). Investigations have expanded to include examinations of the immune modulating effects of specific fatty acids, fat levels, cholesterol, plasma lipoproteins and oxidized fats. Here I will briefly review the literature addressing this rapidly expanding field of research, by describing the immune system including its component cells, the products of these cells and the system's responses.

2.2.3.1 The immune system

The immune system is a collection of tissues, cells and molecules whose prime physiological function is to maintain the internal environment of the body by destroying invading infectious organisms. There are many different defense mechanisms that protect an individual from microorganisms and potentially harmful material. Some of these, including the skin, phagocytic cells, certain chemical substances and enzymes, are active prior to exposure to foreign material. These innate or natural immune mechanisms are not enhanced by previous exposure, nor do they discriminate between most foreign substances. Other defense mechanisms, called acquired or adaptive immunity, have components that are able to recognise variation in structures present on foreign material. The defense mechanism that is generated is therefore able to exclusively eliminate the offending material, with subsequent exposure leading to a more efficient and effective immune response. Properties of natural and acquired immunity are shown in table 2-1.

Table 2-1 Contrasting properties of natural and acquired immunity

	Natural	Acquired
Resistance	Unaltered on repeated infection	Improved by repeated infection (= memory)
Specificity	Generally effective against all organisms	Specific for stimulating organism
Important cells	Phagocytes Natural killer cells	Lymphocytes
Important molecules	Lysozyme Complement Acute phase proteins Interferons	Antibodies Cytokines from lymphocytes

Source: Introducing Immunology (Staines *et al.*,1993)

The immune system is partitioned into two major divisions, cell-mediated and humoral immunity. Cell-mediated immunity is derived from the actions of certain cells, which are primarily derived from bone marrow hematopoietic stem cells. Most important among them are the polymorphonuclear leukocytes (i.e. neutrophils, eosinophils, and basophils/mast cells), monocyte/macrophages,and thymus-derived T lymphocytes. Humoral immunity is conferred by antibodies formed by B-lymphocytes and is found in extra cellular fluids.

Neutrophils comprise approximately 60-75% of circulating leukocytes, their primary function is phagocytosing cells at which they are extremely efficient. They serve as a first line of defense against microbes invading across barriers such as the skin. Eosinophils are also phagocytic and contribute to regulation of allergy and immunity against parasites. Basophils have large numbers of cytoplasmic granules containing vasoactive amines (e.g. histamine and serotonin) and other substances. They mediate immediate hypersensitivity reactions, including certain allergic responses. Both eosinophils and basophils are found in low numbers in the blood because they rapidly migrate into tissues upon release from the bone marrow.

Macrophages ("large eater"), the primary phagocytic cells, are the largest cells in the lymphhatic system. Macrophages in blood are called monocytes, those in tissues are called histiocytes. They serve a central role in nonspecific immunity (e.g. phagocytosis, bacterial and tumoricidal activity, inflammation, and tissue repair) and in regulating molecules called

cytokines, which influence the activity and function of T lymphocytes, B lymphocytes, natural killer (NK) cells and macrophages. These cytokines include molecules like interleukin 1 (IL-1), tumor necrosis factor (TNF), and interferon. Macrophages are also the major producers of immune modulating eicosanoids. Macrophages also serve as "antigen-presenting" accessory cells, which are necessary for T-lymphocyte and B-lymphocyte responsiveness to most antigens.

T lymphocytes serve a central role in immune regulation as T helpers (Th), as inducers and suppressors (Ts) of immune responses, as mediators of delayed-type hypersensitivity (Tdth), and T lymphocyte-mediated cytotoxicity (Tc). T lymphocyte functions are closely involved in many immune responses, including graft rejection, some aspects of anticancer immunity, antifungal responses, and delayed-hypersensitivity. The immunoregulatory functions provided by T lymphocytes are perhaps their most important role with respect to survival of the host.

Natural killer cells are large granular non-T, non-B lymphocytes. They have the ability to kill other cells and are important in destroying some virus-infected cells. They display cytotoxicity toward many tumor cells lines, such as lymphomas, leukemias, and melanomas. They also have a role in the immune surveillance of tissue cells for malignant changes. Animals with depressed NK function are more susceptible to transplanted tumor cells. NK cells have a special function in controlling the growth and proliferation of stem cells in bone marrow that give rise to some of the circulating cells in the blood.

B-lymphocytes arise from precursors in the bone marrow and are the precursors of the cells that synthesize immunoglobulins (plasma cell). B-lymphocytes contain readily detectable surface immunoglobulin (SIg), whereas T lymphocytes do not have surface immunoglobulin. In response to antigen and mitogenic factors provided by T lymphocytes and macrophages, B-lymphocytes proliferate and differentiate to antibody-secreting plasma cells. The isotype of immunoglobulin (Ig) produced (e.g.IgA, IgG) depends on a number of factors including previous exposure, the antigen, and the route of exposure. Therefore, B-lymphocytes are

ultimately responsible for the generation of all antibodies and are the primary cell type responsible for humoral immunity.

2.2.3.2 Dietary fat and lymphocyte functions

2.2.3.2.1 Lymphocyte proliferation

Many studies (Erickson *et al.,* 1983; Morrow *et al.*, 1985; Olson *et al.*, 1987; Alexander and Smythe 1988; Berger *et al.*, 1993; Calder 1998) have found that high-fat diets result in diminished *ex vivo* lymphocyte proliferation compared with low-fat diets, but the precise effect depends upon the level of fat used and its source. Some studies have revealed that high-saturated-fat diets do not affect lymphocyte proliferation compared with feeding low-fat diets (Kollmorgen *et al.*, 1979; Alexander and Smythe 1988) while others have shown that they are suppressive but less so than PUFA-rich diets (Friend *et al.*, 1980; Levy *et al.*, 1982; Morrow *et al.*, 1985; Yaquoob and Calder 1995b). A series of studies in which high saturated-fat diets are used as a comparison with PUFA-rich diets showed that they have different effects from the PUFA-rich diets (Marshall and Johnston, 1985; Kelly *et al.*, 1988). Whether one diet is without effect and the other inhibits the lymphocyte response or whether one is without effect and the other enhances the lymphocyte response is unclear. Most studies agree that saturated fatty acids cause less inhibition than unsaturated fatty acids (Mertin and Hughes, 1975; Calder & Newsholme, 1992a,b)

A large number of studies have investigated the effects of diets rich in linoleic acid upon lymphocyte proliferation. Several studies have reported lower concanavalin A (Con A) or phytohaemagglutinin (PHA)-stimulated T lymphocyte proliferation following the feeding of diets rich in corn or safflower oils to laboratory rodents compared with feeding diets rich in saturated fatty acids (Kollmorgen *et al.*, 1979; Friend *et al.*, 1980; Marshal and Johnston, 1985; Yaqoob and Calder, 1995b). However, some studies have reported no effect of feeding linoleic acid-rich diets upon rodent T-lymphocyte proliferation (Alexander and Smythe, 1988; Berger *et al.,* 1993). It is apparent that the outcome of measures of lymphocyte

proliferation is strongly influenced by the conditions used to culture the cells *ex vivo*, and this may account for the discrepancies in the literature.

There have been few studies of the effects of dietary oleic acid upon lymphocyte functions. Berger *et al* (1993) reported that feeding female rats throughout gestation and lactation a diet containing 100g/kg olive oil did not affect Con A-stimulated proliferation of spleen lymphocytes prepared from the offspring at weaning, this study cultured the lymphocytes in fetal calf serum. In contrast, it has been shown that feeding rats a 200g/kg olive oil diet results in diminished *ex vivo* lymphocyte proliferation if the cells are cultured in autologous serum (Yaqoob *et al.*,1994). Jeffery *et al* (1997) reported that there was a significant inverse relationship between rat spleen lymphocyte proliferation and the ratio of oleic acid to linoleic acid in the diet.

In recent years there has been increased interest in the effects of n-3 PUFA-containing oils upon immune cell functions. Feeding rats diets containing large amounts of linseed oil (rich in α-linolenic acid) suppressed spleen T-lymphocyte proliferation compared with feeding diets rich in hydrogenated coconut oil (Marshall & Johnston, 1985), sunflower oil, or corn oil (Jeffery *et al.*, 1996). Fish oil contains the long chain n-3 PUFAs eicosapentaenoic (EPA) and docosahexaenoic acids (DHA). Feeding fish oil to rabbits (Kelly *et al.* 1988), chickens (Fritsche *et al.*, 1991), rats (Yaqoob *et al.*, 1994), or mice (Yaqoob & Calder 1995a) results in suppressed proliferation of T-lymphocytes compared with feeding hydrogenated coconut, safflower, corn oils.

In general, high fat diets lower T-lymphocyte proliferation compared with low-fat diets. Among high fat diets the order of potency is: Saturated fat < n-6 PUFA-rich oils < Olive oil < Linseed oil < Fish oil.

2.2.3.2.2 Cytotoxic T lymphocyte activity

Mertin (1976) reported that subcutaneous injection of linoleic acid into mice resulted in lowered spleen lymphocyte responses to allogeneic cells, suggesting that linoleic acid lowers

cytotoxic T lymphocyte (CTL) activity. This suggestion is supported by the observation that feeding mice 80 or 200 g/kg safflower oil for four weeks decreases spleen CTL activity compared with feeding a fat-free diet, a low-fat diet (containing corn oil) or diets containing 80 or 200 g/kg hydrogenated coconut oil (Erickson, 1984). Feeding weanling mice 50 or 200 g/kg soybean oil for nine months resulted in lower spleen CTL activity, with the cells from the 200 g/kg group having the lowest activity (Olson *et al.,* 1987). The CTL activity of spleen lymphocytes was lower after feeding mice 100 g/kg fish oil for up to ten weeks than after feeding mice 100 g/kg linseed oil (Fritsche and Johnston, 1990). Feeding chickens diets containing 70 g/kg fish or linseed oil significantly reduced spleen CTL activity compared with diets containing 70 g/kg lard or corn oil (Fritsche and Cassity, 1992).

2.2.3.3 Natural killer cell activity

Erickson & Schumacher (1989) reported that no difference was observed in spleen natural killer (NK) cell activity between mice fed diets containing either 50 or 200 g/kg palm (rich in saturated fatty acids) or safflower oil (rich in n-6 PUFA) or a low-fat diet for four weeks. In contrast to this study, Morrow *et al* (1985) observed that feeding weaning mice 90 g/kg lard (rich in saturated fatty acid) or corn (n-6 PUFA) decreased spleen NK cell activity compared with feeding 10 g/kg corn oil. The type of saturated fatty acid in the rat diet and its position in the dietary triacylglycerol (TAG) have been reported to influence spleen NK cell activity, which was higher if the animals had been fed a diet containing palmitic acid than if they had consumed diets rich in medium chain, lauric, or stearic acids (Jeffery *et al.*, 1997).

Jeffery *et al* (1996) reported that a 200 g/kg linseed oil diet (rich in n-3 PUFA) decreased rat spleen lymphocyte NK cell activity compared with feeding a 200 g/kg sunflower oil (rich in n-6 PUFA). Feeding young mice a diet containing 100 g/kg fish oil for six weeks caused a decrease in spleen NK cell activity compared with feeding 100 g/kg corn oil (Meydani *et al.* 1988), while feeding mice a diet containing 100 g/kg fish oil suppressed spleen NK cell activity compared with a 100 g/kg linseed oil diet (Fritsche & Johnston, 1979). Berger *et al* (1993) reported that feeding female mice a diet containing 100 g/kg fish oil for five months decreased spleen NK cell activity compared with feeding 100 g/kg safflower or olive oil.

Like wise, spleen NK cell activity was lower in mice fed a diet containing 186 g/kg fish oil than those fed a diet containing 100 g/kg fish oil; in turn, the NK cell activity was lower in mice fed the later diet than those fed a 100 g/kg corn oil diet (Lumpkin *et al.*, 1993). Yaqoob *et al* (1994) observed that feeding rats of the high-fat diets resulted in lower NK cell activity compared with feeding the low-fat diet; feeding the fish oil diet resulted in the lowest activity followed by the olive oil and evening primrose oil diets.

Although Berger *et al* (1993) reported no effect of a 100 g/kg olive oil diet on *ex vivo* rat spleen NK cell activity, diets containing 200 g/kg of olive oil significantly reduced this activity (Yaqoob *et al.*, 1994). Jeffery *et al* (1997) observed a significant reverse linear correlation between the level of oleic acid or the ratio of oleic acid to linoleic acid in the rat diet upon spleen lymphocyte NK cell activity.

Thus, it appears that high-fat diets lower NK cell activity compared with low-fat diets. Among high-fat diets the order of potency is: Saturated fat < n-6 PUFA-rich oils < Olive oil < Linseed oil < Fish oil.

2.2.3.4 Cytokine production

2.2.3.4.1 Macrophage- and monocyte-derived cytokines

A number of studies have reported that feeding rodents n-3 PUFA-containing diets results in enhanced production of TNF *ex vivo* (Lokesh *et al.,* 1990; Chang *et al.,* 1992; Somers and Erickson, 1994), although there are reports of decreased production (Billiar *et al.,* 1988; Yaqoob & Calder, 1995a) or no effects (Tappia & Grimble, 1994) following fish oil feeding. Tappia & Grimble (1994) reported that dietary olive and fish oils increase IL-6 production by rat peritoneal macrophages, while Lokesh *et al* (1990) observed increased IL-1 production after feeding fish oil. The most likely reason for the variations in observations are the differing protocols used, studies have varied in the amount of fat given (100 to 300 g/kg) and the duration of feeding time (4 to 10 weeks), the species of animal used (mouse, rat and pig), the type of macrophage used (peritoneal, Kupffer) and its state of activation at harvesting

(resident, elicited), the stimulus used to elicit cytokine production (LPS, calcium ionophore, another cytokine), the type of serum present in *ex vivo* macrophage culture (none, fetal calf, autologous) and its concentration, and the method used to measure cytokine concentration (bioassay, enzyme-linked immunosorbant assay). In agreement with some of these animal experiments, Endres *et al* (1989) and Meydani *et al* (1991) found that fish oil supplementation in humans diminished the ability of peripheral blood monocytes to produce TNF, IL-1α, and IL-2β *ex vivo*, while Meydani *et al* (1991) also reported decreased IL-6 production.

2.2.3.4.2 Lymphocyte-derived cytokines

In contrast to the large number of studies on the effects of dietary lipids on the *ex vivo* production of macrophage-derived cytokines, there have been relatively few studies on lymphocyte-derived cytokines. In the study of Yaqoob & Calder (1995b), weaning mice were fed a low-fat diet, or diets containing 200 g/kg coconut, olive and safflower, or fish oil for eight weeks, the spleen lymphocytes were subsequently stimulated with Con A. This study found the concentration of IL-2 was higher in the medium of cells from mice fed on the low-fat diet or coconut oil; feeding fish oil had no effect on the IL-2 production in the medium (Yaqoob & Calder 1995b). However, mitogen-stimulated spleen lymphocytes from mice fed on olive oil, safflower oil or fish oil produced less IL-4 and IL-10 than those from mice fed on the low-fat or coconut oil diets (Yaqoob & Calder 1995b). Turek *et al* (1994) reported that alveolar lymphocytes from pigs fed diets containing 195 g/kg fish oil or linseed oil lower IL-2 production. In contrast, IL-2 and IL-4 production by Con A-stimulated spleen lymphocytes taken from autoimmune disease-prone mice were higher in the group fed 100 g/kg fish oil than those fed 100 g/kg corn oil; the intracellular levels of messenger RNA for IL-2, IL-4, and transforming growth factor β were also elevated in the fish oil-fed mice (Fernandes *et al.*, 1994).

2.2.3.5 Antigen presentation

Supplementation of n-3 PUFAs in the diet of mice or rats results in a diminished percentage of peritoneal exudate cells bearing the major histocompatibility complex (MHC) II antigens on their surface (Kelly *et al.* 1985; Huang *et al.* 1992). The level of MHC II expression on positive cells was also suppressed by feeding fish oil (Huang *et al.* 1992). Fujikawa *et al* (1992) reported that feeding mice the ethyl ester of EPA for four weeks resulted in diminished presentation of antigen by spleen cells *ex vivo*. These observations suggest that diets rich in n-3 PUFAs will result in diminished antigen presentation. In accordance with animal studies, supplementation of the diet of human volunteers with n-3 PUFAs (approximately 1.56 g/day) for three weeks resulted in a decreased level of MHC II expression on the surface of peripheral blood monocytes (Hughes *et al.,* 1996).

2.2.3.6 *In vivo* measures of cell-mediated immunity

2.2.3.6.1 *In vivo* response to endotoxin and cytokines

Mascioli *et al* (1988) reported that intravenous infusion of a 10% (v/v) lipid emulsion rich in fish oil into guinea pigs significantly enhanced survival to intraperitoneally injected LPS compared with infusion of a safflower oil emulsion. Later, Mascioli and his co-workers (1989) showed that feeding a fish oil-rich diet to guinea pigs for six weeks significantly increased survival following an intraperitoneal injection of LPS compared with animals fed a safflower oil diet. Feeding weanling rats a 100 g/kg fish oil diet for eight weeks significantly decreased a number of responses to intraperitoneal TNF-α; the rise in liver zinc and plasma C3 concentrations, the fall in plasma albumin concentration, and the increase in liver, kidney, and lung protein synthesis rates were all prevented by the fish oil diet (Mulrooney and Grimble, 1993). Feeding fish oil to rats also diminished the anorexic effects of IL-1 and TNF-α compared with feeding n-6 PUFA-containing oils (Mulrooney and Grimble, 1993).

2.2.3.6.2 Delayed-type hypersensitivity

The delayed-type hypersensitivity (DTH) reaction is the result of a cell-mediated response to challenge with an antigen to which the individual has already been primed. Feeding guinea pigs high fat diets reduced the DTH response to a challenge with keyhole limpet haemacyanin (KLH) or tuberculin compared with feeding a low-fat diet (Friend *et al.,* 1980); the response was lower in animals fed 200 g/kg corn oil than those fed 200 g/kg beef tallow. Crevel *et al* (1992) reported that high fat (200 g/kg) diets reduced DTH responses to tuberculin with a low fat diet; no difference was noted between high saturated fat or high n-6 PUFAs diets. Yashino & Ellis (1987) reported that intragastric administration of a fish oil concentrate to rats for fifty days lowered the DTH response to bovine serum albumin compared with administering safflower oil, olive oil, or water. Fowler and his co-workers (1993) observed that addition of ethyl esters of either EPA and DHA to the diet of mice consuming a safflower oil diet reduced the DTH response to tuberculin. These observations suggest n-3 PUFAs significantly reduced DTH response in rodents.

In summary, the animal studies indicate that high-fat diets reduce the DTH response compared with low-fat diets. Among high-fat diets the order of potency is: Saturated fat < n-6 PUFA-rich oils < Fish oil.

2.3 Milk Fat Components

Milk fat contains a number of components such as conjugated linoleic acid (CLA), sphingomyelin, butyric acid, ether lipids, vitamin A, β-cartonene, and vitamin D. These components have different biological activities. Some have the potential to inhibit the process of carcinogenesis, while others may possess antiatherogenic and immunomodulating properties and be beneficial in preventing degenerative disease. The following review will outline recent research on these milk fat components.

2.3.1 Sphingomyelin and metabolites

Sphingomyelin (N-acylsphingosine-1-phosphocholine or ceremide phosphocholine) is a phospholipid comprised of a ceramide backbone. It is predominantly located in the outer leaflet of the plasma membrane in most animal tissues, where it regulates membrane fluidity (Parodi, 1996). In bovine milk, sphingomyelin and other phospholipids account for 0.2 to 1.0 % of the total lipids (Christie *et al.,* 1987), and are associated with the milk fat globule membrane. When milk is processed, the membrane is disrupted and the phospholipids may relocate to the aqueous phase. The degree of transfer depends upon the type and severity of treatment. Sphingomyelin represents about one third of the total milk phospholipids, however this varies according to season and stage of lactation (Christie *et al.,* 1987; Bitman and Wood 1990).

Sphingomyelin, through its biologically active metabolites ceramide and sphingosine, plays an important role in transmembrane signal transduction and cell regulation (Hunnun and Bell 1993; Zhang and Kolesnick 1995). In the pathway of cell regulation, extracellular agonists such as 1, 25-dihydroxylxyritamin D_3 (1,25 $[OH]_2 D_3$), tumor necrosis factor α (TNF-α), gamma-interferon (IFN-γ) and interleukin-1 (IL-1), stimulate cell surface receptors which activate a plasma membrane neutral sphingomyelinase. This in turn hydrolyses sphingomyelin to generate ceramide and phosphocholine (Parodi, 1997). Ceramide acts as a second messenger for the action of the extracellular agonist, transmitting the signal towards the nucleus through multiple downstream targets (Hannun 1994; Hunnun and Bell 1993). Protein kinase C (zeta isoform), ceramide-activated protein phosphatase and ceramide-activated protein kinase are the most important among these targets. These targets have a role in the activation of a number of factors such as the transcription factor NF_kB, which participates in the control of cell proliferation; dephosphorylation of the retinoblastoma gene product (pRB), a tumor suppressor gene that plays an important role in cell-growth suppression and regulation of cell-cycle progression; and downregulation of expression of the proto-oncogene *c*-myc, which plays an important role in both cell proliferation and apoptosis (Parodi 1996). Ceramide acts as a powerful regulator of cell growth by inhibiting cell growth

and inducing differentiation (Hannun 1994). Cancer is usually regarded as a result of an arrest in normal cellular differentiation without loss of proliferative ability.

Sphingosine, the deacylation product of ceramide, is associated with a number of cell regulatory pathways (Parodi 1997). It is a potent inhibitor of protein kinase C (PKC), which is closely associated with tumor progression and metastatic potential, and can modulate the activity of some other protein kinases and enzymes involved in cell regulation (Hunnun and Linardic 1993). Sphingosine also activates the protein product of the retinoblastoma (RB) gene, involved in suppression of cellular growth (Parodi 1996). The RB gene encodes a nuclear phosphoprotein (pRB) and is normally expressed in all body cells at all phases of the cell cycle and plays a role in inhibiting cell cycle progression. The hypophosphorylated form of pRB is the active form and is associated with growth inhibition. Sphingosine induces pRB dephosphorylation, which precedes inhibition of growth and a specific arrest in the G_0/G_1 phase of the cell cycle.

2.3.2 Butyric acid

Butyric acid is a short chain fatty acid (four carbons) present in bovine milk fat at a level of between 7.5 to 13.0 mg butyric acid /100g fat (Parodi 1996,1997). Approximately one third of milk fat triacyglycerols contain one molecule of butyrate. Hydrolysis of butyrate takes place between the stomach and the small intestine by the enzyme Lipase. Colonocytes use butyrate as an important source of energy. Alternatively, butyric acid passes to the portal circulation via the basolateral membrane. The liver then rapidly clears most of the butyric acid, with little reaching other tissues (Young and Gibson, 1994).

Butyric acid has been described as an anticancer agent in many studies (Parodi, 1999). In colon cancer cell lines, butyrate is able to inhibit excessive growth, however it is also able to stimulate proliferation in normal colonocytes. Due to the fact that it is the cells of colonic crypt base that are dividing, and not those at the crypt surface, which remain

differentiated this is not considered pre-neoplastic (Valezquez *et al.*, 1996). One reason explaining the protective effects of dietary fibre against colon cancer is thought to be the generation of butyrate in the colon.

Yanagi *et al* (1993) significantly reduced the incidence of DMBA-induced mammary carcinomas and adenocarcinomas in rats simply by the addition of 6g/100g sodium butyrate to a diet containing 20% safflower oil-based margarine. In *in vitro* studies butyric acid has been shown to strongly inhibit cell growth and also to induce differentiation in a wide variety of neoplastic cells (Prasad 1980). Pouillart *et al.*, (1991) showed that butyric acid can prevent the development of a grafted tumour, reduce leukaemia being transmitted in mice and can also assist non-cancerous cells to mature. Butyric acid is able to regulate the expression of oncogenes and suppressor genes in several cell types. Young and Gibson (1994) have suggested that butyric acid inhibits the activity of urokinase, an enzyme that enables malignant cells to penetrate the substratum, and hence may play a role in preventing the development of tumours by limiting the invasiveness of the tumour cells.

2.3.3 Ether lipids

Bovine milk fat contains small amounts of alkyldiacylglycerols and alkylacylglycerophospholipids, commonly referred to as ether lipids (Parodi, 1997). In these ether lipids the sn-1-position of glycerol is attached via an ether rather than an ester linkage. Hallgren *et al* (1974) reported that the natural lipids in cow's milk contained 0.01% of alkyldiacylglycerols while the phospholipids contained 0.16% of alkylacylglycerophospholipids (Parodi 1996).

Ether lipids have many biological properties including (Berdel 1991; Eibl *et al* 1992):

- cytotoxic and cytostatic effects against tumour cells
- induction of cellular differences in malignant cells
- restriction of neoplastic cell invasion into normal tissues
- stimulation of apoptosis in leukaemic cells
- activation of cytotoxic macrophages
- induction of cytokine release

Ether lipids are thought to exert their biochemical actions by reducing some enzymes associated with mitogenic growth factor-induced signal transduction pathways.

Studies in rodents and humans have shown that 1-0-alkyl-sn-glycerols liberated from dietary ether lipids in the intestinal lumen are readily absorbed without cleavage of the ether bond. They are transported to the liver and other organs where they are directly utilised to synthesise membrane alkylglycerolipids and plasmalogens (Das *et al* 1992).

2.3.4 The fat soluble vitamins

Milk fat is a major dietary source of vitamin A and β-carotene. Vitamin A is a generic discriptor now generally used for compounds, other than carotenoids, that exhibit the biological properties of retinol. Dietary vitamin A in the form of esters is hydrolysed during digestion and is absorbed in the free form from the proximal small intestine. Bile salts, pancreatic lipase and fat aid in the absorption of both vitamin A and β–carotene. β–carotene can either be absorbed intact or cleaved in the intestinal mucosa at the central double bond to form two molecules of retinal which is reduced to retinol. The retinyl esters are hydrolysed and the free retinol binds to a specific retinol-binding protein for transport to peripheral tissues. Vitamin A plays an important role in the function of the retina, it is necessary for growth and differentiation of epithelial tissue and is required for bone growth, reproduction and embryonic development (Parodi, 1996).

Epidemiological studies suggest an effective role for vitamin A supplementation in the prevention of cancer (Tallman and Wiernik, 1992). Vitamin A and its derivatives have been found to have anticarcinogenic and chemopreventive action in cultured cells and organ cultures (Hennekens *et al.*,1986; Moon, 1989). Vitamin A deficiency in animals has been shown to predispose individuals to premalignant changes and to enhance the development of chemically induced cancers (Moon, 1989). Retinoids have been shown to inhibit the promotion stage of tumourigenesis in mouse skin and act as chemopreventive agents of epithelial cancer (Hennekens *et al.*,1986; Moon, 1989; Hill and Grubbs, 1992). However the role of β–carotene in cancer chemoprevention is less well established (Hennekens *et al.*, 1986; Moon, 1989; Krinsky, 1991). Animal studies found that β–carotene can prevent the appearance of skin tumors induced by ultra violet light (UVL), ultra violet light and carcinogen, and carcinogen alone (Parodi, 1996). Supplementation of β–carotene has been

shown to inhibit mammary and lung tumor formation in some animal models (Moon, 1989; Krinsky, 1991).

Although the mechanisms for the anticarcinogenic effects of vitamin A and β–carotene are not completely understood, it is known that retinoids inhibit proliferation and promote differentiation in a number of cell lines. The retinoid molecule binds to a specific cytosolic receptor protein and this complex is translocated to the nucleus, with subsequent modification of gene transcription. β–carotene appears to have an anticarcinogenic role independent of its conversion to vitamin A (Ziegler, 1989). Retinoids protect against the early stages of carcinogenesis, while, β-carotene protects cells against oxidative DNA damage. The anticarcinogenic action of retinoids and carotenoids is closely correlated with enhanced gap-junction cell-to-cell communication, which is caused by stimulation of the synthesis of the gap-junction protein, connexin-43, and its mRNA (Parodi, 1997).

2.3.5 Conjugated linoleic acid (CLA)

2.3.5.1 Introduction

Conjugated linoleic acid (CLA), is a collective name for a mixture of derivatives of linoleic acid (*c*-9, *c*-12-octadecadienoic acid, C18:2) possessing conjugated double bonds. In part of its molecule, a double bond is followed by one single bond and then another double bond. This alteration of double and single bonds is called "conjugation", hence the term "conjugated linoleic acid" (Figure 2-3). Several positional and geometrical isomers are possible. Double bonds can appear in positions 9 and 11 or 10 and 12 of the carbon chain, and these double bonds can have either *cis* (*c*) or *trans* (*t*) orientations. Thus the *c*-9, *t*-11 isomer has a '*c*' double bond at the 9 position and a '*t*' double bond at the 11 position. The *c*-9, *t*-11 and the *t*-10, *c*-12 isomers of CLA have been the most extensively studied and are believed to be the biologically active forms (Ha *et al.*, 1990; Ip *et al.*, 1991; Belury, 1995). The synthesis of CLA requires free linoleic acid, a free-radical generating species and sulfur amino acid-rich proteins. *In vivo*, CLA is an intermediate compound formed during the biohydrogenation of linoleic acid by a specific phospholipid-dependent *c* 9, *t* 11-reductase enzyme found in bacteria residing in the rumen (Kepler *et al.*,1966).

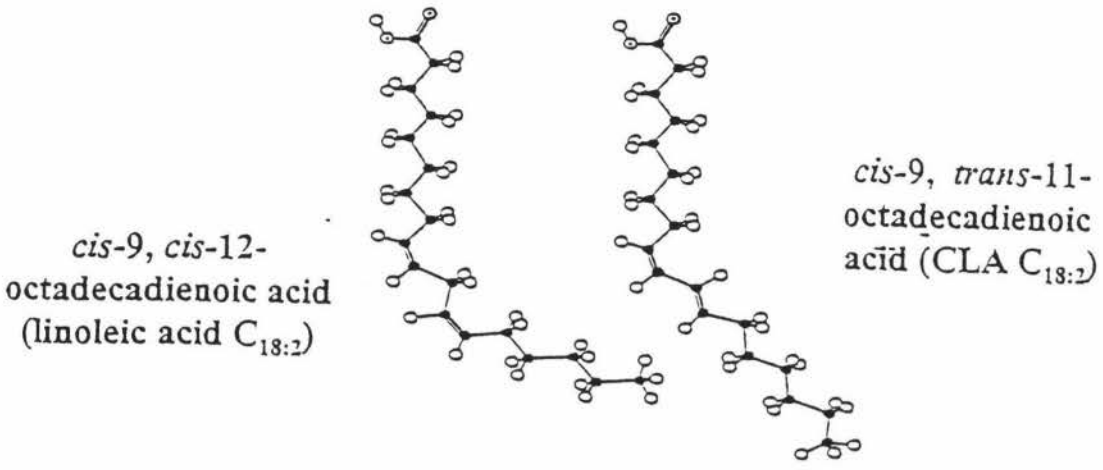

Figure 2-3 Structures of the *cis*-9, *cis*-12 linoleic acid and *cis*-9, *trans*-11 CLA isomers

2.3.5.2 Tissue distribution, occurrence and sources of CLA

CLA has been found in the serum, bile, duodenal juice, breast milk and adipose tissue of humans as well as the adipose of several animal species (Fogerty *et al.*, 1988; Ha *et al.*, 1990; Ip *et al.*, 1991 McGuire *et al.*,, 1996). The serum level of CLA increases in humans consuming foodstuffs rich in CLA (Britton *et al.*, 1992). Huang *et al* (1994) reported a 19-27% increase in plasma CLA levels in subjects consuming a cheddar cheese supplement that provided about 170mg CLA /day. Herbel and Shultz (1995) noted that in 12 human subjects consuming 16 g of linoleic acid (from safflower oil), in addition to their regular diet for 6 weeks, had no appreciable change in plasma CLA levels. An animal study has shown that CLA can be produced from dietary linoleic acid (presumably by the intestinal flora) only if it is supplied as the free fatty acid as opposed to being esterified in triglycerides (Chin *et al.*, 1994b).

Carbon-centered free-radical oxidation of linoleic acid may also make a contribution to CLA concentrations in humans (Iversen *et al.*, 1984). However it is commonly recognised that diet is the major source of CLA in humans. Ha *et al* (1987) found a decline in CLA consumption over the last 30-40 years or so in the U.S.A. and some other western countries. This reduction might be the result of a reduction in the consumption of meat and dairy products as the general public lowers saturated fat and cholesterol intake, and the result of

different trends in the agricultural scene where cows have gone from grazing on grass to being fed more grain based diets.

In ruminant animals, CLA is an intermediate in the biohydrogenation of linoleic acid (Kepler *et al.*, 1966). It was shown by Chin *et al.* (1994), that germ-free rats could not convert free linoleic acid to CLA while conventional rats could, which suggests that the intestinal microflora of non-ruminants may be able to isomerize linoleic acid to CLA to a limited degree.

CLA is known to occur in a wide range of foods including ruminant meats (Ha *et al.*, 1987), milk (Parodi, 1977), other dairy products (Shantha *et al.*, 1995), partially hydrogenated vegetable oils (Banni *et al.*, 1996) and to a much lesser extent certain vegetable oils (Chin *et al.*, 1992).

2.3.5.2.1 CLA in dairy products

The content of CLA in milk fat varies from 0.24 to 2.81% (Riel, 1963). This variation is seasonal, highest values occurring when pastures are lush and rich in PUFAs . Milk fat may contain up to 30 mg CLA/g fat, which is almost entirely the *c*-9, *t*-11 isomer (Parodi, 1996). Fogerty *et al.* (1988) found that two samples of Australian butter contained 9.4 and 11.9 mg/g fat *cis*-9, *trans*-11 CLA. Chin *et al.* (1992) determined the CLA content of 14 types of dairy products, with CLA level ranging from 0.6 mg/g fat for non-fat frozen dairy dessert to 7.0 mg/g fat for condensed milk. Thirteen varieties of natural cheeses were also analysed, and CLA was found to range from 2.9 mg /g fat in Romano to 7.1 mg/g fat in brick cheese. The CLA content of four processed cheeses averaged 5.0 mg/g fat, with the variation between different processed cheeses being very slight. In this study the *c*-9, *t*-11 isomer accounted for about 90% of the total CLA in the various dairy products. Ha *et al.* (1989) reported that processed cheese containing added whey protein concentrate contained about four times the amount of CLA than cheese that was not enriched (8.8 vs. 1.8 mg/g fat). The increased CLA content may be due to heating during processing, free-radical type oxidation of linoleic acid affected by aging, and protein quality (Ha *et al.*, 1989). Shantha *et al.* (1992)

found that processing Cheddar cheese at temperatures of 80° C and 90° C under atmospheric conditions increased CLA content by about 10%, while processing under nitrogen had no effect. The *c*-9, *t*-11 isomer in purchased processed cheese represented 39.7% to 67.9% of total CLA content, while in Cheddar cheese supplemented with whey protein, the proportion of *c*-9, *t*-11 CLA isomer remained constant at about 45%. Shantha *et al.* (1995) studied several dairy products to determine the effects of processing on CLA content. In processed, salted butter, unsalted butter and nonfat yoghurt, the CLA content increased by 1.32 fold, 1.27 fold and -1.2 fold respectively. There was no change in the CLA content of processed low fat yogurt, sour cream or cheese. Storage for 6 weeks at 4° C or 6 months at –20° C also did not alter the CLA content in any of the products examined.

Dhiman *et al.* (1995) reported that the CLA content of milk from grazing cows could be increased by dietary means. They found that CLA and total unsaturated fatty acid contents in milk were higher in the permanent pasture fed group (22.5 mg CLA/ g fat) compared with the 2/3 (13.5 mg CLA/ g fat) and 1/3 (8.5 mg CLA/g fat) pasture fed groups. MacGibbon and Hill (1998) investigated the seasonal variation in the CLA content of milk fat from New Zealand butter obtained from five regions. The mean CLA content of the milk fat was 11 mg/g fat with a range of 7-15 mg/g fat. The summer values tended to be lower than the mid-spring and the mid-autumn values. The New Zealand pasture fed cows produced a greater CLA content than generally reported for milk from sources where pasture was not the predominant feed source. Milk fat extracted from the milk of Friesian herds exhibited a significantly higher CLA level than that from Jersey herds. These two studies have shown that the CLA content of milk fat can be expected to vary largely, between 2 mg/g and 30 mg/g fat (Parodi, 1994). The variation is seasonal, with highest values occurring when pastures are lush and rich in polyunsaturated fatty acids.

2.3.5.2.2 CLA in non-dairy foods

Subcutaneous fat from New Zealand pastured fed lambs was found to contain from 1 to 7 mg/g fat of *c*-9, *t*-11 CLA (Hansen and Czochanska, 1976). Ha *et al.* (1987) detected about 1 mg CLA/g fat in grilled ground beef. Studies in the US reported the CLA content of beef fat

as 3.7, lamb 5.6 and veal 2.7 mg/g fat (Chin *et al.*, 1992), while Australians have reported a higher value of CLA with 13.0 for beef fat, 14.9 for lamb and 7.4 mg/g fat for veal (Fogerty *et al.*, 1988). Chin *et al.* (1992) found that seafood had a CLA content of around 0.5 mg /g fat. They also reported values for corn, olive and coconut oil at about 0.2 mg CLA/g fat, with the *c*-9, *t*-11 isomer accounting for around 45% of the total CLA.

The cited studies indicate that after dairy products the richest source of CLA is meat from ruminant animals. CLA produced by biohydrogenation in the rumen passes to the small intestine where it is absorbed together with other fatty acid of dietary origin, re-esterified, and ultimately circulated to all parts of the animal (Christie, 1979). Chin *et al.* (1992) reported that in lipids isolated from tissues at several sites in rats fed either a control diet alone or supplemented with linoleic acid, CLA concentration increased as a function of linoleic acid feeding. Bacterial flora in the rat and other non-ruminants may convert linoleic acid to CLA albeit to a lesser degree than in ruminants.

2.3.5.3 Health benefits of CLA

2.3.5.3.1 CLA and immune function

Since CLA increases body weight gain and feed efficiency, it follows that CLA feeding during situations of weight loss, may at least retard this process. Such situations of weight loss exist in animals and humans during chronic immune stimulation as a result of vaccinations and infections. Factors which stimulate the immune system, such as vaccinations or endotoxim, stimulate macrophages to produce cytokines, and this causes catabolism of muscle cells (Doyle, 1998). CLA-feeding during immune stimulation would be expected to partially overcome some of the catabolic responses. Miller *et al.* (1994) examined the effect of CLA on endotoxin-induced growth suppression. Mice were fed basal diets containing 2.5% linoleic acid or the basal diet plus 0.5% fish oil or the basal diet plus 0.5% CLA. After 15 days of feeding mice were injected with lipopolysaccharide (LPS). Animals were weighed periodically over the next 72 hours and feed intake was determined. During the first 12 hours after injection body weight loss was independent of dietary

treatment, by 24 hours mice fed a basal diet or fish oil diet lost twice as much body weight as mice fed CLA. During the 24 hours post injection period, CLA not only slowed down the weight loss associated with immune stimulation (i.e. endotoxin injection), but also accelerated the return to pre-injection body weights. Cook *et al* (1993) studied CLA effects in chickens and rats and found that animals fed basal diets experienced weight loss after endotoxin injection. However when animals were fed the basal diets supplemented with 0.5% CLA, endotoxin-induced weight loss was significantly reduced.

CLA has been reported to enhance immune function in several animal studies (Chew *et al.*, 1997; Wong *et al.*, 1997; Sugano *et al.*, 1998). Michal *et al.* (1992) reported that porcine blood lymphocytes cultured with 1.78×10^{-5}, 3.57×10^{-5} and 7.14×10^{-5} M of CLA displayed a dose-dependent enhance of pokeweed mitogen (PWM)-induced lymphocyte blastogenesis, and increased concanavalin A (Con A)- and phytohemagglutinin (PHA)-induced lymphocyte proliferation. Supplementation with CLA also enhanced lymphocyte cytotoxity but suppressed IL-2 production (Wong *et al.*, 1997). Cook *et al.* (1993) showed that rats fed 0.5% CLA for four weeks elicited enhanced PHA-induced lymphocyte blastogenesis. However, dietary CLA had no effect on antibody production in response to sheep red blood cells (SRBC) in chickens or rats. Miller *et al.* (1994) also reported that 0.5% CLA fed to mice for two weeks increased PHA-induced lymphocyte proliferation and the phagocytosis of the fluorescein isothiocyanate-labeled yeast cells by peritoneal adherent cells (macrophages). Wong *et al.* (1997) also studied the effects of CLA on lymphocyte function. Mice were fed 0.1%, 0.3% or 0.9% CLA for three or six weeks. Lymphocyte proliferation, interleukin-2 (IL-2) production and lymphocyte cytotoxicity were assessed using splenic lymphocytes. They found that lymphocyte proliferation in mice fed 0.3% and 0.9% CLA was enhanced in PHA-induced but not in Con A- or lipopolysaccharide (LPS)-induced cultures. Plasma CLA concentrations increased in a dose-dependent manner with CLA feeding. Production of IL-2 was stimulated by CLA. CLA was found to have no effect on lymphocyte cytotoxicity. Chew *et al.* (1997) reported that CLA stimulated PWM-, Con A- and PHA-induced lymphocyte proliferation, lymphocyte cytotoxic activity and macrophage bactericidal activity.

CLA has been shown to have effects on food-induced allergic reactions (Sugano *et al.*, 1998). Sugano *et al.* (1998) found that there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels, although CLA did not appear to affect the release of histamine. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed a 1.0% CLA diet. CLA also reduced serum prostaglandin E_2 ($PG\ E_2$) levels. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA diets. It is reported that the clinical symptoms of food allergy may be induced by the production of chemical mediators such as histamine, leukotriene (LT), and prostaglandin (PG) triggered by allergen-specific immunoglobulin (Ig E) (Metcalfe, 1991). Therefore, this study supports the possibility that CLA might mitigate the food-induced allergic reaction.

2.3.5.3.2 CLA and cancer

There is a substantial body of data linking fat consumption with increased susceptibility to experimentally-induced carcinogenesis in animal models, especially the rat. Several studies have documented increased tumor incidence with increasing amounts of the n-6 fatty acid, linoleic acid, usually supplied as corn oil (Ip *et al.*, 1985). Only the n-3 fatty acids, linolenic (18:3n-3), eicosapentaenoic (20:5n-3) and decosahexaenoic (22:6n-3) acids appear to inhibit tumourigenesis. It is well known that fish oil is a rich source of the n-3 fatty acids.

In contrast to linoleic acid the situation with CLA is quite different. Several studies have provided exciting evidence for CLA's anticarcinogenic properties (Pariza and Hargraves, 1985; Ha *et al.*, 1987; 1990). Initially, crude and partially purified extracts from fried ground beef were found to contain substances that inhibit mutagenesis in bacteria and the initiation of epidermal carcinogenesis in mice by 7, 12-dimethylbenz[a]anthracene (DMBA). This mutagenesis modulator was also detected in extracts of uncooked ground beef and was apparently not destroyed by cooking (Pariza and Hargraves, 1985).

Ha *et al.* (1987) isolated a highly purified fraction of CLA by HPLC which was found to contain four major isomers (*c*-9, *t*-11; *t*-9, *t*-11; *t*-10, *c*-12 and *t*-10, *t*-12) accounting for more than 90% of total CLA. The fraction inhibited cytochrome P-450 enzyme activity *in vitro*. Synthetically prepared CLA (containing all four isomers) was examined for anti-initiation activity in a two stage mouse epidermal carcinogenesis model (Parodi, 1994). CLA was topically applied to the dorsal skin of mice, control mice were treated similarly with linoleic acid or acetone solvent. After initiation with DMBA all mice were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) to promote tumor formation. There was no difference in tumor incidence or yield between linoleic acid-treated mice and solvent-treated mice. In contrast, the CLA-treated mice developed approximately half as many papillomas and exhibited a lower tumor incidence compared with the control mice.

In a further study, Ha *et al.* (1990) showed that synthetic CLA inhibited the initiation of mouse forestomach tumorigenesis by benzo[a]pyrene (BP). In three independent experiments mice treated by gavage with CLA, prior to BP induction, developed only about half as many neoplasms per animal as mice in the control groups given olive oil or linoleic acid. In two of the experiments tumor incidence was also reduced. A significant finding in this study was that although mice were intubated with synthetic CLA, only the *c*-9, *t*-11 isomer was found to be incorporated into forestomach cell phospholipids, indicating this is the biologically active factor. CLA was also found to be an effective antioxidant and was more potent than α-tocopherol and almost as good as butylated hydroxytoluene. The authors suggested that the antioxidant ability of CLA was dependent on the formation of α β-hydroxy acrolein moiety within the CLA molecule (Ha *et al.*, 1990).

Benjamin *et al.* (1990) reported that CLA reduced the induction of mouse forestomach ornithine decarboxylase activity (ODC) induced by the tumor promoter TPA. ODC activity is regarded as a tumor promoter with elevated levels being found in rapidly proliferating tissues. Protein kinase C (PKC) plays a role in many activities, including intercellular communication, cell proliferation and tumor promotion. TPA is a potent activator of PKC. Benjamin *et al.* (1992) found that CLA inhibited PKC activity resulting from TPA activation.

The authors postulated this might explain the anticarcinogenic effects of CLA on the forestomach and other sites (Benjamin *et al.*, 1992).

Ip *et al.* (1991) established a rat model to examine the effect of synthetically prepared CLA. Rats were fed basal corn-oil containing diets, supplemented with CLA (*c*-9, *t*-11 and *t*-9, *c*-11 isomer content was 43.3%; *t*-10, *c*-12 45.3%; *c*-9, *c*-11 1.9%; *c*-10, *c*-12 1.4%; *t*-9, *t*-11 and *t*-10, *t*-12 2.6%; and linoleic acid 4.4%). Animals were fed the various diets commencing 2 weeks prior to DMBA administration and continued on their respective diets for up to six months. Mammary adenocarcinomas induced by DMBA, were inhibited in a dose dependent fashion (32-60%) at CLA doses of 0.5- 1%, with no further benefits at a CLA level of 1.5%. Tumor incidence, tumor multiplicity and tumor weight all decreased in a similar manner. Fibroadenomas also decreased with increasing levels of CLA; thus CLA inhibits the development of both malignant and benign tumors. Analysis of mammary tumor and liver (a non-target tissue) phospholipids revealed that only the *c*-9, *t*-11 isomer of CLA was incorporated in both organs, the level of incorporation increased with dietary intake. Chronic feeding of CLA (for one month) significantly decreased lipid peroxidation in the mammary gland but not in the liver. This study (Ip *et al.*, 1991) was the first study to show that the synthetic CLA was a potent anticarcinogen and also established that chronic CLA feeding was safe and without apparent side-effects.

Ip *et al.* (1994) found that with a lower dose of DBMA, dietary CLA (0.05% to 0.5%) produced a dose-dependent inhibition in mammary tumor yield. As little as 0.1% of CLA was sufficient to cause a significant reduction in the total number of tumors. Feeding CLA from the time of weaning (3 weeks of age) to the time of carcinogen administration (~ 7 weeks of age), which corresponds to the period during which the rat mammary gland matures to the adult form, protected against subsequent tumor development. This observation suggests that the timing of CLA feeding can "imprint" certain biochemical changes which only become apparent later.

Liew *et al.* (1995) reported that CLA inhibited 2-amino-3methylimidazo[4,5-f]quinoline (IQ)-induced DNA adducts and aberrant crypt foci (ACF) in rat colon. The dose of CLA

represented approximately 0.5% of the total diet. CLA was found not to affect ACF size but significantly inhibited by 75% the number of ACF per colon. CLA was also found to be cytotoxic to human cancer cells (Shultz *et al.*, 1992a). Incubation of human malignant melanoma (M21-HPB), colorectal (HT-29) and breast cancer (MCF-7) cells with varying concentrations of CLA (20, 40 and 70 μM) showed significant reductions in cell proliferation compared to control cultures (Shultz *et al.*, 1992a).

2.3.5.3.3 CLA and atherosclerosis

Antiatherogenic effects of CLA have been reported in several animal studies (Nicolosi *et al.*, 1993; Lee *et al.*, 1994). Lee *et al.* (1994) fed rabbits a semi-synthetic high fat diet (14% fat and 0.1% cholesterol) for 22 weeks, elevating total plasma cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) levels to the 1200mg/dl and 500mg/dl range. When half of the animals were fed CLA (0.5g/day/rabbit) for 12 weeks, significant reductions were observed in TC, LDL-C and plasma triacylglyerol concentrations. Although CLA did not affect the high-density lipoprotein cholesterol levels (HDL-C), the decrease in LDL-C resulted in a significant reduction in the LDL/HDL ratio. A tendency for less atherosclerosis in the aortas was noted in the animals fed CLA. Although there was no significant difference between the two dietary groups, the mean values of maximal plaque thickness were consistently lower in the CLA group.

Nicolosi *et al* (1997) reported that CLA significantly reduced total serum cholesterol and the fatty streak area in the arteries of hamsters fed an atherogenic enriched diet. However this study did not find any beneficial effects of CLA on the plasma lipid profile.

2.3.5.3.4 CLA on growth and development

Chin *et al.* (1994) examined the effect of CLA on animal development and growth. Two experiments were carried out in this study. In the first experiment, female rats were fed a control diet or 0.5% CLA-supplemented diet during gestation and lactation. The CLA content of milk was elevated in animals fed the 0.5% CLA supplemented diet. The mean

body weight of the pups from mothers receiving CLA during gestation and lactation were also significantly higher. In the second experiment, rats were fed diets containing 0, 0.25% or 0.5% CLA during gestation and lactation. The CLA content of the milk increased in accordance with the dietary CLA levels. For rats fed a 0.5% CLA-supplemented diet, their pups exhibited significantly higher body weights and greater feed efficiency compared with the control group. Pup weight on day 10 of lactation was also significantly higher. This study demonstrated that CLA could effectively increase body weight gain and feed efficiency.

2.3.5.3.5 CLA and body fat regulation

Many experiments have demonstrated that dietary CLA induces a decrease in body fat levels and an increase in body protein content (Pariza *et al.*, 1996; West *et al.*, 1997; Doyle, 1998). Feeding young pigs a diet containing 0.5% CLA caused an initial reduction in feed intake, but growth and feed efficiency increased later. Compared to control pigs, the CLA-fed animals had less body fat and 5% more lean muscle mass (Doyle, 1998). Mice, rats and chickens fed 0.5% CLA-supplemented diets for 4-8 weeks had body fat reductions of 57-70%, 23% and 22% respectively, while lean body mass was significantly increased by 5-14%, 3% and 4% (Pariza *et al.*, 1996).

Park *et al.* (1997) reported CLA increased the activity of carnitine palmitoyltransferase, (the rate-limiting enzyme for fatty acid β-oxidation), and of hormone-sensitive lipase, which hydrolyzes lipids in adipocytes for release to the blood. The activity of heparin-releasable lipoprotein lipase in 3T3-L1 adipocytes was significantly reduced. This enzyme is involved in the uptake of fatty acids into the adipocytes. All these alterations in enzyme activity are consistent with the reduction in body fat caused by CLA.

CLA fed animals expended more energy than controls and did not demonstrate an increased respiratory quotient at night (Doyle, 1998). Since a lower respiratory quotient is indicative of fat oxidation, the CLA fed mice continued to burn fat at night while the control mice oxidized more carbohydrates (Doyle, 1998).

Although the mechanisms relating CLA with body fat reduction are not entirely clear, part of the effect may be due to CLA increasing lipolysis in adipocytes while at the same time increasing muscle and adipocyte fatty acid oxidation (Pariza *et al.*, 1997).

2.3.5.3.6 CLA and bone metabolism

Some cytokines can play a role in stimulating bone resorption leading to weakening and wasting of bones (Doyle, 1998). Since CLA modulates cytokine production, CLA would be expected to have positive effects on bone health. Watkins *et al.* (1997) examined bone metabolism in chickens and rats. They found dietary CLA increases the ash (mineral) content of bones and may also enhance chondrocyte synthesis of collagen. Diets rich in CLA, as compared to soybean oil, caused a greater rate of bone formation. This is presumed to result from a moderation of the production of PGE_2, which, at high levels, may depress bone synthesis. CLA reduces PGE_2 production in bone and may up-regulate the insulin-like growth factor to support bone formation (Watkins *et al.*, 1997). CLA was also found to prevent a leg deformity which is periodically manifested in chicken flocks (Doyle, 1998).

2.3.5.4 CLA and human studies

Most of the human studies which have been carried out have been aimed at assessing the impact of dietary CLA on plasma CLA concentrations (Britton *et al.*, 1992; Huang *et al.*, 1994). Britton *et al.* (1992) examined the effect of dietary CLA on serum CLA levels. In this study subjects were simply instructed to consume diets containing foodstuffs rich in CLA during one phase, and reduce intake of other foodstuffs. It was found that the serum phospholipid levels increased following consumption of foodstuffs rich in CLA.

Huang *et al.* (1994) conducted a similar study. Nine healthy male subjects followed their routine dietary pattern, except that a cheddar cheese supplement was substituted for other dairy products. 80% of the CLA isomers in the cheese were in the form of *c*9, *t*11- and *t*9, *c*11- isomers. After the supplementation period the plasma phospholipid-esterified CLA concentration was significantly higher compared to the beginning value. Elevated plasma

CLA levels were apparent in 7 of the 9 subjects. Plasma total cholesterol concentrations were not affected during the supplementation period.

Since the bacterial species responsible for CLA production from linoleic acid (LA) in cattle is found in humans, volunteers were fed diets supplemented with safflower oil triacylglycerol-esterified linoleate for six weeks (Herbel *et al.*, 1998). Three-day diet records and fasting blood were obtained initially and during dietary and post-dietary intervention periods. Although LA intake increased significantly during the dietary intervention, there were no significant changes in plasma CLA concentrations.

Thom (1997) investigated the efficacy and tolerability of synthetic CLA (Tonalin) on body composition in humans. A randomized placebo-controlled double-blind study was carried out in 20 healthy subjects. Tonalin and the placebo were given in the form of capsules. The dose amounted to a consumption level of 0.3g of pure CLA per day. Body composition measurements were carried out using infrared technology. A significant reduction of body fat was obtained during the three months treatment of subjects taking Tonalin capsules, while no change was seen in the placebo group. This study suggested that CLA had a favourable effect on body composition through reduced fat deposits in subjects with normal body weight.

Some researchers have investigated CLA concentrations in human milk and infant formulae samples (McGuire *et al.*, 1997). They found that human milk had significantly more CLA than infant formulae (3.8 vs. 1.0 mg/g fat) with 83-92% in the form of the 9-*c*, 11 *t*- isomer. This isomer varied between 52-100% in the infant formula. Formulae made with vegetable oils had 4-fold lower CLA content than those made from animal fats. Park *et al.* (1997) reported that the maternal diet affects human milk CLA content. Breast feeding women who consumed high CLA diets had a higher CLA content in their milk compared with women who consumed low CLA diets.

2.3.5.5 Mechanism of action of CLA

Although CLA has been shown to have a wide spectrum of biological activities, the mechanism for these effects is not clear. There are several possibilities that may help to elucidate the exact mechanism of CLA effects.

Originally CLA was believed to work as an antioxidant. It was more potent than α-tocopherol and as effective as butylated hydroxytoluene (BHT) in inhibiting iron-thiocyanate-induced peroxide formation (Belury, 1995). In addition CLA was shown to be as effective as vitamin E and BHT in inhibiting the formation of thiobarbituric reactive substances (TBARS), a biomarker often used to assess oxidation in biological systems in the mammary gland (Ip *et al.*, 1991). Subsequent work found that CLA only affected malondialdehyde levels and not 8-hydroxy-deoxyguanosine levels, thus excluding the possibility that it was protecting DNA from oxidative damage (Ip *et al.*, 1996). But Van den Berg *et al.* (1995) reported that CLA could not be considered as an antioxidant, since CLA neither acts as a radical scavenger nor is converted into a metal chelator in the Fe^{2+} ion-dependent oxidative reaction. A recent study (Yurawecz *et al.*, 1995) suggests that CLA does not itself possess anti-oxidant properties, but may produce substances (i.e. furan fatty acids) which protect the cell from the detrimental effects of peroxides.

Since CLA is incorporated into cell membrane phospholipids, it may modify their fluidity and exert its effects by altering intracellular events via one or more signal transduction pathways and/or by prostaglandin (PG) synthesis. Since CLA is a polyunsaturated fatty acid, it may compete with linoleic acid in the pathway of eicosanoid synthesis, via the cyclooxygenase or lipoxygenase pathways. However, Ip *et al.* (1996) observed that dietary linoleic acid did not modify the inhibitory effects of CLA on mammary carcinogenesis. Alternatively CLA may inhibit carcinogenesis by being cytotoxic to tumor cells (Shultz *et al.*, 1992a, 1992b; Visonneau *et al.*, 1997). The cytotoxicity may be in part due to the conjugated double bond system that allows for increased electron trapping, which increases the possibility of superoxide anion generation, resulting in cytotoxicity.

The signal transduction hypothesis is particularly attractive, as such small quantities of CLA are needed to elicit a response, therefore it is logical that CLA *in vivo* serves as an "amplifier" of some "cascade" mechanism. Furthermore, CLA may upregulate some key molecule which can serve as the "signal" in a wide variety of biochemical pathways, each of which leads to different biological responses (Khosla, 1997). These intracellular messengers which may be the target for CLA include protein kinase C.

As discussed previously, CLA has some effects on prostaglandin synthesis. Prostaglandin-E_2 (PGE_2), derived from arachidonic acid (a linoleic acid metabolite), is an important regulator of immune function. PGE_2 acts in part by regulating the synthesis, through macrophages and other immune cells, of hormones called cytokines that stimulate the activity of other cells in the immune system. Two cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), are particularly relevant. TNF-α is a key mediator in many chronic diseases including atherosclerosis, carcinogenesis, weight loss and possibly obesity. Therefore, the modulation of cellular responses to these cytokines by CLA, possibly through eicosanoid pathways, presents an attractive hypothesis for at least partly explaining CLA's bewildering multifunctionality.

2.3.5.6 CLA, dairy products and the future

At present most of the data demonstrating the health-associated effects of CLA has been carried out in animals and in tissue culture, and by using synthetically prepared CLA, mostly as the free fatty acid. There are no direct data on whether CLA is as effective in humans. Even if one of several potentially beneficial effects of CLA can be verified in humans, the problem remains of how to utilize this information for the wide variety of human food products in which CLA occurs naturally. Although dairy products are amongst the richest natural sources of CLA, the levels are well below those used to elicit beneficial effects in animals. Ip *et al.* (1994) calculated that the beneficial effects of 0.1% CLA inhibiting mammary carcinogenesis in rats translated to a 70 kg human consuming approximately 3g CLA/day. Since milk provides milligram quantities of CLA per gram of total fat, and

assuming that requirement of CLA was to be met solely by milk consumption, this would necessitate the consumption of more than 25 litres per day.

However, the potential of CLA in humans for disease prevention is enormous and offers the dairy industry a marketing tool. The big challenge for the dairy industry is to find ways to enrich milk and dairy products directly or to produce specialized fats, containing elevated levels of CLA, which would allow for increased dietary intakes of CLA without compromising total fat and cholesterol intakes. This means finding ways for cows to produce CLA-enriched milk. It could be achieved by implementing animal dietary practices that serve to naturally elevate the CLA content of milk and animal fats.

2.4 Summary

Dietary fatty acids have been shown to have some effects on immune system, cardiovascular diseases, and other health aspects. Certain milk fat components contain different biological activities. Sphingomyelin and its metabolites are regarded as tumor suppressor lipids. Butyric acid acts as an antineoplastic agent as does Ether lipid. Conjugated linoleic acid (CLA) is a newly recognised nutrient, which is abundant in milk fat and dairy products. CLA has been shown to exhibit a wide range of health benefits including immunomodulation, inhibition of carcinogenesis, and it displays antiatherogenic, regulation of body fat and growth promoting effects. However, systemic studies of the effect of CLA on immune function are not available. Almost all of the data examining the effects of CLA have been carried out in animals and in tissue culture, and by using synthetically prepared CLA. The effect of natural CLA on the immune system is still unknown. The present study is focused on the immunomodulatory properties of CLA. There are two objectives in this study; one is to investigate the dose effect of synthetic CLA (Tonalin) on immune function in mice; the other is to investigate the effect of natural CLA derived from milk fat on immune function in mice. More study should be undertaken to give us a better understanding of how CLA works.

CHAPTER THREE Experiment One: Dose response experiment using synthetic CLA (Tonalin)

3.1 Introduction

Conjugated linoleic acid (CLA) exerts diverse physiological effects, most of which are favorable to human health. The anti-cancer effect of CLA has been well documented, however, only a few studies are available that describe the effect of CLA on immune function. CLA has been reported to increase mitogen (PWM, PHA and Con A)-induced lymphocyte blastogenesis in rodents and pigs (Michal *et al.*, 1992; Cook *et al.*, 1993; Chew *et al.*, 1997; Wong *et al.*, 1997). Miller *et al.* (1994) reported that 0.5% CLA fed to mice for two weeks increased the phagocytic ability of peritoneal macrophages. Interleukin 2 (IL-2), a cytokine that regulates both T and B lymphocyte proliferation in response to antigens (Magnuson *et al.*, 1987), was reported to be either increased (Wong *et al.*, 1997) or suppressed (Chew *et al.*, 1997) by CLA in two different studies. CLA was found to reduce the catabolic response induced by immune stimulation in mice, rats, and chickens, without adversely affecting immune function (Cook *et al.*, 1993; Miller *et al.*, 1994).

Currently, systemic studies on the effects of CLA on antibody-mediated and other aspects of immunity are not available. The objective of this research was to investigate the effect of CLA on immune function in mice. The effect of different doses of synthetic CLA was also examined.

Because CLA is a fairly common, but usually minor fatty acid that appears mainly in dairy products, the CLA sources employed in all studies so far are the synthetic form. The present study also used synthetic CLA in the form of Tonalin, a commercial product which contains 60% synthetic CLA, with the remainder comprising saturated fatty acid, oleic acid and linoleic acid.

3.2 Materials and Methods

3.2.1 Animals

Animal experimentation in this study adhered to the Massey University guidelines for the care and use of laboratory animals and was approved by the Animal Ethics Committes of Massey University. One hundred and twenty BALB/c male mice (6-8 weeks old) were kept at the small animal production unit (SAPU), Massey University, Palmerston North, New Zealand. They were housed in pairs in metal cages at a temperature of 22 ± 2°C, with a 12 hour light / dark cycle. Animals were fed *ad libitum* with free access to water at all times.

3.2.2 Diets

Animals were fed skim milk powder (SMP) based diets with or without supplementation with CLA. The composition of this diet was: protein 20%, vitamin 5%, mineral 5%, cellulose 1%, fat 8% and filler (cornflour) 62% (Table 3-1). Tonalin was obtained from PharmaNutrients, Inc, U.S.A., and contained 60% pure CLA (Table 3-2). Corn oil or "corn oil + Tonalin" were maintained at 5.3% of the animal diets. All diets were fed *ad libitum* to mice. Prepared diets were kept at 4°C until used.

Table 3-1 Diet composition (% diet weight)

	Dietary treatment (% CLA)					
Component	0 (control)	0.1%	0.25%	0.5%	1.0%	2.0%
SMP	54.32	54.32	54.32	54.32	54.32	54.32
Cellulose	1	1	1	1	1	1
CLA	0	0.1	0.25	0.5	1	2
Cornflour	29.38	29.38	29.38	29.38	29.38	29.38
Mineral mix	5	5	5	5	5	5
Vitamin mix	5	5	5	5	5	5
Corn oil	5.3	5.146	4.915	4.531	3.762	2.223

Table 3-2 Fatty acid composition (% fat) of Tonalin

Fatty acid	%
C16: 0	6.47
C18: 0	4.14
C18: 1	22.19
C18: 2	4.53
CLA isomers: Total	60.0
c-9, *t*-11	22.2
t-10, *c*-12	27.0
c-10, *c*-12	6.0
t-9, *t*-11 and/or *t*-10, *t*-12	4.8

3.2.3 Vaccination

Fluvax vaccine (45 µg per 0.5mL dose), Tetanus vaccine (Tet-Tox, 6Lf per 0.5mL dose) and poliomyelitis vaccine (Polio, 20 µg per dose) were purchased from CSL Limited, Australia. Twenty seven µl of a diluted polio vaccine (80µl polio in 1 mL 0.1M sodium bicarbonate) was administered orally on days 7 and 21. A mixture of Fluvax and Tet Tox (25µl each) was injected subcutaneously into the same mouse on days 7 and 21.

3.2.4 Preparation of Serum

One ml of blood was collected from each mouse and put into a 2 ml eppendorf tube. All samples were left at room temperature for 2 hours after which the samples were centrifuged (Sorvall MC 12 V, DuPont) at 1950 x g for 10 minutes. The serum was removed and placed in another tube and stored frozen at –20°C until required for further use.

3.2.5 Collection of Intestinal Contents

Small intestine (from stomach pylorus (sphincter) to caecum end) of each mouse was removed aseptically and its contents flushed out with 1.5 ml of PBS (phosphate buffered saline) into a 15 ml centrifuge tube. The contents were then centrifuged (Heraeus Sepatech Megafuge 1.0 R) at 500 x g for 10 minutes after which the supernatants were removed and placed in another tube. The samples were stored at –20°C until required for further use.

3.2.6 Preparation of Spleen Lymphocytes

This protocol was a modification of that described by Coligan *et al.* (1991a). Spleens were removed aseptically from the mice and placed in pairs into 2ml of RPMI-1640 media (containing 25 mM Hepes buffer (pH 7.3), 2mM L-glutamine, 50μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 10% heat-inactivated fetal calf serum (FCS)) (purchased from GIBCO Bethesda Research Laboratories (BRL), Life Technologies Ltd., USA) in a 24 well plate (Nunclon Multidishes, Nunc, GIBCO BRL, Life Technologies Ltd., New Zealand). All subsequent procedures were carried out aseptically in sterile airflow cabinets. The spleens were cut into small pieces using scissors and tweezers. They were then homogenised by sucking up and down through a 1ml syringe, after which they were transferred to centrifuge tubes containing 5ml RPMI-1640 media. Samples were centrifuged (Heraeus Sepatech Megafuge 1.0R) for 10 minutes at 125 x g. The supernatants were discarded and the pellets resuspended in 5ml ACK (ammonium chloride / potassium) lysis buffer (0.15M NH_4C1, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2-7.4) and incubated for 5 minutes at room temperature with occasional mixing. An additional 5ml of RMPI-1640 media was added into each tube. The samples were then centrifuged for another 10 minutes at 125 x g and the supernatants discarded and the pellets resuspended in 5ml RPMI-1640 media. The samples were recentrifuged for 10 minutes at 125 x g. Supernatants were discarded and the pellets were resuspended in 5ml RMPI-1640 media (stock solution). One ml of stock solution was removed and placed into tubes containing 4ml RMPI-1640 (1/5 dilution). A small volume was transferred into a FACS tube (Falcon® 6ml polystyrene round bottom tube, Becton Dickinson & Co., NJ, USA) and the lymphocytes enumerated by flow

cytometry (FACSCalibur ™, Becton Dickinson Immunocytometery Systems, San Jose, California, USA). Cell concentration was then adjusted to 2 x 10^6/ml.

3.2.7 The Cell Proliferation Assay

The method for this assay was a modification of that found in the Cell Proliferation Kit by Boehringer Mannheim, USA (Cell Proliferation ELISA, BrdU (colorimeteric), Cat. No.1647229). Fifty µl of the prepared lymphocyte suspension (as described in section 3.2.6) were aliquoted into 6 wells of a 96 well flat bottom plate (Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ). Fifty µl of RMPI-1640 media was added to three wells to serve as 'No Mitogen' control wells, while 50µl of mitogen was added to the remaining three wells to serve as 'with Mitogen' wells. The mitogens used were Concanavallin A (Con A) at 2.5µg/ml, Phytohemagglutinin (PHA) at 15µg/ml, and Lipopolysaccaride (LPS) at 5µg/ml.

One hundred µl of RMPI-1640 media was added to background wells where no lymphocytes were aliquoted. Three wells each of P815 and YAC 1 were aliquoted to act as standards. The plates were incubated at 37°C, 5% CO_2 and 100% humidity for 72 hours before adding the labelling reagent 5-bromo-2'-deoxyuridine (BrdU). Ten µl of BrdU labelling solution was added to each well and the plates were incubated at 37°C for another 16 hours. Each plate was centrifuged at 125 x g for 10 minutes and the labelling medium was removed by flicking off and tapping. The cells were dried using a hair-dryer for approximately 15 minutes. Two hundred µl of FixDenat was added to each well and incubated for 30 minutes at room temperature (RT). The FixDenat was thoroughly removed and 100 µl of anti-BrdU-POD (peroxidase) working solution was added into each well. The plates were incubated at RT for 1 hour, after which the antibody was removed and the plates were washed three times in PBS (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaC1, pH 7.2-7.4) using a plate washer (EL 404, Microplate Autowasher, Bio-Tek instruments, U.S.A.). The wash solution was removed and 100 µl of substrate solution was added per well and the plates were incubated at RT for 20 minutes (Con A, LPS) or 30 minutes (PHA). After incubation, 25 µl of 1M H_2SO_4 (stop

solution) was added to each well and the plates were shaken for 10 seconds. Each plate was read on an ELISA plate reader (KC3 / CERES900C, Bio-Tek instruments, Inc., U. S. A.) at 450 nm (reference wavelength: 690nm). Measurement was carried out within 5 minutes of adding the stop solution.

The Absorbance Change was used to measure the degree of proliferation of the lymphocytes. The formulae is as following:

Absorbance Change = Absorbance of cells With Mitogen – Absorbance of cells Without Mitogen

3.2.8 Cytokine Production

This assay uses the spleen cell preparation described in sections 3.2.6. Eight million cells in 2 ml solution were aliquoted into a 12 well plate (Costar) and 2µl of undiluted Con A (1 mg/ml) of stock was added to each well. The plate was incubated for 48 hours at 37°C, 5% CO_2 and 100% humidity, following which the well contents were transferred into labelled eppendorf tubes and centrifuged (Sorvall MC 12 V, DuPont) at 1950 x g for 10 minutes. Supernatants were transferred into another set of labelled eppendorf tubes and the samples were kept frozen at –20°C until required for further use. (These samples were used in section 3.2.18 and 3.2.19).

3.2.9 The Immunophenotyping Assay (whole blood method)

This assay was adapted from Lloyd *et al.* (1995) and Nicholson *et al.* (1984). Blood was collected into EDTA tubes (Vacutainer® blood collection tubes, Becton Dickson & Co., NJ, USA) and inverted multiple times to prevent clotting. One hundred µl of blood was aliquoted into a 2ml eppendorf tube (three tubes for each sample). Monoclonal antibodies (Mab) were then added to the sides of the tubes:

Tube 1: 5 µl of rat anti mouse CD4 conjugated to FITC (CD4: FITC) and rat anti mouse CD25 conjugated to R. Phycoerythrin (RPE) (CD25: RPE) (both from Serotec, Inc., UK)

Tube 2: 5 µl of rat anti mouse CD8 conjugated to FITC (CD8: FITC) and rat anti mouse CD40 conjugated to R. Phycoerythrin (RPE) (CD40: RPE) (both from Serotec, Inc., UK)

Samples were mixed and incubated on ice for 20 minutes after which they were washed twice with 1 ml PBS. After each wash, samples were centrifuged (Sorvall MC 12 V, DuPont) at 1000 x g for 10 minutes and the supernatants were discarded. One hundred µl of 8% Formaldehyde (in PBS) was added to each tube, mixed thoroughly and incubated for one minute. One ml H_2O was added to each tube and incubated for another 10 minutes to lyze red blood cells. Samples were then centrifuged at 1000 x g for 10 minutes and washed twice with 1ml PBS. After each wash, samples were centrifuged at 1000 x g for 10 minutes and the supernatants discarded. Samples were resuspended in 0.75 ml PBS and analysed by flow cytometry (FACSCalibur ™, Becton Dickinson Immunocytometery Systems, San Jose, California, U.S.A.).

3.2.10 The ELISA Antibody Assay

The method for this assay is a modification of that described by Coligan *et al.* (1991c). Ninety six well flat bottom plates (Nune Immuno plates, Nunc, GIBCO BRL, Life Technologies Ltd., NZ) were coated with 100 µl/well of appropriate antigen in a carbonate coating buffer (15 mM Na_2CO_3, 34mM $NaHCO_3$, pH 9.6) and then covered and incubated overnight at 4°C. Dilutions for antigens are listed below:

Tetanus toxin (Tet-Tox)	1 µg /ml final
Diptheria toxin	10 µg/ml final
Fluvax (Flu)	a 1/100 dilution is needed, final concentration is 0.9 µg/ml
Polio vaccine	a 1/500 dilution is required

Plates were removed from the fridge the following day and washed 3 times using PBST (0.05% Tween 20 in PBS). One hundred µl of samples diluted in sample buffer and standards were aliquoted into the wells in triplicate and plates were covered and incubated overnight at 4°C. The sample buffer used for dilution consisted of 95ml PBS and 5ml heat-inactivated Fetal Calf Serum (FCS). The dilutions for samples and standard are listed below:

Standards:

CT: intestinal washings and serum standards 1→ 7 (1/10000 → 1/640000)

OV: standards 3 → 9 (1/40000 → 1/2560000) (for intestinal contents)

standards 2 → 8 (1/20000 → 1/1280000) (for serum)

Tet-Tox: serum only. Add 100 µl of standard serum to 3.075ml of sample buffer.

Do 9, two fold serial dilutions from this and use the last 7 as the standards.

Diptheria toxin: standards 1 → 7 (1/50 → 1/3200) (serum only)

Fluvax (Flu): standard 1 → 7 (1/200 → 1/12800) (serum only)

Polio vaccine: standard 1 → 7 (1/10 → 1/1280) (intestinal contents only)

(N.B. Pooled samples of serum or intestinal washings were used as standards).

Samples:

CT and OV: undiluted intestinal washings, 1/50 dilution for serum samples

Tet-Tox: 1/500 dilution for serum samples

Diptheria toxin: 1/400 dilution for serum samples

Fluvax: 1/250 dilution for serum samples

Polio: 1 /2 dilution for intestinal contents

The following day the plates were removed and washed 3 times with PBST. Sheep anti-mouse Immunoglobulin (Ig), conjugated to alkaline phosphatase (AKP) (diluted 1/500 in sample buffer) (Silenus Brand, purchased from Amrad Operations P/L, Australia) was added to the wells and the plates were incubated in 37°C in a CO_2 incubator for 1 hour. Plates were then washed 3 times with PBST. One hundred µl of alkaline phosphatase substrate (from AKP substrate kit, Bio-Rad Laboratories, CA, USA) was added to each well. The plates were then incubated at RT for 1 hour before being read on an ELISA plate reader (KC3/ CERES900C, Bio- Tek Instruments, Inc., USA).

3.2.11 Preparation of Peritoneal Macrophages

This method was an adaptation of that described by Coligan *et al* (1994). Following euthanasia, the abdomen of each mouse was sprayed with ethanol before retracting the skin without breaking the peritoneal cavity. Ten ml of RMPI-1640 media was injected (using

10ml syringe with 21G needle) into the cavity and the sides of the cavity gently massaged with finger tips. The peritoneal fluid was then withdrawn through the lower side of the cavity and was aspirated into sterile 10ml siliconised glass Vacutainer® tubes in a sterile airflow cabinet. The fluid was kept on ice prior to centrifugation at 125 x g for 10 minutes at 4°C. The supernatant was then gently tipped off and the pellet resuspended in 10 ml of RMPI-1640, after which it was centrifuged at 125 x g for 10 minutes at 4°C. The supernatant was removed by using a Pasteur pipette and the pellet was resuspended in 1ml of RMPI-1640 media. One hundred µl of the resuspended cells were transferred into FACS tubes containing 400µl of PBS and enumerated by flow cytometry. The samples were adjusted to 1 x 10^6 cells/ml with RMPI-1640 media.

3.2.12 Phagocytosis by Peritoneal Macrophages

The peritoneal macrophages were prepared as described in section 3.2.11. Ten µl of FITC-*E.coli* was added to a 5ml glass tube (noadditive,Vacutainer® blood collection tubes, Becton Dickinson & Co., NJ, USA). One hundred µl of peritoneal macrophages was added to each tube and the mixture was incubated for 20 minutes at 37°C, in a humidified 5% CO_2 incubator. The samples were then immediately placed on ice and 0.5ml of ice cold PBS was added to each tube to stop phagocytosis. Fifty µl of trypan blue was added to the samples which were then analysed by flow cytometry.

3.2.13 Phagocytosis by Peripheral Blood Leukocytes

This method was an adaptation of that described in the PHAGOTEST® test kit (Orpegen Pharma, Germany). One hundred µl of whole blood was added to a 5ml glass tube (no additive, Vacutainer® blood collection tubes, Becton Dickinson & Co., NJ, USA) which had previous been aliquoted with 10µl of FITC labeled *E. coli* (FITC-*E. coli*). The samples were incubated for 30 minutes at 37°C, in a humidified 5% CO_2 incubator. One hundred µl of 8% Formaldehyde was added to the samples which were then incubated for 1 minute at RT. One ml of ice cold water was added to lyse the red blood cells and the samples were thoroughly

mixed. The samples were incubated for 10 minutes at RT and then centrifuged (Heraeus Instruments Megafuge 1.0R) at 1950 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in 0.5ml of PBS. The suspension was transferred into FACS tubes (Falcon® Becton Dickinson & Co., USA) containing 50µl of trypan blue (0.4% w/v and filtered) and the phagocytic activity of the samples analysed by flow cytometry.

3.2.14 Monokine Production

Peritoneal macrophages were prepared as described in section 3.2.11. Cell concentration was adjusted to 1 x 10^6/ml. 1×10^6 cells/ml 24 were incubated with LPS (0.5 mg/ml) for 24 hours at 37°C, 5% CO_2 and 100% humidity. After incubation, the contents of the wells were transferred into eppendorf tubes and centrifuged (Sorvall MC 12 V, DuPont) at 1950 x g for 10 minutes. Supernatants were harvested into another set of labeled eppendorf tubes and subsequently frozen at –20°C until required for further use. (The samples in this section were not analysed in this study).

3.2.15 Respiratory Burst Assay

Peritoneal macrophages were prepared as described in section 3.2.11. One hundred µl of macrophages (1 x 10^6/ml) were aliquoted into 6 wells of a flat bottom 96 well plate (Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ). The plate was then incubated for 2 hours at 37°C/CO_2 atmosphere, following which the plate was washed 3 times with HBSS (Hanks balanced salt solution). One hundred µl of 'SOD' solution (3ml Fe $^{3+}$ Cytochrome C, 20µl PMA (phorbol 12-myristate 13-acetate), 0.74 ml HBSS and 0.24ml SOD (superoxide dismutase)) was added to three wells, one hundred µl of '-SOD' solution (3ml Fe $^{3+}$ Cytochrome C, 20µl PMA and 0.980ml HBSS) was added to another three wells. The plate was incubated for 90 minutes at 37°C/CO_2 atmosphere. After incubation period, the plate was read in a microtiter plate reader at 550 nm (KC3/ CERES900C, Bio- Tek Instruments, Inc., USA). Ten µl dithionate (10mg/ml) was added to each well and the plate was read again in the plate reader.

The amount of O_2^- released was calculated as follows:

a. Determine total Fe3+ cytochrome c reduction (% dithionite reduced) by expressing

the initial reading as a percentage of the reading obtained after dithionite treatment

b. Subtract the absorbance values for the SOD treated samples from the corresponding experimental values (experimental-SOD)
c. O_2^- released = (experimental-SOD) × % dithionite reduced × 10.9 nmol

3.2.16 Nitric Oxide Production Assay

Peritoneal macrophages were prepared as described in section 3.2.11. Fifty µl of macrophages (1 x 10^6/ml) in RMPI- 1640 media were aliquoted into six wells of a flat bottom 96 well plate (sterile) (Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ). Fifty µl of LPS mitogen (5µg/ml) and 50µl of RMPI-1640 media were added to 3 wells. One hundred µl of RMPI- 1640 media was aliquoted to the remaining 3 wells for each sample. The plate was incubated for 24 hours at 37°C/CO_2 atmosphere. Sixty µl of supernatants were transferred into a new non-sterile plate for developing. Thirty µl of Greiss reagent A (see recipe below) was added to each well of the plate. Thirty µl of Greiss reagent B (see recipe below) was added to each well too. The plate was read in a microtiter plate reader at 550 nm (KC3/ CERES900C, Bio- Tek Instruments, Inc., U.S.A.).

Formula for reagent:

Greiss A: Dissolve 1% (w/v) sulfanilamide and 0.1% (w/v) naphthylethylenediamine Dihydrochloride separately in2.5% phosphoric acid (H_3PO_4). Store solution in glass bottles at 4°C.

Greiss B: 2mM $NaNO_2$ (Sigma) in DMEM-5 (stored at 4°C; stable).

3.2.17 NK Cell Activity Assay

YAC-1 cells were labelled at 1 x 10^6 cells/ml overnight with Dye solution 275 (D275, Sigma) in the incubator at 37°C (1µl of D275 was added for every ml of cells in the flask). Spleen lymphocytes were prepared as described in section 3.2.6. At the step when diluted samples of cells were added to flow cytometry tubes, 1µl of 5mg/ml propidium iodide was added and incubated at 37°C for 10 minutes. The YAC-1 target cells were washed twice in RMPI-1640 media. After each wash samples were centrifuged at 125 x g for 10 minutes. The pellet was

resuspended in RMPI-1640 media to get a concentration of 1 x 10^6 cells/ml. Lymphocyte samples were counted on the flow cytometer. Cell number and the percentage of dead cells in the population were recorded. The concentration of cells in the stock solution was determined using the formulae listed below:

a = lymphocyte count from the flow cytometer

b = *a* (x10^6) x 20 = cells/ml in diluted sample

c = *b* x 5 (if the dilution factor is 1/5) = cells/ml of stock solution

The volume of stock solution added to each well was such that it brought the spleen lymphocyte effector cell concentration up to 1 x 10^6 cells/well. The optimum ratio of effector: target cells was 40: 1. Samples were set up in duplicate on a 96 well cytotoxicity plate (N163320, Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ). Twenty five µl of target cells was added to the plate wells first, then the effector cells from the cell proliferation stock solutions. The total well volume was made up to 200µl with RMPI-1640 media. The wells containing effector cells only and target cells only were used as blanks. The plate was centrifuged at 125 x g for 1 minute and then incubated at 37°C/CO_2 atmosphere for 3 hours. Three µl of propidium iodide was added to each well 15 minutes prior to the end of the incubation. Following incubation samples and blanks were transferred to FACS tubes (Falcon® Becton Dickinson & Co., USA) containing 350 µl of PBS and analysed in flow cytometry.

3.2.18 Interferon -γ (IFN -γ) Assay

The method used in this study is an adaptation of that described by Coligan *et al.* (1997). Purified IFN- γ monoclonal antibody (Pharmingen, 0.5mg/ml) was diluted to 4 µg/ml in carbonate coating buffer (0.1mM $NaHCO_3$, pH 8.2). Fifty µl of diluted solution was aliquoted into a 96-well flat-bottom plate (Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ) and covered and incubated at 4°C overnight. The following day the plate were washed 5 times using PBST (0.05% Tween 20 in PBS). One hundred µl of samples ((from section 3.2.8) diluted 1/75 in sample buffer (5ml heat-inactivated Fetal Calf Serum (FCS) in 95ml PBS)), IFN- γ standards (IFN- γ 0.5mg/ml, Pharmingen, 0.78- 100 international reference units (IRU)/ml in sample buffer) and PBS (to act as a control) were

aliquoted into the wells in triplicate and the plates were covered and incubated overnight at 4°C. The following day the plates were washed 5 times with PBST. The biotinylated IFN antibody (0.5 mg/ml, Pharmingen) was diluted to 2 μg/ml in sample buffer. One hundred μl of this diluted biotinylated IFN antibody was added to each well. The plate was covered and incubated at room temperature (RT) for 1 hour and then washed 6 times with PBST. Streptavidin-KP antibody (0.5 mg/ml, Pharmingen) was diluted to 2μg/ml in sample buffer. One hundred μl of this diluted solution was added to each well and covered and incubated at RT for 30 minutes. Subsequently the plate was washed 8 times with PBST. One hundred μl of NPP substrate solution (BioRad cat. No. 1721063 Alkaline Phosphatase Kit) was aliquoted to each well and the plate was incubated at RT for three hours or until colour developed, then read at 405 nm on an ELISA plate reader (KC3/ CERES900C, Bio- Tek Instruments, Inc., USA).

3.2.19 Interleukin 4 (IL-4) Assay

The method used here is an adaptation of that described by Coligan *et al.* (1997). Purified IL-4 monoclonal antibody (Mab, Pharmingen, 0.5mg/ml) was diluted to 4 μg/ml in coating buffer (0.1mM $NaHCO_3$, pH 8.2). Fifty μl of diluted solution was aliquoted into a 96-well flat-bottom plate (Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ) and then covered and incubated at 4°C overnight. The following day the plates were washed 5 times using PBST (0.05% Tween 20 in PBS). One hundred μl of samples ((from section 3.2.8) diluted 1/75 in sample buffer (5ml heat-inactivated Fetal Calf Serum (FCS) in 95ml PBS)), IL-4 standards (IL-4 0.5mg/ml, Pharmingen, 0.78- 100 international reference units (IRU)/ml in sample buffer) and PBS (to act as a control) were aliquoted into the wells in triplicate and the plates were covered and incubated overnight at 4°C. The following day the plates were washed 5 times with

PBST. The biotinylated IL-4 antibody (0.5 mg/ml, Pharmingen) was diluted to 2 μg/ml in sample buffer. One hundred μl of this diluted biotinylated IL-4 antibody was added to each well. The plate was covered and incubated at room temperature (RT) for 1 hour and washed 6 times with PBST. Streptavidin-KP IL-4 antibody (0.5 mg/ml, Pharmingen) was diluted to 2 μg/ml in the sample buffer. One hundred μl of this diluted antibody was added to each well and covered and incubated at RT for 30 minutes. Subsequently the plate was washed 8 times with PBST. One hundred μl of NPP (BioRad cat. No. 1721063 Alkaline Phosphatase Kit) substrate solution was aliquoted to each well and the plate was incubated at RT for three hours or until colour developed, then read on ELISA plate reader (KC3/ CERES900C, Bio- Tek Instruments, Inc., USA) at 405 nm.

3.3 Experimental design

One hundred and twenty BALB/c male mice (6-8 weeks old) were randomly divided into 6 groups each containing 20 mice. After acclimatisation on milk free pellets for 7 days, animals were fed a skim milk powder (SMP)-based diet, containing either 0, 0.1, 0.25, 0.5, 1, or 2% CLA for four weeks.

Mice were immunised orally with 27μl diluted polio vaccine (80 μl polio vaccine in 0.1 mL sodium bicarbonate), and subcutaneously with a mixture of Fluvax and Tetanus toxin vaccine (25μl each) on days 7 and 21. Body weight and food intake were measured weekly. After four weeks of feeding, mice were euthanased by isoflurane overdose. Blood samples were taken by cardiac puncture to obtain 1 ml of blood. The same animals were then used for the harvesting of peritoneal macrophages, spleens and collection of intestinal contents. Immune function was assessed using the following assays (for method details see section 3.2):

a). Cell mediated immunity

- Cell proliferation (spleen lymphocytes)

- Immunophenotyping assay (whole blood method)
- Cytokine production (IFN-γ, IL-4)

b). Humoral immunity

- Antibody responses to antigens/vaccines (ELISA)
- Lymphocyte proliferation (B lymphocytes)

c). Natural killer cell activity

- Cytotoxicity detection

d). Macrophage function

- Phagocytosis (peritoneal macrophages and peripheral blood leukocytes)
- Respiratory burst assay
- Nitric oxide assay

3.4 Statistical analysis

Data were analysed by ANOVA using a General Linear Models (GLM) Procedure of SAS (SAS User's guide, 1990). A split-plot statistical model was used to analyse treatment differences in feed intake and body weight. All other treatment differences were compared using student's t-test to identify significant differences, with $p < 0.05$ being significant and $p < 0.01$ being highly significant. Values in the text were expressed as means ± SE (standard error).

3.5 Results

3.5.1 Body weight and feed intake

Dietary CLA did not significantly affect body weight change (gain) and total food intake during the experimental period (Table 3-3). Feed efficiency (gram of body weight gain per gram of food consumed over this time period) was also not affected by treatment.

Table 3-3 Effect of consumption of diets containing synthetic CLA (Tonalin) on growth performance of mice

	CLA concentration in diet					
	0 (Control)	0.1%	0.25%	0.5%	1.0%	2.0%
Total body weight gain (g)	3.91 ± 0.24	3.88 ± 0.27	3.77 ± 0.30	3.80 ± 0.33	3.60 ± 0.17	3.57 ± 0.37
Total food intake (g)	117.93 ± 2.52	122.01 ± 1.85	123.36 ± 4.31	112.39 ± 4.54	111.70 ± 2.53	116.70 ± 2.23
Feed efficiency	0.031 ± 0.0023	0.032 ± 0.0025	0.030 ± 0.0025	0.032 ± 0.0023	0.032 ± 0.0015	0.030 ± 0.0028

1. Values are means ± SE (n=20).
2. Feed efficiency = gram of body weight gain per gram of food intake

3.5.2 Effects of CLA on spleen weight

A general trend was seen of increasing spleen weight with increasing dietary intake of CLA (Table 3-4). The differences were significant in two groups fed with 1.0% and 2.0% CLA when compared to the control. Similarly, there was a trend of increasing spleen/body weight

ratio with increasing concentrations of dietary CLA. Significant differences were also found between mice fed 1.0 and 2.0% CLA and the mice fed control diets.

Table 3-4 Effect of dietary CLA (Tonalin) on spleen weight and spleen/body weight ratio

Treatment	Spleen weight (g)	Spleen/body weight ratio
Control	0.091 ± 0.0016^{a}	0.0035 ± 0.000045^{a}
0.1% CLA	0.094 ± 0.0023^{a}	0.0037 ± 0.000083^{a}
0.25% CLA	0.097 ± 0.0068^{ab}	0.0039 ± 0.000261^{ab}
0.5% CLA	0.100 ± 0.0068^{ab}	0.0039 ± 0.000189^{ab}
1.0% CLA	0.100 ± 0.0039^{b}	0.0041 ± 0.000136^{b}
2.0% CLA	0.109 ± 0.0056^{b}	0.0044 ± 0.000176^{b}

Values are means ± SE. Within a column, values that do not have a common superscript are significantly different ($p < 0.05$).

3.5.3 The phagocytic activity of peritoneal macrophages

Dietary CLA significantly enhanced the phagocytic activity of peritoneal macrophages to *E. coli* ($p < 0.05$), in a dose-dependent manner. The level of phagocytic activity increased by 6.2% ($p < 0.01$), 5.7% ($p < 0.05$), 7.4% ($p < 0.01$), 9.6% ($p < 0.01$) and 10.1% ($p < 0.01$) in mice fed 0.1%, 0.25%, 0.5%, 1.0% and 2.0% CLA respectively as compared to mice fed control diet (Figure 3-1). No significant differences were observed between these CLA treatment groups.

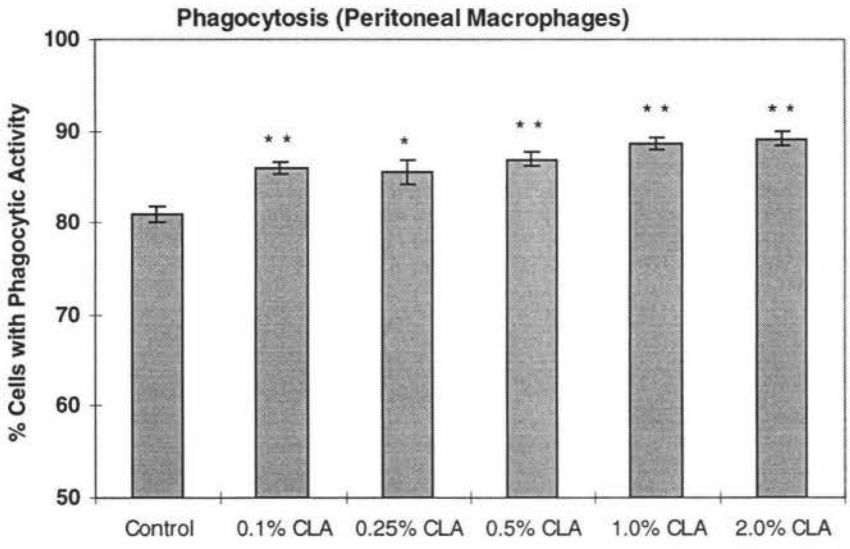

Figure 3-1. Effect of dietary CLA on phagocytosis (Peritoneal Macrophages). Means ± SE (n= 10). Labels above each bar indicate statistical difference when compared to their respective controls: $*p < 0.05$ and $**p < 0.01$

3.5.4 The phagocytic activity of peripheral blood leukocytes

Dietary CLA significantly enhanced the phagocytic activity of peripheral blood leukocytes to *E. coli* ($p < 0.05$), in a dose-dependent manner (Figure 3-2). The phagocytic activities increased 40% ($p < 0.01$), 51% ($p < 0.01$), 61% ($p < 0.01$), 80% ($p < 0.01$) and 100% ($p < 0.01$) in mice fed 0.1%, 0.25%, 0.5%, 1.0% and 2.0% CLA respectively as compared to unsupplemented mice.

Significant differences were observed between several treatment groups ($p < 0.05$). As shown in Figure 3-2, the levels of phagocytic activity in mice fed 1.0% CLA were significantly higher than those of mice fed 0.1% CLA ($p < 0.05$). While the treatment group with 2.0% CLA showed significantly increased phagocytic activity when compared to treatment groups with 0.1%, 0.25% and 0.5% CLA ($p < 0.05$). No significant differences were observed between the three treatment groups fed lower amounts of CLA (0.1, 0.25 and 0.5%). There was also no significant difference in the level of enhancement of phagocytic activity between the two groups fed 1.0% and 2.0% CLA.

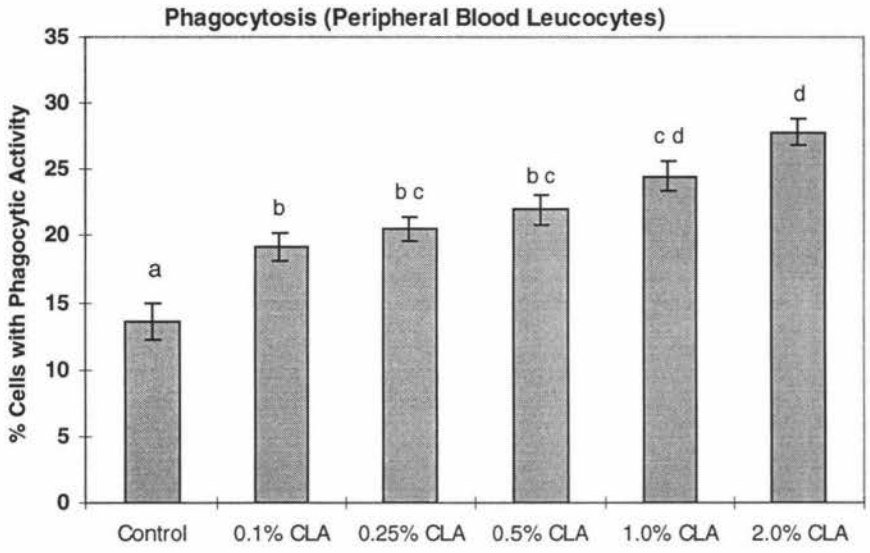

Figure 3-2. Effect of dietary CLA on phagocytosis (peripheral blood leucocytes). Means ± SE (n= 10). Values without a common letter are significantly different from one another at $p < 0.05$.

3.5.5 Respiratory Burst

Dietary CLA did not have any effect on respiratory burst. There were no significant differences in the level of respiratory burst in the treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2.0% CLA) when compared to the control group (Table 3-5), or between these five treatment groups.

Table 3-5 Effect of synthetic CLA on respiratory burst

Treatment	Respiratory Burst (OD)
Control	0.028 ± 0.0028
0.1% CLA	0.031 ± 0.0038
0.25% CLA	0.031 ± 0.0041
0.5% CLA	0.034 ± 0.0072
1.0% CLA	0.024 ± 0.0035
2.0% CLA	0.028 ± 0.0044

Mean ± SE of (n= 10). No significant difference was found between the control (no CLA supplement) and treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2% CLA).

3.5.6 Nitric Oxide Production

Dietary CLA (Tonalin) enhanced nitric oxide production in a dose dependent manner (Figure 3-3). The level of nitric oxide production significantly increased by 55% ($p < 0.05$), 62% ($p < 0.05$) and 80% ($p < 0.05$) in mice fed 0.25%, 0.5% and 1.0% CLA as compared to control mice without CLA supplementation. However mice fed 0.1 and 2.0% CLA did not show significantly enhanced nitric oxide production as compared to the control mice. No significant differences were observed among these five CLA treatment groups.

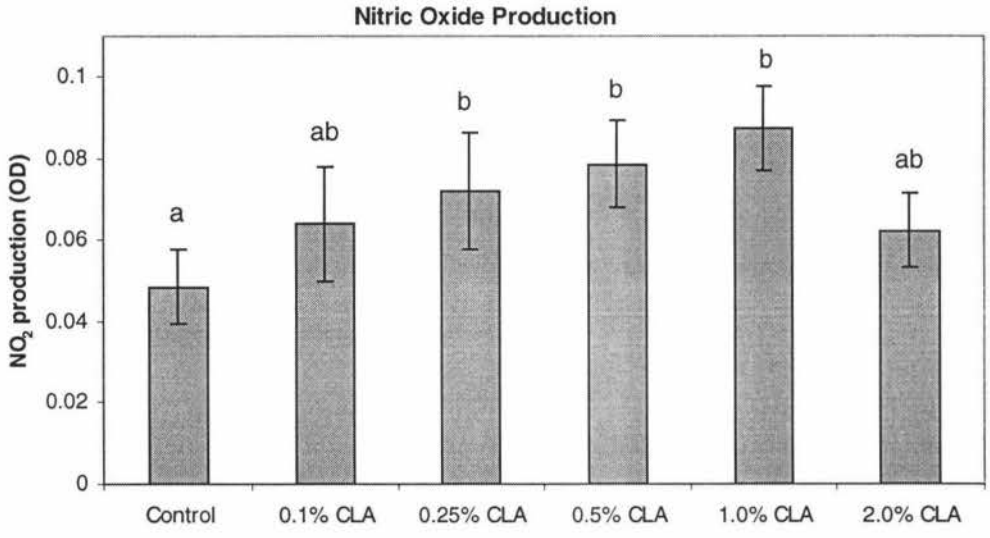

Figure 3-3. Effect of dietary CLA on nitric oxide production. Means ± SE of (n=10). Values without a common letter are significantly different at $p < 0.05$.

3.5.7 NK cell activity--Cytotoxicity detection

The levels of cytotoxic activity in mice fed CLA (from 0.1% to 2%) were higher than mice fed the control diet. The percentage of cytotoxicity significantly increased by 14% ($p < 0.05$) and 12.7% ($p < 0.05$) in mice fed 0.25%, 0.5% CLA as compared to the control mice (without CLA supplementation) (Figure 3-4). No significant differences were observed among the five different CLA treatment groups.

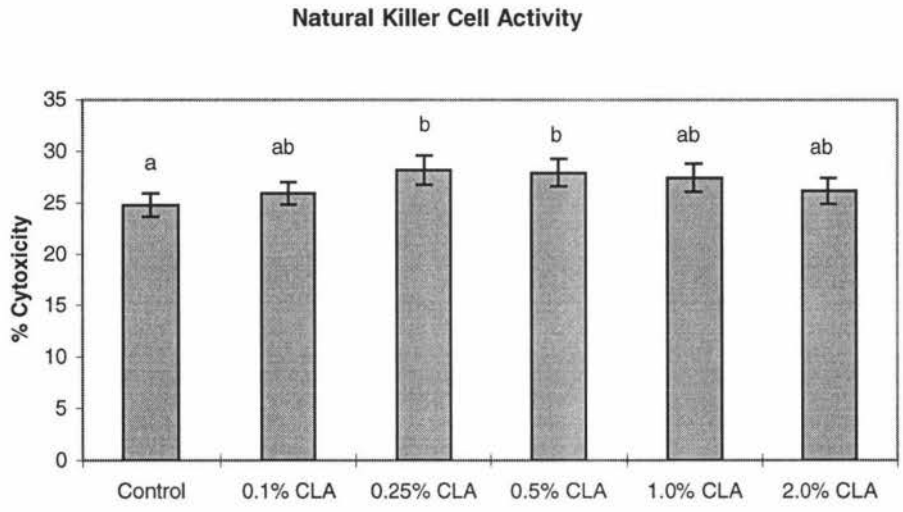

Figure 3-4. Effect of dietary CLA on NK cell activity. Mean ± SE (n=20). Values without a common letter are significantly different at $p < 0.05$

3.5.8 Mucosal and systemic (serum) antibody responses to antigens/ vaccines

Three vaccines were used in this trial: Tetanus toxoid, Polio vaccine and Fluvax vaccine. For Tetanus toxoid, the serum antibody responses were significantly enhanced by CLA in a dose-dependent manner ($p < 0.05$) (Figure 3-5). Serum antibody responses increased 47%, 79.5% ($p < 0.05$) and 118% ($p < 0.01$) in mice fed 0.1%, 0.5% and 2.0% CLA, respectively compared to unsupplemented mice. However no significant differences were observed between the five treatment groups (mice supplemented with 0.1%, 0.25%, 0.5%, 1.0% and 2.0% CLA).

For Polio vaccine (Figure 3-6), a significant dose-dependent increase in mucosal antibody responses was also observed. Mucosal antibody responses increased 58.2%, 93.4% ($p < 0.05$), 102.8% ($p < 0.05$) and 125% ($p < 0.01$) in mice fed 0.1%, o.5%, 1.0% and 2.0% CLA, respectively compared to unsupplemented mice (control). Again there was no significant difference between the different CLA treatment groups.

In contrast to the dose dependent antibody response seen for polio vaccine and tetanus toxoid, enhancement of serum antibody responses to Fluvax were only observed in mice fed low levels of CLA (0.1 and 0.25%) (Figure 3-7). Antibody responses increased 61% in mice fed

0.1% CLA ($p < 0.05$), while only 18% and 9% in mice fed 0.5% and 2.0% CLA (all compared with control mice).

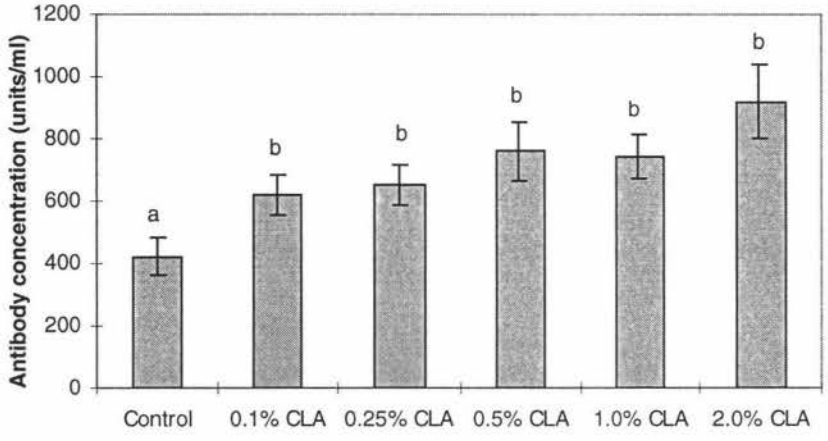

Figure 3-5 Serum antibody responses to Tetanus Toxoid. Mean ± SE (n= 20). Values without a common letter are significantly different from one another at $p < 0.05$

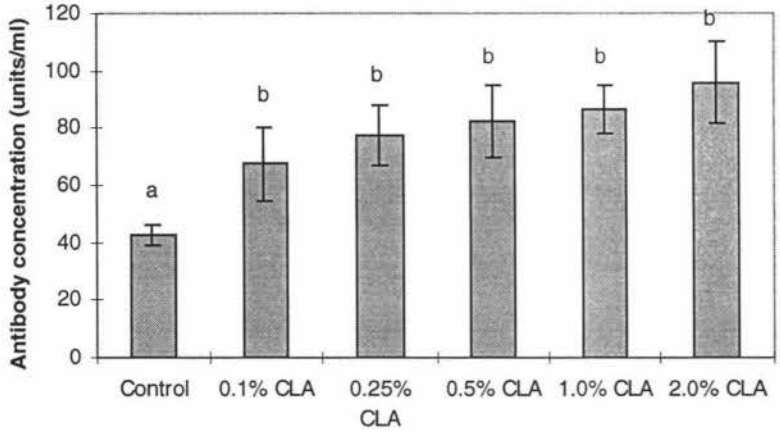

Figure 3-6 Mucosal antibody responses to Polio vaccine. Means ± SE of 20 mice. Values without a common letter are significantly different from one another at $p < 0.05$

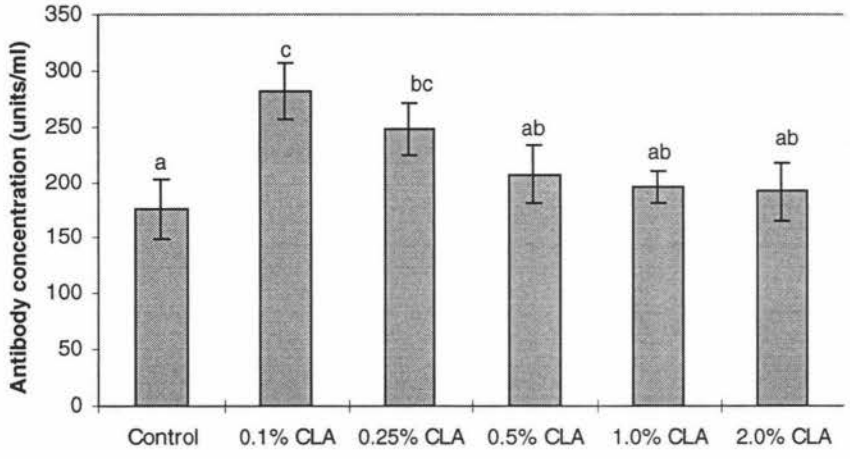

Figure 3-7 Serum antibody responses to Fluvax vaccine. Means ± SE of 20 mice. Values without a common letter are significantly different at from one another $p < 0.05$

3.5.9 Total leukocyte and total lymphocyte levels in peripheral blood

Dietary CLA (Tonalin) did not have any significant effect on total leukocyte or total lymphocyte numbers in peripheral blood (Table 3-6). There was no significant difference in the number of total leukocytes and lymphocyte in the treatment groups compared to the control group, or between the different treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2.0% CLA).

Table 3-6 Effect of synthetic CLA on total lymphocytes and total leukocytes levels in peripheral blood

Treatment	Total lymphocytes (cells × 10^6 / ml)	Total leukocytes (cells × 10^6 / ml)
Control	3.02 ± 0.22	3.16 ± 0.23
0.1% CLA	3.05 ± 0.36	3.26 ± 0.36
0.25% CLA	3.12 ± 0.44	3.31 ± 0.44
0.5% CLA	2.79 ± 0.21	3.00 ± 0.21
1.0% CLA	2.78 ± 0.40	3.03 ± 0.41
2.0% CLA	2.65 ± 0.45	2.83 ± 0.47

Mean ± SE of (n= 10). No significant difference was found between the control (no CLA supplement) and treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2% CLA).

3.5.10 Expression of peripheral blood leukocyte cell surface markers

The effect of dietary CLA on the expression of surface markers is shown in Table 3-8. Five lymphocyte surface markers were measured ($CD4^+$, $CD25^+$, $CD8^+$, $CD3^+$ and CD 40^+), the $CD4^+$ to $CD8^+$ ratio was calculated along with the total numbers of $CD4^+$ & $CD40^+$ cells. Of all these lymphocyte subsets ($CD4^+$, $CD25^+$, $CD8^+$, $CD3^+$, $CD40^+$, $CD4^+$: $CD8^+$, Total $CD4^+$, Total $CD40^+$), no significant differences were observed between the control group (mice without CLA supplemented) and the CLA supplemented groups (mice fed with 0.1, 0.25, 0.5, 1.0, and 2.0% CLA) (Table 3-7).

However the level of $CD8^+$ in the treatment groups showed a slight increase with increasing of dietary CLA intake compared to the control mice, the number of $CD8^+$ lymphocytes increased from 6% to 14% in mice fed from 0.1 to 2.0% CLA, although the change was not significant.

There was no significant difference between the five treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2.0% CLA) for any of the lymphocyte subsets shown in table 3-7.

Table 3-7 Effect of synthetic CLA on the expression of surface markers on peripheral blood leukocytes

Immune measure	CLA (Tonalin) concentration in diet					
	0 (Control)	0.1%	0.25%	0.5%	1.0%	2.0%
$CD4^+$ (%)	42.50 ± 2.78	42.83 ± 1.67	42.15 ± 1.95	43.69 ± 2.53	42.77 ± 3.37	43.33 ± 2.75
$CD25^+$ (%)	4.66 ± 0.22	4.59 ± 0.20	4.59 ± 0.23	4.69 ± 0.30	4.77 ± 0.28	4.99 ± 0.27
$CD8^+$ (%)	12.67 ± 0.52	13.43 ± 0.42	13.69 ± 0.84	14.64 ± 1.42	14.37 ± 1.04	15.05 ± 1.16
$CD3^+$ (%)	62.30 ± 3.26	59.21 ± 3.51	60.94 ± 3.69	58.05 ± 4.09	60.86 ± 3.19	61.09 ± 3.27
$CD40^+$ (%)	30.90 ± 2.45	28.94 ± 2.36	29.69 ± 3.0	26.10 ± 1.65	29.34 ± 4.03	25.30 ± 2.87
CD4 : CD8	3.36 ± 0.20	3.19 ± 0.09	3.13 ± 0.16	3.13 ± 0.24	3.04 ± 0.24	3.0 ± 0.25
Total $CD4^+$ (cell x 10^6)	126.5 ± 9.0	134.3 ± 21.6	128.2 ± 13.7	122.7 ± 12.3	133.6 ± 21.8	118.0 ± 18.4
Total $CD40^+$ (cell x 10^6)	97.8 ± 13.9	87.1 ± 12.8	113.2 ± 25.1	71.8 ± 7.9	77.2 ± 12.6	74.4 ± 20.5

Mean ± SE of (n= 10). No significant difference was found between the control (no CLA supplement) and treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2% CLA).

3.5.11 Cell proliferative response to T and B cell mitogens

Three mitogens were used in cell proliferation assays, phytohemagglutinin (PHA), concanavalin A (Con A) and *E. coli* lipopolysaccharide (LPS). PHA and Con A stimulate T cells, while LPS stimulates B cells (Schultz and Adams, 1978).

PHA-induced lymphocyte blastogenesis in mice fed CLA was enhanced ($p < 0.05$) in a dose-dependent manner (Figure 3-8). All the treatment groups showed enhancement of proliferative responses, with the 0.25, 0.5 & 1.0% groups showing significant enhancement. Lymphocyte proliferation increased 30% ($p < 0.05$), 42.4% ($p < 0.01$) and 29.5% ($p < 0.05$) in mice fed 0.25%, 0.5% and 1% CLA, respectively as compared to mice fed control diet. No significant difference was found between the treatment groups (mice fed 0.1, 0.25, 0.5, 1 and 2% CLA).

The effect of CLA on Con A-induced lymphocyte proliferation was similar to that observed with PHA (Figure 3-9). Mice fed 0.25% CLA significantly enhanced ($p < 0.05$) lymphocyte blastogenic response when compared to the control mice. No significant difference was found among the treatment groups (mice fed 0.1, 0.25, 0.5, 1 and 2% CLA).

CLA did not show any significant effect on LPS-induced lymphocyte blastogenesis (Figure 3-10).

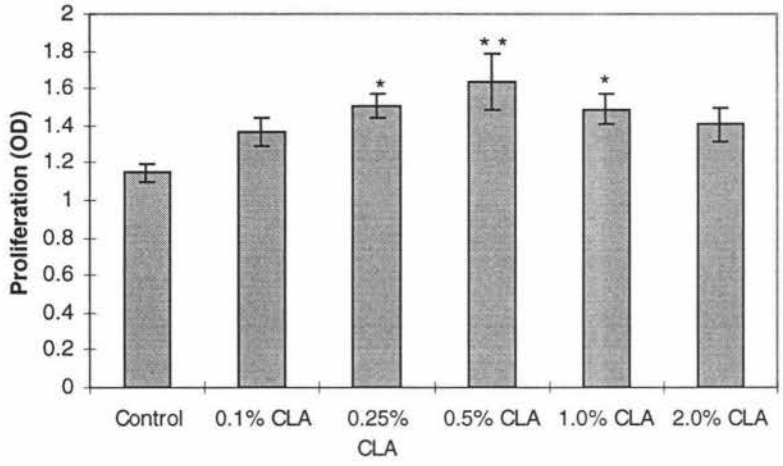

Figure 3-8 Effect of CLA on Splenic lymphocyte proliferation induced by PHA (phytohemagglutinin). Means ± SE (n = 10). Labels above each bar indicate statistical difference when compared to their respective controls: $*p < 0.05$ and $* *p < 0.01$.

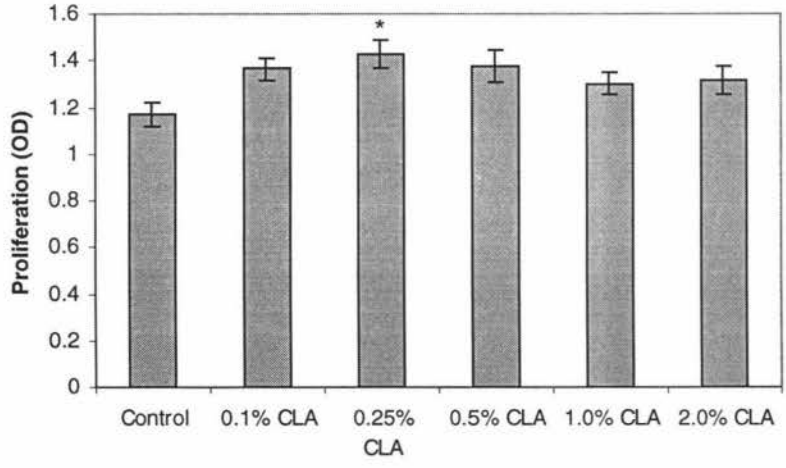

Figure 3-9 Effect of CLA on Splenic lymphocyte proliferation induced by Con-A (Concanavalin A). Means ± SE (n = 10). Labels above each bar indicate statistical difference when compared to their respective controls: $*p < 0.05$.

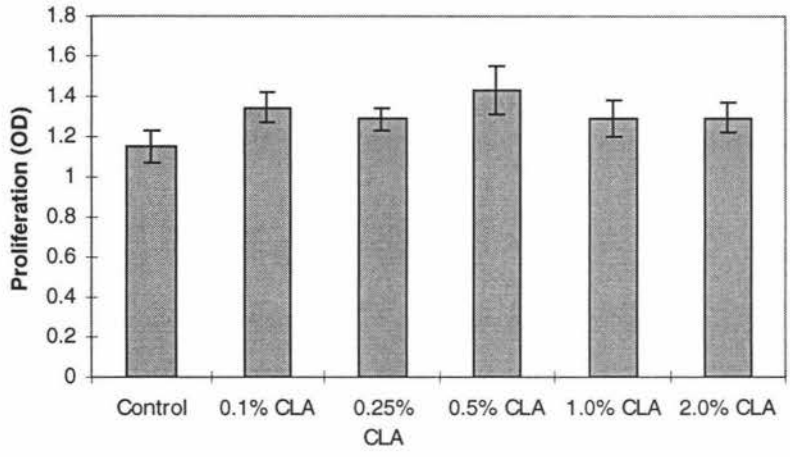

Figure 3-10 Effect of CLA on Splenic lymphocyte proliferation induced by LPS (*E. coli* lipopolysaccharide). Means ± SE of (n = 10). No difference was found between the treatments and their respective control

3.5.12 Interferon-γ (IFN-γ) and Interleukin–4 (IL-4) production

There was no difference in the level of production of IFN-γ in any of the treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2.0% CLA) when compared to the control group. There were also no significant differences between the different treatment levels (0.1, 0.25, 0.5, 1.0 and 2.0% CLA) (Table 3-8).

No significant differences were observed in interleukin-4 (IL-4) production in the treatment groups when compared to the control group. However the IL-4 levels in treatment groups showed a progressive decrease with increasing dietary CLA intake. IL-4 levels decreased 12.9%, 16% and 35.6% in mice fed 0.1%, 0.5% and 2% CLA, as compared to mice fed without CLA supplementation, although the changes were not statistically significant (Table 3-8). There were also no significant differences between the different treatment levels (0.1, 0.25, 0.5, 1.0 and 2.0% CLA).

Table 3-8 Effect of synthetic CLA on interferon-γ (IFN-γ) production and interleukin-4 (IL-4) production

Treatment	IFN-γ production (unit)	IL-4 production (unit)
Control	69.31 ±15.59	170.50 ±44.86
0.1% CLA	69.46 ±19.71	148.40 ±31.92
0.25% CLA	85.25 ±36.35	140.78 ±75.11
0.5% CLA	65.49 ±19.80	143.71 ±35.75
1.0% CLA	78.84 ±20.41	100.22 ±26.55
2.0% CLA	69.09 ±24.18	109.84 ±23.99

Mean ± SE of (n= 10). No significant difference was found between the control (no CLA supplement) and treatment groups (mice fed 0.1, 0.25, 0.5, 1.0 and 2% CLA).

3.6 Discussion

In the present study, the dose effect of different synthetic CLA (Tonalin) on immune function was investigated.

CLA refers to a mixture of the conjugated positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid). The two double bonds in CLA are in positions 9 and 11, 10 and 12 or 11 and 13 along the carbon chain (Parodi, 1994). Each of the bonds can be in the *cis* (*c*) or *trans* (*t*) configuration. Up to nine different positional and geometric isomers of CLA have been reported as minor components in the lipid fraction of many different kinds of food (Ha *et al.*, 1989). However, the *c*9, *t*11 isomer of octadecadienoic acid is the most abundant form amounting to as much as 90% of the total CLA content of dairy products (Chin *et al.*, 1992). The *c*9, *t*11 CLA isomer was reported to be incorporated into the phospholipids of the neoplastic cells (Ha *et al.*, 1987; 1990), suggesting that this isomer may be the most biologically active form.

The isomer composition of synthetic CLA (Tonalin) used in the present study is different to that of naturally occurring CLA. The *c*9, *t*11 CLA isomer which is thought to be biologically active isomer comprises only 37.1% of the CLA in Tonalin, while consists of 90% in

naturally occurring CLA (Chin *et al.*, 1992; Ip *et al.*, 1991). It is been speculated that there might be some biological differences between the two forms (natural and synthetic) of CLA, however no work appears to have been done in this area.

Results of this study showed that dietary synthetic CLA (Tonalin) did not significantly affect final body weight and feed intake during the experimental period (Table 3-4). This finding is consistent with the observations of other studies that dietary CLA did not influence body weight gain and feed intake in rats and mice (Sugano *et al.*, 1997; Wong *et al.*, 1997; Li & Watkins, 1998). However, Chin *et al* (1994b) and Li & Watkins (1998) have reported that feed efficiency (total grams weight gain/total grams feed consumed) was significantly improved for rats fed CLA for six and eight weeks. In the present study feed efficiency was not significantly influenced in mice fed CLA (Tonalin) for four weeks. The discrepancy may be due to differences between animal species and the feeding period.

In the present study, a general trend was observed of increasing spleen weight and spleen/body weight ratio with increasing dietary intake of CLA (Tonalin) (Table 3-4). The differences between two of the dietary treatment groups (1.0 and 2.0% CLA) and the control were significant. The result is consistent to a study by West *et al.* (1998) which also showed that dietary CLA increased spleen weight in mice. However, Sugano *et al.* (1998) reported that CLA feeding to rats did not influence spleen weight. The difference in animal species may also account for this discrepancy.

Two groups of mitogens were used to measure lymphocyte cell proliferative responses, Con A and PHA are T-cell mitogens, LPS is a B-cell mitogen (Schultz and Adams, 1978). CLA (Tonalin) was found to enhance PHA- and Con A-induced lymphocyte blastogenesis (Figure 3-8 and 9). The effect was significant ($p < 0.05$) when CLA (Tonalin) supplementation was at the 0.25%, 0.5% and 1% level compared to the control. These results are consistent with the observations in other studies (Cook *et al.*, 1993; Miller *et al.*, 1994; Chew *et al.*, 1997; Wong *et al.*, 1997). Chew *et al.* (1997) reported from *in vitro* studies that CLA stimulated porcine lymphocyte proliferation when cells were stimulated with both the T-cell mitogens PHA and Con A and with the T-cell dependent B-cell mitogen pokeweed mitogen (PWM).

Wong *et al.* (1997) also reported that synthetic CLA enhanced PHA-induced lymphocyte blastogenesis in mice, and that the stimulation was in a dose-dependent manner (0.3% and 0.9% CLA supplementation showed significant differences when compared with the control). The current study confirms that the stimulation of PHA- and Con A–induced T lymphocyte blastogenesis was dose-dependant at CLA level of 1% or below. No further enhancement was observed when mice were fed CLA (Tonalin) at the 2% level. The reason for the plateau is not clear. There may be a limiting step in the capacity to metabolize CLA to another active product(s) that is essential for enhancing lymphocyte blastogenesis. T lymphocyte blastogenesis represents the first response to an antigenic challenge, while cytotoxic T lymphocytes directly kill neoplastic and virus infected cells (Carter *et al.*, 1986). Therefore, stimulation of cell mediated immunity may be an important protective mechanism against tumor development.

In contrast to T cells mitogens, synthetic CLA (Tonalin) did not show any significant effect on LPS-induced lymphocyte proliferation (Figure 3-10). This result is consistent with a study conducted by Wong and his colleagues (1997) that also reported synthetic CLA did not influence LPS-induced lymphocyte proliferation. It seems that synthetic CLA is able to specifically enhance T cell proliferation, but not B cells. The reason for this is not clear, however, it is possible that certain receptors present on T cells but not B cells are mediators in the stimulation process.

Synthetic CLA (Tonalin) had no significant effect on the relative percentages of peripheral blood lymphocyte subsets, including $CD4^+$, $CD25^+$, $CD8^+$, $CD3^+$ and $CD40^+$. The $CD4^+/CD8^+$ ratio, the levels of total $CD4^+$ and $CD40^+$ were not influenced by supplementation with synthetic CLA. The results agree with the study (Sugano *et al.*, 1998) that there were no effects of synthetic CLA on T-lymphocyte populations ($CD4^+$ and $CD8^+$) of mesenteric lymph nodes (MLN) in rats. When an individual is exposed to a bacterial infection such as *Salmonella* infection, the populations of lymphocyte subsets ($CD4^+$, $CD8^+$) in peripheral blood are usually very high. It may be a beneficial effect for CLA to maintain lymphocyte subsets in a relative stable condition that might protect human body against infectious diseases and cancers.

Mucosal and serum antibody responses to vaccines were significantly enhanced by supplementation with CLA (Tonalin). Further, the serum antibody responses to Tetanus Toxoid were enhanced by CLA (Tonalin) in a dose-dependent manner (Figure 3-5), with the difference between all treatment groups and the control being significant ($p < 0.05$). The mucosal antibody responses to Polio vaccine were also enhanced by CLA (Tonalin) in a dose-dependent manner (Figure 3-6), with again there being significant differences between all treatment groups and the control. The serum antibody responses to Fluvax vaccine were enhanced by CLA (Tonalin) (Figure 3-7), but the stimulation was not dose-dependent. The responses were significant when mice were fed 0.1% and 0.25% CLA (Tonalin) as compared with the control, however other CLA (Tonalin) groups did not show any significant difference when compared with the control group.

Although the antibodies measured here are total antibodies (immunoglobulin, Ig), the mucosal antibodies against Polio vaccine are primarily secretory immunoglobulin A (sIg A), and the serum antibodies against Tetanus toxoid and Fluvax are mainly Ig G and Ig M (Sell, 1987). Ig G is the most abundant immunoglobulin of internal body fluids, particularly extravascular where it combats microorganisms and their toxins (Roitt, 1994). Ig M is a very effective agglutinator, which is produced early in the immune response. It is the effective first line of defense against pathogens. Ig A is the major immunoglobulin in sero-mucous secretions where it defends external body surfaces. From the present results it might be extended that synthetic CLA may increase the levels of IgA, IgG, IgM, which are great helpful for the body to fight against bacterial infections and diseases. The present results have similarities with another study (Sugano *et al.*, 1998) which claimed that dietary synthetic CLA increased the serum concentration of Ig A, Ig G and Ig M in rats. However Sugano *et al* (1998) did not use vaccines in their study, and the difference was only significant in the 1.0% CLA group as compared with the control. In our study the difference was significant even in the 0.1% CLA (Tonalin) group compared to the control. It must be noted that the discrepancy between the studies may be accounted for by other reasons, such as differences in the length of feeding period and different assays for measuring antibody responses.

The mechanism for the antibody responses enhanced by CLA is not clear. It is interesting that although the levels of antibody responses to antigens/vaccines were enhanced by CLA (Tonalin), CLA (Tonalin) did not enhance LPS-induced B-cell lymphocyte blastogenesis. This is backed by current observations that CLA (Tonalin) did not appear to have any significant effect on LPS- induced B lymphocyte blastogenesis. Wong *et al.* (1997) also reported that dietary CLA (synthetic) did not significantly influence LPS-induced lymphocyte proliferation. It is well documented that lipopolysaccharide (LPS) is a T-cell independent B-cell mitogen, while pokeweed mitogen (PWM) is the T-cell dependent B-cell mitogen (Schultz and Adams 1978). Both Michal *et al.* (1992) and Chew *et al.* (1997) reported that CLA enhanced PWM-induced B lymphocyte blastogenesis. It was postulated that CLA might increase T-cell dependent B lymphocyte proliferation accompanied by the increase of T-helper cells. However in the present study, CLA (Tonalin) seemed to have no effect on peripheral blood lymphocyte subsets, such as $CD4^+$, $CD8^+$ and $CD40^+$. $CD4^+$ is a marker of helper T-cell populations which promote activation and maturation of B-cells and cytotoxic T-cells, and control antigen-specific chronic inflammatory reaction through stimulation of macrophages (Roitt, 1994). $CD8^+$ is a marker for the cytotoxic T lymphocyte population (Roitt, 1994). $CD40^+$ is the marker of general B lymphocyte populations and acts as a receptor for *p*39 costimulator (Roitt, 1994). It would thus appear that the observed stimulatory effect of CLA on antibody levels is not mediated through non-specific enhancement of B lymphocyte proliferation (at least not T-cell independent B cells). Plasma cells are the mature cells of the B cell series that synthesise and secrete immunoglobulin. CLA (Tonalin) might stimulate plasma differentiation directly without influencing B cell proliferation, thus enhancing antibody responses to antigens/vaccines. However, this is uncertain.

Macrophages are derived from bone marrow promonocytes which, after differentiation to blood monocytes, finally settle in tissues as mature macrophages where they constitute the mononuclear phagocyte system (Roitt, 1994). They serve a central role in non-specific immunity, such as phagocytosis, bacterial and tumoricidal activity, inflammation and tissue repair (Roitt, 1994). The ability of macrophages to phagocytose particles represents the first step in a series of events that ultimately leads to the elimination of intruding microorganisms,

tumor cells and cellular debris. Following phagocytosis, two independent biochemical pathways contribute to the ability of macrophages to eliminate intracellular pathogens and extracellular targets: enhanced production of reactive nitrogen and oxygen intermediates (Roitt, 1994). Therefore the production of nitric oxide and respiratory burst production of oxygen radicals may be two simple indicators of macrophage cytotoxicity against microorganisms, tumor cells and cellular debris (Roitt, 1994).

It was observed in this study that CLA (Tonalin) enhanced phagocytic activity of peritoneal macrophages and peripheral blood leukocyte in a dose-dependent manner (Figure 3-1 and 2), and the effect was significant ($p < 0.05$) in all treatment groups as compared with the control. CLA was also found to enhance nitric oxide production (Figure 3-3), and the effect was significant when CLA (Tonalin) supplementation was at 0.25, 0.5 and 1.0% level as compared to the control. And CLA (Tonalin) did not have any significant effect on respiratory burst. The results are consistent with a study (Cook *et al.*, 1993) in which CLA was found to enhance the phagocytic activity of macrophages in rats. However Chew and his co-workers (1997) reported that the phagocytic activity of peritoneal macrophages was decreased by the higher concentrations of CLA, and that CLA enhanced the killing ability of macrophages, while the production of superoxides by macrophages (respiratory burst) was not affected by the presence of CLA. The reason for these divergent results is not clear. A critical examination of their experiment (Chew *et al.*, 1997) showed that they were using radioscintillation counts (uptake of [^{3}H]-uridine by bacteria) in measuring phagocytic activity, whereas in both the present study and the study by Cook and his colleagues (1993), flow cytometry was used to determine the level of phagocytosis. Flow cytometric analysis has an important advantage over other traditional techniques, as it discriminates between internalised and membrane-bound particles in phagocytosis (Fattorissi *et al.*, 1989). Only live cells that have internalised particles are reported. Therefore this technique is more reliable and accurate in measuring the level of phagocytosis. It is possible that the use of different methods of measurement may account for the differences in results.

The reason for why CLA (Tonalin) significantly improved phagocytosis and increased nitric oxide production, while did not have any effect on respiratory burst production of oxygen

radicals is not clear. One hypothesis is that CLA triggers specific activating factors by enhancing specific macrophage functions. This growth activation may occur by fatty acid modulation of membrane fluidity. Membrane fluidity affects the mobility of membrane proteins and receptors that are important in signal transduction and cell cycle induction. Synthetic CLA might increase membrane fluidity, thus improving specific receptor-ligand interactions and/or their sugnal transductions. It is understood that foreign particles adhere to the cell membrane of the macrophage through non-specific and specific (C3b) receptors (Roitt, 1994). The enhanced specific receptor-ligand interactions may result in improved macrophage responses, including phagocytosis, nitric oxide production but not respiratory burst. The activation triggered by CLA may be a limiting step, with the effect of CLA maximized at the 2% level, and the effect being dose-dependent when the CLA level is under 1% or 2%.

Natural Killer (NK) cell activity was also observed to be enhanced by supplementation with dietary CLA (Tonalin). The effect was significant at two CLA levels (0.25% and 0.5%) as compared with the control group. NK cells have a wide range of biological activities, including immunosurveillance against neoplastic cells, viral infection, abnormal hematopoietic development and production of lymphokines such as interferons (Meydani *et al.,* 1988). The effect of CLA on NK cell activity has not been reported previously. However, dietary fatty acids have been reported to influence NK cell activity (Meydani *et al.,* 1988; Yamashita *et al.,* 1991; Calder, 1998). Meydani *et al.* (1988) found that mice fed fish oil had lower natural killer cell activity than those fed corn oil. Yamashita *et al.* (1991) found that natural killer cell activity of human peripheral blood lymphocytes decreased after the infusion of the (20:5(n-3)) fatty acid into volunteers. Natural killer cell activity may be decreased following intake of dietary fish oil via a reduction in n-6 derived lipoxygenase products, prostaglandins, and interleukin-1 (Berger *et al.,* 1993). Phosphatidylinositol metabolites may also be important in natural killer cell-mediated cytotoxicity (Brahmi, 1992).

The mechanism of CLA action on NK cell activity is uncertain. One hypothesis is that CLA might have the ability to modulate eicosanoid synthesis. Eicosanoids are a family of oxygenated derivatives of dihomo-γ-linolenic, arachidonic, and eicosapentaenoic acids.

Eicosanoids include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), lipoxins, hydroperoxyeicosatertraenoic acids (HPETE) and hydroxyeicosatraenoic acids (HETE). Cook *et al* (1993) reported that the concentration of arachidonic acid in the fat pad from CLA treated rats was only one-third of the concentration found in unsupplemented rats. CLA may block the conversion of linoleic acid to arachidonic acid, thus decreasing the amount of arachidonic acid entering the lipoxygenase and cyclooxygenase pathways. This will reduce the production of LTB_4, 5-HPETE and PGE_2 in tissue lipids (Figure 3-11). Sugano *et al.* (1998) agreed with this hypothesis when they reported that CLA effectively reduced LTB_4 and PGE_2 production. LTB_4, 5-HPETE and PGE_2 are known to have very important roles in suppressing the activity of natural killer cells (Meydani *et al.*, 1988). Therefore CLA may modulate natural killer cell activity by regulating eicosanoid synthesis.

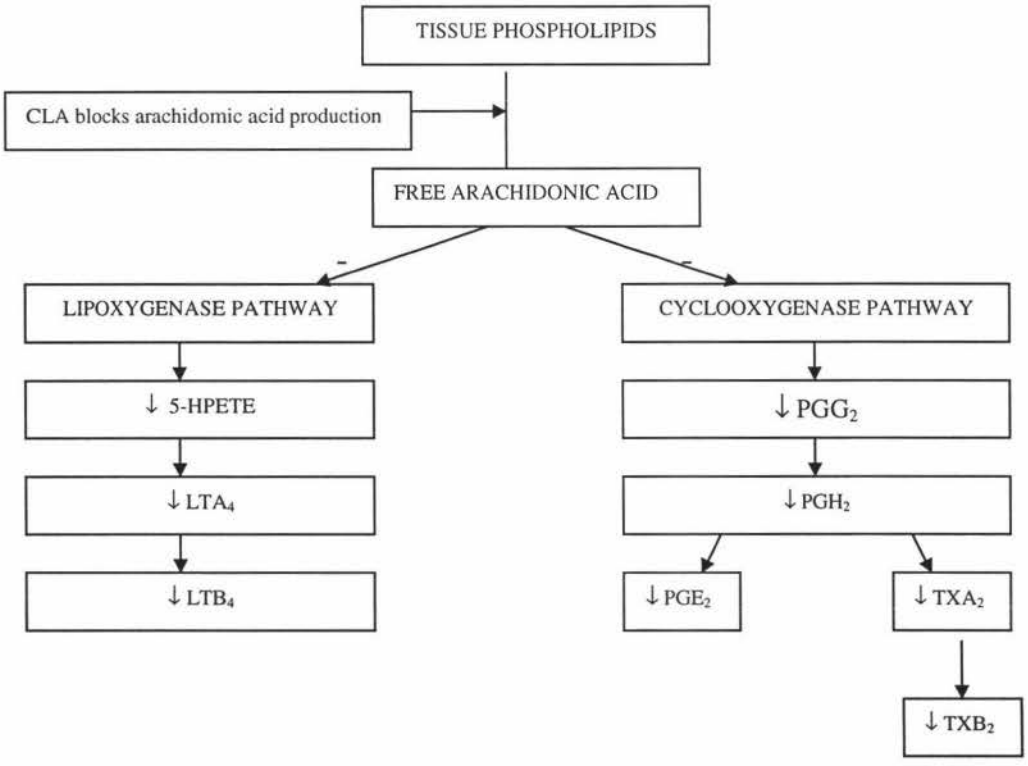

Figure 3-11 Proposed mechanism of action of CLA in regulating eicosanoid synthesis. HEPETE, hydroxyperoxyeicosatertraenoic acid; HETE, hydreoxyeicosatertraenoic acid.

IFN-γ and IL-4, are both very important cytokines. Cytokines are a group of signalling molecules involved in cellular communication. They are produced by many different cells and are usually glycoproteins (Weir and Stewart, 1993). IFN-γ is an important immunomodulatory cytokine that regulates natural, cell-mediated, and humoral immunity by eliciting a number of biological responses in many different cell types. IFN-γ is produced by activated T lymphocytes and natural killer (NK) cells. In man and mouse, T cells of either the helper (CD4) or cytolytic (CD8) phenotypes can produce the IFN-γ (Weir and Steward, 1993). Interleukin 4 (IL-4) causes activation, proliferation, and differentiation of B cells, and is also a growth factor for T cells and mast cells and macrophages (Hamblin, 1993). IL-4 is a switch factor for IgE and IgG production by B lymphocytes. The major source of IL-4 is the activated T_{H2} subset of CD4 T cells in mice and the equivalent population in humans (Hamblin, 1993).

It was observed that CLA (Tonalin) did not show any significant effect on gamma-interferon (IFN-γ) production. CLA slightly decreased interleukin 4 (IL-4) production in a dose-dependent manner, although the difference was not significant in the treatment groups as compared with the control. IFN-γ has potent immunoregulatory effects on a variety of cells including activation of macrophages, induction of FcγRI on macrophages and FcγRII on granulocytes, enhanced production of IgG2a by B lymphocytes, and enhancing NK cell activity (Hamblin, 1993). The major effects of IL-4 are activation and differentiation of B cells, enhancing proliferation of PHA-activated mature lymphocytes (Roitt, 1994). It would appear that the observed stimulatory effect of CLA on T lymphocyte proliferation and macrophages and NK cell activity might not be mediated through IFN-γ and IL-4 production. Similar work on the effect of CLA on IFN-γ and IL-4 level is not available. However, the production of IL-2, another cytokine that regulates both T and B lymphocyte proliferation, has been reported to be enhanced in mice (Wong *et al.*, 1997) or suppressed (Michal *et al* , 1992; Chew *et al* , 1997) by CLA.

Two important biological activities of IL-4 are induction of expression and release of low affinity IgE receptor and stimulation of IgG1 and IgE synthesis and inhibition of IgM, IgG3, $IgG2_a$, and $IgG2_b$ synthesis. The present study found that CLA slightly reduced IL-4

production and may therefore diminish IgE level. IgE is an allergen-specific immunoglobulin. Food allergy reaction is initiated by the production of IgE. Sugano *et al.* (1998) reported that CLA increased the production of IgA and IgG. IgA acts as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor by competing with the binding of the allergen to the receptor on the surface of target cells such as mast cells and basophils (Metcalfe, 1991). Although IgA, IgG and IgE levels were not measured in the present study, according to the results in the current study, such as low IL-4 levels, high antibody responses, it may be postulated that CLA may have some effect in reducing allergic responses.

3.7 Summary

In summary, synthetic CLA (Tonalin) was found to enhance a range of immune functions in mice, including stimulating T lymphocyte proliferation, enhancing macrophages activities (phagocytosis and nitric oxide production), increasing mucosal and systemic antibody responses to antigens/vaccines (Polio, Tetanus toxin and Fluvas), and promoting NK cell cytotoxic activity. On the other hand, CLA (Tonalin) slightly decreased IL-4 production, which may be postulated to regulate IgE synthesis, therefore reducing allergic responses. The enhancing effect of CLA on macrophages was dose dependent. Overall, 0.25% CLA showed a wider immunoregulating activity than any other CLA concentration and was regarded as the most suitable CLA level for achieving optimal immunoregulating effects.

CHAPTER FOUR Experiment Two: Effect of milk fat derived CLA on immune function

4.1 Introduction

CLA is known to occur in a wide variety of foods, principally dairy products and other foods derived from ruminant animals. CLA appears to be a minor, naturally occurring product of microbial lipid metabolism. A number of bacterial species that reside in the rumen generate CLA from linoleic acid using certain of their enzymes (the linoleate isomerases) (Chin *et al.*, 1992).

Cis-9, *trans*-11 CLA is the main isomer produced by *Butyrivibrio. fibrisolvens* and most of the other rumen microorganisms involved in linoleic acid metabolism, although other CLA isomers may also be formed. About 90% of total CLA in milk fat is in the *cis*-9, *trans*-11 form, and this isomer is regarded as the most biologically active form of CLA (Ha *et al.*, 1992).

Milk fat is the richest natural dietary source of CLA. The amount of CLA in milk fat reportedly varies from 0.24% to 2.81% (Riel, 1963), with the fluctuation being seasonal. In New Zealand, the CLA level in milk fat is higher in the spring and autumn because in these seasons cows can be fully pastured. And New Zealand pasture fed cows have been reported to produce higher CLA levels in milk fat than overseas sources where grain feeding predominates (MacGibbon and Hill, 1998).

As discussed in chapter II, all of the studies on CLA that have been reported to date have used synthetically prepared CLA. In the present study, natural CLA derived from milk fat was used to investigate the effect of natural CLA (derived from milk fat) on immune function in mice. Dose response of natural CLA was examined.

4.2 Materials and Methods

4.2.1 Animals

The animal experimentation was according to the Massey University guidelines for the care and use of laboratory animals and was approved by the Massey University Animal Ethics Committe. One hundred and twenty BALB/c male mice (6-8 weeks old) were kept at the small animal production unit (SAPU), Massey University, Palmerston North, New Zealand. They were housed in pairs in metal cages at a temperature of $22 \pm 2^{o}C$, with a 12 hour light / dark cycle. Animals were fed *ad libitum* with free access to water at all times.

4.2.2 Diets

Animals were fed a skim milk powder (SMP) based diet. There were five treatment diets and one control diet. The compositions of the diets are shown in Table 4-1. Tonalin was purchased from PharmaNutrients, Inc. U.S.A. All milk fat preparations were provided by the NZ Dairy Research Institute (NZ DRI). The fatty acid compositions of the milk fat are shown in Table 4-2. The actual CLA levels calculated for each diet are shown in Table 4-3. All the diets were fed *ad libitum* to mice. Prepared diets were kept at $4^{o}C$ until being used.

Table 4-1 Diet composition (% diet weight)

Component (%)	Dietary Treatment					
	Control	Milk fat	Fractionated milk fat	Tonalin	Milk fat + Tonalin	CLA-enriched milk fat
SMP	52.63	52.63	52.63	52.63	52.63	52.63
Cellulose	1	1	1	1	1	1
Mineral mix	5	5	5	5	5	5
Vitamin mix	5	5	5	5	5	5
Corn oil	8	0	0	8	0	0
Milk fat	0	8[a]	8[b]	0	8[a]	8[c]
Tonalin	0	0	0	0.2625	0.2625	0
Corn flour	28.37	28.37	28.37	28.1075	28.1075	28.37

a : Ordinary milk fat
b: 1st stage fractionated milk fat
c: 2nd stage fractionated milk fat, CLA enriched to 2.1% in fat.
All milk fat were provided by NZ Dairy Research Institute (NZ DRI)

Table 4-2 Fatty Acid Composition (% fat) of milk fat and Tonalin

Fatty acid	Ordinary milk fat	Fractionated milk fat (1st stage)	CLA enriched milk fat (2nd stage)	Tonalin
C16:0	28.9	18.6	15.3	6.6
C18:0	10.4	7.9	15.6	2.4
C18:1	22.1	31.4	37.5	13.8
C18:2	1.5	1.7	4.4	0.8
C18:2 (conjugated)	1.4	1.5	2.1	74.6

Table 4-3 CLA concentration in each diet (% diet weight)

Diet	CLA level (%)
Control	0
milk fat	0.112
Fractionated milk fat (1^{st} stage)	0.12
Tonalin	0.21
milk fat + Tonalin	0.322
CLA enhanced milk fat (2^{nd} stage)	0.168

4.2.3 Vaccination

ADT (Tetanus vaccine (Tet-Tox) and Diphtheria vaccine), Fluvax vaccine (45μg/per dose) , and poliomyelitis vaccine (Polio, 20μg/per dose) were purchased from CSL Limited, Australia. Cholera Toxin (CT, 10μg/per dose) and Ovalbumin (OV, 1mg/per dose) were purchased from Sigma chemical Co., Australia. Twenty seven μl of a mixture of CT, OV and polio vaccines (80μl of polio vaccine in 1mL CT and OV mixture) was administered orally. Fifty μl of a mixture of Fluvax / ADT (25μl each) was injected subcutaneously into each mouse.

4.2.4 Methods

Most of the methods were the same as **Chapter Three** Section 3.2.4 to 3.2.19.

In addition to Tet Tox, diptheria, Fluvax and Polio vaccines, cholera toxin (CT) and ovalbumin (OV) were also used for immunisation. For ELISA, concentrations/or dilutions of different antigens used for coating plates were: cholera toxin (CT, 10 μg/ml), ovalbumin (OV, 138 μg/ml), Tetanus toxin (Tet-Tox, 1μg/ml), diptheria toxin (10 μg/ml), Fluvax (Flu, 0.9 μg/ml) and Polio vaccine (1/500 dilution). The details of this assay were descried in section **3.2.10**.

4.3 Experimental Design

One hundred and twenty male BALB/c mice (6-8 weeks old) were randomly divided into 6 groups of 20. After acclimisation on milk free pellets for 7 days, animals were fed a skim milk powder (SMP)-based test diet *ad libitum*. There were five treatment diets and one control diet. The control diet contained skim milk powder (SMP) only. The test diets contained ordinary milk fat; fractionated milk fat (1st stage); Tonalin; Ordinary milk fat + Tonalin, and CLA enriched milk fat. Diet details are shown in Tables 4-1, 2,and 3. Animals were fed these diets for 28 days, during which the mice were weighed weekly and food intake was measured once a week.

Mice received both oral and subcutaneous vaccinations. The vaccination schedule is shown in Table 4-4.

Table 4-4 Vaccination Schedule.

Day	Vaccination	Procedure
7	1st	• 27 µl of a mixture of CT, Ovalbumin and Polio vaccine (80µl of polio vaccine in 1mL CT and Ovalbumin vaccine) was administrated orally. • 50 µl of a mixture of ADT and Fluvax vaccines (25µl each) was administrated subcutaneously.
14	2nd	• 25 µl of a mixture of CT and Ovalbumin vaccines was used for oral immunisation.
21	3rd	• 27 µl of a mixture of CT, Ovalbumin and Polio vaccine (80µl of polio vaccine in 1mL CT and Ovalbumin vaccine) was administrated orally. • 50 µl of a mixture of ADT and Fluvax vaccines (25µl each) was administrated

After feeding for four weeks, mice were euthanased by isoflurane overdose. Blood samples were obtained through cardiac puncture to obtain 1 ml of blood. The same animals were then used for harvesting of peritoneal macrophages, spleens, and intestinal contents. Immune function was assessed using the following assays (for method details see section 3.2):

a). Cell mediated immunity

- Cell proliferation (spleen lymphocytes)
- Immunophenotyping assay (whole blood method)
- Cytokine production (IFN-γ, IL-4)

b). Humoral immunity

- Antibody responses to antigen/vaccines (ELISA)
- Lymphocyte proliferation

c). Natural killer cell activity

- Cytotoxicity detection

d). Macrophage function

- Phagocytosis (peritoneal macrophages and peripheral blood)
- Respiratory burst assay
- Nitric oxide assay

4.4 Statistical Analysis

Data were analysed by ANOVA using a General Linear Model (GLM) Procedure of SAS (SAS User's guide, 1990). A split-plot statistical model was used to analyse treatment differences in feed intake and body weight. All other treatment differences were compared using student's t test to identify significant differences, $p < 0.05$ was significant and $p< 0.01$ was highly significant. Values in the text were expressed as mean ± SE (standard error).

4.5 Results

4.5.1 Body weight and food intake

Natural CLA derived from bovine milk fat and synthetic CLA (Tonalin) did not significantly affect body weight change (gain) and food intake during the experimental period (Table 4-5). Feed efficiency (total weight gain/total food consumed) was not significantly influenced by dietary CLA.

Table 4-5 Effect of consumption of diets containing natural and synthetic CLA on growth performance of mice

	Diet					
	Control	Milk fat	Fractionated milk fat	Tonalin	Milk fat +Tonalin	CLA enriched milk fat
Total body weight gain (g)	4.13 ± 0.23	4.51 ± 0.24	4.36 ± 0.19	3.56 ± 0.12	3.52 ± 0.15	3.81 ± 0.14
Total food intake (g)	109.48 ± 1.37	108.79 ± 1.78	108.53 ± 1.92	108.23 ± 1.71	106.02 ± 2.16	109.13 ± 1.32
Feed efficiency	0.038 ± 0.0021	0.042 ± 0.0021	0.041 ± 0.0021	0.031 ± 0.0014	0.031 ± 0.0017	0.035 ± 0.0013

1. Values are means ± SE (n=20).
2. Feed efficiency = gram of body weight gain per gram of food intake

4.5.2 Effects of natural and synthetic CLA on spleen weight and spleen/body weight ratio

CLA derived from bovine milk fat increased spleen weight and spleen/body weight ratio, the effect was significant in mice fed 'Tonalin + milk fat' as compared with mice fed the control diet (Table 4-6). No significant differences were observed among the five treatment groups

(mice fed with milk fat, Fractionated milk fat, Tonalin, milk fat + Tonalin and CLA enriched milk fat.

Table 4-6 Effect of natural and synthetic CLA on spleen weight and spleen/body weight ratio

Treatment	Spleen weight (g)	Spleen/body weight ratio
Control	0.097 ± 0.0022^{a}	0.0038 ± 0.00007^{a}
Milk fat	0.102 ± 0.0041^{a}	0.0038 ± 0.00017^{a}
Fractionated milk fat	0.0916 ± 0.0047^{a}	0.0035 ± 0.00018^{a}
Tonalin	0.099 ± 0.0016^{a}	0.0041 ± 0.00011^{ab}
Tonalin + milk fat	0.113 ± 0.0045^{b}	0.0043 ± 0.00015^{b}
CLA enriched milk fat	0.101 ± 0.0034^{a}	0.0038 ± 0.00010^{a}

Values are means ± SE. Within a column, values that do not have a common superscript are significantly different ($p < 0.05$).

4.5.3 The phagocytic activity of peritoneal macrophages

Natural CLA (derived from bovine milk fat) and synthetic CLA (Tonalin) significantly enhanced the phagocytic activity of peritoneal macrophages to *E. coli* ($p < 0.01$) (Figure 4-1). The level of phagocytosis increased by 18% ($p < 0.01$), 16% ($p < 0.01$), 18% ($p < 0.01$), 16% ($p < 0.01$) and 18% ($p < 0.01$) in mice fed 'ordinary milk fat', 'fractionated milk fat' 'Tonalin', 'ordinary milk fat + Tonalin' and 'CLA enriched milk fat' as compared to mice fed the control diet. No significant difference was observed between the treatment groups.

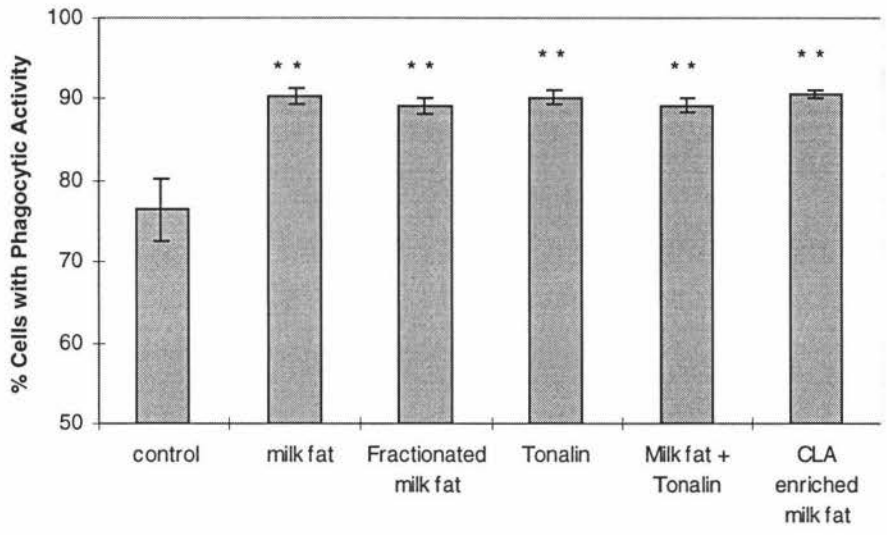

Figure 4-1. Effect of milk fat derived CLA on Phagocytosis (Peritoneal Macrophages). Mean ± SE (n=10). Labels above each bar indicate statistical difference when compared to their respective control: * $p < 0.05$ and * * $p < 0.01$.

4.5.4 The phagocytic activity of peripheral blood leukocytes

CLA enhanced the phagocytic activity of peripheral blood leukocytes to *E. coli* (Figure 4-2). The effect was significant ($p < 0.01$) in mice fed test diets ('ordinary milk fat', 'fractionated milk fat', 'Tonalin', 'ordinary milk fat + Tonalin' and 'CLA enriched milk fat) as compared with the mice fed the control diet. No significant difference was observed between the treatment groups.

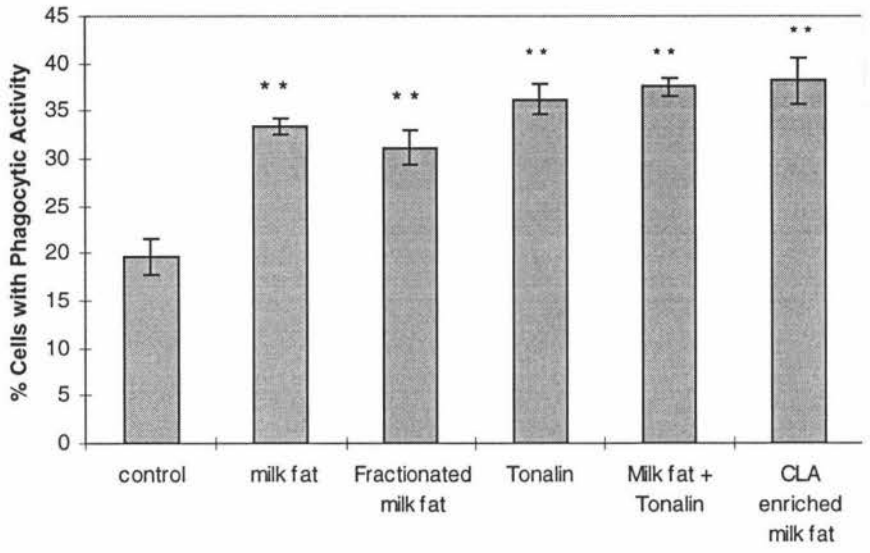

Figure 4-2. Effect of milk fat derived CLA on Phagocytosis (Peripheral blood leukocytes). Mean ± SE (n=10). Labels above each bar indicate statistical difference when compared to their respective control: * $p < 0.05$ and * * $p < 0.01$.

4.5.5 Respiratory Burst

Natural CLA derived from bovine milk fat and synthetic CLA (Tonalin) did not show any effect on respiratory burst. There were no significant differences in the level of respiratory burst activity in any of the treatment groups when compared to the control group (Table 4-7), or between the five treatment groups.

Table 4-7 Effect of natural and synthetic CLA on respiratory burst

Treatment	Respiratory Burst (OD)
Control (SMP)	0.032 ± 0.0018
Milk fat	0.028 ± 0.0020
Fractionated milk fat	0.034 ± 0.0034
Tonalin	0.031 ± 0.0016
Tonalin + milk fat	0.029 ± 0.0021
CLA enriched milk fat	0.032 ± 0.0026

Mean ± SE of (n= 10). No significant difference was found between the control (SMP) and treatment groups.

4.5.6 Nitric Oxide Production

CLA was observed to stimulate nitric oxide production. The nitric oxide production was significantly increased by 79% ($p < 0.05$) and 89% ($p < 0.05$) in mice fed 'fractionated milk fat' and 'ordinary milk fat + Tonalin' as compared to mice fed control diet (Figure 4-3). Other treatment groups also showed increased nitric oxide production but the differences were not significant. No differences were observed between the five treatment groups.

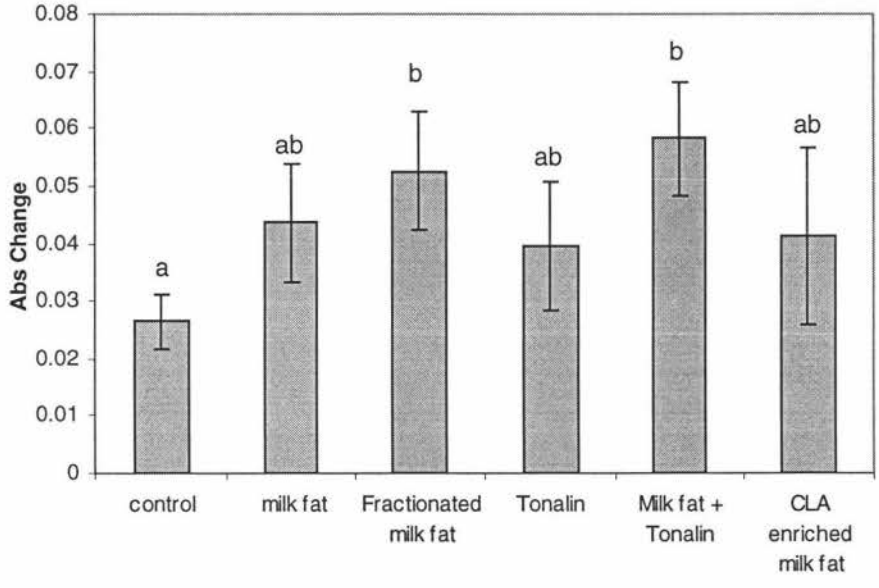

Figure 4-3 Effect of milk fat derived CLA on nitric oxide production. Means ± SE of (n=10). Values without a common letter are significantly different from one another at $p < 0.05$.

4.5.7 NK Cell Activity – Cytotoxicity Detection

There was no significant difference in Natural Killer (NK) cell activity in treatment groups as compared to control group (Figure 4-4). However significant differences were observed between treatment groups ($p < 0.05$). The level of NK cell activity in groups fed 'Tonalin' and 'CLA enriched milk fat' were significantly lower than in the group fed 'ordinary milk fat' ($p < 0.05$). There was no difference between the other treatment groups.

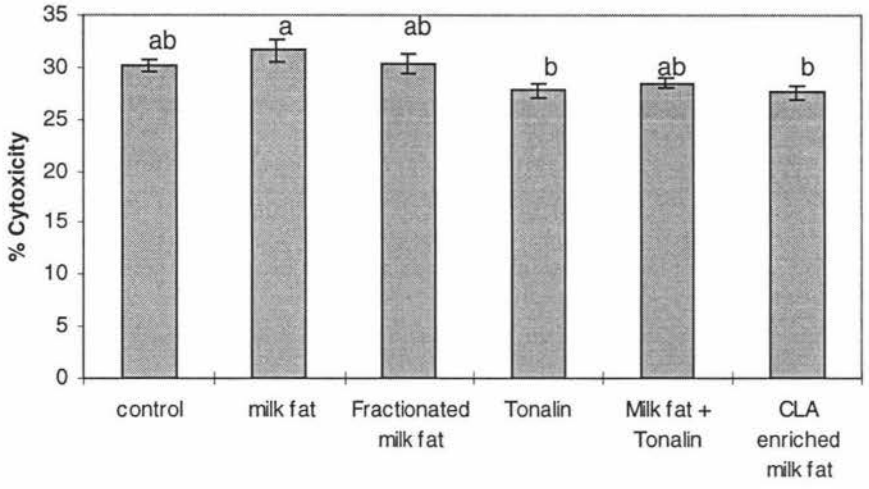

Figure 4-4. Effect of milk fat derived CLA on NK cell activity. Mean ± SE (n=20). Values without a common letter are significantly different from one another at $p < 0.05$.

4.5.8 Mucosal and Systemic Antibody Responses to Antigens / Vaccines

4.5.8.1 Mucosal antibody responses to CT, OV and Polio Vaccine

In response to cholera toxin (CT), a significant increase ($p < 0.05$) in mucosal antibody production was observed in mice fed 'CLA enriched milk fat' when compared to mice fed control diet (Figure 4-5). There was no significant difference between the five treatment groups.

Mucosal antibody responses to Ovalbumin (OV) were enhanced in all the treatment groups (Figure 4-6). Mucosal antibody responses significantly increased by 161% ($p < 0.05$), 190% ($p < 0.05$) and 312% ($p < 0.01$) in mice fed 'fractionated milk fat', 'milk fat + Tonalin' and 'CLA enriched milk fat' respectively as compared to mice fed control diet. No differences were observed between the treatment groups.

In response to Polio vaccine, an increase in mucosal antibody responses was observed in all treatment groups as compared to the control (Figure 4-7), and the differences between all treatment groups and the control were significant ($p < 0.05$). There were no significant differences between the treatment groups.

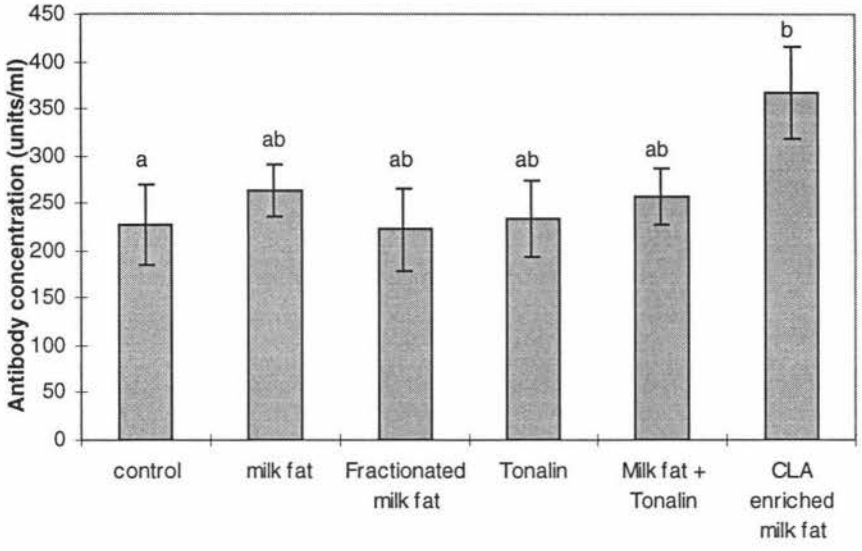

Figure 4-5 Mucosal antibody responses to cholera toxin (CT). Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$.

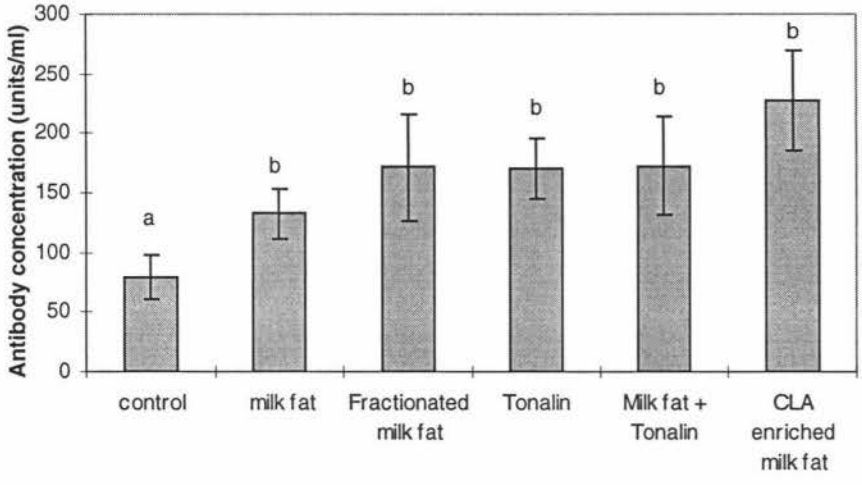

Figure 4-6 Mucosal antibody responses to ovalbumin (OV). Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$

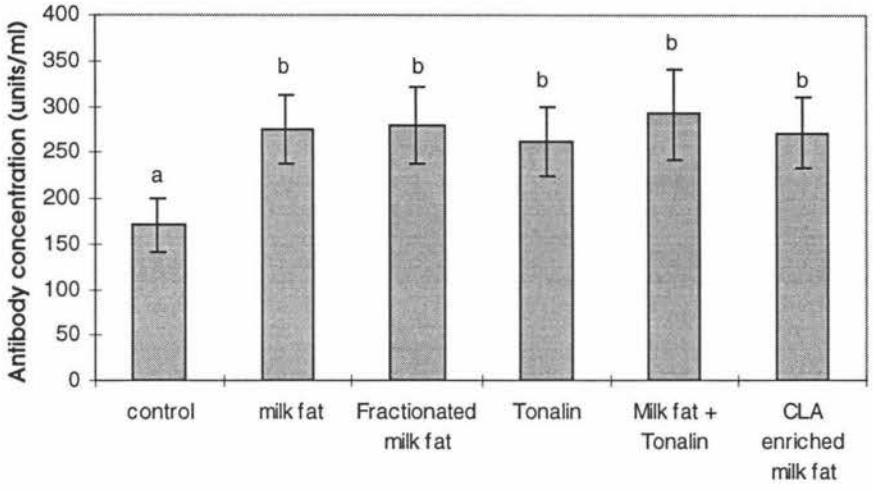

Figure 4-7 Mucosal antibody responses to Polio vaccine. Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$

4.5.8.2 Systemic antibody responses to CT and OV

Serum antibody responses to cholera toxin (CT) were significantly higher in mice fed 'milk fat' as compared to the mice fed the control diet (Figure 4-8). Other treatment groups also showed a slight increase in serum antibody responses to CT, but the differences were not significant. No significant differences were observed between the treatment groups.

All treatment groups were observed to have increased serum antibody responses to ovalbumin (OV) as compared to the control group (Figure 4-9), but the difference was only significant in mice fed 'milk fat' and 'ordinary milk fat + Tonalin' ($p < 0.05$). No differences were found between the treatment groups.

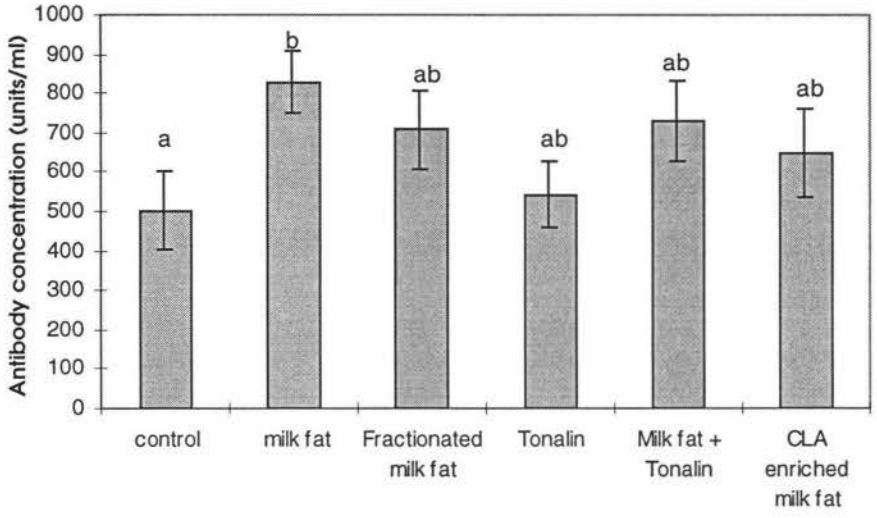

Figure 4-8 Serum antibody responses to cholera toxin (CT). Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$.

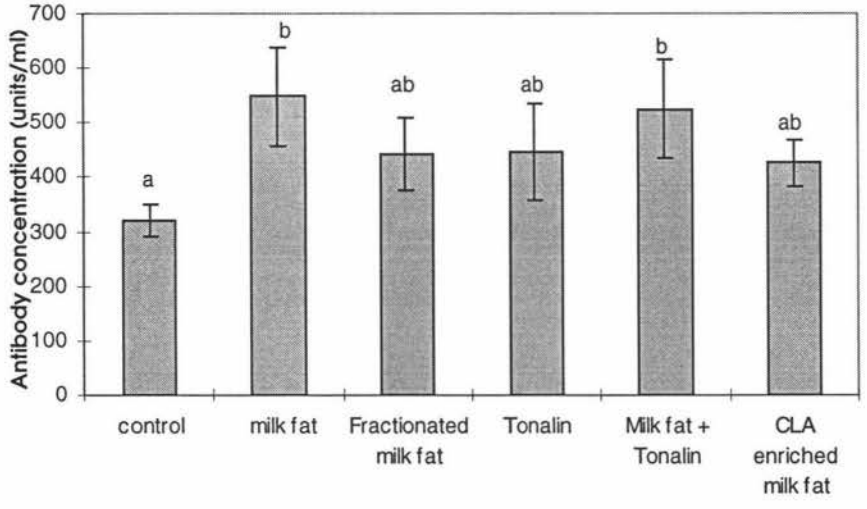

Figure 4-9 Serum antibody responses to ovalbumin (OV). Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$.

4.5.8.3 Serum antibody responses to Fluvax, Diphtheria and Tetanus Toxoid vaccines

Serum antibody responses to Fluvax increased 96% and 100% in mice fed 'fractionated milk fat ' and 'Tonalin' diets as compared to mice fed control diet, and the differences were significant ($p < 0.05$). No significant difference was observed in other treatment groups when

compared to the control groups (Figure 4-10). No significant difference was observed among the treatment groups.

CLA increased serum antibody responses to Diptheria vaccine, and the differences were significant ($p < 0.05$) in mice fed 'milk fat' and 'Tonalin' when compared to mice fed control diet (Figure 4-11). There was no significant difference between the treatment groups.

Milk fat derived CLA and synthetic CLA (Tonalin) were observed to enhance serum antibody responses to Tetanus Toxoid (Tet Tox) vaccine (Figure 4-12). The serum antibody levels to Tet Tox significantly increased 45% ($p < 0.05$) and 78% ($p < 0.01$) in mice fed 'fractionated milk fat' and 'Tonalin' diets as compared to mice fed control diet. Other treatment groups also showed significant increases in serum antibody responses to Tet Tox when compared to the control. There was no significant difference between the treatment groups.

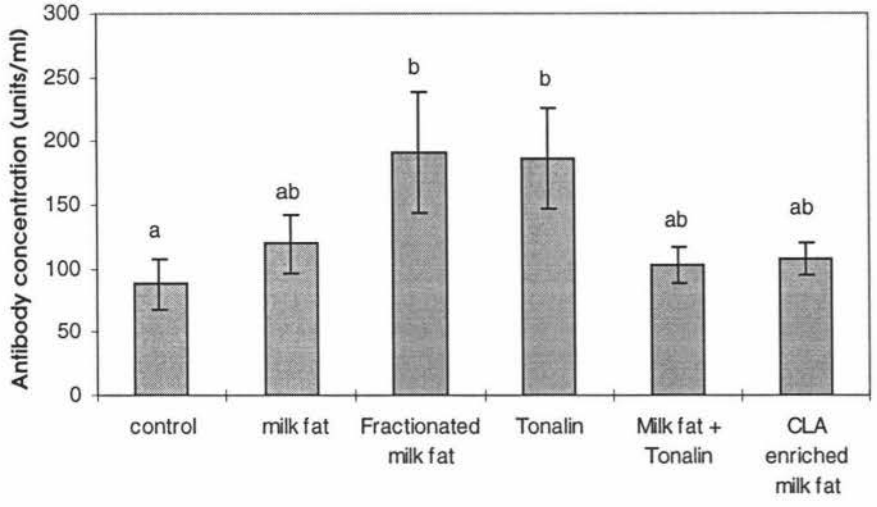

Figure 4-10 Serum antibody responses to Fluvax. Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$

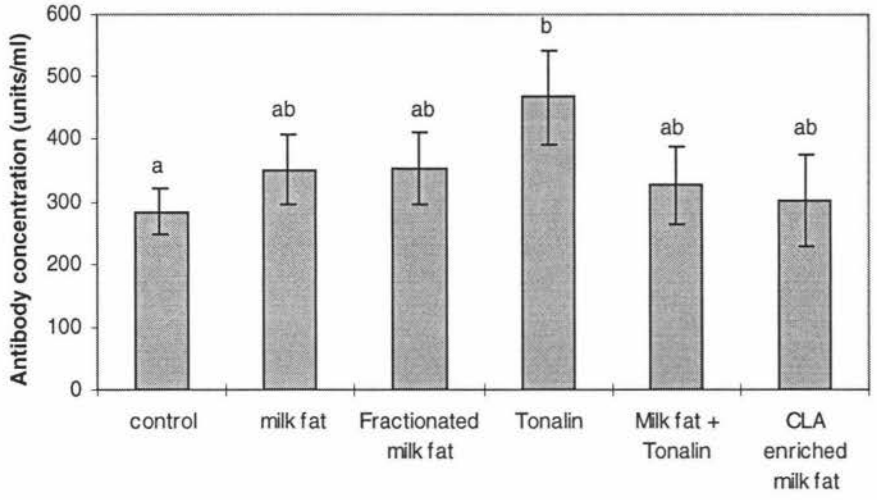

Figure 4-11 Serum antibody responses to Diptheria. Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$

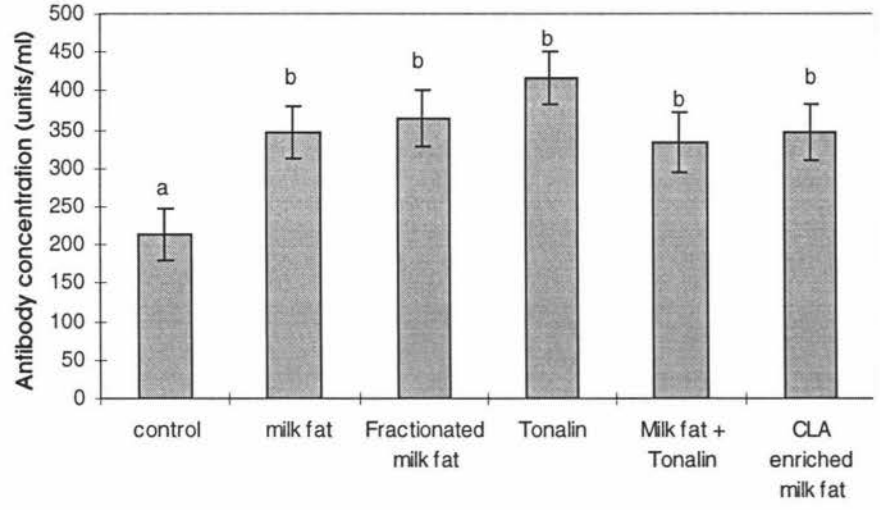

Figure 4-12 Serum antibody responses to Tetanus Toxoid (Tet Tox). Means ± SE (n = 20). Values without a common letter are significantly different from one another at $p < 0.05$

4.5.9. Total leukocytes and total lymphocytes in peripheral blood

Milk fat derived CLA and synthetic CLA (Tonalin) did not have any significant effect on total leukocyte or total lymphocyte numbers in peripheral blood (Table 4-8). There was no significant difference in the number of total leukocytes and lymphocytes in the treatment groups compared to the control group, or between the different treatment groups.

Table 4-8 Effect of natural and synthetic CLA on total lymphocytes and total leukocytes in peripheral blood

Treatment	Total lymphocytes (cells × 10^6 / ml)	Total leukocytes (cells × 10^6 / ml)
Control (SMP)	4.02 ± 0.31	4.20 ± 0.32
Milk fat	4.13 ± 0.28	4.29 ± 0.29
Fractionated milk fat	3.83 ± 0.39	4.10 ± 0.41
Tonalin	3.77 ± 0.29	4.07 ± 0.32
Tonalin + milk fat	3.55 ± 0.40	3.82 ± 0.41
CLA enriched milk fat	3.80 ± 0.52	4.07 ± 0.54

Mean ± SE of (n= 10). No significant difference was found between the control (SMP) and treatment groups.

4.5.10 Expression of peripheral blood leukocyte cell surface markers

Mice treated with different milk fats showed an increase in the number of $CD4^+$ lymphocytes when compared to the control group, however, the increase was only significant in the group fed with 'milk fat + Tonalin' group ($p < 0.05$) (Table 4-9). No significant differences were observed between the treatment groups.
No differences were observed in the number of $CD8^+$ and $CD3^+$ lymphocytes between the different treatment groups and the control.

Natural CLA derived from milk fat significantly enhanced the level of expression of the $CD25^+$ population in peripheral circulation. The level of $CD25^+$ expression significantly increased by 60% ($p < 0.05$) and 63% ($p < 0.05$) in mice fed 'milk fat + Tonalin' and 'CLA enriched milk fat' as compared with mice fed control diet, however, no significant difference was observed between the treatment groups.

Natural CLA and synthetic CLA (Tonalin) were observed to increase the level of $CD40^+$ lymphocytes. The level of $CD40^+$ population increased by 39% ($p < 0.05$) and 45% ($p < 0.05$) in mice fed the 'fractionated milk fat' and 'Tonalin' diets as compared to the mice fed control diet. No significant differences were observed between other treatment groups and the control.

There was a slightly increase in the of $CD4^+$ / $CD8^+$ ratio in mice fed 'milk fat' as compared to mice fed control, however the difference was not significant. No difference was observed between the other treatment groups and the control.

The number of total $CD4^+$ cells increased 13%, 11% and 11% in mice fed 'milk fat', 'milk fat +Tonalin' and 'CLA enriched milk fat' as compared to mice fed with control diet, but the differences were not significant. No differences were observed between the treatment groups.

The number of total $CD40^+$ cells increased 32%, 21% and 9% in mice fed 'milk fat', 'fractionated milk fat' and 'CLA enriched milk fat' when compared to the mice fed with control diet, however the increases were not significant.

Table 4-9 Effect of natural CLA (derived from milk fat) and synthetic CLA on the expression of surface markers on peripheral blood leukocytes

Immune measure	Diet					
	Control	Milk fat	Fractionated milk fat	Tonalin	Milk fat +Tonalin	CLA enriched milk fat
$CD4^+$ (%)	36.04 ± 1.79^{a}	39.49 ± 1.68^{ab}	38.37 ± 3.55^{ab}	36.86 ± 1.87^{ab}	44.47 ± 1.42^{b}	41.89 ± 1.94^{ab}
$CD25^+$ (%)	2.86 ± 0.17^{a}	4.05 ± 0.40^{b}	3.94 ± 0.57^{b}	3.20 ± 0.17^{ab}	4.69 ± 0.84^{bc}	4.68 ± 0.83^{bc}
$CD8^+$ (%)	11.85 ± 0.92	11.52 ± 0.56	13.40 ± 1.30	13.95 ± 1.65	17.59 ± 2.68	14.60 ± 1.38
$CD3^+$ (%)	48.50 ± 2.59	50.55 ± 2.66	49.65 ± 3.51	47.75 ± 2.02	55.14 ± 2.09	55.88 ± 2.92
$CD40^+$ (%)	24.23 ± 3.00	31.68 ± 1.85	33.81 ± 3.39	35.15 ± 2.74	26.79 ± 1.57	29.00 ± 2.07
CD4 :CD8	3.14 ± 0.16	3.52 ± 0.18	2.93 ± 0.23	2.88 ± 0.25	2.94 ± 0.27	3.09 ± 0.31
Total $CD4^+$ (cells x 10^6)	143.59 ± 10.08	162.79 ± 11.46	151.65 ± 22.87	139.14 ± 12.74	160.05 ± 18.79	159.86 ± 23.28
Total $CD40^+$ (cells x 10^6)	100.10 ± 16.34	132.72 ± 13.63	121.83 ± 11.50	133.43 ± 14.54	94.22 ± 12.12	109.94 ± 15.66

Mean ± SE of (n= 10).
Within a row, values without a common superscript (a, b, c) letter are significantly different at $p < 0.05$

4.5.11 Cell Proliferative Responses to T and B Cell Mitogens

The same three mitogens, T cells mitogens phytohemagglutinin (PHA), concanavalin A (Con A) and B cell mitogen *E. coli* lipopolysaccharide (LPS) were used in this experimentation.

4.5.11.1 Cell Proliferation Response to PHA

Natural CLA derived from milk fat and synthetic CLA was observed to enhance PHA-induced lymphocyte blastogenesis (Figure 4-13). The lymphocyte proliferation increased 17% ($p < 0.05$) and 33% ($p < 0.01$) in mice fed fractionated milk fat with enriched CLA and ordinary milk fat, respectively as compared to the mice fed skim milk powder (control) only. No significant differences were found between the treatment groups (mice fed milk fat, Fractionated milk fat, Tonalin, milk fat + Tonalin and CLA enriched milk fat).

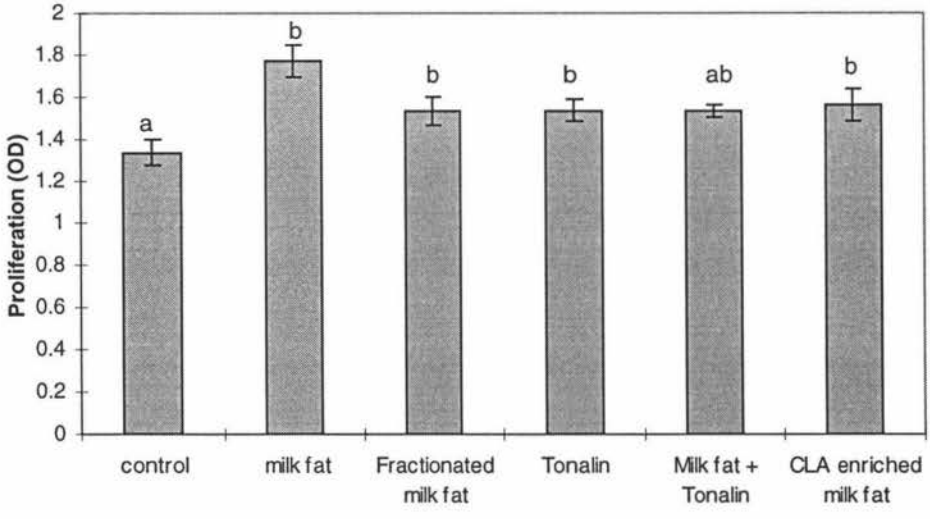

Figure 4-13. Effect of milk fat derived CLA on splenic lymphocyte proliferation induced by PHA (phytohemagglutin). Means ± SE (n = 10). Values without a common letter are significantly different from one another at $p < 0.05$

4.5.11.2 Proliferation Response to Con A

Con A-induced lymphocyte blastogenesis was significantly enhanced ($p < 0.05$) in mice fed natural CLA derived from milk fat and synthetic CLA (Tonalin) compared to the control mice (Figure 4-14). Lymphocyte proliferation increased 20% ($p < 0.05$) and 26% ($p < 0.01$) in mice fed milk fat and Tonalin diets, as compared to mice fed the control diet. No differences were observed between these treatment groups.

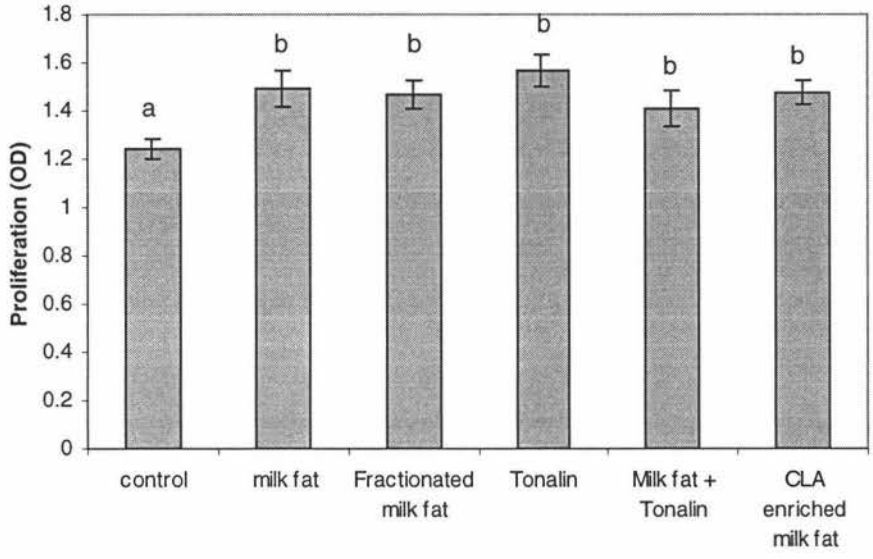

Figure 4-14. Effect of milk fat derived CLA on splenic lymphocyte proliferation induced by Con A (Concanavalin A). Means ± SE (n = 10). Values without a common letter are significantly different at $p < 0.05$

4.5.11.3 Proliferation Response to LPS

Neither natural CLA (derived from milk fat) nor synthetic CLA (Tonalin) had any effects on LPS-induced lymphocyte blastogenesis (Figure 4-15). There were no significant differences in treatment groups when compared to the control. No significant differences were observed between the treatment groups.

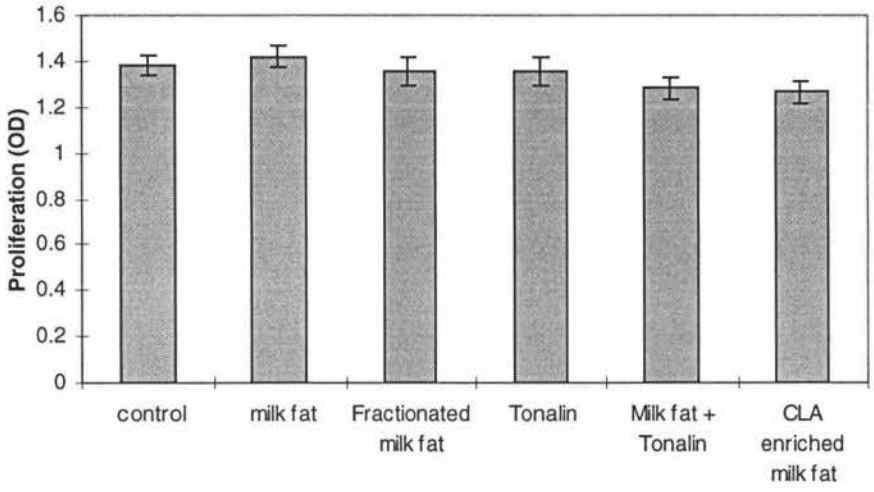

Figure 4-15. Effect of milk fat derived CLA on splenic lymphocyte proliferation induced by LPS (*E.coli* lipopolysaccharide). Means ± SE (n = 10). No differences were found between the treatment groups and their respective control.

4.5.12 Interferon-γ (IFN-γ) Production and Interleukin-4 (IL-4) Production

Milk fat derived CLA and synthetic CLA (Tonalin) were observed to enhance IFN-γ production (Table 4-10). The level of IFN-γ production increased by 46%, 54% and 104% in mice fed 'Tonalin', 'CLA enriched milk fat' and 'milk fat + Tonalin', respectively as compared to mice fed with the control diet, although the increase was only significant ($p < 0.01$) in mice fed 'milk fat + Tonalin'. The levels of IFN-γ production were significant higher in mice fed 'milk fat + Tonalin' as compared to mice fed 'milk fat' and 'fractionated milk fat' ($p < 0.05$).

A trend of reduction for IL-4 production was observed. The number of IL-4 production decreased 8% and 25% in mice fed 'milk fat + Tonalin' and 'CLA enriched milk fat' as compared to mice fed control diet (Table 4-10), but the differences were not significant. No differences were observed between the treatment groups.

Table 4-10 Effect of synthetic CLA on interferon-γ (IFN-γ) production and interleukin-4 (IL-4) production

Treatment	IFN-γ production (unit)	IL-4 production (unit)
Control	50.91 ±4.64[a]	78.33 ±17.40
milk fat	54.56 ±3.67[a]	85.20 ±19.57
Fractionated milk fat	53.39 ±5.10[a]	82.49 ±19.11
Tonalin	73.96 ±11.67[ab]	52.27 ±7.93
milk fat + Tonalin	103.84 ±18.47[b]	72.18 ±10.81
CLA enhanced milk fat	77.35 ±11.67[ab]	59.18 ±10.60

Means ± SE (n= 10).

Within a column, values without a common superscript (a, b, c) letter are significantly different at $p < 0.05$

4.6 Discussion

In the present study, the effect of bovine milk fat derived CLA on immune function was investigated in mice.

CLA is an intermediary product of ruminal biohydrogenation of polyunsaturated fatty acids. CLA is found predominately in food products from ruminant animals; dairy products are the major source in the human diet. CLA is formed as a result of incomplete biohydrogenation of dietary fatty acids in the rumen (Kelly *et al*, 1998). Typical concentrations of CLA in overseas milk fat are 3-6 mg/g of fat, but the levels of CLA in milk can vary widely during different seasons (Kelly and Bauman 1996). In the present study, all milk fats were extracted from New Zealand milk by New Zealand Dairy Research Institute (NZ DRI). MacGibbon

and Hill (1998) reported that the CLA content of milk fat in New Zealand is 7-15 mg/g of fat, with the mid-spring and mid-autumn values of CLA in milk fat tending to be higher than those found in the summer, because in these seasons cows can be fully pasture fed. The main New Zealand dairy season is from September (spring) to April (autumn). The present trial used current season (spring) milk. The milk fat used in this experiment contained 1.4% CLA/g fat.

The results from this study indicate that CLA from milk fat enhances a range of immune functions in mice. This was shown by increased T lymphocyte proliferation (induced by mitogens PHA and Con A), enhanced phagocytic activity, and increased mucosal and systemic antibody responses to vaccines.

It was observed that natural CLA derived from milk fat and synthetic CLA (Tonalin) did not affect weekly change in body weight and feed intake (4-5). Although each mouse was vaccinated (using vaccines Tet Tox, Fluvax, Cholera toxin, Polio and Ovalbumin) for three times (once a week), no reduction of body weight was observed. The present results were consistent with the results in previous chapter and other studies (Sugano *et al.*, 1997; Wong *et al.*, 1998). Similar studies on the natural CLA derived from milk fat are not available. However Klasing *et al.* (1987) reported that endotoxin (LPS) injection caused weight loss in chicks. Endotoxin served as an immune stimulant. The weight loss was the result of increased catabolic responses induced by immune stimulation (Klasing *et al.*, 1987). Body weight loss usually occurred within 24 hours of the endotoxin injection (Miller *et al.*, 1994). Return to pre-injection body weight occurred in 72 hours post endotoxin injection. Cook *et al.* (1993) and Miller *et al.* (1994) both reported that synthetic CLA reduces the catabolic response induced by immune stimulation (LPS injection) in mice, rats and chicken without adversely affecting immune function. The discrepancy may be due to differences of immune stimulant used in the studies and different observing frequency.

The natural CLA from milk fat and synthetic CLA (Tonalin) were both observed to increase PHA and Con A-induced T lymphocyte proliferation, but neither showed any effect on LPS-induced B lymphocyte blastogenesis. The effect of synthetic CLA (Tonalin) has been

discussed in the previous chapter. The CLA content from milk fat is only 0.112%, while in the Tonalin group, the CLA level is 0.21%. It seems that the natural CLA in low concentration could shows strong biological activities, such as mitogen-induced lymphocyte blastogenesis. The reason for this phenomenon is not clear. One possibility may be the different value of *c*9, *t*11 isomers in these two kinds of CLA. In milk fat, more than 90% of the CLA is in this form (*c*9, *t*11)(Chin *et al*, 1992). However, only 37.1% of CLA in Tonalin is in the form *c*9, *t*11. The *c*9, *t*11 CLA isomer is believed to be the most active form because apparently only this isomer is incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers (Ha *et al*, 1990). A number of reports showed that there was an association between this isomer (*c*9, *t*11) and several important biological activities including anticarcinogenesis (Ip *et al*, 1991; 1996; Belury *et al*, 1996), antiatherogenesis (Nicolosi *et al*, 1997), and anabolism (Miller *et al*, 1994). Until now, the effect of natural CLA on immune function has not been previously reported. Although the CLA content in two of the treatment groups (fractionated milk fat and CLA enriched milk fat) is higher than that of ordinary milk fat, no further stimulation of lymphocyte blastogenesis was evident. The fact may indicate a limiting step in converting natural CLA to some active products that are essential to stimulate immune function, or there is something else in the milk fat that has the stimulating effect on immune function.

It was observed that natural CLA derived from milk fat significantly increased the expression of the $CD25^+$ lymphocyte subset population in peripheral blood (Table 4-9), while synthetic CLA did not have this effect. However, the two types of CLA did not show any effect on other lymphocyte subsets, such as $CD4^+$, $CD8^+$, $CD3^+$, and $CD40^+$. The ratio of $CD4^+$ to $CD8^+$ was not influenced by supplementation with CLA. $CD25^+$ is a marker of interleukin 2 (IL-2) receptor α chain (Hamblin, 1993). The high affinity IL-2 receptor is formed from two noncovalently linked polypeptides, α and β chain. The α chain of IL-2 receptor contains a smaller intracytoplasmic section (55kDa peptide) and is recognized by the anti-TAC monoclonal antibody and is referred as CD25 (Hamblin, 1993). IL-2 regulates both T and B lymphocyte proliferation in response to antigens, activates macrophages, and stimulates T cells to produce other cytokines. IL-2 interacts with cells by binding to this high affinity receptor (Roitt, 1994). An adequate number of high affinity receptors are mandatory for the

mitogenic action of IL-2 (Roitt, 1994). The numbers of these receptors on the cells increase under the action of IL-2 and of antigen. As antigen is cleared, the receptor numbers decline and, with that, the cellular responsiveness to IL-2. The increased CD25 population in peripheral blood may indicate that the numbers of IL-2 receptor may increase and IL-2 production may be enhanced. Enhanced IL-2 production may stimulate T cell proliferation and B lymphocyte growth and differentiation. This is consistent with the present finding that T lymphocyte blastogenesis was promoted by CLA.

The natural CLA from bovine milk fat and synthetic CLA (Tonalin) both significantly increased mucosal and serum antibody responses to different antigens/vaccines, including Ovalbumin, Cholera toxin, Polio vaccine, Fluvax and Tetanus Toxin. Whether they are using the same pathway to promote antibody responses is not sure. For natural CLA derived from milk fat, there might be an association with IL-2 production. It is well known that proliferation and maturation of B cell responses are mediated by cytokines, including IL-2, IL-4, IL-5 and IFN-γ (Roitt, 1994). In the present study, CLA was observed to increase antibody responses, but did not influence B lymphocyte blastogenesis, and there was no change in the total numbers of B cells (as indicated by the total $CD40^+$ number). These results seem contradicting. A possible explanation is that CLA increases IL-2 production, IL-2 might stimulate B cell maturation only and thus increase the frequency at which B cells proliferate into plasma cells. Increased numbers of plasma cells will secret more antibodies (immunoglobulins). Therefore, the antibody responses are increased, while the number of B cells is not affected.

The natural CLA derived from milk fat was observed to enhance IFN-γ production, but did not show any effect on interleukin 4 (IL-4) production (Table 4-10). The level of IFN-γ enhancement was significantly enhanced in the group that milk fat and Tonalin were presented together as compared to the control group. Probably there was an interaction between natural CLA derived from milk fat and synthetic CLA from Tonalin, which brought a combination effect in increasing IFN-γ production. IFN-γ counteracts the effects of IL-4 on B lymphocyte and thus inhibits the activity of T_{H2} cells. IFN-γ is produced by T lymphocytes from blood and lymphoid tissues upon stimulation with specific antigens, mitogens, or

alloantigens (Hamblin, 1993). Both CD4+ and CD8+ T lymphocytes and NK cells can produce IFN-γ. The mechanism for the increase of IFN-γ by natural CLA derived from milk fat is uncertain. Several possibilities may account for this. One possibility is that natural CLA first stimulates T lymphocyte blastogenesis and promotes CD4+ and CD8+ activity. IFN-γ production may thus be improved by the enhancing CD4+ and CD8+ T lymphocytes. Another possibility is that natural CLA derived from milk fat may increase the number of IL-2 receptors and thus increase the activity of IL-2. IL-2 may stimulate T cells to produce other cytokines including IFN-γ. Another explanation may be that other milk fat components, such as spingomyelin, butyric acid, may have some effect in enchancing IFN-γ production.

Although there was no difference in natural killer (NK) cell activity in treatment groups as compared with the control, the level of NK cell activity was significantly lower in the group fed 'Tonalin' only when compared with the group fed 'ordinary milk fat'. One possibility is that natural CLA is more active than synthetic CLA in stimulating NK cell activity. It is possible that natural CLA derived from milk fat has a stronger activity than synthetic CLA in suppressing eicosanoid synthesis, thus reducing LTB_4 and PGE_2 production. It is well documented that these eicosanoids show strong effects in repressing NK cell activity (Hwang, 1989). Another possible explanation is associated with the IL-2 activity. Natural CLA derived from milk fat stimulates IL-2 production and IL-2 can feed back and activate NK cells.

It was noticed that natural CLA derived from milk fat and synthetic CLA (Tonalin) significantly enhanced macrophage phagocytic activity. Modulation of macrophage activity could, in turn, affect immune responsiveness because of this cell's antigen-presenting role. The biochemical mechanisms of the actions of the two types of CLA on macrophages are not fully understood. As discussed in previous chapter, synthetic CLA may increase membrane fluidity and improve receptor-ligand interaction, thus enhancing macrophage activity. Another possibility is that CLA might mediate with the regulation of cytokines, such as IL-2, IFN-γ and possibly TNF-α. IL-2, IFN-γ and TNF-α are produced by activated T lymphocytes. CLA might increase IL-2, IFN-γ and TNF-α production by enhancing T lymphocyte blastogenesis, or CLA could increase the efficacy of lymphocyte membrane

receptors to interact with these cytokines. Both IFN-γ and TNF-α have strong effect in activating macrophages, therefore, macrophage activity such as phagocytosis could have been improved by this mechanism.

4.7 Summary

In summary, natural CLA derived from milk fat was found to have some effects on immune function. The natural CLA can stimulate T lymphocyte blastogenesis, increase antibody responses to vaccines/antigens, increase the population of $CD25^+$ cells in peripheral blood, and enhance macrophage phagocytic activity. Even at low concentration, natural CLA derived from milk fat showed a potent effect in enhancing growth of immune cells and promoting immune activities.

CHAPTER FIVE General Discussion and Conclusion

Conjugated linoleic acid (CLA) is a collective term for positional (positions 9 to 12) and geometrical (*cc*, *ct*, *tc*, and *tt*) isomers of linoleic acid. CLA is present mainly in dairy products and other foods derived from ruminants such as cattle, sheep and goats (animals which have four compartments in their stomachs and which chew the cud) (Ha *et al*, 1989; Chin *et al*, 1992). CLA appears to be a naturally-occurring product of microbial lipid metabolism, it is produced in ruminants as a first intermediate in the biohydrogenation of dietary linoleic acid by a linoleic acid isomerase from the rumen bacteria *Butyrivibrio fibrisolvens* (Kepler and Tore, 1967).

cis–9, *trans*-11 CLA is the main isomer produced by *Butyrivibrio.fibrisolvens* and most of the other rumen microorganisms involved in linoleic acid metabolism, although other CLA isomers including the *trans*-10, *cis*-12 isomer may also be formed (Parodi, 1994). The *cis*-9, *trans*-11 isomer is the most abundant form in milk and dairy products (Chin *et al*, 1992; Parodi, 1977, 1994), representing 92% of the total octadecadienoic acid isomers.

CLA has been associated with diverse biological activities, including anticarcinogenic activity (Belury, 1995; Ha *et al*, 1990; Ip *et al*, 1991, 1994; Parodi, 1994; Shultz *et al*, 1992a and 1992b) and inhibition of development of atherosclerosis in animals (Lee *et al*, 1994; Nicolosi *et al*, 1993). CLA was also reported to be an effective antioxidant with an activity similar to butylated hydroxytoluene and greater than that of vitamin E (Ha *et al.*, 1990; Ip *et al.*, 1991), although some evidence refutes this hypothesis (Van den Berg *et al.*, 1995).

Most of the physiological effects produced by CLA are beneficial to human health. One of the most important is the anticancer activity. Synthetic CLA has been reported to reduce the incidence and severity of papillomas in mice (Ha *et al.*, 1987). CLA reduced forestomach neoplasia when mice were fed with CLA (Ha *et al.*, 1990). CLA also showed a protective effect against 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary adenocarcinomas in rats (Ip *et al.*, 1991). Shultz *et al* (1992 a, b) reported that CLA significantly inhibited the

in vitro growth of human malignant melanoma, colorectal and mammary cancer cells. The inhibition was dose- and time-dependent and CLA was especially inhibitory against mammary cancer cells.

Since the immune system is central to defense against cancer, it is plausible to suggest that the anticancer activity of CLA may be mediated through enhanced immune function. The immune system also plays a very important role in fighting infection. However, systemic studies of the effect of dietary CLA on immune function are not available. Until now, few studies were available describing the effects of CLA on immune function. CLA has been reported to increase concanvalin A (Con A)- and phytohemagglutinin (PHA)-induced lymphocyte proliferation (Michal *et al*, 1992). CLA also protects mice, rats and chickens against the catabolic effects (weight loss) induced by immune stimulation (Cook *et al*, 1993; Miller *et al*, 1994). But the effect of CLA on antibody-mediated (systemic and mucosal) and other aspects of immunity remain to be determined.

CLA is not the only fatty acid known to inhibit carcinogenesis. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are representative of n-3 polyunsaturated fatty acids (PUFA) in fish oil, also fit the category (Cave, 1991). However, CLA differs from fish oil fatty acids distinctly in that fish oil is usually required at levels of approximately 10%, whereas CLA at levels of 1% or less is sufficient to produce a significant protective effect against cancer (Ip *et al*, 1994).

CLA was found to be antihypercholesterolemic and antiatherogenic in rabbits. These effects are also evident when CLA is given at dietary levels of less than 2 percent energy (Lee *et al.*, 1994; Nicolosi *et al.*, 1993).

It seems that CLA is able to exert beneficial effects at a low dietary level, however, will this also be the case for CLA in stimulating of the immune system? Is there a CLA level that has an optimal effect in modulating immune function? It is important to note that CLA employed in studies so far is a synthetic one and therefore the effect of natural CLA on the immune system remains unknown. So there are many uncertainties and questions that lie before us.

Thus this project was carried out with the objective to investigate the immunomodulatory properties of CLA (both synthetic and natural CLA).

The aim of the first trial was to examine the dose effect of synthetic CLA (Tonalin) on immune function in mice. Synthetic CLA was analysed by using GC assay method and results showed that 37.1% of synthetic CLA was in the form of *c*-9, *t*-11 CLA isomer. It is well known that over 90% of natural CLA from milk fat is in this isomer (*c*-9, *t*-11) (Ha *et al.*, 1992). The difference of the two types CLA on immune function will be discussed later.

Results from first trial showed that synthetic CLA enhanced PHA- and Con A-induced T lymphocyte blastogenesis. The effect was significant when CLA was present at three levels (0.25%. 0.5% and 1%), as compared with the control. However synthetic CLA did not stimulate LPS-induced B lymphocyte blastogenesis. The mechanism for this phenomenon is not clear. T lymphocytes are very important in cell-mediated immunity and represent the first response to an antigenic challenge. T cells including T helper cells and cytotoxic T lymphocytes may be activated to kill neoplastic and virus infected cells. Although synthetic CLA stimulated splenic T lymphocyte proliferation, it had no influence on peripheral blood lymphocyte subsets, including $CD4^+$, $CD8^+$, $CD3^+$, $CD25^+$ and $CD40^+$. $CD4^+$ is the marker for T helper cells, and $CD8^+$ is the marker of cytotoxic T lymphocytes. Synthetic CLA had no effect on total leukocytes and total lymphocytes in blood. So, it is hard to put these results together that synthetic CLA increased T lymphocyte blastogenesis, while at the same time did not have any effect on lymphocyte subsets in peripheral blood.

The mucosal and systemic (serum) antibody responses to vaccines /antigens were significantly enhanced by synthetic CLA (Tonalin). For the two vaccines (Polio vaccine and Tetanus Toxoid), the antibody responses were dose-dependent. However, enhancement of serum antibody responses to Fluvax vaccine were only observed in mice fed low levels of CLA (0.1% and 0.25%). Antibodies that are involved in the serum responses are primarily of the IgG and IgM isotypes. The mucosal immune responses are mediated mainly by secretory IgAs (sIgA). These results indicated that synthetic CLA was able to promote IgG, IgM and IgA production.

Synthetic CLA significantly enhanced phagocytic activity of peripheral blood leukocytes and peritoneal macrophages in a dose-dependent manner. Nitric oxide production was also improved by CLA, and a trend of dose-dependent increase was observed when CLA level was below 1%. Nitric oxide production is an index of the cytotoxic activity of macrophages against microorganisms, tumor cells and cellular debris. Respiratory burst, another index of macrophage cytotoxicity, was observed to have no significant difference in mice fed test diets as compared to mice fed the control diet.

Natural killer (NK) cells, which provide an early host response to viral, parasitic, and bacterial infection, are important components of the natural immune system. NK cells have been shown to provide resistance to some of these infections and to play roles in tumour surveillance and in the regulation of hematopoiesis (Meydani *et al.*, 1988). Synthetic CLA was observed to enhance NK cell cytotoxic activity in present study, and the effect was significant in two CLA levels (0.25% and 0.5%) compared to the control.

Two cytokines, gamma interferon (IFN-γ) and interleukin 4 (IL-4), were examined in this study. Synthetic CLA did not show any effect on IFN-γ production and a trend of reduction of IL-4 production was observed but the difference was not significant. IL-4 is a switch factor for IgE synthesis by B lymphocytes and stimulates IgE synthesis. Although IgE levels were not measured in the present study, the concentration of IgE in peripheral circulation was postulated to decrease as the result of the decreased IL-4 production produced by synthetic CLA. The hypothesis was proved in a recent study (Sugano *et al*, 1998), which reported that synthetic CLA decreased IgE level in serum, spleen and MLN lymphocytes of rats. It is well known that food allergy is initiated by the production of allergen-specific IgE. IgA and IgG, both serve as antiallergenic factors. So, by reducing IgE levels, combined with increasing IgA and IgG concentrations, synthetic CLA may exert a special effect in mitigating the food-induced allergic reaction.

The mechanism of how synthetic CLA stimulates and regulates immune function is not clear. Two hypotheses may give an explanation. One is that synthetic CLA modulates eicosanoid

synthesis. Eicosanoid products, such as PGE_2, LT_4, HETE, hold a strong immune regulatory effect on immune cells, such as T lymphocytes, NK cells and macrophages. Thus, the immunomodulatory effect of synthetic CLA was mediated with other substances, in an indirect way. Another hypothesis is that CLA increases membrane fluidity. This would increase the efficiency of immune cells in antigen recognition and improve receptor-ligand interactions, resulting in enhanced immune cell activities, such as enhanced T lymphocyte blastogenesis, phagocytosis and antibody responses. This is a direct way.

Cytokines are a group of signaling molecules involved in cellular communication. In the present study, it seems that the immunomodulatory effect of CLA on immune cells was not mediated through IFN-γ production as expected. It has been suggested that some cytokines, such as tumor necrosis factor alpha (TNF-α), IL-1 and IL-2 hold a key role in explaining the effect of CLA on immune function (Pariza *et al.*, 1996). TNF-α is particularly relevant. TNF-α has two different cellular receptors that occur almost on every cell in the body. TNF-α can be synthesised by cells that are not only part of the immune system, but also other cells such as fat cells and cells in the brain (Pariza *et al.*, 1996). However since TNF-α, IL-1 and IL-2 were not measured in present study, such a hypothesis could only be confirmed in future study.

In general, results from the first trial indicated that synthetic CLA enhances a variety of immune functions in mice. However, it is not known which synthetic level has the optimal effect in modulating immune function. Looking back at the results and observing the effect of different CLA levels on immune function, it can be concluded that synthetic CLA at the 0.25% level is able to enhance a range of immune functions and the enhancing effect in most situations is significant as compared with the control.

The aim of the second trial was to examine the effect of natural CLA derived from milk fat on immune function in mice. Six diets were used in this trial and a skim milk powder based diet was used as a control. The five treatment diets were: three natural CLA diets (CLA levels are 0.112%, 0.12% and 0.168%), one synthetic CLA diet (0.21% CLA), and one diet which was mixed with natural CLA and synthetic CLA (0.322% CLA).

Both natural CLA derived from milk fat and synthetic CLA from Tonalin stimulated PHA- and Con A-induced T lymphocyte proliferation, but did not show any effect on LPS- induced B lymphocytes blastogenesis. CD25 is a marker of interleukin 2 (IL-2) receptor α chain (Hamblin, 1993). Natural CLA derived from milk fat increased $CD25^+$ cell populations in peripheral blood circulation, which may indicate that both the numbers of IL-2 receptors and IL-2 production were increased. The possible mechanism may be the incorporation of natural CLA into lymphocytes and the prolonged activation of protein kinase C (PKC), thus enhancing IL-2 production. Enhanced IL-2 production may stimulate T cell proliferation. This hypothesis is consistent with the finding in this study that T lymphocyte blastogenesis was promoted by CLA.

The vaccines/antigens Ovalbumin, Cholera toxin, Polio vaccine, Fluvax and Tetanus toxin were used in the second trial. Both natural and synthetic CLA were observed to significantly increase mucosal and systemic (serum) antibody responses to these vaccines.

Natural CLA increased IFN-γ production but did not influence IL-4 production. Synthetic CLA did not significantly increase IFN-γ and IL-4 production. In the second trial, IFN-γ production was significantly stimulated in the diet containing both natural and synthetic CLA, compared with the control. This finding indicated a positive interaction between the natural CLA and synthetic CLA, thus having a combined effect in enhancing IFN-γ production.

Both natural and synthetic CLA did not show any significant effect on NK cell activity compared to the control in the second trial. However the level of NK activity was significantly higher in groups fed 'milk fat' than that in groups fed 'Tonalin' only.

The phagocytic activities of peritoneal macrophages and peripheral blood leukocytes were enhanced by both natural and synthetic CLA. Interestingly, the increases in phagocytosis were not dose-dependent. This finding differs from the results from last trial, in which a dose-dependent manner was observed.

Results from the second trial showed that natural CLA in low concentrations (such as 0.112% CLA) expressed strong effects in modulating the activities of immune cells. According to the results from first trial, synthetic CLA at the same level (0.1% CLA) did not show such potent biological activities.

One possible explanation is the big differences in percentage of *c*-9, *t*-11 isomer between the two types of CLA. About 90% of total CLA in milk fat is in the form of *c*-9, *t*-11-isomer, as rumen microorganisms preferentially isomerize *c*-9, *c*-12-octadecadienoic acid to *c*-9, *t*-11-octadecadienoic acid (Hughes, *et al*, 1982). In contrast, the synthetic CLA used here contained eight different isomers, with the *c*-9, *t*-11 isomer accounting for only 37.1% of the total CLA. Since the *c*-9, *t*-11 isomer is the only form incorporated in membrane phospholipids, the c9, t11 isomer is regarded as the most biologically active form of CLA (Ha *et al.*, 1992).

Synthetic CLA in the second trial was found to enhance a range of immune functions, such as T lymphocyte proliferation, phagocytosis and antibody response. This finding is consistent with the results in the first trial.

The results from the two trials presented here suggest that both synthetic and natural CLA are able to enhance a range of immune functions in mice. An optimally functioning immune system plays an important role in protecting against cancers and infectious disease. Milk fat and dairy products containing high levels of CLA, may improve immune performance and enhance resistance to cancers and infectious diseases. It also improves responses to vaccines, which is important in children, the elderly and in malnourished individuals.

In the future, the following studies should be taken:

1) To verify the effect of CLA on the immune system over time. The present study was carried out over 4 weeks and the effects of CLA in the long term are unknown.
2) To examine the immunomodulatory effects of natural CLA derived from milk fat in humans and to determine the optimal dose of CLA for maximal human health.
3) To elucidate the mechanism of how CLA acts on the immune system.
4) To examine the anticarcinogenic effects of natural CLA derived from milk fat.

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