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Fatty Acids and The Immune System: Dose Response Studies With *n*-3 Polyunsaturated Fatty Acids

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Abstract

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By Samantha Kew

Considerable interest in fish oil was initially generated by epidemiological studies in Eskimos showing the beneficial effect of consuming fish and fish oil on coronary heart disease. More recently the long chain *n*-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), found in fish oil have been found to be beneficial in many inflammatory and autoimmune diseases suggesting that they are anti-inflammatory and immunosuppressive. Studies investigating the effects of *n*-3 PUFA on immune function have been conducted in both animals and humans. However, most of the studies in animals have used large amounts of fish oil, such that the dose of *n*-3 PUFA was >20 g/100g of total fatty acids, while in humans doses of *n*-3 PUFA of >2.5 g/day have most frequently been used. This is at least ten-times the habitual intake of these fatty acids in the UK diet. The immunomodulatory effects observed in the studies have often been contradictory. The majority of studies in humans have used capsules as the vehicle for *n*-3 PUFA administration. The situation regarding the immunological effects of α-linolenic acid (α-LNA), the precursor of EPA and DHA, is very unclear. Studies using >10.0 g/day have shown inhibitory effects on the limited number of immune parameters measured. This level is greatly in excess of habitual intake. Thus, studies using more realistic amounts of the *n*-3 PUFA, α-LNA, EPA and DHA are required. The dose-response relationships between fatty acid intake, at these lower levels, and immune function must be addressed. In addition, an understanding of the effects of consumption of these fatty acids as part of the normal human diet, rather than in capsular form would also be advantageous.

The studies in this thesis have involved administration of different doses of EPA and DHA to mice (for 7 days) rats (for 6 weeks), and humans (for 6 months). In the animal studies EPA or DHA was fed at 2.2 or 4.4 g/100g total fatty acids, while in the human study EPA+DHA was supplied at 0.7 g or 1.5 g/day. The human study also included two doses of α-LNA (5.0 g and 10.0 g/day). Several aspects of both innate and acquired immunity were investigated. These include the measurement of lipopolysaccharide- and mitogen-elicited cytokines (TNF-α, IL-6, IL-1β, IL-2, IFN-γ, IL-4, and IL-10), monocyte and neutrophil phagocytic and oxidative burst activity, lymphocyte proliferation and the delayed type hypersensitivity response. In addition, the fatty acid compositions of plasma in both animals and humans, as well as blood mononuclear cells in humans and spleen cells in animals were determined.

There were significant dose-dependent increases in the content of *n*-3 PUFA in rat plasma and rat and mouse spleen cells, when feeding EPA or DHA at 2.2 g/ and 4.4 g/day. There was a significant effect of the position of EPA within dietary triacylglcerol (TAG) on its incorporation into the phospholipid (PL) fraction in murine spleen cells, with a significant increase in EPA content when fed in the *sn*-2 position. In rats this was only detected when the PL molecular species were analysed. In terms of rat lymphocyte functions there was no effect of EPA or DHA feeding on the production of cytokines, while lymphocyte proliferation was dose-dependently decreased when EPA was fed in the *sn*-2 position of dietary TAG compared to the *sn*-1(3) position. In terms of murine macrophage function there was no effect of dietary DHA, while dietary EPA was found to dose-dependently decrease the number of macrophages performing phagocytosis. However, when EPA was fed in the *sn*-2 position there was an increased ability for active macrophages to engulf bacteria, while when fed in the *sn*-1(3) position this was not seen.

There was dose-dependent enrichment of plasma PL in both EPA and DHA after EPA+DHA intervention in humans, while there was maximal enrichment in α-LNA and EPA, but no change in DHA content after intervention with 5.0 g α-LNA /day. There was no change in plasma PL arachidonic acid or α-LNA content in any of the intervention groups. With respect to mononuclear cell PL there was no change in α-LNA content after α-LNA intervention, while EPA content increased to the same extent after the higher doses of both α-LNA and EPA+DHA. DHA content increased after the higher dose of EPA+DHA. There was a significant decrease in AA in all treatment groups but this was not dose-dependent. With respect to immune function there were no significant effects of any of the interventions on the measures of innate and acquired immune function investigated. However, there were significant relationships between the fatty acid composition of mononuclear cell PL and many of the immune cell functions investigated.

It is concluded that while long-chain n-3 PUFA, especially EPA, do possess immunomodulatory effects, modest increases in their consumption by healthy humans do not alter immune function as assessed in this study. This is despite significant changes in immune cell fatty acid composition.

Table of Contents

List of Tables

List of Figures

To

Mum and Dad

Acknowledgements

This thesis is the result of three years spent in Southampton, in a lab I will miss, but have the feeling I will visit again. The work contained in this thesis is the culmination of many hours spent with three people in particular and I would like to thank them before I go any further.

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To my friends Jenbo and Paul, here is to many more evenings drinking!

While to Chrissy, well what can I say, sweets? You are the best, the one and only, and always will be to me.

To my parents, I am a student no longer, and thank you both for making that possible.

Don't Quit

When things go wrong as they sometimes will,

When the road you're trudging seems all up hill,

When funds are low and debts are high,

And instead of a smile you have to sigh,

When care is pressing you down a bit,

Rest in God's love – and never quit.

Life can be strange with its twists and turns,

And many a failed man's turned away

When with God's help he'd have won the day.

Don't give up though the pace seems slow –

For you may suceed with another go –

Success is failure turned inside out,

The silver glint in the cloud of doubt –

You never can tell how close you are -

The goal may be near when it seems so far.

So turn to the Lord when you're hardest hit:

Put your trust in Him – and never quit

Abbreviations

α-LNA	α-Linolenic acid
AA	Arachidonic acid
APC	Antigen presenting cell
ATP	Adenosine triphosphate
B-cell	B-lymphocyte
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
CE	Cholesterol ester
CFU	Colony forming unit
CGMP	Cyclic guanidine monophosphate
CM	Chylomicron
CMR	Chylomicron remnant
Con A	Concanavalin A
COX	Cyclooxygenase
CPT	Carnitine pamitoyl transferase
DAG	Diacylglycerol
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DTH	Delayed type hypersensitivity
EPA	Eicosapentaenoic acid
FABP	Fatty acid binding protein
$FADH_2$	flavin adenine dinucleotide (reduced form)
fMLP	N-formyl-MetLeuPhe
γ-LA	γ- Linolenic acid
HDL	High density lipoprotein
HETE	Hydrooxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acids acid
HSL	Hormone sensitive lipase
ICAM	Intracellular adhesion molecule
IDL	Intermediate density lipoprotein

IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IP_3	1,4,5-trisphosphate
LA	Linoleic acid
LDL	Low density lipoprotein
LOX	Lipooxygenase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LT	Leukotriene
MA	Myristic acid
MAG	Monoacylglyceride
MC	Mononuclear cell
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MUFA	Monounsaturated fatty acid
NADH	Nocotinamide adenine dinucleotide phosphate
NADPH	Nocotinamide adenine dinucleotide phosphate (reduced form)
NEFA	Non-esterified fatty acid
NF	Nuclear factor
NF-AT	Nuclear factor of activated T-lymphocytes
NK	Natural killer cells
OA	Oleic acid
PA	Palmitic acid
PAF	Platelet activating factor
PBMNC	Peripheral blood mononuclear cells
PBL	Peripheral blood lymphocytes
PC	Phosphotidylcholine
PE	Phosphotidylethanolamine
PG	Prostaglandin
PHA	Phytohaemagglutinin
PI	Phosphotidylinositol

PIP_2	Phosphotidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristic 13-aceteate
PS	Phosphotidylserine
PUFA	Polyunsaturated fatty acid
SA	Stearic acid
SFA	Saturated fatty acid
TAG	Triacylglycerol
Tc	Cytotoxic T lymphocyte
TCR	T cell receptor
T-cells	T-lymphocytes
TGF	Transforming growth factor
Th	T Helper lymphocyte
Th1	Th lymphocyte 1
Th2	Th lymphocyte 2
TNF	Tumour necrosis factor
TX	Thromboxane
VLDL	Very low density lipoprotein

CHAPTER 1. INTRODUCTION

In this chapter it is intended to describe the fundamentals of the immune system, the nomenclature of fatty acids and their biochemistry and role in nutrition, the roles of fatty acids in cells and the influence of fatty acids on the immune system. Finally the aims of the studies described in this thesis will be given.

1.1 The Immune System

This section provides an overview of the immune system, its components and the way in which it functions. The literature reviewed in this section is limited due to the enormity and complexity of the subject matter. The reader is referred to several good texts for further information on the immune system (Abbas *et al.,* 1994; Roitt, 1997; Kuby, 1997).

1.1.1 Immunity : An Overview

Infectious diseases occur as a result of the invasion of the body by pathogens. These include viruses, bacteria, fungi and parasitic organisms. Animals have many mechanisms which work co-operatively to defend and protect against such invaders. This protection against infection is termed immunity. There are two main components of immunity: innate immunity and acquired immunity.

Innate Immunity

The innate immune system is the very first line of defence against infection. It includes several physical barriers which extend throughout the body, such as the skin and mucosal membranes. The physiological and nutritional state of the body greatly influences the integrity of these barriers. An array of cells in the blood and tissues also contribute to this type of immunity and these include mononuclear phagocytic cells, granulocytes and natural killer cells. These cells can produce a variety of mediators, such as cytokines and eicosanoids which go on to elicit an inflammatory response. A variety of blood-borne chemicals and molecules, including the

complement system, also contribute to this first line of defence against infection. The innate immune system is non-specific and has no memory of past immune challenges. It has a low activation threshold (Hansson, 1997) and responds rapidly to a variety of stimuli, for example bacterial endotoxins.

Acquired Immunity

Other defence mechanisms involved in immunity are enhanced by exposure to a pathogen, and are specific for it. These mechanisms are part of acquired immunity. Foreign substances and pathogens induce or stimulate the cells of this system, so that with each successive exposure there is an amplified and more rapid response. The ability of the system to 'remember' the source of the stimulation (termed an antigen) aids in the response.

The acquired immune system has evolved to include a number of co-operative interactions between so-called effector cells, such as mononuclear phagocytes and lymphocytes, and cellular mediators. This part of the immune system enhances and complements the actions of the components of the innate immune system. Acquired immunity can be divided into two components: the humoral and cell-mediated immune responses. This division is based upon the main types of cells that are involved and the function of the response. Humoral immunity involves the production and release of antibodies from B-lymphocytes, while the key cell in the cell-mediated immune response is the T-lymphocyte, of which there are several types. When functioning correctly this system has the ability to distinguish between self and non-self. Dysfunction of this system may give rise to an auto-immune disease.

1.1.2 The Cells of the Immune System

All immune cells derive from bone marrow. Upon leaving the marrow they may be found circulating in the blood or lymph, or as scattered cells in nearly all tissues, or as collections of cells within lymphoid organs (thymus, spleen, lymph nodes and gut associated lymphoid tissue). The principal cells of the immune system are dendritic cells, mononuclear phagocytes (monocytes and macrophages), T- and B-

lymphocytes, natural killer cells and granulocytes (neutrophils, eosinophils and basophils). These cells are collectively termed as leukocytes or white blood cells. Their paths of maturation and interrelationship are shown in *Table 1.1*. There are different proportions of these cells in the peripheral blood (*Table 1.1*), and these proportions can differ between species (*Table 1.1*).

Table 1.1. The proportions of leukocytes in peripheral blood from different species

Leukocyte		Human[1]	Mouse[2]	Rat[2]
Neutrophils	(% Leukocytes)	50-70	12-44	9-34
Eosinophils	(% Leukocytes)	0-3	0-5	0-6
Basophils	(% Leukocytes)	0-1	0-1	0-1.5
Monocytes	(% Leukocytes)	1-10	0-15	0-5
Lymphocytes	(% Leukocytes)	20-40	54-85	65-84
T lymphocytes	(% Lymphocytes)	70	-	-
B Lymphocytes	(% Lymphocytes)	10-20	-	-

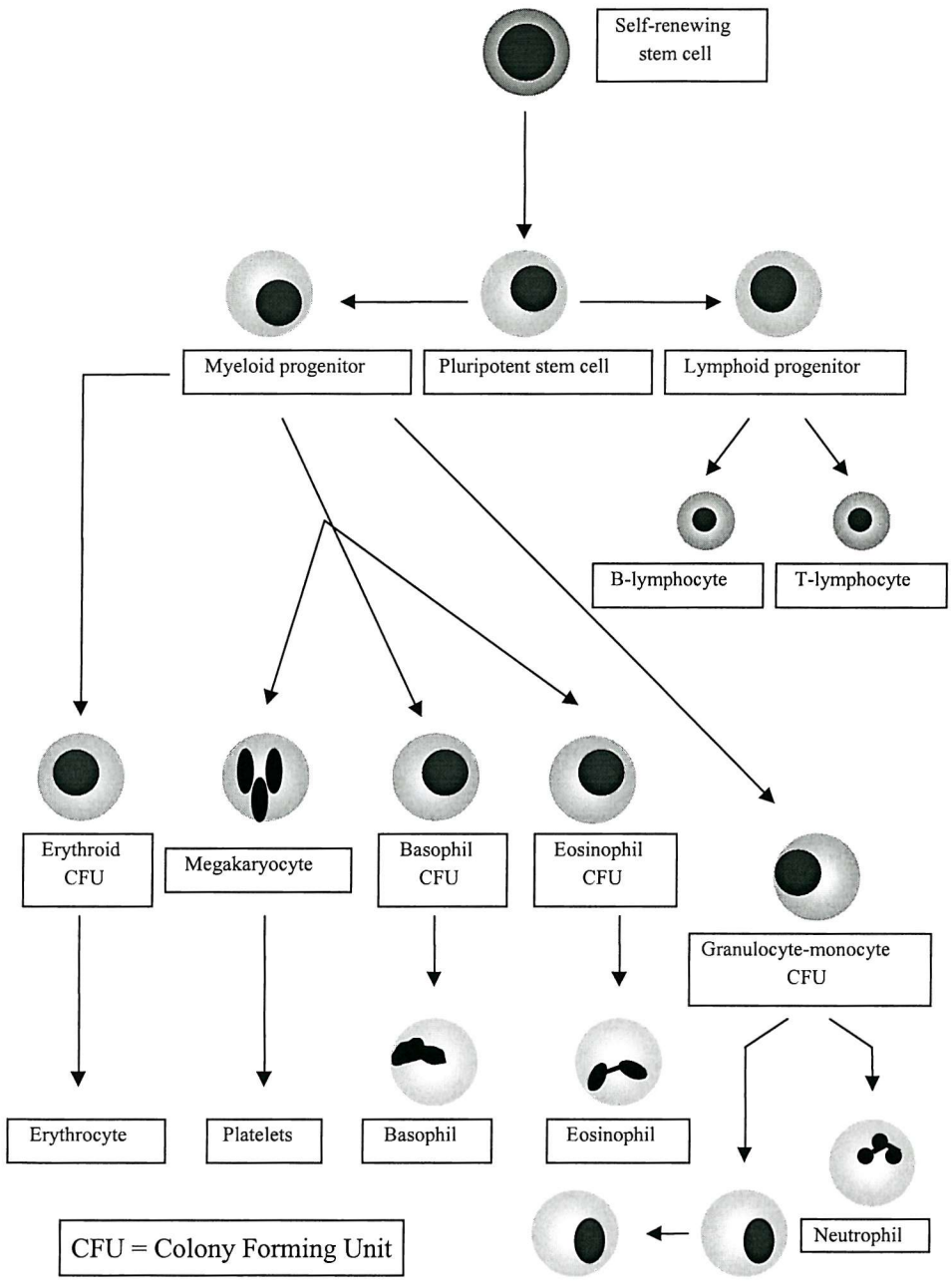
[1]Taken from Abbas *et al.* (1993)
[2]Taken from Dittmer (1961)

1.1.2.1 Mononuclear Phagocytes

The primary function of this group of cells is phagocytosis. These cells develop from bone marrow monoblasts into promonocytes, which then enter the peripheral blood circulation as monocytes, where they remain for between 36 and 104 h (Hughes, 1998). When a monocyte enters the blood it is incompletely differentiated and is 10 to 20 μm in diameter. It has well-developed Golgi apparatus, several lysosomal granules and phagocytic vacuoles and a single-bean shaped nucleus.

When a monocyte settles in a tissue (e.g. liver, lung, and peritoneal cavity) it becomes differentiated and matures into a macrophage. Macrophages are larger than monocytes, being 10 to 80 μm in diameter, and may live for months or even years. Macrophages have an oval-shaped nucleus, dense granules in the cytoplasm as well as endocytic vesicles, mitochondria and lysosomes and have many pseudopodia, which protrude from the cell surface. The tissue location of a macrophage gives rise to a specific name being assigned to it; in the central nervous system for example the macrophage is called a microglial cell, while in the liver it is termed a Kupffer cell.

*Figure 1.1.*The development of immune cells (Adapted from Abbas *et al.*, 1993)



Macrophage Activation

Macrophages are very important as part of the innate immune system and function before and during the development of the acquired immune response. The stimulus to

the differentiation of a monocyte is important to the function of the macrophage which develops. There are several types of mature macrophage. The 'resident' macrophage is a cell with low functional activities as it has not yet encountered a stimulus whether this be endogenous (a dying cell) or exogenous (foreign material) in origin. When a resident macrophage encounters a stimulus it is activated and differentiates further. Resident macrophages which become activated by immunological factors such as lymphocyte-derived cytokines or bacterial products such as endotoxin (lipopolysaccharide) possess microbicidal and tumouricidal activity while those stimulated by non-specific inflammatory factors, termed inflammatory macrophages, have highly secretory activities.

The activation of a macrophage involves an extracellular signal, such as bacterial lipopolysaccharide or a cytokine, such as interferon-γ, binding to a receptor, and the subsequent generation of a second messenger via a transmembrane signal. The second messenger then initiates the covalent modification of intracellular proteins, and the resultant enhanced binding of nuclear regulatory factors leads to the alteration in gene transcription and thus production of certain proteins. It is the modulation of cell products, which results in the alteration in cell function. The process of macrophage activation shares many intracellular signalling pathways with the process of lymphocyte activation (section 1.1.2.2.).

The functions that mononuclear phagocytes perform will now be described in more detail.

Phagocytosis

Phagocytosis is the process by which particles, pathogens (e.g. bacteria) or pathogen-infected cells (e.g. a virus-infected cell) are bound to specific or non-specific receptors on the macrophage plasma membrane and are then engulfed. This is achieved by the formation of a phagocytic vesicle from the macrophage plasma membrane pseudopodia which move to surround the particle.

The binding of particles and pathogens to the macrophage is facilitated by the presence of receptors on the macrophage plasma membrane. These receptors bind the Fc portion of immunoglobulins or the C3 components of complement which coat

foreign particles in a process termed opsonisation. The expression of the receptors for Fc and C3 on the macrophage plasma membrane is influenced by inflammation, during which the number and affinity of these receptors is increased.

Macrophages can also phagocytose non-opsonised material, such as bacteria, dying erythrocytes and yeast. This occurs due to the interaction between sugars or lectins on the particle surface and the receptors and sugars on the macrophage plasma membrane.

Once a phagocytic vesicle is formed and internalised, the membrane of the vesicle fuses with a lysosome. The contents become acidified and portions of the membrane and its associated receptors are recycled back to the cell surface. The acidic nature of the vesicle interior allows the digestion of the phagocytosed material by hydrolytic enzymes supplied by the lysosome.

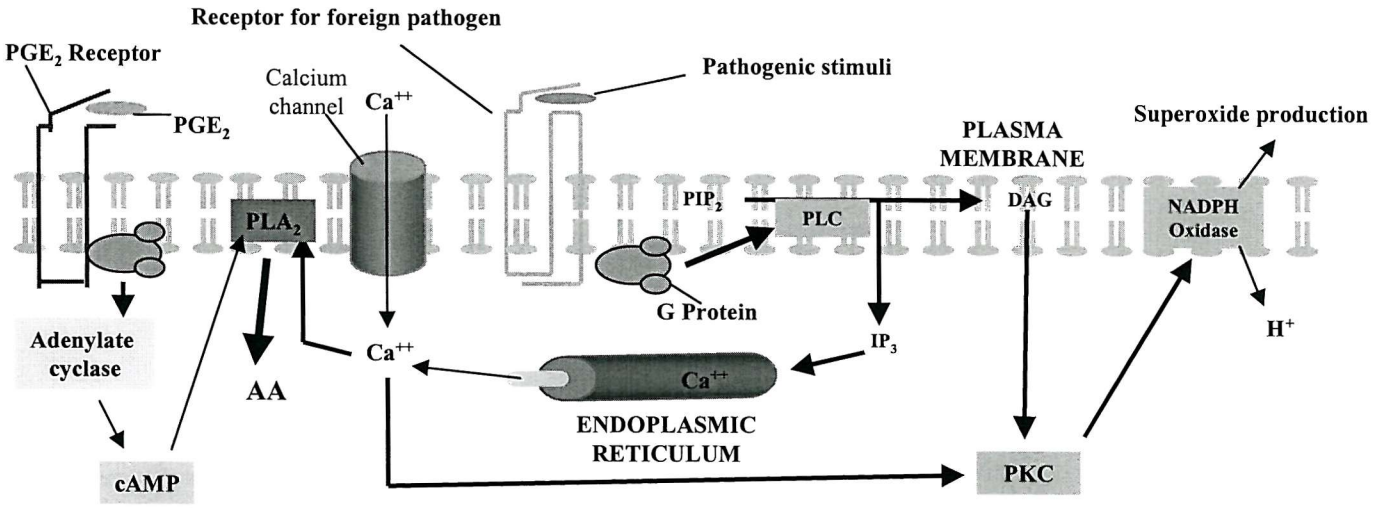
Respiratory Burst

The respiratory burst is the name given to the rapid increase in the production of reactive oxygen species including superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet -}$) and hydrogen peroxide (H_2O_2). These agents form part of the bactericidal and tumouricidal activity of macrophages (and also of neutrophils, see section 1.1.2.5.). The ingestion of particles or the binding of a certain agents by macrophages can lead to a sharp increase in the consumption of oxygen and in the activity of a membrane-associated oxidase that is dependent on NADPH (*Figure 1.2.*). The increase in activity of this enzyme leads to a rise in the production of reactive oxygen species. Although the respiratory burst is intimately connected with phagocytosis, it is not essential for phagocytosis, and can be mounted in response to cytokine stimulation in the absence of phagocytosis. The products of the respiratory burst may be released into both in the vacuolar and extracellular environments. Since reactive oxygen species are damaging to cells, the macrophage must be protected against them. This is achieved by the presence of the enzymes catalase and glutathione peroxidase.

Secretion

Macrophages contain many vacuoles, called lysosomes. These vacuoles contain a number of substances which enable the macrophage to perform its many and varied functions. These substances include enzymes such as lysozyme (which digests bacterial cell walls), neutral proteases such as elastase and collagenase (which break down damaged extracellular matrix), and acid hydrolases such as proteases, lipases and phosphatases (which digest the constituents of the cell membrane). The production and secretion of polypeptide mediators such as interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and bioactive lipids such as the arachidonic acid derivatives (prostaglandins, leukotrienes and thromboxanes), allow interaction between the macrophage, its environment and other immune cells. The production of polypeptide mediators is the result of macrophage activation using similar mechanisms to those of the T-lymphocyte (see *Figure 1.4.)*. The production of lipid mediators is also achieved via receptor stimulation but is the result of the activation of membrane–bound enzymes e.g. PLA_2 (section 1.1.3.2..) as a result of a rise in intracellular Ca^{2+} or cAMP concentrations.

Figure 1.2. The activation of a macrophage to elicit a respiratory burst.



The binding of a foreign pathogen (e.g. an opsonised bacterium) results in the activation of receptor associated G proteins which generates the production of intracellular signals e.g. IP_3 and DAG that activate PKC, both indirectly and directly, respectively. The activation of PKC results in the stimulation of the enzyme NADPH oxidase which then generates the production of reactive oxygen species extracellularly (e.g. superoxide), and hydrogen ions intracellulary. The Na^+/H^+ transport system in the membrane expels this ion appropriately.

DAG, Diacylglycerol; PIP_2, Phosphotidylinositol-4,5-bisphosphate; IP_3, Inositol-1,4,5-trisphosphate; PLC, Phospholipase C; PKC, Protein kinase C; cAMP, cyclic Adenosine monophosphate

Reactive species such as nitric oxide (NO) are also produced by macrophages during an immune response (Snyder and Bredt, 1992). NO has cytotoxic properties and appears to regulate lymphocyte function (Snyder and Bredt, 1992). The presence of enzymes, mediators and of reactive oxygen species allows the macrophage to effectively destroy dead and invading material as discussed above, and allows the regeneration of injured tissue.

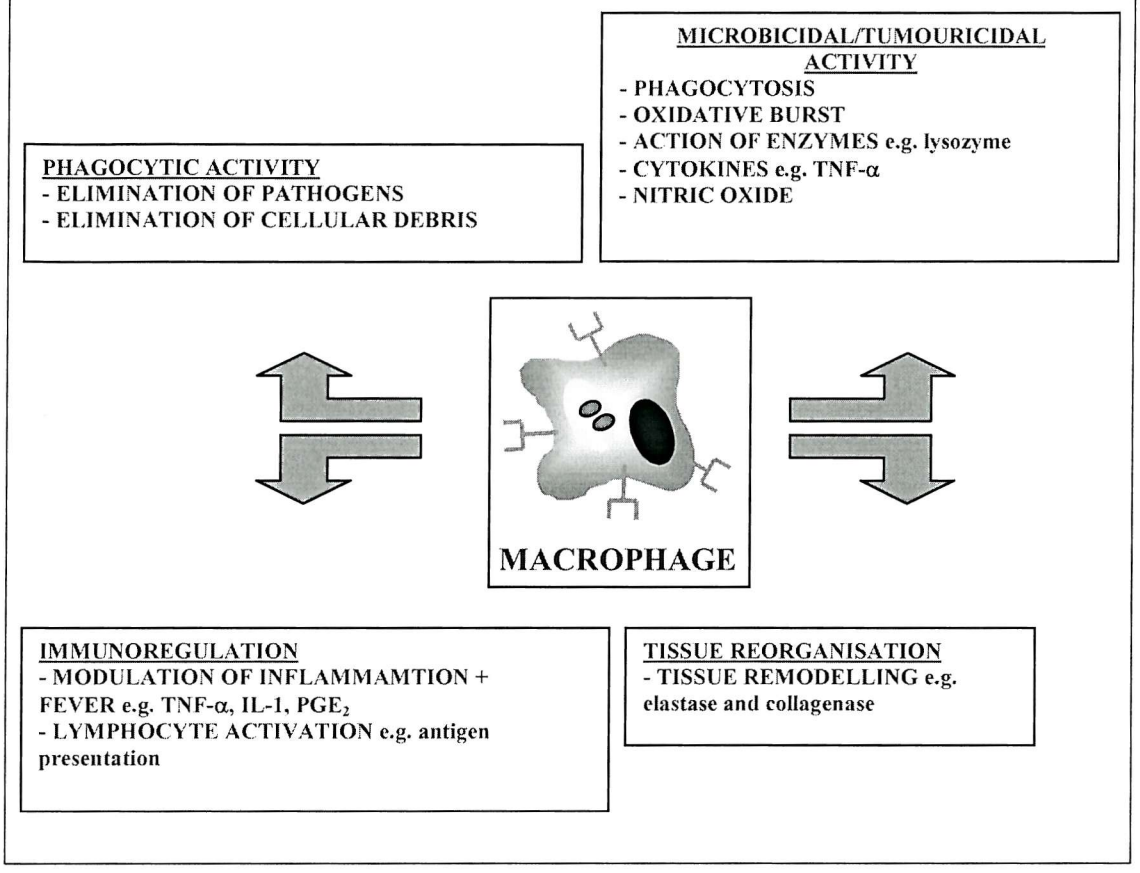
Immunoregulation

The role of the macrophage in the acquired immune system is to function both as an accessory cell (a cell which helps the activity of other cell types) and an effector cell (a cell which carries out a function). Macrophages may act as accessory cells when they act as antigen presenting cells (APC). This function is achieved by the phagocytosis of a pathogen, its digestion and the subsequent expression of antigenic peptides on the macrophage cell surface. Antigen is displayed on certain molecules on the macrophage surface. These are termed the major histocompatibility molecules class I and II (MHC-I and -II) (section 1.1.2.2). Since antigen on these receptors triggers a T-lymphocyte response, macrophages participate in the promotion of T-lymphocyte activation and the secretion of cytokines. In turn these lymphocyte-derived cytokines (e.g. IFN-γ) may positively feed back, such that the macrophage is further activated. In this way macrophages play a role in cell-mediated immunity. Macrophages act as effector cells when they perform processes such as phagocytosis.

Summary of Macrophage Activities

The functions performed by macrophages are essential components of both the innate and the acquired immune responses (*Figure 1.3.*). Macrophages are intimately involved in the defence of the body from various immune challenges and in the clearance of dead cells and invading pathogens and microbes. They are also involved in the subsequent tissue remodelling and wound healing which occurs. Thus the macrophage can be considered an integral part of the innate and acquired immune responses.

Figure 1.3. Functions of the macrophage



1.1.2.2 Lymphocytes

Lymphocytes are the key cells involved in the acquired immune response. Lymphocytes are a heterogeneous population of cells, which vary in diameter from 6 to 20 μm and have large nuclei. The cytoplasm of most lymphocytes, with the exception of the natural killer cell, is devoid of granules. There are several subsets of lymphocytes and they are classified according to the site in the body in which they differentiate further and mature. The precursors to all lymphocytes originate in the bone marrow (*Figure 1.1*). Lymphocyte precursor cells that differentiate in this tissue are termed B-lymphocytes (B-cells). Precursor cells that migrate to the thymus where they subsequently differentiate and mature are called T-lymphocytes (T-cells). The T-lymphocyte population is further sub-divided into functionally mutually exclusive groups. These include the helper T (Th)-cells and the cytotoxic T (Tc)-cells. The

basis of this classification is the surface glycoproteins, called cluster of differentiation (CD) molecules, that the cell possesses, and which act as receptors. T-lymphocytes express the CD3 receptor, while Th-cells also express the CD4 receptor and Tc-cells the CD8 receptor. The CD molecules on the surface of a lymphocyte are intimately involved in the activation of the cell, since these accessory molecules respectively recognise antigen presented by the MHC II and MHC I complexes on an APC and subsequently facilitate the transduction of T-cell receptor (TCR):CD3 mediated signals (Viellette *et al.,* 1988).

T-Lymphocyte Activation

The resting T-lymphocyte which is in G_0 phase of the cell cycle needs to be activated in order to perform its functions. During activation the cell undergoes several changes as it enters the cell cycle: it enlarges, the number of organelles increases, as does the cytoplasm and amount of RNA. The activation process requires the presence of accessory cells (for example the macrophage), which possess co-stimulatory receptors (for example MHC), to bind to the TCR, and to secrete co-stimulatory cytokines (Fernandes *et al.,* 1998).

T-lymphocytes recognise peptide antigens that are presented by an accessory cell (e.g. a macrophage, in its capacity as an APC) via surface receptors (e.g. MHC-II). The TCR is closely associated with the CD3 molecule on the plasma membrane of the T-lymphocyte. These are the key surface molecules involved in recognition of specific antigen by MHC-restricted lymphocytes. There are also a number of other cell surface proteins, called accessory molecules, that participate in the activation of the T-cell (*Figure 1.4.*). These include some CD molecules and other cell surface proteins such as intracellular adhesion molecule-1 (ICAM-1). Accessory molecules serve to strengthen the binding of T-lymphocyte to the accessory cell during this activation process, while others function to transduce signals to the interior of the cell (Veillette et al 1988; Berry and Nishizuka, 1990; Bach, 1990). The T-lymphocyte enters G_1 of the cell cycle and then progresses into the S phase, due to the stimulation of the accessory cell, and increases the expression of a number of co-stimulatory surface molecules, such as adhesion molecules and other CD molecules. In addition to this, an array of cellular mediators, such as cytokines, are produced.

The increase in the expression of surface molecules and production in cellular mediators is due to the generation of intracellular signals, and the subsequent transient changes in the transcription of several genes, the products of which function to promote the differentiation and proliferation of the T-lymphocytes as well as other cell types, including the B-lymphocyte, macrophage and natural killer cell.

Signal Transduction and Intracellular Messengers in T-lymphocyte Activation

The specific events in T-lymphocyte activation are shown in *Figure 1.4.* The TCR:CD3 stimulated series of events involved in signal transduction are numerous, but begin with the activation of phopholipase C (PLC) which acts upon phosphotidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane of the T-lymphocyte. The products of this reaction are inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), and their release leads to an increase in cytoplasmic concentrations of calcium, from both internal stores (endoplasmic reticulum) and external sources (via a plasma membrane calcium channel), and to the phosphorylation of many membrane-bound and cytoplasmic proteins. Enzymes such as PLC can be activated by phosphorylation catalysed by proteins such as tyrosine kinases. The enzyme protein kinase C (PKC) is activated by the increased presence of DAG and calcium in the cytoplasm and this is a very important step in the activation process. PKC activators, such as phorbol esters and calcium ionophores, initiate a similar response, in terms of intracellular events, to that seen when T-lymphocyte-APC binding occurs (Valge *et al.,* 1988; Berry and Nishizuka, 1990). There is considerable evidence for the involvement of PKC and calcium-dependent, cyclic AMP-dependent and cyclic GMP-dependent kinases in the phosphorylation of amino acid residues, such as serine and threonine, on cytoplasmic proteins, and the intracellular domains of CD3 and CD4 and other molecules which are involved in transcriptional activity (Dumont *et al.,* 1989; Berry and Nishizuka, 1990; Rao, 1991).

Another type of enzyme involved in T-lymphocyte activation is calcineurin, a calcium/calmodulin-regulated serine/threonine phosphatase (Crabtree and Clipstone 1992). The activity of this enzyme causes the dephosphorylation of other proteins such as transcription factors, for example the nuclear factor of activated T-cells (NF-

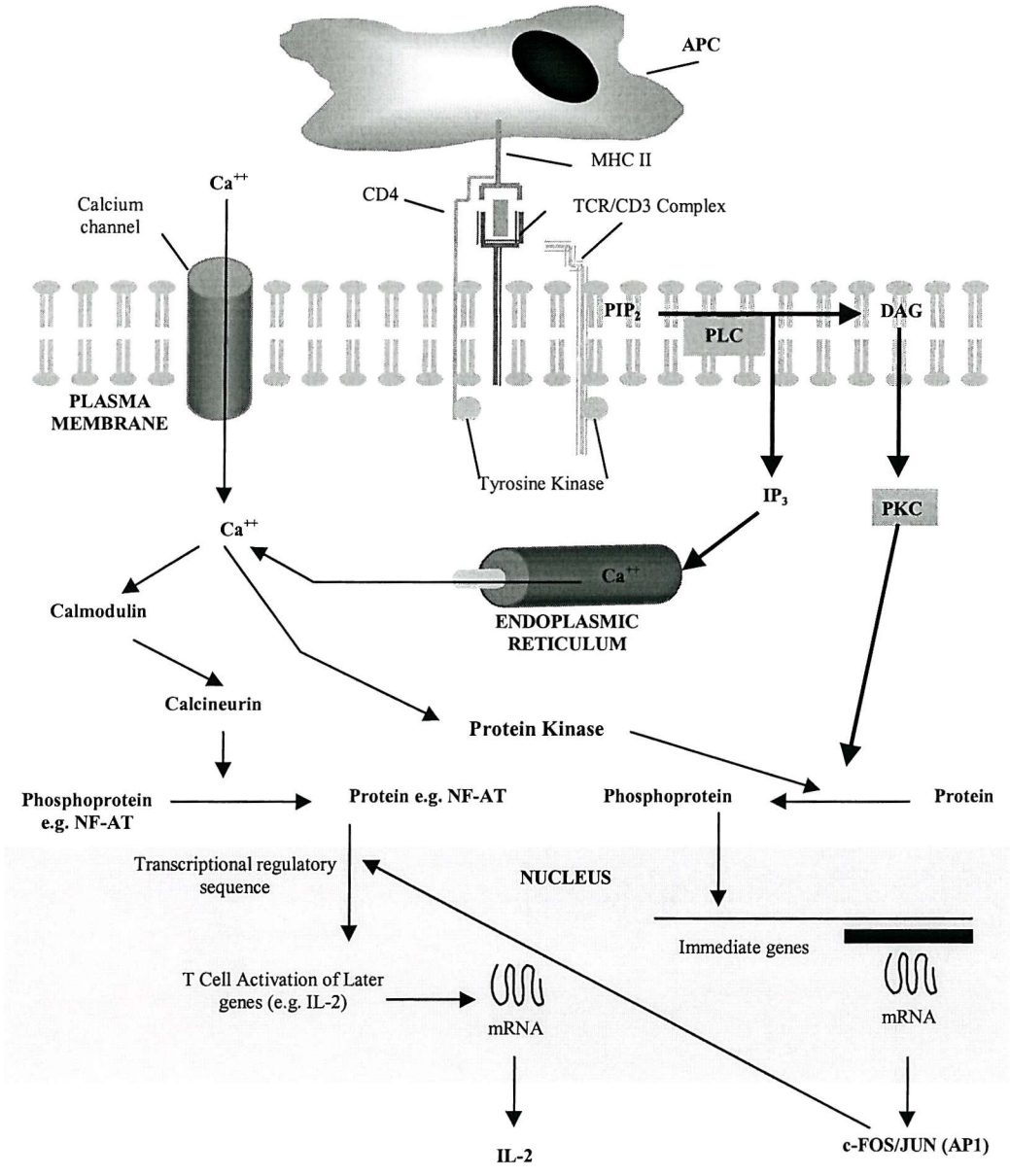
AT), which is than translocated into the nucleus where it binds to specific DNA sequences resulting in the transcription of the adjacent IL-2 gene (Crabtree and Clipstone, 1992). The transcription of immediate genes for the transcription factors c-fos, Jun (AP-1) and c-myc may play a role in the transcriptional regulation of other later genes such as IL-2 and its receptor (Rao, 1991). There are a number of other cellular responses to T-lymphoyte activation. The interior of the cell becomes more alkaline as the exchange of ions via the Na^+/H^+ transporter increases, and there is an increase in the efflux of K^+ via the Na^+/K^+ ATPase (Rao, 1991). There are also changes in membrane function with an increased influx of glucose, amino acids and nucleosides.

Central to the cell-mediated immune response is an increase in the number of T-lymphocytes. This increase in lymphocyte number is termed proliferation and is one of the first responses when T-lymphocyte activation occurs. Proliferation enables the immune system to produce a faster and more efficient response to an immune challenge. The principal cytokine involved in T-lymphocyte proliferation is IL-2.

The Th Lymphocyte

The naïve Th lymphocyte, when it initially encounters an antigen and is activated will secrete the cytokine IL-2. This causes the Th cell to differentiate into a Th0 lymphocyte, which may further differentiate into one of two distinct populations of cells. These Th lymphocytes are termed the Th1 and Th2 lymphocytes. The mode of activation, which is dependent on the cytokines produced in the local environment (for example by the activating accessory cell), will determine the type of Th cell that is formed. A population of T-lymphocytes exposed to an environment with an increased concentration of IL-12 (and IFN-γ) will have an increased proportion of Th1 cells, while a population exposed to a higher concentration of IL-4 will have a greater number of Th2 cells.

Figure 1.4. Intracellular signalling pathways in T-cell activation.



The binding of a peptide-MHC complex to a TCR complex generates intracellular signals (e.g. IP_3, DAG) that transiently increase the transcription of several genes that are quiescent in unstimulated T cells. This in turn leads to the transient production of proteins that are essential for the T cell mitosis and function (e.g. cytokines).
mRNA, messenger RNA; NF-AT, Nuclear factor of activated T-lymphocytes; DAG, diacylglycerol; PIP_2, phosphotidylinositol 4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C.

Th lymphocytes themselves produce a specific profile of cytokines (*Figure 1.5.*), whose function it is to promote the differentiation and proliferation of Th cells and the activity of other cells. The Th1 cells produce IL-2 and IFN-γ, which activate a variety of cells including macrophages, natural killer cells, and Tc cells. These cells are the principal effectors of cell-mediated immunity. The Th2 cells tend to produce cytokines that induce B-lymphocyte differentiation and antibody production. These include cytokines such as IL-4, which stimulates immunoglobulin (Ig)-E production, and IL-5, an eosinophil-activating and monocyte-macrophage activating mediator. In addition to these, IL-13 is also secreted which ultimately results in the appearance of plasma cells (derived from B-lymphocytes) which produce large quantities of IgG antibodies specific for the antigen first presented to the Th2 cell by a macrophage (Mossman and Coffman, 1989) (*Figure 1.5.*).

Unlike the innate and acquired components of the immune system which complement each other in their function and production of mediators, the Th1 and Th2 systems counterbalance each other. The Th1 cells of the immune system predominantly secrete the cytokines IL-2 and IFN-γ, while the Th2 cells secrete IL-4, IL-5 and IL-10. It has been found that IL-10, produced by Th2 cells inhibits the secretion of IFN-γ by Th1 cells (D'Andrea *et al.,* 1993). Similarly IFN-γ inhibits IL-4 and IL-10 production (D'Andrea *et al.,* 1993). Thus, the two systems reciprocally inhibit each other (*Figure 1.5.*). The impact of the cytokines produced by the different Th-cell systems on the other cells of the immune system is such that the immune response is driven in different directions. The Th1 system is more driven to initiate cell-mediated immunity, and so targets the destruction of bacteria, viruses and fungi. In addition, the Th1 cell system is involved in the delayed-type hypersensitivity response and inflammation. The Th2 system is more driven to destroy pathogens such as parasitic worms, and is also involved in allergy and antibody production. The balance between these two systems is very important in some disease states. For example, the Th2 system has been found to be dominant over the Th1 in asthma and other allergic conditions (Panyani *et al.,* 1999), while the Th 1 system is dominant in chronic inflammatory diseases such as rheumatoid arthritis (Romagnani *et al.,* 2000).

1.1.2.3 Natural Killer Cells

Natural killer (NK) cells are also part of the lymphocyte population. They are found in the circulation and in the spleen. NK cells do not undergo thymic maturation. They contain large cytoplasmic granules. They are capable of lysing tumour- or virus-infected cells, and can be regarded as a more primitive form of Tc cells, since they lack a specific TCR for antigen recognition. However, since they are not constrained by MHC molecules and are not antige*n*-specific, their killing is very immediate and targets a broader range of pathogens. This does have a consequence, however, in that their killing is random and not all infected cells are eliminated. It is thought that the purpose of NK cells is to kill virus-infected cells until Tc cells can be activated, which usually occurs in response to cytokines such as IFN-γ, IL-2 and several other cytokines such as IL-12 and IL-4.

NK cells can be activated by the cytokines IL-2, IL-12, IFN-γ and TNF-α. However, the activation of the killing mechanism of an NK cell can be achieved through the recognition, by CD16 on their cell surface, of an aggregated IgG coating on the surface of a target cell. The occupancy of CD16 by IgG activates NK cells to synthesise and secrete TNF-α and IFN-γ. This will also induce the discharge of intracellular granules by exocytosis and induce target cell DNA fragmentation and apoptosis. NK cell granules, like those of Tc cells, contain pore-forming proteins and cytotoxins. NK cells form a part of the innate immune response.

1.1.2.4 B-Lymphocytes

B-lymphocytes play a key role in the functioning of the humoral branch of the acquired immune system. These cells are responsible for the production of antibodies, which function to neutralise and eliminate the antigen that induced their formation. Antibodies (immunoglobulins (Ig)) have their own nomenclature, and the type of antibody produced will depend on the stimulus and anatomical site of the lymphocytes involved. Cytokines determine the types of antibodies produced by B-lymphocytes (Callard, 1989), by selectively promoting Ig heavy-chain class switching and by stimulating B-lymphocyte proliferation. The cytokine profiles which lead to the differential production of antibodies are summarised in *Figure 1.6.*

Figure 1.5. Differentiation and activities of Th lymphocytes (adapted from Calder, 1997).

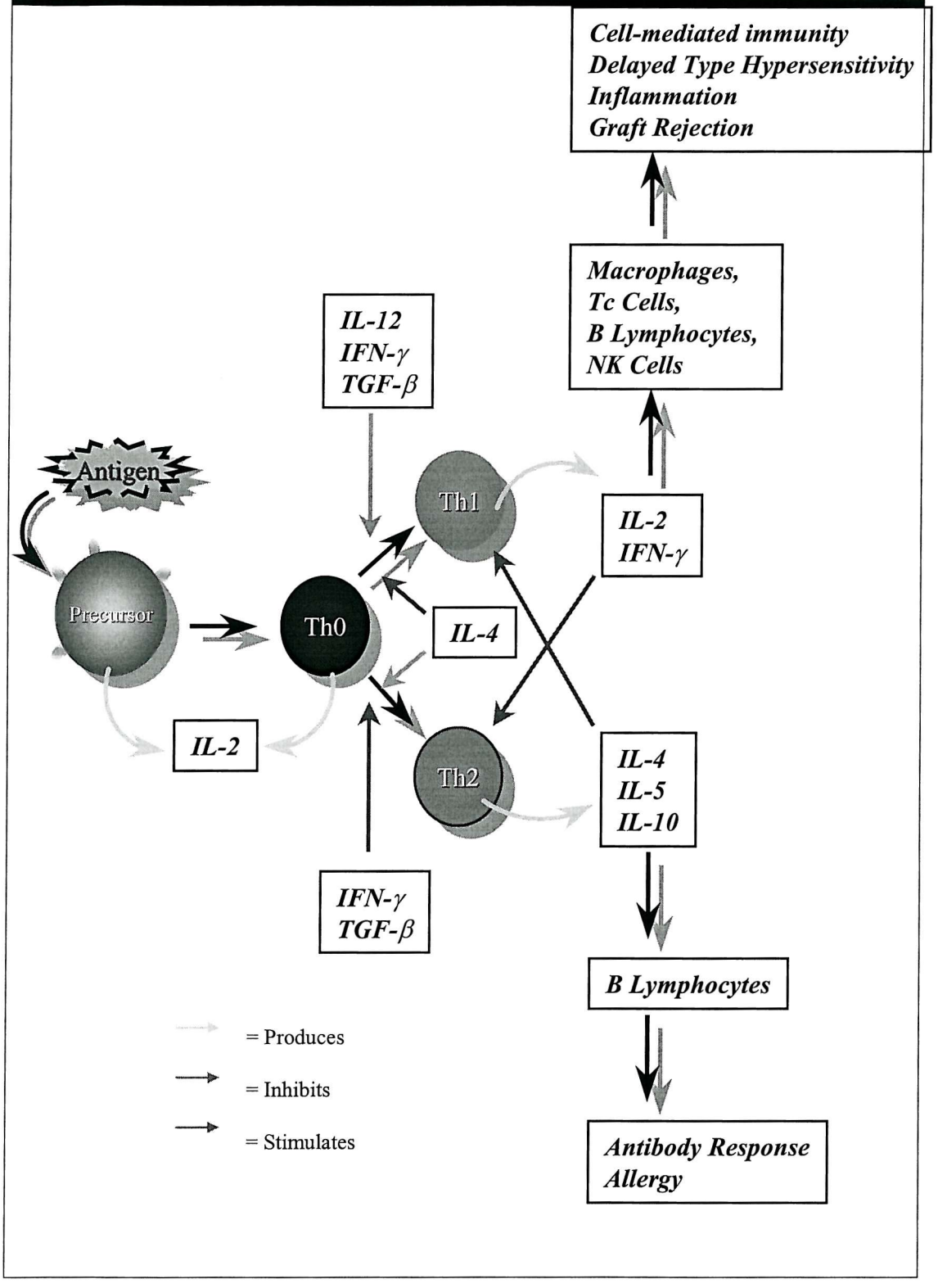
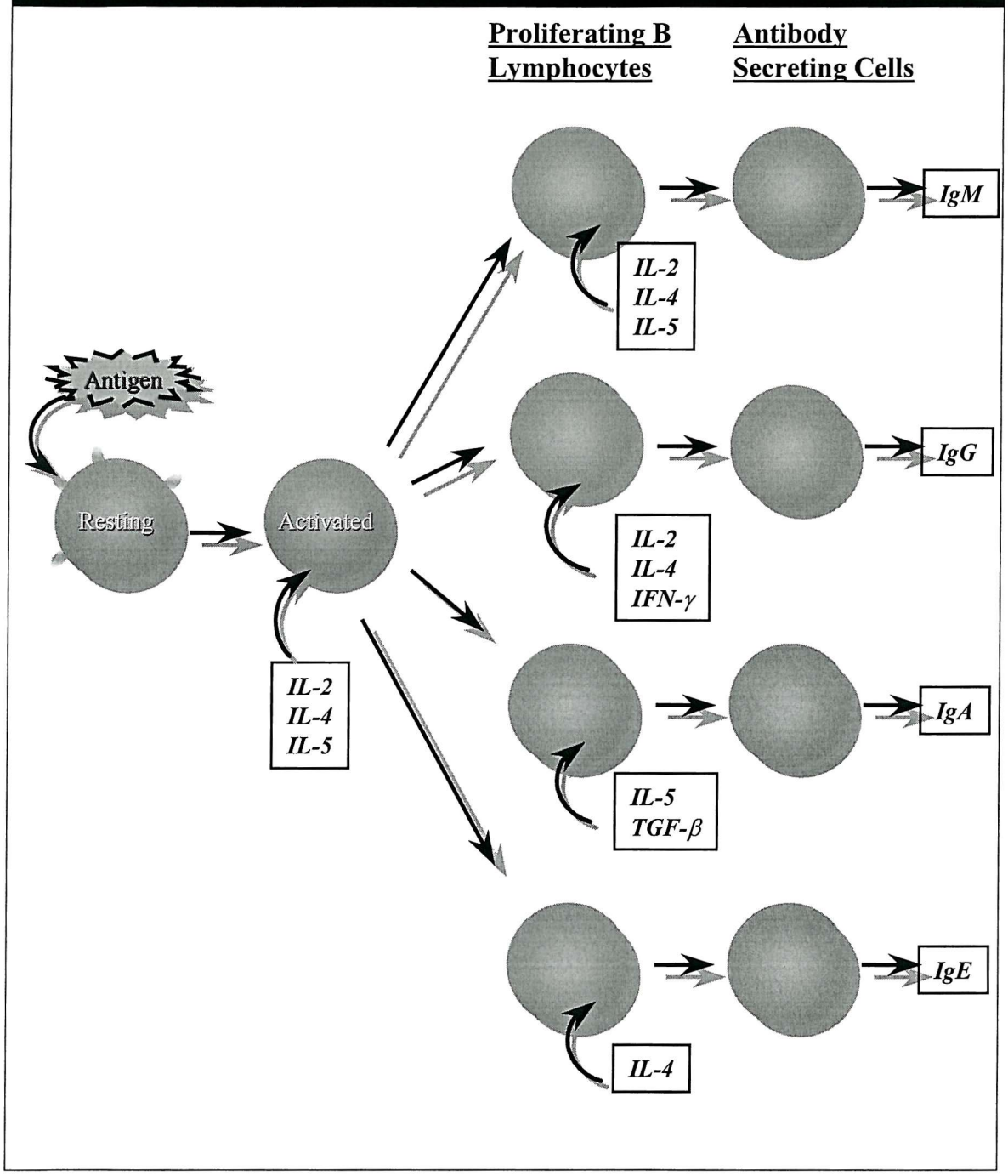


Figure 1.6. B Lymphocyte activation and differentiation (adapted from Calder, 1998)



1.1.2.5 Granulocytes

Granulocytes, are 12 to 15 μm in diameter, have large amounts of cytoplasm and contain many cytoplasmic granules. One of their main functions is to eliminate microbes and dead cells and this is achieved through the process of phagocytosis and

the respiratory burst. Granulocytes respond to many T lymphocyte-derived cytokines and can be considered important in the acquired immune response. There are three types of granulocyte in the peripheral blood: neutrophils, eosinophils and basophils.

Neutrophils

Neutrophils are also termed polymorphonuclear cells, due to their multilobed nuclei. The neutrophil is the most numerous of the granulocytes and responds rapidly to chemotactic stimuli, which are released by micro-organisms, injured tissues and macrophages as well as components of the complement system. Chemotactic agents include such cellular mediators as leukotriene (LT) B_4 and TNF-α, and these stimulate the neutrophil to migrate through tissue to the site of origin of the chemotoxin. Despite their origin and morphology being somewhat different from the macrophage, they do have some similarities. They have many granules, which contain a variety of enzymes including lysozyme (an enzyme which can lyse bacterial cell walls), in addition to the enzymes that induce the respiratory burst. Neutrophils possess Fc receptors IgG and for complement proteins. It is these receptors which mediate the adhesion of opsonised bacteria to the neutrophil surface and its subsequent phagocytosis. The bacterium is then destroyed in one of the vacuoles of the cell. This destruction is facilitated by the respiratory burst (section 1.1.2.1.) which is an important part of the activity of the neutrophil. The production of such a damaging range of enzymes and oxygen metabolites can also lead to the damage of local tissue and sometimes an inappropriate inflammatory response.

Neutrophil Activation - Oxidative Burst and Chemotactic Response

The pathways of neutrophil activation which will elicit an oxidative burst in response to a stimulus use similar signalling pathways as those involved in the activation of the macrophage oxidative burst (*Figure 1.2.*). The chemotactic response also uses these pathways of activation stimulated in response to chemotaxins such as LTB_4. The cellular response seen due to the resultant series of intracellular events includes the assembly of elements of the cytoskeleton and cell contraction.

Eosinophils

Eosinophils have bilobed nuclei and once released from the bone marrow they migrate to tissues such as the lung where they may reside for several weeks. These cells are similar to neutrophils in that they move towards chemotactic stimuli derived from lymphocytes and mast cells. Eosinophils secrete the antibody IgE and express surface receptors for this class of antibody. As such they are thought to defend the body from helminthic parasites (worms) which induce the production of this antibody. These cells contain specialised granule proteins, which are effective at killing helminths when released onto the surface of this invading parasite. Eosinophils often succeed where macrophages and neutrophils have failed. The important difference is that the invading pathogen is not engulfed by the eosinophil but its cell wall is digested and the organism is destroyed. Eosinophils are also found at the site of hypersensitivity or allergic reactions. Eosinophils are stimulated by IL-5, which is produced by Th2 cells. Thus, T-lymphocytes may be responsible for the eosinophil accumulation which occurs during allergic reactions.

Basophils

Basophils are a type of motile phagocytic cell, which express receptors for IgE and will bind free cytosolic IgE antibodies. These bound antibodies may then encounter antigens, which subsequently stimulate the basophils to secrete their granule contents, which are involved in an allergic or immediate hypersensitivity response. Basophils contain histamine in their cytoplasmic granules, which they release into the surrounding tissue. Basophils are similar to mast cells in this respect.

1.1.3 Cellular Mediators of the Immune System

1.1.3.1 Cytokines

Cytokines are one of the principal cellular mediators within the immune system. They are a diverse group of soluble proteins, which are produced during the response of activated cells of the immune system (such as T cells and macrophages which are

responding to an antigen). They serve to regulate and mediate immune and inflammatory responses.

Cytokines are not stored within a cell but are synthesised de novo when gene transcription is upregulated in response to a stimulus. When released, a cytokine may act on a wide range of cell types rather than on just a single type of cell, and they can act in an inhibitory or a stimulatory fashion (or both depending upon the target cell). A cytokine may also have multiple effects on a target cell. The cytokine molecule may act in an autocrine manner by influencing the cell that produced it, or in a paracrine manner by influencing a nearby cell. Alternatively, the cytokine may be released into the circulation where it acts as an endocrine mediator by targeting a remote cell. The roles of some important cytokines are summarised in *Table 1.2.*

1.1.3.2 Eicosanoids

Eiocosanoids are a group of oxygenated derivatives of dihomo-γ-linolenic acid, arachidonic acid (AA) and eicosapentaenoic acid (EPA). The principal eicosanoids include the prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), lipoxins, hydroperoxyeicosatetraenoic acids (HPETEs) and hydrooxyeicosatetraenoic acids (HETEs). The cyclo-oxygenase enzymes produce the PG and TX, while the LT are synthesised by the lipoxygenase enzymes. These mediators are not stored within the cell but are made de novo from the precursor fatty acids in the cell membranes in response to a stimulus (e.g. antigen-antibody complex, bacterial endotoxin or cytokine) (Calder, 1995).

The PG_2 and TX_2 series and LT_4 series are formed from AA and these are considered to be biologically more potent than their counterparts (PG_3 and TX_3 series and LT_5 series) synthesised from EPA (*Figure 1.7.*). The amounts and types of mediator produced depend on the availability of AA (or EPA) in the cell membrane, the activity of phospholipase A_2 (which acts on phosphotidylcholine (PC)) and phospholipase C (which acts on phosphotidylinositol-4,5-bisphosphate (PIP_2)) and DAG lipase (section 1.3.2.3.). It is these enzymes which release the substrate fatty acid for the cyclo-oxygenase and lipoxygenase enzymes to metabolise.

Table 1.2. Summary of the effects of a selection of cytokines

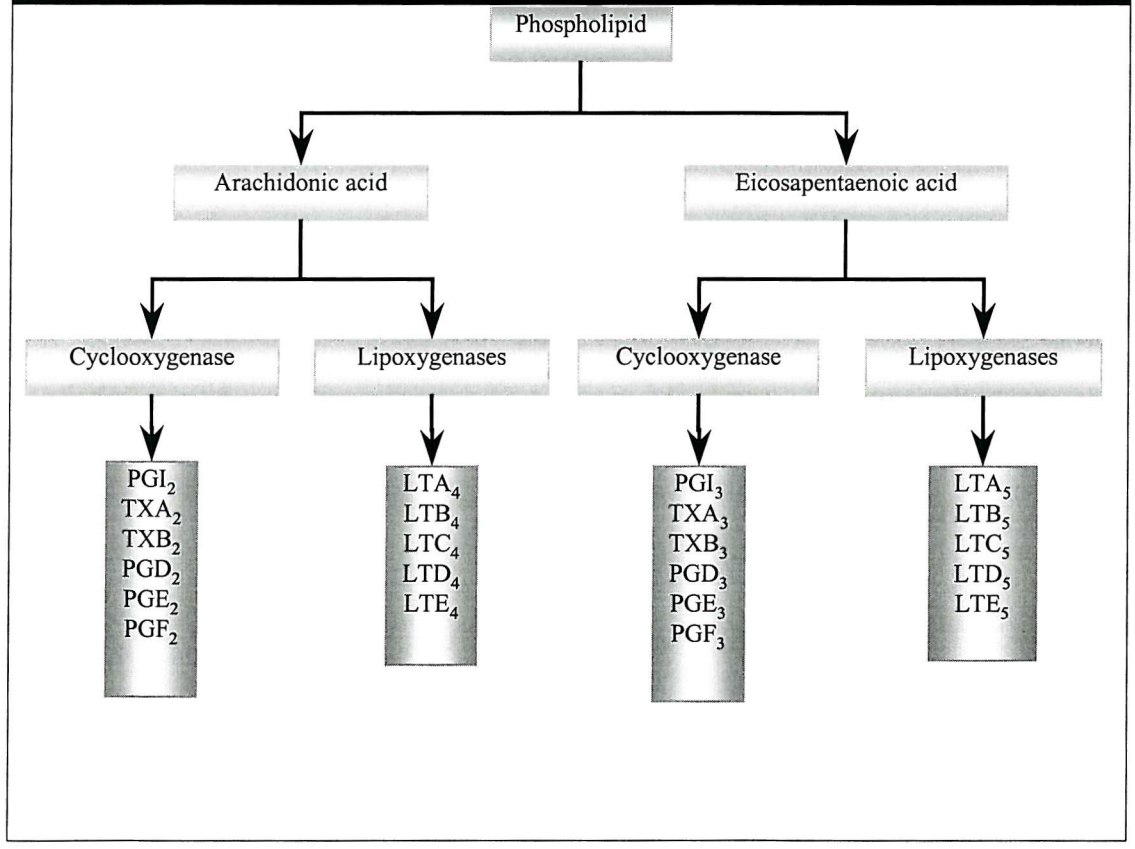
Cytokine	Cell Source	Cell Target	Effects
TNF-α	Activated monocytes and macrophages, neutrophils, T-(CD4) cells and NK cells	Neutrophils, monocytes, macrophages	Tumouricidal; Chemoattractant to neutrophils; Upregulates adhesion molecule expression by neutrophils, macrophages and endothelial cells; Phagocytosis by macrophages; T- and B-cell function; MHC expression and production of IL-1 and IL-6; Mediates systemic effects of infection e.g. fever via effect on hypothalamus.
IL-1(α/β)	Activated monocytes, macrophages and B cells	T- and B-cells	Upregulates T- and B-cell proliferation and release of IL-2 and IL-6; Mediates systemic effects of infection e.g. acute phase response via effects on liver.
IL-6	Activated monocytes, macrophages, T- and B-cells and eosinophils	T- and B-cells	Induces B-cell differentiation and maturation to plasma cells; T-cell activation and differentiation.
IL-2	Activated Th cells, especially Th1 cells	Th1 cells and others	Upregulates the Th1 (cell-mediated) immune response.
IL-4	Activated Th cells, especially Th2 cells	B-cells	Promotes B-cell proliferation, differentiation and production of IL-5 and IL-6; Upregulates B-cell expression of the MHC II complex; Inhibits activation of NK cells induced by IL-2.
IFN-γ	Activated Th and Tc cells and NK cells, B cells	T- and B-cells, macrophages and monocytes	Antiviral and anti-parasitic effects; Inhibits the differentiation and proliferation of Th2 cells, B-cells growth and production of immunoglobulins; Required for the expression of the IL-2 receptor on T-cells and modulates T-cell growth and functional differentiation and MHC II expression; Is intimately involved in the cell-mediated immune response, causes macrophage activation; Induces the secretion of TNF-α by macrophages.
IL-10	T-(CD8) cells, T-(CD4) cells:Th0, Th1 and Th2 cells, monocytes	T- and B-cells, macrophages	Inhibits synthesis of IFN-γ, IL-10 and TNF-α, in the Th1 but not Th2 cells - this activity is antagonised by IL-4; The reduction in IFN-γ production is via suppression of IL-12 synthesis by accessory cells; Suppression of IL-1, IL-6 and TNF-α production and antigen presentation by macrophages but not B cells.

The biosynthesis and release of extracellular lipid mediators from precursors in the cell membrane are important for the functioning of the inflammatory and immune responses (section 1.1.3.3.). When a polyunsaturated fatty acid (PUFA) (such as AA or EPA) is released by the action of PLA_2 one of two types of cyclo-oxygenase enzyme (COX) will metabolise the released fatty acid to form a PG. The COX I enzyme is found equally in the endoplasmic reticulum and nuclear envelope, while COX II is found predominantly in the nuclear envelope (Serhan *et al.*, 1996). COX I

produces PG constitutively, while COX II is only active under certain circumstances (Serhan *et al.,* 1996). COX II is induced by inflammatory and immune stimuli (Serhan *et al.,* 1996). Thus, it would seem that there are two somewhat distinct biosynthetic pathways for the production of PG.

The process of LT biosynthesis is reviewed by Parker (1987). There are three main lipoxygenase (LOX) enzymes, and these are primarily soluble enzymes. They include 5-LOX which is prominent in monocytes, macrophages, granulocytes and mast cells, 12-LOX which is predominantly found in platelets and 15-LOX which is found in leukocytes and other cell types such a tracheal epithelial cells (Parker, 1987). The products of 5-LOX are particularly important in the immune system and have been found to be of particular importance in allergic and inflammatory responses. One of the most important LT with regard to the immune system is LTB_4.

Figure 1.7. Synthesis of eicosanoids from AA and EPA



Cells of the immune system are important producers of eicosanoids (Goldyne and Stobo 1981). The PGs and LTs derived from AA and EPA are involved in

modulating the intensity and duration of an immune response as they influence the inflammatory response and cytokine production by monocytes, macrophages and T lymphocytes (*Figure 1.8.*). They have pro-inflammatory effects since they elicit vasodilatation and vascular permeability as well as inducing fever (*Table 1.3.*). The LTs are chemoattractants for immune cells such as the neutrophils and promote the movement of leukocytes to sites of immune and inflammatory activity (*Table 1.3.*).

Table 1.3. The effects of arachidonic acid metabolites on immune function

4-series Leukotrienes	**PGE_2**
↑ Vasoconstriction	↑ Fever
↑ Vascular permeability	↑ Pain
↑ Leukocyte chemotaxis	↑ Vasodilatation
↑ Neutrophil activity (e.g. superoxide production)	↑ Vascular permeability
↑ Pro-inflammatory cytokine production	↓ Pro-inflammatory cytokine production
↑ Th-1 cytokine production	↓ Th1 cytokine production
↑ NK cell activity	↑ IgE production by B cells
↓ Lymphocyte proliferation	↓ Lymphocyte proliferation
	↓ NK cell activity
	↓ MHC II expression

1.1.3.3 The Role of Eicosanoids in Inflammation and the Immune Response

The effects of eicosanoids on the cells of the immune system are wide-ranging. Prostaglandins, predominantly PGE_2, can influence the functioning of B-lymphocytes, macrophages, T-lymphocytes and NK cells and are intimately involved in the cell-mediated and humoral immune responses, as well as in inflammation (*Table 1.3.*; *Figure 1.8.*). These effects are extensively reviewed by Goldyne and Stobo (1981), Goodwin and Cueppens (1983) and Roper and Phipps (1994).

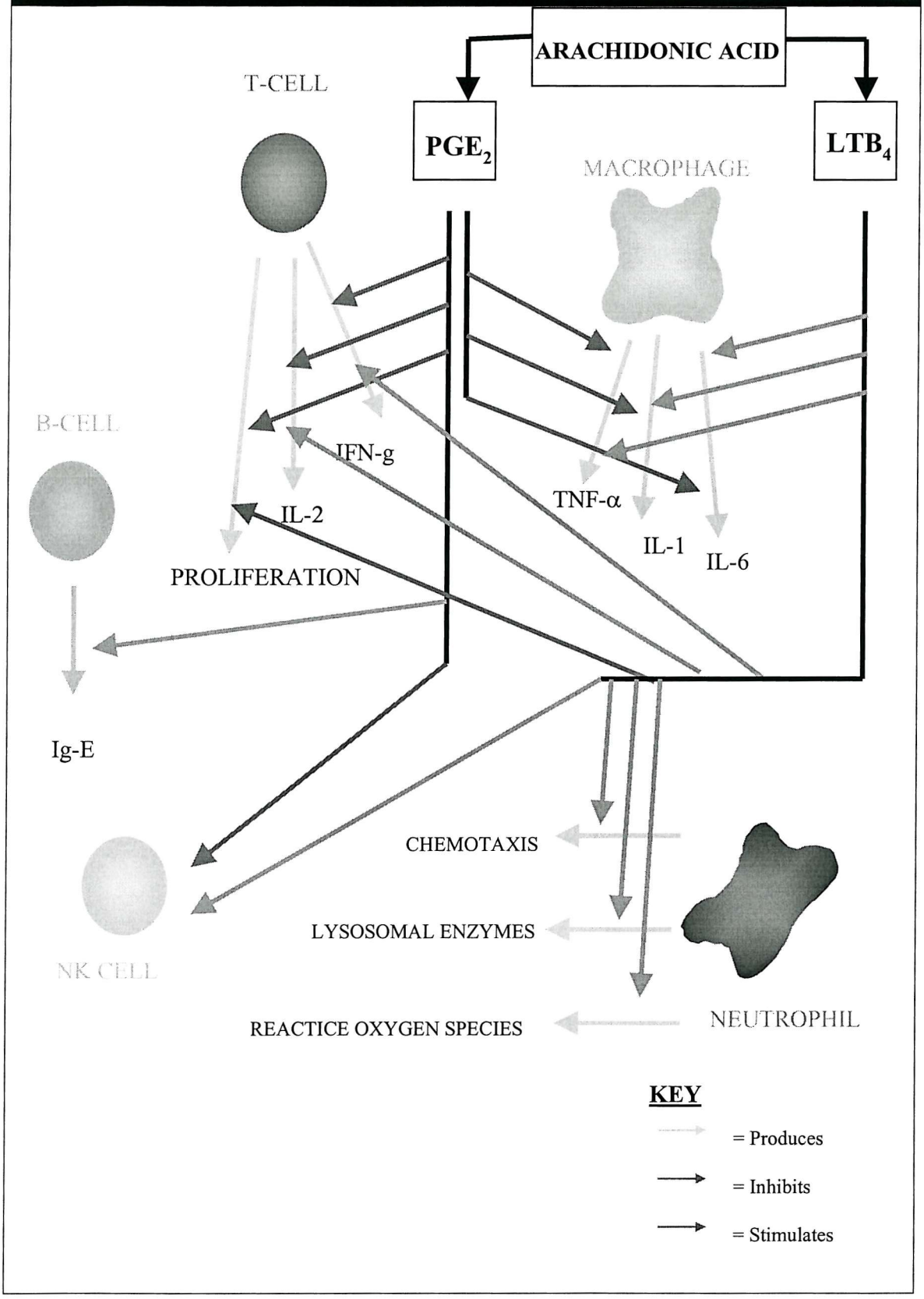
It has been shown experimentally that macrophages are the primary producers of PGE_2 and that lymphocytes have an extremely limited capacity to produce this prostaglandin (see Goldyne and Stobo, 1981 for references), as they are thought to have limited COX enzyme activity. The production of PGE_2 by macrophages and monocytes is thought to have a key role in the regulation of immune cell function.

Through its effects on B-lymphocytes, PGE_2 modulates the humoral response primarily by causing an inhibition of the production of IgM, inhibition of B-lymphocyte proliferation and inhibition of B-lymphocyte activation events (Roper and Phipps, 1994). However, PGE_2 promotes IgE production by B-lymphocytes (Roper and Phipps, 1994). In the T-lymphocyte population the proliferative response is reduced by PGE_2 (Goodwin *et al.,* 1974). It is thought that this is due to the reduction in IL-2 production by T-lymphocytes in the presence of PGE_2 (Wu & Meydani, 1998). This PG also inhibits the production of IFN-γ (Betz and Fox, 1991), and the ability of NK cells and Tc cells to kill target cells (Wolf and Droege, 1982). PGE_2 does not have the same inhibitory effect on Th2 cells and so the production of IL-4 is not reduced (Novak and Rothenberg, 1990); this helps to further augment the humoral immune response.

In addition to producing PGE_2 macrophages are also susceptible to its effects. PGE_2 has been found to inhibit the expression of MHC II by macrophages (Snyder *et al.,* 1982). This has a profound effect on the APC capacity of the immune system. PGE_2 has also been found to reduce the production of IL-1, which is necessary for antigen presentation by macrophages, and the production of TNF-α (Bray *et al.,* 1976, Kunkel *et al.,* 1982).

It has been established that the effects of PGE_2 on immune cells are mediated via a cAMP-dependent mechanism. This is achieved via the binding of PGE_2 to a cell receptor, activation of a G protein and stimulation of adenylate cyclase and a consequential rise in cAMP, which can modulate the intracellular signalling pathways of the cell. A rise in cAMP is known to be associated with inhibitory effects on immune cells (Lewis, 1983). Numerous investigations have shown that the actions of PGE_2 can be mimicked by cAMP-elevating drugs (see Roper and Phipps 1994 for references). In the T-lymphocyte it is has been established that the inhibitory effects of a rise in cAMP due to the PGE_2 are attributable to modulation of calcineurin and its signalling cascades involved in T-lymphocyte activation and regulation of gene transcription (Paliogianni *et al.,* 1993).

Figure 1.8. The pathways by which PGE_2 and LTB_4 modulate the immune system



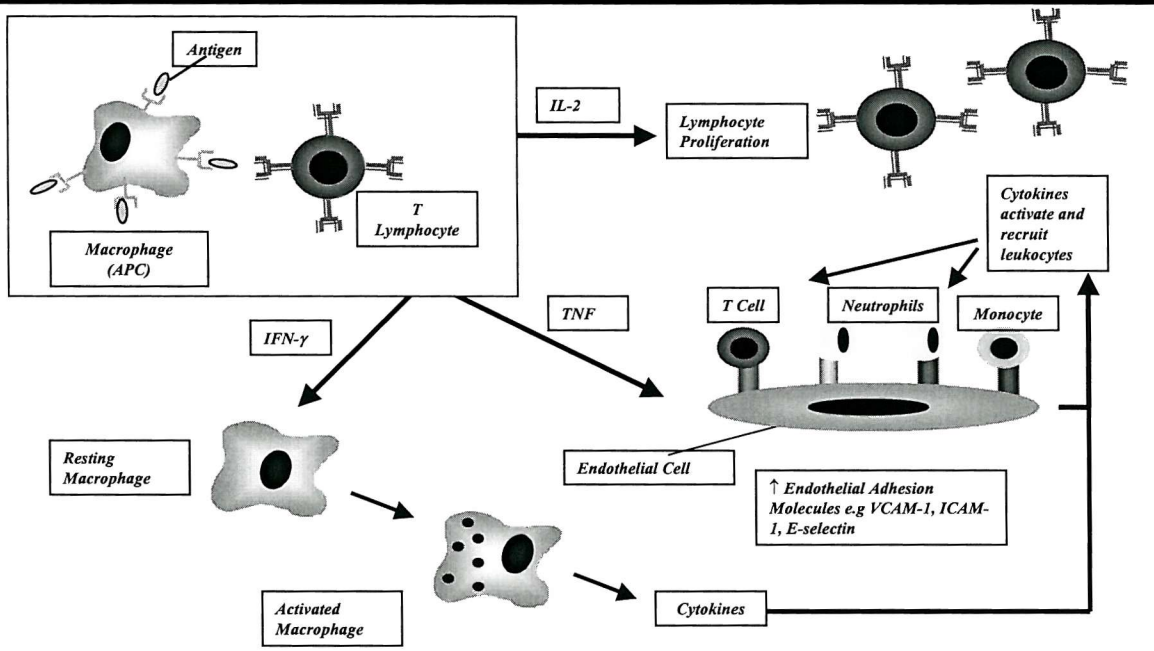
1.1.4 The Delayed Type Hypersensitivity Skin Reaction

So far this chapter has examined the function of immune cells in isolation, although it has been indicated that they communicate by producing cytokines and eicosanoids and by cell-to-cell interactions. Physiologically the cells function in an integrated manner and a good example to illustrate this is the delayed type hypersensitivity (DTH) response.

The skin provides one of the three major interfaces between the environment and the body. Human skin can cover a surface area of 1.6 – 1.85 m^2 and is 3-5 mm in depth (Groth, 1976). The skin comprises several layers: the epidermis, dermis (outer and reticular) and subcutis. A DTH reaction is mounted after a foreign antigen penetrates the epidermis and migrates down to the reticular layer of the dermis, which provides structural support for the skin and also contains specialised resident macrophages, called Langerhans cells, as well as mast cells and an extensive vascular network.

When a new antigen is encountered, either by natural infection or by vaccination, there is a sensitisation reaction, which involves the presentation of antigen by Langerhans cells to naïve T-lymphocytes resulting in antigen-specific CD4 T-lymphocyte activation, expansion and differentiation (*Figure 1.9.*). If the antigen is of a viral nature then there will be CD8 T cell activation. The ability of T-lymphocytes to cross the endothelium increases, due to an increase in vascular permeability and leukocyte adhesion molecules (VCAM-1 and ICAM-1) influenced by cytokines (IFN-γ, TNF-α), and this, together with an increase in number of T-lymphocytes, confers a greater probability of leukocyte encounter with an antigen at the site of exposure (*Figure 1.9.*). If the individual is already sensitised to the antigen then it is unlikely that Langerhans cells will play an important part in antigen presentation. It is more likely that vascular endothelium which expresses MHC molecules and releases co-stimulating molecules (e.g. TNF-α), may play a role in antigen presentation to memory T cells. This has been shown to be possible *in vitro* (Khalfoun *et al.*, 1996a). Monocytes may also be involved in antigen presentation to T cells, bypassing the endothelium if the antigen is non-specific and inflammatory. Monocytes are also intimately involved in antigen elimination.

Figure 1.9. The initiation and progression of a DTH response. (adapted from Abbas *et al.*, 1994).



DTH reactions are initiated by T cell recognition of an MHC-associated antigen on anAPC. This results in the activation and multiplication of T cells by IL-2. Other cytokines are also produced by T cells. These cytokines e.g. TNF-α and IFN-γ, act to recruit and activate other leukocytes such as macrophages, and to stimulate endothelial cells to vasodilate, to upregulate the expression of adhesion molecules, and produce pro-inflammatory mediators and vasodilator agents e.g. NO.

They are activated by IFN-γ, the most potent macrophage-activating cytokine, and thus one of the most important cytokines in DTH (*Figure 1.9.*).

A DTH response consists of three phases (*Figure 1.9.).* During the first 15 hr post exposure to an antigen there is an increase in the number of T-lymphocytes, neutrophils and monocytes, and an increase in the production of cytokines and expression of cell surface receptors, such that there is a self-perpetuating cell-mediated immune response at the site of the antigen (*Figure 1.9.*). This response is also associated with vasodilatation of nearby blood vessels, leakage of fibrinogen from the vasculature and a deposition of fibrin which allows greater movement of leukocytes, but also gives rise to the characteristic DTH swelling and hardening of the skin around the site of exposure - the induration. After 48–96 hr the antigen will be eliminated depending on the individual's immune system integrity. However, the response can be associated with tissue injury where there is a prolonged immune response, hence the term hypersensitivity

1.2 Fatty Acids: Nomenclature, Synthesis, Dietary Sources and Transport

This section aims to introduce fatty acids, and explain their nomenclature, biochemistry and the way in which they are transported around the body.

1.2.1 Nomenclature of Fatty Acids

Fatty acids are a class of compounds that contain a hydrocarbon chain and a terminal carboxyl group. The systematic name for a fatty acid is derived from the number of carbon atoms in the hydrocarbon chain; in addition to this the number of double bonds and their position is reflected in the nomenclature. This leads to the classification of several groups of fatty acids. Those with no double bonds and thus complete saturation of the carbon chain with hydrogen are termed saturated fatty acids (SFA). Those fatty acids which are not completely saturated are grouped as unsaturated fatty acids of which there are two types. Those with one double bond are termed monounsaturated fatty acids (MUFA) and those with more than one double

bond are termed polyunsaturated fatty acids (PUFA). Important fatty acids and their dietary sources are listed in *Table 1.4.*

1.2.2 Biosynthesis of Fatty Acids

The pathways for the biosynthesis of polyunsaturated fatty acids are shown in *Figure 1.10.* The introduction of double bonds into a hydrocarbon chain is achieved through the activity of desaturase enzymes. The enzymes involved are specific for the position in the chain at which the double bond is to be inserted. Thus, for the conversion of stearic acid (18:0) to oleic acid (18:1*n*-9) the enzyme Δ^9-desaturase is involved. This enzyme is universally present in both plants and animals. It is plants alone however which can then go on to convert oleic acid to linoleic acid (18:2*n*-6) using the Δ^{12}-desaturase enzyme by inserting a double bond between the existing double bond at the 9-position and the methyl terminus of the carbon chain. The Δ^{15}-desaturase enzyme, again only present in plants, can convert linoleic acid to α-linolenic acid (18:3*n*-3). Since linoleic and α-linolenic acids can only be synthesised by plants, they must be consumed in the mammalian diet. Thus, these two fatty acids are referred to as the essential fatty acids.

Animal cells can convert α-linolenic acid into eicosapentaenoic acid (EPA; 20:5*n*-3), docosapentaenoic acid (DPA; 22:5*n*-3) and docosahexanoic acid (DHA; 22:6*n*-3) (*Figure 1.10.).* This is achieved via a series of desaturase and elongation enzymes. A similar series of reactions also allows the conversion of dietary linoleic acid to arachidonic acid (20:4*n*-6). Arachidonic acid, EPA and DHA are not strictly essential, although their intake may become very important in essential fatty acid deficiency.

The *n*-9, *n*-6 and *n*-3 families of PUFA are not metabolically interconvertible in mammals. Long chain PUFA, such as EPA and DHA can be synthesised, at the very start of the food chain, by unicellular algae in phytoplankton and other marine plants. These fatty acids are then transferred through the food chain, via the consumption of oily fish, marine mammal tissues and fish oils, to the human population.

Table 1.4. Fatty acid nomenclature and dietary sources (adapted from Calder, 1998)

Systematic name	**Trivial name**	**Shorthand notation**	**Sources**
Saturated Fatty Acids			
Dodecanoic	Lauric	12:0	De novo synthesis Coconut oil
Tetradecanoic	Myristic	14:0	De novo synthesis Milk
Hexadecanoic	Palmitic	16:0	De novo synthesis Milk, egg, animal fats, meat, cocoa butter, palm oil, fish oils
Octadecanoic	Stearic	18:0	De novo synthesis Milk, egg, animal fats, meat, cocoa butter
Monounsaturated Fatty Acids			
9-Hexadecanoic	Palmitoleic	16:1n-7	Desaturation of palmitic acid Fish oils
9-Octadecanoic	Oleic	18:1n-9	Desaturation of stearic acid Milk, egg, animal fats, meat, cocoa butter, vegetable oil, especially olive oil
Polyunsaturated Fats			
9,12-Octadecadienoic	Linoleic	18:2n-6	Cannot be synthesised in mammals Milks, eggs, animal fats, meat, green leaves, most vegetable oils especially corn, sunflower, safflower and soyabean oils
9,12,15-Octadecatrienoic	α-Linolenic	18:3n-3	Cannot be synthesised in mammals Green leaves, some vegetable oils especially rapeseed, soyabean and linseed oils
6,9,12-Octadecatrienoic	γ-Linolenic	18:3n-6	Synthesised from linoleic acid Evening Primrose oil
5,8,11-Eicosatrienoic	Mead	20:3n-9	Synthesised from oleic acid
8,11,14-Eicosatrienoic	Dihomo-γ-linolenic	20:3n-6	Synthesised from linoleic acid
5,8,11,14-Eicosatetraenoic	Arachidonic	20:4n-6	Synthesised from linoleic acid
5,8,11,14,17-Eicosapentaenoic	Eicosapentaenoic	20:5n-3	Synthesised from α-linolenic acid Fish oils
4,7,10,13,16,19-Docosapentaenoic	Docosapentaenoic	22:5n-3	Synthesised from α-linolenic acid
4,7,10,13,16,19-Docosahexaenoic	Docosahexaenoic	22:6n-3	Synthesised from α-linolenic acid Fish oils

1.2.3 Fatty Acids and the UK Diet

Currently the adult UK diet provides approximately 40% of the total energy intake as fat (COMA Report on the Dietary Reference Values, 1991). Dietary fat is of four main types; 90 % as triacylglycerol, 4-8 % as phospholipids, 1.5 % as glycolipids and < 0.5 % as sterols. The level of total fat intake and the intake of the different types of fatty acids vary somewhat from the recommended intakes (*Table 1.5.*). Most adult Western diets provide between 8 and 15 g/d of essential fatty acids (COMA Report on Dietary Reference Values, 1991).

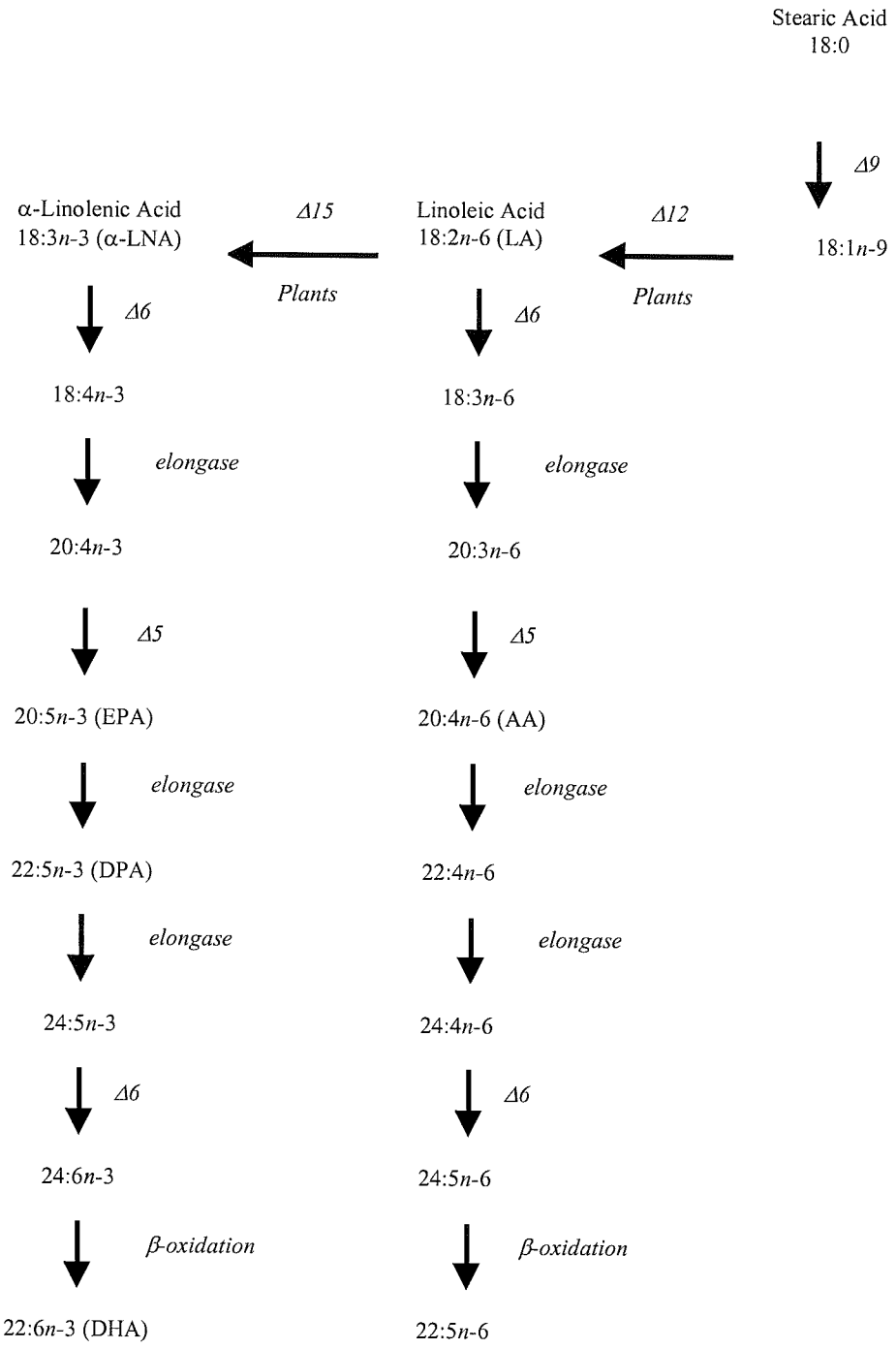
Table 1.5. The actual and recommended fatty acid intakes of the UK population

	Average intake		**Recommended intake (1991)**		**Recommended intake (1999)**	
	% Energy	**g/day (men)**	**% Energy**	**g/day (men)**	**% Energy**	**g/day (men)**
Saturated fat	15.4	39	11	27	-	-
MUFA	11.6	28	13	33	-	-
PUFA	5.8	15	6.5	15	7.5	19
n-6 PUFA	5.1	13	6.0	15	6.0	15
n-3 PUFA	0.8	2	-	-	1.0	3
Very long chain *n*-3 PUFA	<0.1	<0.3	-	-	0.5	1
Total fatty acids	Approx 40	100	<35	88	<35	88

Data are taken from the British Nutrition Foundation (1992) (based on the Department of Health and Social Secruity COMA Report, 1991) and (1999).

The data in *Table 1.5.* are taken from the British Nutrition Foundation (BNF) Task Force Report (1992) and are based on the British Adults Survey which was carried out in the 1980s by Gregory *et al.* (1990). The BNF (1999) states that these are the most recent data of this type available.

Figure 1.10. The pathways for biosynthesis of PUFA



α-LNA - α-Linolenic Acid; AA - Arachidonic Acid; DHA – Docosahexaenoic Acid; DPA - Docosapentaenoic acid; EPA - Eicosapentaenoic Acid; LA - Linoleic Acid

1.2.4 Digestion, Absorption and Transport of Dietary Fatty Acid

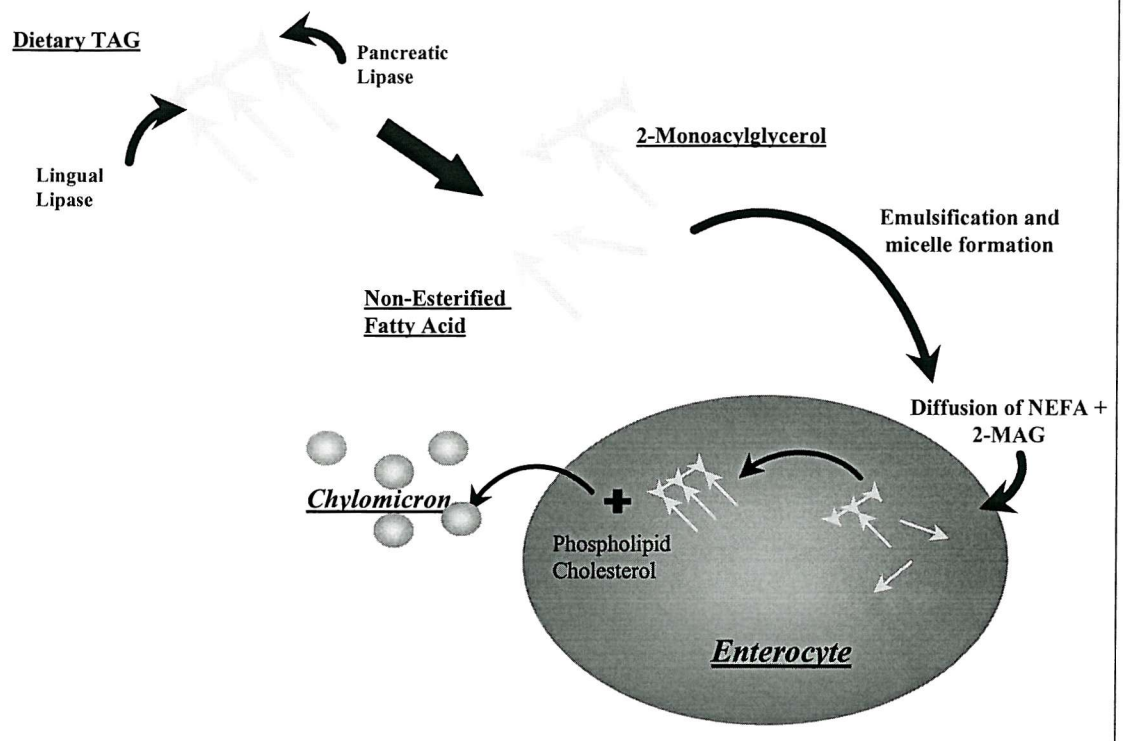
1.2.4.1 Digestion and Absorption

The primary type of fat in the diet is triacylglycerol (TAG). These molecules are composed of three fatty acids esterified to a glycerol backbone. In order for the fatty acids to be absorbed by the body the ester linkages must be hydrolysed. The small intestine is the main site of digestion of dietary TAG. Enzymes, mainly pancreatic lipase, released by the pancreas hydrolyse the TAG molecules mainly at the *sn*-1(3) ester bonds, releasing two free fatty acids (NEFA) and a 2-monoacylglycerol (MAG) molecule (Pufal *et al.,* 1995) (*Figure 1.11.*). Bile salts secreted into the small intestine act as detergents and emulsify the TAG to facilitate TAG hydrolysis. The 2-MAG and NEFA liberated also form micelles due to the presence of the bile salts. Micelles allow the movement of non-polar lipids to the intestinal lining where they collapse and create a locally high concentration of MAG and NEFA. The intestinal lining is composed of enterocytes, which absorb the lipid, via diffusion, leaving the bile salts in the gut lumen from where they are later absorbed. Absorption involves the binding of NEFA to a high affinity cytosolic protein called fatty acid binding protein (FABP) inside the enterocyte, where TAG molecules are reformed from the pool of 2-MAG and NEFA. TAG then associates with phospholipids, cholesterol and cholesterol esters and these are then complexed with apolipoproteins to form chylomicrons (CM).

CM leave the enterocyte by exocytosis and enter the lymphatic system (*Figure 1.12.*). From here they enter the circulation through the left subclavian vein. TAG carried in CM is hydrolysed by lipoprotein lipase (LPL) which is found on the surface of endothelial cells which line capillaries. LPL preferentially cleaves at the *sn*-1(3) positions of the TAG (Wang *et al.*, 1982). Thus the fatty acid at the *sn*-2 position must isomerize to the *sn*-1(3) position in order to be hydrolysed (Braun and Severson, 1992). The NEFA released by the action of LPL creates a locally high concentration of free fatty acids and some of these diffuse or are transported (see section 1.3.1.) into nearby cells for utilisation or storage. The activity of LPL is controlled by the hormone insulin, such that expression in capillaries running

through adipose tissue is high in the fed state (Frayn, 1996). This serves to direct dietary TAG to adipose tissue for storage (as TAG). In contrast, in the fasted state or during exercise, adipose tissue LPL activity is low and so the capacity of adipose tissue to access TAG from the circulation is low (Frayn, 1996).

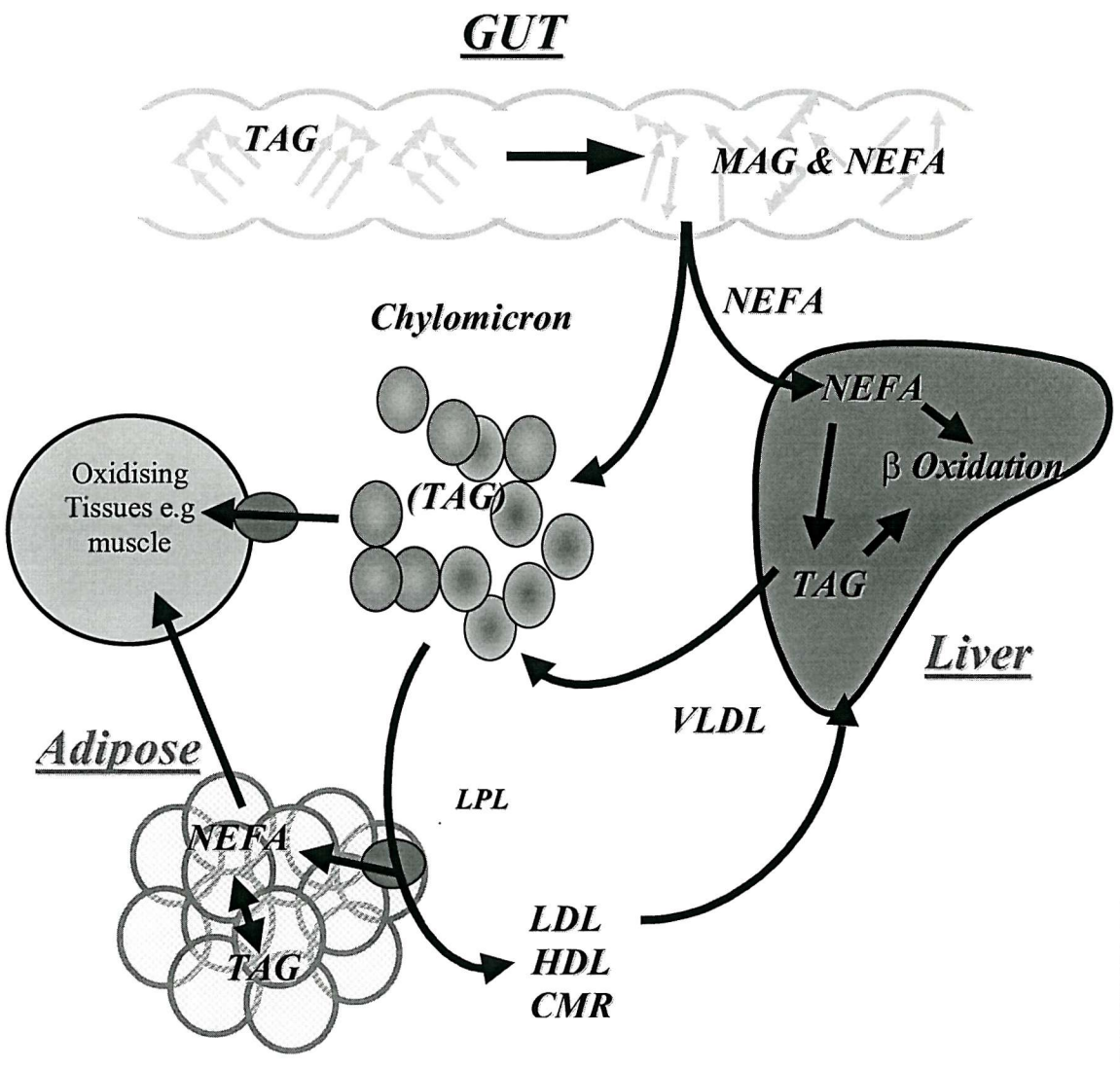
Figure 1.11. The digestion and absorption of triacylglycerol fatty acids



MAG – Monoacylglycerol; NEFA – Non-esterified fatty acid; TAG – Triacylglycerol.

In response to hormones such as adrenaline and glucagons, hormone-sensitive lipase (HSL) in adipose tissue functions to hydrolyse stored TAG and so allows the release of NEFA into the circulation (Frayn, 1996). This provides a supply of energy, via fatty acid oxidation, to number of tissues. NEFA mainly travel in the circulation bound to albumin, and are absorbed into cells via simple diffusion or through the activity of a transporter (Stremmel *et al.,* 1992) (section 1.3.1.).

Figure 1.12. The pathways of lipid transport



CMR – Chylomicron remnant; HDL – High density lipoprotein; LDL – Low density lipoprotein; LPL – Lipoprotein lipase; MAG – Monoacylglycerol; NEFA – Non-esterified fatty acid; TAG – Triacylglycerol; VLDL – Very low density lipoprotein

1.2.4.2 Lipid Transport – Lipoproteins

TAG and other lipid molecules are extremely hydrophobic and in order to be transported around the body they must be packaged into lipoproteins. Lipoproteins consist of a central non-polar, lipid core (TAG and cholesterol esters) surrounded by a layer of phospholipids, free cholesterol and associated proteins (apoproteins). This

outer layer solubilizes the complex. There are four main classes of lipoproteins, differing in size, lipid composition, density and associated apoproteins: high-density lipoproteins (HDL), low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and chylomicrons (CM). Each lipoprotein has a specific role in lipid transport in the circulation (*Table 1.6.*) and there are different pathways for dealing with exogenous and endogenous lipids. Apolipoproteins regulate lipoprotein metabolism, structure and destination, and allow lipoproteins to be internalized by cells via receptor-mediated endocytosis (de Sanctis *et al.*, 1995).

Table 1.6. Classification and properties of lipoproteins (adapted from Benyon, 1998)

Lipoprotein	Major constituents	Apoproteins	Source and function	Mechanism of lipid delivery
CM	90% TAG	A, B-48, C, E	Transport of dietary TAG	Hydrolysis by lipoprotein lipase
Chylomicron remnant		B-48, E		Receptor-mediated endocytosis by liver
VLDL	65% TAG	B-100, C, E	Transport of endogenously synthesised TAG from the liver to peripheral tissues	Hydrolysis by lipoprotein lipase
LDL	50% cholesterol 25% protein	B-100	Carries cholesterol to peripheral tissues	Receptor-mediated endocytosis by liver and other tissues
HDL	55% protein 25% phospholipid	A	Formed in the liver: Reverse cholesterol transport removes cholesterol from tissues and takes it to the liver Provides apoproteins for CM and VLDL metabolism	Transfer of CE to LDL

CMR – Chylomicron remnant; HDL – High density lipoprotein; LDL – Low density lipoprotein; LPL – Lipoprotein lipase; MAG – Monoacylglycerol; NEFA – Non-esterified fatty acid; TAG – Triacylglycerol; VLDL – Very low density lipoprotein

CM are the largest of the lipoproteins and are formed in the absorptive cells (enterocytes) lining the small intestine (*Figure 1.12.*). They transport TAG and cholesterol from the gut to various tissues, and their size is dependent on the quantity of lipid ingested in a meal. The fatty acids released from CM by lipases are used for energy or are stored in adipocytes until needed. As TAG in CM is processed and NEFA released, there is a concurrent change in the apolipoproteins of the CM, and the subsequent complex is called a chylomicron remnant (CMR). These particles travel in the circulation to the liver where they are removed by hepatocytes and

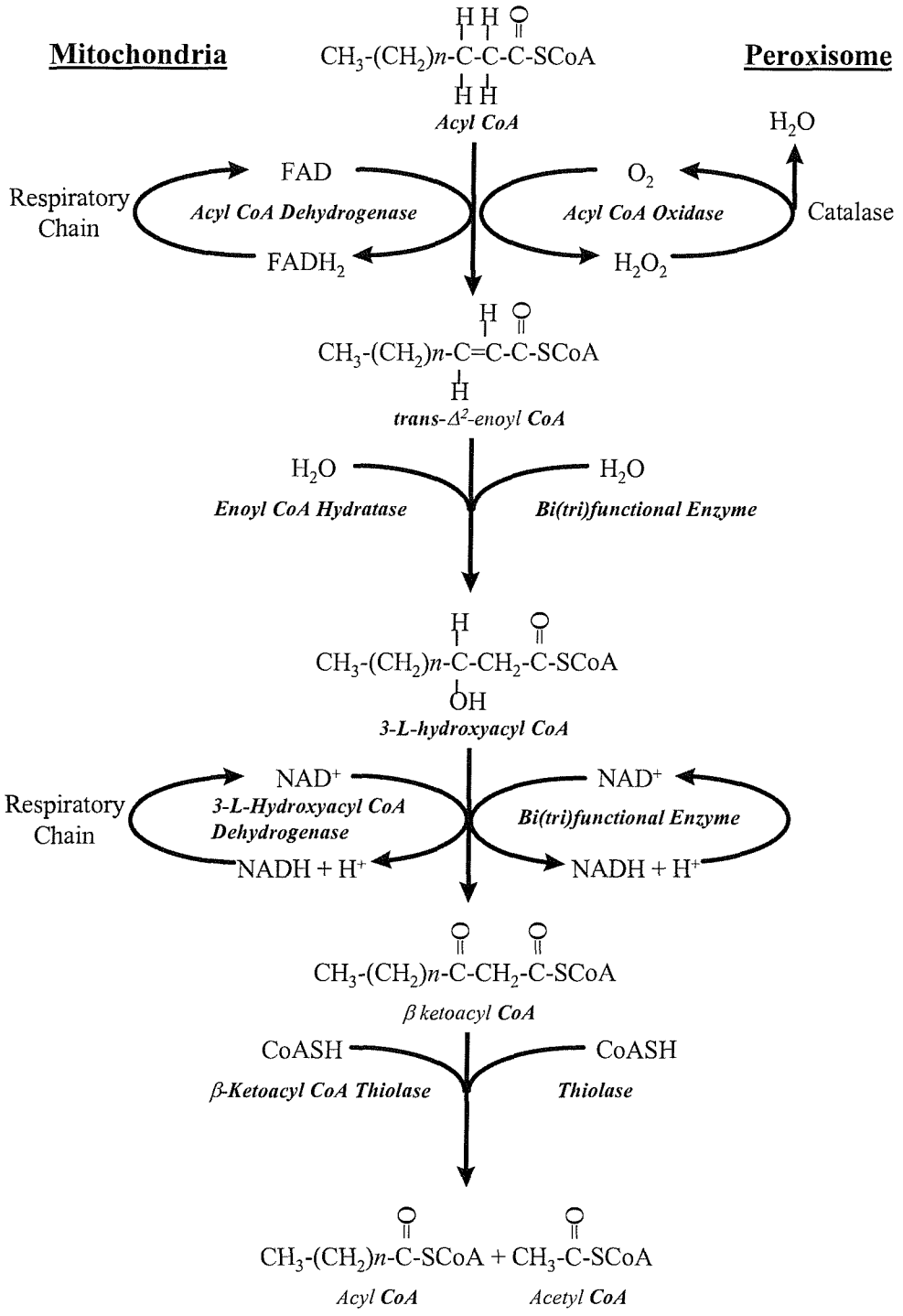
provide the basic components for the formation of very low-density lipoprotein (VLDL). This complex is released by the liver (and intestine) and its main function is to carry TAG. As VLDL is metabolised, it loses cholesterol, phospholipids and apolipoproteins to high-density lipoproteins (HDL), which reciprocally exchange certain apolipoproteins back to VLDL. HDL also functions to transport cholesterol from peripheral tissues to the liver. Low-density lipoprotein (LDL) is the main means of cholesterol transport around the body, and is produced by the intra-vascular catabolism of VLDL via an intermediary complex, intermediate-density lipoprotein (IDL).

1.2.5 The Oxidation of Fatty Acids

In order to utilise fatty acids as a source of energy they must be transferred into the mitochondria of the cell where the process of oxidation takes place. Fatty acids must first be activated to their CoA thioesters by an acyl-CoA synthetase so that they can be transported into a mitochondrion to be oxidized.

Transferase molecules on both faces of the inner mitochondrial membrane facilitate the transport of acyl CoA into the mitochondrial matrix where β-oxidation occurs. These enzymes are carnitine palmitoyl transferase I (CPTI) (outside) and CPTII (inside). The β-oxidation of fatty acyl CoA in the mitochondrial matrix involves the sequential removal of two carbon units from the carboxyl end of the molecule by a variety of enzymes, which act sequentially and repeatedly to generate acetyl CoA at each cycle (*Figure 1.13.*). This process also results in the generation of NADH and $FADH_2$ which are then used in electron transfer reactions involved in the production of ATP. Acetyl CoA is shuttled to the citric acid cycle, which is the final pathway for the oxidation of fuel molecules. This cycle involves the complete oxidation of acetyl CoA to yield carbon dioxide and to generate ATP via the electron transport chain.

Figure 1.13. The β-oxidation pathway



To allow unsaturated fatty acids to enter the oxidation pathway two extra enzymes are needed. These are aconitate Δ-isomerase and 3-hydroyacyl-CoA epimerase and allow the enzymes of β-oxidation to act upon PUFA. Fatty acids with an odd number of carbons are metabolised by the β-oxidation pathway until propionyl-CoA is formed. This metabolite is then carboxylated and transformed into succinyl-CoA, an intermediate of the citric acid cycle. The process of β-oxidation can also occur in the peroxisome, but this is not coupled to the production of ATP, and involves a different set of enzymes.

Peroxisomal β-oxidation seems to be more involved with the metabolism of substrates which include long chain fatty acids, such as erucic acid which, when it has passed twice through the peroxisomal β-oxidation, forms oleic acid which a much better substrate for mitochondrial β-oxidation. Peroxisomes can also metabolise long chain fatty acid derivatives, such as the prostaglandins and certain drugs. The metabolites produced are then passed to the mitochondria for further oxidation or excreted.

1.3 Fatty Acids and Cell Composition and Function

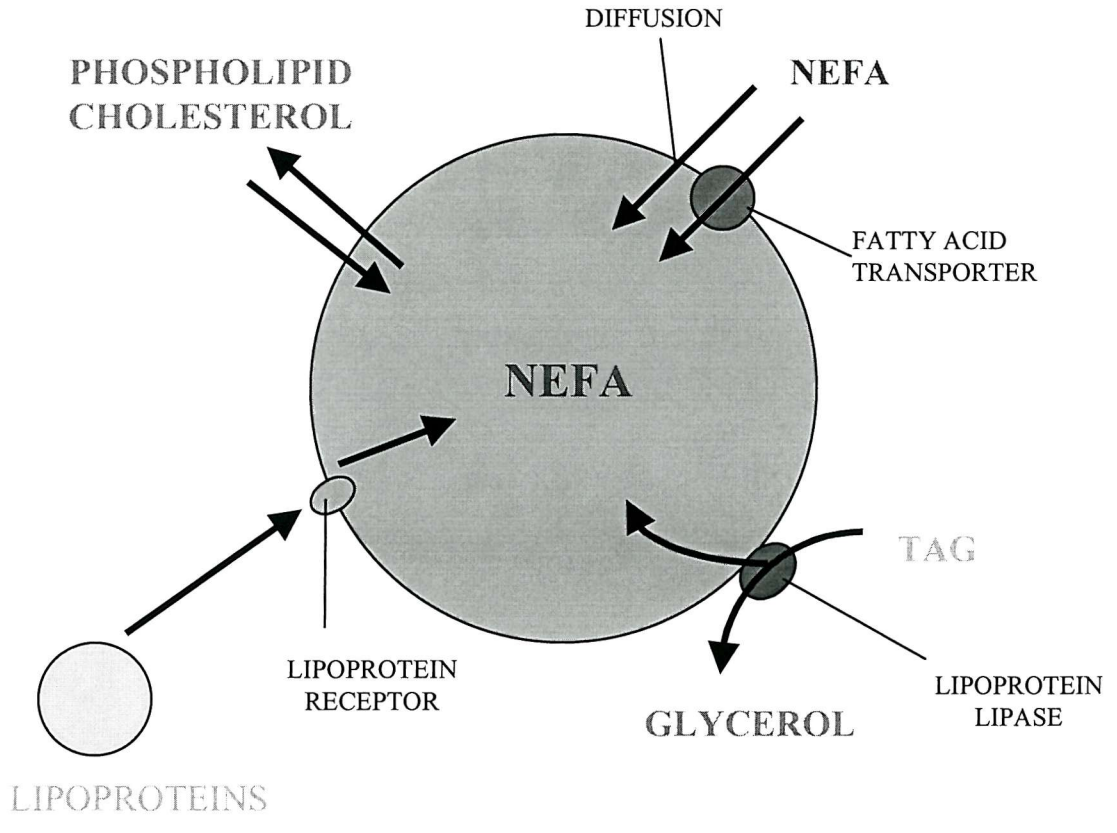
This section will examine how fatty acids are taken into cells from the circulation and their subsequent fates within that cell. Fatty acids may be used as structural components of the membrane, as a source of energy or as a substrate for the production of cellular mediators. The influence of fatty acids on cell function will also be addressed.

1.3.1 Fatty Acid Entry into Immune Cells – the Utilisation of Extracellular Lipids

Experimental evidence indicates that cells of the immune system are not able to desaturate fatty acids very readily (Chapkin *et al.,* 1993). However, immune cells contain high proportions of PUFA. Thus, it is likely that the source of these fatty acids is the external cellular environment (e.g. plasma). Cells can obtain fatty acids from two main lipid pools in the circulation: NEFA and lipoproteins. It is thought

that NEFA are the primary source of extracellular lipid available to immune cells. However, it has also been shown that immune cells in culture can utilise lipid directly from TAG, phospholipid and cholesterol as well as from lipoproteins (Spector *et al.,* 1981) (*Figure 1.14.*). It has also been established that the cell storage lipid and the cell membranes of immune cells in culture reflect the fatty acid composition of the culture medium (Spector *et al.,* 1981); this suggests that the cell membrane composition is dynamic. The mechanisms by which fatty acids enter cells are addressed below.

Figure 1.14. Mechanisms of entry of extracellular fatty acids into a cell



NEFA – non-esterified fatty acids; TAG – Triacylglycerol

Uptake from the NEFA Pool

The uptake of NEFA into the cell was for many years considered to be a purely unregulated passive process of diffusion from albumin-bound NEFA into the membrane (Spector and Steinberg, 1965). The uptake process received little attention until it was found that NEFA were in fact taken up from a small unbound plasma pool, and that this transport showed the kinetics of a facilitated transport process, for example counter-transport and saturation (Stremmel, 1988). The process of uptake involves the dissociation of the fatty acid from albumin and its entry into the plasma membrane as it binds to and is translocated by a Na^+/long chain fatty acid transporter and then released at the inner surface. Here it binds to a cytosolic fatty acid binding protein (FABP).

The uptake of NEFA by immune cells has been shown to occur in culture. Several studies using radioactively labelled NEFA have show that cultured lymphocytes and macrophages can take up and incorporate fatty acids into their membranes (Schroit and Gailly, 1979; Lokesh and Wrann, 1984; Calder *et al.,* 1990; Calder *et al.,* 1994a; Anel *et al.,* 1990).

Lipoprotein lipase has been demonstrated to be active in macrophages (Khoo *et al.,* 1981; Chait *et al.,* 1982; Mahoney *at al.,* 1982; Stray *et al.,* 1990), lymphocytes (Calder *et al.,* 1994b) and natural killer cells (de Sanctis *et al.,* 1994). This suggests that these cells are able to access NEFA directly from TAG in the circulation (or lymph). When TAG was added to the culture medium of lymphocytes or macrophages it was shown to be broken down with the subsequent uptake of some NEFA into the cells (Khoo *et al.,* 1981; Mahoney *at al.,* 1982; Calder *et al.,* 1994b).

Uptake of Entire Lipoproteins

The presence of HDL receptors (Alam *et al.,* 1989), VLDL receptors (Takahashi *et al.,* 1992), LDL receptor-related proteins (Kriger and Hertz, 1994) and CD36/LDL receptors (Endemann *et al.,* 1993) and LDL scavenger receptor (Kreiger *et al.,* 1993) on the surface of the macrophage, and LDL receptors (Suziki *et al.,* 1990) on the lymphocyte surface has been established. Flow cytometry was used to demonstrate the uptake and internalisation of HDL, LDL, CM and VLDL by lymphocytes by

receptor-mediated endocytosis (de Sanctis *et al.,* 1994). Presumably immune cells would have access to NEFA from the lipid fractions carried in lipoproteins following the intracellular degradation of these lipoproteins.

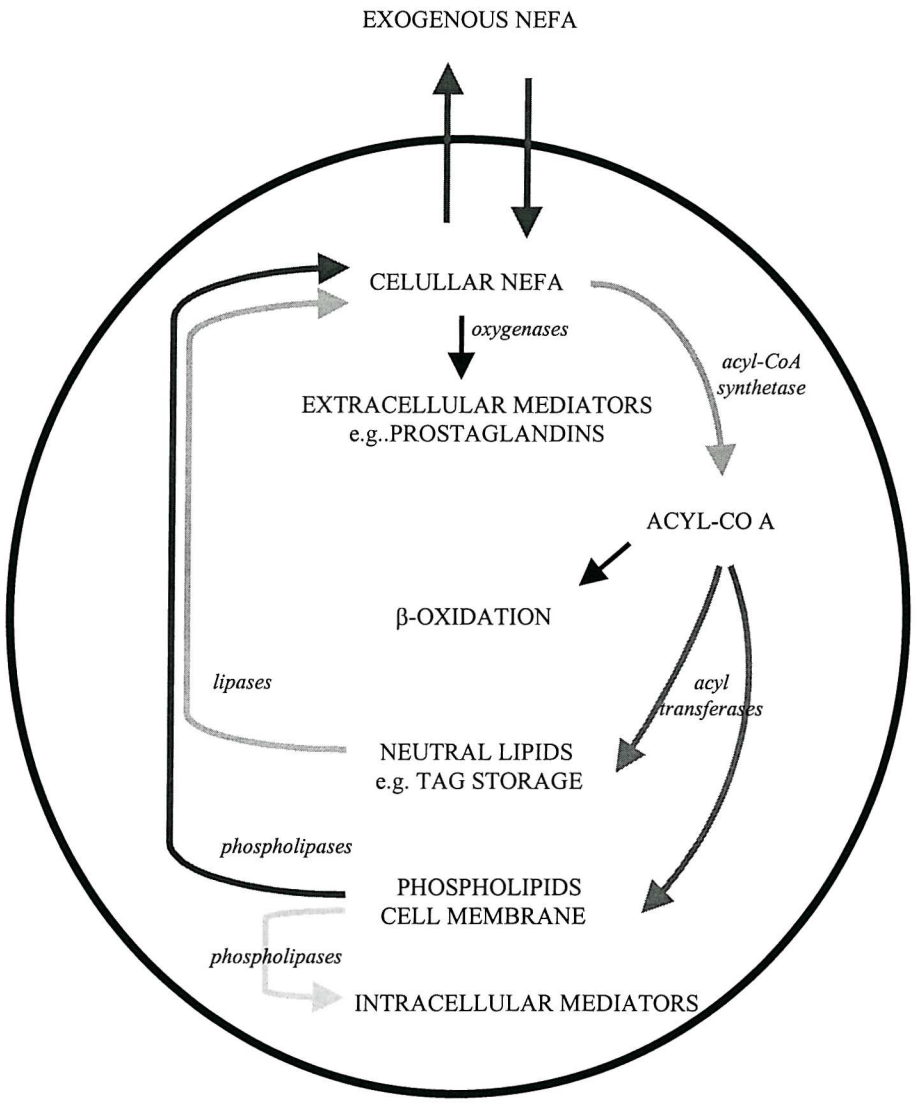
Direct Exchange of NEFA between the Cell Membrane and Extracellular Environment

Evidence suggests that there is some direct exchange of lipid between the phospholipids of plasma lipoproteins and cell membranes; this has been demonstrated in cell culture (Reed, 1968; Kook and Rubeinstein, 1970; Illingworth *et al.,* 1973). The latter paper also suggests that, not only do cells release phospholipid into the culture medium over time, but that this release is greatly increased by the presence of LDL and HDL, enhancing the exchange of the lipids. The uptake of phospholipids and cholesterol from liposomes to cultured cells suggests that direct exchange of the fatty acids in these lipid fractions is possible (Huang and Pagano, 1975).

1.3.2 Intracellular Turnover and Fates of Fatty Acids

When a fatty acid enters a cell it has one of four main fates (*Figure 1.15.)*. It may be used as an immediate source of energy, stored as TAG (which serves as a storage form of NEFA), incorporated into the cell membrane phospholipids or metabolised to an inter- or intracellular mediator. The cell membranes of immune cells are continually undergoing turnover as the processes of cell division and phagocytosis are carried out. As a result of this there is a very dynamic aspect to the turnover of fatty acids within a cell. The membrane also acts as a store, especially for unsaturated fatty acids which are the precursors for signalling molecules such as eicosanoids.

Figure 1.15. The fates of NEFA within a cell.



Fatty Acids as Fuels

Energy is generated from fatty acids by the process of β-oxidation (section 1.2.5.). It has been demonstrated that both lymphocytes and macrophages have the ability to oxidise fatty acids via β-oxidation (Ardawi and Newsholme, 1984; Newsholme and

Newsholme, 1989). It has been estimated that fatty acid oxidation might provide as much as 25% of the energy requirements of these cells (Spolarics *et al.*, 1991).

Fatty Acids as Components of Cell Membranes

Cell Membranes

The function of cell membranes is several-fold. They serve to compartmentalise the cell contents and are the site of activity of many enzymes, transporters of ions and metabolites, and hormone receptors and the site of production of many inter- and intracellular signalling molecules. The fluid mosaic model of membrane structure (Singer and Nicholson, 1972) describes a membrane bilayer composed of amphipathic lipids, arranged such that the outer faces of the membrane are polar and the interior, non-polar. Phospholipids, with their polar phospholipid 'heads' and non-polar fatty acid 'tails' are the main building blocks of the membrane, and their composition plays a key role in determining the structure and physiochemical properties of the lipid bilayer (Brenner, 1984). The cell membrane also contains some cholesterol and TAG, as well as the many and varied functional proteins, which act as enzymes, trans-membrane channels and receptors. These proteins may be peripheral and so bound to the charged surface of the bilayer, or integral and inserted into the bilayer.

There are several types of phospholipids that can be found in a cell membrane. These include phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin. Phospholipids are distributed asymmetrically in biological membranes. For example in human erythrocyte membranes the outer leaflet contains predominately PC, sphingomyelin and PE, while the inner leaflet contains mostly PS and PI (Bretscher, 1972).

Cell Membrane Composition

The composition of the cell membrane with regard to phospholipids is usually characteristic of the cell type. It may change during the progression through the cell cycle, with age, in response to stimuli or to changes in the environment or diet

(Stubbs and Smith, 1984). There is an appreciable turnover of phospholipids in the cell membrane, as has be shown by the incorporation of radioactive fatty acids into cell membranes in short term experiments (Spector and Steinberg, 1965), and the culturing of cells with different fatty acids (section 1.3.1.).

The asymmetric distribution of phospholipids species, and their constituent fatty acids, in biological membranes may influence the functioning of the membrane through modifications in membrane fluidity and the type of inter- and intracellular mediators produced. The asymmetry of the membrane is also important when the requirement and position of cytosolic and membrane enzymes are considered, since differences in phospholipid and fatty acid leaflet composition could lead to differential activity of these enzymes on opposite sides of the membrane perhaps due to changes in fluidity. It has been shown that, in general, integral membrane proteins are up-regulated with increasing membrane fluidity while the activity of peripheral membrane proteins is down-regulated due to the less stable association with the membrane (Fernandes *et al.,* 1998). There may be a degree of metabolic drive within the cell to maintain the composition of the membrane such that these enzymes and receptors function optimally and thus retain the integrity of the cell and its functions (Stubbs and Smith, 1984). This principle has been shown experimentally in an *in vitro* experiment where rat lymphocytes were cultured with different fatty acids and the proliferative capacity of the lymphocytes measured. The inhibitory effects of unsaturated fatty acids on lymphocyte proliferation could be partially or totally relieved by the addition of myristic or palmitic acids (Calder *et al.,* 1991). This suggests that a change or an imbalance in the composition of the membrane, which might affect fluidity (section 1.3.3.), can affect the function of the cell. The degree to which cells try to retain their composition and the mechanism by which they can achieve this requires further investigation. From many earlier experiments (reviewed by Stubbs and Smith, 1984) it appears that there is a constancy in the proportion of SFA in cell membranes, and that the substitution of PUFA into membrane phospholipids is at the expense of other PUFA (often AA).

In unstimulated human T-lymphocytes the composition of the cell membrane was altered after a 24 hr culture in the presence of 10% fetal calf serum (Anel *et al.,* 1990). It was found that there was enrichment of the cells with oleic acid, DPA and

DHA with a decrease in the proportions of AA and linoleic acid, and this was believed to be due to a tendency for lymphocytes to equilibrate their fatty acid composition with their environment. The fatty acid composition of T-lymphocytes, stimulated with phytohemagglutinin showed a similar change in composition to the quiescent cells, and the modification of the cell membrane became more marked as the incubation period continued for 48 and 72 hr (Anel *et al.*, 1990).

Changes in total fatty acid composition may be attributable to discrete changes in the fatty acid composition of different parts of the membrane. For example, further investigation of the fatty acid composition of specific lipid classes in the experiments of Anel *et al.* (1990) established that there was a decrease in linoleic acid and this was mainly in the PC fraction, and a decrease in AA in the TAG, PC and PE fractions while there was an increase in DPA which was observed in the TAG, PE and PS fractions and an increase in DHA in the PC and PE fractions (Anel *et al.*, 1990). There appeared to be no appreciable changes in CE, DAG or NEFA in the cells when they were activated and cultured for 72 hr. In peritoneal macrophages, activated with IFN-γ and cultured in fetal calf serum, there was an increase in linoleic acid in PE (Jackson *et al.*, 1992). This was combined with a decrease in stearic acid and AA in the PC species, and palmitic acid in the PI species (Jackson *et al.*, 1992). It has been shown that the fatty acid compositions of different lipid fractions of the membrane are modulated to different degrees when a cell is exposed to different fatty acid environments. When human peripheral blood lymphocytes were cultured with DHA it was found that the DHA was incorporated to a greater extent in the PC>PE>PI=PC (Bechoua *et al.*, 1998). Since PC is considered an important source of DAG compared to other PL species such as PE; it is thought that modulation of PL species will influence the production of intracellular mediators such as DAG. Given that phospholipid species are distributed asymmetrically in the cell membrane, the relationship between the fatty acid composition of PL and the function of the cell membrane and the cell itself must be very complex.

Within the cell membrane there are domains where lipid-protein and lipid-lipid interactions may be highly specific. Some functional proteins require the presence of specific lipid around hydrophobic regions of their structure, and thus are considered to be sensitive to their fatty acid environments. These proteins include adenylate

cyclase, and the Na^+/K^+-ATPase (Poon *et al.,* 1981), as well as a number of receptors such as that for PGE_2 (Opmeer *et al.,* 1984). Mechanisms by which the membrane fatty acid composition can affect protein function include changes in membrane fluidity (section 1.3.3.) and fatty-acid dependent effects on the conformation of the protein complex.

Fatty Acids in the Formation of Signalling Molecules

Eicosanoid Production

This process is described in section 1.1.3.

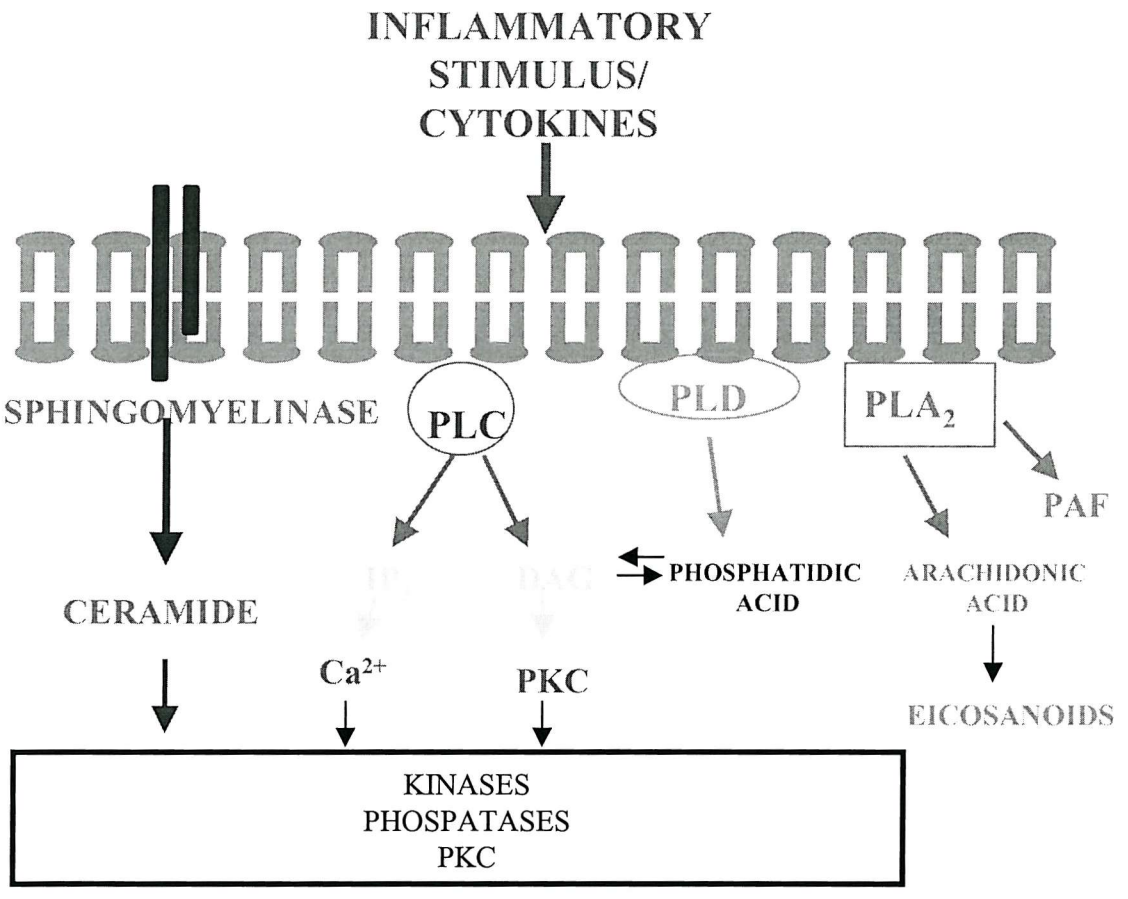
Intracellular Signalling Molecules

The generation of intracellular lipid mediators such as DAG or ceramide is ubiquitous to all cells but appears to be very important in the activation of immune cells (e.g. section 1.1.2.2.). The activation of specific phospholipases within cells via extracellular stimuli is critical in the generation of many of these intracellular mediators (*Figure 1.16.*). These phospholipases include PLA_2, PLC, phospholipase D (PLD) and sphingomyelinase. Each phospholipase is thought to be activated independently to give specific lipid-derived second messengers. Each lipid mediator will go on to generate signal cascades within the cell (Sumida *et al.,* 1993) (section 1.1.2.).

There are numerous lipid mediators (or second messengers) generated including NEFA (e.g. AA), prostaglandins (e.g. PGE_2), phosphatidic acid, platelet activating factor (PAF), DAG, IP_3 and ceramide. Different mediators are involved in different signalling pathways, and thus the type of second messenger released will profoundly influence the intracellular events which occur, and which ultimately lead to activation of nuclear transcription factors and the up- and down- regulation of gene transcription (Brenner, 1984). The mediator DAG is thought to be a positive effector in cell function while ceramide is generally thought to be a negative effector, except in the lymphocyte where ceramide has been found to have a positive role in proliferation (Jolly *et al.,* 1997). In addition, it is now known that fatty acids released from the cell membrane can directly bind to transcription factors (Clarke *et al*, 1999),

or act in an autocrine fashion and influence the activity of enzymes which released them. Thus modulation of the fatty acid composition of the membrane may ultimately influence gene transcription within an immune cell (see section 1.3.3.).

Figure 1.16. Phospholipase activity in the cell membrane after an extracellular stimulus



1.3.3 Changes in Fatty Acid Composition Can Affect Immune Cell Function

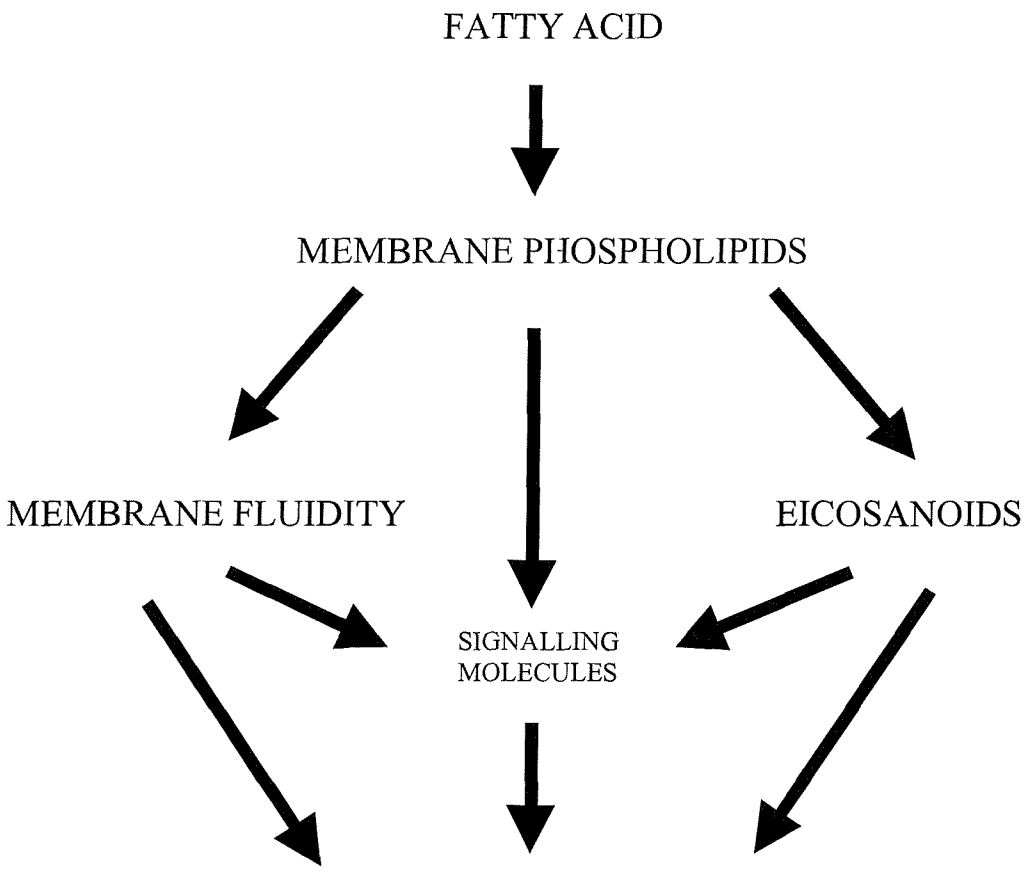
Changes in membrane fatty acid composition may affect the functioning of the cell. The mechanisms by which this can occur are interrelated but are based upon changes in the fatty acid composition of the membrane phospholipids (*Figure 1.17.*)

Fatty Acids and Membrane Fluidity

The physical properties of fatty acids determine the 'fluidity' of a membrane. These properties are dependent on chain length and degree of unsaturation of a fatty acid (Stubbs and Smith, 1984). Membrane fluidity comprises two parts. The first is the order of the membrane, which relates to the packing of the fatty acyl chains. The most ordered membrane is that in which all fatty acids are in the *trans* configuration. The second is the rotational or lateral movement of the fatty acyl chains. The presence of a double bond within a fatty acid chain renders it inflexible. As a result the fatty acyl chain will not pack as well with other chains and this leads to an increase in the average surface area per fatty acyl chain. The presence of a *cis* double bond increases the rate and range of movement of an acyl chain. Thus, when assessing the effect of unsaturated fatty acids on the membrane several factors must be considered: the number of double bonds, their position along the hydrocarbon chain and configuration (i.e. *cis* or *trans*), and the chain length.

The packing that fatty acids can attain is reflected in their melting point. The melting point is lowered with increasing number of double bonds present in the fatty acids as well as with decreasing chain length. Thus, fatty acids with short chain length and a high level of unsaturation will have a very low melting point. The position of the double bond in the chain is also important in this respect. The nearer the double bond to the centre of the fatty acid chain the lower the melting point.

Figure 1.17. Interrelationship between changes in cell fatty acid composition and cell function

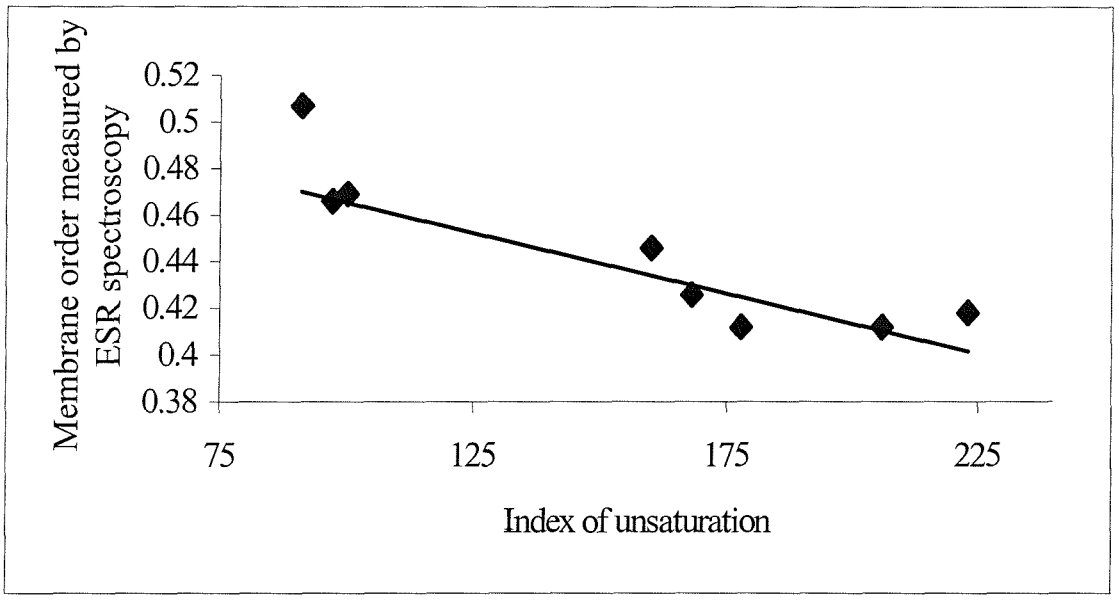


The fatty acid composition of the cell membrane, and therefore its degree of unsaturation, affects its fluidity. The fewer the number of double bonds, and thus the more saturated the membrane, the more rigid the membrane will be. This has been shown experimentally in macrophages (Mahoney *et al.,* 1980) and lymphocytes (Calder *et al.,* 1994a) (*Figure 1.18.*).

Cholesterol is also a key regulator of membrane fluidity (Stubbs and Smith, 1984). Cholesterol inserts into the lipid bilayer in such a way that it stearically blocks large

motions of fatty acyl chains and so decrease membrane fluidity (Stubbs and Smith, 1984). It has been shown that when human peripheral blood mononuclear cells (PBMNC) or isolated T-lymphocytes are activated there is a decrease in the ratio of cholesterol to phospholipids, with the concurrent increase in the proportion of unsaturated fatty acids in the cell membrane (Anel *et al.,* 1990). However, this was not the case for macrophages where no change in the phospholipid:cholesterol ratio was observed upon activation (Mahoney *et al.,* 1980). Thus, the influence of cholesterol on the fluidity of the membrane may be very important when considering the effects of unsaturated fatty acids on cellular function

Figure 1.18. The relationship between the degree of fatty acid unsaturation of the lymphocyte membrane and membrane fluidity. Drawn from data of Calder *et al.* (1994a).



Feeding animals diets of differing fatty acid composition has been shown to alter the fatty acid composition of immune cells (Yaqoob and Calder, 1995a). The same occurs in humans when unusual fatty acids are provided in the diet (Gibney and Hunter, 1993; Yaqoob *et al.,* 1995a). As indicated earlier such changes may influence the activity of proteins within the membrane. Receptor and enzyme functions have been shown to be modulated by alterations in the presence of certain fatty acids in immune cell membranes and are reviewed by Murphy, (1990) (section

1.3.3.). Examples include PGE_2 receptors (Opmeer, 1984), the TNF-α receptor in macrophages (Tappia *et al.,* 1997), and the Na^+/K^+ATPases present in lymphocytes (Poon *et al.,* 1981).

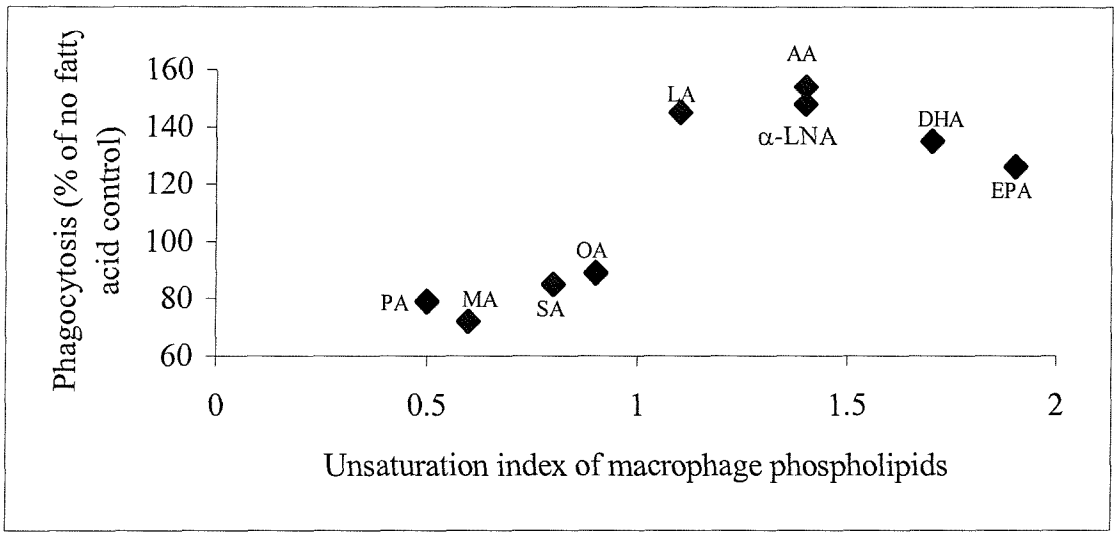
In addition to the membrane functions discussed so far there is another cellular process, which needs to be addressed: phagocytosis. Phagocytosis involves the extension of the cell membrane pseudopodia around an invading pathogen or dead/decaying cell and its subsequent endocytosis and degradation (*Figure 1.19.*) (see section 1.1.1.2.). The cell membrane plays a key role in this function and any changes in membrane composition may alter the cells capacity to phagocytose. The unsaturation of membrane phospholipids has been shown to affect the phagocytic activity of murine peritoneal macrophages (*Figure 1.18.*) (Calder *et al.,* 1990). When compared to macrophages cultured under standard conditions the rate of phagocytosis was increased by an increase in the unsaturation index of the membrane phospholipids, and thus perhaps by an increase in membrane fluidity. It is worth noting that the cells cultured with the EPA and DHA showed decreased phagocytosis compared to those cultured with the other long chain PUFA (linoleic, α-linolenic and arachidonic acids). It may be that there is a certain point at which the phagocytic activity of macrophages becomes inhibited by the membrane becoming too fluid. The inhibitory effect of increasing saturated fatty acid content of the macrophage membrane on phagocytosis has also been shown by Mahoney *et al.* (1980), who went on to demonstrate that this phenomena was reversible by adding unsaturated fatty acids to the culture medium.

Fatty Acids and the Modulation Signalling Pathways and Gene Expression

The fatty acid composition of the cell and organelle membranes may modulate gene expression by influencing the signal cascades which occur inside the cell as a result of a stimulus. The subsequent transcription of genes in the nucleus may become up- or down regulated and with respect to immune function this results in modulation of the production of cytokines or cell division. Although eicosanoids are known to regulate gene expression, some of the effects of fatty acids seem to be exerted in an eicosanoid-independent manner. There are two ways in which this could occur. The first involves the modulation of signal transduction pathways themselves (*Figure*

1.20.) and the second involves the binding of fatty acids and/or their derivatives directly to nuclear transcription factors (NF) thus altering their activity (*Figures 1.21.*).

Figure 1.19. The relationship between the degree of unsaturation of membrane phospholipids of macrophages and the ability of macrophages to perform phagocytosis. Drawn from data of Calder *et al.* (1990).



MA, Myristicic acid; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; α-LNA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

Fatty acids can modulate the signal transduction pathways of the cell at multiple stages. The primary influence of fatty acids is the modification of the cell membrane not only with respect to fluidity but also with regard to the fatty acid composition of phospholipid species (see section 1.3.2.). There is a considerable amount of experimental evidence which has shown that the introduction of different fatty acids *in vitro* can modulate immune cell membrane fatty acid composition (see section 1.3.2.). This is also true for the fatty acid composition of phospholipids of lymphocytes (Huang *et al.*, 1992) and macrophages (Marangoni *et al.*, 1993) after feeding diets rich in different proportions of fatty acids such as those found in fish oil.

The modulation of the cell membrane will then influence signalling mechanisms further downstream via the modulation of membrane-bound enzymes. The enzyme PLC is situated in the membrane and its activation is critical in the signal transduction of the TCR:CD3 complex when it binds an antigen (Rao, 1991). The PLCγ-1 isoform is activated by phosphorylation by tyrosine kinases (Liao *et al.,* 1993). The activity of PLC may be influenced by the fatty acid composition of the membrane in two ways: via modulation of the activity of PLC itself or the activity of tyrosine kinases. The tyrosine kinases of the cell are associated with specific areas of the membrane (Rao, 1991) and it may be that their activity requires a certain phospholipid composition and/or fluidity, and thus the fatty acid composition of the diet could modulate tyrosine kinase activity. In a study by Sanderson and Calder (1998) the tyrosine phosphorylation state of PLCγ-1 in activated lymphocytes was significantly altered according to the fatty acid composition of the diet.

The production of IP_3 by rat lymphocytes in response to Con A stimulation was also reduced by the feeding of fish or olive oil diets (Sanderson and Calder, 1998a). The modulation of the PLC enzymes, which produce IP_3, may be a mechanism that contributes to the modulation of the concentration of intracellular free Ca^{2+} from intracellular stores. The rise in cytosolic free Ca^{2+} in a cell can also be achieved through the opening of Ca^{2+} channels in the extracellular membrane. The long chain PUFA α-LNA, EPA and DHA act directly on the receptor-operated Ca^{2+} channel in the JURKAT T-cell line: when these cells were cultured with these fatty acids and stimulated via CD3 there was no increase in intracellular Ca^{2+} concentration (Chow *et al.,* 1990; Breittmayer *et al.,* 1993). This suggests that when the cell membrane is more unsaturated there is modulation of the receptor-operated Ca^{2+} channel or that fatty acids regulate the channel or receptor directly.

The fatty acid composition of DAG has been shown to affect the activity of PKC. DAG containing two oleic fatty acids or two arachidonic acids rather than two SFA or one SFA and one PUFA results in greater PKC activity (Kishmoto *et al.,* 1980). Likewise PKC is more active in the presence of PUFA-rich PS than PUFA-poor PS (Bell and Sargent, 1987). The type of fatty acid in the diet also affects the amount of DAG produced upon cell stimulation. DAG generation was decreased after Concanavalin A (Con A)-stimulation of lymphocytes from mice fed EPA or DHA

compared to those mice fed safflower-oil (Fowler *et al.,* 1993; Jolly *et al.,* 1997). This study also showed that EPA and DHA feeding reduced the production of ceramide in activated lymphocytes (Jolly *et al.,* 1997).

The cells of the immune system possess many transcription factors including NF kappa B (NFκB), NF of activated T cells (NFAT), AP-1 and various oncogenes (myc, fos, jun) (section 1.1.2.) as well as steroid hormone receptors and specific NF for cellular mediators (e.g. NF-IL-6) and adhesion molecules (NF-ICAM). The transcription factor NFκB regulates the synthesis of many cytokines (TNF-α, IL-1, -2, -6) and receptors such as IL-2R (Mukaida *et al.*, 1994). NFκB consists of three proteins and is activated when one of these, the inhibitory kappaB is phosphorylated and dissociates from the protein. The remaining dimer then translocates to the nucleus and binds to the relevant response element on target genes (*Figure 1.21.*). Since PKC phosphorylates inhibitory kappaB and PKC activity is modulated by the presence of certain PUFA perhaps via reduced PLC activity (Sanderson and Calder, 1998a), which results in reduced DAG and IP_3 generation and thus a reduced rise in intracellular Ca^{2+}, it would seem that the phosphorylation of inhibitory kappaB can be modulated by fatty acids. However, NFκB can also be activated by ceramide independently of PKC. It has been shown in murine lymphocytes that ceramide production is reduced by EPA and DHA feeding (compared to SFA and arachidonic acid feeding) (Jolly *et al.,* 1997) and thus this PKC independent mechanism may also contribute to the reduction in cell functional responses.

Figure 1.20. Mechanisms by which the incorporation of PUFA into cell membranes may influence downstream signalling events (adapted from Miles and Calder, 1998).

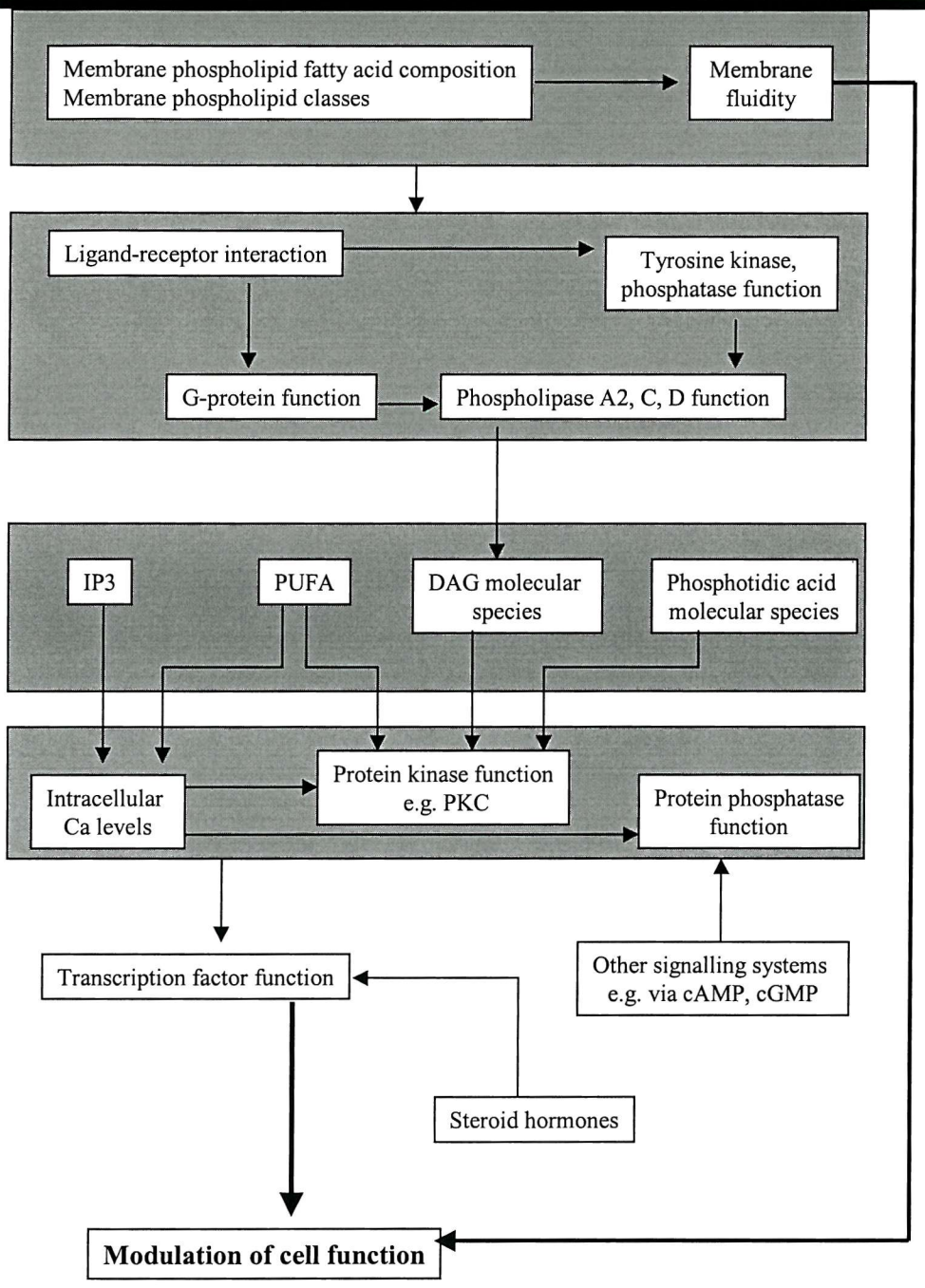
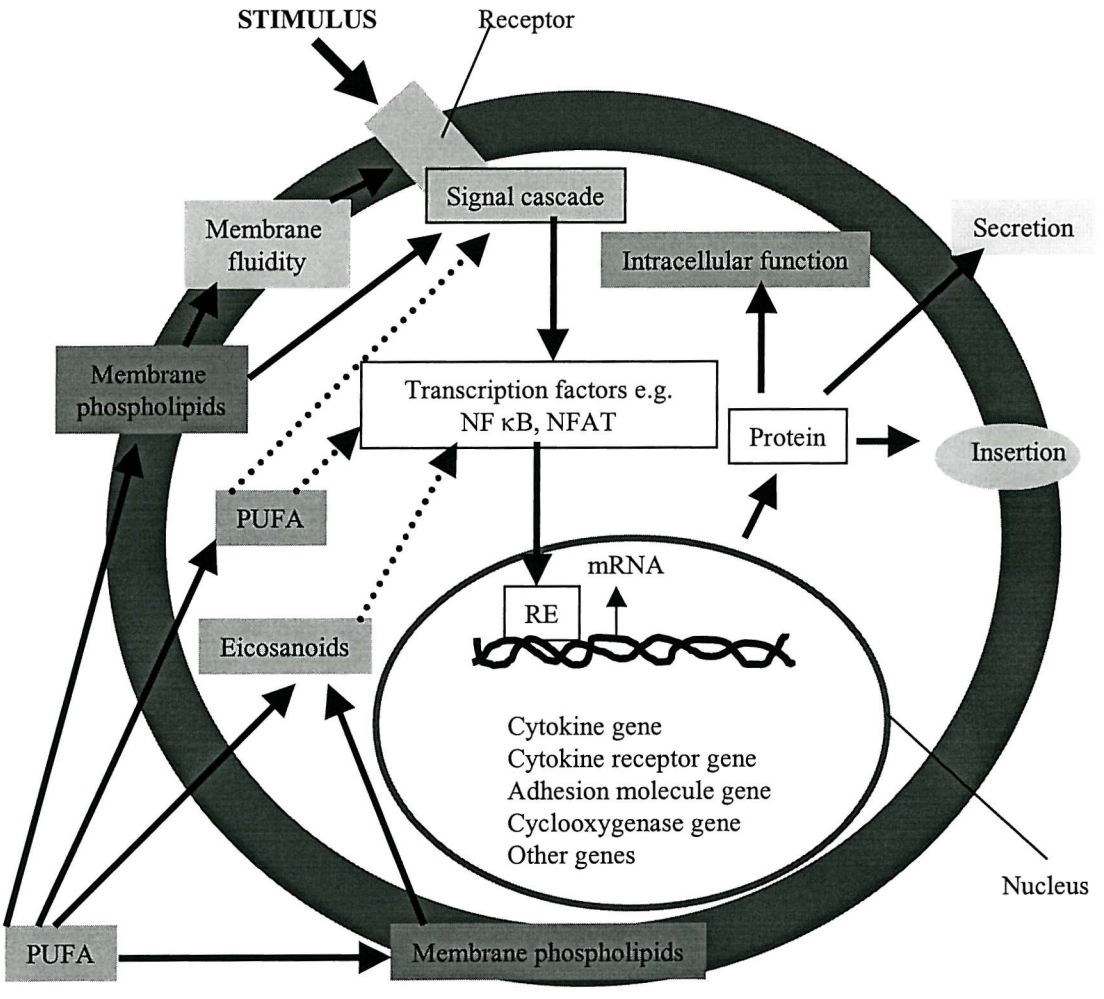


Figure 1.21. The mechanisms by which PUFA may influence gene expression (adapted from Miles and Calder, 1998); RE, response element.



Fatty Acids and Eicosanoids

The release of a fatty acid from the membrane, either by the action of PLA_2 on PC or by PLC and DAG lipase on PIP_2, may result in it becoming a substrate for the COX and LOX enzymes. The principal substrate is arachidonic acid but EPA can also be used as a substrate for eicosanoid synthesis. The pathways for the metabolism of arachidonic acid and EPA have been discussed already in section 1.1.3.2. The

products made from different fatty acids have slightly different biological effects. For example, the products of EPA metabolism are less potent in their actions than those from arachidonic acid (Lee *et al.,* 1992). EPA and DHA competitively inhibit the oxygenation of arachidonic acid by COX enzymes. Thus, enriching cell membranes with fatty acids such as EPA and DHA will have an effect on immune cell function by modulating the eicosanoids produced. When the diet of healthy subjects was supplemented with fish oil (providing 3.2 g EPA + 2.2 g DHA/ d) for 6 weeks the production of LTB_4 was reduced with a concomitant increase in the production of LTB_5 (Lee *et al.,* 1985). The production of PGE_2 has also been shown to be decreased after fish oil feeding in humans (Meydani *et al.,* 1991).

1.4 Fatty Acids and the Immune System

This section aims to review the experimental evidence, which has accumulated from a variety of studies where the fatty acid composition and/or the function of immune cells have been altered. The methods used to investigate these parameters will be outlined and the section will then go on to compare the many *in vitro* experiments and their results. The results of dietary feeding studies in both animals and humans will then be examined. Each sub-section will give a comprehensive overview of the evidence in that area and it is hoped that differences in experimental results will be resolved to some extent. The long chain PUFA, α-LNA, EPA and DHA will be examined in the greatest detail, since these are the fatty acids of most relevance to the studies described later in this thesis.

1.4.1 Historical Background to the Present Work

Epidemiological studies have shown that there are differences in the incidence of certain diseases among different populations. For example, it has been noted that there is a lower incidence of inflammatory and autoimmune diseases such as psoriasis, multiple sclerosis and type 2 diabetes mellitus in the Eskimo (*Table 1.7.*) compared with sex- and age-matched groups living in Denmark population (Kromann and Green, 1980). The traditional Eskimo diet was rich in seal meat and whale blubber and contained about 7 g/d of *n*-3 PUFA (Bang and Dyerberg, 1972). It

had a PUFA to SFA ratio of 0.84, as compared to a typical Danish diet with a ratio of 0.24. It was postulated that the immunomodulatory effects of the *n*-3 fatty acids were responsible for the reduction in the occurrence of inflammatory diseases in the Eskimo population (de Deckere *et al.,* 1993).

The fatty acids in the diet interact with the cells of the immune system in several ways. This is predominantly thought to be via the manipulation of the lipid composition of their environment, leading to changes in their fatty acid composition which in turn will affect membrane fluidity and the generation of eicosanoids and cell signalling mediators (section 1.3.3.). The effects of the different fatty acids upon immune cell function will be discussed in the rest of this section.

Table 1.7. Distribution of inflammatory and autoimmune diseases in Greenland Eskimos (1950-1974). (Taken from Kromann and Green, 1980)

Disease	**Number of cases***	**Expected number of cases***
Cancer (all types)	46	53
Stroke	25	15
Epilepsy	16	8
Peptic ulcer	19	29
Multiple sclerosis	0	2
Psoriasis	2	40
Bronchial asthma	1	25
Diabetes mellitus	1	9
Acute myocardial infarction	3	40

*(per 1000 people)

1.4.2 Methods Used to Investigate the Effects of Fatty Acids on the Immune System

There are several techniques which are used to ascertain the status and response of the immune system; these techniques often involve measures of the function of immune cells taken from the bloodstream and examined *ex vivo* (*Table 1.8.*). The states of components of the immune system can also be assessed *in vivo* using several techniques. These include:

- measurement of the levels of circulating concentrations of Ig subclasses, complement proteins, cytokines and cytokine receptors

- measurement of the circulating antibody response to immunisation
- measurement of the concentration of secretory IgA in saliva
- measurement of the delayed-type hypersensitivity response to intradermal application of antigens to which the individual has already been exposed so assessing the cell-mediated immune response, in terms of the size of the swelling (termed induration) around the area of application at a period (usually 48 hr) after the application.

Table 1.8. The techniques used to assess immune cell functions *ex vivo*.

Cell type	Measurement technique
Lymphocytes	Proliferation - Lymphocytes can be made to divide in response to a variety of stimuli. These substances are called mitogens and include concanavalin A (Con A) and phytohemagglutinin (PHA) which stimulate T-lymphocytes and protein A which stimulates B-lymphocytes. The lymphocyte proliferative response is often measured by following the incorporation of [^{3}H] thymidine into the DNA of cells.
Lymphocytes and Monocytes	Cytokine production – These immune cells produce cellular mediators such as cytokines when they are stimulated by a mitogen (lymphocytes) or bacterial lipopolysaccharide (LPS) (monocytes). The concentrations of these mediators can be measured in the culture medium after lymphocytes, monocytes or peripheral blood mononuclear cells (PBMNCs – which include lymphocyte and monocyte cell populations) have been cultured for varying lengths of time.
NK Cells	Tumouricidal capacity – NK cell activity is measured in terms of the ability of the NK cells to kill specific target cells. For example human NK cells will target the K562 tumour cell line.
Neutrophils and Monocytes	Phagocytic Activity – Neutrophils and monocytes can engulf foreign particles or dead cells. If the target particle or cell is labelled, for example with a fluorescent marker, the capacity of the neutrophil or monocyte to perform phagocytosis can be measured.
Neutrophils and Monocytes	Chemotaxis – The movement of neutrophils and monocytes towards chemoattractants, such as LTB_4, FMLP, autologous serum and plasma and PAF can be measured.
Neutrophils and Monocytes	Respiratory Burst – The production of superoxide and hydrogen peroxide by these cells can be measured. Stimuli used to induce the respiratory burst include bacteria, zymosan (bacterial cell walls), latex particles, PAF, phorbol esters (e.g. PMA) and FMLP.

FMLP – Formyl-methionylleucylphenylalanine; LTB_4 - Leukotriene B_4; NK cell – Natural Killer cell; PAF – Platelet activating factor; PHA, Phytohemagglutinin; PMA – Phorbol myristic acetetae.

1.4.3 *In vitro* Studies of Fatty Acids on Cells of the Immune System

1.4.3.1 T-Lymphocyte Functions

Lymphocyte Proliferation

A number of *in vitro* studies have investigated the effects of fatty acids on lymphocytes in culture. The results of these experiments are summarised in *Table 1.9.* The consensus of these experiments is that fatty acids inhibit lymphocyte proliferation and that unsaturated fatty acids, such as EPA and AA, are more inhibitory than the shorter chain, saturated fatty acids, such as SA and PA (Calder *et al.,* 1991).

The order of potency of the saturated fatty acids appears to be SA>PA>MA (Calder, 1995). The differences observed in the potencies of different fatty acids observed by different experimenters can most likely be explained by the diversity of the experimental conditions, which are indicated in *Table 1.9.* The use of different stimuli and the different forms of presentation of the fatty acid, differences in the types of lymphocyte (animal and human) and the anatomical site of origin of the lymphocyte, may all contribute to the difference observed in potencies of the fatty acids. Variability between lymphocytes from different subjects in the same experiment also contributes to the differences observed (Virella *et al.,* 1991).

A comprehensive experiment, which investigated the effects of the concentration of fatty acids, duration of exposure of the cells to fatty acids and the time of addition of fatty acids to the culture medium helped to establish the importance of the culture conditions on the outcome of the experiment (Calder *et al.,* 1991). Fatty acid concentration profoundly influences the cell response. Calder *et al.* (1991) observed that above a certain concentration (about 5 μM) the fatty acids tested were predominantly inhibitory, while below this concentration there can be inhibitory or stimulatory effects depending on the fatty acid. The longer the cells are exposed to the fatty acid in the culture the more inhibitory the effect, and this occurred whether the fatty acid is added before, at the same time or after the Con A stimulus (Calder *et*

al., 1991). Many of the other experiments summarised in *Table 1.9.* have also demonstrated a dose-dependent effect of certain fatty acids on the proliferation of lymphocytes (e.g. Soyland *et al.,* 1993).

The inhibition of proliferation of lymphocytes by unsaturated fatty acids is not dependent on an eicosanoid pathway since inhibitors of phospholipase A_2, cyclo-oxygenase and lipoxygenase enzymes do not prevent this inhibition (Calder *et al.,* 1992a; Soyland *et al.,* 1993; Rotondo *et al.,* 1994; Khalfoun *et al.,* 1996b). In addition to this PGE_2 levels have been observed not to be different from the control (Calder *et al.,* 1991) or not correlated with (Calder *et al.,* 1992a) the fatty acid inhibition of lymphocyte proliferation.

T-Lymphocyte Cytokine Production

Another measure of lymphocyte function is the production of cytokines (*Table 1.10.*). There is only limited literature on the effect of fatty acids on T-lymphocyte-derived cytokine production, and work in this area was not conducted until relatively recently. The level of the cytokine IL-2 has been measured in the medium of cells cultured with various fatty acids, perhaps since it is the principal cytokine involved in lymphocyte proliferation (*Table 1.10*). The only study to investigate the effects of saturated fatty acids on IL-2 production found that they had little or no effect (Calder and Newsholme, 1992a,b).

Table 1.9. Effects of fatty acids on lymphocyte proliferation *in vitro*

Cell Type	Stimulus	Fatty acid	Form	Effect	Reference
Human PBL	PHA	PA, SA, OA, LA, AA	Ethanol	↓ SA>OA>LA>AA>PA	Mertin *et al.* (1975)
Human PBL	PHA	PA, SA, OA, LA, AA	BSA	↓OA>PA>AA>LA>SA	Weyman *et al.* (1977)
Human PBL/murine spleen lymphocytes	PHA	MA, PA, SA, OA, LA, AA	Ethanol	↓ MA>OA>PA>SA	Tsang *et al.* (1977)
Human PBL	Con A	OA, LA, AA, DGLA, α-LA	Ethanol	0.1-5.0μg/ml dose-dependent↑; >5.0 μg/ml dose-dependent↓ AA>Others	Kelly & Parker (1979)
Murine spleen lymphocytes	Con A/PHA	PA, SA, OA, LA	BSA	Dose-dependent↓ OA>LA>PA>SA	Buttke *et al.* (1984)
Human PBL (IL-2 dependent)	IL-2	PA, OA, DGLA, AA, EPA	Ethanol	Dose-dependent ↓ DGLA=AA>EPA; no effect of PA or OA	Santoli *et al.* (1990)
Rat lymph node lymphocyte	Con A	EPA, AA, LA, OA, DHA,SA, α-LNA, PA, MA	BSA	EPA>AA>LA>OA>DHA >SA>α-LNA>PA>MA	Calder *et al.* (1991)
Human PBL	Anti-CD3	AA, EPA	Ethanol	Dose-dependent ↓ EPA>AA	Virella *et al.*, (1991)
IL-2-dependent cell line	Con A	DGLA, AA, EPA, OA	Ethanol	↓ DGLA>AA; no effect EPA, OA	Borofsky *et al.* (1992)
Human PBL	Con A	MA, PA, SA, OA, LA, α-LA, AA, EPA, DHA	BSA	Dose-dependent ↓ EPA>AA>LA>OA>DHA > SA> α-LA>PA>MA	Calder and Newsholme (1992a)
Human PBL/rat spleen lymphocytes	PHA/Con A	EPA, DHA		↓	Brouard & Pascaud (1993)
Human PBL	PHA	MA, PA, OA, α-LA, AA, EPA, DHA	BSA	Dose-dependent ↓ DHA>EPA>AA>LA; No effect of α-LA, OA, MA, PA	Soyland *et al,* (1993)
Rat lymph node/ thymus/ spleen lymphocytes	Con A	MA, PA, SA, OA, LA, α-LA, AA	TAG	Dose-dependent ↓ AA>α-LA>LA; No effect of PA, MA, SA, OA	Calder *et al.* (1994b)
Human PBL	PHA	LA, α-LA, GLA, AA, EPA, DHA	DMSO	Dose-dependent ↓ GLA>α-LA>EPA>AA>DHA>LA	Devi & Das (1994)
Murine thymus lymphocytes	IL-1	DGLA, AA, EPA, LA, α-LA, γ-LA, PA, OA	Ethanol	0-100μM Dose-dependent ↓ DGLA>AA>EPA>LA>α-LA>γ-LA No effect of PA or OA at 100μM AA>EPA>DGLA>LA>	Rotondo *et al,* 1994
Human PBL	PHA	AA, DHA, EPA	BSA	Dose-dependent ↓ EPA>DHA>AA	Khalfoun *et al,* (1996b)
Human PBL	PHA/Con A	γ-LNA, EPA, DHA	Ethanol	Dose-dependent ↓ EPA>DHA>γLNA	Purasiri *et al.* (1997)

α-LNA, α-linolenic acid; γ-LNA, γ-linolenic acid; AA, arachidonic acid; BSA, bovine serum albumin; Con A, concanavalin A; DGLA, dihomo γ-linolenic acid; DHA, docosahexaenoic acid; DMSO, dimethyl sulphoxide; EPA, eicosapentaenoic acid; LA, linoleic acid; LPS, lipopolysaccharide; MA, myristic acid; OA, oleic acid; PBL, PA, palmitic acid; Peripheral blood lymphocyte; PHA, phytohaemagglutinin;. SA, stearic acid

The longer chain, unsaturated fatty acids have been more thoroughly investigated and these seem to concur in that α-LNA, EPA and DHA significantly inhibit IL-2 production, by up to 90% (Virella *et al.,* 1991; Calder and Newsholme, 1992a,b; Purasiri *et al.,* 1997), while the effect of AA is a little more unclear. The study of Purasiri *et al.* (1997) also measured IFN-γ and reported that there was a decrease in production of IFN-γ when cells were cultured with DHA but there was no effect of GLA or EPA.

Table 1.10. Effects of fatty acids on lymphocyte IL-2 production

Cell Source	Stimulus	Fatty Acid	Form	Effect	Reference
Rat lymph nodes	Con A	MA, PA, SA, OA, LA, α-LNA, AA, EPA, DHA	BSA	No effect of MA, SA, PA; ↓ EPA> DHA>AA>LA>α-LNA> >OA	Calder and Newsholme (1992b)
Human blood	Con A	MA, PA, SA, OA, LA, α-LNA, AA, EPA, DHA	BSA	No effect of MA, PA, SA; Time-dependent ↓ EPA>AA>LA>α-LNA>DHA>OA	Calder and Newsholme (1992a)
Human blood	PHA	LA, α-LA, GLA, AA, EPA, DHA	DMSO	Dose-dependent ↓ LA>DHA>AA>EPA >α-LNA>GLA	Devi & Das (1994)
Human blood	Anti-CD3	EPA, AA	Ethanol	↓ EPA; AA no effect	Virella *et al.* (1991)
Human blood	PHA/ Con A	GLA, EPA, DHA	Ethanol	Significant ↓ EPA=DHA>GLA	Purasiri *et al.* (1997)

α-LNA, α-linolenic acid; GLA, γ-linolenic acid; AA, arachidonic acid; BSA, bovine serum albumin; Con A, concanavalin A; DGLA, dihomo γ-linolenic acid; DHA, docosahexaenoic acid; DMSO, dimethyl sulphoxide; EPA, eicosapentaenoic acid; LA, linoleic acid; LPS, lipopolysaccharide; MA, myristic acid; OA, oleic acid; PBL, PA, palmitic acid; Peripheral blood lymphocyte; PHA, phytohaemagglutinin;. SA, stearic acid

T-Lymphocyte Surface Molecule Expression

Adhesion molecules are involved in many cell-cell interactions. It has been shown that EPA and DHA can reduce the surface expression of L-selectin (CD62L) and leukocyte-function-associated antigen 1 (CD11a and CD18) on activated human peripheral blood lymphocytes (Khalfoun *et al.,* 1996a). In contrast, the presence of AA in the culture medium had no effect on the surface molecule expression of these cells. This study also showed a reduction in the adhesion between endothelial cells left untreated or stimulated with cytokine or lipopolysaccharide, and human lymphocytes which had been cultured with EPA or DHA.

Lymphocyte-Mediated Cytolysis

The efficiency of cytotoxic lymphocyte (Tc) killing of target cells in vitro is thought to be reduced by OA, LA and AA, since there is a marked reduction in the ability of rat Tc cells to release the contents of granules into the extracellular medium when these fatty acids are present (Richieri & Kleinfeld 1990). In contrast, there was no effect of SA on this process. The presence of EPA or DHA in culture medium suppresses the activity of NK cells from human peripheral blood (Yamashita et al., 1986; Yamashita et al., 1991; Purasiri et al., 1997) or mouse spleen (Yamashita et al., 1988) (*Table 1.11*).

Table 1.11. Effects of fatty acids on NK cell-mediated cytolysis

Cell	Stimulus	Fatty Acid	Form	Effect	Reference
Human PBL	^{51}Cr-labelled K562 cells	Tri-EPA	TAG emulsion	Dose-dependent ↓	Yamashita *et al.* (1986)
Murine spleen cells	^{51}Cr-labelled YAC-1 cells	Tri-EPA	TAG emulsion	Dose-dependent ↓	Yamashita *et al.* (1988)
Human PBL	^{51}Cr-labelled K562 cells	Tri-EPA, Tri-DHA	TAG emulsion	Dose-dependent ↓; DHA>EPA	Yamashita *et al.* (1991)
Human PBL	^{51}Cr-labelled K562 cells	EPA, DHA, GLA	Ethanol	EPA=DHA > γ-LA	Purasiri *et al.* (1997)

γ-LA, γ-linolenic acid; EPA, eicosapentaenoic acid; Tri-EPA, EPA as TAG; DHA, docosahexaenoic acid; TAG, triacylglycerol

Summary of the Effects of Fatty Acids on T-Lymphocytes in vitro

In summary, it appears that incubation of immune cells with fatty acids *in vitro* has the ability to modulate their function. Incubation of lymphocytes with the longer chain PUFA results in a greater reduction of *in vitro* proliferation of lymphocytes compared to incubation with the shorter chain and more saturated fatty acids. This is true for a range of stimuli and cell types and for different modes of fatty acid addition. The inhibition of proliferation is dependent on the dose of the fatty acid. Studies of the effect of fatty acids on IL-2 production show that the longer chain

PUFA reduce IL-2 production while shorter chain more saturated fatty acids have little or no effect on the production of this mediator. Since the cytokine IL-2 is intimately involved in the initiation and continuation of the proliferative response (section1.1.2.2.), it is reasonable to postulate that the inhibition of lymphocyte proliferation by PUFA is due to a decrease in the concentration of IL-2. However since the shorter chain, more saturated fatty acids can also inhibit proliferation to some extent without a concurrent decrease in IL-2, there may be a second mechanism that is IL-2 independent by which fatty acids modulate the proliferative response.

The incubation of lymphocytes with different fatty acids also results in the modulation of cell adhesion and the killing capacity of NK cells. The longer chain fatty acids EPA and DHA are very effective at reducing these cell functions.

1.4.3.2 B-Lymphocyte Functions

There is very little experimental data in this area of fatty acids and immune function. One study has shown that there are differential effects of fatty acids on the production of Ig molecules by B lymphocytes. The presence of the unsaturated fatty acids, AA or LA at concentrations less than 100 μM did not affect the production of IgM, IgG, IgA and IgE by cultured rat mesenteric lymph node lymphocytes (Yamada *et al.,* 1996). At a concentration of 1 mM there was a distinct inhibition of the production of these Ig, except IgE which was increased. When these cells were incubated with α-LA, EPA or DHA an increase in the production of IgE was also seen (Yamada *et al.,* 1996). These effects seem to link in with the pattern of cytokine production by Th1 and Th2 cells. The production of IgG is promoted by Th1-type cytokines and the production of IgE is promoted by Th2-type cytokines. Unsaturated fatty acids inhibit the production of IL-2 (section 1.4.3.1.), a key cytokine in the Th1 response, and thus inhibit this response. This alters the balance between these two pathways and thus could lead to a positive effect on the Th2 response and so to an increase in IgE production (Calder, 1998).

1.4.3.3 Monocyte Functions

Monocyte Cytokine Production

The production of cytokines by monocytes has been measured in only one *in vitro* study. EPA and DHA both inhibited the production of IL-1β by human PBMNC stimulated with LPS, while EPA, DHA and γ–LA inhibited TNF-α production, and had no effect on IL-6 (Purasari *et al.,* 1997).

Phagocytosis

Early experiments showed that the presence of different fatty acids, namely PA, OA, EPA and DHA in the culture medium could influence phagocytosis by macrophages (Schroit and Gailly, 1979; Mahoney *et al.,* 1980; Lokesh and Wrann, 1984). A more recent study has shown that there is a positive correlation between the phagocytosis of unopsonised and opsonised zymosan and the % PUFA, and the ratio of unsaturated to saturated fatty acids of macrophage phospholipids (Calder *et al.,* 1990) (*Figure 1.18.*). Macrophages enriched with palmitic or myristic acid had a 28% and 21% decrease in their ability to phagocytose unopsonised zymosan, while those enriched with PUFA had a 25-55% enhanced phagocytic ability (Calder *et al.,* 1990) (*Figure 1.18.*).

Expression of Surface Molecule Expression

When murine thioglycollate-elicited peritoneal macrophages were cultured with EPA or DHA the cells were found to be less adherent to artificial surfaces than those cultured with saturated fatty acids (Calder *et al.,* 1990). The presence of α-LNA had no effect on cell adhesion. The macrophages cultured with more saturated fatty acids (OA, SA, PA and MA) had an increased ability to adhere to tissue culture plastic (Calder *et al.,* 1990). Another study has shown that the ability of macrophages to present antigen may be affected by the fatty acid in which the cells are cultured: EPA was shown to inhibit this function (Fujikawa *et al.,* 1992). Since adhesion molecules are intimately involved in the process which results in the presentation of antigen, these observations together suggest that the long chain PUFA have an inhibitory

effect on the expression of cell adhesion molecules, in addition to the expression of MHC-II.

In a more recent study the expression of cell surface markers such as intracellular adhesion molecule-1 (ICAM-1), and the MHC-II molecule which are critical in the initiation of an immediate immune response has been measured. The expression of ICAM-1 was found to be inhibited in a dose-dependent manner when unstimulated human monocytes are cultured with EPA (Hughes *et al.,* 1996). The same experiment with the DHA saw an increase in ICAM-1 expression. This study also showed that when human monocytes are cultured in the presence of IFN-γ (which activates the cells and thus upregulates the expression of MHC class-II molecules), both EPA and DHA inhibit the expression of MHC II (Hughes *et al.,* 1996). This inhibition has also been seen in mice peritoneal macrophages cultured with *n*-3 PUFA, with DHA having a more potent effect (Khair-El-Din *et al.,* 1995). When the two fatty acids are combined at a ratio similar to that found in some fish oils (60% EPA and 40% DHA) there is no inhibition of expression of MHC class-II molecules on unstimulated monocytes, but this is not the case for activated monocytes where a significant inhibition of expression was seen (Hughes and Pinder, 1997). In addition, there was an inhibition of ICAM-1 expression for both stimulated and unstimulated monocytes cultured with the combination of EPA and DHA (Hughes & Pinder, 1997).

Summary of the Effects of Fatty Acids on Monocyte Function in vitro

The observations of the effects of the longer chain PUFA on monocyte cytokine production, ability to phagocytose and the expression of cell surface markers including adhesion molecules, together suggest, that these fatty acids have strong modulatory effects on these parameters of monocyte function *in vitro*. The inhibitory effects on cytokine production and expression of surface molecules are seen in both monocytes and lymphocytes, and it may be that these are due to a similar effect of the long chain PUFA on cell signalling mechanisms. The increased ability of macrophages cultured with PUFA to phagocytose may well be attributed to changes in cell membrane fluidity (section 1.3.3.).

1.4.4 *The Effects of Dietary Fatty Acids on Immune Cell Functions Tested* ex vivo

In vitro studies have shown that the functions of immune cells can be modulated by fatty acids (section 1.4.2.). However, the conditions of these experiments are somewhat artificial. For example, the concentrations of fatty acids used may not be physiological, although these experiments have provided a starting point for further work. The addition of specific fatty acids to the diet will enable us to establish whether or not the same or similar effects on the immune system could be achieved through this more indirect (but more physiological) route of exposure of immune cells to fatty acids. The experiments related to these dietary studies have been performed with animals and human volunteers.

In many of the studies that have been carried out there are several aspects of fat intake that have been addressed. These include the amount of fat in the diet and the effects of the different types of fat i.e. SFA, MUFA, and *n*-6 and *n*-3 PUFA, and the ratio of these different fatty acids e.g. the *n*-6: *n*-3 PUFA. These studies have elucidated the differential effects of fat in the diet on the immune system and an overview of these experimental results will be given. The *n*-3 PUFA α-LNA, EPA and DHA will be dealt with in the most detail since these are the fatty acids of most relevance to the studies described later in this thesis.

1.4.4.1 Lymphocyte Functions

Lymphocyte Proliferation - Animal studies

Many studies in this area have investigated the effects of different types of fat in the diet on immune function (reviewed by Calder, 1998), and the results of these studies will be outlined. It was not until more recently that the effects of individual fatty acids were studied in controlled conditions, and those concerned with the *n*-3 PUFA will be examined in the greatest detail.

High Fat vs Low Fat Feeding

The effect of 'high' fat versus 'low' fat feeding has been investigated in many animal studies dating back to the 1960s (Calder and Yaqoob, 2000). It has been shown that feeding high-fat diets (20% fat by weight) to rats and mice inhibits *ex vivo* lymphocyte proliferation when compared to very low-fat feeding (<5% fat by weight) (e.g. Yaqoob *et al.,* 1994).

Effects of Feeding n-6 PUFA-Rich Diets

Several studies have shown that feeding rats a diet rich in corn, safflower or sunflower oils (rich in *n*-6 PUFA i.e. LA) results in the inhibition of proliferation of rat splenocytes compared to feeding a diet high in SFA such as lard, tallow or hydrogenated coconut oil, or a low-fat diet (Kollmorgen *et al*, 1979; Erickson *et al.,* 1980; Levy *et al.,* 1982; Locniskar *et al.,* 1983; Morrow *et al.,* 1985; Marshall and Johnstone 1985; Yaqoob *et al.,* 1994). In contrast some studies have shown that there is no effect of a diet rich in *n*-6 PUFA (Cathcart *et al.,* 1995), or even an enhancement of proliferation (Ossman, 1980). Since a high-fat diet rich in SFA has the least effect on lymphocyte proliferation (compared with low-fat feeding) it is often used as a control when investigating the effects of diets rich in other fatty acids such as *n*-6 and *n*-3 PUFA.

Effects of Feeding n-3 PUFA-Rich Diets

Feeding a high-fat diet rich in linseed oil (called flaxseed oil in North America) compared to a diet containing hydrogenated coconut oil (Marshall and Johnstone, 1985) or sunflower oil (Jeffrey *et al.,* 1996) resulted in a decrease in rat splenocyte proliferation. In chickens a linseed oil-rich caused an inhibition of lymphocyte proliferation diet compared to corn, lard or canola oil-rich diets (Fritsche *et al.,* 1991).

The effects of fish oil on lymphocyte proliferation are well documented (*Table 1.12.*). In most studies a fish oil diet was compared to feeding a high-fat diet rich in hydrogenated coconut, safflower, corn or linseed oils (Kelley *et al.,* 1988, Yaqoob *et al.,* 1994; Yaqoob and Calder, 1995a; Sanderson *et al.,* 1995). These studies all conclude that when fish oil contributes to between 5% and 20% of fat in the diet

there is a significant inhibition of the ability of lymphocytes to proliferate (*Table 1.12*).

Table 1.12. Effects of dietary *n*-3 PUFA on lymphocyte proliferation

Cell Source	Diet used	Effect	Reference
Rat spleen	10% linseed oil, 3 weeks	Inhibition	Marshall and Johnstone (1985)
Mouse spleen	20% fish oil, 8 weeks	Inhibition	Alexander and Smythe (1988)
Rabbit spleen	7.6% fish oil, 20 weeks	Inhibition	Kelley *et al.* (1988)
Rabbit blood	7.6% fish oil, 20 weeks	Inhibition	Kelley *et al.* (1988)
Chicken spleen	7% linseed or fish oil, 3 weeks	Inhibition	Fritsche *et al.* (1991)
Rat spleen	20% fish oil, 10 weeks	Inhibition	Yaqoob *et al.* (1994)
Rat lymph node	20% fish oil, 10 weeks	Inhibition	Yaqoob *et al.* (1994)
Rat thymus	20% fish oil, 10 weeks	Inhibition	Yaqoob *et al.* (1994)
Rat blood	20% fish oil, 10 weeks	Inhibition	Yaqoob *et al.* (1995)
Rat spleen	15% rapeseed oil, 8 weeks	Inhibition	Calder *et al.* (1995)
Rat spleen	20% fish oil, 12 weeks	Inhibition	Sanderson *et al.* (1995)
Mouse spleen	20% fish oil, 8 weeks	Inhibition	Yaqoob and Calder, (1995a)
Rat spleen	20% linseed oil, 10 weeks	Inhibition	Jeffrey *et al.* (1996)
Pig lymph node	5% fish oil, 6 weeks	Inhibition	Thies *et al.* (1999)

Effects of Controlled Addition of Individual Dietary Fatty Acids

More recent studies have gone on to look at changes in individual fatty acids in the diet rather than of dietary oils which contain blends of one or more fatty acids.

Previous studies (*Table 1.12.*) have not established whether the effects of fish oil on proliferation are attributable to EPA or DHA or both. Some recent studies have addressed this question (*Table 1.13.*). These studies have been useful since they have helped to determine the differential effects of the component fatty acids of fish oil. Feeding a diet containing 10 g/kg of EPA or DHA plus 20 g/kg of safflower oil as fat, reduced spleen lymphocyte proliferation by 80% as compared to a diet containing 30 g/kg safflower oil (Jolly *et al.,* 1997). This study showed no differential effects of EPA and DHA. A more recent study has gone on to elucidate the level of *n*-3 PUFA in the diet, which will modulate lymphocyte proliferation. The levels of EPA or DHA used were 6.6 or 4.4 g/100 g of fatty acid, the *n*-6: *n*-3 PUFA ratio was kept constant by the addition of α-LNA to replace EPA or DHA, and the fat content was 178 g /kg. It was found that at 4.4 g/100g of fatty acids there was inhibition of proliferation, and this was equal for both EPA and DHA (Peterson *et al.,* 1998a). The inhibition of proliferation was however only 30-35% compared with

a control diet containing α-LNA. This study has helped to establish a better lower limit for the amount of EPA and/or DHA which must be consumed to have an immuno-modulatory effect.

Table 1.13. The effects of controlled addition of individual fatty acids on lymphocyte proliferation.

Cell source	Diet	Effect	Reference
Monkey blood	30% energy as fat; SFA, MUFA, PUFA constant; LA replaced by EPA+DHA (1.3 or 3.3 % energy) or α-LNA (3.5 or 5.3% energy); 14 weeks	No effect of α-LNA Increased proliferation as level of EPA+DHA increased	Wu *et al.* (1996)
Rat spleen	17.8% fat containing low (17.8g/kg fat) or high (35g/kg fat) proportions of total PUFA and varying *n*-6:*n*-3 PUFA (total SFA and MUFA constant); 6 weeks	Proliferation decreased as *n*-6:*n*-3 PUFA ratio of low-PUFA diet decreased Little effect of *n*-6:*n*-3 PUFA ratio of high-PUFA diet At *n*-6:*n*-3 ratio of 100 or 20, proliferation lower for high-PUFA than low-PUFA-fed animals	Jeffrey *et al.* (1997c)
Mouse spleen	30 g SO/kg v. 20g SO/kg plus 10g arachidonic acid or EPA or DHA/kg; 10 days	No effect of arachidonic acid Proliferation decreased by replacement of LA with either EPA or DHA	Jolly *et al.* (1997)
Rat spleen	178g/kg fat with SFA, MUFA, total PUFA and *n*-6:*n*-3 constant; α-LNA (4.4 g/100g fatty acids) replaced with either EPA or DHA	Proliferation decreased by inclusion of either EPA or DHA in the diet	Peterson *et al.* (1998a)

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PA, palmitic acid; OA, oleic acid; LA, linoleic acid; α-LNA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SO, safflower oil.

The effect of varying the total PUFA content in the diet has also been investigated. This was achieved by varying the total amount of PUFA (17.5 or 35 g/100g fatty acids) and the *n*-6: *n*-3 PUFA ratio (Jeffrey *et al.*, 1997b). The latter was achieved by replacing a proportion of palmitic acid with LA or α-LNA. The ratios achieved were 100, 20, 10, 5 and 1. This study showed that as the *n*-6:*n*-3 PUFA ratio in the 17.5 g/100 g fatty acid diet decreased (that is *n*-6 PUFA decreased and *n*-3 PUFA increased) there was a decrease in the proliferation of rat spleen lymphocytes. In the high PUFA diet (35 g PUFA/100 g fatty acids) there was no significant effect of changing the *n*-6:*n*-3 PUFA ratio. This study implies that dietary α-LNA reduces

lymphocyte proliferation but that this effect is dependent on its level relative to LA and the total PUFA content of the diet.

Lymphocyte proliferation - Human studies

The effects of variations in dietary fat upon lymphocyte proliferation in humans are somewhat more difficult to conclude. The reduction in dietary fat intake from 40% to 25% caused an increase in the ability of peripheral blood mononuclear cells (PBMNC) to proliferate (Kelley *et al.,* 1992b), the same effect as seen in animals. Thus a decrease in fat content of the diet has an enhancing effect on some aspects of the immune system. For the 25% fat diet, there was, however, no effect of increasing the amount of *n*-6 PUFA consumed (from 3.5% energy to 12.9% energy) on lymphocyte proliferation (Kelley *et al.,* 1992b), which contradicts animal experiments.

The effects of *n*-3 PUFA on lymphocyte proliferation in humans are not very well defined due to a lack of studies which can be directly compared, although on the whole supplementation with α-LNA, EPA or DHA seems to induce an inhibition of lymphocyte proliferation. When a diet rich in linseed oil, providing 18 g of α-LNA/d, was fed to healthy young men for 8 weeks a significant decrease in the proliferative response to PHA was seen (Kelley *et al.,* 1991). The *n*-6: *n*-3 PUFA ratio of this diet was 0.7 in the linseed oil diet to 24 in the control diet. The effect of α-LNA observed in this study agrees with that seen in animal studies (Jeffrey *et al.,* 1997b). A study in which blackcurrant seed oil was fed to healthy volunteers, aged 65 and over, for 2 months showed that there was an increase in proliferation of PBMNC (Wu *et al.,* 1999). Since this oil is very high in both α-LNA (14.5%) and γ-LNA (15.0%), a further study is required to compare these fatty acids and elucidate their effects. Since dietary α-LNA has been shown to have inhibitory effects on human PBMNC proliferation it may be that γ-LNA has enhancing effects on this function.

As part of a typical American diet containing 35-40% energy as fat, 2.4 g/d of encapsulated *n*-3 fatty acid was fed to 'older' women (aged 51-68 y) for 12 weeks and there was a 36% decrease in the proliferation of PBMNC in response to PHA.

There was, however, no effect on cells from younger women (aged 23 to 33 y) (Meydani *et al.,* 1991). In a more recent study, the mitogenic response of PBMNC was lowered by 24% for those subjects who consumed a low fat (26.4% fat as energy), low cholesterol diet supplemented with 1.23 g/d (0.54% energy) of *n*-3 fatty acids derived from fish oil (Meydani *et al.,* 1993). Another study, where 4.7 g *n*-3 PUFA/d were fed as 18 g/d of fish-oil concentrate to healthy individuals consuming a typical Western diet, showed a 70% decrease in mitogenic response 10 weeks after the end of the supplementation period (Endres *et al.,* 1993). However, during the supplementation period there was only a slight (non-significant) decrease in proliferation. No inhibition of proliferation was seen for patients with inflammatory skin disease when fed 6 g of *n*-3 PUFA/d (Soyland *et al.,* 1994). This suggests that the activation state of the immune system may be an important factor in the effect of feeding *n*-3 PUFA on lymphocyte proliferation. A very recent study where 3.2 g/d of EPA + DHA was fed to males and females reported no significant effects on lymphocyte proliferation in whole blood or PBMNC cultures (Yaqoob *et al.,* 2000).

Lymphocyte-Derived Cytokine Production

Studies have also shown that there is modulation in the production of cytokines by lymphocytes, although more studies need to be done to clarify the exact effects of fatty acids on this aspect of immune function.

The production of IL-2 by pig alveolar lymphocytes has been shown to be reduced by the feeding of fish oil and linseed oil (Turek *et al.,* 1994). More recently in a study where mice were fed either EPA or DHA (20 g SO/kg plus 10g EPA or DHA /kg) a decrease in IL-2 production by spleen lymphocytes was seen (Jolly *et al.,* 1997). A study by Yaqoob and Calder (1995a) revealed little effect of a fish oil diet on the production of the cytokines IL-2, IL-4, IL-10 or IFN-γ by murine spleen lymphocytes. In a study by Wu *et al.* (1996), where EPA+ DHA (1.3 or 3.3 % energy) or α-LNA (3.5 or 5.3 5 energy) was fed, an increase in IL-2 production was seen in monkeys, contradicting previous animal (and human) studies. This has been attributed to a difference in the level of vitamin E in the diet. In humans the picture is a little clearer, in that a larger number of studies have been carried out, and concur that the *ex vivo* production of IL-2 by PBMNC is diminished after healthy

individuals had supplemented their diet with fish oil (*Table 1.14.*). The *ex vivo* production of IFN-γ by human cells was also reduced after fish oil feeding (Gallai *et al.*, 1993).

Table 1.14. Effects of dietary *n*-3 PUFA on *ex vivo* production of lymphocyte-derived cytokines by human PBMNC.

Cell	Details of diet	Effect	Reference
PBMNC	Encapsulated *n*-3 PUFA (2.4 g/d) 12 weeks	Decrease in IL-2	Meydani *et al.* (1991)
PBMNC	Low-fat, low-cholesterol + *n*-3 PUFA (1.23 g/d) 24 weeks	Decrease in IL-2	Meydani *et al.* (1993)
PBMNC	Fish oil (6 g/d = 3.1 g EPA/d + 1.9 g DHA/d) 24 weeks	Decrease in IL-2 Decrease in IFN-γ	Gallai *et al.* (1993)
PBMNC	Fish oil concentrate (18 g/d = 2.8 g EPA + 1.9 g DHA) 6 weeks	No change in IL-2	Endres *et al.* (1993)
PBMNC Whole Blood	2.1 g EPA + 1.1 g DHA/d	No effect on IL-2 No effect on IL-10 No effect on IFN-γ	Yaqoob *et al.* (2000)
PBMNC	18.0 g α-LNA/d	No effect on IL-2	Kelley *et al.* (1993)

α-LNA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.; PBMNC, Periphreal Blood Mononuclear cells; PUFA, polyunsaturated fatty acids;

Sub-Populations of Cell Types

The proportions of different immune cell types, namely T-, B-, CD4+ and CD8+ cells, from rat spleen and thymus, were unchanged by feeding fish oil (Yaqoob *et al.*, 1994a; Sanderson *et al.*, 1995). In the human it has been shown that a fish oil enriched, low cholesterol, low fat diet alters the proportions of CD4+ and CD8+ cells (Meydani *et al.*, 1993). However, other studies with fish oil in humans have failed to confirm this (Yaqoob *et al.*, 2000)

Lymphocyte Surface Molecule Expression

There are only a limited number of studies in this area. The most recent studies with fish oil feeding to rats, have shown that dietary lipids can affect the expression of functionally important adhesion molecules (e.g. ICAM-1) on lymphocytes from rats

(Sanderson & Calder 1998b). Sanderson & Calder (1998b) also showed that these alterations in surface molecule expression can affect the ability of lymphocytes to bind to macrophages and endothelial cells. The effects of dietary MUFA and PUFA on adhesion molecule expression are summarised in *Table 1.15*.

Table 1.15. The effect of dietary MUFA and *n*-3 PUFA on the expression of surface molecules.

Cell Source	Details of diet	Effect	Reference
Rat spleen or thymic lymphocytes	High fat diets rich in olive oil and fish oil	Decrease in the number of lymphocytes bearing IL-2 receptor and transferrin receptor post Con A stimulation and in the level of expression of the transferrin receptor per cell	Yaqoob *et al.* (1994)
Rat spleen lymphocytes	High fat diets rich in fish oil	Decrease in the level of CD2 and CD11a expression on fresh cells; decrease in the level of expression of CD2, CD11a and ICAM-1 on lymphocytes post Con A stimulation	Sanderson *et al.* (1995)
Rat spleen lymphocytes	High fat diets rich in olive oil and fish oil	Decrease in the expression of IL-2 receptor following mitogenic stimulus	Sanderson *et al.* (1995)
Rat spleen lymphocytes	High fat diets rich in fish oil	Decrease in the level of CD18 and CD44 expression on fresh cells; decrease in the level of expression of CD18 and CD62L on lymphocytes post Con A stimulation. Decrease in the expression of adhesion molecules seen as a decrease in the adhesion of lymphocytes to macrophage monolayers and untreated endothelial cells, and a decrease in Con A stimulated lymphocyte adhesion to TNF-α-stimulated endothelial cells	Sanderson & Calder (1998b)
Human blood lymphocytes	6 g *n*-3 PUFA ethyl esters/d	Decrease in the % of IL-2 receptor-positive cells and level of IL-2 receptor expression post PHA stimulation	Soyland *et al.* (1994)

Con A, concanavalin A; ICAM, intracellular adhesion molecule; PHA, Phytohemagglutinin; PUFA, polyunsaturated fatty acids

Summary of the Effects of n-*3 PUFA on* ex vivo *Lymphocyte Functions*

It is apparent that diets rich in different fats modulate the immune system. It is thought that SFA have the least effect on proliferation followed by *n*-6 PUFA-rich oils (e.g. safflower oil rich in LA), olive oil (high in MUFA e.g. oleic acid), linseed oil (high in α-LNA) and fish oil (high in *n*-3 PUFA EPA and DHA) (Calder, 1998). This has been taken further and the effects of different individual fatty acids have now begun to be investigated. Investigations so far with dietary *n*-3 PUFA have shown that there are no differential effects of EPA and DHA, but that both of these *n*-3 PUFA have modulatory effects on lymphocyte proliferation. The ratio of *n*-3 to *n*-6 PUFA has been shown to be important in determining the effects of *n*-3 PUFA on immune function. At present it appears that a level of 4.4 g/100g of *n*-3 fatty acids has an immunomodulatory effect in animals. The lower level for humans remains to be established. Measurement of cytokine levels in culture have shown that dietary *n*-3 PUFA have the ability to modulate the production of these mediators as well as the expression of cell surface molecules (e.g. adhesion molecules) in the lymphocyte population.

1.4.4.2 Natural Killer Cell Activity

Natural Killer Cell Activity - Animal Studies

Recent studies have shown that feeding increased proportions of α-LNA (*n*-6 : *n*-3 ratio of 100, 20 and 10) has an inhibitory effect on the activity NK cell from rat spleen (Jeffrey *et al.,* 1997b), while feeding 40% of fatty acids as oleic acid had the same effect (Jeffrey *et al.,* 1997a). The effects of fish oil on NK activity have also been investigated. Fish oil feeding was found to inhibit NK activity in rats (Yaqoob *et al.*, 1994) and in mice (Meydani *et al.*, 1988). EPA but not DHA (at 4.4 g/100 g fatty acids) reduced rat spleen NK cell activity (Peterson *et al.,* 1998b). This suggests that EPA rather than DHA is responsible for the effect of fish oil on NK cell activity.

Natural Killer Cell - Activity Human Studies

The effects of a decreased fat intake have been shown to have a positive impact on the activity of NK cells (Barone *et al.,* 1989). The effects of *n*-3 PUFA feeding on the activity of human natural killer cells have been investigated in 3 different studies reported in the last three years. When 3.2 g EPA + DHA was fed to healthy young adults there was no reported effect on blood NK cell activity after 4, 8 or 12 weeks (Yaqoob *et al.,* 2000). However, when 1 g EPA + DHA was fed to elderly subjects there was a significant decrease in blood natural killer cell activity (Thies *et al.,* 2001a). This suggests that the age of a subject may have an influence on the effect of dietary *n*-3 PUFA observed. This latter study also found that 0.7 g DHA/d and 2 g α-LNA had no effect on NK cell activity. Kelley *et al.* (1999) found that after 12 weeks of feeding 6 g DHA/d there was a 15% decrease in NK activity, while at 8 weeks there was no effect. With regard to dietary DHA, NK cell activity is subject to modulation, but the level at which this is achieved needs to be further investigated. The concentration of DHA to exert the immunomodulatory effects of fish oil and EPA + DHA containing diets is not clear. In the animal studies, discussed above, EPA mimics the effects of fish oil or EPA + DHA, but DHA is less active. This question needs to be addressed with regard to humans.

1.4.4.3 Macrophage Functions

Macrophage-Derived Cytokine Production

The literature in this area is somewhat contradictory, and requires careful consideration of the precise experimental conditions in which the data were collected. Factors to be considered include the total amount of fat in the diet, the duration of feeding and the species of animal used. Also important are the type of macrophage used (alveolar, peritoneal, Kupffer, peripheral blood) and the state of its activation (monocyte, resident or elicited macrophage), the stimulus used to elicit the macrophage, and the stimulus used to induce cytokine production and its concentration, the duration of culture and the type of serum present in the culture. These are discussed in detail in a review by Calder (1997).

Macrophage–Derived Cytokine Production - Animal studies

A number of studies have investigated the production of TNF-α, IL-1 and IL-6 by macrophages from animals fed different types of dietary fat (*Table 1.16; Table 1.17.*). The results of different studies have contradicted each other but the factors described above may well account for the differences observed. The production of TNF-α may have a correlation with the state of activation of the macrophage (Wallace *et al.,* 2000). Most studies have concurred that the production of TNF-α by resident macrophages is enhanced by *n*-3 PUFA (Hardardottir and Kinsella, 1992; Watanabe *et al.,* 1991; Lokesh *et al.,* 1990; Chang *et al.,* 1992). With regard to elicited macrophages three studies have observed that there is no effect of fish oil when the cells were elicited with CFA (complete Freund's adjuvant) (Hardardottir *et al.,* 1992; Watanabe *et al.,* 1991; Turek *et al.,* 1991). In the case of thioglycollate-elicited macrophages, it is a little more difficult to find such a relationship. Several groups have reported an increase in TNF production (Hubbard *et al.,* 1994a, Somers and Erickson, 1994), a reduction (Yaqoob & Calder 1995b; Renier *et al.,* 1993; Tappia and Grimble 1994), or no effect (Somers and Erickson, 1994; Tappia and Grimble, 1994) with fish oil feeding. Another group has shown no effect of α-LNA on TNF-α production (Hubbard *et al.,* 1994b). The overall picture is that feeding fish oil enhances IL-1 production by resident macrophages, while in thioglycollate-elicited macrophages it causes a reduction in IL-1 and IL-6 production (*Table 1.16; Table 1.17.*).

Macrophage–Derived Cytokine Production - Human studies

There are many studies which have determined the effects of feeding n-3 PUFA on cytokine production by human blood monocytes. These are summarised in *Table 1.18.* It is apparent that there are conflicting results obtained in these studies, since investigators have found that there are decreases, or no change, in cytokine production. Several factors could explain this, and these include the dose of fatty acid, cell culture conditions and factors such the nature of the medium in which the cytokine is measured. Several studies have measured cytokine concentrations in the cell lysate or the supernatant, while some have combined these. Further consideration

of these studies has shown that feeding n-3 PUFA may well have differential effects on the intra- and extracellular concentrations of monocyte cytokines.

Macrophage-Derived Eicosanoid Production

The production of eicosanoids has been shown to be modulated by the increased incorporation of fish oil or its constituent fatty acids EPA and DHA in to the diet. A number of different studies have been conducted and they concur that there is a decrease in the production of the 2-series prostaglandins and 4-series leukotrienes after fish oil feeding (Lokesh *et al.,* 1986; Brouard and Pascaud, 1990; Hardardoittar *et al.,* 1992; Fritsche *et al.,* 1993; Lee *et al.,* 1985; Endres *et al.,* 1989;Yaqoob and Calder, 1995b). It has also been shown that as the production of 2-series prostaglandins and 4-series leukotrienes decreases, there is a concomitant increase in the EPA-derived eicosanoids (that is the 3-series prostaglandins and 5-series leukotrienes) (Chapkin *et al.,* 1992).

Table 1.16.. Summary of animal experiments investigating *ex vivo* IL-1 production by macrophages (adapted from Calder, 1997)

Species, Strain	Gender	Details of Diet	Cell Type	Ex vivo stimulus	Culture Conditions	Assay Type	Observations	Reference
Rat Sprague-Dawley	Male	150g/kg corn, safflower or fish oil; 2 or 6 weeks	Kupffer	LPS (1μg/ml)	5% FCS	Bioassay	FO ↓ production v. SO; FO and SO ↓ production v. CO	Billiar *et al.* (1988)
Mouse, CD-1	Male	100g/kg olive, corn or fish oil; 4 weeks	Resident peritoneal	LPS (1,000μg/ml)	Serum-free	Bioassay	FO ↑ production v. OO and CO	Lokesh *et al.* (1990)
Mouse, C3H/HeN	Male	50g/kg corn, safflower or fish oil; 3 weeks	Resident peritoneal (24 h following experimental haemorrhage or sham operation)	LPS (1μg/ml)	10% FCS	Bioassay	No difference in production after sham op; production lower after haemorrhage in CO and SO; FO maintained levels following haemorrhage	Ertel *et al.* (1993)
Mouse, Swiss	Female	150g/kg palm, corn or fish oil; 6 weeks	Resident peritoneal	LPS (0.1μg/ml)	Serum-free	RIA	FO ↑ intracellular IL-1α v other diets; no effect on IL-1β or secreted IL-1α	Blok *et al.* (1996)
Mouse, MF1	Male	Low fat (25g/kg corn oil) or 200 g/kg coconut, olive, safflower or fish oil; 8 weeks	Thioglycollate-elicited peritoneal	LPS (10μg/ml)	10% FCS	ELISA (IL-1α)	No significant effect but trend toward lower production with FO	Yaqoob & Calder (1995b)
Mouse, C57Bl6	Male	Low fat (25g/kg corn oil) or 200 g/kg coconut, olive, safflower or fish oil; 12 weeks	Thioglycollate-elicited peritoneal	LPS (10μg/ml)	10% FCS	ELISA (IL-1β)	FO↓ production	Wallace *et al.* (2000a)
Rat, Wistar	Male	Chow or 100g/kg butter or coconut, olive, corn, or fish oil; 4 or 8 weeks	Thioglycollate-elicited peritoneal	TNF (2.5 ng/ml)	10% FCS	Bioassay	No effect at 4 weeks; at 8 weeks, FO=CO<butter<chow<OO=CNO	Tappia & Grimble (1994)

Table 1.17. Summary of animal experiments investigating *ex vivo* IL-6 production by macrophages (adapted from Calder, 1997)

Species, Strain	Gender	Details of Diet	Cell Type	Ex vivo stimulus	Culture Conditions	Assay Type	Observations	Reference
Rat, Wistar/Kyoto	Male	Infusion of saline or of 20% (v/v) safflower or fish oil (9g fat/kg body weight per day);4 days	Peripheral blood mononuclear cell	LPS	5% FCS	ELISA	FO ↓ production	Grimm *et al.* (1994)
Mouse, MF1	Male	Low fat (25g/kg corn oil) or 200 g/kg coconut, olive, safflower or fish oil; 8 weeks	Thioglycollate-elicited peritoneal	LPS (10μg/ml)	10% FCS	ELISA	FO ↓ production	Yaqoob & Calder (1995b)
Mouse. C57Bl6	Male	Low fat (25g/kg corn oil) or 200 g/kg coconut, olive, safflower or fish oil; 12 weeks	Thioglycollate-elicited peritoneal	LPS (10μg/ml)	10% FCS	ELISA	SO ↑ production; FO ↓ production	Wallace *et al.* (2000a)

CNO, Coconut oil ;Fetal calf serum; FO, Fish oil; LPS, lipopolysaccharide; OO, Olive oil ;SO, Safflower oil,

Table 1.18. Summary of human experiments investigating *ex vivo* production of monocyte cytokines (adapted from Calder, 2000)

Subjects	Details of supplementation per day	Duration (weeks)	Cell Type	Stimulus	Nature of medium	Observations	Reference
Males aged 21-39 y (n=9)	2.8 g EPA + 1.9 g DHA	6	PBMNC	LPS or S. epidermidis	Cell lysate + culture medium	n.s ↓ 15% TNF, n.s ↓ 22% IL-1β to S. epidermidis n.s ↓ 43% TNF, n.s ↓ 22% IL-1β to LPS	Endres *et al.* (1989)

Females
(a) aged 22-33 y or
(b) aged 51-68 y
n= 6
	1.7 g EPA + 0.7 g DHA	4, 8, 12	PBMNC	LPS or S. epidermidis	Cell lysate + culture medium	For (b) ↓ 70% TNF-α
						For (a) ↓ 58% TNF-α
						For (b) ↓ 90% IL-1β
						For (a) ↓ 48% IL-1β
							Meydani *et al.* (1991)

Male mean age 27 y mean age 24 y n=9/8	1 g EPA + 0.6 g DHA 2 g EPA + 0.2 g DHA	7	PBMNC	LPS, PHA or PPD	Cell lysate or culture medium	No effect on TNF-α or IL-1β in culture medium for (a) or (b) However for dose (b) there was a decrease in IL-1β in cell lysate	Molvig *et al.* (1991)
Males and females Mean age 65 y n=11	Oily fish (8 servings/week) as part of a low fat diet (2.5% energy from fat)	24	PBMNC	LPS or S. epidermidis	Cell lysate + culture medium	With LPS ↓ 7% TNF-α ↓ 40% IL-1β With S. epidermidis ↓ 35% TNF-α ↓ 25% IL-1β	Meydani *et al.* (1993)
Males and females Aged 20-50 y n=15	3.1 g EPA + 1.9 g DHA	4, 12, 24	PBMNC	LPS	Culture medium	↓ 21% TNF-α ↓ 25% IL-1β	Gallai *et al.* (1993)
Males and females Aged 61-72 y n=7	3.0 g EPA + DHA	16	PBMNC	LPS	Culture medium	No effect	Cannon *et al.* (1995)
Males and females Aged 24-52 y n= 16	0.34 g EPA + 0.19 g DHA	12	Monocytes	LPS	Culture medium	No effect	Schmidt *et al.* (1996)

Table 1.18. Summary of human experiments investigating *ex vivo* production of monocyte cytokines (adapted from Calder, 2000) (continued....)

Subjects	Details of supplementation per day	Duration (weeks)	Cell Type	Stimulus	Nature of medium	Observations	Reference

Males
Aged 22-44 y
n=15
	13.7 g of α-LNA as spread + cooking oil
	as (a) + 1.6 EPA + 1.1 g DHA
	1.6 g EPA + 1.1 g DHA
		4	PBMNC	LPS	Cell lysate + culture medium	For (c) ↓ 70% TNF-α
						↓ 78% IL-1β
						For (a) ↓ 27% TNF-α
						↓ 30% IL-1β
							Caughey *et al.* (1996)

Males Aged 21-87 y n= 14/15	0.8 g EPA + 0.2 g DHA 1.6 g EPA + 0.3 g DHA 2.4 g EPA + 0.5 g DHA	26, 52	Whole Blood	LPS	Culture medium	No effect of supplementation on IL-1β or TNF-α, but no baselines given ∴ difficult to conclude	Blok *et al.* (1997)
Males Aged 20-49 y n= 7	6 g DHA (low fat diet – 30% energy)	8, 12	PBMNC	LPS	Culture medium	↓ 30% TNF-α ↓ 40% IL-1β	Kelley *et al.* (1999)
Males and females Aged 21-70 y n=8	2.1 g EPA + 1.1 g DHA	4, 8, 12	PBMNC and Whole blood	LPS	Culture medium	No significant effects of supplementation on IL-1β or TNF-α	Yaqoob *et al.* (2000)

α-LNA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL-1β, interleukin-1β; LPS, bacterial lipopolysaccharide; MC, mononuclear cells; PHA, phytohaemagglutinin; PPD, purified protein derivative of *Mycobacterium tuberculosis*; TNF-α, tumour necrosis-α.

Production of Reactive Oxygen Species and Nitric Oxide by Macrophages - Animals

The production of superoxide, hydrogen peroxide and nitric oxide (NO) is very important in the killing of pathogens that infiltrate the body, although an inappropriate production of these can be damaging to the host and may have implications in the pathogenesis of disease. Dietary intervention with different *n*-3 PUFA has produced conflicting data. Feeding rats a diet rich in α-LNA has been shown to lower superoxide production by peritoneal macrophages in response to PMA, but not to affect production in response to *Listeria monocytogenes* (Babu *et al.,* 1997). A diet enriched with 100 g/kg of linseed oil or fish oil had no effect on NO production by elicited rat peritoneal macrophages (Hubbard *et al.,* 1994b). In contrast, feeding fish oil (100 g/kg) has been shown to increase NO production by mouse macrophages (Renier *et al.,* 1993) and to increase both superoxide and nitric oxide production by rat macrophages in response to LPS (Berger *et al.,* 1993, Hubbard *et al.,* 1994a; Joe *et al.,* 1994).

Production of Reactive Oxygen Species and Nitric Oxide by Macrophages - Humans

Although one study showed that production of reactive oxygen species and NO by human monocytes is not affected by fish oil feeding (Halvorsen *et al.,* 1997), a 30 - 55% decrease in reactive oxygen species production was observed when 6 g EPA + DHA/d were provided (Fisher *et al.,* 1990).

Macrophage-Mediated Phagocytosis - Animals

There are conflicting observations as to whether feeding different fats, especially fish oil, has a modulatory effect on phagocytosis. Studies have shown no effect of fish oil feeding on macrophage and monocyte phagocytic capacity (D'Ambola *et al.,* 1991; Turek *et al.,* 1994). This lack of effect was also observed for rats fed a diet enriched with linseed oil (Babu *et al.,* 1997). However, in the healthy mouse it has been shown that the ability of Kupffer cells to phagocytose *Salmonella typhimurium* was reduced by fish oil feeding (Eicher and McVey 1995), although the capacity of the

cells to kill the bacteria was not affected. If the Kupffer cells were taken from a previously infected mouse there was no effect of previous diet on either phagocytosis or bacterial killing.

Macrophage-Mediated Phagocytosis - Humans

There is only one study to date, interpreting the effects of feeding *n*-3 PUFA on macrophage-mediated phagocytosis in humans. No effect of dietary intervention with 3.8 g EPA or 3.8 g DHA per day for 7 weeks on phagocytosis by blood monocytes was observed (Halvorsen *et al.,* 1997).

Expression of Macrophage Cell Surface Markers

MHC Expression

The expression of the MHC II complex by the macrophage is very important for the activation of T-lymphocytes. Inclusion of *n*-3 PUFA in the diet of mice and rats has been shown to have caused a reduction in the percentage of macrophages from the peritoneum which expressed MHC II (Kelley *et al.,* 1985, Mosquera *et al.,* 1990, Huang *et al.,* 1992). The last study also showed a decrease in the level of expression of MHC II on positive cells (Huang *et al.,* 1992). This was also observed for thioglycollate-elicited peritoneal macrophages from rats (Sherrington *et al.,* 1995a). In humans, a decrease in the level of MHC II expression on peripheral blood monocytes has been observed after fish oil feeding (Hughes *et al.,* 1996).

Adhesion Molecule Expression

A decrease in the levels of expression of ICAM-1 on peripheral blood monocytes was seen after feeding 1.5 g n-3 PUFA/d to healthy humans for 3 weeks (Hughes et al., 1996).

Macrophage Chemotaxis

The chemotaxis of monocytes has also been observed to be modulated by dietary fatty acids. A dose of 4 g *n*-3 PUFA/d for 6 weeks was found to significantly reduce

the chemotaxis of human monocytes in response to autologous serum (Schmidt *et al.*, 1992). More recently, when a lower dose of *n*-3 PUFA was fed (0.65 g/d) for 12 weeks, there was no significant effect on monocyte chemotaxis (Schmidt *et al.*, 1996).

Summary of the Effects of Fatty Acids on ex vivo *Monocyte and Macrophage Functions*

It is clear that dietary *n*-3 PUFA have modulatory effects on the production of cytokines by macrophages. The observations so far suggest that feeding fish oil has an inhibitory effect on the production of TNF-α, IL-1 and IL-6, in both humans and animals, although there are some contradictory findings. The effect of feeding α-LNA on the production of these cytokines remains to be established in animals, with limited experiments in humans showing an inhibitory effect on cytokine production.

The effects of *n*-3 PUFA on macrophage functions have also been investigated and observations suggests that these fatty acids are capable of eliciting functional changes in these cells. There is evidence to suggest that feeding fish oil causes inhibition of MHC II expression and thus of the ability of the cells to present antigen. The importance of the expression of MHC II molecules is clear since it has been shown that T-lymphocyte proliferative response is proportional to the number of MHC II molecules on antigen-presenting cells (Janeway *et al.*, 1984) and that the percentage of MHC II molecules and the density of this receptor on the cell surface can alter the degree of responsiveness of the immune system of an individual (Janeway *et al.*, 1984).

Dietary fish oil has also been shown to inhibit the expression of adhesion molecules in both animals and humans and to decrease the ability of cells to move towards a stimulus. The ability of monocytes and macrophages to perform phagocytosis has been shown to be subject to modulation in animals. The only study to investigate phagocytosis of monocytes in humans, reported no effect. Further work is needed in this area to confirm this.

There is extremely limited literature on the effects of α-LNA on macrophage cytokine production and function. At present there are no studies which have investigated the effects of this fatty acid on macrophage chemotaxis, phagocytosis or expression of cell surface molecules.

1.4.4.4 Neutrophil Functions

There is rather limited literature concerning dietary fish oil and neutrophil function. There were no studies with dietary α-LNA and neutrophil function until very recently when a study with this fatty acid in humans was reported.

Chemotaxis – Human Studies

Although high doses of EPA+DHA decrease neutrophil chemotaxis studies with lower doses have resulted in conflicting results (*Table 1.19.*). It appears that more work is needed to determine the lower threshold at which *n*-3 PUFA can modulate neutrophil chemotaxis.

Table 1.19. Effects of *n*-3 PUFA on neutrophil chemotaxis

Cell Source	Details of diet	Stimulus	Observation	Reference
Human blood	3.2 g EPA + 2.2 g DHA/d, 6 weeks	LTB_4	↓ in chemotaxis	Lee *et al.* (1985)
Human blood	5.3 g *n*-3 PUFA, 6 weeks	fMLP	↓ in chemotaxis	Schmidt *et al.* (1989)
Human blood	1.3, 4, 9 g *n*-3 PUFA, 6 weeks	fMLP	↓ in chemotaxis	Schmidt *et al.* (1991)
Human blood	0.55 g *n*-3 PUFA,	Autologous serum	↔ in chemotaxis	Schmidt *et al.* (1996)
Human blood	9.4 g EPA + 5 g DHA/d, 3 or 10 weeks	LTB_4, PAF	↓ in chemotaxis	Sperling *et al.* (1993)
Human blood	4.1 g α-LNA, 4, 8, 12 weeks	fMLP	↔ in chemotaxis	Healey *et al.* (2000)
Human blood	≤2.25 g EPA + DHA, 4, 8, 12 weeks	fMLP	↔ in chemotaxis	Healey *et al.* (2000)

α-LNA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; fMLP, N-formyl-MetLeuPhe; LTB_4, leukotriene B_4; PUFA, polyunsaturated fatty acids.

Reactive Oxygen Species – Human Studies

The production of reactive oxygen species by human neutrophils appears to be subject to modulation by dietary EPA and DHA (*Table 1.20.*), although further work is needed to establish the levels at which this can be achieved. The only study reported to date using α-LNA showed no effect on neutrophils superoxide production.

Table 1.20. Effects of *n*-3 PUFA on production of reactive oxygen species by neutrophils

Cell Source	Details of diet	Stimulus	Observation	Reference
Human blood	2.16 g EPA/d, 7 weeks	fMLP, PMA	↓ in superoxide production	Thompson *et al.* (1991)
Human blood	0.55 g EPA + DHA/d, 8 weeks	Zymosan or phorbol ester	↔ in hydrogen peroxide production	Varming *et al.* (1995)
Human blood	4.1 g α-LNA, 4, 8, 12 weeks	fMLP	↔ in superoxide production	Healey *et al.* (2000)

α-LNA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; fMLP, N-formyl-MetLeuPhe; PMA, phorbol 12-myristic 13-acetetate.

Neutrophil Phagocytosis– Human Studies

The effect of fish oil, EPA, DHA or α-LNA feeding on neutrophil phagocytosis has not been investigated in humans. The effect of feeding these fatty acids on this function in neutrophils needs to be investigated.

1.4.5 *The Effects of Dietary* n-*3 PUFA on the Immune Response Tested* in vivo

There are few means by which the *in vivo* immune response can be evaluated; however, the delayed-type hypersensitivity response (DTH response) is one such method. It is beneficial to look at the *in vivo* response because the measurements discussed so far have only been concerned with cells in isolation, and do not reflect the complexity of the cellular interactions involved in the immune response.

The DTH Response

The effects of dietary fatty acids on the DTH response have been studied in both animals and humans. The studies performed with animals have shown that a high fat diet reduces the DTH response as compared to a low fat diet (Friend *et al.,* 1980; Crevel *et al.,* 1992). These studies also showed that *n*-6 PUFA rich diets have a greater suppressive effect than SFA-rich diets (Friend *et al.,* 1980; Crevel *et al.,* 1992). A study with fish oil feeding in mice has shown that the DTH response is decreased when compared to a MUFA- or *n*-6 PUFA-rich diet (Yoshino and Ellis, 1987). More recently, a study in beagle dogs showed a reduced DTH response as the *n*-6: *n*-3 PUFA ratio of the diet decreased from 31 to 5.4 to 1.4. The ratio was altered by a replacement of LA with EPA or DHA. Thus the overall potency of the fatty acids in reducing the DTH response is SFA<*n*-6 PUFA< fish oil. A more recent study has shown that increasing the amount of α-LNA compared to LA in the diet decreases the graft vs host response (another measure of cell-mediated immunity) (Jeffrey *et al.,* 1996).

In humans there was no effect of a reduction in fat intake (40 to 25-30 %) on the DTH response, assessed as described in section 1.1.4. (Kelley *et al.,* 1992b). This study also showed that was there no effect of increasing the level of *n*-6 PUFA in the diet to 3.2 or 9.1% energy on the DTH response. However, if the diet is enriched with α-LNA, at a level of 18 g/d, by consuming a diet rich in linseed oil, a non-significant 23% decrease in DTH induration score was seen (Kelley *et al.,* 1991). A diet low in fat and cholesterol and supplemented with 1.25 g *n*-3 PUFA/d has been shown to cause a 45% reduction of the DTH induration index in men and women (Meydani *et al.,* 1993). The feeding of 6 g/d of DHA alone had no effect on the DTH response (Kelley *et al.,* 1999), and this suggests that the modulation seen with fish oil feeding is due more to the influence of EPA than DHA. Given the large number of human studies evaluating the effect of *n*-3 PUFA on *ex vivo* human immune cell functions it is surprising that there are so few measuring *in vivo* immune status.

1.5 The Aims of the Studies Described in this Thesis

The literature reviewed in section 1.4 strongly suggests that *n-3* fatty acids exert effects on a range of inflammatory and immune cell functions and the consensus is that these effects are of an inhibitory nature. Inhibition of the function of T-lymphocytes, NK cells, monocytes and macrophages, and neutrophils by dietary *n-3* PUFA has been widely reported. These effects are frequently termed anti-inflammatory or immunosuppressive and have been used to support the use of long chain *n-3* PUFA (as fish oil) as therapeutic agents in various diseases involving inflammation or an apparently active immune system. In fact, long chain *n-3* PUFA have been shown to be beneficial in these sorts of disorders, for example arthritis (Volker and Garg, 1996), Chron's disease (Belluzzi and Miglio, 1998), ulcerative colitis (Rodgers, 1998), psoriasis (Ziboh, 1998), lupus (Clark *et al.*, 1989), and asthma (Broughton *et al.*, 1997) and in patients receiving organ transplants (Homan van der Heide, 1993). This use of *n-3* PUFA is taking advantage of their anti-inflammatory/immunosuppressive effects. However, given that the immune system normally functions to protect the host against pathogens (section 1.1), and that immune deficiency makes individuals significantly more susceptible to infectious diseases and some cancers, and less able to cope with some challenges, an *n-3* PUFA induced suppression of immune function could be seen as being detrimental to human health. Despite this, long chain *n-3* PUFA are considered beneficial to health (de Deckere *et al.*, 1998; BNF 1992, 1999), being particularly protective against cardiovascular disease (de Deckere *et al.*,1998; BNF 1992, 1999). On this basis there have been numerous recommendations to increase the intake of *n-3* PUFA (e.g. de Deckere *et al.*, 1998; BNF 1992, 1999). Since it is now recognised that atherosclerosis (the disease which underlies CVD) has an inflammatory component (and perhaps even an autoimmune component) (Ross ans Stier, 1999), the benefits of *n-3* PUFA here might relate, at least partly, to their anti-inflammatory effects. Nevertheless, it seems that a state of immunosuppression should be avoided in the general population.

As described in section 1.4, studies investigating the effects of *n-3* fatty acids on immune function have been conducted in both animals and humans. There are a large number of studies using fish oil, and these have examined many immune parameters

(see section 1.4.). Most animal studies have used large amounts of fish oil (often up to 20% by weight of the diet) such that the *n-3* fatty acids provided over 30% of fatty acids and over 10% of dietary energy. In comparison, current human intakes of long chain *n-3* fatty acids provide, 0.1% of energy, and even a substantial increase in intake (as recommended by the BNF, (1999)) would result in them providing ≤ 1.0 % of energy. Only the most recent animal studies have investigated a more realistic (in human terms) amount of long chain *n-3* PUFA in the diet of rodents: for example Peterson *et al.* (1998) used EPA or DHA at a level of 4.4. % of fatty acids (1.7% of energy). There are no animals studies of fish oil derived fatty acids and immune function which have used lower amounts of these fatty acids as part of a high fat diet. Human studies investigating immune function have used a wide range of *n-3* PUFA levels, providing fish oil as capsules or as a liquid to drink; levels used have been between 0.55 g and 14.4 g EPA+DHA/ day with most studies providing more than 2.5 g/d. This is ten times the current UK intake of these fatty acids (*Table 1.6.*) and 2.5 times the most extreme recommendation for their intake (BNF 1999). Studies using more realistic levels of these fatty acids are required (e.g. levels between current intake and the BNF recommendation and levels at around the BNF recommendation). Despite the large numbers of studies of fish oil-derived *n-3* PUFA and human immune function (section 1.4.), it remains difficult to get a clear view of the nature of their effects. This is because many of the studies have been poorly designed, most have used small numbers of subjects, often a heterogeneous mix, most have used large amounts of *n-3* PUFA, and technical differences between studies which have reported on different aspects of immune function do not allow for an easy comparison (see Calder, 2001 for a review). Thus, it is difficult to state unequivocally what the effects of dietary long chain *n-3* PUFA are on any aspect of human immune function or upon the immune response as a whole. Therefore, there is a need for carefully designed large studies comparing the effects of different, but realistic, doses of long chain *n-3* PUFA on a variety of immune functions and on the immune response as a whole. The situation regarding the immunological effects of α-LNA is even more unclear. Studies in laboratory animals using large doses of α-LNA (as 60% of fatty acids or 24% of energy) have shown inhibitory effects on the limited number of parameters measured; however, there are few or no animal studies looking at α-LNA and cytokine production, phagocytosis, or respiratory burst. With

respect to human immune function two studies using very high doses have been reported. Kelley *et al.* (1991) reported inhibition (23%) of lymphocyte proliferation and DTH after providing 18 g α-LNA/ day. Caughey *et al.* (1996) reported 30% inhibition of TNF-α and IL-1β production after 14 g α-LNA/ day. These levels of α-LNA are greatly in excess of habitual intake (0.5 to 1.5 g/d) and provide little useful information about the immunological effects or detriments of smaller increase in α-LNA intake, which might occur in response to recommendations to increase *n-3* PUFA intake. Some recent studies have used lower doses of α-LNA (2 or 4 g/d) (Healy *et al.*, 2000; Thies *et al.*, 2001). However, there have been no dose response studies of the immunological impact of α-LNA in humans.

Aims of the Animal and Human Studies in this Thesis

This thesis is divided into two main sections: chapters 2 and 3 which describe interventions with dietary EPA and DHA in laboratory animals, and chapters 4, 5 and 6 which describe a dietary intervention with *n-3* PUFA in humans. The first set of aims described here will outline the purpose of the animal studies, while the second set of aims will outline the purpose of the human intervention. The hypotheses for the work conducted will then be stated.

The literature reviewed so far with respect to feeding studies conducted with animals has shown that fish oil (i.e. a preparation rich in EPA and DHA) can have marked immunomodulatory effects. Levels as low as 4.4 g EPA or DHA /100 g of total fatty acids (which is 20-25% of the level found in fish oil) have recently been shown to have modulatory effects on the immune system (Peterson *et al.*, 1998a). However, there is little information regarding the lowest dose of EPA and/or DHA to have an effect on the immune system. The first aim of the animal work is to elucidate the responses of the murine and rat immune systems to different doses of EPA or DHA. These two fatty acids will be fed independently to try to unravel their separate effects on the immune system, if any. This is an avenue that has not yet been explored to any great extent. The second aim is to investigate the effect of lower doses of EPA and DHA than have been used previously. The third aim of the animal investigations is to establish whether EPA and/or DHA fed in different positions of dietary TAG (i.e. in

the *sn*-2 or *sn*-1(3) position) could influence the incorporation of these fatty acids into lipid pools and whether the immune system is affected to different extents by different isomers. The idea behind this is explored in chapters 2 and 3.

The literature reviewed with respect to human intervention studies has demonstrated that feeding fish oil, i.e. a preparation rich in EPA and DHA, (predominately in capsular forms), or α-LNA (predominately at very high levels (>14 g/ day)), has significant effects on the function of the immune system. The first aim of this study was to introduce these fatty acids into the human diet in a more subtle way, through their incorporation into a staple part of the diet, margarine. This will be very advantageous since it will determine whether an immuno-modifying level of EPA, DHA and α-LNA can be introduced into the diet. The second aim is to determine whether the administration of *n-3* PUFA through food can result in the enrichment of these fatty acids in the lipid pools of the body, as has been seen in previous studies in humans with capsules. The relationship between the precursor *n-3* PUFA, α-LNA, and its metabolites EPA and DHA with respect to enrichment of lipid pools will also be considered.

The third and last aim of the human intervention study is to introduce the *n-3* PUFA in the margarine at lower doses than have been used in most previous studies of human immune function. In human studies conducted so far the effects of low levels of EPA and DHA, and especially of α-LNA, have not been satisfactorily investigated, nor have dose-response relationships been determined with respect to *n-3* PUFA and immune function. Thus, this study aims to investigate the effects of different doses of *n-3* PUFA on human immune function. The relatively large consumption of fish oil in capsular form in previous human studies (ranging from 1.23 g/d to 18 g/d) has had many draw backs for example unpleasant taste, stomach upsets and expense and so the outcomes of this study may enable the general population to increase intakes of *n-3* PUFA with out any of these considerations.

1.5.1 Hypotheses

- Consuming increasing doses of EPA or DHA will result in a dose-dependent enrichment of these fatty acids in animal tissues

- The postion of EPA or DHA in dietary TAG will result in differential enrichment of these fatty acids in animal tissues
- The postion of EPA and DHA in dietary TAG will have differential effects on the murine and rat immune systems
- The effects of EPA on the immune system will be greater than those of DHA in animals
- Consuming increasing doses of α-LNA or EPA+DHA will result in a dose-dependent enrichment of these fatty acids in human plasma and immune cells
- There will be dose-dependent effects of α-LNA and EPA+DHA on human immune function
- The effects of dietary α-LNA on human immune function will be less significant than those of EPA+DHA

CHAPTER 2. THE EFFECTS OF STRUCTURED TRIACYLGLYCEROLS ON BLOOD AND SPLEEN LEUKOCYTE LIPID COMPOSITION AND IMMUNE FUNCTION IN RATS

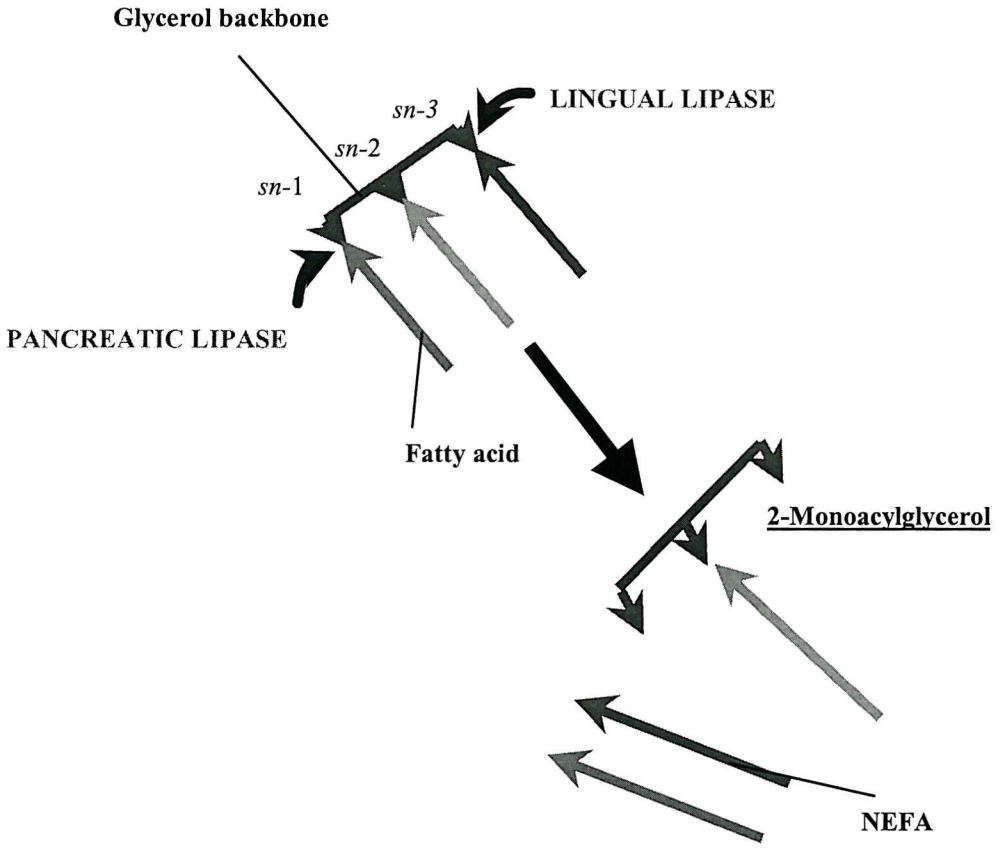
2.1 INTRODUCTION

A triacylglycerol (TAG) molecule consists of 3 fatty acids esterified to a glycerol 'backbone'. The three positions on the glycerol at which fatty acids can be esterified are termed *sn*-1, *sn*-2 and *sn-3.* The type and position of fatty acids in dietary TAG may influence the physical properties of the TAG molecule and are often characteristic of a particular fat or oil (*Table 2.1.*). Dietary TAG is ingested and partly hydrolysed in the gastrointestinal tract by lipases (lingual and pancreatic) which remove two of the fatty acids, usually from the *sn*-1 and *sn-3* positions, from the glycerol backbone (*Figure 2.1.).* This results in the liberation of non-esterified fatty acids (NEFA) and a monoacylglycerol (MAG) with the fatty acid at the *sn*-2 position still esterified (2-MAG). The digestion and absorption of dietary TAG are described in more detail in section 1.2.4.

Table 2.1. The characteristic positions of fatty acids in TAG molecules of different fat types. (Taken from British Nutrition Foundations Report (1992))

	Cows' milk fats	**Human milk fats**	**Animal depot fats**
sn-1	Random	Unsaturated fatty acids	Saturated fatty acids
sn-2	Random	Saturated fatty acids	Unsaturated fatty acids and short chain fatty acids
sn-3	Short chain fatty acids	Unsaturated fatty acids	Random

Figure 2.1. Schematic diagram of a TAG molecule and its hydrolysis



Those fatty acids in the *sn*-1(3) position of dietary TAG which are hydrolysed to NEFA can be lost in the faeces due to the formation of calcium salts (Brink *et al.*, 1995). Palmitic acid (PA) in the *sn*-2 position was absorbed more rapidly from the gastrointestinal tract than PA in the *sn*-1(3) position by preterm infants (Carnielli *et al.*, 1995). When fatty acids with chain lengths >20 were fed in the *sn*-2 position they appeared in higher proportions in blood chylomicron (CM) than when fed (at the same level in the diet) at the *sn*-1(3) position in dietary TAG (Redgrave *et al.*, 1988). These observations support the concept that there is a less efficient absorption of fatty acids found at the *sn*-1(3) position of dietary TAG than at the *sn*-2 position. Those NEFA which are absorbed into the enterocyte are randomly re-esterified to the

2-MAG molecules which have been absorbed (*Figure 2.2.*). Therefore, fatty acids in the *sn*-2 position are preferentially retained at this position once absorbed by the enterocyte but those in the *sn*-1(3) positions are not (Small, 1991).

The TAG molecules formed in the enterocytes then associate with phospholipids (PL), cholesterol and cholesterol ester and these are then complexed with apolipoproteins to form CM. CM enter the circulation via the lymphatic system and the left subclavian vein. An enzyme called lipoprotein lipase (LPL) then cleaves TAG preferentially at the *sn*-1(3) position when CM contact the surface of endothelial cells (Wang *et al.*, 1982). The fatty acid at the *sn*-2 position must isomerize to the *sn*-1(3) position in order to be hydrolysed from CM TAG and taken up as NEFA (Braun and Severson, 1992). The NEFA released are then absorbed into nearby cells for utilisation or storage. However, intact TAG molecules can also be absorbed (as components of lipoproteins) by cells by a receptor-mediated process (see section 1.3.1.), thus increasing the likelihood that a fatty acid in the *sn*-2 position of dietary TAG will be retained at this position through absorption and metabolism to incorporation into cells.

Due to the mode of breakdown of TAG, that is the fatty acid at *sn*-1(3) being randomized after absorption while the fatty acid at *sn*-2 remains constant (Pufal *et al.*, 1995), it would seem that there may be several physiological and biochemical implications of substituting different fatty acids in the *sn*-2 rather than *sn*-1(3) position of dietary TAG. In the last ten years the effects of substituting saturated fatty acids, especially PA, at different positions on dietary TAG have been examined.

The hypothesis that fatty acids in the *sn*-2 position may be metabolised differentially compared to those in the *sn*-1(3) position has been investigated in a study where piglets were fed sow's milk (70% PA in the *sn*-2 position) or formulae (containing similar total saturated fatty acids (SFA) but oleic acid (40-60%), linoleic acid (LA) (22-25%) and α-linolenic acid (α-LNA) (approx. 5%) at the *sn*-2 position, and thus PA at the *sn*-1(3) positions). The sow's milk-fed animals showed larger amounts of PA esterified to the plasma TAG at the *sn*-2 position compared to those fed formulae, where PA was concentrated at the *sn*-1(3) position (Innis *et al.*, 1995). This suggests that that there is retention of this fatty acid at the *sn*-2 position. This may have

implications when the diet, of infants in particular, is considered. Human milk contains PA at the *sn*-2 position while in cow's milk PA is predominantly at the *sn*-1(3) position (*Table 2.1)*. In another study, higher levels of PA were found in the plasma TAG and NEFA of infants fed formula enriched with 19% of this saturated fatty acid at the *sn*-2 position than in infants fed PA in the *sn*-1(3) positions (Carnielli *et al.,* 1995). A study in Sprague Dawley rats which were fed PA at either the *sn*-2 or *sn*-1(3) positions of dietary TAG agrees with the concept of retention of PA at the *sn*-2 position (Aoyama *et al.,* 1996).

2.1.1 Aims of This Chapter

The effects of positional isomerism of the longer chain unsaturated fatty acids in dietary TAG on their absorption and metabolism have not yet been examined in detail. With the growing interest in fish oil (which contains eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and its effects on the human health (see section 1.4), this is an area that should be addressed.

The composition of an oil with regard to EPA and DHA varies with the source of the oil. The position in a TAG molecule that the EPA or DHA normally occupies varies between the type of fish or marine mammal fat studied, as do their relative proportions. For example, in seal oil EPA is predominantly found in the *sn*-1(3) position, while in fish oil EPA is found in the *sn*-2 position (Yoshida *et al.,* 1999). This study aims to determine whether positional isomerism of EPA and DHA in dietary TAG causes functional changes by their differential incorporation into cells and tissues, namely those of the immune system. The effects of fish oil, EPA and DHA on the immune system are reviewed in Chapter 1.

The evidence for the concept that fatty acids in the *sn*-2 position in dietary TAG may be retained at this position through digestion and absorption to the level of cellular uptake was described earlier in this introduction and is summarised in *Figure 2.2.* This figure demonstrates that the uptake of NEFA and 2-MAG (as well as TAG) by a cell can influence the composition of PL formed in the cell with the fatty acid at the *sn*-2 position in a phospholipid being that which was at this position in dietary TAG. This is important when the production of certain lipid mediators, such as

prostaglandins, is considered. These lipid mediators are generated from the fatty acid in the *sn*-2 position of phospholipids in the cell membrane. Since the type of fatty acid (e.g.arachidonic acid (AA) versus EPA) used to generate prostaglandins and other eicosanoids influences the potency of the mediator formed (see section 1.1.3.2.), the fatty acid present at the *sn*-2 position in dietary TAG, and thus in the membrane phospholipids, is of great importance.

To test the hypothesis that a long chain fatty acid (EPA or DHA) in the *sn*-2 position in dietary TAG is retained through to uptake by a cell, the compositions of blood lipid fractions and immune cells will be investigated, along with the functional activity of these cells.

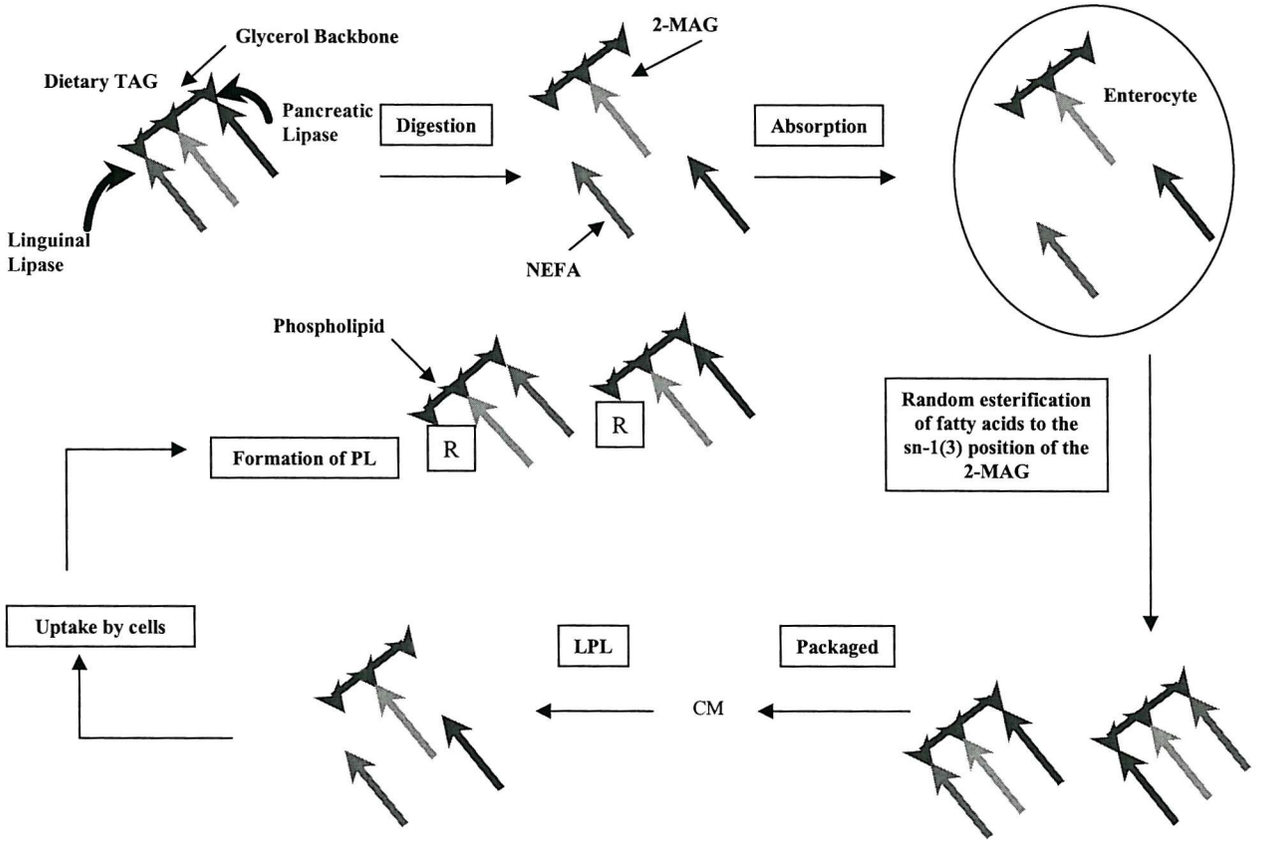
Hypotheses:

- Consuming increasing doses of EPA or DHA will result in a dose-dependent enrichment of these fatty acids in animal tissues
- The postion of EPA or DHA in dietary TAG will result in differential enrichment of these fatty acids in animal tissues
- The postion of EPA and DHA in dietary TAG will have differential effects on the rat immune system
- The effects of EPA on the immune system will be greater than those of DHA in animals

The outcome variables to be investigated will be:

- Plasma lipid levels and fatty acid composition
- Spleen leukocyte PL and diacylglycerol fatty acid composition
- Spleen leukocyte PL molecular species
- Spleen lymphocyte proliferation
- Production of prostaglandin E_2 by cultured spleen leukocytes
- Production of Th1 and Th2 cytokines (Interleukin (IL)-2, IL-4, IL-10, Interferon (IFN-γ) by cultured spleen leukocytes

Figure 2.2. The handling of dietary TAG from the point of ingestion to uptake by cells, showing the retention of fatty acids at the *sn*-2 position



CM= chylomicron; LPL = lipoprotein lipase; MAG = monoacylglycerol; NEFA = non-esterifed fatty acid; PL = phospholipid
R= Phosphate group; TAG = triacylglycerol;

2.2 Methods

2.2.1 Chemicals

Acetic acid, concanavalin A (Con A), boron trifluoride (14% in methanol), bovine serum albumin (BSA), chloroform, ether, foetal calf serum (FCS), formaldehyde, glutamine, Histopaque, heparin, hexane, methanol, penicillin, potassium chloride, HEPES-buffered RPMI medium (glutamine-free), sodium chloride, streptomycin and sodium azide were all obtained from Sigma Chemical Co. (Poole, UK). [6-^{3}H] Thymidine (2 Ci/mmol) was obtained from Amersham International (Amersham, UK). Fluorescein isothiocyanate-labelled goat anti-mouse IgG (GAM-FITC) was obtained from Serotec (Kidlington UK). Monoclonal antibodies to rat CD4 (W3/25), CD8 (OX8), B cells (CD19; OX12), and monocytes (CD14; OX42) were gifts from the Sir William Dunn School of Pathology, University of Oxford.

Cytokine concentrations were determined using Biosource ELISA kits and prostaglandin E_2 concentrations were determined using Neogen ELISA kits purchased from Appligene-Oncor-Lifescreen (Watford, UK). Plasma triacylglycerol and total cholesterol concentrations were determined using diagnostic kits purchased from Sigma chemical Co., Poole, UK). Plasma NEFA and non-esterified cholesterol concentrations were determined using enzyme-based diagnostic kits (Boehringer, Mannheim, Germany)

2.2.2 Animals and Diets

Weanling male Lewis rats were housed individually for a period of 6 weeks prior to sacrifice. During this time they were given free access to water and one of 9 experimental diets (n=8 per diet) provided by Unilever Research Colworth Laboratory, Sharnbrook, UK.

Each diet contained 178 g/kg lipid and the levels of all other dietary components were identical (*Table 2.2*).

Table 2.2. Composition of the diets

Component	Level (g/kg diet)
Casein	182
Starch	520
Fibre (Solkafloc)	60
AIN-76 mineral mix	42
DL-methionine	4
AIN-76 vitamin mix	12
Choline bitartrate	2
Total lipid	178

The diets contained similar amounts of palmitic acid (approx. 22g/100 g total fatty acids), stearic acid (approx. 4 g/100 g total fatty acids), oleic acid (approx. 36 g/100 g total fatty acids), and linoleic acid (31 g/100 g total fatty acids) and the ratio of *n-3/n-6* PUFA was kept constant (*Table 2.3.*). The diets also contained less than 1.0 g/100g of 12:0 or 14:0. In all diets the amount of *n-3* PUFA was similar (approx. 4.4 g/100 g total fatty acids). The diets differed in the proportions of the different *n-3* PUFA (α-LNA, EPA, DHA) they contained and in the position (*sn*-2 or *sn*-1(3)) of EPA and DHA within the dietary TAG (*Table 2.3.*). In the 4.4 diets EPA or DHA replaced α-LNA, while the other components remained the same. In the 2.2 diets, EPA or DHA replaced approximately 2.2 g/100 g total fatty acids of α-LNA, while the other components remained the same. The diets were powdered and were stored at -20°C and provided fresh to the rats every two days.

The rats were killed in the fed state by an overdose of CO_2. Blood was collected into heparin by cardiac puncture and kept at room temperature. Plasma was then collected by centrifugation for 10 min at 1000rpm. Plasma was stored at -20°C until analysis. At sacrifice various tissues, including spleen and thymus, were dissected out and weighed.

Table 2.3. Fatty acid composition of the diets

Diet	16:0	18:0	18:1 *n-9*	18:2 *n-6*	18:3 *n-3*	20:5 *n-3*	22:6 *n-3*	*n-6/ n-3*
Control	22.7	4.3	36.5	30.4	4.2	0.0	0.0	7
2.2 EPA *sn*-2	21.6	4.3	33.5	31.4	2.3	2.1	0.7	7
4.4 EPA *sn*-2	20.6	4.2	32.1	30.1	0.3	4.4	1.4	7
2.2 EPA *sn*-1(3)	20.6	4.3	34.1	32.0	2.4	2.1	0.3	7
4.4 EPA *sn*-1(3)	19.7	4.3	33.6	30.3	0.3	4.2	0.7	7
2.2 DHA *sn*-2	20.9	4.2	35.1	33.3	2.3	0.2	1.9	7
4.4 DHA *sn*-2	20.5	4.1	34.2	33.9	0.2	0.4	3.7	7
2.2 DHA *sn*-1(3)	21.3	4.3	33.8	32.4	2.4	0.3	2.3	7
4.4 DHA *sn*-1(3)	21.7	4.3	31.5	32.7	0.3	0.7	4.5	7

Values are expressed as g/100 g total fatty acids.

2.2.3 Blood Lipid Concentrations

Plasma Triacylglycerol Concentration

Plasma TAG concentrations were measured using the Sigma Diagnostics triglyceride (GPO-Trinder) kit (procedure 337). The assay is based on the method of McGowan *et al.* (1983) involving the hydrolysis of TAG by LPL to release glycerol which is then measured by coupled enzyme reactions. The concentration of glycerol was measured by following the change in absorbance at 540 nm. The increase in absorbance is directly proportional to the total glycerol concentration (i.e. free glycerol plus that released from the action of LPL on TAG present in the sample). It was assumed that free glycerol would make only a minor contribution to total glycerol in these samples.

Plasma Non-Esterified Fatty Acid Concentration

Plasma non-esterified fatty acid (NEFA) concentrations were determined using the Boehringer Mannheim Free Fatty Acids Half-micro Test (Cat No 1 383 175). This procedure is based on the method described by Shimizu *et al.* (1980), using a series

of coupled reactions, which use NEFA as the limiting reagent. The absorbance at 546 nm of a red dye formed in the final of these reactions is proportional to the concentration of NEFA in the original sample.

Plasma Total Cholesterol Concentration

Plasma total cholesterol concentrations were determined using the Sigma Diagnostics cholesterol kit (procedure No. 352). The method used is modified from Allain *et al.* (1974) using a series of coupled enzyme reactions. The absorbance at 500 nm of a red dye, the oxidation of which is the end-product to these reactions, is proportional to the concentration of total cholesterol in the original sample. Total cholesterol in plasma includes all of the cholesterol and cholesterol ester (which is hydrolysed by cholesterol esterase to release the cholesterol and fatty acids present) found in various lipoproteins.

Plasma Free (Non-Esterified) Cholesterol Concentration

Plasma non-esterified cholesterol concentrations were measured using the Boehringer Mannheim cholesterol kit (Cat No. 139 050). The method is based on the method of Rőschlau *et al.* (1974). The assay utilises a series of coupled enzyme reactions, which begin with the oxidation of cholesterol and end with the formation of a lutidine-dye. The concentration of the dye is proportional to the amount of non-esterified cholesterol and is measured by an increase in absorbance at 405 nm.

2.2.4 Spleen Leukocyte Preparation

The spleen was freed of adipose tissue and gently ground into RPMI medium through a fine mesh using the plunger from a 10 ml syringe. The cell suspension was filtered through lens tissue and leukocytes were collected by centrifugation (1000 rpm for 10 min). The cells were resuspended in RPMI medium and purified by centrifugation on Histopaque (density 1.077 g/ml) (2000 rpm for 15 min). The leukocytes were then washed once in RPMI medium. The leukocytes were counted using a Coulter cell counter (model Z1; Beckman Coulter, UK) and the cell concentration was adjusted as required.

2.2.5 Analysis of Leukocyte Subpopulations

Flow cytometry was used to measure the presence of markers on the surface of rat spleen leukocytes. The cells (1 x 10^7) were collected by centrifugation (1000 rpm) and washed three times in PBS supplemented with BSA (1 g/l) and sodium azide (0.65 g/l) (mPBS). They were then resuspended in mPBS and incubated for 20 min at 4°C, in a 96 well microtiter plate, with monoclonal antibodies (Mabs) to rat cell surface markers (*Table 2.4.*). Incubation with a Mab to the human C3b activator protein (OX21) was used as a negative control. After this, the cells were washed twice with mPBS, resuspended in mPBS and incubated with GAM-FITC for 20 min at 4°C. The cells were then washed twice with mPBS and suspended in FACS-Fix (2 ml formaldehyde in 100 ml PBS). They were examined for fluorescence using a Becton Dickinson FACScalibur fluorescence-activated cell sorter. Fluorescence data were collected on 2 x 10^4 viable cells. Results were analysed using Cellquest software and are expressed in terms of the percentage marker-positive cells and median fluorescence intensity (MFI). Typical profiles are shown in *Figure 2.3.*

Table 2.4. Stains used to identify the different mononuclear cell subsets

Cell Type	Stain
Negative Control	anti-human C3b, activator protein
Helper T (Th) Cell	Mouse-anti-rat-CD4
Cytotoxic T (Tc) cell	Mouse-anti-rat -CD8
B Cell	Mouse-anti-rat -CD19
Monocyte	Mouse-anti-rat -CD14

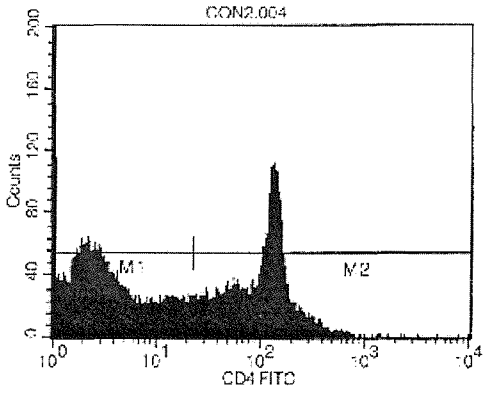
2.2.6 Lymphocyte Proliferation

Spleen leukocytes were cultured, in duplicate, at 37°C in an air/CO_2 (19:1) atmosphere in a 96-well round-bottomed plate at a density of 5 x 10^5 cells/well and a total culture volume of 200 µl in RPMI medium supplemented with 2 mM glutamine, antibiotics (penicillin and streptomycin) and 10% (v/v) FCS and various concentrations of Con A. After 48 h of culture [6-H^3] thymidine was added to each well (20 µl; 0.2 µCi/well) and the cells were incubated for a further 18 h. The cells were then harvested onto glass fiber filters and washed and dried using a Skatron

Cell Harvester (Lier, Norway). Radioactive incorporation was determined by liquid scintillation counting.

Figure 2.3. Typical profiles of spleen leukocyte subpopulations analysed by flow cytometry.

i



The profile from each sample was first displayed in a dot plot such that the leukocytes in the sample could be observed. The gated populations were then displayed in histograms. *Figure 2.3. i.* The un-gated leukocytes stained for different cell surface markers (see *Table 2.4*) were displayed in histograms. The population was then divided into two sections using 'markers' (M1 and M2), and the percentage of cells expressing a cell surface marker (i.e. cells in the M2 region) was determined. The level of expression of the marker (i.e. the mean fluorescence intensity in the M2 region) was also determined.

2.2.7 Cytokine and PGE_2 Production

Spleen leukocytes were cultured at 37°C in an air/CO_2 (19:1) atmosphere in a 24-well culture plate at a density of 5 x 10^6 cells/well and a total culture volume of 2 ml in RPMI medium supplemented with 2 mM glutamine, antibiotics (penicillin and streptomycin) and either 10% (v/v) FCS or 2.5% (v/v) autologous plasma and 2.5 μg/ml Con A. After 24 h of culture, the plates were centrifuged and the medium was collected and frozen at -20 °C for later analysis. Cytokine and PGE_2 concentration were determined using ELISA kits according to the manufacturer's instructions. The percentage coefficient of variation for the cytokines measured were (for intra-assay):

6.0 (IL-2), 4.1 (IFN-γ), and 5.8 (IL-10); (for inter-assay): 7.2 (IL-2), 4.9 (IFN-γ), and 7.0 (IL-10). The samples were diluted as appropriate, with the ranges of detection being 5 to 1500 pg/ml (IL-2), 13 to 1400 pg/ml (IFN-γ), 5 to 1000 pg/ml (IL-10) and 0.5 to 10.0 ng/ml (PGE_2).

2.2.8 Fatty Acid Analysis by Gas Chromatography

Lipid Extraction

Total lipid was extracted from plasma or spleen leukocytes using chloroform/methanol/water (2/1/0.8 vol/vol/vol). The sample was homogenized in 4 ml of methanol containing 100 μg/ml of the antioxidant butylated hydroxytoluene (BHT) and sealed under nitrogen. The sample was then vortexed and incubated at room temperature for 30 min. At the end of this time 2 ml chloroform and 1.1 ml water were added to reach a final ratio of 2:1:0.8 of methanol:chloroform:water and once again sealed under nitrogen and vortexed. A further 2 ml of chloroform and 2 ml of water were added to the sample which again was sealed under nitrogen and vortexed. The lower non-aqueous phase was then collected after centrifugation (10 min, 2500 rpm). The procedure was repeated from the point of addition of chloroform and water with the water being replaced by KCl (0.88 g/100 ml H_20) (Bligh and Dyer, 1957). The non-aqueous phase was then stored at –80°C.

Separation of Lipid Classes

Lipid extracts were dried under nitrogen and resuspended in a few drops of chloroform. Samples were then deposited (as a spot) onto a 20 x 20 cm thin layer chromatography (TLC) plate (K6 silica gel; layer thickness 250 μm; Whatman). The TLC plate was run in a tank containing hexane/ether/acetic acid (90:30:1 vol/vol/vol) as the elution phase. After approximately 30 min the plate was removed from the solvent tank and the separated lipid fractions were visualised under a UV light. Plasma extracts were separated into phospholipid (PL), triacylglycerol (TAG), cholesterol ester (CE), cholesterol, NEFA and identified by standards run previously (Rf values = PL: 0.18, TAG: 0.74 and CE: 0.95 (% coefficient of variance was 0.04,

0.01 and 0.02 respectively)). The fractions were then collected separately into individual tubes. The efficiency of the extraction and recovery of each fraction from the TLC plate was investigated. The % recovery from these steps was found to be: PL: 40.8, TAG: 43.6 and CE: 27.4.

Saponification and Methylation of Fatty Acids

Fatty acid methyl esters were formed by reaction with 2 ml methanolic boron trifluoride for one hour at 80°C. Samples were then cooled and 2 ml of hexane (containing BHT) and 1 ml saturated NaCl solution were added. The tubes were closed under nitrogen and vortexed. The layers were separated by centrifugation (10 min, 2500 rpm) and the top layer containing the fatty acids was collected.

Gas Chromatography

The fatty acid composition of each sample was determined by gas chromatography (GC) in a Hewlett-Packard (Hewlett-Packard; Avondale, PA, USA) 6890 gas chromatograph fitted with a 25 m x 0.32 mm SGE BPX70 capillary column with a film thickness of 0.25 μm. Helium at 1 ml/min was used as the carrier gas, and the split/splitless injector is used in the split mode with a split ratio of 20:1. Injector and detector temperatures were 280°C and 275°C respectively and the column oven temperature was held at 170°C for 12 min and then programmed to increase by 5°C/min until it reached 200°C. The detector was a flame ionising detector (FID). The separation was recorded with HP GC Chem Station software (Hewlett-Packard; Avondale, PA, USA). Fatty acid methyl esters were identified by comparison with a series of standards run previously. An example of a trace is shown in *Figure 2.4*. The retention time for a particular fatty acid was adjusted regularly by analysis of a standard sample (*Table 2.5.*). All samples were run on the same machine.

Figure 2.4. An example of a GC standard trace. The retention times are shown in *Table 2.6.*

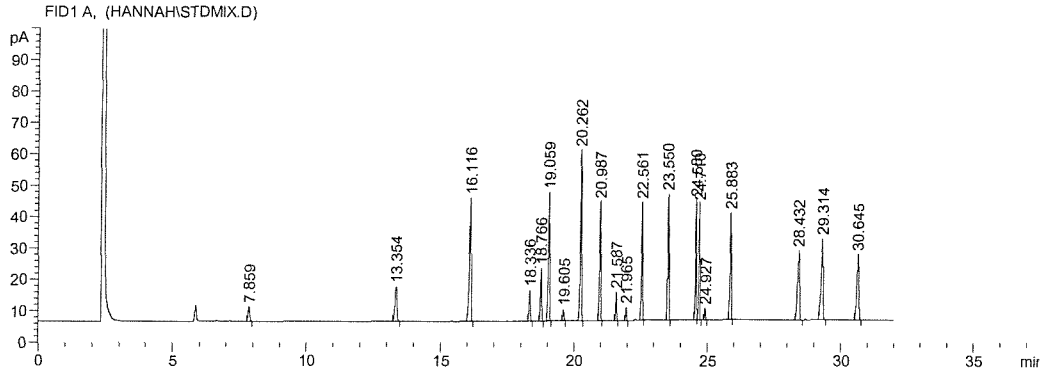


Table 2.5. Retention times used to differentiate and identify fatty acid methyl esters

Fatty acid methyl ester	Retention Time (min)
14:0	7.85
16:0	13.35
17:0	16.12
t18:1	18.34
18:0	18.77
18:1	19.10
t18:2 *n-6*	20.62
18:2 *n-6*	20.99
18:3 *n-6*	21.58
18:3 *n-3*	22.56
20:1	23.55
20:2 *n-6*	24.58
20:3 *n-6*	24.71
20:4 *n-6*	24.93
22:0	25.88
20:5 *n-3*	28.43
24:0	28.43
24:1	29.31
22:5 *n-3*	30.13
22:6 *n-3*	30.65

2.2.9 *Mass Spectrometric Analysis of Spleen Phospholipid Species*

Molecular species compositions of phospholipid were analyzed by ESI-MS using a Quattro II triple quadrupole mass spectrometer (Micromass UK, Manchester, UK), by colleagues in the Departemtn of Child Health, Southampton General Hospital, Southampton.

Total lipid was extracted from spleen leukocytes according to Bligh and Dyer. Spleen leukocyte phospholipids were fractionated into PC and acidic phospholipid fractions by standard procedures (see section 2.2.8).

Lipid extracts were dissolved in 20 µl of chloroform-methanol (1:2 vol/vol) containing 5 mM NaOH and 5 µl introduced by rheodyne valve injection into a flow of methanol-chloroform-water (80:10:10 vol/vol) pumped at 10 µl/min into the capillary inlet of the mass spectrometer. PC molecular species were detected as sodiated adducts under positive conditions, $(M+Na)^+$, whereas phosphatidyethanolamine (PE) and phosphatidylinositol (PI) molecular species were detected as their molecular ions under negative conditions, (MH).

Spectra were obtained by signal averaging (typically 1 min) and processed by use of Masslynx software (Micromass UK, Manchester, UK). After conversion to centroid format according to area and correction for ^{13}C isotope effects and for reduced response with increasing *m/z* values, the PC species were expressed as a percentage of the total present in the sample.

2.2.10 Statistical Analysis

Data shown are mean ± SEM of 8 animals fed each diet. Statistical analysis was performed using one-way analysis of variance and a post-hoc least significance test. These analyses were performed using SPSS Version 10.0 (SPSS Inc., Chicago, IL); in all cases a value for P of less than 0.05 was taken to indicate statistical significance.

Significant differences are expressed using superscripts such that:

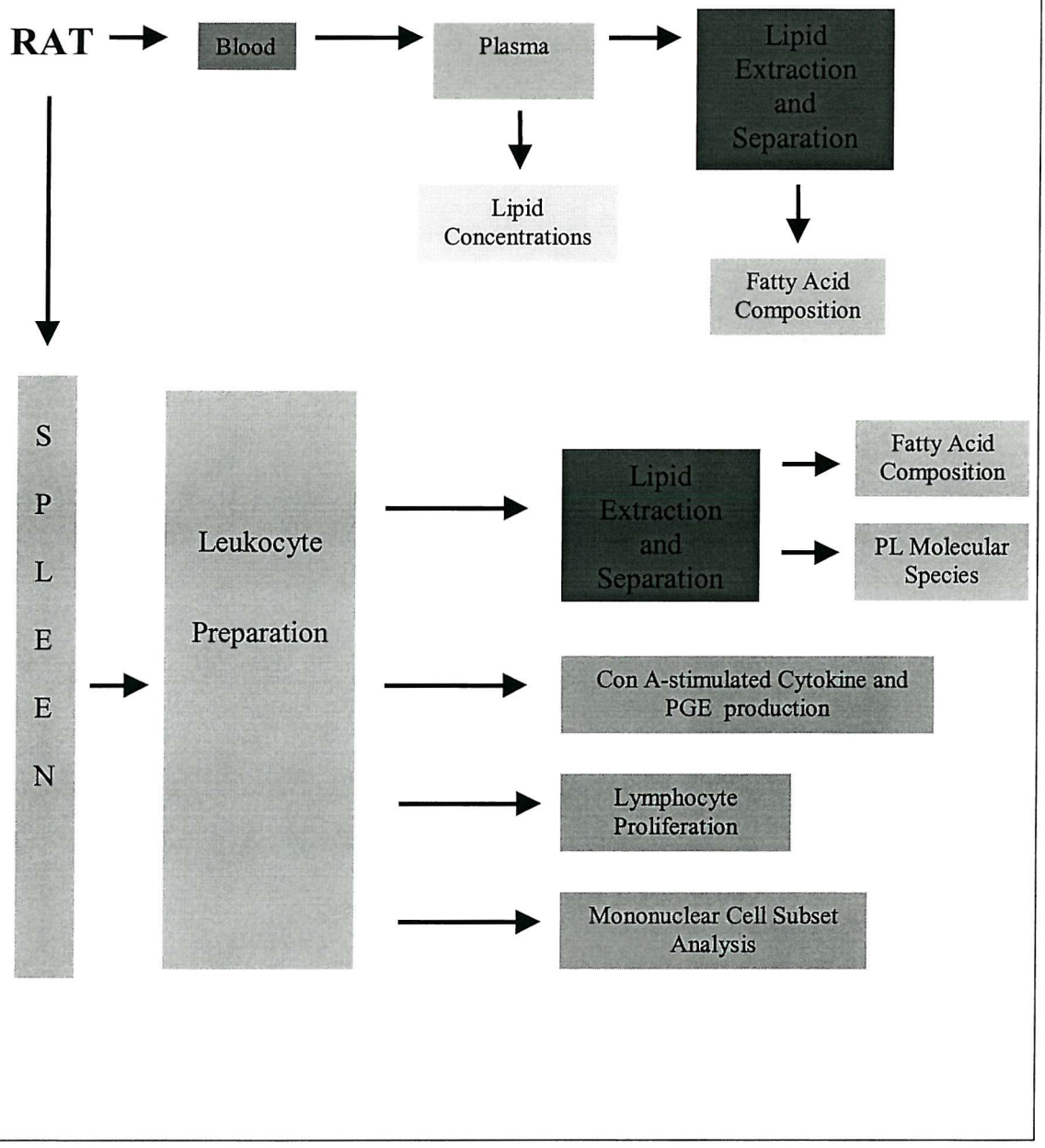
a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same level)
c. Indicates that the 2.2 g/100 g fatty acid diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

Comparison between the effects of EPA and DHA was also made but will not reported here.

2.2.11 Summary of Investigations in This Chapter

The investigations described in this chapter are summarised in *Figure 2.5*.

Figure 2.5. Summary of the investigations performed in this chapter.



2.3 RESULTS

2.3.1 Body Weights of Animals Fed the Structured TAG Diets

There were no significant differences in the initial body weights of the animals in the different groups. However, after 6 weeks the animals fed on the 2.2 EPA *sn*-2 diet had a significantly greater weight gain than those fed the *sn*-1(3) diet containing this fatty acid at this level (*Table 2.6.*). Animals fed the 2.2 DHA *sn*-2 diet had a significantly decreased weight gain compared to those fed the *sn*-1(3) diet containing this fatty acid at this level. This was reflected in the slightly lower total body weight of the animals.

Table 2.6. Weight gain and body weights of animals fed structured TAG diets

Diet	Initial Body Wt (g)	Final Body Wt (g)	Weight Gain (g)
Control	231.8 ± 7.6	412.6± 6.7	180.7± 6.0
2.2 EPA *sn*-2	225.5 ± 5.4	427.2± 7.3	201.7± 7.1[b]
4.4 EPA *sn*-2	217.1 ± 6.7	418.1± 6.5	200.9± 5.8
2.2 EPA *sn*-1(3)	230.8± 7.0	413.4± 9.4	182.6± 8.1
4.4 EPA *sn*-1(3)	232.0± 7.10	414.7± 9.1	182.7± 11.2
2.2 DHA *sn*-2	228.5± 9.6	395.5± 8.4	167.0± 9.5[b]
4.4 DHA *sn*-2	224.1± 8.2	410.6± 10.5	186.4± 9.3
2.2 DHA *sn*-1(3)	221.5± 7.3	416.1± 8.3	194.6± 6.6
4.4 DHA *sn*-1(3)	225.8± 8.6	429.8± 8.4	204.0± 11.1

Data are the mean ± SEM of 8 animals
Significant differences are expressed as:
b Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

2.3.2 Lymphoid Tissue Weights of Animals Fed Structured TAG Diets

There was no effect of diet upon the weight of the thymus (as % of body weight) (*Table 2.7.*). There were no differences in spleen weight (as % of body weight) among the animals fed the different diets, except that those fed the 2.2 DHA *sn*-2 diet had significantly larger spleens than control rats (*Table 2.7.*).

Table 2.7. Weights of lymphoid organs of animals fed structured TAG diets (expressed as a % of total body weight)

Diet	Thymus	Spleen
Control	0.349 ±0.05	0.166±0.003
2.2 EPA *sn*-2	0.337 ±0.03	0.179±0.004
4.4 EPA *sn*-2	0.300 ±0.03	0.187±0.004
2.2 EPA *sn*-1(3)	0.304 ±0.02	0.173±0.005
4.4 EPA *sn*-1(3)	0.308 ±0.04	0.165±0.004
2.2 DHA *sn*-2	0.267 ±0.02	0.179±0.007[a]
4.4 DHA *sn*-2	0.297 ±0.03	0.171±0.005
2.2 DHA *sn*-1(3)	0.326 ±0.03	0.175±0.003
4.4 DHA *sn*-1(3)	0.286 ±0.02	0.175±0.004

Data are the mean ± SEM of 8 animals
Significant differences are expressed as:
a. Indicates a significant difference from the control diet

2.3.3 Concentrations of Blood Lipids

There were no significant effects of diet on the plasma concentrations of total cholesterol (control group = 3.28 ± 0.20 mM), free cholesterol (control group = 0.148 ± 0.40 mM), TAG (control group = 0.165 ± 0.02 mM) or NEFA (control group = 0.95 ± 0.01 mM) (data not shown).

2.3.4 Fatty Acid Composition of Plasma and Spleen Leukocytes

The complete fatty acid compositions of the plasma and spleen leukocytes are shown in *Tables 2.8.- 2.12.* The changes are complex and so the fatty acids of main interest (i.e. arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)) will be examined in more detail than the other fatty acids, although any major changes in the latter will be described.

Table 2.8. Fatty acid composition of plasma PL from animals fed structured TAG diets*

Diet	16:0	18:0	18:1 *n-9*	18:2 *n-6*	20:4 *n-6*	20:5 *n-3*	22:5 *n-3*	22:6 *n-3*	AA/EPA
Control	23.7 ±0.8	25.0 ±1.0	6.6 ±0.3	21.1 ±1.5	17.2 ±0.9	0.3 ±0.1	0.9 ±0.3	4.4 ±0.3	45.3 ±1.5
2.2 EPA *sn*-2	24.0 ±1.2	23.0 ±0.7[a]	6.1 ±0.2	24.7 ±1.0[ac]	13.0 ±0.4[a]	1.1±0.1[ac]	1.2 ±0.2	4.4 ±0.5[c]	11.8 ±0.9[a]
4.4 EPA *sn*-2	24.4 ±1.1	23.4 ±0.7[a]	5.7 ±0.3	21.1 ±0.8	13.5 ±0.9[a]	1.8 ±0.1[a]	1.6 ±0.2[a]	6.3 ±0.8[a]	7.8 ± 0.5[a]
2.2 EPA *sn*-1(3)	24.2 ±0.6	24.0 ±0.7	6.3 ±0.3	21.8 ±1.0	14.8 ±0.5[a]	1.1±0.1[ac]	1.3 ±0.1	4.3 ±0.2	14.3 ±0.9[a]
4.4 EPA *sn*-1(3)	23.2 ±1.0	24.0 ±0.3	6.1 ±0.4	21.5 ±0.7	14.8 ±0.6[a]	2.1 ±0.1[a]	1.7 ±0.3[a]	5.3 ±0.6	7.2 ±0.6[a]
2.2 DHA *sn*-2	23.6 ±1.3	24.1 ±0.7[c]	5.5 ±0.7	22.5 ±1.7[c]	15.0 ±1.5[c]	0.4 ±0.1[a]	0.5 ±0.1	5.6 ±0.4	26.3 ±7.0[a]
4.4 DHA *sn*-2	25.5 ±1.7	21.4 ±0.5[a]	6.1 ±0.9	28.0 ±0.5[ab]	11.5 ±0.1[ab]	0.6 ±0.1[a]	0.6 ±0.1	6.3 ±0.7[a]	26.6 ±6.5[a]
2.2 DHA *sn*-1(3)	23.3 ±0.6	21.7 ±1.2[a]	6.0 ±0.3	26.2 ±0.3[a]	14.2 ±0.6[a]	0.5 ±0.2[a]	0.5 ±0.2	6.8 ±0.7[a]	29.0 ±3.5[a]
4.4 DHA *sn*-1(3)	24.1 ±0.0	21.8 ±0.2[a]	5.6 ±0.1	22.7 ±0.5	14.4 ±0.6[a]	0.8 ±0.1[a]	0.9 ±0.1	8.2 ±0.1[a]	20.3 ±4.2[a]

*Values are the mean ± SEM of 8 animals
*Values are g/100g of total fatty acid

a. indicates a significant difference from the control diet
b. indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. indicates that the 2.2 g/100 g fatty acids diet is significantly different from the 4.4 g/100g fatty acids diet (for the same fatty acid and the same positional isomer)

Table 2.9. Fatty acid composition of plasma TAG from animals fed structured TAG diets*

Diet	16:0	16:1	18:0	18:1 *n-9*	18:2 *n-6*	20:4 *n-6*	20:5 *n-3*	22:5 *n-3*	22:6 *n-3*
Control	23.4 ±0.8	1.1 ±0.1	5.3 ±2.0	30.6 ±3.1	28.0 ±0.3	2.6 ±0.6	0.6 ±0.1	0.9 ±0.4	1.3 ±0.3
2.2 EPA *sn*-2	23.3 ±1.1	1.2 ±0.1	4.1 ±0.9	33.2 ±1.7	29.0 ±0.8[c]	1.0 ±0.1[a]	2.0 ±0.2[ac]	1.5 ±0.7[c]	2.2 ±0.4[ac]
4.4 EPA *sn*-2	22.6 ±2.1	1.4 ±0.1	3.1 ±0.1	27.7 ±1.9	26.2 ±1.4	2.1 ±1.0	4.2 ±0.6[a]	2.7 ±1.3[ac]	4.1 ±0.8[a]
2.2 EPA *sn*-1(3)	21.2 ±0.8	1.4 ±0.1	3.0 ±0.2	32.4 ±1.3	30.0 ±0.7	1.4 ±0.1	2.5 ±0.2[ac]	2.0 ±1.0[a]	2.4 ±0.3[a]
4.4 EPA *sn*-1(3)	22.4 ±0.9	1.5 ±0.5	4.3 ±0.9	32.0 ±0.8	27.0 ±0.3	1.7 ±0.4	3.7 ±0.4[a]	2.7 ±1.2[a]	2.8 ±0.4[a]
2.2 DHA *sn*-2	21.4 ±0.8	1.1 ±0.1	4.0 ±0.5	32.5 ±1.0[c]	29.0 ±0.9	1.5 ±0.1	0.9 ±0.0	1.0 ±0.1	5.5 ±0.5[ac]
4.4 DHA *sn*-2	21.5 ±1.3	1.1 ±0.2	3.5 ±0.8	27.4 ±1.7	31.0 ±0.5[a]	1.2 ±0.3[a]	1.6 ±0.3	1.4 ±0.1[d]	8.6 ±0.7[a]
2.2 DHA *sn*-1(3)	23.2 ±1.3	1.2 ±0.2	3.1 ±0.1	31.2 ±0.8	30.0 ±1.0	1.3 ±0.1	0.9 ±0.1	1.2 ±0.0	5.6 ±0.7[ac]
4.4 DHA *sn*-1(3)	21.2 ±0.1	1.2 ±0.1	3.3 ±0.2	29.6 ±0.9	29.1 ±0.8	1.3 ±0.1	1.3 ±0.1	1.5 ±0.2[d]	8.2 ±0.6[a]

*Values are the mean ± SEM of 8 animals
*Values are g/100g of total fatty acid

a. indicates a significant difference from the control diet
b. indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. indicates that the 2.2 g/100 g fatty acids diet is significantly different from the 4.4 g/100g fatty acids diet (for the same fatty acid and the same positional isomer)

Table 2.10. Fatty acid composition of plasma CE from animals fed structured TAG diets*

Diet	**16:0**	**16:1**	**18:0**	**18:1 *n-9***	**18:2 *n-6***	**20:4 *n-6***	**20:5 *n-3***	**22:5 *n-3***	**22:6 *n-3***
Control	8.0 ±0.4	1.3 ±0.5	0.9 ±0.2	7.2 ±0.3	20.8 ±0.6	53.6 ±2.1	0.7 ±0.0	n.d	2.4 ±0.5
2.2 EPA *sn*-2	7.9 ±0.7	1.1 ±0.2	0.7 ±0.1	7.3 ±0.4	23.5 ±0.8	49.1 ±0.8	5.9 ±0.4[ac]	n.d	2.4 ±0.4
4.4 EPA *sn*-2	8.9 ±0.8	1.0 ±0.3	0.8 ±0.0	7.1 ±0.5	21.4 ±1.2	45.6 ±2.2[a]	9.6 ±0.6[a]	3.4 ±0.1[a]	2.3 ±0.5
2.2 EPA *sn*-1(3)	8.4 ±0.7	1.5 ±0.5	1.0 ±0.4	8.1 ±0.8	21.2 ±1.1	49.9 ±3.1	5.4 ±0.4[ac]	n.d	2.2 ±1.2
4.4 EPA *sn*-1(3)	8.1 ±0.6	1.3 ±0.4	0.7 ±0.0	7.2 ±0.3	20.5 ±0.3	44.1 ±1.4[a]	9.3 ±0.2[a]	4.2 ±0.1[a]	2.1 ±0.5
2.2 DHA *sn*-2	8.8 ±0.2	1.4 ±0.2	0.6 ±0.1	9.3 ±0.9	24.6 ±0.8[a]	49.5 ±2.4[c]	1.8 ±0.1[c]	3.8 ±0.1[a]	2.4 ±0.2
4.4 DHA *sn*-2	10.2 ±1.2[a]	1.5 ±0.2	0.9 ±0.2	11.1±0.7[ab]	27.5 ±3.2[a]	37.3 ±4.1[ab]	5.3 ±2.7[a]	n.d	2.6 ±0.5
2.2 DHA *sn*-1(3)	8.3 ±0.3	1.0 ±0.3	0.9 ±0.0	7.7 ±0.6	26.5 ±0.3[a]	49.3 ±1.2	1.9 ±0.2[c]	n.d	2.7 ±0.3
4.4 DHA *sn*-1(3)	9.2 ±0.5	1.6 ±0.3	0.9 ±0.0	8.4 ±0.6	26.2 ±1.0[a]	46.3 ±1.3[a]	3.2 ±0.3[a]	n.d	2.7 ±0.1

*Values are the mean ± SEM of 8 animals
*Values are g/100g of total fatty acid

a. indicates a significant difference from the control diet
b. indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. indicates that the 2.2 g/100g fatty acids diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

Table 2.11. Fatty acid composition of spleen leukocyte PL from animals fed structured TAG diets*

Diet	16:0	18:0	18:1 *n-9*	18:2 *n-6*	20:4 *n-6*	20:5 *n-3*	22:5 *n-3*	22:6 *n-3*	AA/EPA
Control	24.5 ±1.4	23.4 ±1.5	8.2 ±1.0	17.6 ±2.4	18.1 ±0.9	0.4 ±0.1	0.9 ±0.1	3.4 ±0.6	38.4 ±0.1
2.2 EPA *sn*-2	25.4 ±1.2	21.7 ±0.9[a]	8.0 ±1.3	20.9 ±2.5	14.2 ±0.9[a]	1.2±0.5[ac]	1.8 ±0.4[a]	3.8 ±0.6	11.9 ±2.1[a]
4.4 EPA *sn*-2	24.4 ±0.7	21.7 ±1.1[a]	7.5 ±1.2	18.3 ±1.8	14.1 ±0.7[a]	1.8 ±0.1[a]	2.3 ±0.4[a]	4.9 ±1.0	7.80 ±0.7[a]
2.2 EPA *sn*-1(3)	24.2 ±0.4	21.4 ±1.2[a]	9.4 ±1.4	17.1 ±2.1	15.7 ±0.6[a]	1.1±0.1[ac]	2.2 ±0.4[a]	3.2 ±0.6	16.6 ±9.2[a]
4.4 EPA *sn*-1(3)	23.4 ±0.6	20.5 ±2.2[a]	8.2 ±1.3	17.8 ±2.3	14.4 ±0.4[a]	2.0 ±0.1[a]	2.5 ±0.5[a]	3.7 ±1.1	7.10 ±0.8[a]
2.2 DHA *sn*-2	23.0 ±0.8	21.1 ±1.4[a]	8.4 ±1.3	17.6 ±2.4	15.8 ±0.9[ab]	0.6 ±0.2	0.8 ±0.1	4.7 ±0.5	30.2 ±15.3
4.4 DHA *sn*-2	25.1 ±1.0	20.4 ±0.7[a]	8.4 ±1.5	17.4 ±2.4	13.3 ±1.1[a]	0.5 ±0.1	1.0 ±0.5	5.8 ±0.5[a]	28.3 ±12.6
2.2 DHA *sn*-1(3)	22.9 ±0.5	20.5 ±1.5[a]	7.6 ±1.6	22.4 ±3.4	14.5 ±0.5[a]	0.5 ±0.1	0.9 ±0.5	5.9 ±1.0[a]	38.0 ±14.6
4.4 DHA *sn*-1(3)	24.9 ±0.8	21.4 ±0.4[a]	7.3 ±1.7	23.9 ±2.4	14.2 ±0.4[a]	0.7 ±0.1	0.9 0.7	7.1 ±1.0[a]	19.5 ±2.9[a]

*Values are the mean ± SEM of 8 animals

*Values are g/100g of total fatty acid

a. indicates a significant difference from the control diet

b. indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

c. indicates that the 2.2 g/100g fatty acids diet is significantly different from the 4.4 g/100g fatty acids diet (for the same fatty acid and the same positional isomer)

Table 2.12. Fatty acid composition of spleen leukocyte DAG from animals fed structured TAG diets*

Diet	16:0	18:0	18:1 *n-9*	18:2 *n-6*	20:4 *n-6*	20:5 *n-3*	22:5 *n-3*	22:6 *n-3*
Control	22.0 ±1.4	20.8 ±3.1	10.7 ±2.6	20.0 ±1.2	16.7 ±1.2	1.5 ±1.5	1.2 ±0.3	3.7 ±0.5
2.2 EPA *sn*-2	22.0 ±2.2	20.3 ±2.8	9.8 ±3.7	24.4 ±0.8[ab]	13.0 ±0.3[a]	1.2 ±0.5	1.5 ±0.3	4.0 ±0.5
4.4 EPA *sn*-2	23.5 ±1.2	20.9 ±2.5	7.7 ±2.0	20.6 ±0.7	13.3 ±0.8[a]	2.1 ±0.9	1.9 ±0.4	5.7 ±0.8[a]
2.2 EPA *sn*-1(3)	21.3 ±1.8	17.5 ±3.0[a]	12.7 ±2.9	20.6 ±0.7	14.4 ±0.6	1.6 ±0.7[c]	2.1 ±0.4	3.2 ±0.5
4.4 EPA *sn*-1(3)	20.3 ±2.1	17.4 ±3.1[a]	10.0 ±1.9	18.3 ±1.6	12.9 ±1.5[a]	2.7 ±1.1[a]	2.6 ±0.4[a]	4.5 ±0.5
2.2 DHA *sn*-2	21.9 ±1.4	12.4 ±2.5[a]	9.9 ±2.8	20.6 ±1.6[c]	15.3 ±1.2[c]	0.5 ±0.2	2.1 ±1.2	5.1 ±0.4[a]
4.4 DHA *sn*-2	22.9 ±1.9	10.6 ±2.5[a]	10.9 ±3.0	25.5 ±1.6[a]	12.1 ±0.6[a]	0.7 ±0.1	0.9 ±0.2	6.3 ±0.4[a]
2.2 DHA *sn*-1(3)	21.9 ±1.0	10.8 ±2.7[a]	11.4 ±3.4	23.8±1.6[a]	14.4 ±0.5	0.5 ±0.1	0.9 ±0.2	5.7 ±0.8[a]
4.4 DHA *sn*-1(3)	22.3 ±1.4	10.5 ±2.9[a]	11.4 ±3.4	22.5 ±0.4	14.1 ±0.5	0.9 ±0.1	1.1 ±0.2	6.8 ±0.9[a]

*Values are the mean ± SEM of 8 animals
*Values are g/100g of total fatty acid

a. indicates a significant difference from the control diet
b. indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. indicates that the 2.2 g/100g fatty acids diet is significantly different from the 4.4 g/100g fatty acids diet (for the same fatty acid and the same positional isomer)

2.3.4.1 Fatty Acid Composition of Plasma Phospholipids, TAG and Cholesterol Ester

Changes in Plasma PL

There were no changes in PA or OA content of the plasma PL compared to the control group (*Table 2.8.*). There was, however, a decrease in the proportion of SA in plasma PL compared to the control group in some groups fed EPA or DHA (*Table 2.8.*). This decrease was significant for the EPA *sn*-2 diets but not for the *sn*-1(3) diets. Thus, there appears to be an effect of the position of EPA in dietary TAG on the content of SA in plasma PL. The decrease in SA after DHA feeding was significant for the 4.4 DHA *sn*-2 diet and for the DHA *sn*-1(3) diets at both doses. This suggests an effect of dose when DHA is in the *sn*-2 position, while in the *sn*-1(3) position both doses are equipotent.

The changes in LA were complex (*Table 2.8.*). In plasma PL there was no change in LA content after feeding EPA, except for the 2.2 *sn*-2 EPA group where there was a significant increase compared to the control. There was an increase in LA in plasma PL compared to the control in the 4.4 *sn*-2 DHA and 2.2 *sn*-1(3) DHA groups. Feeding either EPA or DHA resulted in a significant decrease in AA in plasma PL. This was more pronounced for the *sn*-2 EPA diets compared to the *sn*-1(3) EPA diets. The decrease in AA was dose-dependent for the *sn*-2 DHA diets, but not for the *sn*-1(3) DHA diets. There was also a significant effect of the position of DHA at the 4.4 level with AA content being significantly less for the DHA *sn*-2 group compared to the *sn*-1(3) group.

There was a dose-dependent increase in EPA (400 and 500 %) and DPA (30 to 100%) in the plasma PL for the EPA-fed animals and the magnitude of this increase was irrespective of position of EPA in dietary TAG (*Table 2.8*). In this fraction there was also a dose-dependent decrease in the AA/EPA ratio for the animals fed EPA, and this was independent of the position of EPA in dietary TAG (*Table 2.8*). There was an increase in DHA (50%) in the plasma PL after EPA feeding at the 4.4 level. The increase in DHA was greatest for the 4.4 EPA *sn*-2 diet and this was significant compared to the control group and the 2.2 EPA *sn*-2 group (*Table 2.8*). The DHA-fed

animals also showed a dose-dependent increase in EPA in plasma PL fractions (50 to 100 % increases). The content of DPA in plasma PL tended to decrease in animals fed DHA. There was a 50% decrease in the AA/EPA ratio of plasma PL for the animals fed DHA compared to the control. There was a dose-dependent increase in the proportion of DHA in plasma PL for the DHA-fed animals (up to 80%) but this was independent of position of DHA in dietary TAG.

Changes in Plasma TAG

There were no changes in PA or OA content of the plasma TAG compared to the control group (*Tables 2.9.*). There was, however, a non-significant decrease in SA in plasma TAG when EPA or DHA were fed (*Table 2.9.*). There was a small decrease in LA for both the 4.4 EPA groups while in the 2.2 EPA groups there was a small increase; these changes were not significant. For the DHA-fed animals there was an increase in LA in plasma TAG for all groups compared to the control and this was significant for the 4.4 *sn*-2 DHA group. All diets resulted in a significant decrease in AA in plasma TAG, although this was significant only for the 2.2 EPA *sn*-2 and the 4.4 DHA *sn*-2 diets.

There was a dose-dependent increase in EPA (230 to 600%) in plasma TAG and this was independent of position of EPA in dietary TAG (*Table 2.9.*). An increase in DHA content after EPA feeding was also seen in the plasma TAG (up to 300 % increase) and the 4.4 *sn*-2 EPA group was significantly different from the control and 2.2 *sn*-2 EPA groups (*Table 2.9*). The DHA-fed animals also showed a dose-dependent increase in EPA in plasma TAG (up to 170% increase). There was also a dose-dependent increase in DPA compared to the control group when DHA was fed (*Table 2.9*). There was a dose-dependent increase in the proportion of DHA in plasma TAG for the DHA-fed animals (up to 160%) but this was independent of position of DHA in dietary TAG.

Changes in Plasma CE

The fatty acid composition of the cholesterol ester (CE) fraction also changed according to the diet. There was no change in SA content, but there were increases in

both OA and LA in some DHA-fed animals (*Table 2.10.*). In contrast, there was no change in the content of OA or LA in CE of EPA-fed animals. When EPA or DHA was fed in the diet there was a dose-dependent decrease in the content of AA (*Table 2.10*). This decrease was significant for the 4.4 EPA groups regardless of EPA position in dietary TAG, while for the DHA-fed groups there was a significant effect of the position of DHA at the 4.4 level, and of dose when DHA was in the *sn*-2 position. The content of EPA and DPA in CE increased dose-dependently for the EPA-fed animals (*Table 2.10.*). DHA feeding also caused a dose-dependent increase in EPA content of CE (*Table 210*). Inclusion of EPA or DHA in the diet did not change the DHA content of the CE fraction (*Table 2.10*).

2.3.4.2 Fatty Acid Composition of Spleen Cell Phospholipids and Diacylglycerol

There were no significant changes in the PA or OA content of spleen cell PL and DAG when EPA or DHA were fed in the diet (*Tables 2.11.* and *2.12.*). The proportion of SA in both fractions was significantly decreased when either EPA or DHA was fed with no apparent effect of dose or position (except for the EPA *sn*-2 diets where there were no changes in SA in the spleen DAG). There was a non-significant increase in LA in spleen PL when EPA was fed in the *sn*-2 position, but no change when it was in the *sn*-1(3) position. There was a non-significant increase in LA in spleen PL for both doses of DHA at the *sn*-1(3) position, but no change for DHA at the *sn*-2 position. In the DAG fraction there was an increase in LA content in the 2.2 EPA *sn*-2 group and this was significant compared to the control and 2.2 EPA *sn*-1(3) group (*Tables 2.12.*). Thus, there was an effect of the position of EPA in dietary TAG when fed at the 2.2 level. For the DHA-fed groups there was a dose-dependent increase in LA content of the DAG fraction, and this was significant for the *sn*-2 groups. The content of LA was significantly increased for the 4.4 DHA *sn*-2 and the 2.2 DHA *sn*-1(3) groups compared to the control (*Tables 2.12).*

In both fractions there was a decrease in AA content compared to the control when either EPA or DHA was fed (*Tables 2.11.* and *2.12.*). There was a significant effect of position of DHA when fed at the 2.2 level on AA in spleen PL (*Tables 2.10.* and *2.11.*). In the DAG fraction the decrease in AA content was significant for both EPA

sn-2 groups compared to the control, while in the EPA *sn*-1(3) groups the reduction in AA was only significant at the 4.4 level. The feeding of EPA resulted in dose-dependent increases in EPA (50 to 100% in DAG and 200 to 400% in PL) and DPA in both spleen lipid fractions (*Tables 2.11.* and *2.12.*). Feeding DHA did not alter the content of EPA or DPA in either fraction. There were dose-dependent decreases (60 to 70 %) in the AA/EPA ratio in the PL fraction for those animals fed EPA and this was independent of position of EPA in dietary TAG (*Table 2.12.*) Feeding DHA also resulted in a dose-dependent decrease in this ratio but to a much lesser extent (25%) than when EPA was fed. In both the PL and DAG fractions there was a dose-dependent increase in DHA when DHA was fed (30 to 100%). The increase in DHA after DHA feeding was more marked than when EPA was fed (11 to 30 %).

2.3.4.3 Summary of the Changes in Fatty Acid Composition for the Different Spleen Leukocyte and Plasma Lipid Fractions

The fatty acid composition of the diet modulated the fatty acid composition of each of the plasma and cell lipid fractions studied. The changes in fatty acid composition are summarised in *Table 2.13.* and *Table 2.14.* All lipid fractions studied were altered by dietary EPA and DHA. A number of dose-dependent effects of EPA and DHA were identified, including effects on EPA and DHA content, the content of their metabolites, and, interestingly, on the content of other fatty acids. The effect of position of EPA and DHA in dietary TAG was much less obvious and it may be that there is little difference between consuming EPA or DHA in the *sn*-2 or *sn*-1(3) position of dietary TAG.

Table 2.13. The effects of EPA in dietary structured TAG upon the fatty acid composition of different lipid fractions

Fatty acid	Spleen PL	Spleen DAG	Plasma PL	Plasma TAG	Plasma CE
16.0	↔	↔	↔	↔	↔
18.0	↓	↓ (except *sn*-2 diet)	↓ dd	↓	↔
18.1 *n-9*	↔	↔	↔	↔	↔
18.2 *n-6*	↑ *sn*-2 ↔ sn1(3)	↑ *sn*-2 ↓ *sn*-1(3)	↑ *sn*-2 ↔ *sn*-1(3)	↓ dd	↔
20.4 *n-6*	↓	↓ dd	↓	↓	↓ dd
20.5 *n-3*	↑ dd	↑ dd	↑ dd	↑ dd	↑ dd
22.5 *n-3*	↑ dd	↑ dd	↑ dd (*sn*-1(3)>*sn*-2)	↑ dd	↑ dd
22.6 *n-3*	↔	↔	↔	↑ dd	↔

dd = dose-dependently

Table 2.14. The effects of DHA in dietary structured TAG upon the fatty acid composition of different lipid fractions

Fatty acid	Spleen PL	Spleen DAG	Plasma PL	Plasma TAG	Plasma CE
16.0	↔	↔	↔	↔	↔
18.0	↓	↓	↓ dd	↓	↔
18.1 *n-9*	↔	↔	↔	↔	↑ dd (except 4.4 *sn*-2)
18.2 *n-6*	↑	↑	↔ *sn*-2 ↑ *sn*-1(3)	↑	↑ dd
20.4 *n-6*	↓	↓ dd	↓	↓	↓ dd
20.5 *n-3*	↔	↓	↑ (smaller than EPA diet)	↑ dd (smaller than EPA diet)	↑ dd (smaller than EPA diet)
22.5 *n-3*	↔	↔	↔	↔	↔
22.6 *n-3*	↑ dd	↑ dd	↑ dd	↑ dd	↔

dd = dose-dependently

2.3.5 Spleen Leukocyte Phospholipid Molecular Species

Spleen leukocyte PL was separated into its constituent molecular species by mass spectrometry and the fatty acid composition of these was investigated. The compositions of the phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS) species were determined. Due to constraints in the analyses of these samples, the composition of phosphatidylethanolamine was not able to be investigated. There were changes in the composition of the phospholipid molecular species according to diet.

Phosphatidylcholine Molecular Species Composition

The PC molecular species were the most interesting of those analysed (*Tables 2.15* and *2.16.*). This is because this species was present in the greatest amount in the phospholipid extracted from the spleen cell preparation and so yielded mass spectra of high intensity, which could be studied in detail.

Overall, it appeared that feeding EPA led to a decrease in AA and an increase in EPA and DHA in PC species. However, the changes in AA were not always in the same species as the increase in content of EPA or DHA. After feeding DHA there was an increase in this fatty acid in some PC species.

The greatest reduction in AA-containing PC species was seen in 18:0/20:4 PC (diacyl) which decreased by over 50% when EPA or DHA was fed (*Table 2.15*). This one of the two major AA-containing PC species. There was also a significant reduction in 18:1/20:4 PC (alkylacyl) where EPA was fed, and a non-significant reduction in 18:0/20:4 (alkylacyl) and 18:1/20:4 (diacyl) PL (*Table 2.15*). The second major AA-containing PC species (16:0/20:4) was largely unaffected by the presence of EPA or DHA in the diet. There was a small effect of position of EPA in dietary TAG on the reduction of AA-containing PC species: EPA in the *sn*-1(3) position reduced the total content of AA containing PC species to a greater extent than when fed in the *sn*-2 position (*Table 2.15*).

EPA containing PC species were rare. Nevertheless, there was a significant increase in 16:0/20:5 PC, EPA was fed (*Table 2.15*). There was a much more marked increase

in this species when the *sn*-2 4.4 EPA diet was fed. The small increase in 18:0/20:5 which accompanied EPA feeding was not significant. Feeding EPA in the *sn*-2 position also increased the amount of 16:0/22:6 PC (*Table 2.15.*). The total of EPA containing species increased for all the EPA-fed groups compared to the control (100 to 200% increase) but this was only significant for the 4.4 EPA *sn*-2 diet (200% increase).

Two DHA containing PC species were identified. An increase in 16:0/22:6 DHA was seen after feeding DHA, but this was not significant compared to the control group. There was no increase in DHA in the 18:0/22:6 PC after DHA feeding. There was no change in the content of EPA containing species after DHA feeding.

There were also changes in the content of some of the other PC species measured (*Tables 2.16.)*. There was a significant increase in 16:0/18:2 PC and 18:0/18:2 PC when either EPA or DHA was fed. There was also a significant decrease in the content of 18:0/20:3 PC for the 2.2 EPA *sn*-1(3) group compared to the 4.4 EPA *sn*-1(3) group. There was a decrease in the content of 16:0/18:1 PC after EPA was fed in the *sn*-2 position, and this was significant at the 4.4 level compared to the control group.

Phosphatidylinositol and Phosphatidylserine Molecular Species Composition

The PI and PS content of the PL was much less than that of PC and due to the sample preparation the intensity of the traces was very weak, and background contamination was high. As a result the molecular species composition of these PL was more difficult to interpret. However, the major PI and PS species could be identified.

The major PI species were 18:0/20:4, 18:0/22:6 and 18:1/20:4 and 16:0/20:4, 18:0/18:2 and 18:0/18:1 were also present. The differences in content of these between the different diets could not be established. The major PS species were 18:0/18:2, 18:0/18:1 and 18:0/20:4, but again the differences in content of these between the different diets could not be established.

Table 2.15. Composition of plasma phosphatidylcholine molecular species containing fatty acids of interest*

Diet	16:0/20:4	16:0/20:5	16:0/22:6	18:0/20:4 alkylacyl	18:0/20:4 diacyl	18:0/20:5	18:0/22:6	18:1/20:4 alkylacyl	18:1/20:4 diacyl	Total AA molecular species	Total EPA molecular species
Control	6.45 ±1.03	0.14 ±0.01	0.80 ±0.23	0.44 ±0.22	6.51 ±0.36	0.39 ±0.02	0.42 ±0.43	1.81 ±0.33	2.11 ±0.005	17.3 ±0.7	0.54 ±0.03
2.2 EPA *sn*-2	5.92 ±0.35	0.52 ±0.10[a]	1.13 ±0.14	0.30 ±0.06	3.63 ±0.27[a]	0.47 ±0.14	0.39 ±0.05	1.27 ±0.20	2.18 ±0.25[b]	13.3 ±0.6	0.99 ±0.07
4.4 EPA *sn*-2	5.22 ±0.87	0.97 ±0.29[a]	1.34 ±0.25[ab]	0.14 ±0.05	3.39 ±0.02[a]	0.50 ±0.03	0.52 ±0.15	1.54 ±0.05[a]	2.06 ±0.32	12.4 ±1.1	1.47 ±0.26[a]
2.2 EPA *sn*-1(3)	6.04 ±0.72	0.51 ±0.16[a]	0.80 ±0.09	0.19 ±0.18	2.57 ±0.02[a]	0.41 ±0.17	0.28 ±0.04	1.39 ±0.18	1.60 ± 0.06	11.7 ±0.7[a]	0.92 ±0.33
4.4 EPA *sn*-1(3)	4.61 ±0.65	0.58 ±0.27[a]	0.59 ±0.09	0.23 ±0.07	2.86 ±0.57[a]	0.39 ±0.08	0.38 ±0.07	0.81 ±0.20[a]	1.60 ± 0.10	10.1 ±1.3[a]	0.97 ±0.18
2.2 DHA *sn*-2	4.98 ±1.25	0.08 ±0.08	1.02 ±0.18	0.17 ±0.12	3.39 ±1.29[a]	0.37 ±0.11	0.32 ±0.02	1.49 ±0.41	1.49 ±0.87	11.5 ±3.7[a]	0.46 ±0.18
4.4 DHA *sn*-2	5.36 ±0.35	0.21 ±0.02	1.19 ±0.09	0.28 ±0.01	4.17 ±0.29[a]	0.26 ±0.05	0.33 ±0.09	1.09 ±0.24	1.40 ±0.21	12.3 ±0.6	0.48 ±0.03
2.2 DHA *sn*-1(3)	5.36 ±0.39	0.14 ±0.09	1.00 ±0.18	0.43 ±0.08	4.49 ±0.66	0.29 ± 0.15	0.32 ±0.02	1.30 ±0.32	0.94 ±0.12	12.5 ±0.1	0.45 ±0.07
4.4 DHA *sn*-1(3)	4.89 ±113	0.17 ±0.00	1.35 ±0.03	0.32 ±0.01	3.28 ±0.32[a]	0.36 ±0.01	0.31 ±0.04	0.98 ±0.39	1.40 ±0.44	10.6 ±2.3[a]	0.54 ±0.01

*Values are the mean ± SEM of 3 animals
*Values are g/100g of PC

a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

Table 2.16. Composition of plasma phosphatidylcholine molecular species containing the other fatty acids measured*

Diet	16:0/16:0	16:0/18:1	16:0/18:2	16:0/18:1	18:1/18:2	18:0/18:2	18:0/18:1	18:0/20:3
Control	27.90 ±1.12	4.00 ±0.36	13.84 ±0.83	19.11 ±0.63	3.69 ±0.32	8.15 ±0.02	2.89 ±0.34	1.23 ±0.74
2.2 EPA *sn*-2	25.21 ±2.06	3.01 ±0.62	18.84 ±0.18 [a]	17.68 ±0.74	4.94 ±1.16	11.16 ±0.72 [a]	2.29 ±0.06	1.00 ±0.17
4.4 EPA *sn*-2	24.37 ±.0.66	3.62 ±0.39	18.03 ±0.74 [a]	17.22 ±0.01 [a]	5.58 ±0.05	10.80 ±0.47 [a]	2.78 ±0.24	1.87 ±0.15
2.2 EPA *sn*-1(3)	25.84 ±1.13	3.50 ±0.30	18.54 ±0.84 [a]	18.35 ±1.09	5.20 ±0.45	11.35 ±0.34 [a]	2.95 ±0.68	0.50 ±0.04 [c]
4.4 EPA *sn*-1(3)	28.27 ±2.89	3.51 ±0.47	18.16 ±1.75 [a]	19.27 ±0.15	4.36 ±0.58	10.62 ±0.52 [a]	2.71 ±0.93	1.04 ±0.06
2.2 DHA *sn*-2	27.67 ±3.57	1.40 ±0.76[c]	17.03 ±1.12 [a]	21.56 ±3.70	5.75 ±1.44	8.39 ± 2.05	3.30 ±0.62	1.54 ±0.38
4.4 DHA *sn*-2	24.30 ±1.49	3.10 ±0.36	17.52 ±1.11 [a]	18.23 ±1.71	5.66 ±0.77	11.30 ± 0.89 [a]	3.94 ±0.53	1.60 ±0.17
2.2 DHA *sn*-1(3)	23.90 ±0.18	2.95 ±0.38	17.78 ±0.46 [a]	18.66 ±0.19	5.96 ±0.76	11.13 ± 0.96 [a]	4.03 ±0.16	1.28 ±0.59
4.4 DHA *sn*-1(3)	24.20 ±21.17	2.67 ±0.05	19.01 ±1.41 [a]	19.25 ±0.41	5.25 ±0.22	11.23 ±0.17 [a]	4.33 ±0.33	1.23 ±0.13

*Values are the mean± SEM of 3 animals

*Values are g/100g of PC

a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. Indicates that the 2.2 g/100g fatty acids diet is significantly different from the 4.4 g/100g fatty acids diet (for the same fatty acid and the same positional isomer)

2.3.6 Analysis of Spleen Cell Subpopulations

There was no effect of diet on the % of CD3+ (T cells), CD4+ (Th cells) or CD19+ (B cells) cells in the spleen (*Table 2.17.*). There was a significantly lower % of CD8+ positive cells in the spleen when DHA was fed in the *sn*-1(3) position at 2.2 g/100 g of total fatty acids compared to the control and the same fatty acid at the 4.4 level. The % of monocytes was greater after feeding the 2.2 EPA *sn*-2 diets compared to the control (and also compared to the respective DHA diet) (*Table 2.17.*).

Table 2.17. Proportions of cells found in the spleen (expressed as percentage of total leukocytes)*

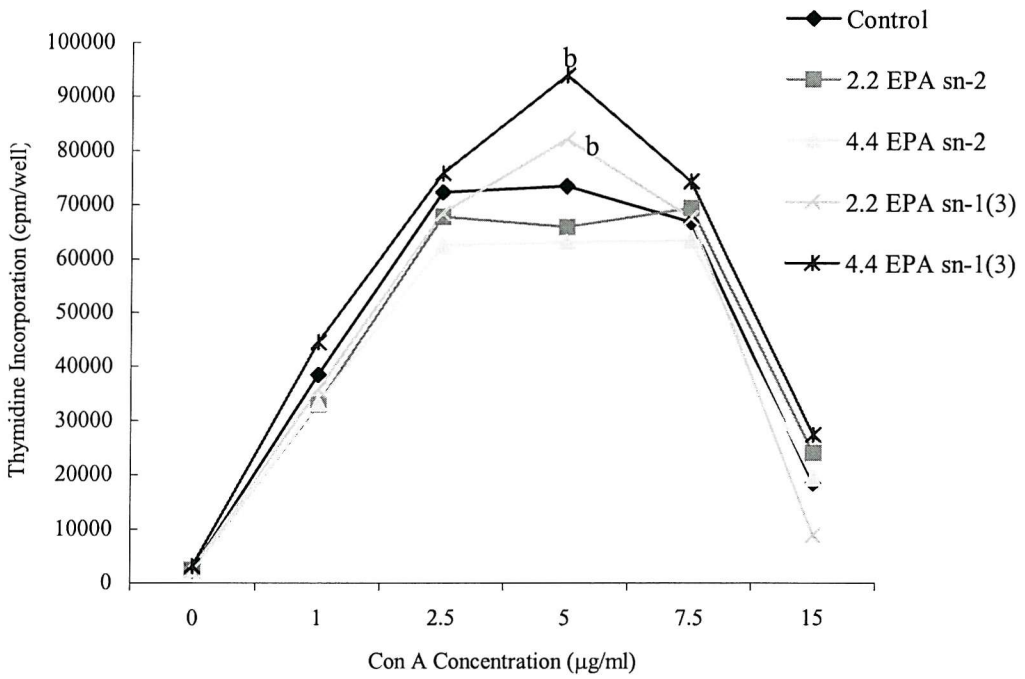
Diet	CD3+	CD19+	CD14+	CD4+	CD8+
Control	44.95 ± 5.5	30.57 ± 2.1	7.60 ± 0.7	40.56 ± 2.9	17.50 ± 1.0
2.2 EPA *sn*-2	45.11 ± 4.0	28.9 ± 3.1	11.70 ± 2.8[a]	41.04 ± 2.6	17.69 ± 1.0
4.4 EPA *sn*-2	45.54 ± 1.8	28.29 ± 2.0	9.40± 0.9	37.00 ± 2.4	16.69 ± 1.3
2.2 EPA *sn*-1(3)	44.19 ± 1.8	28.89 ± 2.1	8.70 ± 0.8	42.60 ± 3.1	15.98 ± 0.9
4.4 EPA *sn*-1(3)	45.76 ± 3.2	27.44 ± 2.1	8.40 ± 1.1	40.80 ± 4.1	15.79 ± 0.8
2.2 DHA *sn*-2	46.42 ± 2.2	29.20 ± 2.3	7.40 ± 0.5	38.59 ± 1.7	14.64 ± 0.8
4.4 DHA *sn*-2	47.70 ± 2.1	26.20 ± 2.3	8.80 ± 0.7	43.00 ± 0.9	16.40 ± 0.9
2.2 DHA *sn*-1(3)	44.72 ± 2.7	25.80 ± 1.8	8.10 ± 1.2	37.70 ± 3.4	13.90 ± 1.0[ac]
4.4 DHA *sn*-1(3)	44.40 ± 1.5	25.40 ± 2.9	9.30 ±0.7	42.00 ± 1.2	17.50 ± 2.2

*Values are the mean ± SEM of 8 animals
Significant differences were expressed as:
a Indicates a significant difference from the control diet
c Indicates that the 2.2 g/100 g fatty acids diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

2.3.7 Proliferative Response of Lymphocytes to Con A

There was an effect of feeding EPA upon lymphocyte proliferation compared to the control diet. There was a significant increase in peak proliferation of the cells from animals fed the *sn*-1(3) diets compared to the *sn*-2 diets (*Figure 2.6.*). There was no significant effect of feeding DHA upon lymphocyte proliferation (*Figure 2.7*).

Figure 2.6. The proliferation of spleen lymphocytes from animals fed structured TAG diets enriched with EPA

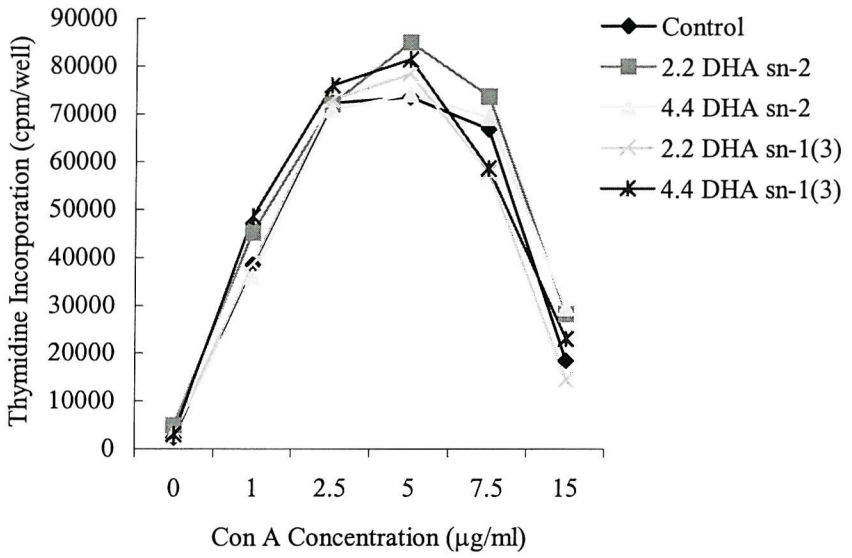


Data are the mean of 8 animals
Cells were cultured in FCS
Significant differences are expressed as:
b Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

2.3.8 Production of Cytokines by Cultured Spleen Leukocytes

The production of cytokines by Con A-stimulated cells was markedly increased (between 50 and 150 times higher) compared to the production by unstimulated cells (*Tables 2.18* to *2.20*). There were no differences in the production of cytokines from the unstimulated cells between any of the dietary groups.

Figure 2.7. The proliferation of spleen lymphocytes from animals fed structured TAG diets enriched with DHA



Data are the mean of 8 animals
Cells were cultured in FCS

There were small differences in the production of cytokines by Con A-stimulated cells for the different dietary groups. In autologous plasma (AP) cells from the animals fed DHA *sn*-2 at the 2.2 level produced significantly more IL-2 than those from control animals. When in foetal calf serum (FCS) the cells from the animals fed the 4.4 EPA *sn*-1(3) diets produced significantly more IL-2 than those from animals fed the 4.4 EPA *sn*-2 diets (*Table 2.18.)*. There was no significant effect of diet on the production of IFN-γ or IL-10 when the cells were cultured in FCS (*Table 2.19.* and *Table. 2.20.*). The level of IL-10 measured when the cells were cultured with AP was significantly higher for the DHA *sn*-2 diet compared with *sn*-1(3) diet at the 2.2 level (*Table. 2.20.)*. In AP there was a significantly greater amount of IFN-γ in the supernatant of the DHA 2.2 *sn*-2 dietary group than the control. The production of IL-4 was also measured in the culture medium for the different dietary groups, but the levels were too low to be detected (<5 pg/ml).

Table 2.18. The production of IL-2 by spleen lymphocytes from animals fed structured TAG diets

Diet	IL-2 AP (U/ml)		IL-2 FCS (U/ml)	
	- Con A	+ Con A	- Con A	+ Con A
Control	16.2 ± 11.5	19196 ± 2683	16.00 ± 13.5	24696 ± 4798
2.2 EPA *sn*-2	39.5 ± 39.5	20158 ± 1766	115.0 ± 5.40	23034 ± 3006
4.4 EPA *sn*-2	0.00 ± 0.00	24463 ± 2117	15.60 ± 15.6	20920 ± 3535
2.2 EPA *sn*-1(3)	11.8 ± 8.37	22801 ± 2690	58.20 ± 30.9	29810 ± 3283
4.4 EPA *sn*-1(3)	72.9 ± 5.12	22721 ± 3427	6.600 ± 5.30	26638 ± 3990[b]
2.2 DHA *sn*-2	23.7 ± 21.2	30935 ± 7146[a]	0.000 ± 0.00	34818 ± 10738
4.4 DHA *sn*-2	50.5 ± 29.9	22439 ± 4288	0.000 ± 0.00	22772 ± 5460
2.2 DHA *sn*-1(3)	49.0 ± 49.0	22105 ± 1781	0.000 ± 0.00	32775 ± 3154
4.4 DHA *sn*-1(3)	6.00 ± 0.00	24555 ± 1462	0.000 ± 0.00	27705 ± 2195

Data are mean ± SEM of 8 animals

AP = autologous plasma; FCS = foetal calf serum; Significant differences are expressed as:

a. Indicates a significant difference from the control diet

b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

Table 2.19. The production of IFN-γ by spleen lymphocytes from animals fed structured TAG diets

Diet	IFN-γ AP (pg/ml)		IFN-γ FCS (pg/ml)	
	- Con A	+ Con A	- Con A	+ Con A
Control	19.0 ± 16.8	3029 ± 324	0.00 ± 0.00	2963 ± 336
2.2 EPA *sn*-2	15.4 ± 15.4	3558 ± 438	1.13 ± 1.56	3064 ± 503
4.4 EPA *sn*-2	9.60 ± 8.00	3319 ± 778	0.00 ± 0.00	3309 ± 546
2.2 EPA *sn*-1(3)	11.4 ± 11.4	3656 ± 590	3.50 ± 5.30	3268 ± 483
4.4 EPA *sn*-1(3)	1.40 ± 2.50	2873 ± 513	0.00 ± 0.00	2799 ± 261
2.2 DHA *sn*-2	11.4 ± 6.20	4922 ± 1005[a]	38.0 ± 20.2	3962 ± 943
4.4 DHA *sn*-2	0.00 ± 0.00	4150 ± 491	0.00 ± 0.00	3667 ± 567
2.2 DHA *sn*-1(3)	0.00 ± 0.00	3628 ± 555	0.00 ± 0.00	4094 ± 721
4.4 DHA *sn*-1(3)	16.8 ± 12.1	4084 ± 386	0.00 ± 0.00	3806 ± 353

Data are mean ± SEM of 8 animals

AP = autologous plasma; FCS = foetal calf serum; Significant differences are expressed as:

a. Indicates a significant difference from the control diet

Table 2.20. The production of IL-10 by spleen lymphocytes from animals fed structured TAG diets

Diet	IL-10 AP (pg/ml)		IL-10 FCS (pg/ml)	
	- Con A	+ Con A	- Con A	+ Con A
Control	2.36 ± 5	198 ± 30	5.60 ± 3	144 ± 29
2.2 EPA *sn*-2	1.45 ± 6	224 ± 46	18.2 ± 9	147 ±18
4.4 EPA *sn*-2	6.30 ± 1	152 ± 14	6.59 ± 4	162 ± 28
2.2 EPA *sn*-1(3)	10.23 ± 6	162 ± 32	19.23 ± 8	90 ± 20
4.4 EPA *sn*-1(3)	12.56 ± 12	174 ± 12	5.23 ± 3	120 ± 9
2.2 DHA *sn*-2	9.99 ± 6	236 ± 50[b]	11.56 ± 6	133 ± 7
4.4 DHA *sn*-2	18.32 ± 8	150 ± 23	13.06 ± 5	127 ± 26
2.2 DHA *sn*-1(3)	20.23 ± 11	123 ± 12	17.0 ± 12	117 ± 15
4.4 DHA *sn*-1(3)	5.60 ± 13	126 ± 20	6.39 ± 2	125 ± 19

Data are mean ± SEM of 8 animals

AP = autologous plasma; FCS = foetal calf serum; Significant differences are expressed as:

b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

2.3.9 Production of PGE_2 by Cultured Spleen Leukocytes

There were no differences in the production of PGE_2 by unstimulated cells between the dietary groups. Con A stimulatin resulted in greatly increased production of PGE_2 (*Table 2.21*). There were no significant differences in the production of PGE_2 by Con A-stimulated cells between the dietary groups, except when 4.4 EPA was fed in *sn*-1(3) position. There was a decrease (approximately 50%) in the production of PGE_2 when the cells from animals fed this diet were cultured with either AP or FCS (*Table 2.21.*). There was no clear relationship between the membrane content of AA, EPA, or the AA/EPA ratio and the ability of the lymphocytes to produce PGE_2.

There was a non-significant effect of DHA on PGE_2 production, with a dose-dependent decrease when the cells were cultured with FCS. This effect appeared to be greater if DHA was in the *sn*-1(3) position than when in the *sn*-2 position (*Table 2.21.*).

Table 2.21. The production of PGE_2 by spleen lymphocytes for animals fed structured TAG diets

Diet	PGE_2 (ng/ml) - AP		PGE_2 (ng/ml) - FCS	
	- Con A	+ Con A	- Con A	+ Con A
Control	0.12 ± 0.13	6.49 ± 0.60	1.50 ± 0.23	6.68 ± 2.40
2.2 EPA *sn*-2	1.23 ± 0.45	6.05 ± 1.17	0.36 ± 0.45	6.39 ± 1.53
4.4 EPA *sn*-2	0.56 ± 0.23	6.84 ± 1.81	0.96 ± 0.16	5.21 ± 1.83
2.2 EPA *sn*-1(3)	1.45 ± 0.65	6.43 ± 1.46	1.20 ± 0.91	6.12 ± 2.08
4.4 EPA *sn*-1(3)	1.50 ± 1.22	3.46 ±.0 78[a]	0.63 ± 0.13	3.00 ± 0.57
2.2 DHA *sn*-2	1.63 ± 0.90	7.52 ± 1.57	0.87 ± 0.56	5.37 ± 1.03
4.4 DHA *sn*-2	0.96 ± 0.30	4.57 ± 0.93	0.15 ± 0.63	4.42 ± 1.28
2.2 DHA *sn*-1(3)	1.00 ± 0.41	5.62 ± 1.20	0.61 ± 0.26	4.41 ± 1.02
4.4 DHA *sn*-1(3)	0.98 ± 0.36	5.20 ± 1.52	1.45 ± 0.13	2.91 ± 0.41

Data are mean ± SEM of 8 animals
AP = autologous plasma; FCS = foetal calf serum
Significant differences are expressed as:
a. Indicates a significant difference from the control diet

2.4 Discussion

2.4.1 Changes in Fatty Acid Composition

The fates of the fatty acids of interest (i.e. EPA, DPA and DHA) are shown pictorially in Figures 2.8 and 2.9.

After EPA feeding

There was a strong tendency for EPA to be sequestered into the spleen leukocyte PL fraction. There was also an accumulation of DPA in this lipid fraction, although there was no increase in the content of DHA (*Figure 2.8.)*. In contrast, the content of EPA, DPA and DHA increased in the plasma PL fraction after EPA feeding (*Figure 2.9.)*. In the spleen leukocyte DAG and the plasma TAG fractions there was an increase in EPA, DPA and DHA contents after EPA feeding (*Figure 2.8.; Figure 2.9.)*, while in the plasma CE fraction there was no increase in the content of DHA but a very large increase in the content of EPA.

After DHA feeding

There was an increase in the content of DHA in both of the spleen leukocyte lipid fractions for the rats fed the DHA-rich diets (*Figure 2.8.)*, suggesting that this fatty acid can be readily incorporated into leukocytes when fed in the diet. Plasma TAG from the DHA-fed rats was greatly enriched with DHA, while the PL and, to a greater extent, the CE fractions were enriched with EPA (*Figure 2.9.*).

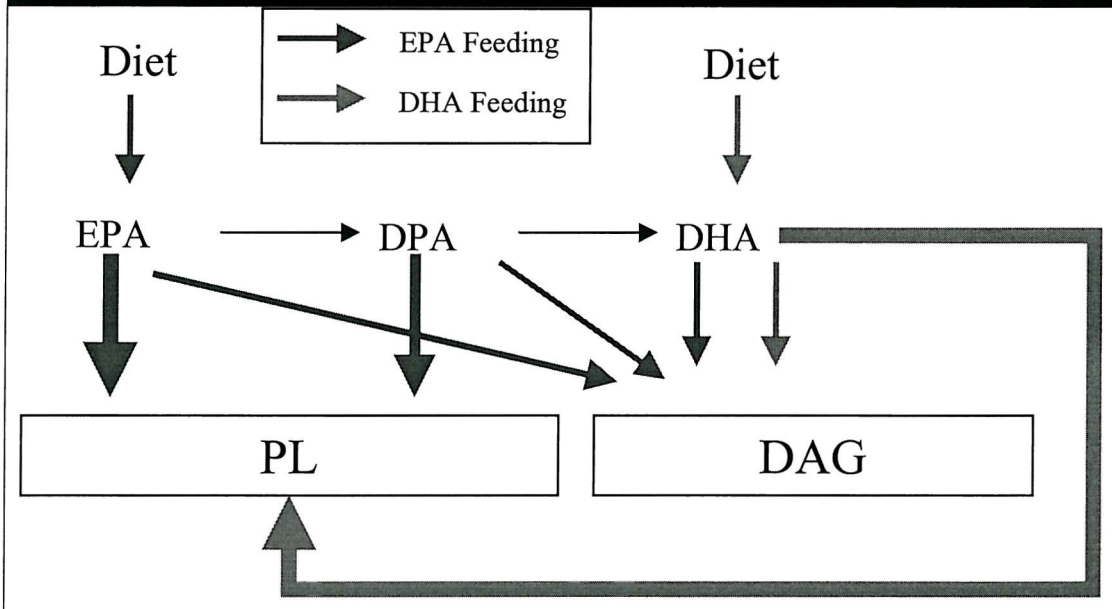
Plasma Fatty Acid Composition

After EPA feeding

There appeared to be no effect of the position of EPA in dietary TAG on the changes in fatty acid composition in any of the plasma lipid fractions which occurred when EPA was fed, except in the proportions of DPA and LA (*Table 2.13.*). There was a tendency for the increase in DPA to be greater when EPA was fed in the *sn*-1(3)

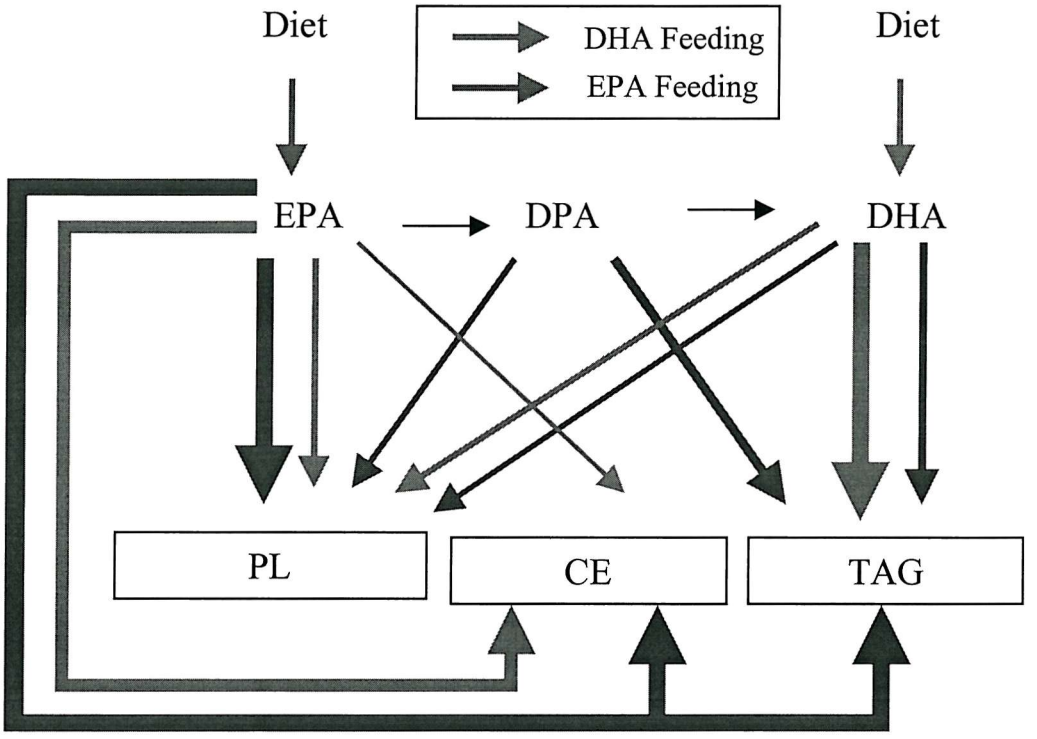
position rather then the *sn*-2 position. This implies that the EPA in the *sn*-1(3) position is more available for use in the synthesis of longer chain *n-3* PUFA than EPA at the *sn*-2 position. The content in LA was influenced by position in that when EPA was fed in the *sn*-2 position the increase of LA in plasma PL was greater than when EPA was fed in the *sn*-1(3) position. A mechanism as to why LA might increase after EPA feeding is discussed in chapter 3 (see section 3.4.1).

Figure 2.8. The fate of EPA and DHA within the spleen leukocyte lipid fractions



The level of incorporation of a fatty acid into a lipid fraction is represented by the width of the line depicting the pathway of enrichment (not to scale)

Figure 2.9. The fate of EPA and DHA within the plasma lipid fractions



The level of incorporation of a fatty acid into a lipid fraction is represented by the width of the line depicting the pathway of enrichment (not to scale)

There was a significant effect of the dose of EPA fed on the fatty acid composition of plasma lipid fractions. The enrichment in EPA and DPA was dose-dependent for all lipid fractions studied, while in plasma TAG and PL but not CE, the increase in DHA was dose-dependent. The increases in EPA in plasma fractions seen in this study, are comparable to those seen in rat serum after feeding 4.4 g EPA/100 g fatty acids for 6 weeks (Peterson *et al.,* 1998b). Unfortunately, any changes in DPA were not reported by Peterson *et al.* (1998b). An absence of a significant increase in DHA after EPA feeding was observed in the study by Peterson *et al. (*1998b). However, in the current study there was an increase in DHA when fed at the 4.4 g/100g fatty acids level in the plasma TAG and PL fractions, but not at either dose in the CE fraction.

After DHA feeding

There was no effect of the position of DHA in dietary TAG on the changes in fatty acid composition of any of the lipid fractions which occurred when DHA was fed (*Table 2.14*), except again where LA was concerned. The increase in LA in plasma PL was greater when DHA was fed in the *sn*-1(3) position compared to the *sn*-2 position. A mechanism as to why LA might increase after DHA feeding is discussed in chapter 3 (see section 3.4.1).

There was a significant effect of the dose of DHA upon the changes in fatty acid composition of the plasma lipid fractions studied. The increase in DHA in plasma PL and TAG, but not in CE, was dose-dependent. There was no change in the DPA content in any of the lipid fractions when DHA was fed. However, there was an increase in EPA in all lipid fractions and this was dose-dependent for TAG and CE. The 1.5 to 2-fold increase in DHA in plasma (TAG and PL) seen in this study after DHA feeding was markedly less than the 4-fold increase in this fatty acid seen by Peterson *et al.* (1998b) in rat serum after feeding 4.4 g DHA/100 g fatty acids for 6 weeks. It is notable that the content of DHA in the control animals in this study was much greater than that of the control rats in the study of Peterson *et al.* (1998b). An increased capacity for enrichment with DHA could account for the 4-fold increase in content of DHA seen in the study of Peterson *et al.* (1998b).

Handling of the fatty acids of interest

The changes in EPA content in plasma TAG and PL after EPA feeding suggest that there is transport of this fatty acid in the circulation from the gut in chylomicrons (dietary) and from the liver as VLDL (endogenous). There is conversion of EPA to DPA and DHA in the liver and these fatty acids can then be released as TAG in VLDL. The absence of an increase in the content of DHA in the CE and PL fractions after EPA feeding, and the large increase in this fatty acid in the plasma TAG fraction suggests that this fatty acid when synthesised or repackaged (from dietary sources supplied in CM) in the liver is mainly released as TAG in VLDL.

The large increase in EPA in the plasma CE fraction suggests that this fatty acid upon reaching the liver in chylomicrons (from dietary sources) or HDL (esterified to

cholesterol) is repackaged and re-released esterified to cholesterol (therefore from endogenous sources). This implies that this fatty acid, compared to DPA and DHA, may be important in cholesterol metabolism. The increase in EPA (mainly in CE but also to a small extent in the TAG and PL fractions) after DHA feeding suggests that there is some retro-conversion of DHA to EPA (probably in the liver) since EPA was not included in the diet. The EPA formed is then sequestered to a greater extent into the plasma CE fraction and this again indicates that EPA is important in cholesterol metabolism. These fatty acids may be transported to peripheral tissues. The observations of changes in plasma fatty acid composition seen in this study are in agreement with a previous study where the fatty acid composition of plasma PL, TAG and CE of rats were investigated (Gibson *et al.,* 1992).

The fate of dietary EPA and DHA depends on the handling of the fatty acids not only at the gut level but later on, in the liver and further still in the peripheral tissues such as adipose tissue (see section 2.1). It would seem that the effects of feeding EPA and DHA upon the incorporation of these fatty acids into different plasma lipid fractions are different and that there is little or no effect of position of these fatty acids in dietary TAG on changes in the fatty acid composition of plasma TAG, CE or PL.

Spleen Leukocyte Phospholipid Composition

After EPA feeding

There was no effect of the position of EPA in dietary TAG on the changes in fatty acid composition of PL or DAG in spleen leukocytes seen after feeding EPA (*Table 2.13*), except for the contents of DPA and LA. The increase in LA seen in both lipid fractions was greater when EPA was fed in the *sn*-2 position compared to the *sn*-1(3) position of dietary TAG. An increase in LA in spleen leukocytes was also seen after feeding rats a diet containing 4.4 g EPA/100 g fatty acids for 6 weeks (Peterson *et al.,* 1998b). A mechanism which might explain why LA might increase after EPA feeding is discussed in Chapter 3 (see section 3.4.1). The increase in DPA was also more marked when EPA was fed in the *sn*-1(3) position compared to the *sn*-2 position. This suggests that EPA in the *sn*-1(3) position is more readily available for elongation and subsequent incorporation into spleen leukocyte PL.

There was an effect of the level of EPA in the diet on the changes in fatty acid composition of the spleen leukocyte lipid fractions seen after feeding EPA. The increases in EPA and DPA after EPA feeding were dose-dependent. There was no significant increase in DHA when EPA was fed (except in the DAG fraction for the 4.4 *sn*-2 EPA diet compared to the control). The increases in EPA seen in the spleen leukocyte total PL fraction were slightly greater than those seen when 4.4 g EPA/100 g fatty acids was fed to rats (Peterson *et al.,* 1998b). However, the content of EPA in the leukocyte PL of the control rats in the study of Peterson *et al.* (1998b) was approximately half that of the rats in this study although the control diet was exactly the same. The decrease in AA, although seen in both lipid fractions, was dose-dependent for the DAG fraction only, but was comparable to the decrease in AA seen in spleen leukocytes in the study of Peterson *et al. (*1998b).

After DHA feeding

There was no effect of the position of DHA in dietary TAG on the changes in fatty acid composition of PL or DAG in spleen leukocyte membrane seen after feeding DHA (*Table 2.14*).

However, there was an effect of the dose of DHA fed on the changes in fatty acid composition of the spleen leukocyte lipid fractions seen after feeding DHA. The increases in DHA after DHA feeding were dose-dependent for both PL and DAG. Notably there was no increase in EPA in spleen leukocyte lipid fractions after DHA feeding despite increases in EPA in the plasma lipids in these groups. Similar to the plasma PL fraction, the 1.5 to 2-fold increase in DHA seen in this study after DHA feeding was markedly less than the four-fold increase in this fatty acid seen by Peterson *et al.* (1998b) in rat spleen leukocytes after feeding 4.4 g DHA/100 g fatty acids. It is notable that the content of DHA in spleen leukocyte PL, in the control animals in this study was markedly greater than that of the rats in the study of Peterson *et al.* (1998b). An increased capacity for enrichment with DHA could account for the four-fold increase in content of DHA seen in the study of Peterson *et al. (*1998b). As for the plasma fractions, the decrease in AA seen in both leukocyte lipid fractions when DHA was fed was dose-dependent. The magnitude of change in

AA was similar to that seen in rat spleen leukocytes by Peterson *et al.* (1998b) after feeding 4.4 g DHA/100 g fatty acids.

Handling of the fatty acids of interest

The increases in EPA seen in the plasma fractions (including PL), after EPA feeding were mirrored in the PL and DAG fractions of the spleen leukocytes. However, the increases in plasma EPA after DHA feeding were not seen in the PL and DAG fractions of spleen leukocytes. This suggests that the source of EPA (i.e. exogenous versus endogenous) is important in determining the enrichment of cell membranes in EPA.

The increases in DHA in leukocyte PL and DAG after DHA feeding suggest that enrichment of the diet in this fatty acid leads to incorporation of this fatty acid into the membrane of spleen leukocytes. However, it again appears that the source of the fatty acid (i.e. exogenous versus endogenous) is important. Plasma TAG was enriched in DHA after EPA feeding (with no increase in plasma PL or CE) but there was no increase in DHA in spleen lipid fractions measured after EPA feeding. Thus, it may be that the plasma fraction (in lipoproteins) which is enriched in EPA and DHA is important in determining the enrichment of leukocytes in these fatty acids i.e. only enrichment of plasma PL with long chain fatty acids can lead to enrichment of these fatty acids in spleen leukocytes in the rat. This might because there is direct exchange of PL between cell membranes and lipoproteins (see section 1.3.).

Spleen Leukocyte Phospholipid Species Composition

Three PL classes were investigated: PC, PI and PS. The changes in PC molecular species, which were present in the greatest amount in the membrane were examined to a greater extent than those in the PI and PS molecular species. In these latter two types of PL the changes in the composition of the molecular species could not be determined satisfactorily.

The composition of the PC molecular species observed in the spleens from control rats is in close agreement with murine spleen PC composition (Dombrofsky *et al.*, in press).

There was enrichment of EPA in PC after EPA feeding and this was almost exclusively in the 16:0/20:5 molecular species. The greatest, and most significant, increase in this species after EPA feeding was in the 4.4 *sn*-2 EPA group. Thus, in this type of PL there appears to be a significant effect of the position of EPA in dietary TAG, since the increase in this species after feeding the 4.4 EPA *sn*-1(3) diet was the same as seen after feeding both of the 2.2 diets. Thus, although the increases in EPA in total PL after EPA appeared to have no dependence on the position of EPA in dietary TAG, it appears that certain phospholipid species are altered in a position-specific manner. This suggests that EPA at the *sn*-2 position in dietary TAG may be retained to a certain degree and is then directed into this type of PL.

The increases in DHA PC species were almost exclusively in the 16:0/22:6 species and were not dependent on position of DHA in dietary TAG but were dose-dependent. There was also an increase in DHA after EPA was fed in the *sn*-2 position at 4.4 g/100 g total fatty acids (not seen in the total PL) and this was again in the in the 16:0/22:6 species.

Although EPA feeding decreased the levels of some AA containing species this was not universal and EPA failed to alter the level of some AA containing PC species. This is indicative of a complex set of metabolic changes which link dietary plasma and cell PL fatty acid composition. However, it is important to note that there are some PL species which are much more resistant to change than others.

2.4.2 Changes in the Subpopulations of Spleen Leukocytes

There were no changes in the populations of spleen leukocytes observed after feeding EPA or DHA. This is in agreement with several other studies where either fish oil or EPA and DHA have been fed to rats (Sanderson *et al.*, 1995, Yaqoob *et al.*, 1995b, Peterson *et al.*, 1998a).

2.4.3 Changes in the Proliferative Response, Cellular Mediator and PGE_2 Production

In the present study there was a decrease in proliferation of lymphocytes compared to the control when EPA was fed in the *sn*-2 position. When EPA was fed in the *sn*-1(3) position there was an increase in the proliferation of lymphocytes compared to the control. These differences in lymphocyte proliferation between the position of EPA in dietary TAG were found to be significant. There was no effect of DHA feeding on the proliferation of lymphocytes in this study.

Previous studies of fish oil feeding have found an inhibitory effect on lymphocyte proliferation. Feeding fish oil (high in EPA and DHA) to rats or mice as part of a high fat diet resulted in a decrease in the proliferation of spleen lymphocytes (Yaqoob *et al.*, 1994; 1995b). When fed to mice as part of low fat diet (30 g/kg) both EPA and DHA (10 g/kg diet) resulted in a decrease in spleen lymphocyte proliferation, and their effects were equipotent (Jolly *et al.*, 1997). A more recent study where 4.4 g or 6.6 g EPA+DHA/100 g of total fatty acids was fed showed a decrease in rat spleen lymphocyte proliferation (Peterson *et al.*, 1998a). Another study by the same authors demonstrated that feeding 4.4 g of EPA or DHA/100 g of total fatty acids could decrease rat spleen lymphocyte proliferation compared to a control diet (containing 4.4 α-LNA/100 g of total fatty acids) (Peterson *et al.*, 1998b). The present study suggests that there is an effect of EPA on lymphocyte proliferation and that the position of EPA in the dietary TAG is also important. When EPA is in the *sn*-2 position, there is a significant decrease on lymphocyte proliferation compared to when EPA is in the *sn*-1(3) position. On the other hand DHA did not effect lymphocyte proliferation in the present study and this is in contrast to the study of Peterson *et al.* (1998b). The present study indicates that EPA is more active in the inhibition of the proliferative response than DHA. A recent human study agrees with this finding; this study showed that providing humans with 750 mg DHA/day did not affect lymphocyte proliferation whereas providing 800 mg EPA + 250 mg DHA/day decreased proliferation (Thies *et al.*, 2001b).

The mechanism by which EPA and/or DHA can modulate lymphocyte proliferation was investigated in mice by Jolly et al. (1997). It was shown that there was a

decrease in the amount of DAG released within lymphocytes upon Con A stimulation after EPA or DHA feeding. DAG is an important intracellular lipid mediator involved in T-lymphocyte proliferation, and is predominantly released from membrane PC PL. DAG activates PKC which initiates a key signalling pathway in T-lymphocytes (Rao, 1991). As such, the modulation of the amount of DAG may well be responsible for the decrease in proliferation seen (Jolly *et al.,* 1997).

In the present study there was a dose-dependent enrichment of spleen PC and DAG with EPA after EPA feeding and a dose-dependent enrichment in DHA after DHA feeding. However, the total amounts of DAG produced were not measured. The changes in fatty acid composition are comparable to those seen in other rat studies (see above), although these studies only report total spleen leukocyte fatty acid composition. There are no details of fatty acid composition of spleen PL or DAG given in the study of Jolly *et al.* (1997).

The composition of DAG may be important in determining its ability to activate PKC and in the current study the composition of DAG was changed according to diet. This altered content and composition of DAG may be a mechanism by which *n*-3 PUFA alter lymphocyte proliferation.

In the present study it was observed that EPA in different positions of dietary TAG resulted in different effects on spleen leukocyte PC molecular species. This may account for the different effects of EPA in the *sn*-2 versus *sn*-1(3) position on lymphocyte proliferation. This suggests that analysis of PL molecular species is important, since although there was no effect of the position of EPA in dietary TAG on total PL enrichment in EPA, there was an effect on PC molecular species composition. These results also indicate that PC composition is important in the regulation of proliferation of lymphocytes as Jolly *et al.* (1997) suggest.

IL-2 is intimately involved in the proliferative response of T-lymphocytes (see section 1.1.). Thus, a change in the production of IL-2 is expected to be associated with a change in the proliferation of T-lymphocytes. However, there were no significant changes in IL-2 production in the present study, as have been observed in combination with a decrease in proliferation in some other studies after EPA and DHA or fish oil feeding (Jolly *et al.,* 1997; Wallace *et al.,* 2001). There were also no

significant changes in IL-10 or IFN-γ production. There was however a decrease in the production of PGE_2, with the level of this mediator significantly reduced when EPA was fed in the *sn*-1(3) position at the 4.4 level compared to the control. It is interesting that the proliferative response of the cells from animals fed this diet was markedly increased compared to the other EPA-containing diets and the control diet (there was no change in PGE_2 production for these diets). This suggests that the concentration of PGE_2 may influence the proliferative response, with a decrease in production of this eicosanoid resulting in increased proliferation of lymphocytes, and this is in agreement with previous studies (Wu and Meydani, 1998). However, the absence of a change in IL-2 production by the cells from animals fed EPA in the *sn*-1(3) position at the 4.4 level suggests that the action of PGE_2 on lymphocyte proliferation is via an IL-2-independent mechanism. The absence of modulation of the production of cytokines may be responsible for the small effects of EPA feeding on lymphocyte proliferation in the present study.

It appears that the modulation of lymphocyte signalling pathways after EPA feeding which would be responsible for the changes in cell functions (cytokine production and proliferation) are different. One explanation of the differences between the present study and that of Jolly *et al.* (1997) is that the fatty acid EPA (especially in the *sn*-2 position) is sequestered into PC (and not PI or PS) and that PC is not as involved in the modulation of cytokine production as it is in lymphocyte proliferation.

Although the level of DHA was increased in PL and in PC it appears to have little or no effect on lymphocyte function in the present study. This indicates that EPA rather than DHA is the important fatty acid in fish oil with regard to the modulation of lymphocyte function.

2.5 Conclusion

There was no effect of the position of EPA in dietary TAG on the incorporation of EPA into plasma and spleen leukocyte lipid fractions. However, the dose of EPA in dietary TAG greatly influenced the degree to which the various lipid fractions were enriched. When the spleen PL classes were investigated there was an effect of the

position of dietary TAG on the incorporation of EPA into PC. When EPA was at the *sn*-2 position (4.4 level) there was a significant increase in the content of EPA-containing PC species compared to when EPA was fed in the *sn*-1(3) position of dietary TAG. This implies that there is retention of EPA at the *sn*-2 position of dietary TAG from the point of ingestion through to the incorporation into certain cells of the body, and that the incorporation may be very specific to certain (phospho)lipid species.

In terms of the effects of position of EPA on immune function there was no effect of EPA position in dietary TAG on the production of cytokines by lymphocytes, while there was an effect of position on lymphocyte proliferation. When EPA was fed in the *sn*-2 position there was a dose-dependent decrease compared to the control while EPA in the *sn*-1(3) position dose-dependently increased proliferation compared to the control diet. Thus it seems that feeding EPA in the *sn*-2 position compared to the *sn*-1(3) position may have effects on incorporation into the spleen leukocyte PC and that subsequently there is an effect of position of EPA on certain lymphocyte functions.

There was no effect of DHA, regardless of dose or position in dietary TAG, on the function of spleen lymphocytes. Thus, it appears that the more active fatty acid in fish oil with regard to EPA and DHA is in fact EPA.

CHAPTER 3. THE EFFECTS OF DIETARY STRUCTURED TRIACYLGLYCEROLS ON MURINE MONOCYTE AND NEUTROPHIL FUNCTION

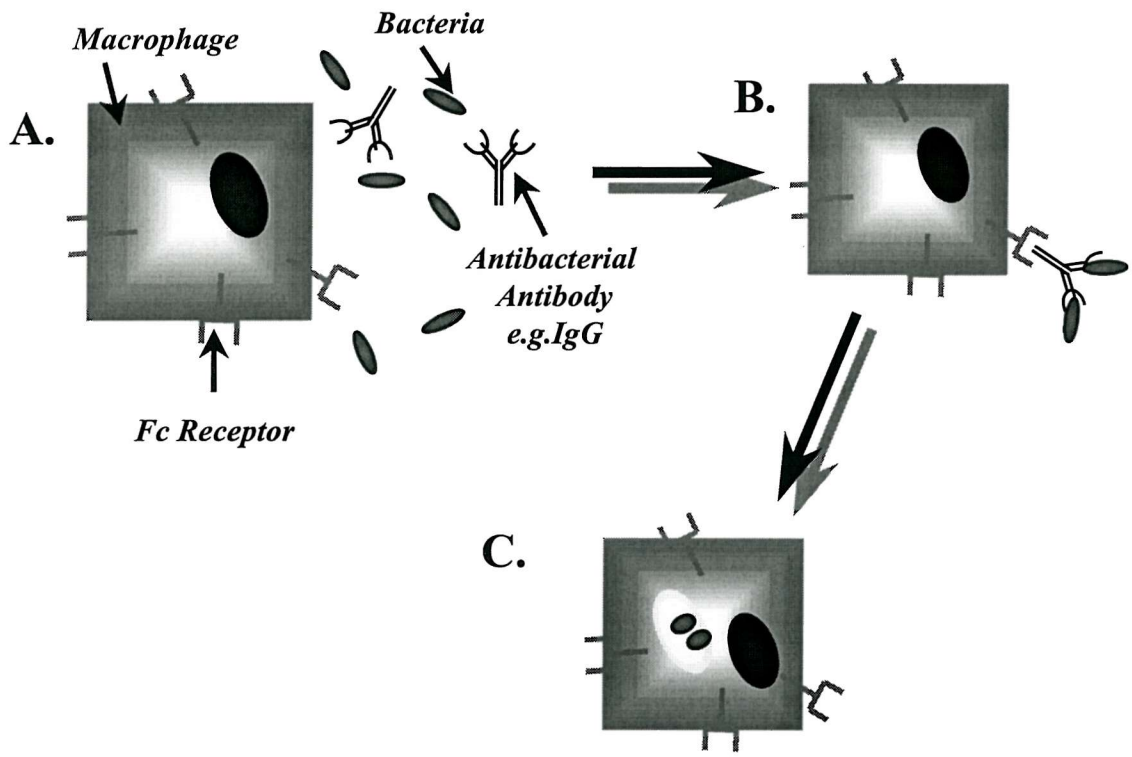
3.1. Introduction

Phagocytosis is a cellular process that involves the extension of the cell membrane pseudopodia around an invading pathogen or a dead/decaying cell and its subsequent endocytosis and breakdown (*Figure 3.1.*) (section 1.1.2.1.). Since this process requires a fluid cell membrane, changes in membrane composition may alter the capacity of a cell to carry out phagocytosis (section 1.3.3.). It has been shown that changing the degree of fatty acid unsaturation of the macrophage membrane has a modulatory effect on their ability of macrophages to perform phagocytosis *in vitro* (Schroit *et al.,* 1979; Mahoney; 1980; Calder *et al.,* 1990). As the cell membrane becomes more saturated there is a decrease in its fluidity, and it is this phenomenon which may be responsible for the altered phagocytosis (see section 1.3.3.).

Several dietary studies with animals have shown that the fatty acid composition of the macrophage is subject to changes according to the fatty acid composition of the diet (Lokesh *et al.*, 1986; Brouard and Pascaud, 1990; Surette *et al.*, 1995). However, conflicting effects of dietary fatty acids on macrophage phagocytosis have been observed (section 1.4.3.2.). Dietary fish oil was shown to have no effect on the phagocytic activity of alveolar macrophages from the rabbit (D'Ambola *et al.,* 1991) or pig (Turek *et al.,* 1994). Likewise, a diet enriched with linseed oil, and thus high in α-linolenic acid (α-LNA), was found to have no effect on phagocytosis by rat peritoneal macrophages (Babu *et al.,* 1997) or pig alveolar macrophages (Turek *et al.,* 1994). In contrast, the ability of Kuppfer cells from mice to phagocytose *Salmonella typhimurium* was reduced after feeding a diet enriched with fish oil (17% by weight), although there was no effect on phagocytosis by splenocytes (Eicher & McVey, 1995). The composition of fish oil with respect to its content of DHA and

EPA may well be important as a contributing factor to the differential effects of dietary fish oil on phagocytosis observed to date.

Figure 3.1. The phagocytic activity of a macrophage in response to bacteria



A. The macrophage can bind bacteria directly on the Fc receptor and phagocytose them.
B. However the binding of antibody (or complement protein) to bacteria, a process called opsonization, markedly increases phagocytosis.
C. The macrophage has engulfed the bacteria and formed an internal phagocytic vacuole, where the bacteria will be killed and then digested.

Relatively little is known about the effects of dietary fish oil on neutrophil function in animals (see section 1.4.4.4.). There are no animal studies which have investigated the fatty acid composition of neutrophils after fish oil feeding, although significant fatty acid composition changes in neutrophils have been observed in humans fed fish oil (Sperling *et al.*, 1993; Healy *et al.*, 2000). One *in vitro* study has shown that the ability of human neutrophils to perform phagocytosis is decreased when the cells are cultured with EPA and DHA (Sipka *et al.*, 1989). Overall, it is not clear what effect dietary fish oil has on neutrophil function, especially on phagocytosis.

Thus, the effect of dietary fatty acids on macrophage and neutrophil phagocytosis is unclear. It is possible that long chain *n*-3 fatty acids, as found in fish oil, will have the greatest effect on phagocytosis since they have the greatest enhancing effect on membrane fluidity (Calder *et al.*, 1990). However, despite the fluidising effect of EPA and DHA on cell membranes these fatty acids decreased the phagocytic capacity of macrophages compared to macrophages cultured with other long chain PUFA, such as arachidonic acid and α-LNA (Calder *et al.*, 1990). EPA had a greater effect than DHA. These observations suggest that it might be possible to have a membrane which is 'too fluid' for optimal phagocytosis to occur. Thus, it is hypothesised that a diet rich in EPA and DHA will modulate monocyte/macrophage and neutrophil phagocytosis.

3.1.1. Aims of This Chapter

The study described in this chapter aimed to investigate the effects of the active fatty acids of fish oil, EPA and DHA, on the functional responses of monocytes and neutrophils of mice. It set out to establish whether EPA and DHA fed in different positions in dietary triacylglycerol (TAG) are incorporated differentially into murine immune cells and whether the ability of monocytes and neutrophils to phagocytose *E. coli* is affected as a result. In addition, the production of IL-6 was investigated, as changes in the production of cellular mediators may contribute to changes in cell function, and fish oil has been shown to affect IL-6 production by murine macrophages (see section 1.4). Although there have been many studies of the effects of dietary *n*-3 PUFA on production of IL-6, the effect of the position of these fatty acids in dietary TAG has not been studied previously. The reader is referred to Chapter 2 for further background information to the study in this chapter.

Hypotheses:

- Consuming increasing doses of EPA or DHA will result in a dose-dependent enrichment of these fatty acids in animal tissues
- The postion of EPA or DHA in dietary TAG will result in differential enrichment of these fatty acids in animal tissues

- The postion of EPA and DHA in dietary TAG will have differential effects on the murine immune system
- The effects of EPA on the immune system will be greater than those of DHA in animals

The parameters to be investigated in this chapter will be:

- Spleen cell phospholipid and triacylglycerol fatty acid composition
- Production of IL-6 by lipopolysaccharide stimulated whole blood
- Phagocytic activity of monocytes and neutrophils
- the number of cells able to perform phagocytosis
- capacity of a phagocytosing cells to engulf *E. coli*

3.2. Methods

3.2.1. Chemicals

Chloroform, glutamine, heparin, lipopolysaccharide (LPS), methanol, HEPES-buffered RPMI medium (glutamine-free), potassium chloride, penicillin, sodium chloride and streptomycin were obtained from Sigma Chemical Co. (Poole, UK). IL-6 concentrations were determined using Biosource ELISA kits purchased from Appligene-Oncor-Lifescreen (Watford, UK). The phagocytic activity of cells was determined using kits obtained from Becton Dickinson (Oxford, UK).

3.2.2. Animals and Diets

Weanling male C57Bl/6 mice were housed individually for a period of 7 days prior to sacrifice. During this time they were given free access to water and to one of the 9 experimental diets described in section 2.1.2. The mice were killed in the fed state by an overdose of CO_2. Blood was collected into heparinised tubes by cardiac puncture and kept at room temperature. The spleen was removed and frozen in liquid nitrogen before storage at –80 °C for fatty acid analysis.

3.2.3. IL-6 Production

Whole blood was diluted 1 in 10 with HEPES-buffered RPMI medium supplemented with 2 mM glutamine and antibiotics (penicillin and streptomycin). A total volume of 1 ml of diluted blood was cultured with 10 μg/ml LPS at 37°C in an air/CO_2 (19:1) atmosphere in a 24-well culture plate. The plates were centrifuged (1000 rpm, 5 min) after either a 24 or 48 hr culture period. The supernatants were then frozen at -20°C until analysis. IL-6 concentrations were determined using ELISA kits according to the manufacturer's instructions. The intra-assay % coefficient of variation was 4.2 (n=16), while the inter-assay % coefficient of variation was 5.0 (n=20) . The samples were diluted as appropriate, since the sensitivity of the standard curve was 13 to 1000 pg/ml.

3.2.4. Measurement of Leukocyte Phagocytic Activity

Monocyte and neutrophil phagocytic activity in whole blood were determined using Phagotest kits supplied by Becton Dickinson using the method described in section 5.2.4., except that the test and control tubes were incubated for a period of 60 min (determined during preliminary time course experiments) rather than 10 min.

3.2.5. Fatty Acid Composition Analysis of Spleen Cells

Lipid Extraction

Spleen samples were homogenised and total lipid extracted using the method of Folch *et al.* (1957) (methanol/chloroform (2:1 vol/vol) containing 100 μg/ml of the antioxidant butylated hydroxytoluene (BHT)). The sample was closed under nitrogen, vortexed and incubated at room temperature for 2 hr. At this point 2 ml KCl (0.88 g/100 ml water) was added and the sample closed under nitrogen, vortexed and centrifuged for 10 min at 2000 rpm. The top aqueous layer was then aspirated and discarded. The lower no*n*-aqueous layer was passed through filter paper and stored at -80°C.

Separation of Lipid Classes

This was carried out in using the method set out in section 2.2.8.

Saponification and Methylation of Fatty Acids

This was carried out in using the method set out in section 2.2.8.

Gas Chromatography

The fatty acid composition of the TAG and PL were determined by gas chromatography as described in section 2.2.8.

3.2.6. Statistical Analysis

Data shown are mean ± SEM of 5 mice fed each diet. Statistical analysis was performed using one-way analysis of variance and a post-hoc least significance test. These analyses were performed using SPSS Version 10.0 (SPSS Inc., Chicago, IL); in all cases a value for P of less than 0.05 was taken to indicate statistical significance. A comparison between the effects of EPA and DHA were also performed but are not reported here.

Significant differences are expressed using superscripts such that:

a Indicates a significant difference from the control diet

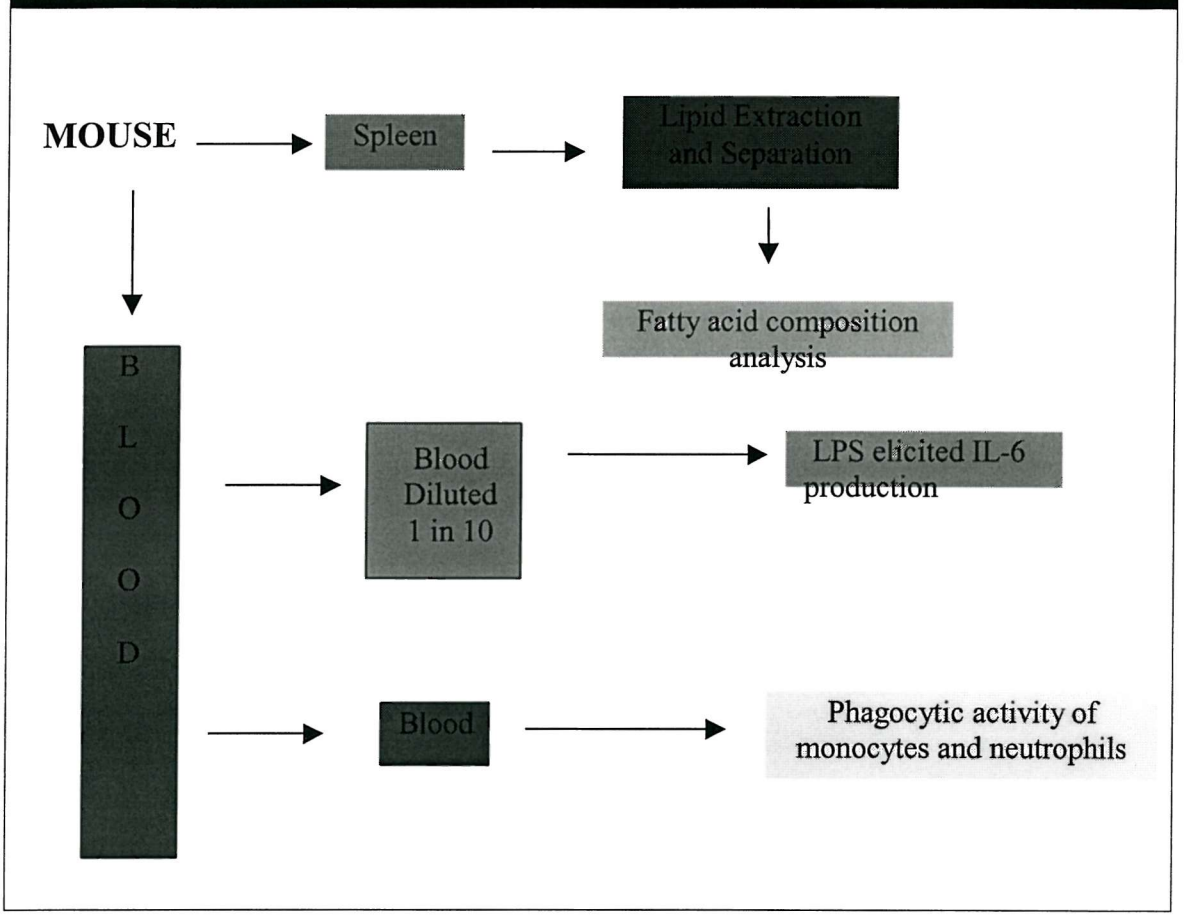
b Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same level)

c Indicates that the 2.2 g/100 g fatty acid diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

3.2.7. Summary of Investigations Used in this Chapter

The investigations described in this chapter is summarised in *Figure 3.2*.

Figure 3.2. Summary of the investigations described in this chapter



3.3. Results

3.3.1. Body Weights of Mice Fed Structured TAG Diets

There were no significant differences in the weight of the animals in the different dietary groups at the start of the feeding period (*Table 3.1*). There were no significant differences in the growth of the mice over the feeding period or in their final body weight (*Table 3.1.*).

Table 3.1. Body weights and weight gain of mice fed structured TAG diets

Diet	Initial Body Weight*	Final Body Weight*	Weight Gain*
Control	21.0 ±0.95	24.58 ±0.94	3.58 ±1.07
2.2 EPA *sn*-2	23.4 ±0.51	25.60 ±0.43	2.20 ±0.75
4.4 EPA *sn*-2	20.2 ±1.06	22.50 ±1.14	2.30 ±0.44
2.2 EPA *sn*-1(3)	19.6 ±0.75	23.96 ±0.42	4.36 ±0.84
4.4 EPA *sn*-1(3)	20.8 ±1.06	22.68 ±0.58	1.88 ±0.59
2.2 DHA *sn*-2	21.4 ±0.68	25.24 ±0.76	3.84 ±0.54
4.4 DHA *sn*-2	21.2 ±0.73	26.08 ±0.83	4.88 ±0.40
2.2 DHA *sn*-1(3)	20.4 ±1.12	25.62 ±0.79	5.22 ±0.77
4.4 DHA *sn*-1(3)	20.0 ±0.89	24.32 ±0.89	4.08 ±0.91

*Values are expressed in g

3.3.2. Fatty Acid Composition of Spleen Cell Phospholipids

The fatty acid compositions of the spleen cell phospholipids (PL) are shown in *Table 3.2.* The changes are complex and so the fatty acids of main interest (i.e. arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)) are also presented separately (*Table 3.4.*).

There was an absence of α-LNA in spleen PL of control mice, despite a fairly high level of α-LNA in the diet. There was a significant decrease in the proportion of stearic acid (SA) in spleen PL after feeding diets containing EPA or DHA, compared to the control diet and this was independent of dose or position of EPA or DHA (*Table 3.2*). The proportion of linoleic acid (LA) in spleen PL was significantly increased after feeding diets rich in EPA and this effect was dose-dependent for DHA in the *sn*-2 position compared to the control diet (*Table 3.2*). This increase was more marked in the DHA-fed animals and was dependent on the position of DHA within the dietary TAG molecule when DHA was fed at a level of 4.4 g/100 g fatty acid diets. Each of the experimental diets resulted in a significant increase in the proportion of 20:2 *n*-6 compared to the control, where it was not detected.

Arachidonic Acid

The proportion of AA was significantly decreased compared to the control when either EPA or DHA was included in the diet (*Table 3.2.*). The decrease in AA was greater when EPA was in the *sn*-2 position than in the *sn*-1(3) position, but the position of DHA had no effect.

Eicosapentaenoic Acid

The proportion of EPA in the spleen PL of control mice was greater than expected, and this could be attributed to conversion of α-LNA in this diet to EPA. Including DHA in the diet significantly decreased the proportion of EPA in spleen PL compared to the control and compared to the equivalent EPA-rich diet (*Table 3.2.*). Among the four diets which included EPA, only the one with 4.4 g EPA/100 g fatty acids with EPA in the *sn*-2 position resulted in an increased proportion of EPA in spleen PL (*Table 3.2.*). Thus, there was an effect of position of EPA in dietary TAG on its incorporation into spleen PL. This diet also caused the largest decrease in AA content.

AA/EPA Ratio

Feeding EPA in the *sn*-2 position led to a dose-dependent decrease by more than 50% in the ratio of AA to EPA in spleen cell PL (*Table 3.2.*). The AA/EPA ratio was significantly increased (by 100%) when DHA was included in the diet compared to the control and the corresponding EPA-rich diet (*Table 3.2.*).

Docosapentaenoic Acid

Animals fed EPA had significantly increased DPA in their spleen cell phospholipids compared to the control and the DHA diets (*Table 3.2*; *Figure 3.3.*, *3.4.*). This suggests conversion of EPA to DPA in these animals. There was little effect of EPA dose or position in dietary TAG on the content of DPA

Docosahexaenoic Acid

There was no difference between the control and EPA-fed animals with regard to the proportion of DHA in spleen cell PL (*Table 3.2.*). There was however a significant increase in the proportion of DHA compared to the control and EPA diets, when mice were fed DHA (*Table 3.2.*). This increase in DHA content was independent of DHA dose or its position in dietary TAG.

Table 3.2. Fatty acid composition of spleen cell PL fraction for animals fed structured TAG diets

Diet	14:0	16:0	18:0	18:1 *n*-9	18:2 *n*-6	20:2 *n*-6	20:3 *n*-6	20:4 *n*-6	20:5 *n*-3	22:5 *n*-3	22:6 *n*-3	AA/EPA
Control	ND	27.64±1.74	18.07±0.57	10.01±0.29	11.03±0.19	ND	1.28±0.20	18.1±0.53	2.59±0.22	1.98±0.29	6.54±0.49	7.07±0.37
2.2 EPA *sn*-2	0.52±0.07	28.31±0.84	16.83±0.23[a]	9.54±0.19	11.93±0.26	0.89±0.24[c]	1.21±0.23[c]	12.9±0.40[abc]	2.33±0.11[c]	4.62±0.17[a]	7.09±0.24	5.57±0.16
4.4 EPA *sn*-2	0.55±0.1	29.18±0.95	16.79±0.32[a]	10.10±0.36	12.17±0.31[a]	0.64±0.45	1.09±0.28[b]	11.5±0.25[ab]	3.6±0.10[ab]	4.79±0.30[a]	6.81±0.44	3.20±0.12[b]
2.2 EPA *sn*-1(3)	0.39±0.04	28.17±0.74	16.97±0.54[a]	9.66±0.11	12.19±0.15[a]	0.87±0.22[c]	1.20±0.33	14.5±0.58[a]	2.21±0.13	4.58±0.11[a]	6.52±0.08	6.65±0.47
4.4 EPA *sn*-1(3)	0.51±0.52	26.69±0.39	16.22±0.38[a]	10.07±0.21	12.01±0.42[a]	0.73±0.46	0.34±0.24	14.6±0.58[a]	2.55±0.46	4.48±0.29[a]	5.99±0.28	6.69±0.22
2.2 DHA *sn*-2	0.28±0.08[b]	28.72±0.69	17.03±0.33[ac]	11.02±1.33[bc]	13.28±0.57[a]	1.00±0.48	1.41±0.47[b]	12.5±0.45[ac]	0.87±0.17[a]	1.61±0.18	10.15±0.80[a]	16.32±2.5[abc]
4.4 DHA *sn*-2	0.43±0.03	28.38±0.54	16.09±0.22[a]	9.56±0.11	13.69±0.21[ab]	0.95±0.21	1.43±0.35	11.2±0.35[a]	0.87±0.06[a]	1.52±0.06	11.74±0.22[a]	13.08±0.70[ab]
2.2 DHA *sn*-1(3)	0.60±0.09	26.54±0.85	16.67±0.11[a]	9.36±0.18	13.23±0.42[a]	0.93±0.18	1.29±0.39[c]	12.8±0.25[a]	0.75±0.55[a]	1.65±0.13	10.47±.0.50[a]	17.45±1.03[ac]
4.4 DHA *sn*-1(3)	0.53±0.08	26.94±1.07	16.13±0.35[a]	9.35±0.13	12.76±0.16[a]	0.88±0.43	1.16±0.51	12.0±0.23[a]	1.02±0.62[a]	1.71±0.13	11.54±0.61[a]	11.92±0.63[a]

Values are the mean ± SEM of 8 animals fed on each diet
Values expressed as g/100 g total fatty acids; ND – not detectable
Significant differences are expressed as:

a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. Indicates that the 2.2 g/100 g fatty acids diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

Table 3.3. Fatty acid composition of spleen cell TAG fraction for animals fed structured TAG diets

Diet	16:0	16:1	18:0	18:1 *n*-9	18:2 *n*-6	18:3 *n*-3	20:1 *n*-9	20:2 *n*-6	20:3 *n*-6	20:4 *n*-6	20:5 *n*-3	22:5 *n*-3	22:6 *n*-3
Control	26.25±0.47	3.5±0.56	4.62±0.28	37.8±1.35	21.28±0.52	1.41±0.22	0.72±0.05	ND	0.39±0.07	1.06±0.75	ND	0.33±0.001	0.79±0.40
2.2 EPA *sn*-2	27.57±1.24[bc]	4.61±0.36	4.57±0.32	35.4±1.38	22.77±1.13	1.02±0.14[ac]	0.62±0.07	0.18±0.001[a]	0.38±0.24	0.54±0.03	1.08±0.55[a]	0.38±0.04	0.69±0.05[b]
4.4 EPA *sn*-2	25.12±0.47	4.83±0.51	4.43±0.37	37.4±1.09	22.92±0.62	0.50±0.06[a]	0.76±0.06	0.18±0.00[a]	0.39±0.03	0.41±0.04[a]	1.08±0.37[a]	0.41±0.05	0.65±0.04
2.2 EPA *sn*-1(3)	24.82±0.31	3.97±0.20	4.73±0.37	37.4±.0.75	24.06±0.74[a]	0.96±0.22[a]	0.73±0.06[c]	0.15±0.00[a]	0.20±0.09	0.30±0.04[a]	0.47±0.12[a]	0.31±0.04	0.36±0.02[a]
4.4 EPA *sn*-1(3)	25.41±0.45	4.11±0.26	4.67±0.23	38.0±1.27	22.82±0.81	0.44±0.06[a]	0.95±0.09	0.14±0.02[a]	0.27±0.07	0.30±0.07[a]	0.53±0.29[a]	0.45±0.06	0.46±0.08[a]
2.2 DHA *sn*-2	25.97±0.48	4.65±0.60	4.06±0.31	36.2±0.87	23.84±0.77[a]	1.06±0.04[ac]	0.49±0.03	0.17±0.04[a]	0.14±0.03[a]	0.30±0.11[a]	1.18±0.46[a]	0.15±0.04	0.91±0.07[c]
4.4 DHA *sn*-2	25.89±028	4.48±0.29	4.35±0.29	35.3±.0.26	23.76±0.46[a]	0.45±0.03[a]	0.45±0.12	0.26±0.11[a]	0.15±0.05[a]	0.53±0.14	0.40±0.31[a]	0.20±0.05	1.35±0.08[a]
2.2 DHA *sn*-1(3)	26.16±0.34	4.89±0.46[a]	4.17±0.29	35.34±0.77	22.67±0.30	0.96±0.03[ac]	0.4±0.09	0.14±0.01[a]	0.18±0.04	0.46±0.23[a]	0.16±0.07[a]	0.12±0.43	0.92±0.04[c]
4.4 DHA *sn*-1(3)	26.44±0.48	4.67±0.45	4.47±0.30	34.3±0.39[a]	22.38±0.75	0.34±0.04[a]	0.6±0.04	0.26±0.14	0.20±0.02	0.24±0.03[a]	0.22±0.14[a]	0.16±0.03	1.36±0.07[a]

Values are the mean ± SEM of 8 animals fed on each diet
Values expressed as g/100 g total fatty acids; ND – not detectable
Significant differences are expressed as:

a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. Indicates that the 2.2 g/100 g fatty acids diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

3.3.3. Fatty Acid Composition of Spleen Cell Triacylglycerols

The fatty acid compositions of the spleen cell TAG are shown in *Table 3.3.* The changes are complex and so the effects on the fatty acids of main interest (i.e. AA, EPA, DPA and DHA) are summarised separately (*Table 3.5.*).

There were few significant changes in the proportions of palmitic acid, stearic acid or oleic acid in spleen cell TAG after feeding the different diets (*Table 3.3.*). There was an increase in the proportion of linoleic acid after feeding each diet compared to the control, but this was only significant for the 2.2 EPA *sn*-1(3), and the 2.2 and 4.4 DHA *sn*-2 diets. There was a significant increase in the proportion of 20:2 *n*-6 for each of the experimental diets compared to the control diet.

α-Linolenic Acid

There was a significant decrease in the proportion of α-LNA after feeding each of the diets containing EPA or DHA compared to the control (*Table 3.3.*). This may be expected since the control diet contained 4.4 g α-LNA/100 g fatty acids whereas the other diets contained 0 or 2.2 g α-LNA/100 g fatty acids. The proportion of α-LNA in spleen cell TAG was inversely related to the amount of EPA or DHA in the diet. Thus the 2.2 g/100 g fatty acid EPA and DHA diets (which also contained 2.2 g α-LNA /100 g fatty acids) had a greater proportion of α-LNA than the respective 4.4 g/100 g fatty acid diets.

Arachidonic Acid

Including EPA or DHA in the diet led to a significant decrease in proportion of AA in spleen cell TAG compared to the control diet (*Table 3.3*). This decrease was significant for each diet, except the 2.2 g/100 g fatty acid EPA *sn*-2 and 4.4 g/100 g fatty acid DHA *sn*-2 diets, and was independent of dose and position of EPA or DHA.

Eicosapentaenoic Acid

EPA was not detected in the spleen cell TAG of control mice. There was a significant increase in the proportion of EPA in spleen cell TAG after feeding diets containing EPA or DHA compared to the control. The increase was greatest after feeding the EPA *sn-2* diets compared to *sn*-1(3) diets, but these were not significantly different from one another. The increases in EPA content after feeding the DHA-rich diets suggests that retro-conversion of DHA to EPA took place.

Docosapentaenoic Acid

There were no significant differences in the proportion of DPA in spleen cell TAG among the different DHA groups. However, there was significantly more DPA after feeding the EPA diets compared to the respective DHA diets. This suggests that there was conversion of α-LNA and EPA to DPA.

Docosahexaenoic Acid

There was a dose-dependent increase in the proportion of DHA in the TAG fraction of spleen PL after feeding the DHA-rich diets. There was no change in DHA composition for the *sn*-2 EPA-fed animals compared to the controls. However, when EPA was fed in the *sn*-1(3) position there was a significant decrease in DHA content compared to the control.

3.3.4. Summary of Changes in Fatty Acid Composition

Spleen Cell Phospholipids

The inclusion of EPA and DHA in the diet resulted in marked changes in the fatty acid composition of the spleen phospholipids, which are summarised in *Table 3.4.* However, no significant effects of the position of EPA or DHA in dietary TAG, or of the dose of these fatty acids in the diet on the fatty acid composition of spleen cell phospholipids was observed except for one diet. Among the four diets, which included EPA, only the one with 4.4 g EPA/100g fatty acids with EPA in the *sn*-2

position resulted in an increased proportion of EPA in spleen PL (*Table 3.2*). Thus, there was an effect of position of EPA in dietary TAG on its incorporation into spleen PL. This diet also caused the largest decrease in AA content.

Table 3.4. A summary of the effects of EPA or DHA on the fatty acid composition of spleen cell phospholipid fractions compared to the control

Fatty Acid	**Change in enrichment of phospholipid fraction**	
	EPA diets	DHA diets
Stearic acid (18:0)	↓↓	↓↓
Linoleic acid (18:2)	↑↑	↑↑
Arachidonic acid (20:4)	↓↓↓	↓↓↓
Eicosapentaenoic acid (20:5)	↑	↓↓↓
Docosapentaenoic acid (22:5)	↑↑↑	↔
Docosahexaenoic acid (22:6)	↔	↑↑↑

Spleen Triacylglycerols

The changes in the fatty acid composition of spleen TAG were less profound than those seen in phospholipids. When EPA was included in the diet there was an increase in the content of EPA compared to the control. There appeared to be better incorporation of EPA into the TAG for the *sn*-2 EPA diets compared to the *sn*-1(3) diets. This was not significant but suggests that the fatty acid (EPA) at the *sn*-2 position may be more highly conserved than when in the *sn*-1(3) position.

Table 3.5. A summary of the effects of EPA or DHA on the fatty acid composition of the spleen cell triacylglycerol fraction compared to the control

Fatty Acid	**Change in enrichment of phospholipid fraction**	
	EPA diets	DHA diets
Linoleic acid (18:2*n*-6)	↑ (ns)	↑ (*sn*-2)
α-Linolenic (18:3*n*-3)	↓↓	↓↓
Arachidonic acid (20:4*n*-6)	↓↓	↓↓
Eicosapentaenoic acid (20:5*n*-3)	↑↑↑ (*sn*-2>*sn*-1(3))	↑↑
Docosapentaenoic acid (22:5*n*-3)	↔	↓
Docosahexaenoic acid (22:6*n*-3)	↓ (*sn*-1(3))	↑↑

ns = not significant

3.3.5. Phagocytic Activity by Peripheral Blood Monocytes

Phagocytic activity is expressed in two ways: the ability of cells to phagocytose (% of cells) and the number of bacteria ingested by each macrophage (capacity of the cell to phagocytose, i.e. the median fluorescence). DHA did not significantly affect phagocytosis by blood monocytes (*Table 3.6.*). However, EPA did affect monocyte phagocytosis (*Table 3.6.*).

Table 3.6. The effects of structured TAG on the phagocytic activity of peripheral blood monocytes

Diet	% Positive cells	Median Fluorescence Intensity (MFI)	Index of Activity (% Positive cells x MFI)
Control	29.60 ±6.09	350.87 ±90.02	17052 ±2975
2.2 EPA *sn*-2	23.70 ±8.55	423.75 ±98.05[a]	16415 ±881
4.4 EPA *sn*-2	19.13 ±1.38	620.24 ±72.58[b]	17645 ±2761[b]
2.2 EPA *sn*-1,3	20.36 ±2.32[c]	448.57 ±35.73	14294 ± 2459
4.4 EPA *sn*-1,3	14.18 ±0.71[a]	335.15 ±24.55	8875 ± 1414
2.2 DHA *sn*-2	27.30 ±6.37	577.80 ±148.9	19515 ±3885
4.4 DHA *sn*-2	27.20 ±9.85	369.80 ±121.3	20844 ±9014
2.2 DHA *sn*-1,3	30.64 ±7.69	397.60 ±73.4	28578 ±7953
4.4 DHA *sn*-1,3	20.42±5.81	409.60 ±80.4	15590 ±5654

Significant differences are expressed as:

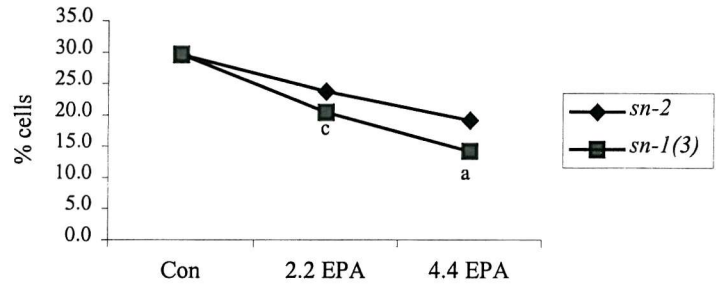
a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)
c. Indicates that the 2.2 g/100 g fatty acids diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

Effect of Feeding Eiocosapentaenoic Acid

There was a dose-dependent linear decrease in the percentage of monocytes which performed phagocytosis ($r = -0.637$ for the relationship between EPA content of the diet in the *sn*-1(3) position and the % positive cells ($p<0.05$); $r = -0.376$, for the relationship between EPA content of the diet in the *sn*-2 position and the % positive cells ($p<0.05$)). This was significant after feeding the 4.4 g/100 g fatty acid *sn*-1(3) diet compared to the control diet (*Figure 3.3.*). The *sn*-1(3) diets were also more inhibitory than the *sn*-2 diets, but this effect was not significant. There was an effect of the position of EPA in dietary TAG upon the capacity of the cells to phagocytose (*Figure 3.4.*). There were no differences between the 2.2 g/100 g fatty acid EPA diets and the control diet with respect to the median fluorescence. However, when EPA was fed in the *sn*-2 position at a dose of 4.4 g/100 g fatty acids, there was an increase

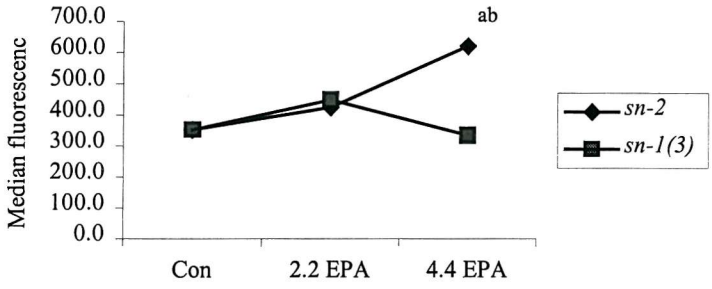
in the number of *E. coli* that the monocytes could ingest. This was significantly more than the number ingested by the monocytes from mice fed the diet containing EPA at position *sn*-1(3) and a dose of 4.4 g/100 g fatty acids. There was a significant difference between the *sn*-2 and *sn*-1(3) diets at the 4.4 g/100 g fatty acid level in the index of activity of monocytes. This reflects the difference in the number of cells that a monocyte performing phagocytosis was able to engulf.

Figure 3.3. The percentage of monocytes performing phagocytosis



Significant differences are expressed as:
a Indicates a significant difference from the control diet
c Indicates that the 2.2 g/100g fatty acids diet is significantly different from the 4.4 g/100g fatty acids diet (for the same fatty acid and the same positional isomer)

Figure 3.4. The activity of monocytes (a measure of their capacity to engulf *E. coli*)



Significant differences are expressed as:
a Indicates a significant difference from the control diet
b Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same diet and same dose)

3.3.6. Phagocytic Activity by Peripheral Blood Neutrophils

Phagocytic activity is expressed in two ways: the ability of cells to phagocytose (% of cells) and the number of bacteria ingested by each neutrophil (capacity of the cell to phagocytose, i.e. the median fluorescence). DHA did not significantly affect phagocytosis by blood neutrophils (*Table 3.7.*). However, EPA did affect neutrophil phagocytosis (*Table 3.7.*).

Table 3.7. The effects of the structured TAG on the phagocytic properties of peripheral blood neutrophils

Diet	% Positive cells	Median Fluorescence Intensity (MFI)	Index of Activity (% Positive cells x MFI)
Control	61.10 ±5.46	272.37 ±29.85	31206 ±7810
2.2 EPA *sn*-2	54.35 ±5.32	306.95 ±19.50[b]	29876 ±14604
4.4 EPA *sn*-2	46.46 ±3.26	369.64 ±34.88[b]	26465 ±3380
2.2 EPA *sn*-1,3	53.55 ±1.83	263.30 ±39.80	22390 ±425
4.4 EPA *sn*-1,3	44.29 ±0.67[a]	199.15 ±29.52	14150 ±1808
2.2 DHA *sn*-2	62.80 ±4.29	310.50 ±62.16	35068 ±15460
4.4 DHA *sn*-2	62.40 ±8.60	305.80 ±95.50	38856 ±17128
2.2 DHA *sn*-1,3	66.30 ±8.55	405.00 ±83.63	39328 ±11053
4.4 DHA *sn*-1,3	56.00 ±7.91	253.80 ±59.48	27160 ±8821

Significant differences are expressed as:

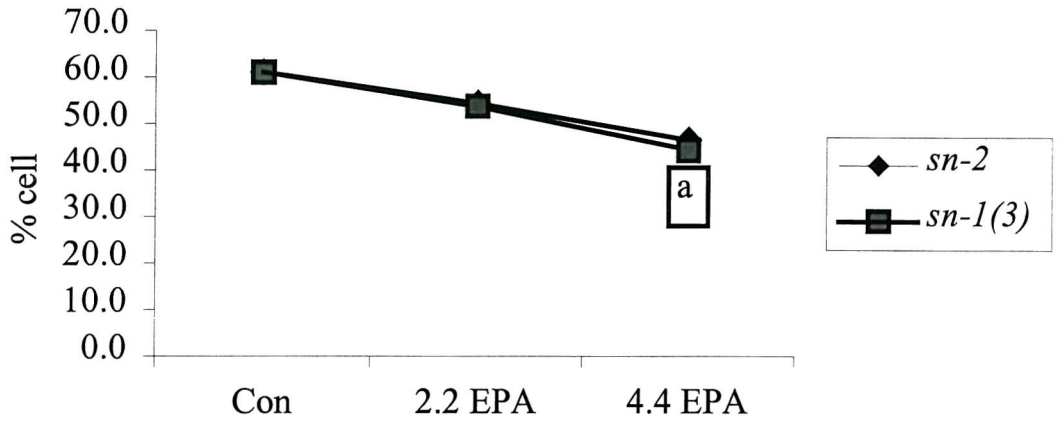
a. Indicates a significant difference from the control diet
b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty aicd and same dose)

Effect of Feeding Eiocosapentaenoic Acid

There was a dose-dependent linear decrease in the percentage of neutrophils performing phagocytosis ($r = -0.686$, for the relationship between EPA content of the diet in the *sn*-1(3) position and the % positive cells ($p<0.05$); $r = -0.564$ for the relationship between EPA content of the diet in the *sn*-2 position and the % positive cells ($p<0.05$)) and this was independent of the position of EPA within the dietary TAG (*Figure 3.5.*). The effect of EPA on the activity of the neutrophils was similar to that on monocytes. EPA fed in the *sn*-2 position at the dose of 4.4 g/100 g fatty acids increased the capacity for the ingestion of *E. coli* by neutrophils that were performing phagocytosis (*Figure 3.6.*). However, EPA fed in the *sn*-1(3) position at the same dose significantly inhibited this capacity. There was a 50% decrease in the index of activity for EPA at the 4.4 g/100 g fatty acids level in the *sn*-1(3) position compared to the control, and a 40% decrease compared to the same level of EPA when fed in the *sn*-2 position. These changes were not significant but reflect the

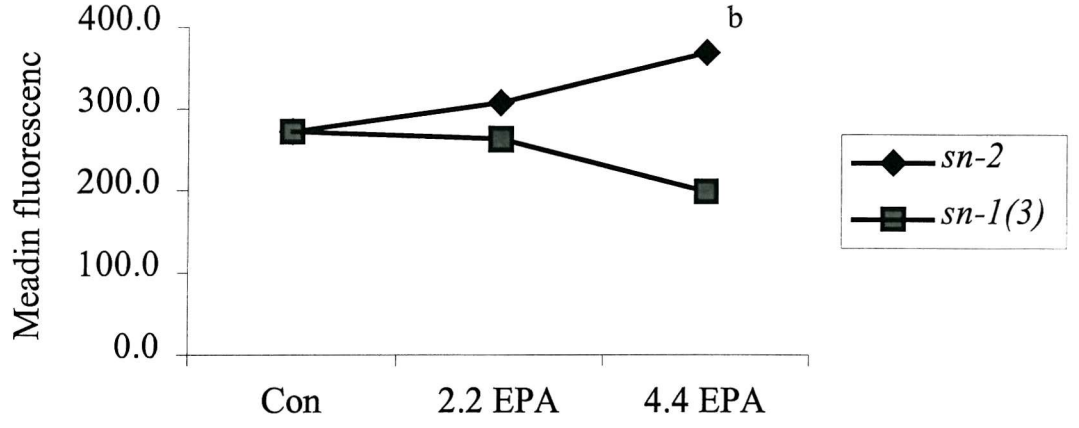
decrease in the ability of monocytes to engulf bacteria when EPA is fed in the *sn*-1(3) position.

Figure 3.5. The percentage of neutrophils performing phagocytosis



Significant differences are expressed as:
a. Indicates a significant difference from the control diet

Figure 3.6. The activity of neutrophils (a measure of their capacity to engulf *E. coli*).

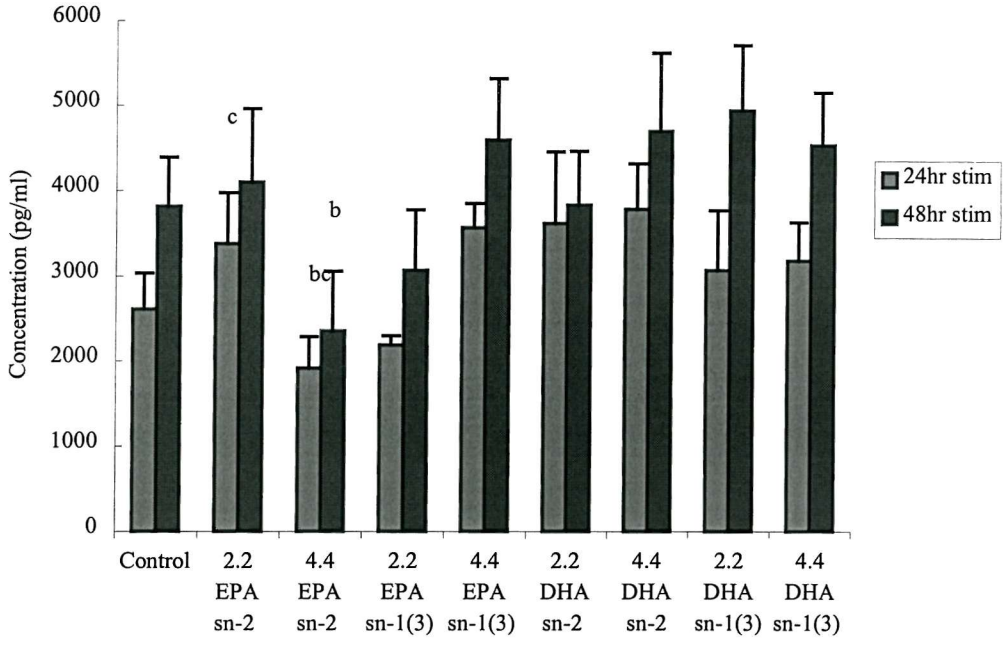


Significant differences are expressed as:
b. indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same diet and same dose)

3.3.7. Production of IL-6 by PBMNC in Whole Blood Culture

There were a number of significant effects of feeding EPA on the production of IL-6 (*Figure 3.7.*). EPA fed in the *sn*-2 position at the dose of 4.4 g/100 g fatty acids significantly reduced production of IL-6 after a 24 hr culture period compared to EPA fed at the level of 2.2 g/100 g fatty acids and in the same position (*Figure 3.7.*). EPA at the *sn*-2 position, 4.4 level also significantly inhibited IL-6 production compared to EPA in the *sn*-1(3) position at the 4.4 g/100 g fatty acids.

Figure 3.7. LPS stimulated IL-6 production by blood mononuclear cells after 24 and 48 hr culture periods.



Significant differences are expressed as:

b. Indicates that the *sn*-2 diet is significantly different from the *sn*-1(3) diet (for the same fatty acid and same dose)

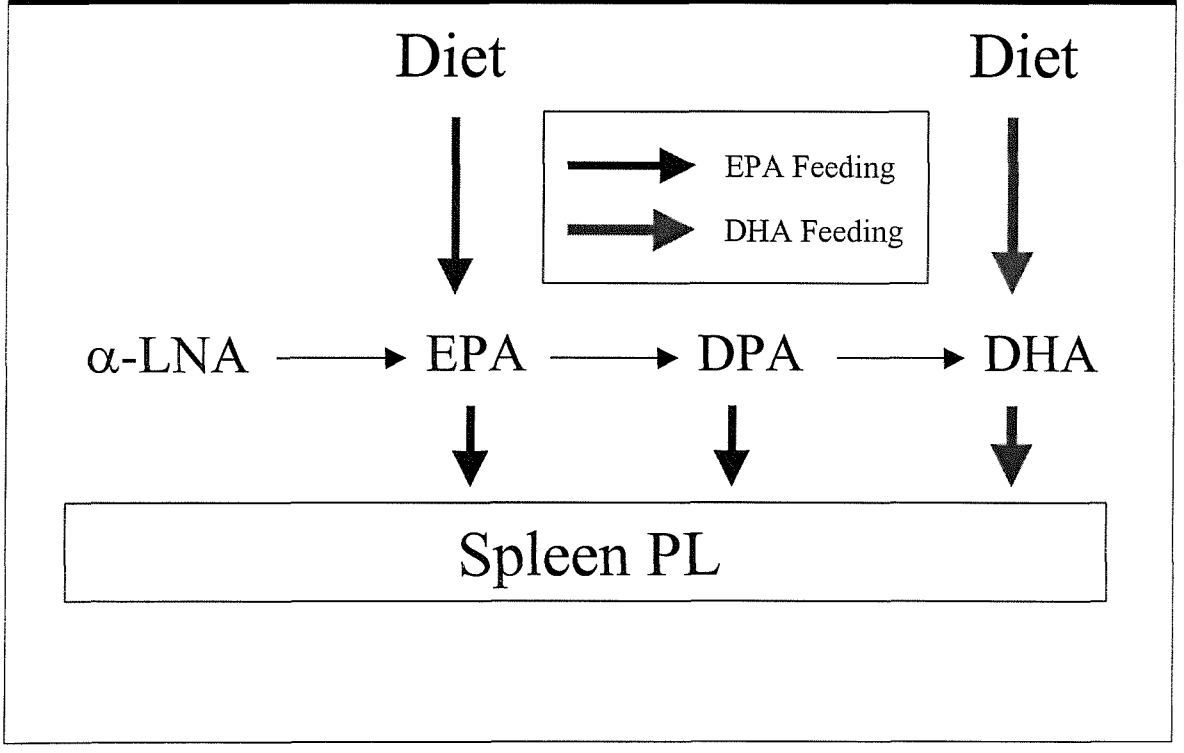
c. Indicates that the 2.2 g/100g fatty acids diet is significantly different from the 4.4 g/100 g fatty acids diet (for the same fatty acid and the same positional isomer)

3.4. Discussion

3.4.1. Changes in Fatty Acid Composition of Spleen Cells

It appears that significant incorporation of DHA (and EPA) into spleen cell membrane phospholipids is attainable even at a dose as low as 2.2 g/100g fatty acids. In addition, it seems that incorporation of DHA is independent of its position in dietary TAG, while that of EPA may be slightly increased when fed it is in the *sn*-2 position. The fates of dietary EPA and DHA, in terms of spleen cell phospholipids are summarised pictorially in *Figure 3.8.*

Figure 3.8. The fate of fatty acids within the spleen cell phospholipid fractions for mice fed an EPA-rich or DHA-rich diet.



Spleen cell Phospholipid Composition

After EPA feeding

The proportion of EPA in the spleen cell phospholipids was similar to the control except when EPA was fed at the *sn*-2 position and at a dose of 4.4 g /100g fatty acids diet. After feeding this diet the proportion of EPA was significantly greater than after feeding each of the other diets. This suggests that when fed in the *sn*-2 position EPA is more readily available for incorporation into immune cell phospholipids (at least in mice). This was also seen in rats fed the same diets for PC but not in total PL (see Chapter 2). Therefore, this suggests that enrichment of a particular fatty acid in all PL subtypes is not the same. Since longer chain fatty acids are almost always incorporated into the *sn*-2 position of membrane phospholipids (Budowski *et al.*, 1985), a higher content of a PUFA at this position in the diet may make more of the fatty acid available for incorporation into membranes. Fatty acids in the *sn*-2 position of dietary TAG are retained at this position in 2-monoacylglycerol after digestion (Pufal *et al.*, 1995) and subsequently incorporated into membranes as such. When EPA was fed there was a decrease in the proportion of arachidonic acid in spleen cell PL, while there was no change in the content of DHA compared to the control diet as in rats. However, there were very significant increases in DPA compared to the control group but these were not affected by dose or by position of EPA in dietary TAG.

After DHA feeding

There were changes in the composition of DHA in the spleen cell PL. The observed increases in DHA and decreases in AA (similar to those seen in the EPA-fed animals) compared to the α-LNA control were dose-dependent but were independent of position of DHA in dietary TAG. When DHA was fed there was a decrease in the EPA content of spleen cell PL compared to the control as also seen in rats (see Chapter 2).

Changes in other fatty acids measured

Feeding a diet enriched with EPA caused an increase in LA, 20.2 *n*-6 and DPA in spleen cell PL, regardless of the dose of EPA or its position in dietary TAG. The

mice fed DHA-rich diets in this study displayed an increase in LA and 20:2 *n*-6 in spleen PL that was independent of the dose or position of the DHA in the dietary TAG. The increase in LA after EPA and DHA feeding was also observed in rats (see Chapter 2). However, the fatty acid 20:2 *n*-6 was not measured in that study.

Feeding a fish oil-rich diet (containing 6.1 g EPA and 4.3 g DHA/100 g fatty acids) resulted in the same changes in EPA and also an increase in the amount of DHA in the membrane of rat macrophages (Brouard and Pascaud, 1990). Feeding 4.4 g EPA or DHA /100 g fatty acids resulted in similar changes in the fatty acids composition of rat spleen cell PL (Peterson et al., 1998b) as seen in this study in mice (and rats, see section 2.4.4.). Furthermore, Brouard and Pascaud (1990) reported that the changes in fatty acid composition observed in macrophages after feeding these diets were comparable to those changes seen in splenocyte membranes from the same animals. Thus, the changes in the fatty acid composition of the spleen cells observed in this study most likely also occurred in the monocyte/macrophages (and neutrophils) which were observed to show functional changes.

Brouard and Pascaud (1990) have reported that rats fed a diet rich in linseed oil (containing 15 g α-LNA/100g fatty acids) displayed similar changes in the membrane fatty acid composition of peritoneal macrophages to those seen in the present study. Since the control diet and the 2.2 g/100 g fatty acid experimental diets contained some α-LNA the changes in fatty acid composition seen in this study suggest that in mice there is conversion of α-LNA to EPA and of both these fatty acids to DPA.

The changes in proportions of fatty acids such as DPA, LA, AA and 20:2 *n*-6 may be attributable to a number of different factors. Chapkin *et al.* (1987) have shown that murine macrophages lack the ability to convert LA to AA. Instead macrophages in culture converted LA to 20.2 *n*-6, via elongation reactions (*Figure 3.9.)*. The inability to convert LA to AA has been attributed to a low Δ6 desaturase activity within macrophages. The studies of Chapkin *et al.* (1987, 1990) also showed that there was a decrease in the AA content of macrophages as the concentration of EPA and DPA in the membrane increased.

It has been shown experimentally that α-LNA competes with LA for the Δ6 desaturase enzyme (Brenner and Peluffo, 1966) and thus inhibits LA metabolism (Morrhuauer and Holman, 1963). The affinity of Δ6 desaturase for α-LNA is high (Km = 29-33μM). Since LA is not being metabolised to AA, utilisation of AA from the membrane phospholipids would result in a reduction of this fatty acid in the membrane unless it could be replenished from another fatty acid pool. It has been postulated by Chapkin *et al.* (1987) that macrophages obtain AA from other sources such as the diet, via lipoproteins, or from AA metabolised from dietary LA and supplied to them by other cells (Goldyne and Stobo, 1982). The competition from EPA and DHA for incorporation into the membrane will also contribute to the decreased proportion of AA in the membrane after EPA and DHA feeding.

A subsequent study by Chapkin *et al.* (1990) observed that murine macrophages were able to convert radioactively labelled EPA to DPA but lacked the ability to convert DPA to DHA (*Figure 3.10.*) and again this was attributed to low Δ6 desaturase activity. The low activity of this enzyme in the macrophage population may therefore account for the increase in DPA after EPA feeding observed in this study. The increases in DPA (but not DHA) seen in mouse spleen cells in this study were also seen in rat spleen cells and rat plasma (see section 2.3.4.). Thus it appears that mice and rats are similar in this respect after EPA feeding in both plasma and spleen cell composition. Thus it may be that the absence of an increase in DHA in mouse spleen cells for the EPA-fed animals may be due to an inhibition of the liver Δ6 desaturase enzyme by EPA in addition to the low activity of this enzyme in different cells of the body e.g. the liver. The content of EPA was increased in the diet and has been shown to inhibit the elongation of DPA to DHA in humans by inhibiting the Δ6 desaturase enzyme (Vermunt *et al.*, 1999). The high affinity of α-LNA for Δ6 desaturase may also contribute to the block on the conversion of DPA to DHA.

Figure 3.9. The metabolism of linoleic acid

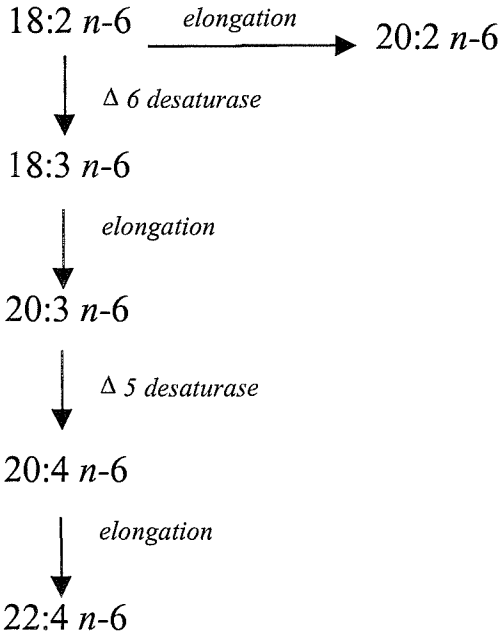
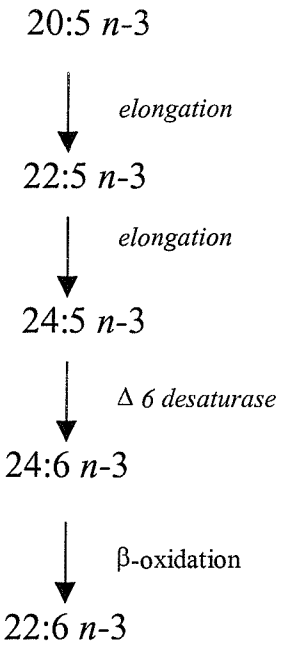


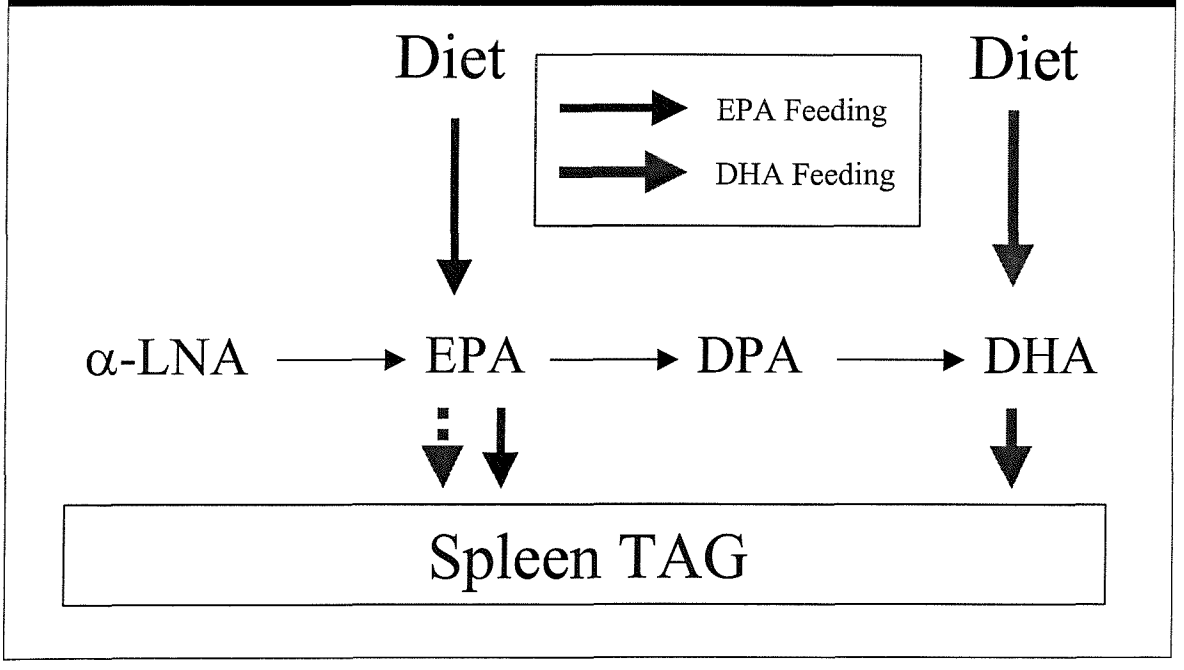
Figure 3.10. The metabolism of eicosapentaenoic acid



Spleen Cell Triacylglycerol Composition

The composition of the splenocyte TAG showed an increase in EPA for the EPA-fed animals and this seemed to be more effective when EPA was fed in the *sn*-2 position compared to the *sn*-1(3) position. There was no change in DHA when EPA was fed, while DHA content was dose-dependently increased compared to the control for the spleen cells from the DHA-fed animals. Thus, it appears that storage of EPA and DHA in neutral lipids in the splenocyte differs slightly from that in the membrane phospholipids. There appears to be little incorporation of DPA into the TAG fraction, although there was twice as much in the EPA-fed animals compared with the DHA-fed animals. This may be due to conversion of EPA (or α-LNA), suggesting this conversion occurs for fatty acids with different metabolic fates. There was a dose-dependent decrease in the content of α-LNA with EPA and DHA feeding and this is probably attributable to the decrease in α-LNA (from 2.2 to 0.5 g/100 g fatty acid) content of the diet with the 4.4 g/100 g fatty acid EPA or DHA diets. It appears that this fatty acid can be stored in the TAG of the leukocyte while in the membrane it was undetectable. The fates of dietary EPA and DHA in spleen cell TAGs are summarised pictorially in *Figure 3.11.*

Figure 3.11. The fate of fatty acids within the spleen cell TAG fractions for mice fed an EPA-rich diet.



3.4.2. Changes in Phagocytic Activity by Murine Monocytes and Neutrophils

The results of cell culture studies have shown that changes in fatty acid composition of macrophages or neutrophils affects phagocytic activity due to changes in membrane properties. However, few studies with *n*-3 PUFA have been conducted. The only study with these fatty acids (α-LNA, EPA and DHA) and macrophages demonstrated a decrease in phagocytic activity as the content of these fatty acids increases in the macrophage membrane (Calder *et al.*, 1990), while in neutrophils increasing the content of EPA and DHA in the membrane also resulted in a decrease in phagocytic activity (Sipka *et al.*, 1989).

In the current study there were significant effects of feeding EPA on the phagocytic function of both monocytes and neutrophils. There were no significant effects of DHA on phagocytosis. When EPA was fed there was a dose-dependent decrease in the number of monocytes and neutrophils that performed phagocytosis. This was coupled with an increase in the ability of the active cells to ingest *E. coli* when EPA was fed at the *sn*-2 position, but not when fed at the *sn*-1(3) position. It is notable that the feeding of this diet also resulted in the greatest increase in the proportion of EPA in the splenocyte phospholipids. This may have profound effects when the composition of fish oil in the diet is considered.

Previous studies have found that feeding fish oil as 17% (by weight) of the diet for 4 weeks modulates murine macrophage phagocytosis, by reducing the percentage of cells performing phagocytosis (Eicher and McVey, 1995). A diet containing 10.5% fish oil fed for 4 weeks did not alter the ability of porcine alveolar macrophages to ingest latex spheres (Turek *et al.*, 1994). The number of macrophages performing phagocytosis was not investigated in this latter study. Fish oil fed as 5 g/kg body weight/d for 7 days had no effect on the ability of rabbit macrophages to perform phagocytosis (D'Ambola *et al.*, 1991). The results of the present study suggest that the different results obtained in these studies may be attributable at least in part to the EPA content of the fish oil used (and so to the dose of EPA fed (rather than the n-3 PUFA)), and more importantly to its position within the TAG molecule. This study indicates that the influence of the DHA in the diet is less important with regard to

phagocytosis. The present study suggests that, although EPA in the *sn*-2 position of dietary TAG decreased the number of monocytes and neutrophils performing phagocytosis, there may be a compensatory increase in the capacity of the actively phagocytosing cells. Thus, overall there may be little effect of EPA-feeding (in the *sn*-2 position) on phagocytic activity. If however the EPA in the fish oil is predominantly in the *sn*-1(3) position there is no such compensation by the phagocytosing monocytes and neutrophils, leading to an overall decrease in phagocytosis.

3.4.3. Changes in IL-6 Production by Murine PBMNC in Whole Blood Culture

There were significant changes in the production of IL-6 Furthermore differences in production after feeding EPA and DHA were observed. A diet enriched with EPA had a more inhibitory effect on IL-6 production compared to a diet rich in DHA. This was dependent on the position of the fatty acid in the dietary TAG, in that EPA in the *sn*-2 position had a significant inhibitory effect on IL-6 production, compared to EPA in the *sn*-1(3) position. It has previously been shown that feeding a diet rich in fish oil (10% by weight) to rats for 4 or 6 weeks significantly increased the production of IL-6 compared to feeding a chow control diet (Tappia and Grimble, 1994, 1995). In contrast feeding mice a diet containing 20% by weight of fish oil for the same length of time resulted in a marked decrease (50 %) in the production of IL-6 (Yaqoob and Calder, 1995b). Thus it may be that there were differences in the position of EPA in dietary TAG in these studies.

From the present study it is possible to postulate that the decrease in production of IL-6 may be more attributable to an increase in EPA in the *sn*-2 position compared to the *sn*-1(3) position and that EPA feeding has greater modulatory effects on IL-6 production compared to DHA feeding.

In the previous chapter no effect of EPA (in any position or dose) was found on cytokine production by lymphocytes in rats. The difference in the outcome of the two studies may to due to several factors. There may be a difference in the level of EPA enrichment needed in a particular cell to elicit a modulation in cytokine production,

or a difference in the level of enrichment in EPA needed in immune cells in different species. More likely the difference in the studies is attributable to a difference in incorporation in EPA (especially at the *sn*-2 position of dietary TAG) into the different PL subtypes in different cell types (i.e. lymphocytes compared to macrophages).

3.5. Conclusion

In the present study each of the experimental diets could induce profound changes in the fatty acid composition of the spleen phospholipids (in agreement with other studies, such as Brouard and Pascaud, (1990)) and TAG and this was mainly dose-dependent. The only effect of position was observed for EPA in dietary TAG. In the spleen cell PL fraction where there was an increased content of EPA after EPA was fed in the *sn*-2 position, at the 4.4 g level. This effect of position on incorporation of EPA into spleen cell PL was not observed in rats in chapter 2 for the total PL fraction but was observed for the PC species, which is the major PL subtype in the macrophage membrane. The most significant changes in fatty acid composition were seen in the proportions of fatty acids in the phospholipid fraction where there were changes in the amounts of AA, EPA, DPA and DHA after *n*-3 PUFA feeding.

A change in fatty acid composition is associated with change in phagocytic activity. This study has shown that the position of EPA but not DHA in dietary TAG has an effect on phagocytic activity. Therefore, maintenance of the position of EPA in 2-MAG, through digestion, absorption and incorporation into cell membranes might be a factor in determining the effect of dietary *n*-3 PUFA on phagocytosis. The effects were dose-dependent and were more profound for EPA in the *sn*-2 position. Feeding levels as low as 2.2 g EPA/100 g fatty acids in the diet can have modulatory effects on monocyte and neutrophil phagocytosis.

It appears that EPA has greater modulatory effects than DHA with respect to this function and this agrees with studies of macrophage phagocytosis using these fatty acids in culture (Calder *et al.*, 1990), where EPA had a greater effect than DHA (*Figure 1.18.*). This also agrees with the results of chapter 2 where DHA had little effect on lymphocyte function.

The mechanisms by which the dietary fatty acids exert their influence on cell function in this study are several fold. First and foremost there is the change in the fatty acid composition of the membrane, which subsequently changes membrane unsaturation. An increase in unsaturation, as has been discussed in section 1.3.3., has profound effects on membrane fluidity. It has been shown *in vitro* that compared to a membrane enriched with AA or α-LNA, the long-chain PUFA EPA and DHA have slightly inhibitory effects on phagocytosis. Thus, there appears to be a contribution of fatty acid composition to the phagocytic activity of the macrophages and neutrophils in this study. When EPA was fed in the *sn*-2 position there was a decrease in the number of cells performing phagocytosis, with a rise in the number of bacteria ingested per active cell. This could be due to an effect of membrane fluidity on membrane function or membrane movement and/or cell signalling. The latter seems to be more likely when the impact on cytokine production of feeding EPA in the *sn*-2 position, as opposed to the *sn*-1(3) position, is considered as well as the increase in incorporation of EPA into spleen PL when fed at the *sn*-2 position compared to the *sn*-1(3) position.

The observed opposing effects on the percentage of active cells and mean fluoresence suggests that there is a mechanism to increase the phagocytic activity of active cells when the total number of active cells is decreased. It is unclear how such a mechanism would operate. However, it may be that there is more than one type of monocyte (or neutrophil) present, and that these have different phagocytic activities. Thus, the effects seen in this study may be the result of an effect of some diets on only one of these populations while other diets might affect both populations.

CHAPTER 4. THE MODIFICATION OF DIETARY INTAKE OF *n*-3 POLYUNSATURATED FATTY ACIDS BY HEALTHY HUMAN SUBJECTS

4.1. Introduction

The Consumption of Fatty Acids in the UK Diet

During the past thirty or forty years there has been a change in the total fat intake of many populations in the Western world, including that of the United Kingdom (British Nutrition Foundation, 1992). According to the Adult Survey the diet of the average adult male in the United Kingdom contains a greater amount of total fat and a greater proportion of this fat as saturated fatty acids (SFA) (section 1.2.3.) than the recommended intakes (British Nutrition Foundation, 1992). Despite this, since 1970 there has been a 40% decrease in the absolute amount of SFA consumed in the diet, and a 20% decrease in the consumption of monounsaturated fatty acids (MUFA) (British Nutrition Foundation, 1992). Along with this there has been an increase in the amount of total polyunsaturated fatty acids (PUFA) consumed in the diet (British Nutrition Foundation, 1999). Thus, the ratio of PUFA to SFA in the diet has risen. In the United Kingdom this ratio is approximately 0.35 while in the United States of America it is 0.63. Many Western populations consume the majority of dietary PUFA as *n*-6 PUFA with little consumption *n*-3 PUFA (British Nutrition Foundation 1992, 1995; Yam *et al.,* 1996). This is most likely due to an increase in the availability of linoleic acid in margarine and cooking oils over the period since 1970. As a result of this the ratio of *n*-6 to *n*-3 PUFA in the United Kingdom diet is about 6.00, although this has been subject to change over the last 10 years. The Government has made recommendations for the consumption of the different types of fatty acids in the UK diet (section 1.2.3.).

Increasing the Intake of n-3 Polyunsaturated Fatty Acids in the UK Diet

Over the last 10 years there has been a large impetus in the UK to try to alter the type of fat, and especially the type of PUFA that is consumed in the diet (Department of Health, 1991). *N*-3 PUFA are believed to have a multitude of health benefits (de Deckere *et al.,* 1998; British Nutrition Foundation, 1999) and it is now considered that not only is *n*-3 PUFA intake too low but that of *n*-6 is too high (de Deckere *et al.,* 1998). Thus, increased consumption of n-3 PUFA should be coupled to a decrease in the intake of *n*-6 PUFA. This is starting to be achieved, as there has been a slight increase in the intake of *n*-3 PUFA and decrease in *n*-6 PUFA intake in the UK population in the last 10 years (*Table 4.1*). This has led to an alteration in the *n*-6 to *n*-3 PUFA ratio of the UK average diet to less than 6.

Table 4.1 Trends in the intake of *n*-3 and *n*-6 fatty acids in the UK population

PUFA	**Intake (g/day)**	
	1991	**1995**
Total *n*-3	**1.61**	**1.8**
18:3 *n*-3	1.39	1.55
18:4 *n*-3	<0.01	0.02
20:3 *n*-3	<0.01	0.03
20:4 *n*-3	0.06	0.07
20:5 *n*-3	0.04	0.06
21:5 *n*-3	0.06	0.02
22:5 *n*-3	0.01	0.05
22:6 *n*-3	0.07	0.10
Total *n*-6	**10.66**	**10.2**
18:2 *n*-6	10.48	10.04
Ratio of *n*-6 to *n*-3 fatty acids	**6.62:1**	**5.67:1**

(Taken from British Nutrition Foundation, 1999)

Since changes in the intake of the long chain *n*-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may be limited by the availability of foodstuffs containing these fatty acids (apart from oily fish), one way to increase their intake may be through the fortification of staple food products. This would also reduce the need for individuals to consume capsules containing *n*-3 PUFA, which are expensive and usually have to be consumed in relatively large numbers to attain the desired intake of *n*-3 PUFA. In addition, the use of α-linolenic acid (α-LNA) as a source of *n*-3 PUFA requires investigation since it is a cheaper source of *n*-3 PUFA,

and is also more easily incorporated into the diet. It is found in large amounts in oils such as rapeseed oil, which lack any noticeable flavour or smell and are also less prone to oxidation than oils containing long chain *n*-3 PUFA.

Over the last decade several initiatives have been taken to increase the availability of *n*-3 PUFA in the food chain. These initiatives have included enriching manufactured foods ranging from bread and biscuits to beverages such as orange juice (Lovegrove *et al.,* 1997) or increasing the intake of long chain *n*-3 PUFA in animal feed in order to enrich meat and diary products in these fatty acids (Leskanich *et al.,* 1997; Farrell *et al.,* 1998). There have been calls by the Government and nutrition organisations to further investigate these avenues of enrichment and to establish the effects on the human health.

Benefits of Increased Intake of n-3 PUFA

The recommendations for the increased intake of *n*-3 PUFA in the diet have been based on the impact of these fatty acids seen in epidemiological studies performed since the 1970s (see section 1.4.1.). These studies demonstrated that in populations such as the Inuit of Greenland, which consume a large quantity of sea mammals and cold water fish and thus have a high intake of the long chain PUFA EPA and DHA, there is a decrease in the occurrence of coronary heart disease (CVD), compared to a sex- and age- matched individuals in Denmark (Dyerberg *et al.,* 1975). The Inuit population also has a lower incidence of inflammatory and autoimmune diseases such as psoriasis, arthritis, diabetes mellitus and glomerular nephritis (Kromann and Green, 1980). Dietary intervention studies in the last 15 years have also linked an increase in the consumption of the *n*-3 PUFA, EPA and DHA which are obtained from oily fish, with a reduction in CVD at a population level in samples of the British (Burr *et al.,* 1989), American (Daviglus *et al.,* 1997; Simopoulous *et al.,* 1991) and Western European (Kromhout *et al.,*1985) populations. There is also growing evidence that an increase in the consumption of the *n*-3 PUFA α-LNA may also reduce the occurrence of CVD (de Lorgeril *et al.,* 1994, Dolecek *et al.,* 1992). The mechanisms by which *n*-3 PUFA have their effects on the development of CVD are numerous. CVD can be considered as a chronic inflammatory disorder (Davies *et al.,* 1993). One aspect of CVD development is the accumulation of cholesterol-rich

macrophages in the arterial wall, which leads to the formation of a plaque and hardening of the arterial wall (Ross and Stier, 1999). Thus, some of the beneficial effects of *n*-3 PUFA in CVD may be on inflammatory cell adhesion and function, such as inflammatory mediator production, and could lead to a delay the formation of these plaques and thus the development of atherosclerosis. The *n*-3 PUFA family has also been found to have beneficial effects on blood lipids (Harris *et al.*, 1996).

Immunomodulation with n-3 PUFA

The type and quantity of fat in the diet have been shown in animal and human studies to modulate many aspects of immune function (see section 1.4.). The consumption of *n*-3 PUFA has been shown to modulate several indices of immune function including lymphocyte proliferation, cytokine production, leukocyte cell surface marker expression, cell adhesion, macrophage function and the delayed type hypersensitivity (DTH) response. It is notable that the studies with long chain *n*-3 PUFA in this area have only been conducted through supplementing the diet with capsules. No investigations with foods rich in long chain *n*-3 PUFA have been conducted, while the effects of α-LNA rich foods have been investigated but only with very large doses.

However, despite a large literature in this field, the effects of *n*-3 PUFA, and especially α-LNA, have not been satisfactorily established with respect to the immune system in healthy free-living individuals. The observations made to date are often conflicting, especially at the lower levels of intake (0.5 to 3.0 g/d of EPA+DHA) and more investigation into the effects of the different types of *n*-3 PUFA and of the dose response of their effects on the immune system is needed. The dose-response relationship with respect to α-LNA and its metabolites EPA and DHA also requires investigation. Findings from such studies have implications for the optimum functioning of the immune system in healthy individuals.

4.1.1. Aims of the Study

As a result of the known beneficial impacts of *n*-3 PUFA on human health and the need to investigate some effects of *n*-3 PUFA further (e.g. effects on immune

function), coupled with the need to increase the intake of *n*-3 PUFA in the UK diet, which has so far proved quite a task, several aims for this study were developed. These aims include:

1. to ascertain whether enriching the diet of free-living healthy human volunteers in α-LNA, EPA and DHA through foodstuffs leads to an enrichment of these fatty acids in various body pools
2. to investigate the immunological effects of EPA and DHA at levels which can be reasonably readily incorporated into the diet
3. to investigate the immunological effects of α-LNA at levels that can be more reasonably readily incorporated into the diet
4. to ascertain a better estimate of the relationship between the amount of α-LNA and of EPA and/or DHA in exerting immunological effects
5. to establish if there are any differences due to gender in the enrichment of lipid pools in *n*-3 PUFA or in the immunomodulatory effects of these fatty acids

The outcome of this dietary intervention study will provide a more realistic picture of the level of EPA and DHA and of their precursor fatty acid α-LNA, which may be introduced into the human diet through food supplementation to achieve immunomodulatory effects.

The results from the study will be dealt with in this and the following two chapters. This chapter will examine the modification of fatty acid intake, the compliance of the subjects and the changes in fatty acid composition of plasma and PBMNC phospholipids. Chapter 5 will examine the effects of increased consumption of n-3 PUFA on various parameters of innate immunity, including phagocytosis and oxidative burst activity by neutrophils and monocytes and the production of cellular mediators by monocytes. Chapter 6 will examine the effects of the increased consumption of *n*-3 PUFA on parameters of acquired immunity, including lymphocyte proliferation, production of cellular mediators by these cells and the DTH response.

4.1.2. Aims of This Chapter

Hypothesis

- Consuming increasing doses of α-LNA or EPA+DHA will result in a dose-dependent enrichment of these fatty acids in human plasma and immune cells

In the current chapter the following will be examined:

- the characteristics of the subjects involved in the study
- the modulation of fatty acid intake by the different treatment groups
- the compliance of the subjects to the dietary interventions (data supplied by colleagues at Reading University)
- the changes in the fatty acid composition of:
    - plasma phospholipids
    - peripheral blood mononuclear cell phospholipids

4.2. Methods

4.2.1. Chemicals

Butylated hydroxytoluene, chloroform, Histopaque, heparin, hexane, HEPES-buffered RPMI medium (glutamine free), glutamine, methanol, penicillin, potassium chloride, sodium chloride and streptomycin were all obtained from Sigma Chemical Co., Poole, UK. The sources of reagents used to generate data at the University of Reading are not described here, but in all cases were high quality reagents purchased from standard suppliers (e.g. Sigma Chemical Co., Poole, UK).

4.2.2. Screening Characteristics

This study was undertaken in collaboration with University of Reading, where members of the Hugh Sinclair Unit of Human Nutrition at the Department of Food

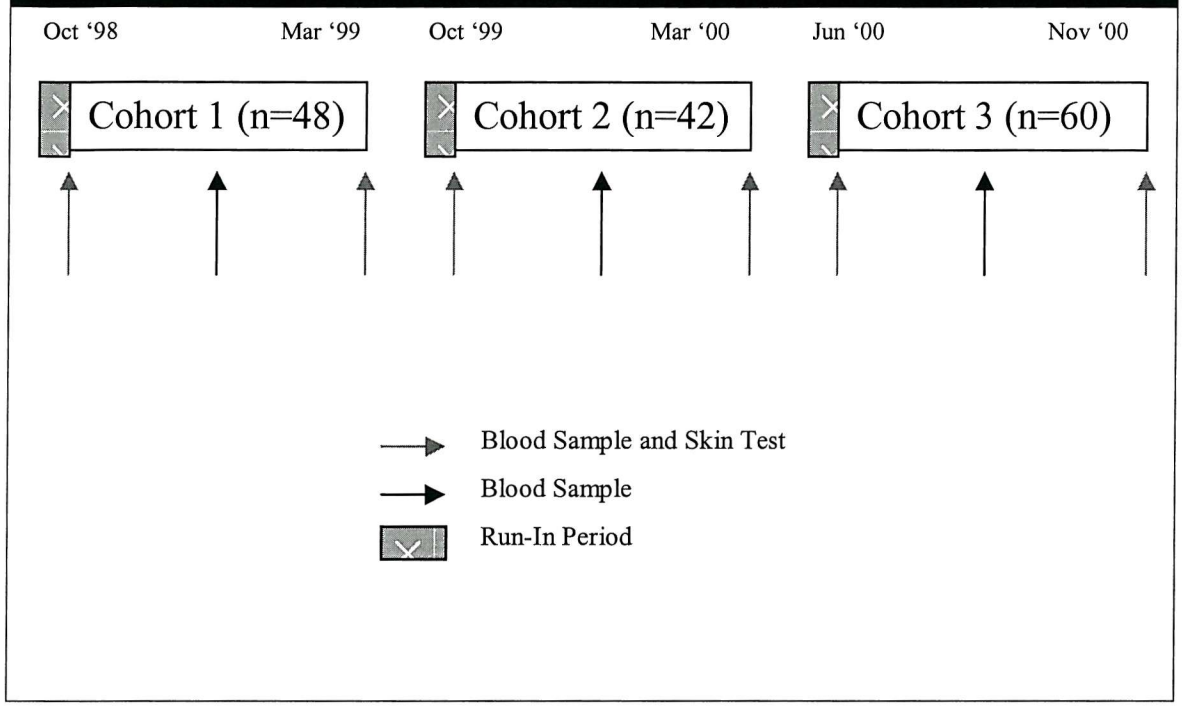
Sciences and Technology were responsible for the recruitment of suitable subjects. The screening process involved the measurement of height and weight and the analysis of a fasted blood sample for the concentrations of cholesterol, triacylglycerol (TAG) and glucose. Suitable candidates for entry into the study had a cholesterol concentration of between 4.5 and 8.0 mM, which is slightly raised compared to the normal range (3.6 to 6.5 mM), a TAG concentration in the range 1.3 to 1.8 mM, a glucose concentration less than 6.5 mM, and a body mass index (BMI) (weight/height^2) less than 32 kg/m^2.

4.2.3. Study Design and Modification of Dietary Intake

The study involved the recruitment of approximately 150 subjects. Each subject taking part in the study consumed a 'placebo' margarine for 4 weeks 'run-in' period prior to the six month intervention period during which 25 g per day of a specially formulated 'test margarine' and a supplement of three 1 g capsules per day were included in the diet.

There were five different test margarines and 25 to 30 subjects were assigned to consume each of these. The subjects were organised into 3 cohorts which each ran for 7 months (one-month baseline and 6 months dietary intervention) (*Figure 4.1.*). Within each cohort between 8 and 10 subjects were randomly assigned to each of the five dietary groups.

Figure 4.1. The time scale over which the dietary intervention where carried out



The five treatment groups varied according to the level of EPA and DHA or α-linolenic acid (α-LNA) which they consumed (*Table 4.2*). The placebo treatment group was to consume 0.2 g EPA+DHA and 1.5 g α-LNA per day, the current UK dietary intakes. The medium EPA+DHA treatment group was to consume 0.7 g EPA+DHA per day. The high EPA+DHA treatment group were to consume 1.5 g EPA+DHA pre day. Due to manufacturing constraints it was not possible to supply 1.5 g of EPA+DHA solely in 25 g of margarine. Thus, in order to attain 1.5 g of EPA+DHA per day, it was necessary to supplement the diet of the subjects in this group supplying with capsules containing 0.8 g EPA+DHA per day. In order to keep the study blinded the remaining groups were asked to consume capsules containing a control oil, which contained no EPA, DHA or α-LNA. The medium and high ALNA treatment groups were to consume 5 g and 10 g α-LNA per day respectively plus 3 control capsules (*Table 4.2.*). The fatty acid composition of the pre-baseline treatment and capsules resembled the current UK diet (as in the placebo group).

Table 4.2. Amounts of the major PUFA in the treatment group [a]

	Placebo	**5.0 g α-LNA**	**10.0g α-LNA**	**0.7 g EPA+DHA**	**1.5 g EPA+DHA**
EPA+DHA	0.2	0.2	0.2	0.7	1.5
α-LNA	1.5	5.0	10.0	1.5	1.5
LA	12	8.5	3.5	11.5	10.7
AA	0.5	0.5	0.5	0.5	0.5
LA/α-LNA	8.0	1.7	0.4	7.6	7.1
AA/EPA+DHA	2.5	2.5	2.5	0.7	0.3

[a] g/25g margarine + capsules + background diet; AA = Arachidonic Acid; LA = Linoleic Acid; Data were provided by Unilever, The Netherlands

The margarines were based on blends of different oils (*Table 4.*3.) in order to achieve the required level of the different fatty acids in the treatment groups and were supplied by Unilever, Vlaardingen, The Netherlands. The differences in the fat blends between the different treatment groups were mainly in the proportions of rapeseed (rich in α-LNA) and RoPUFA oils. RoPUFA is an oil blend containing high concentrations of the fatty acids EPA and DHA. The margaines were 85% fat and 15% water. The fatty acid compositions of the treatment margarines are shown in *Table 4.4.*

*Table 4.*3. Fats used to formulate the test margarines

	Placebo	**5.0 g α-LNA**	**10.0g α-LNA**	**0.7 g EPA+DHA**	**1.5 g EPA+DHA**
Hardstock*	15.5	15.4	15.9	11.1	11.0
Flaxseed Oil	0.0	28.3	72.4	0.00	0.00
RoPUFA [+]	0.00	0.00	0.00	8.80	8.80
Rapeseed Oil	6.9	14.0	0.00	0.00	0.00
Safflower Oil	24.1	0.00	0.00	0.00	0.00
Sunflower Oil	47.1	42.3	0.00	73.8	73.8
High Oleic Sunflower Oil	6.40	0.00	11.7	6.30	6.30

* Hardstock = combination of palm oil and palm kernel oil; [+] RoPUFA = fish oil provided by Hoffman La-Roche, Switzerland; Data supplied by Unilever, The Netherlands
g/100 g margarine

The fatty acid compositions of the placebo and fish oil capsules are shown in *Table 4.5.* The placebo capsules contained no *n*-3 fatty acids, while the EPA+DHA capsules contained the amounts of EPA and DHA needed to attain the dose of 1.5 g EPA+DHA /d when three capsules were consumed daily along with 25 g/d of the EPA+DHA enriched margarine.

Table 4.4. Fatty acid composition of test margarines

Fatty Acid	**Ru*n*-In**	**Placebo**	**5.0 g α-LNA**	**10.0g α-LNA**	**0.7 g EPA+DHA**	**1.5 g EPA+DHA**
6:0	0.00	0.00	0.00	0.00	0.00	0.00
8:0	0.16	0.16	0.17	0.17	0.11	0.11
10:0	0.17	0.17	0.18	0.18	0.13	0.13
12:0	3.33	2.29	2.42	2.58	1.69	1.69
14:0	1.00	1.02	1.02	1.07	1.19	1.19
16:0	13.6	13.6	13.6	13.2	12.6	12.6
16:1	0.10	0.07	0.09	0.05	0.57	0.57
18:0	3.76	3.71	3.80	3.76	4.19	4.19
18:1 cis	23.4	23.6	23.5	23.7	23.0	23.0
18:1 trans	0.33	0.34	0.35	0.39	0.26	0.26
18:2	52.5	51.4	36.4	12.9	50.8	50.8
18:3 *n*-3	0.96	0.93	17.4	40.7	0.30	0.30
20:0	0.31	0.31	0.26	0.17	0.30	0.30
20:1	0.25	0.24	0.24	0.14	0.30	0.30
20:2	0.02	0.02	0.03	0.03	0.04	0.04
20:5 *n*-3	0.00	0.00	0.00	0.00	0.87	0.87
22:0	0.50	0.49	0.39	0.20	0.60	0.60
22:1	0.03	0.25	0.06	0.11	0.23	0.23
22:6 *n*-3	0.00	0.00	0.00	0.00	1.33	1.33
24:0	0.16	0.00	0.16	0.09	0.20	0.20

g/100 g total fatty acids; Source Data: Unilever, The Netherlands

The margarines also contained emulsifiers, flavourings and stabilisers (*Table 8.1.*) which were added during manufacture to allow the margarines to have a four-week shelf life. All margarines contained the same amount of each of these additives which are typically added to all margarines sold in the UK. Vitamin E was added in varying levels (*Table 4.6.*) depending on the number of double bonds in the fatty acids used in the formulation, according to Muggli (1994) (see section 8.1.2.).

Table 4.5. Fatty Acid Composition of Capsules

Fatty Acid	Placebo	Ro PUFA[+]
10:0	0.10	0.10
12:0	0.20	0.20
14:0	0.70	5.50
16:0	34.9	16.1
16:1	0.00	5.90
18:0	4.60	3.10
18:1	35.3	11.6
18:2	20.9	2.30
18:3 *n*-3	0.00	0.10
18:3 *n*-6	0.00	1.60
18:4 *n*-3	0.00	2.70
20:1	0.00	0.40
20:2	0.00	0.30
20:3 *n*-6	0.00	0.10
20:4 *n*-6	0.00	1.00
20.5	0.00	11.0
22:0	0.00	0.10
22:1	0.00	2.10
22:5	0.00	1.10
22:6 *n*-3	0.00	17.2
24:1	0.00	0.50

g/100 g total fatty acid; Data supplied by Hoffman La Roche, Switzerland; [+] RoPUFA = fish oil provided by Hoffman La-Roche, Switzerland

Table 4.6. Vitamin E Content of Margarines

	Placebo	5.0 g α-LNA	10.0g α-LNA	0.7 g EPA+DHA	1.5 g EPA+DHA
Total TE for 25 g margarine / d	10.7	13.3	13.2	12.9	13.3
Total TE for 3 g capsules / d	0.77	0.77	0.77	0.77	1.89
Total TE for margarine + capsules / d	11.4	14.0	14.0	13.7	15.2

TE = Tocopherol Equivalent which is the estimated Vitamin E required calculated according to Muggli (1994). (Section 7.1.).
Data: Margarine Vitamin E measured by Unilever, The Netherlands; Capsule Vitamin E data analysed by Hoffman La-Roche

4.2.4. Collection of Samples for Analyses

At baseline (0 month), and after 3 months and 6 months of intervention a fasting blood sample (40 ml) was taken into heparinised vacuutainer tubes and stored at

room temperature for no longer than 4 hr during which time it was transported from Reading to Southampton by courier. At baseline and 6 months a delayed-type hypersensitivity test was applied as described in section 6.2.6.

4.2.5. Peripheral Blood Mononuclear Cell and Plasma

Whole blood was layered onto an equivalent volume of Histopaque (density 1.077 g/ml) and peripheral blood mononuclear cells (PBMNC) were collected from the interface after centrifugation at room temperature (2000 rpm, 15 min). Plasma was collected from the top layer and some used for cell function experiments; the remainder was frozen at -20 °C for later fatty acid composition analysis.

The PBMNC were washed with Hepes-RPMI medium (containing penicillin, streptomycin and 0.75 mM glutamine) and then relayered onto an equal volume of Histopaque. The PBMNC were collected from the interface after centrifugation, and again washed with RPMI medium. Finally the PBMNC were resuspended in medium, counted using a Coulter cell counter (model Z1; Beckman Coulter, UK) and adjusted to the appropriate cell concentrations for use in various experiments. The remainder of cells were centrifuged to a pellet (2000 rpm, 10 min) and frozen at -20 °C for fatty acid composition analysis.

4.2.6. Fatty Acid Composition Analysis

Total lipid was extracted from plasma and mononuclear cells using the method described in section 2.2.7. Each sample was then separated into phospholipid (PL), triacylglycerol (TAG) and cholesterol ester (CE) by thin-layer chromatography (TLC) according to the method described in section 2.2.7. The fatty acid compositions of the different fractions were determined by gas chromatography (GC), performed as described in section 2.2.7.

The phospholipid fatty acid compositions of plasma and PBMNC are described in this chapter, while the fatty acid compositions of plasma triglyceride and cholesterol ester are tabulated in section 8.2. for reference, but are not described further.

4.2.7. Statistical Analysis

Data are the mean ± SEM of approximately 30 subjects per treatment group (see *Table 4.7.* for details), these being most subjects in all 3 cohorts. Statistical analyses were initially performed using a two-way analysis of variance to establish the effects of treatment and time and the interaction between them. Where treatment or time was significant, one-way analysis of variance and a post-hoc least significance test with a Bonferonni correction were then performed to identify differences between groups at a given time point and differences within a treatment group over time. In all cases a value of $P<0.05$ was taken to indicate statistical significance and the statistical package SPSS Version 10.0 (SPSS Inc., Chicago, IL) was used.

Significant differences within a treatment group over time are expressed as:

a. Significantly different from 0 month
b. Significantly different from 3 month
c. Significantly different from 6 month

Significant differences between treatment groups at a given time point are expressed as:

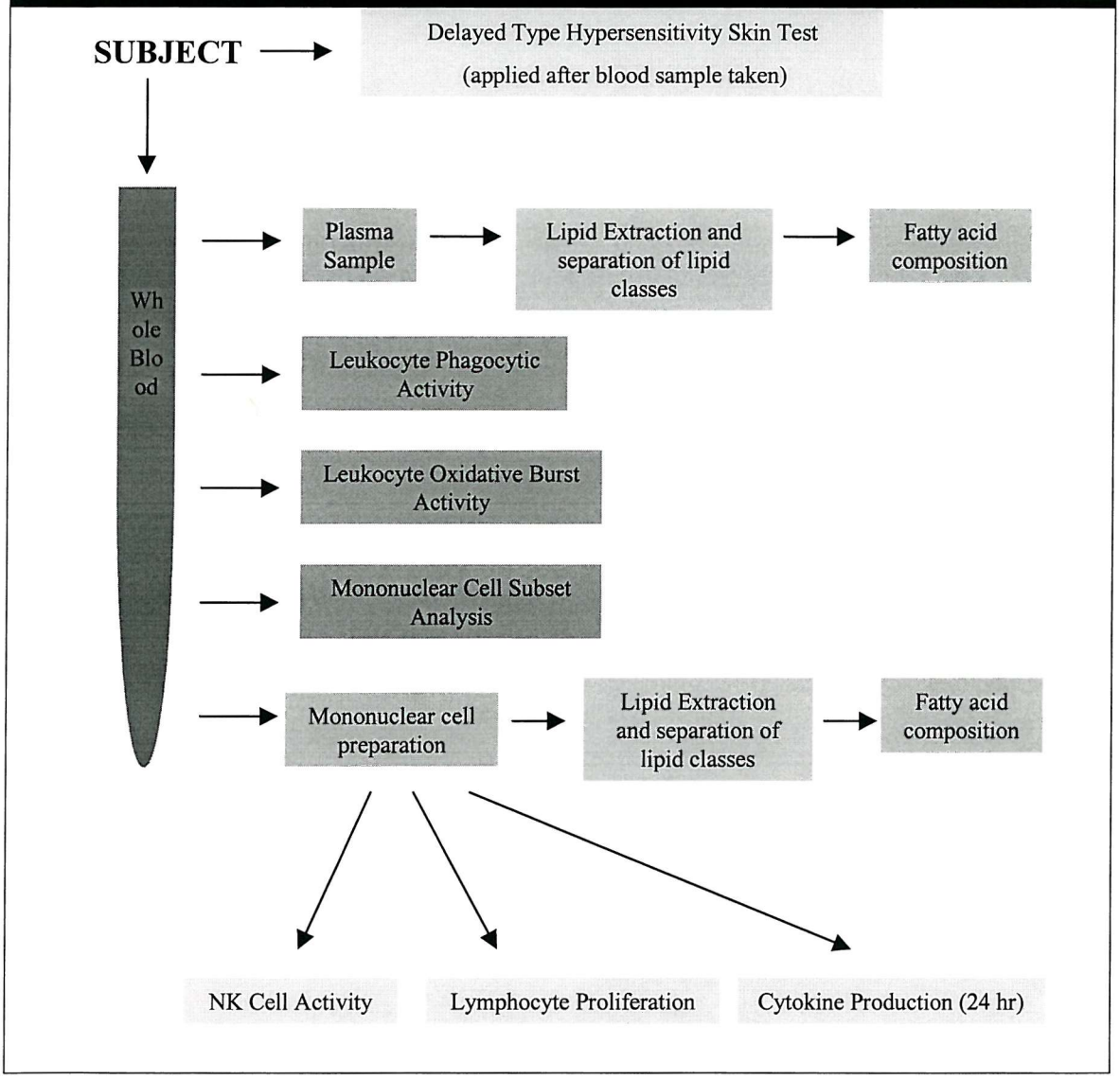
A. Significantly different from the placebo treatment
B. Significantly different from the 5.0 g α-LNA treatment
C. Significantly different from the 10.0 g α-LNA treatment
D. Significantly different from the 0.7 g EPA+DHA treatment
E. Significantly different from the 1.5 g EPA+DHA treatment

Independent t-tests were also performed where a one–way analysis could not be e.g. to establish differences between males and females. Data with a $^{+}$ indicates significant differences between males and females.

4.2.8. *Summary of Investigations Performed*

Several measures of immune cell function were also made in this study. The following two chapters (Chapters 5 and 6) give details of these. However, a summary of the experiments is shown in *Figure 4.2*. Details of these are discussed in Chapters 5 and 6.

Figure 4.2. Summary of experiments performed in this study.



4.3. Results

4.3.1. Characteristics of the Treatment Groups

There were no significant differences between the treatment groups for any of the inclusion criteria (*Table 4.7.*). The intakes of energy and nutrients by the subjects were determined for each of the treatment groups at 0 month of intervention, by colleagues at the University of Reading using FoodBase (Institute of Brain Chemistry and Human Nutrition, London, 1993) (*Table 4.8.*). There were no significant differences between any of the groups in the intake of energy, nutrients or the fatty acids of interest (α-LNA, AA, EPA, DHA).

Table 4.7. Characteristics of the Different Treatment Groups.

Treatment	**Placebo**	**5.0 g α-LNA**	**10.0 g α-LNA**	**0.7 g EPA+DHA**	**1.5 g EPA+DHA**
n	30	30	29	30	31
Sex Males	18	17	17	17	18
Females	12	13	12	13	13
Age (years)	55.0 ±2.0	52.0 ±2.0	54.0 ±2.0	53.0 ±2.0	54.0 ±2.0
BMI (kg/m^2)	25.8 ±0.6	26.3 ±0.6	26.5 ±0.6	27.2 ±0.6	26.1 ±0.6
TAG (mM)	1.62 ±0.12	1.50 ±0.10	1.45 ±0.10	1.49 ±0.10	1.58 ±0.12
Total Cholesterol (mM)	5.75 ±0.17	5.72 ±0.17	5.84 ±0.18	5.62 ±0.17	5.64 ±0.17
Glucose (mM)	5.28 ±0.11	5.28 ±0.10	5.15 ±0.10	5.22 ±0.12	5.21 ±0.09

Data were supplied by colleagues at University of Reading

4.3.2. Changes in Weight for the Different Treatment Groups

There was no difference in the body weight at baseline among the treatment groups. There was a mean weight change of 1.3 kg (SEM ±0.2) over the study period with no difference in weight gain between any of the treatment groups (*Table 4.9.*).

Table 4.8. Intake of energy and nutrients in each treatment group at baseline (n=98)

Treatment	**Placebo (n=21)**	**5.0 g α-LNA (n=20)**	**10.0 g α-LNA (n=19)**	**0.7 g EPA+DHA (n=19)**	**1.5 g EPA+DHA (n=19)**
Energy (MJ)	11.4 ±3.2	9.50 ±2.6	10.4 ±3.0	10.9 ±3.2	11.1 ±3.4
% Energy as					
Protein	17.2 ±3.1	16.2 ±2.0	16.1 ±3.2	16.0 ±1.8	16.8 ±1.9
Carbohydrate	46.2 ±5.3	47.4 ±6.5	46.8 ±7.8	46.4 ±5.9	45.7 ±6.3
Alcohol	3.60 ±4.0	3.70 ±3.9	3.30 ±4.7	2.70 ±2.7	4.10 ±3.8
Fat	33.0 ±4.6	32.4 ±6.4	33.8 ±5.2	34.9 ±4.2	33.4 ±6.2
SFA	11.9 ±2.4	11.5 ±3.8	11.7 ±2.7	12.3 ±2.6	11.9 ±3.2
MUFA	10.1 ±1.7	9.80 ±1.8	10.3 ±1.9	11.0 ±2.1	10.2 ±1.9
PUFA	5.60 ±1.6	5.30 ±1.2	5.80 ±1.6	5.40 ±1.4	6.00 ±1.9
Total *n*-6 PUFA *	14.6 ±5.5	11.3 ±4.6	13.8 ±5.1	13.9 ±6.8	15.6 ±8.5
18:2 *n*-6 *	14.2 ±5.5	11.6 ±4.6	13.4 ±5.0	13.6 ±6.7	15.3 ±8.5
20:4 *n*-6 *	0.22 ±0.1	0.17 ±0.1	0.18 ±0.1	0.20 ±0.1	0.21 ±0.1
Total *n*-3 PUFA *	2.20 ±0.8	1.90 ±1.1	2.00 ±0.9	2.00 ±0.8	2.11 ±0.8
18:3 *n*-3 *	1.60 ±0.6	1.30 ±0.4	1.40 ±0.5	1.50 ±0.7	1.60 ±0.7
20:5 *n*-3 *	0.21 ±0.1	0.20 ±0.3	0.20 ±0.2	0.13 ±0.1	0.16 ±0.1
22:6 *n*-3 *	0.28 ±0.2	0.24 ±0.3	0.24 ±0.2	0.21 ±0.1	0.21 ±0.1

MJ = megajoules; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; * = g/day
Data supplied by colleagues at University of Reading

Table 4.9. Weight change from Baseline over 6 months of intervention.

Treatment	Placebo	5.0 g α-LNA	10.0g α-LNA	0.7 g EPA+DHA	1.5 g EPA+DHA
Change in Weight (kg)	1.2 ±0.1	1.5 ±0.5	0.8 ±0.3	1.1 ±0.4	2.2 ±0.6

Data were supplied by Colleagues at University of Reading

4.3.3. Subject Compliance as Measured by the Returned Packaging

Each subject was asked to complete a 'margarine checklist' on a daily basis in order to ascertain the actual consumption of the margarine. These were returned along with the empty margarine packets and the empty capsule packets so that the amount of margarine and number of capsules not consumed could be calculated. The compliance of the subjects was measured by analysis of these factors and the results are shown in *Table 4.10.* There were no statistical differences in the amount of margarine consumed each day. It was calculated that over 90% of the 25 g of margarine required to be consumed was eaten each day among the treatment groups. There was a compliance of over 90% for the capsules except in the 1.5 g EPA+DHA group where compliance was slightly below this.

Table 4.10. Compliance as measured by the returned empty cartons and packets

Treatment	Margarine Checklist*	Margarine Packets*	Capsule packets*
Placebo	97.5 ±4.5	93.8 ±10.3	90.8 ±15.7
5.0 g α-LNA	95.7 ±6.1	97.6 ±5.20	94.5 ±8.60
10.0 g α-LNA	96.4 ±7.0	92.6 ±10.4	91.3 ±12.4
0.7 g EPA+DHA	95.7 ±6.7	91.9 ±11.9	93.6 ±11.3
1.5 g EPA+DHA	96.8 ±6.3	93.7 ±11.7	87.7 ±20.7

* % total calculated intake

Data supplied by colleagues at University of Reading

4.3.4. Analysis of the Dietary Intake of the Subjects Over the Intervention Period

The intake of energy and nutrients by subjects was determined for each treatment group at 5 months into the intervention by colleagues at the University of Reading,

using FoodBase (Institute of Brain Chemistry and Human Nutrition, London, 1993) (*Table 4.11.*). In summary, the target intakes of *n*-3 PUFA achieved in all groups. Although there was a significant increase in the % energy derived from fat and PUFA during the intervention compared to 0 month (33 to 36%) the increase was uniform across all groups including the placebo.

4.3.5. Modulation of Plasma Phospholipid Fatty Acid Composition

There were no differences between the treatment groups in the proportions of any of the fatty acids in plasma PL at baseline, except for DHA (*Table 4.12.*). This fatty acid was present in a greater proportion in the placebo and 5.0 g α-LNA groups at baseline compared to the 10.0 g α-LNA group and each of the EPA+DHA groups.

Two-way ANOVA established that there was a significant effect of treatment on the proportions of α-LNA ($P<0.001$), ARA ($P<0.005$), EPA ($P<0.003$) and DHA ($P<0.001$) and a significant effect of time on the proportion of α-LNA, EPA and DHA (all $P<0.001$) and DPA ($P<0.009$). The effects of treatment and time were investigated further.

There were significant effects of each of the treatments and of time on the fatty acid composition of plasma PL when one-way ANOVAs were performed (*Table 4.12* and *Table 4.13*).

Table 4.11. Intake of energy and nutrients in each treatment group during the intervention (dietary assessment at 5 months) (n=146)

Treatment	**Placebo (n=21)**	**5.0 g α-LNA (n=20)**	**10.0 g α-LNA (n=19)**	**0.7 g EPA+DHA (n=19)**	**1.5 g EPA+DHA (n=19)**
Energy (MJ)	12.1 ±2.8	9.3 ±2.3	10.4 ±2.4	11.0 ±2.5	10.8 ±2.8
% Energy as					
Protein	16.1 ±2.5	15.5 ±1.7	15.4 ±2.5	15.4 ±1.9	16.1 ±2.1
Carbohydrate	43.3 ±5.6	45.3 ±4.8	43.7 ±6.1	45.0 ±5.1	44.1 ±5.4
Alcohol	4.30 ±5.9	3.30 ±4.3	3.20 ±3.6	3.30 ±3.3	3.90 ±4.1
Fat	36.2 ±4.1	36.0 ±5.0	37.8 ±5.1	36.3 ±4.9	35.8 ±5.0
SFA	11.9 ±2.0	11.5 ±2.6	12.3 ±2.6	11.8 ±2.7	11.3 ±2.7
MUFA	10.5 ±1.2	10.1 ±1.8	10.9 ±1.8	10.5 ±2.1	10.2 ±2.2
PUFA	8.20 ±1.4	8.40 ±1.1	8.80 ±1.6	8.20 ±1.3	8.90 ±1.9
Total *n*-6 PUFA*	^{BC}23.2 ±4.6	^{ADE}16.4 ±3.9	^{ADE} 13.4 ±3.0	^{BC} 21.2 ±4.3	^{BC} 21.5 ±5.1
18:2 *n*-6 *	^{BC} 22.9 ±4.5	^{ADE} 16.2 ±3.9	^{ABDE} 13.1 ±2.9	^{BC} 20.8 ±4.2	^{BC} 21.1 ±5.1
20:4 *n*-6 *	0.23 ±0.1	0.14 ±0.1	0.18 ±0.1	0.19 ±0.1	0.20 ±0.1
Total *n*-3 PUFA*	^{BCE}2.20 ±0.6	^{ACDE}5.00 ±0.8	^{ABDE} 10.0 ±0.9	^{BCE}2.20 ±0.7	^{ABCD}3.30 ±0.7
18:3 *n*-3 *	^{BC}1.50 ±0.4	^{ADE} 4.50 ±0.6	^{ADE} 9.49 ±0.8	^{BC}1.30 ±0.5	^{BC}1.40 ±0.5
20:5 *n*-3 *	^{DE} 0.22 ±0.1	^{DE}0.16 ±0.1	^{DE} 0.15 ±0.1	^{ABC} 0.30 ±0.1	^{ABC} 0.66 ±0.1
22:6 *n*-3 *	^{BCDE}0.30 ±0.2	^{ADE}0.21 ±0.1	^{ADE} 0.19 ±0.1	^{ABCE}0.47 ±0.2	^{ABCD}1.02 ±0.1

MJ = Megajoules; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; * = g/day
Data supplied by colleagues at University of Reading
Significant differences are expressed as:

A. Significantly different from the placebo treatment
B. Significantly different from the 5.0 g ALNA treatment
C. Significantly different from the 10.0 g ALNA treatment
D. Significantly different from the 0.7 g EPA+DHA treatment
E. Significantly different from the 1.5 g EPA+DHA treatment

The Change in Absolute Composition of the Fatty Acids of Interest in Plasma PL

There were significant changes in the proportions of the fatty acids which were the subject of the dietary treatments (α-LNA, EPA and DHA) (*Table 4.12*) and these changes are graphically represented in *Figures 4.3.* to *4.5*. The changes in AA and DPA will also be described in detail, as these are important in the metabolism and incorporation into PL of the fatty acids under study. Some of these changes were significant over time or compared to the placebo group, and are discussed in detail. These changes reflected the composition of the intervention treatments, and thus indicate compliance of the subjects with respect to the intake of the margarines over the 6 months intervention period and subsequently increased incorporation of dietary fatty acids into this lipid pool.

α-Linolenic Acid

There was a significant increase in the proportion of α-LNA in the both the α-LNA treatment groups (70 % and 120 % increases, respectively). This was dose-dependent and was maximal at 3 months (*Figure 4.3.*). There were no significant changes in α-LNA content in any of the other treatment groups.

Arachidonic Acid

There were no significant changes in the content of AA over the intervention period 0 to 6 months for any of the treatment groups. However, there was a tendency for the content of AA to decrease in the 1.5 g EPA+DHA group at 3 months compared to the content at baseline and 6 months (*Table 4.12.*).

Table 4.12. The fatty acid composition of plasma phospholipids at different times of dietary intervention (n = 150)

Treatment	Time (m)	18:3 *n*-3	20:4 *n*-6	20.5 *n*-3	22:5 *n*-3	22:6 *n*-3
Placebo	0	0.34 ±0.02	8.90 ±0.45	0.99 ±0.14	2.01 ±0.19	^{CD}4.48 ±0.24
	3	^{BC}0.29 ±0.03	^{E}9.83 ±0.41	1.19 ±0.23	1.89 ±0.14	^{E} 4.41 ±0.33
	6	^{BC}0.29 ±0.03	9.54 ±0.53	1.27 ±0.23	2.25 ±0.13	^{CDE} 4.40 ±0.24
5.0 g α-LNA	0	0.31 ±0.02 ^{bc}	8.42 ±0.29	1.05 ±0.13^{bc}	2.04 ±0.16	4.29 ±0.21
	3	^{AC} 0.50 ±0.04^{a}	8.62 ±0.29	2.04 ±0.26^{a}	2.25 ±0.11	^{E} 4.08 ±0.17
	6	^{AE} 0.46 ±0.03^{a}	9.16 ±0.37	2.00 ±0.23^{a}	2.14 ±0.10	^{E} 4.16 ±0.24
10.0 g α-LNA	0	0.31 ±0.02 ^{bc}	8.77 ±0.37	0.92 ±0.12 ^{bc}	1.84 ±0.15 ^{c}	^{A}3.46 ±0.18
	3	^{ABDE} 0.80 ±0.09^{a}	8.53 ±0.33	2.23 ±0.30 ^{a}	2.22 ±0.18	^{A}3.48 ±0.22
	6	^{AE}0.76 ±0.08^{a}	9.30 ±0.50	2.14 ±0.30	2.45 ±0.15^{a}	^{AE}3.67 ±0.26
0.7 g EPA+DHA	0	0.38 ±0.02	9.07 ±0.28	0.98 ±0.13	1.69 ±0.16^{b}	^{A}3.43 ±0.14^{bc}
	3	^{C} 0.35 ±0.05	9.21 ±0.43	1.69 ±0.22	2.43 ±0.22^{a}	^{E} 4.81 ±0.28^{a}
	6	0.30 ±0.03	9.51 ±0.41	1.78 ±0.30	2.00 ±0.14	^{AE}5.04 ±0.20^{a}
1.5 g EPA+DHA	0	0.36 ±0.03	8.96 ±0.30 ^{b}	1.12 ±0.11 ^{bc}	1.98 ±0.20	3.90 ±0.18^{bc}
	3	^{C} 0.28 ±0.02	^{E}7.83 ±0.23^{a}	2.13 ±0.32^{a}	2.05 ±0.10	^{ABCD} 6.31 ±0.35^{a}
	6	^{BC} 0.29 ±0.02	8.40 ±0.30	2.25 ±0.30^{a}	2.07 ±0.10	^{ABCD} 6.61 ±0.27^{a}

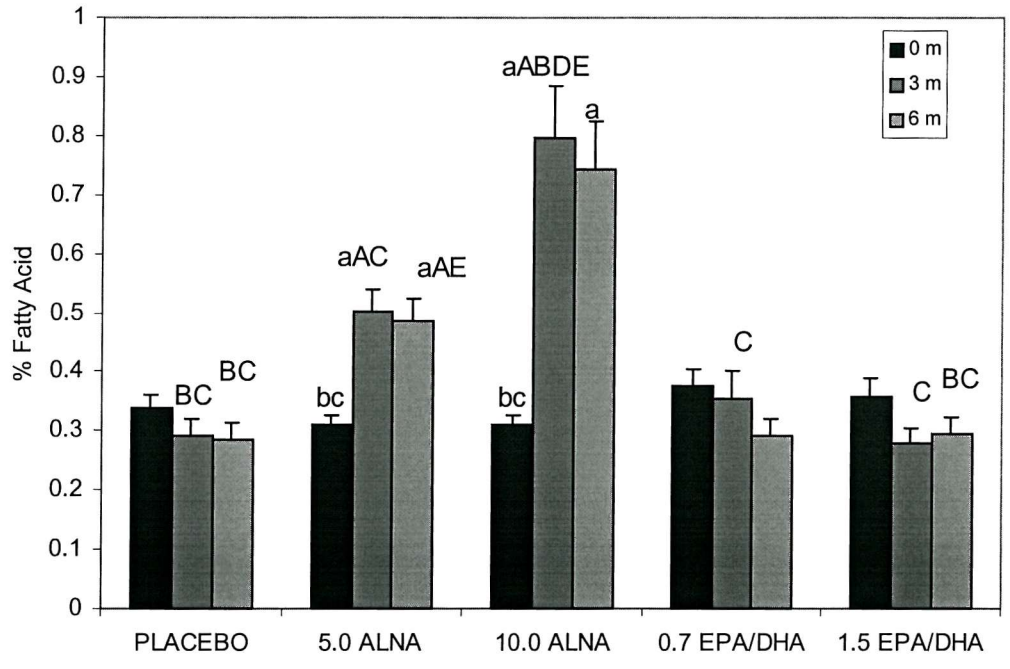
Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

Table 4.13. The composition of plasma phospholipids of the remaining fatty acids measured (n = 150)

Treatment	Time (m)	16:0	18:0	18:1 *n*-9	18:2 *n*-6	18:3 *n*-6	20:1 *n*-6	20:3 *n*-6	22:0
Placebo	0	26.56 ±0.51 [b]	15.85 ±0.64 [bc]	9.92 ±0.50	21.38 ±0.96	0.92 ±0.71	0.39 ±0.03	3.46 ±0.16 [c]	1.31 ±0.12
	3	28.87 ±0.66 [a]	13.36 ±0.35 [a]	9.88 ±0.35	21.23 ±0.90	0.14 ±0.02	0.33 ±0.03	3.33 ±0.22 [c]	[BE]1.20 ±0.12
	6	27.37 ±0.32	13.24 ±0.24 [a]	11.07 ±0.84	21.08 ±0.90	0.19 ±0.03	0.38 ±0.09	4.57 ±0.37 [ab]	1.16 ±0.14
5.0 g α-LNA	0	27.19 ±0.34	16.12 ±0.71 [bc]	9.68 ±0.49	22.10 ±0.49	0.18 ±0.02	0.44 ±0.03 [c]	3.57 ±0.18	1.59 ±0.18 [c]
	3	27.48 ±0.49	14.11 ±0.26 [a]	9.93 ±0.31	21.85 ±0.96	0.18 ±0.03	0.35 ±0.04	3.29 ±0.17	[A]1.23 ±0.09
	6	27.50 ±0.54	13.29 ±0.28 [a]	10.06 ±0.37	23.46 ±0.65	0.17 ±0.02	0.29 ±0.03 [a]	3.60 ±0.30	1.04 ±0.08 [a]
10.0 g α-LNA	0	27.70 ±0.58	15.22 ±0.66 [bc]	10.67 ±0.49	22.57 ±0.52	0.19 ±0.02	0.44 ±0.03	3.41 ±0.22	1.38 ±0.13
	3	27.25 ±1.10	12.79 ±0.60 [a]	10.22 ±0.48	20.83 ±1.05	0.18 ±0.03	0.38 ±0.04	2.80 ±0.17	1.50 ±0.17
	6	27.54 ±0.42	13.05 ±0.60 [a]	11.03 ±0.49	21.63 ±0.51	0.14 ±0.02	0.33 ±0.04	3.72 ±0.45	1.28 ±0.19
0.7 g EPA+DHA	0	28.18 ±0.56	14.76 ±0.73 [c]	10.38 ±0.42	22.14 ±0.50	0.22 ±0.02	0.48 ±0.04 [c]	3.62 ±0.17	1.53 ±0.17
	3	27.80 ±0.60	13.83 ±0.38	9.76 ±0.28	22.24 ±0.65	0.20 ±0.03	0.37 ±0.04	3.44 ±0.17	1.34 ±0.10
	6	27.27 ±0.41	12.60 ±0.55 [a]	9.65 ±0.40	21.88 ±0.64	0.16 ±0.02	0.32 ±0.05 [a]	3.90 ±0.25	1.11 ±0.12
1.5 g EPA+DHA	0	27.50 ±0.47	14.55 ±0.76	10.07 ±0.44	22.70 ±0.96	0.87 ±0.68	0.44 ±0.02	3.20 ±0.15	1.47 ±0.15
	3	28.94 ±0.47	13.84 ±0.26	9.80 ±0.54	20.63 ±0.83	0.20 ±0.02	0.39 ±0.05	2.93 ±0.33	[A]1.80 ±0.19
	6	27.41 ±0.42	12.75 ±0.47	9.41 ±0.32	21.40 ±0.67	0.80 ±0.67	0.34 ±0.04	3.63 ±0.39	1.56 ±0.19

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

Figure 4.3. The proportion of α-LNA in plasma PL in the different treatment groups



Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;
A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

Eicosapentaenoic Acid

There was no significant change in EPA in the placebo treatment group. There were changes in the proportions of the EPA and DHA in the PL of subjects in the groups receiving these fatty acids. There was an increase in the proportion of EPA in both the 0.7 g and 1.5 g EPA+DHA groups (70% and 100% increase respectively) and this was dependent on the amount of EPA in the treatment and was maximal at 3 months (*Figure 4.4.*). The proportion of EPA in the plasma PL of subjects in the two α-LNA groups also significantly increased (100 % increase) and this was independent of the dose of α-LNA in the treatment and was maximal at 3 months (*Figure 4.4.*).

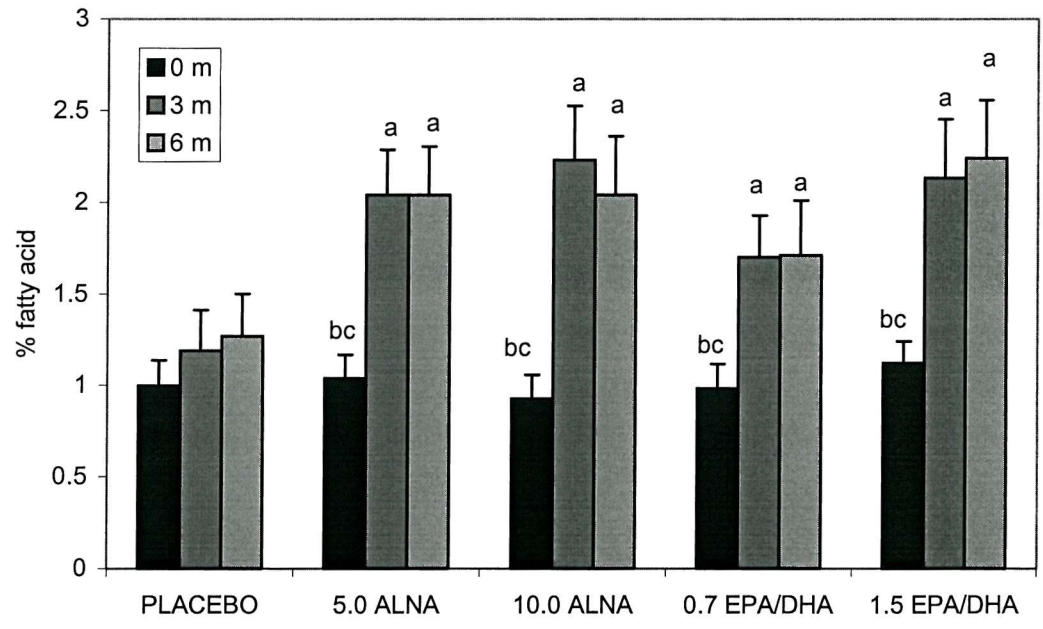
Docosapentaenoic Acid

There was a small but significant increase in the content of DPA in the 10.0 g α-LNA treatment group from baseline to 6 months (*Table 4.12*). A similar increase in DPA content was also seen in the 0.7 g EPA+DHA group (*Table 4.12*). There was no change in DPA content in the placebo, 1.5 g EPA+DHA or 5.0 g α-LNA treatment groups.

Docosahexaenoic Acid

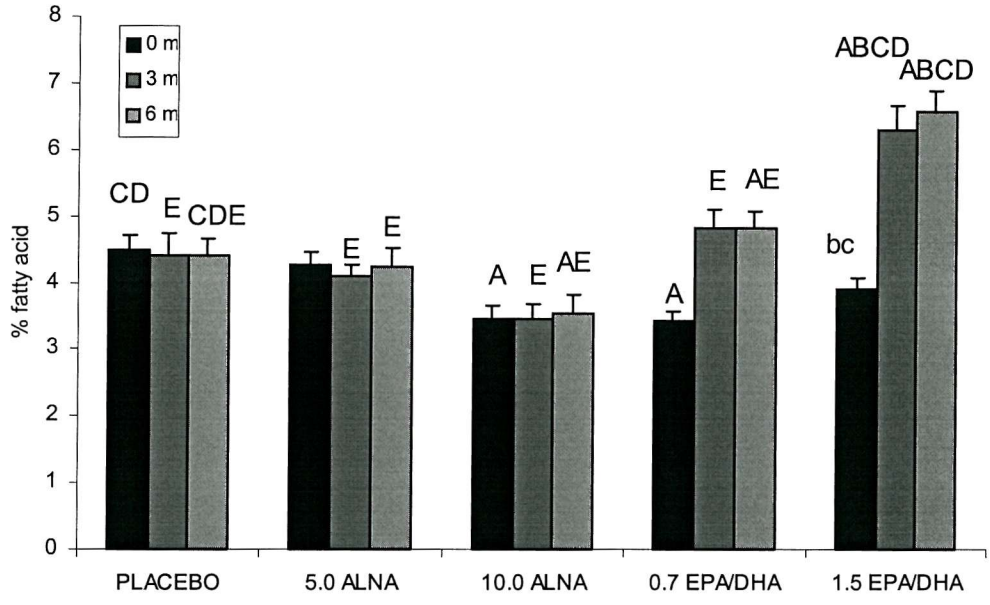
There was no significant change in the DHA content of the placebo or α-LNA treatment groups. There was a significant increase in the proportion of DHA in the plasma PL in subjects in the 0.7 g and 1.5 g EPA+DHA groups. This was dose-dependent with 90% increase in the 1.5 g EPA+DHA dietary group, and a 70% increase in the proportion of DHA for the 0.7 g EPA+DHA group (*Figure 4.5.*).

Figure 4.4. The proportion of EPA in plasma PL in the different treatment groups



Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;

Figure 4.5. The proportion of DHA in plasma PL in the different treatment groups



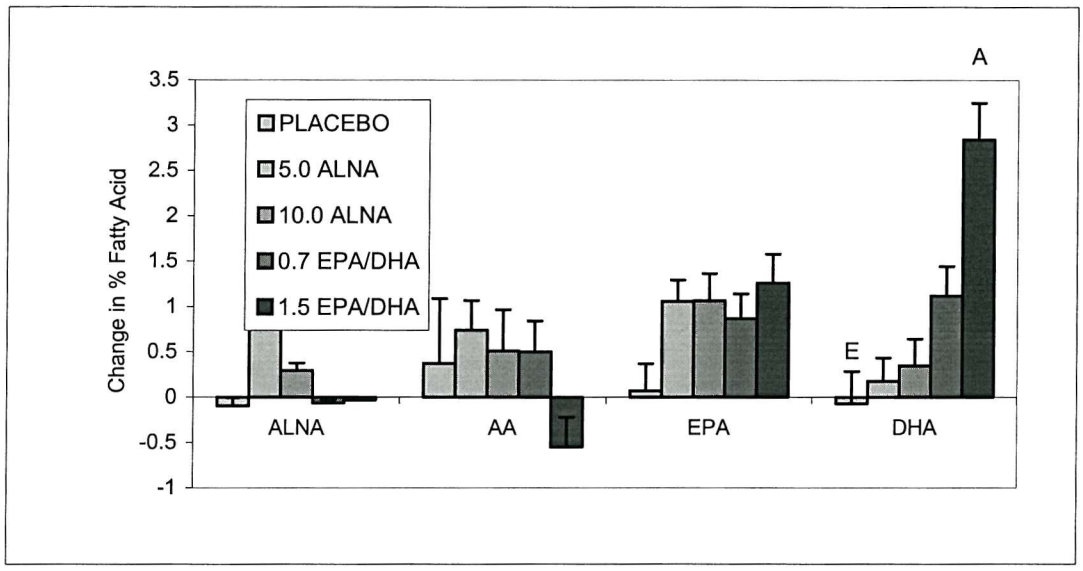
Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;
A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

The Overall Changes From 0 to 6 month in the Composition of the Fatty Acids of Interest

Changes in % Fatty Acids

The changes in the absolute contents of α-LNA, AA, EPA and DHA from 0 to 6 months are shown in *Figure 4.6.* Only the change in the proportion of DHA for the 1.5 g EPA+DHA treatment was significantly different from the placebo group. However, there were non-significant changes in the absolute content of α-LNA in the PL of the individuals receiving this fatty acid and in the content of EPA in all treatment groups except the placebo. There was a small increase in AA in all treatment groups except the 1.5 g EPA+DHA where there was a decrease.

Figure 4.6. The mean change in the proportions of α-LNA, AA, EPA and DHA in plasma PL in the different treatment groups from 0 to 6 months



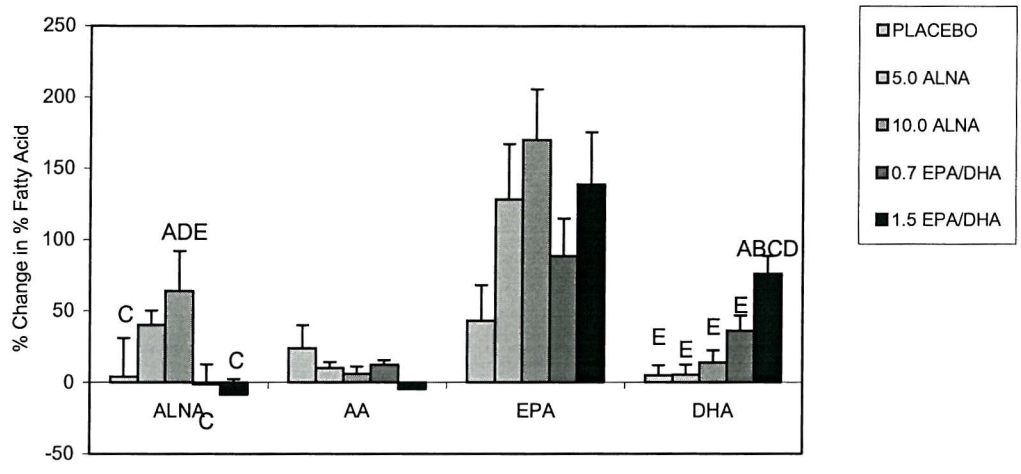
Significant differences are expressed as:
A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

% Changes in % Fatty Acids

There were significant effects of treatment on the % change in the content of α-LNA, EPA and DHA. There was a 65% increase in the content of α-LNA in the 10.0 g α-LNA group compared to the placebo and EPA+DHA groups and this was significant (*Figure 4.7.*). There was also a 45% change in α-LNA in the 5.0 g α-LNA group but this did not reach significance. There were no significant effects of treatment on the % change in AA. Interestingly the only group in which the % change in this fatty acid was negative was in the 1.5 g EPA+DHA group. The % change in EPA was not significant for any of the treatment groups when compared. However, there was a positive change in the content of this fatty acid in plasma PL in both of the α-LNA groups (130 to 170%) and in the EPA+DHA groups (100 to 150 %), while in the placebo group there was only a small change (30%). The % increase in the content of

DHA (75%) was significant for the 1.5 g EPA+DHA group compared to all the other treatment groups. There was also a 50% increase in DHA content in the 0.7 g EPA+DHA group but this was not significant.

Figure 4.7. The % change in the proportions of α-LNA, AA, EPA and DHA in plasma PL in the different treatment groups from 0 to 6 months



Significant differences are expressed as:
A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

The Changes in the Proportion of the Other Fatty Acids Measured in Plasma PL

There were some changes in the proportions of the other fatty acids measured (*Table. 4.13.*). There was a significant decrease in stearic acid (18:0) in all dietary groups except the 1.5 g EPA+DHA group from 0 to 6 months. There was a significant change in the proportion of di-homo-γ-linoleic acid (20:3 *n*-6) in the placebo group, where there was a significant increase in this fatty acid from 3 to 6 months of the intervention period. There was a decrease in the proportion of 22:0 in the 5.0 g α-LNA group over the 6 months of intervention, and a decrease in 20:1 *n*-6 in this and the 0.7 g EPA+DHA groups from 0 to 6 months. There were no significant changes in any of the other fatty acids measured.

Changes in the Ratios of the Different Types of Fatty Acids

There were no differences in the ratio of SFA to PUFA in the plasma PL of subjects consuming the different treatments at any time during the intervention period (*Table 4.14.*). There was no significant change in the proportion of *n*-6 to *n*-3 PUFA in the placebo or α-LNA treatment groups from 0 to 6 months, although there was a decrease at 3 months in both the α-LNA groups. There was a significant decrease in the ratio of *n*-6 to *n*-3 PUFA for the EPA+DHA treatment groups over the 0 to 6 month intervention and this was significant for the 1.5 g EPA+DHA group.

Table 4.14. The ratio of several types of fatty acids in plasma phospholipids

Treatment	Time (m)	SFA/PUFA	*n*-6 PUFA/ *n*-3 PUFA	AA/EPA
Placebo	0	1.01 ±0.03	6.09 ±0.40	14.8 ±2.46
	3	1.13 ±0.06	6.68 ±0.54	11.7 ±2.20
	6	1.01 ±0.05	6.08 ±0.45	12.1 ±2.50
5.0 g α-LNA	0	1.03 ±0.02	6.12 ±0.27	13.7 ±2.35 [bc]
	3	1.02 ±0.04	5.44 ±0.39	9.14 ±2.17 [a]
	6	0.96 ±0.02	6.93 ±0.71	9.87 ±2.50 [a]
10.0 g α-LNA	0	1.04 ±0.03	6.75 ±0.33	15.2 ±2.16 [b]
	3	1.01 ±0.03	5.06 ±0.38	10.7 ±3.50 [a]
	6	0.99 ±0.03	6.69 ±1.31	12.7 ±3.84
0.7 g EPA+DHA	0	1.04 ±0.02	7.13 ±0.46	14.5 ±2.08
	3	0.98 ±0.02	5.41 ±0.45	16.4 ±4.17
	6	0.98 ±0.03	6.37 ±0.60	15.4 ±3.66
1.5 g EPA+DHA	0	1.02 ±0.03	6.08 ±0.34 [c]	10.7 ±1.55
	3	1.12 ±0.05	4.87 ±0.54	12.9 ±2.93
	6	0.99 ±0.03	4.93 ±0.56 [a]	13.7 ±3.55

Significant differences are expressed as:
a. Significantly different from 0 month
b. Significantly different from 3 month
c. Significantly different from 6 month

There were significant differences in the AA/EPA ratio (*Table 4.14.*). This ratio was decreased in both of the α-LNA treatment groups at 3 and 6 month compared to the baseline measurement and was significant at 3 months in both groups. There was no significant change in the ratio of AA and EPA in the EPA+DHA treatment groups, although oddly there was a tendency for this ratio to increase. In the placebo group there was a decrease in the AA/EPA ratio but this was not as great as for the α-LNA groups.

There were no differences in any of these ratios between males and females at any time or with any treatment.

4.3.5.1. Gender Differences in the Incorporation of Fatty Acids into Plasma Phospholipid

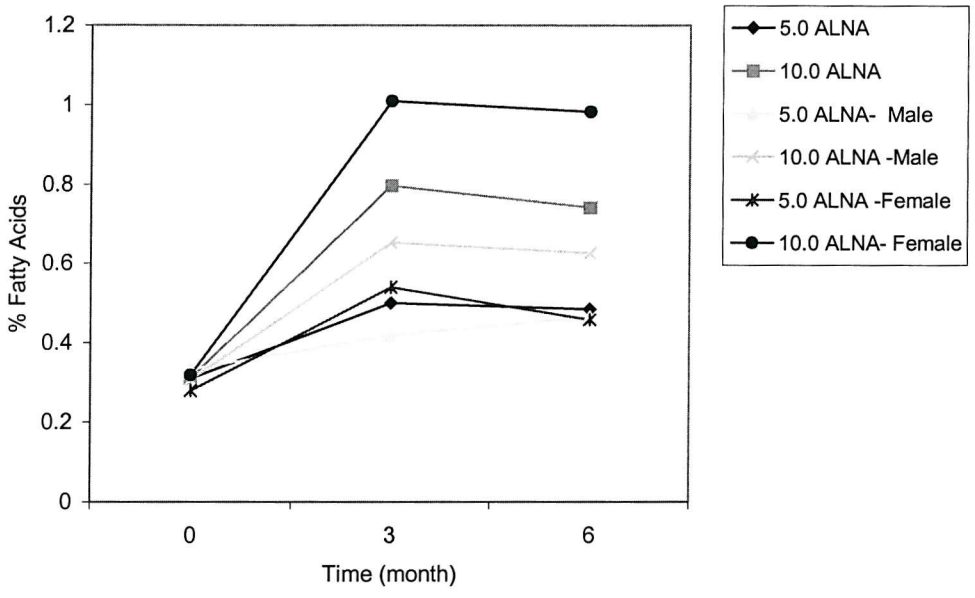
Statistical analyses established a difference between males and females in the modulation of plasma PL by the intervention treatments.

There were several differences between males and females, with respect to the incorporation of the fatty acids of interest (α-LNA, AA, EPA and DHA) during the intervention period. This has implications with respect to the biosynthesis of fatty acids and their incorporation into the lipid pools of the body.

α-Linolenic Acid

There were differences in the incorporation of α-LNA into the plasma PL according to gender (*Figure 4.8.*). The proportion of this fatty acid in plasma PL was greater for women compared to men when they were provided with 10.0 g α-LNA per day. The difference in incorporation was significant at 6 months (P=0.039 at 6 months) but not at 3 months. The incorporation into plasma PL for the 5.0 g α-LNA treatment group tended to be different for men compared to women at 3 months but was not significant (P=0.098). Any difference in incorporation was negligible at 6 month of intervention for the 5.0 g α-LNA treatment group. When these data were calculated as a change in α-LNA proportion from 0 to 6 months again there was a significant difference between males and females in the 10.0 g α-LNA/day group (P=0.044).

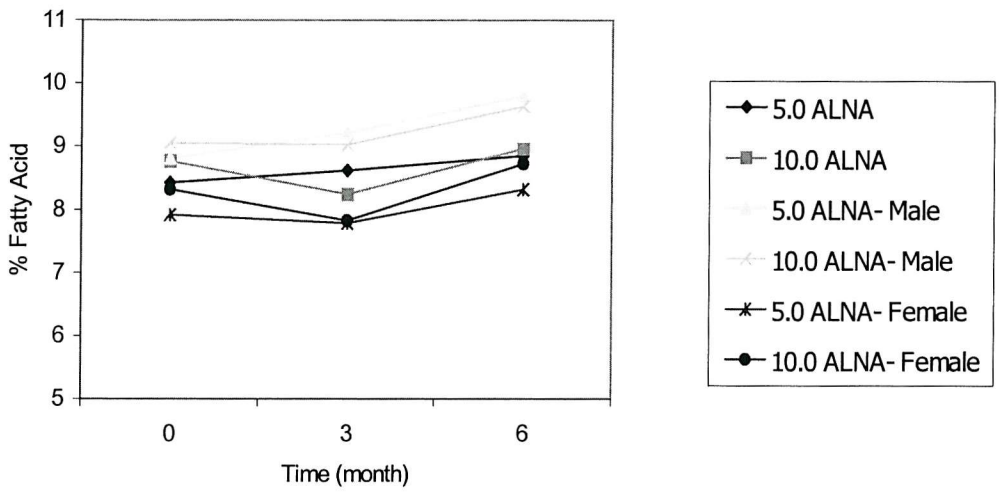
Figure 4.8. Gender differences in incorporation of α-LNA into plasma PL after α-LNA intervention



Arachidonic Acid

There were no significant differences in the AA content of the plasma PL between genders either before or after the intervention with EPA+DHA. However, there were significant differences in the content of this fatty acid between men and women after intervention with α-LNA (*Figure 4.9.*). There was a significant difference in the AA content at 3 months for both doses of α-LNA (P=0.021 for 5.0 g α-LNA; P=0.045 for 10.0 g α-LNA), while at 6 months there was only a significant difference for the 5.0 g α-LNA group (P=0.047). Women had less AA in plasma PL than men. However, when these differences in content of AA were considered as a change in content from 0 to 6 months there was no difference between men and women.

Figure 4.9. Gender differences in incorporation of AA into plasma PL after α-LNA intervention



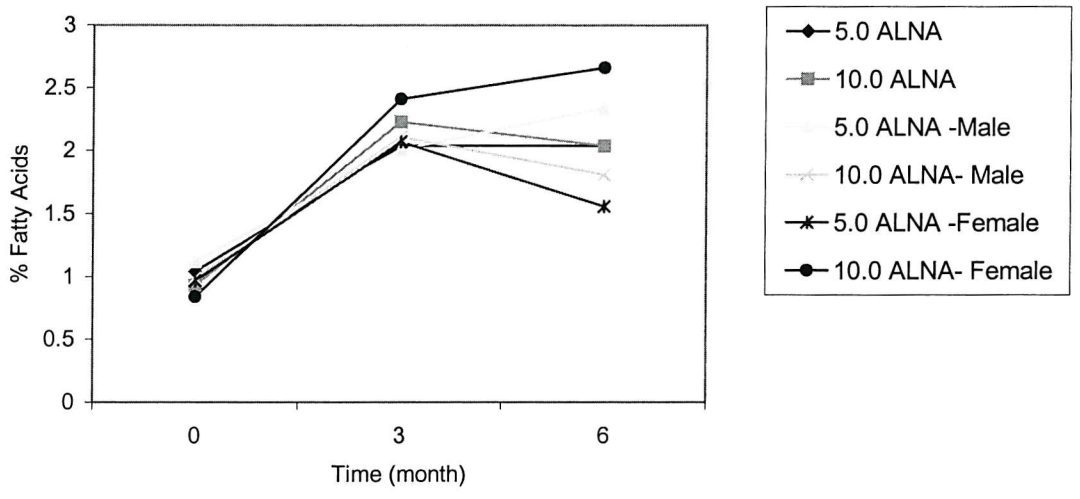
Eicosapentaenoic Acid

The incorporation of EPA into plasma PL after EPA+DHA intervention was increased to the same extent at 3 and 6 months for both EPA+DHA groups in both genders. There were no differences between males and females.

The difference in incorporation of EPA into plasma PL for the α-LNA treatment groups was more marked between males and females. There was a similar increase in content of EPA in both α-LNA treatment groups between 0 and 3 months. However, at 6 months of intervention there was a divergence in the content of EPA between the 2 genders with females having a greater increase in EPA content compared to males after intervention with 10.0 g α-LNA (*Figure 4.10.*). This implies that there may be a difference in the capacity to elongate α-LNA and then to incorporate EPA between the two genders. When the change in EPA content between 0 and 6 months was calculated after α-LNA intervention there was a tendency for the increase to be significantly different between males and females for the 5.0 g α-LNA treatment ($p=0.067$) with males having greater incorporation than females. There was also a

significant difference for the 10.0 g α-LNA treatment (p=0.041), but with females having greater incorporation compared to males (*Figure 4.10.*).

Figure 4.10. Gender differences in incorporation of EPA into plasma PL after α-LNA intervention.



Docosahexaenoic Acid

There were no differences in the incorporation of DHA into plasma PL between the different genders at 0, 3 or 6 months. However, when the change in DHA content from 0 to 6 months was calculated there was a significant difference in incorporation between men and women in the 1.5 g EPA+DHA intervention group (p=0.040): males incorporated DHA into plasma PL to a lesser extent than females.

4.3.6. Modulation of PBMNC Phospholipid Fatty Acid Composition

There were no differences between the treatment groups in the proportions of any of the fatty acids in PBMNC PL at baseline (*Table 4.15*; *Table 4.16*).

Two-way ANOVA established that there was a significant effect of treatment on the proportions of EPA ($P<0.001$), AA/EPA ratio ($P<0.001$) and DHA ($P<0.007$) and a significant effect of time on the proportions of AA ($P<0.0001$), EPA ($P<0.001$) and DHA ($P<0.01$). The effects of treatment and time were investigated further.

There were significant effects of each of the treatments and of time on the fatty acid composition of PBMNC PL when one-way ANOVA were performed (*Table 4.15.* and *Table 4.16.*).

The Changes in the Absolute Composition of the Fatty Acids of Interest in PBMNC PL

There were changes in the proportions of the fatty acids of interest AA, EPA and DHA (*Table 4.15.*) and these are represented graphically in *Figures 4.11.-4.13.*

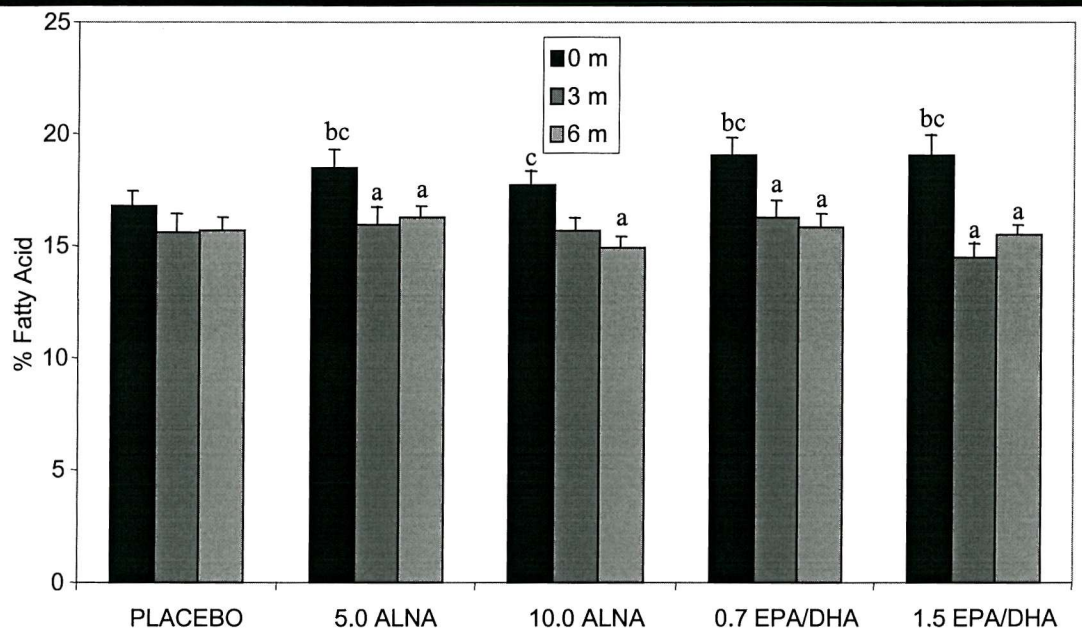
α-Linolenic Acid

There were no changes in the proportion of α-LNA after intervention with this fatty acid at either dose of treatment. There was also no change in the proportion of α-LNA in any of the other treatment groups over the intervention period.

Arachidonic Acid

There was a significant decrease in the content of AA in all treatment groups from 0 to 6 months of intervention, except in the placebo group, where the decrease did not reach significance (*Figure 4.11.*). There appeared to be no significant effect of the dose of EPA+DHA consumed, while the 10.0 g α-LNA treatment resulted in a greater decrease in AA than did the 5.0 g α-LNA treatment. The reduction in AA was maximal at 3 months in all treatment groups.

Figure 4.11. The proportion of AA in PBMNC PL in the different treatment groups

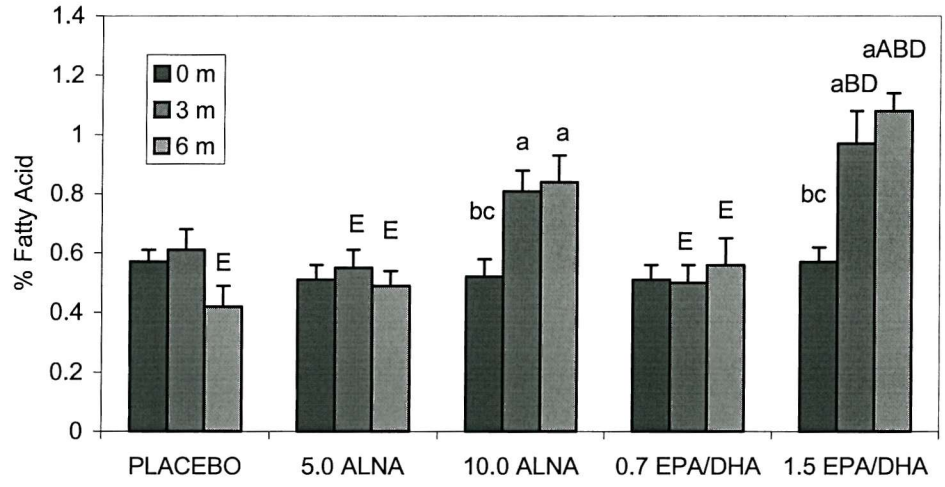


Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;

Eicosapentaenoic Acid

There was a significant increase in EPA at 3 months in the 1.5 g EPA+DHA group (95 % increase) and this level of EPA enrichment was also seen at 6 months (*Figure 4.12.*). There was also a significant increase in the content of EPA in the 10.0 g α-LNA group (75 % increase) and this change was also maximal at 3 months. There was no change in the EPA content for any of the other treatment groups including the 0.7 g EPA+DHA group. Thus, there appears to be an effect of dose of EPA and α-LNA consumed on the content of EPA in PBMNC PL.

Figure 4.12. The proportion of EPA in PBMNC PL in the different treatment groups



Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;
A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

Docosapentaenoic Acid

There were no differences in the content of DPA in any of the treatment groups over time.

Docosahexaenoic Acid

There was a non-significant increase (25%) in the content of DHA in the 1.5 g EPA+DHA treatment group, while there was no change in DHA content in the 0.7 g EPA+DHA group. Thus, there appears to be an effect of the dose of DHA consumed on the incorporation of this fatty acid into PBMNC PL (*Figure 4.13.*).

Table 4.15. The fatty acid composition of PBMNC phospholipids at different times of dietary intervention (n = 150)

Treatment	Time (m)	α18:3 *n*-3	20:4 *n*-6	20.5 *n*-3	22:5 *n*-3	22:6 *n*-3
Placebo	0	0.30 ±0.05	17.54 ±0.63	0.57 ±0.04	3.25 ±0.42	2.76 ±0.15
	3	0.25 ±0.04	15.62 ±0.83	0.61 ±0.07	3.19 ±0.30	2.37 ±0.24
	6	0.24 ±0.05	15.68 ±0.62	[E]0.42 ±0.07	2.47 ±0.19	[E] 2.27 ±0.27
5.0 g α-LNA	0	0.28 ±0.05	18.50 ±0.81 [bc]	0.51 ±0.05	3.49 ±0.39	3.22 ±0.24
	3	0.24 ±0.04	15.91 ± 0.84 [a]	[E] 0.55 ±0.06	2.82 ±0.15	2.37 ±0.18
	6	0.24 ±0.03	16.23 ±0.51 [a]	[E] 0.49 ±0.05	3.72 ±0.24	[E] 2.27 ±0.14
10.0 g α-LNA	0	0.25 ±0.05	17.67 ±0.69 [c]	0.62 ±0.06 [bc]	3.22 ±0.44	2.52 ±0.21
	3	0.31 ±0.04	15.64 ±0.62	0.81 ±0.07 [a]	3.29 ±0.26	[E] 2.11 ±0.18
	6	0.24 ±0.04	14.89 ±0.53 [a]	0.84 ±0.08 [a]	3.18 ±0.33	[E] 1.97 ±0.11
0.7 g EPA+DHA	0	0.27 ±0.03	19.07 ±0.73 [bc]	0.51 ±0.05	2.81 ±0.35	2.88 ±0.21
	3	0.24 ±0.04	16.27 ±0.73 [a]	[E] 0.50 ±0.06	2.77 ±0.17	2.83 ±0.15
	6	0.34 ±0.06	15.88 ±0.55 [a]	[E] 0.56 ±0.09	2.32 ±0.25	2.74 ±0.16
1.5 g EPA+DHA	0	0.22 ±0.05	19.10 ±0.86 [bc]	0.57 ±0.05 [bc]	2.92 ±0.44	2.64 ±0.29
	3	0.26 ±0.04	14.51 ±0.68 [a]	[BD] 0.97 ±0.11 [a]	3.05 ±0.28	[C] 3.04 ±0.22
	6	0.22 ±0.03	15.52 ±0.41 [a]	[ABD] 1.08 ±0.06 [a]	2.67 ±0.28	[ABD] 3.27 ±0.20

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

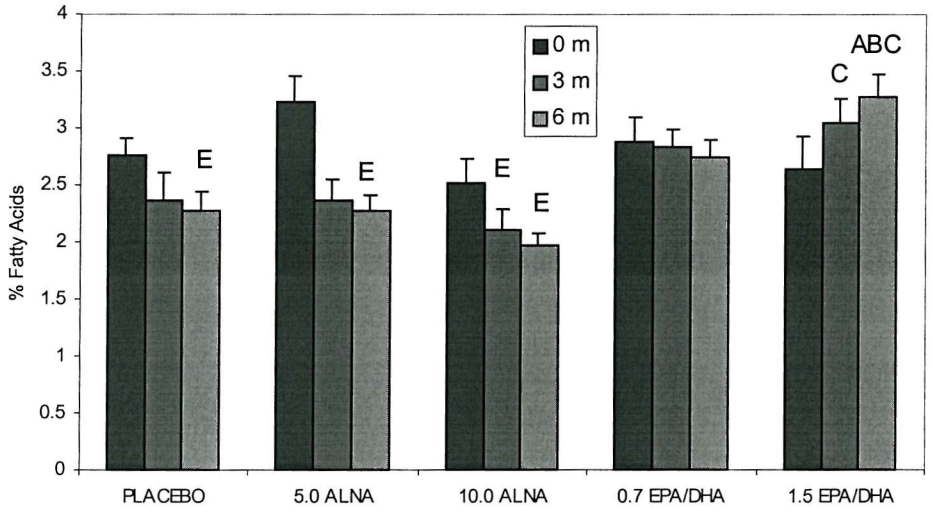
Table 4.16. The composition of PBMNC phospholipids of the remaining fatty acids measured (n = 150)

Treatment	Time (m)	16:0	18:0	18:1 *n*-9	18:2 *n*-6	20:3 *n*-6	22:0
Placebo	0	17.62 ±0.71	20.69 ±0.69	17.34 ±1.12	8.57 ±0.28	2.14 ±0.13	0.74 ±0.11
	3	19.31 ±0.65	20.04 ±0.61	17.62 ±0.80	9.03 ±0.23	1.89 ±0.15	0.83 ±0.14
	6	18.70 ±0.71	20.71 ±0.96	19.42 ±1.25	8.26 ±0.32	1.86 ±0.11	0.70 ±0.07
5.0 g α-LNA	0	16.55 ±0.59 [bc]	20.76 ±0.67	17.34 ±1.10	9.23 ±0.27	2.16 ±0.15	0.82 ±0.09
	3	19.26 ±0.69 [a]	20.14 ±0.65	17.13 ±0.84	9.21 ±0.27	1.99 ±0.19	0.60 ±0.05
	6	19.15 ±0.77 [a]	20.65 ±0.70	16.40 ±0.48	8.50 ±0.32	1.89 ±0.14	0.69 ±0.09
10.0 g α-LNA	0	17.66 ±0.75	19.78 ±0.59	17.57 ±1.01	9.28 ±0.44 [c]	2.14 ±0.12	0.87 ±0.12
	3	19.05 ±0.91	20.76 ±0.64	18.48 ±1.01	8.74 ±0.28	1.87 ±0.13	0.47 ±0.06
	6	20.24 ±0.79	22.17 ±0.77	17.24 ±0.80	[E]7.76 ±0.39 [a]	1.73 ±0.13	0.71 ±0.15
0.7 g EPA+DHA	0	16.31 ±0.63 [bc]	21.48 ±0.70	17.56 ±0.96	8.72 ±0.29	2.18 ±0.14	0.85 ±0.14
	3	18.95 ±0.71 [a]	21.21 ±0.57	17.24 ±0.99	9.26 ±0.23	1.87 ±0.14	0.57 ±0.07
	6	20.26 ±0.82 [a]	21.69 ±0.57	15.76 ±1.09	8.50 ±0.32	1.89 ±0.11	0.71 ±0.11
1.5 g EPA+DHA	0	18.21 ±1.79	21.34 ±0.90	17.15 ±0.83	9.21 ±0.29	2.11 ±0.19	1.07 ±0.19
	3	19.61 ±0.65	20.32 ±0.60	18.30 ±0.91	9.33 ±0.32	1.82 ±0.11	0.80 ±0.11
	6	19.97 ±0.62	20.79 ±0.50	16.82 ±0.63	[C]9.29 ±0.25	1.85 ±0.08	0.78 ±0.14

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

There were no significant changes in the composition of the placebo group, although there was a decrease in the DHA content in both of the α-LNA treatment groups. The content of DHA was also significantly different at 3 and 6 month of intervention between the α-LNA and placebo treatment groups compared to the 1.5 g EPA+DHA group.

Figure 4.13. The proportion of DHA in PBMNC PL in the different treatment groups



Significant differences are expressed as:
A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

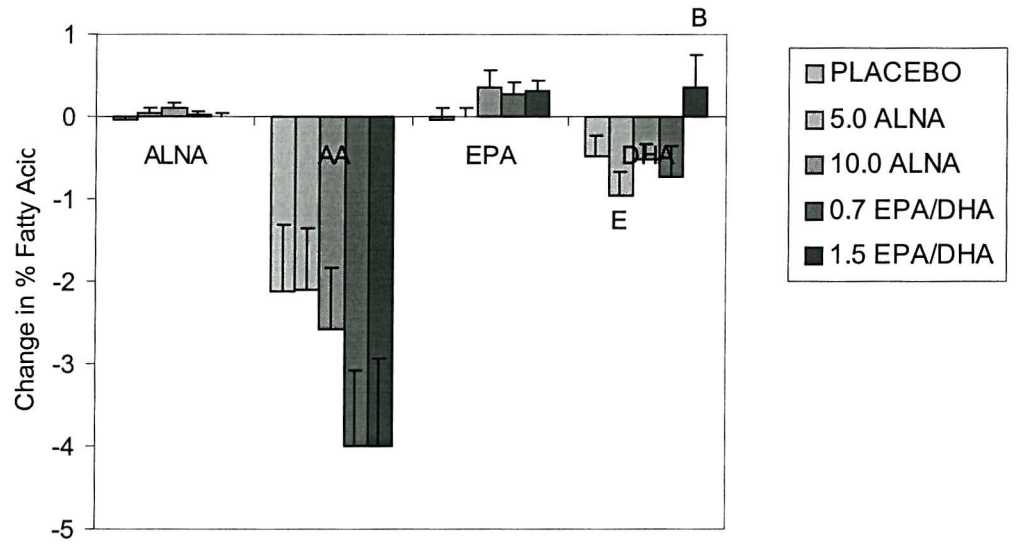
The Overall Changes from 0 to 6 Months in the Composition of the Fatty Acids of Interest

Changes in the % Composition of the Fatty Acids of Interest

There were no significant differences in the content of α-LNA, AA or EPA between any of the treatment groups when the changes in absolute fatty acid content were calculated (*Figure 4.14.*). However, there was a decrease in AA content in all treatment groups with a greater decrease in the EPA+DHA groups. There was an

increase in the mean DHA content only in the 1.5 g EPA+DHA group, and this was significantly different from the 5.0 g α-LNA group.

Figure 4.14. The change in the proportions of α-LNA, AA, EPA and DHA in PBMNC PL in the different treatment groups

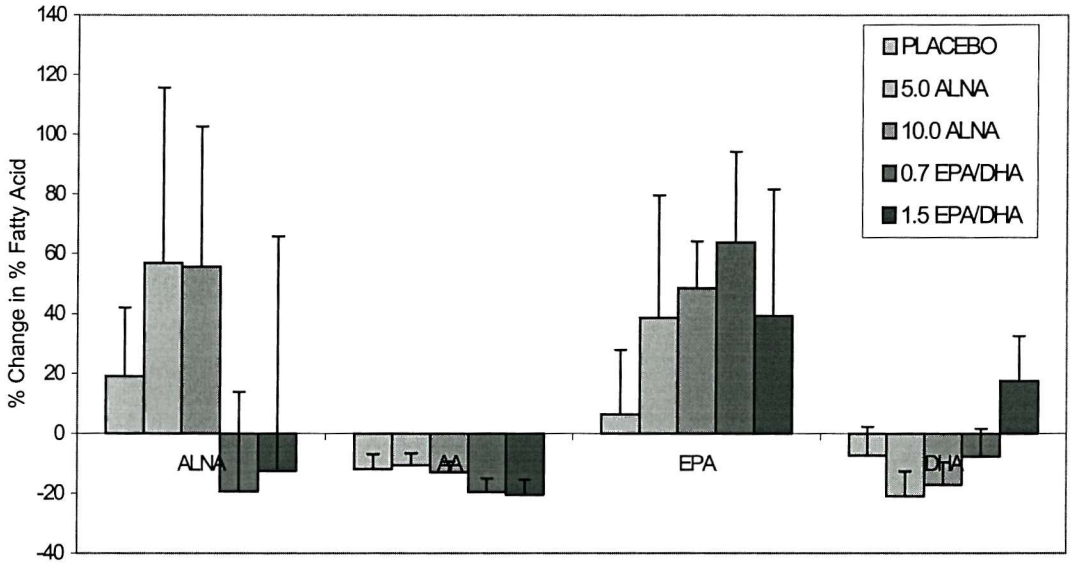


Significant differences are expressed as:
B. Significantly different from the 5.0 g ALNA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

% Change in the % Fatty Acids

There were no significant differences in the % change in the proportion of the fatty acids of interest (*Figure 4.15.*). However, there was a trend for an increase in % of α-LNA for both of the α-LNA treatment groups. There was no effect of the dose of α-LNA and this change was approximately 60% in both groups. The % of EPA increased in both the α-LNA and EPA+DHA groups. There was little effect of dose of α-LNA or EPA on the % increase in EPA in PBMNC PL. The % change in DHA was only positive for the 1.5 g EPA+DHA group (approximately 20%). In all other treatment groups the % change in DHA was negative and was greatest in the α-LNA groups.

Figure 4.15. The % change in the proportions of α-LNA, AA, EPA and DHA in PBMNC PL in the different treatment groups



The Changes in Composition of the Other Fatty Acids Measured

There were some changes in the composition of the other fatty acids measured (*Table. 4.16*). There was an increase in the proportion of palmitic acid (16:0) in all treatment groups over the intervention period. This increase was significant for both the EPA+DHA and the 5.0 g α-LNA treatment groups. The content of linoleic acid (18:2 *n*-6) was significantly decreased in the 10.0 g α-LNA treatment group from baseline to 6 months. There was no change in this fatty acid for any of the other treatment groups. There was a decrease in the content of 20:0 over the intervention period for all groups but this was not significant. There were no changes in the contents of stearic acid (18:0), oleic acid (18:1 *n*-9), di-homo-γ-linoleic acid (20:3 *n*-6) or 22:0.

The Ratios of the Different Types of Fatty Acids

There was a significant increase in the SFA to PUFA ratio in the PBMNC PL for all groups except the 1.5 g EPA+DHA group (*Table 4.17.*). There was a tendency

towards a decrease in the *n*-6 to *n*-3 PUFA ratio in all treatment groups, except the placebo group where there was a non-significant increase from 0 to 6 months. There were no significant differences between males and females in any of these ratios.

The ratio of AA to EPA was significantly decreased in the 1.5 g EPA+DHA and 10.0 g α-LNA treatment groups. The AA/EPA ratio was decreased by about 50% from 0 to 3 months and remained at this level at 6 months of intervention in both these groups. There was no significant change in this ratio for the other three treatments.

Table 4.17. The ratio of several types of fatty acids in PBMNC phospholipids

Treatment	Time (m)	SFA/PUFA	*n*-6 PUFA/ *n*-3 PUFA	AA/EPA
Placebo	0	1.09 ±0.05 [c]	4.21 ±0.28	31.58 ±3.05
	3	1.18 ±0.08	4.16 ±0.36	26.69 ±4.59
	6	1.33 ±0.06 [a]	5.40 ±0.44	33.75 ±3.12
5.0 g α-LNA	0	1.07 ±0.05[c]	5.17 ±0.47	36.79 ±3.71
	3	1.25 ±0.06	4.64 ±0.21	40.01 ±7.67
	6	1.29 ±0.05 [a]	5.07 ±0.30	[E]41.45 ±7.07
10.0 g α-LNA	0	1.10 ±0.06 [c]	5.40 ±0.61	42.41 ±3.52 [bc]
	3	1.34 ±0.08	4.32 ±0.35	22.51 ±2.05 [a]
	6	[E]1.49 ±0.09 [a]	4.59 ±0.39	24.43 ±3.74 [a]
0.7 g EPA+DHA	0	1.08 ±0.04 [c]	5.12 ±0.41	40.78 ±3.90
	3	1.26 ±0.07	4.72 ±0.32	40.03 ±6.44
	6	1.34 ±0.04 [a]	4.77 ±0.35	[E]42.31 ±6.61
1.5 g EPA+DHA	0	1.09 ±0.06	5.24 ±0.56	36.56 ±2.78 [bc]
	3	1.34 ±0.09	3.79 ±0.26	20.21 ±1.32 [a]
	6	[C]1.19 ±0.06	4.61 ±0.29	[AD] 14.95 ±0.84 [a]

Significant differences are expressed as:
a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

4.3.6.1. Gender Differences in the Incorporation of Fatty Acids into PBMNC Phospholipid

In the treatment groups where a change in the composition of the fatty acids of interest (AA and EPA, but not DHA) was observed there were also differences in these changes between men and women.

Arachidonic Acid

The content of AA in the 10.0 g α-LNA treatment group (35% decrease) was found to be different between men and women (p=0.034), at 6 months of intervention. Females had less AA at 6 months intervention compared to males but not at baseline. This difference was not seen in the 1.5 g EPA+DHA group where there was also a significant decrease in the content of AA (25 % decrease). When the change in AA content from 0 to 6 months was considered there was found to be no difference in the decrease in AA by gender according to treatment group.

Eicosapentaenoic Acid

The 1.5 g EPA+DHA group saw the most marked increase in EPA content (100% increase) from 0 to 6 months and content of EPA observed at 6 months tended to be different between men and women (p=0.080). Women had a greater content of EPA than men at 6 months but not at baseline. The content of EPA at 6 months in the 10.0 g α-LNA treatment group was not found to be different between men and women (p=0.303). However, the increase in EPA content for this group was less marked (60% increase). Again when the change in EPA content was calculated there was no difference in the incorporation of EPA between male and females for either the 1.5 g EPA+DHA or 10.0 g α-LNA treatment groups.

4.4. Discussion

4.4.1. Characteristics of the Subjects, Compliance and Analysis of Intakes of Energy and Nutrients

There was a small increase in body weight in each of the treatment groups from 0 to 6 months. However, there was no difference in weight gain between any of the groups. The increase in weight in all groups may be due to the observed increase in energy derived from fat in the diet over the intervention period in all groups including the placebo. This phenomenon has also been observed in a similar intervention study where 1.4 g EPA+DHA/day (0.9 g EPA + 0.5 g DHA/day) was consumed for 22 days in specially manufactured food (Lovegrove *et al.*, 1997). As

in the study of Lovegrove *et al.* (1997) the nature of the food provided in this study as the *n*-3 PUFA vehicle, may have contributed to the higher energy intake and increase in body weight observed. The margarine was 85 % fat and the amount consumed (25 g) was greater than that normally consumed by these subjects.

4.4.2. Changes in Fatty Acid Composition

The changes in the composition of the fatty acids of interest (LA, α-LNA, AA, EPA, DPA and DHA) are summarised in *Table 4.18*. This table shows that the changes in the composition of the diet were not always reflected in the compositions of both plasma and PBMNC PL. The marker of an increased supply of *n*-3 PUFA in the diet is a change in fatty acid composition of the target cell or tissue and changes in cell function are most likely related to changes in cell composition. Thus, it is surprising how few studies which report the effects of *n*-3 PUFA on human immune cell function do not also report the fatty acid composition of the plasma and cells under study. There are several studies with *n*-3 PUFA which do report the fatty acid composition of plasma and PBMNC PL and the results of the present investigation will be compared to these, and where appropriate other studies which have investigated the effects of dietary *n*-3 PUFA on changes in fatty acid composition.

Increased Consumption of α-Linolenic Acid

After intake of 5.0 g and 10.0 g α-LNA/day there was a dose-dependent increase in the α-LNA content of plasma PL, and this was maximal after 3 months of intervention. An increase in α-LNA was not observed in the PBMNC PL with either dose of α-LNA. This suggests that this fatty acid does not enrich all PL pools in the body to the same extent.

There were also increases in the EPA content of plasma PL after α-LNA intervention. This enrichment was the same for both doses of α-LNA and was maximal at 3 months of intervention. This suggests that dietary α-LNA is at some point elongated (probably in the liver) to EPA and carried in lipoproteins (in the PL fraction at least) around the body. The increase in EPA in the PBMNC PL after 10.0

g α-LNA/day suggests that this PL pool is subject to a modulation in EPA content after consuming 10.0 g α-LNA (but not 5.0 g α-LNA) in the diet. This indicates that dietary α-LNA, above a certain dose, can act as a source of EPA for some cell types. DPA is an intermediary fatty acid in the elongation of EPA to DHA (see *Figure 1.10*). There were changes in the content of this fatty acid in some lipid pools after the α-LNA interventions. There was a significant increase in DPA content in plasma PL after the 10.0 g/day α-LNA intervention. This change indicates that there is the capacity for conversion of α-LNA to DPA (via EPA) after consuming increased amounts of α-LNA in the diet, and that the DPA synthesised can enrich the PL fraction of plasma. This conversion is dependent on the dose of α-LNA supplied and this study shows that a 5.0 g dose of α-LNA is insufficient to provide enough substrate for conversion. However, there was no increase in the content of DPA in PBMNC PL after α-LNA interventions and so the enrichment with DPA synthesised from α-LNA, is different in different lipid pools.

It is notable that there was no increase in the content of DHA, the next fatty acid in the elongation pathway, in either of the PL fractions investigated, after α-LNA intervention. This suggests that although α-LNA can be elongated to DPA it cannot be further elongated to DHA. In fact, the content of DHA in PBMNC PL decreased after α-LNA intervention. This suggests that there is some inhibition of the synthesis of DHA and its incorporation into these cells when individuals consume much larger amounts of α-LNA in the diet than at the current UK intake of 2.0 g/d, which was that consumed in the placebo group

Table 4.18 Summary of the changes in composition of the fatty acids of interest in plasma and PBMNC PL and ratios of fatty acids between 0 and 6 month

	LA	**α-LNA**	**AA**	**EPA**	**DPA**	**DHA**	**SFA/PUFA**	***n*-6/*n*-3**	**AA/EPA**
Plasma PL									
Placebo	↔	↔	↑	↔	↔	↔	↔	↔	↓
5.0 g α-LNA	↔	↑ *	↑	↑ * F>M	↔	↔	↔	↔	↓ *
10.0 g α-LNA	↔	↑↑ * F>M	↑	↑ * F>M	↑ *	↔	↔	↔	↓ *
0.7 g EPA+DHA	↔	↔	↑	↑	↑ *	↑ * F>M	↔	↓ *	↔
1.5 g EPA+DHA	↔	↔	↓ F>M	↑	↔	↑↑ *	↔	↓ *	↔
PBMNC PL									
Placebo	↔	↔	↓	↔	↔	↔	↑ *	↑	↔
5.0 g α-LNA	↔	↔	↓ * F>M	↔	↔	↓ *	↑ *	↔	↔
10.0 g α-LNA	↓ *	↔	↓ * F>M	↑ * F>M	↔	↓ *	↑ *	↓	↓↓ *
0.7 g EPA+DHA	↔	↔	↓ * F>M	↔	↔	↔	↑ *	↓	↔
1.5 g EPA+DHA	↔	↔	↓ * F>M	↑↑ * F>M	↔	↑ *	↑	↓	↓↓ *

*M = Male; F = Female; * = significant change in composition*

Comparison with the Literature

The changes in the content of α-LNA in plasma PL seen in this study were similar to those seen in several other studies where α-LNA was supplied through foodstuffs, mainly in margarines and cooking oils, enriched in linseed oil. After a dose of 9.2 g α-LNA/day was consumed for 2 weeks (Mantzioris *et al.*, 2000) or a dose of 15.4 g α-LNA/day (but not 3.7 g α-LNA/day) was consumed for 4 weeks (Li *et al.*, 1999) there was a marked increase (3- to 7-fold) in the α-LNA content of plasma PL. By comparison the increase in the current study at 3 months was 1-fold for 5 g α-LNA/day and 2-fold for 10.0 g α-LNA/day. A positive correlation between the α-LNA content of the diet and the level of α-LNA in plasma PL after 13 g α-LNA/day has also been reported (Mantzioris *et al.*, 1995).

In the studies of Kelley *et al.* (1993), Caughey *et al.* (1996) and Mantzioris *et al.* (2000) there were 3- to 5-fold increases in the α-LNA content of PBMNC PL after providing extra α-LNA in the diet. However, the dose of α-LNA used in these studies was greater than that in the present study (13.7 g α-LNA/day (Caughey *et al.*, 1996) and 18 g α-LNA/day (Kelley *et al.*, 1993)) although Mantzioris *et al.* (2000) used a similar dose to that used here (9.2 g α-LNA/day). The larger doses used in the former studies compared to the current study could explain the absence of an increase in α-LNA in PBMNC PL in the present study. It may also be that the greater content of α-LNA in PBMNC PL at baseline compared to that in the earlier studies, especially that of Mantzioris *et al.* (2000), may also contribute to the differences between this study and others. In the latter study the content of α-LNA in PBMNC PL was approximately 0.02 % of total fatty acids at baseline compared to 0.2 % of total fatty acids in the present study. This may reflect the nature of the habitual diets in the UK and Australia where the Mantzioris *et al.* (2000) study was conducted. The Australian diet is composed of a different fatty acid composition compared to the UK diet (Cashel *et al.*, 1994; Mann *et al.*, 1995).

The increase in EPA in plasma PL seen after intervention with both doses of α-LNA in the present study was also observed in several of the other studies already cited. Providing 13.7 g α-LNA/day (Mantzioris *et al.*, 1994), 15.4 g α-LNA/day (but not

3.7 g α-LNA/ day) (Li *et al.*, 1999) or 9.2 g α-LNA/day (Mantzioris *et al.*, 2000) also considerably increased (2- to 3-fold) the EPA content of plasma PL. In comparison in the current study there was a 0.5- to 1-fold increase in plasma PL EPA after 5 g or 10 g α-LNA/day. A positive correlation between the α-LNA content of the diet and plasma PL content of EPA after 13 g α-LNA/day has also been reported (Mantzioris *et al.*, 1995). As in this study after α-LNA intervention there was an increase (2-fold) in the EPA content of PBMNC PL. This was seen at doses of 13.7 g α-LNA/day (Caughey *et al.*, 1996) and 9.2 g α-LNA/day (Mantzioris *et al.*, 2000). A dose of 4.1 g α-LNA/day resulted in a small increase in EPA in neutrophil PL (Healy *et al.*, 2000). The content of α-LNA in neutrophil PL was not reported in this last study.

The changes in DPA and DHA observed in this study in both plasma PL and PBMNC PL have also been reported in several other α-LNA intervention studies. A 50% increase in DPA in plasma PL has been seen after intervention with 15.4 g α-LNA/day (Li *et al.*, 1999). This level of enrichment was seen in the current study after intervention with 10.0 g α-LNA/day. An increase in DPA in PBMNC PL was seen after 18 g α-LNA (Kelley *et al.*, 1993), while there was no increase in DPA observed in the present study and this is most likely due to the different doses of α-LNA used in that a higher level of α-LNA than 10.0 g/day is required to enrich DPA in PBMNC PL. The content of DPA was not been reported in any other studies of α-LNA intervention. The content of DHA was unchanged in plasma PL (Mantzioris *et al.*, 1994; Li *et al.*, 1999) as seen in this study, although a negative correlation between the α-LNA content of the diet and the DHA content of plasma PL has also been reported (Mantzioris *et al.*, 1995). The DHA content in PBMNC PL was also either unchanged (Caughey *et al.*, 1996) or decreased (by approximately 30%) (Kelley *et al.*, 1993) after α-LNA intervention. This agrees with the present study where the DHA content of PBMNC PL decreased by approximately 30% after both doses of α-LNA. The maximum duration of the studies cited here was 4 weeks, except that of Kelley *et al.* (1993) which was 8 weeks. It was proposed that the elongation of α-LNA to DHA is an extremely slow process and that this accounted for the absence of an increase in DHA with α-LNA intervention (Li *et al.*, 1999).

However, the current study, which was 6-times as long as those cited, suggests that this is not the case and that there is inhibition at some stage in the elongation process (probably at the conversion of DPA to DHA).

Increased Consumption of Eicosapentaenoic Acid

The increase in EPA in plasma PL occurred to the same extent at both levels of EPA+DHA intervention and was maximal at 3 months. This suggests that even at a dose of 0.26 g EPA/day (supplied in the 0.7 g EPA+DHA/day treatment) this lipid pool can be enriched in this fatty acid. However, there was only an increase in EPA content in PBMNC PL after EPA+DHA intervention at a dose of 0.59 g EPA/day (supplied in 1.5 g EPA+DHA/day treatment). This indicates that there is an effect of dose on the enrichment of EPA in PBMNC PL.

There was also a change in DPA content in plasma PL after EPA+DHA intervention. This suggests that the EPA consumed in the diet can be elongated to DPA. Whether this DPA is further elongated to DHA cannot be determined in this study since the presence of DHA in the EPA+DHA intervention could be responsible for the enrichment of DHA seen in this lipid pool.

Comparison with the Literature

Changes in the content of EPA after EPA+DHA or fish oil interventions have been observed in several studies. A dose-dependent increase in EPA in plasma PL was observed after providing 1.5 g, 3 g or 6 g of fish oil/day for 12 weeks (Blonk *et al.*, 1990). Providing 2.1 g EPA/day for 12 weeks (Yaqoob *et al.*, 2000) or 0.72 g EPA/day for 12 weeks (Thies *et al.*, 2001a) also resulted in an increased content (2- to 10-fold depending on EPA dose) of EPA in plasma PL. In the present study the increase in EPA was approximately 2-fold. Thus, the effect of 0.5 g EPA/day in the current study is very similar to that observed for 0.72 g EPA/day by Thies *et al.*, (2001a).

Immune cells readily increase their content of EPA after providing EPA in the diet in capsules. Increases of between 1- and 20-fold have been observed in a number of cell types and lipid fractions after EPA doses ranging from 0.34 to 9.4 g/day (Lee *et al.*,

1985; Endres *et al.,* 1989; Fisher *et al.,* 1990; Molvig *et al.,* 1991; Sperling *et al.,* 1993; Gibney and Hunter, 1993; Caughey *et al.,* 1996; Schmidt *et al.,* 1996; Kelley *et al.,* 1999; Yaqoob *et al.,* 2000; Healy *et al.,* 2000; Thies *et al.,* 2001a). The current study found a 1-fold increase after providing 0.59 g EPA/day. Since 0.26 g EPA did not result in a change in the content of EPA in PBMNC PL, but a dose of 0.34 g EPA/day was enough to elicit an increase in the EPA content of monocytes (Schmidt *et al.,* 1993), further investigation into the lowest dose of EPA required to modulate the composition of the PL of specific cell types is needed. These earlier studies concur that a change in EPA content is achieved at 4 weeks of intervention, and for those studies which had a time course beyond 4 weeks, the changes observed were maximal or near-maximal at 4 weeks.

Few studies have performed interventions with EPA through foodstuffs as opposed to capsules. Those that have used *n*-3 PUFA-enriched foodstuffs have used consumables such sausages and savoury dips (Mantzioris *et al.,* 2000) or other specially manufactured foods (Lovegrove *et al.,* 1997). Both of these studies were investigating the effects of *n*-3 PUFA on biochemical changes in the body other than immune function and so the fatty acid composition of PBMNC was not investigated. However, changes in plasma PL were reported and concur that consuming foodstuffs enriched with EPA+DHA rather than consuming these fatty acids in capsules still results in the enrichment (2- to 3-fold) of plasma PL with EPA. The bioavailability of EPA from microencapsulated fish oil-enriched foods has also been shown not to differ from that of traditional fish oil capsules (Wallace *et al.,* 2000b).

Increased consumption of Docosahexaenoic Acid

There was an effect of the dose of DHA (+EPA) on the enrichment of DHA in plasma PL. There was a greater increase in the content (3-fold) of DHA after 0.91 g DHA/day (supplied in the 1.5 g EPA+DHA/day treatment) than with the 0.44 g DHA/day (supplied in the 0.7 g EPA+DHA/day treatment) where the increase was 2-fold.

This effect of DHA dose was also seen in the PBMNC PL where there was an approximate 20% increase in DHA only at 0.91 g DHA/day (supplied in the 1.5 g EPA+DHA/day treatment).

Changes in the content of DHA in plasma PL after EPA+DHA or fish oil interventions have been observed in several studies. A dose-dependent increase in DHA in plasma PL was observed after 1.5 g, 3 g or 6 g of fish oil/day for 12 weeks (Blonk *et al.*, 1990). Providing 1.1 g DHA/day for 12 weeks (Yaqoob *et al.*, 2000) or 0.85 g or 0.40 g of DHA/day for 12 weeks (Thies *et al.*, 2001a) also resulted in a 30 to 80% increase in the content of DHA in plasma PL.

Immune cells do not increase their content of DHA after DHA intervention as readily as they increase EPA content after EPA intervention or as readily as plasma PL increases DHA content after DHA intervention. Increases of between 15 and 80% have been observed in a number of immune cell types and lipid fractions after DHA doses ranging from 0.19 g/day to 6.0 g/day (Lee *et al.*, 1985; Endres *et al.*, 1989; Fisher *et al.*, 1990; Molvig *et al.*, 1991; Sperling *et al.*, 1993; Gibney and Hunter, 1993; Caughey *et al.*, 1996; Schmidt *et al.*, 1996; Kelley *et al.*, 1999; Yaqoob *et al.*, 2000; Healy *et al.*, 2000; Thies *et al.*, 2001a). These studies suggest that a change in DHA content can be achieved, but that this change is not as uniformly distributed over different immune cell types and lipid fractions as EPA enrichment has been found to be.

As with EPA interventions, few studies have been performed using DHA-enriched foodstuffs rather than capsules. Those that have used foodstuffs have used consumables such sausages and savoury dips (Mantzioris *et al.*, 2000) or other specially manufactured foods (Lovegrove *et al.*, 1997) enriched with EPA and DHA. Changes in plasma PL were reported and concur that consuming foodstuffs enriched with EPA+DHA rather than consuming these fatty acids in capsules still results in a 2- to 3-fold enrichment of plasma PL in DHA. The bioavailability of DHA from microencapsulated fish oil-enriched foods has also been shown not to differ from that of fish oil capsules (Wallace *et al.*, 2000b).

Changes in the Composition of Arachidonic Acid

The changes in the content of this fatty acid were quite different in the two pools studied. Suprisingly there was a small increase in AA in plasma PL in all treatment groups except the 1.5 g EPA+DHA/day group where there was a decrease. This may be because of the increased intake of *n*-6 PUFA in all the treatment groups, with the increased *n*-3 to *n*-6 PUFA intake in the 1.5 g EPA+DHA/day offsetting this change and thus resulting in a decrease in the content of *n*-6 PUFA in this treatment group. The PBMNC PL displayed the greatest changes in AA content after intervention. Although there was a small decrease in AA content in the placebo group, this was non-significant, while in all other groups there was a larger, significant decrease in AA in the PL fraction of PBMNC. Thus, it appears that increasing the consumption of *n*-3 PUFA in the diet may have more impact on the composition of some PL pools than others.

Changes in the content of AA in plasma PL after EPA+DHA or fish oil interventions have been observed in several studies. A dose-dependent decrease in AA in plasma PL was observed after 1.5 g, 3 g or 6 g of fish oil/day for 12 weeks (Blonk *et al.*, 1990). Providing 2.1 g EPA + 1.1 g DHA/day for 12 weeks (Yaqoob *et al.*, 2000) also resulted in an decreased content of AA in plasma PL (approximately 20%), while providing 0.72 g EPA + 0.28 g DHA/day for 12 weeks resulted in a very small and non-significant decrease (approximately 5%) in AA content (Thies *et al.*, 2001a). The decrease in AA seen in the group consuming 1.5 g EPA+DHA/day in the current study was not significant (approximately 5%); this is probably due to the low doses of EPA used (0.26 g and 0.59 g/day in the 0.7 g EPA+DHA and 1.5 g EPA+DHA/day treatment groups, respectively).

Decreases in the content of AA in plasma PL after α-LNA intervention have been reported after providing 13.7 g α-LNA/day (Mantzioris *et al.*, 1994), 15.4 g α-LNA/day (but not 3.7 g α-LNA/ day) (Li *et al.*, 1999) and 9.2 g α-LNA/day (Mantzioris *et al.*, 2000) in the diet. As after EPA+DHA intervention, a decrease in AA in plasma PL was not seen in the current study after α-LNA intervention. This is probably due the dose of α-LNA used, which resulted in an enrichment of EPA in plasma PL equal to that seen after the low doses of EPA used in the intervention.

A decrease in AA content in PBMNC PL is observed in most intervention studies with EPA and DHA. The extent of the decrease depends on the dose of EPA provided, the duration of administration, the cell type and the lipid fraction investigated. Providing 0.26 g or 0.59 g of EPA/day in the diet resulted in a decrease in AA in this study. This is in contrast to the absence of a change in AA content after providing 0.34 g of EPA/day (Schmidt *et al.,* 1993) or 0.06 to 0.3 g of EPA/day (Healy *et al.,* 2000) for 12 weeks. However, in the latter two studies monocytes and neutrophils were investigated, respectively, while in the present study PBMNC were investigated, and this may contribute to the differences observed. The background diet (i.e. supply of AA precursors) may also influence these changes in fatty acid composition.

The differences between males and females in the content of the fatty acids of interest in the different lipid pools

It appears that females may have a greater capacity to incorporate α-LNA from the diet into plasma PL. Females may also have a greater capacity to incorporate EPA, synthesised from α-LNA, into plasma PL. This may be a consequence of females having an increased capacity to convert α-LNA to EPA compared to males, or of males having an increased storage or oxidation of dietary α-LNA, such that there will be less substrate (i.e. α-LNA) available for elongation.

In contrast, there appears to be no difference between genders in the capacity to incorporate EPA into plasma PL after EPA is supplied in the diet. This suggests that the source of EPA (i.e. endogenous v. exogenous) may be important in terms of its incorporation into plasma PL. The incorporation of EPA into PBMNC PL was different with gender, but only after EPA was supplied in the diet and not when it was synthesised from α-LNA, again, suggesting that the source of EPA (i.e. endogenous v. exogenous) is a factor in determining the enrichment of lipid pools in the body.

The content of AA in PBMNC also appears to be a different between genders. Females had less AA in their PBMNC PL than males. When the differences between

gender in *n*-3 PUFA and AA content in PBMNC PL are considered simultaneously it appears that females replace AA with *n*-3 PUFA more readily than males do.

Few studies to date have specifically investigated the differences between males and females in terms of the incorporation of α-LNA, EPA or DHA into lipid pools or in terms of the differences in metabolism of these fatty acids. Further more, it is difficult to obtain data on this from other studies published which look at incorporation of these fatty acids as part of other investigations. This is due to several factors including the small numbers of subjects studied and the use of predominantly male subjects in many studies (especially those designed to investigate immune function). One study has investigated the differences in content of AA, EPA and DHA in groups of males (n=6) and females (n=6). This established a small, but significant difference in the content of AA after intervention with 0.48 g of EPA + 0.28 g of DHA/day, with males having a greater content of AA than females (Marangoni *et al.,* 1993). This difference was not seen in the present study. No differences in the content of EPA or DHA were found between males and females in the study of Marangoni *et al.* (1993), while in this study significant differences were found. The differences between the results of the present study and that of Marangoni *et al.* (1993) perhaps arise from the differences in background diet, in the dose of EPA and DHA used and the larger number of subjects used in the current study, which was at least 10-times greater. Any differences in the content of AA, EPA or DHA between males and females in PBMNC PL were not investigated in the study of Marangoni *et al.* (1993).

Changes in the Content of the Other Major Fatty acids Investigated

The changes in the other major fatty acids identified (i.e. palmitic (16:0), stearic (18:0), oleic (18:1 *n*-9) and linoleic (18:2 *n*-6) acids) were different in the pools investigated. There appears to be a differential increase or decrease in these fatty acids to compensate for the changes in contents of α-LNA, AA, EPA, DPA and DHA. The content of 18:0 in plasma PL decreased in all treatment groups while there was no change in any of the other fatty acids in this pool.

The changes observed in the PBMNC PL were different for the five treatment groups. After EPA+DHA or α-LNA treatment there was a significant increase in the content 16:0 in PBMNC PL. The content of 18:2 *n*-6 was decreased after α-LNA, but was unchanged in EPA+DHA and placebo groups. This perhaps reflects the large decrease in the intake of linoleic acid seen when the diet was enriched in α-LNA (*Table 4.11*).

Changes in the Ratios of the Different Types of Fatty Acids Investigated

The changes in the composition of the different fatty acids in the PL pools investigated led to changes in the ratios of SFA to PUFA, *n*-6 to *n*-3 PUFA and AA to EPA. These changes are summarised in *Table 4.18*.

The ratio of SFA/PUFA was unchanged in the plasma PL despite the changes in fatty acid composition. This ratio in PBMNC PL was increased in all groups. The ratio of these two types of fatty acids is important with respect to membrane fluidity and cell function (see section 1.3.3.). Thus, changes in these fatty acids in the PBMNC PL could be important in determining the effects of the dietary modulation on cell function in this study and these will be discussed in the next two chapters.

The changes in the ratio of *n*-6 to *n*-3 PUFA were more complex. There was no change in this ratio over the intervention period in the plasma PL except in the EPA+DHA groups where there was a significant decrease in *n*-6 PUFA compared to *n*-3 PUFA. This reflects the increase in EPA, DPA and DHA in this fraction. A decrease in the ratio of *n*-6 to *n*-3 PUFA was also observed in the PBMNC PL after both EPA+DHA treatments (dose-dependently) and also after the 10.0 g α-LNA/day treatment. Again, this reflects the increase in EPA and DHA and decrease in AA in this fraction. These findings also suggest an effect of the dose of dietary *n*-3 PUFA on the ratio of *n*-6 to *n*-3 PUFA and this is likely to be important with respect to the production of eicosanoids (see section 1.1.3.). The impact of the interventions on the production of these lipid mediators will be discussed in the following chapters.

The changes in AA to EPA were again quite complex. There was a significant decrease in the ratio of these fatty acids in the plasma PL fraction after α-LNA

treatment but not after EPA+DHA treatment. This reflects the greater increase in EPA in this fraction after α-LNA intervention. This ratio decreased in PBMNC PL only in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups. This reflects the significant increase of EPA into this fraction in these two groups and again suggests an effect of the dose of *n*-3 PUFA on the relative content of these fatty acids in the membrane PL of PBMNC. The content and ratio of these fatty acids in PBMNC PL is again important with respect to the production of eicosanoids and other mediators of the immune system (see section 1.1.3.).

The SFA to PUFA, *n*-6 to *n*-3 and AA to EPA ratios have not been reported in many studies of α-LNA, EPA or DHA interventions *per se*, since the composition of all fatty acids measured are not always published, especially with respect to PBMNC PL. However, the decreases in *n*-6 to *n*-3 and AA to EPA ratios seen in plasma PL after α-LNA intervention in this study have been observed after providing 15.4 g α-LNA/day (Li *et al.*, 1999). After intervention with 1.8 g EPA + 1.0 g DHA/day similar decreases in the *n*-6 to *n*-3 and AA to EPA ratios in plasma PL were observed as in this study (Leaf *et al.*, 1995).

4.5. Conclusion

The incorporation of α-LNA, EPA and DHA into a staple of the diet (margarine) can result in the enrichment of plasma PL in these fatty acids. The changes in plasma PL fatty acid composition indicate that consuming a diet containing at least the 0.7 g EPA+DHA/day can significantly increase the proportion of EPA and DHA in plasma PL. A diet containing 5.0 or 10.0 g α-LNA/day resulted in dose-dependent increases in α-LNA in plasma PL. The consumption of α-LNA resulted in an increase in the proportion of EPA in the plasma PL fraction with the 5.0 g α-LNA/day achieving the same level of enrichment in EPA as the diet containing 10 g α-LNA/day or 0.7 g EPA+DHA/day (0.26 g EPA). Thus, at least 10-times the amount of α-LNA compared to EPA must be consumed in order to achieve the same enrichment in EPA (in plasma PL at least).

The occurrence of EPA in plasma PL after consuming increased levels of α-LNA, suggests that α-LNA can be converted to EPA in humans, presumably in the liver. The elongation of EPA to DPA is also apparent with a rise in DPA for the 10.0 g α-LNA/day treatment, which was not as marked in the 5.0 g α-LNA/day group. There was no increase in DHA after increased intake of α-LNA suggesting that this last step in the elongation pathway is somehow inhibited. This could be due to inhibition (or low activity) of the enzyme Δ6 desaturase (see *Figure 1.10.*), which is involved in the elongation of DPA to DHA. One study in humans using stable isotopes has shown that α-LNA conversion to longer PUFA is inhibited by diets rich in EPA (1.0 g/day) and DHA (0.6 g/day) (Vermunt *et al.,* 1999). Thus, an increase in EPA after increased α-LNA intake may be responsible for the inhibition of the Δ6 desaturase enzyme. The fact that there was no effect of the dose of α-LNA on the enrichment of plasma PL in EPA suggests that above a certain dose of α-LNA, there is an alternative route for α-LNA disposal rather than its elongation to EPA. It has been shown the oxidation of α-LNA might be slightly increased by diets rich in EPA and DHA (Vermunt *et al.,* 1999). Thus, the increase in EPA seen in plasma PL synthesised from dietary α-LNA may reach a certain level and may well than cause feedback inhibition on the conversion of α-LNA to EPA i.e. start to channel α-LNA through the oxidation pathway rather than the elongation pathway.

The changes in the fatty acid composition of PBMNC PL suggest that the changes in plasma PL can be passed on to cell membranes but that there is an effect of dose of *n*-3 PUFA on their enrichment. A level of at least 1.5 g EPA+DHA/day (providing 0.59 g EPA + 0.91 g DHA/day) is needed in the diet to establish significant changes in the composition of EPA and DHA in the PL of PBMNC. A level of at least 10.0 g α-LNA/day is needed to elicit significant changes in the content of EPA and DPA in PBMNC PL. An increase in the content of α-LNA in PBMNC PL at the doses used here was not achievable. Thus, provision of even large amounts of α-LNA is not a useful way to enrich PBMNC in long-chain PUFA, and in fact it may even lead to depletion of DHA. The higher doses of *n*-3 PUFA fed in this study also achieved significant alterations in the AA to EPA ratio and this is important with respect to immune function and the production of lipid mediators, such as eicosanoids.

Finally, the results of this study raise questions with regard to public health nutrition. In adult humans it appears that there is limited conversion of DPA (and its precursors) to DHA. This raises questions about how DHA is synthesised in humans and whether or not DHA is in fact essential in the human diet. It also has implications for the inclusion of α-LNA into foods intended for babies and young children as well as pregnant women. These groups have an increased requirement for DHA due to factors such as brain growth and development (Jorgenson *et al.*, 2001; Moriguchi *et al.*, 2000). DHA is found in large amounts in brain tissue and the retina, and without this fatty acid mental retardation and visual impairment have been observed. It would seem that α-LNA or EPA cannot substitute for pre-formed DHA in the diet. However, further work is needed in this area to elucidate this satisfactorily.

CHAPTER 5. THE EFFECTS OF INCREASED DIETARY INTAKE OF n-3 POLYUNSATURATED FATTY ACIDS ON MEASURES OF INNATE IMMUNITY IN HEALTHY SUBJECTS

5.1. Introduction

Section 1.4. described how changes in the consumption of *n-3* polyunsaturated fatty acids (PUFA) have been reported to modulate the function of several types of cell involved in the innate immune response. These cells include mononuclear phagocytes (i.e. monocytes and macrophages), neutrophils and natural killer cells. It is thought that the modulation of cell function is brought about by an alteration in the supply of fatty acids to the cells and the subsequent alteration in their fatty acid composition, especially of the membrane phospholipids, and thus in cell function (section 1.3.).

Summary of the Changes in Immune Cell Fatty Acid Composition Observed with α-LNA, EPA and DHA Intervention

It has been established in several studies that the fatty acid composition of the cells involved in the innate immune response can be modulated by the changes in fatty acids supplied in culture. Studies have reported modulation of the total lipid profile of macrophages (Schroit *et al.,* 1979; Lokesh *et al.,* 1984; Lokesh *et al.,* 1988) as well as of the phospholipid fraction (Mahoney *et al.,* 1977; Schroit *et al.,* 1979; Calder *et al.,* 1990) and the neutral lipid fraction (Calder *et al.,* 1990). Dietary lipid modulation (e.g. adding *n-3* fatty acids) has also been reported to modify the total fatty acid composition of monocyte and macrophages in several species including humans (Lee *et al.,* 1985; Endres *et al.,* 1989; Halvorsen *et al.,* 1997); changes in the dietary supply of *n-3* fatty acids in the human diet also modify the fatty acid composition of the phospholipids of blood monocytes (Gibney and Hunter, 1993; Marangoni *et al.,* 1993). The fatty acid composition of human neutrophils can also be modulated by changes in dietary fatty acid intake (Sperling *et al.,* 1993; Gibney and

Hunter, 1993; Healy *et al.,* 2000). No study has reported the changes in the fatty acid composition of natural killer cells but changes in the composition of peripheral blood mononuclear cells (PBMNC), of which NK cells are contributor, have been reported a number of times (Molvig *et al.,* 1991; Caughey *et al.,* 1996; Kelley *et al.,* 1999; Yaqoob *et al.,* 2000; Thies et al., 2001b). The studies quoted here represent only some of the dietary studies which have been performed investigating the effect of altered dietary supply of fatty acids in the human diet on immune function. The studies quoted stand apart from others in the literature, as they are the only studies to report the fatty acid composition of the cells being investigated.

An important consideration regarding changes in fatty acid composition of PBMNC PL (as measured in this study; see Chapter 4) and changes in immune cell function, would be whether changes in the fatty acid composition of PBMNC are reflected across the various types of immune cell e.g. T- and B-lymphocytes, monocytes, NK cells and neutrophils. There is one study which has investigated this, and this compared the changes in the fatty acid composition of phospholipids of neutrophils, monocytes, T-lymphocytes and B-lymphocytes after dietary intervention with 0.9 to 1.2 g eicosapentaenoic acid (EPA) + 1.4 to 4.2 g docosahexaenoic acid (DHA)/day (Gibney and Hunter, 1993). This study showed that the extent of the observed increase in both EPA and DHA and of the decrease in arachidonic acid (AA) was the same in all four cell types. The effects of α-linolenic acid (α-LNA) intervention on different cell types has not yet been investigated, but will be presumed to be similar to that seen after EPA and DHA intervention. Thus, changes in PBMNC PL observed in this study can be thought to occur in T-and B-lymphocytes, monocytes and neutrophils. The changes in fatty acid composition of PBMNC PL were measured in the present study and are described and discussed in Chapter 4. They are summarised here.

Summary of the Changes in Fatty Acid Composition of PBMNC PL in the Present Study

There were significant changes in the composition of PBMNC PL only with respect to certain fatty acids (see section 4.3.5.). There were marked changes in the content AA, EPA and DHA. There was a significant decrease in AA in all treatment groups.

There was a significant increase in EPA in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day treatment groups. There were no changes in the content of α-LNA in any of the groups, while there was a decrease in DHA in the α-LNA groups and an increase in DHA in the 1.5 g EPA+DHA/day group. Changes in other types of fatty acids were also observed. In all groups the proportion of 16:0 increased while there was no change in any other fatty acid in any groups except the 10.0 g α-LNA where there was a decrease in 18:2 *n*-6. The ratio of SFA to PUFA increased in all groups. The ratio of *n*-6 to *n-3* PUFA decreased in both EPA+DHA treatment groups, but the change was significant only for the 1.5 g EPA+DHA/day group. There was also a decrease in this ratio in the 10.0 g α-LNA/day but not in the 5.0 g α-LNA/day group. There was a decrease in the ratio of AA to EPA in the 10.0 g α-LNA/day and the 1.5 g EPA+DHA/day groups.

Summary of the Changes in Immune Function Observed with α-LNA, EPA and DHA Intervention

The modulation of monocyte/macrophage, neutrophil and NK cell functions after manipulation of dietary fatty acids has been discussed in Chapter 1 (section 1.4.). The overall outcome of these experiments is that increased consumption of α-LNA and the longer chain *n-3* PUFA, EPA and DHA, has modulatory effects on the functioning of these immune cells. Dietary α-LNA, EPA and DHA have been demonstrated to have inhibitory effects on the production of the cytokines tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β by human monocytes/PBMNC (Endres *et al.,* 1989; Meydani et al 1991; Meydani *et al.,* 1993; Gallai *et al.,* 1993; Caughey *et al.,* 1996; Kelley *et al.,* 1999) although there are some contradictory findings (Molvig *et al.,* 1991; Schmidt *et al.,* 1996; Yaqoob *et al.,* 2000).

There are many reasons for the conflicting data, especially at the lower levels of EPA and DHA intervention, on the effects of these long chain PUFA on the production of cytokines by human monocytes. Different studies have used different experimental conditions, as well as different doses of *n-3* PUFA. Many studies which report an inhibition of cytokine production used the higher doses e.g. > 5.2 g EPA+DHA/ day (Gallai *et al.,* 1993; Kelley *et al.,* 1999), while studies which reported no effect used

the lower doses e.g. < 1 g/ day (Schmidt *et al.,* 1996). There are several other key factors, which may contribute to the differences observed with *n-3* PUFA feeding. One is the difference in the nature of the sample in which the cytokines are assayed i.e. in cell lysate alone, culture medium alone or a combination of these. This leads to some confusion as to whether the cytokine measured is extra- or intra-cellular. The cell culture conditions used are also important since Cooper *et al.* (1993) showed that the concentration of the stimulant used to elicit cytokine production can affect the apparent impact of dietary EPA and DHA on cytokine production. This study showed that fish oil did not affect the concentration of TNF in lipopolysaccharide (LPS) -stimulated whole blood, but that the concentration of IL-1 and IL-6 were decreased by fish oil if a LPS concentration was <1 ng/ml was used to stimulate the cultures, while >10 ng/ml resulted in no such inhibition by fish oil. There is very little known about the effects of α-LNA on the functioning of innate immune cells, since there is only one study using this fatty acid and this showed an inhibition of TNF-α and IL-1β production with 13.7 g/day for 4 weeks (Caughey *et al.,* 1996).

The effects of dietary EPA and DHA on other monocyte functions, such as phagocytosis have been investigated in only one human study where no change was seen with 3.8 g EPA or DHA/day (Halvorsen *et al.,* 1997). Studies which have investigated the effects of α-LNA, EPA and DHA on oxidative burst activity by neutrophils shown that these fatty acids are inhibitory (Thompson *et al.,* 1991) or have no effect (Varming *et al.,* 1995; Healy *et al.,* 2000) at the doses given.

Thus, the literature reviewed with respect to human intervention studies has demonstrated that feeding fish oil (i.e. a preparation rich in EPA and DHA, predominately in capsules), or α-LNA (at a very high levels (approximately 14 g/day)), can have effects on the function of the cells of the innate immune system. In human studies to date the effects of low levels of EPA and DHA, and more especially of α-LNA, have not been satisfactorily investigated, nor have dose-response relationships been determined with respect to the *n-3* PUFA (α-LNA, EPA and DHA) and innate immune function in humans.

Despite the interest in fatty acids and immune function, relatively little is known about the relationship between the habitual intake of fatty acids (which is reflected in

the fatty acid composition of cells of free-living individuals) and immune function. Caughey *et al.* (1996) demonstrated that were statistically significant inverse relationships between PBMNC EPA content and both TNF-α and IL-1β production after LPS stimulation. TNF-α and IL-1β production decreased as the PBMNC EPA content increased to approximately 1% of total fatty acids, after which an increase in EPA had little effect on cytokine production. The current study provides an opportunity to investigate the relationships between intake of fatty acids, the fatty acid composition of PBMNC PL and immune cell function.

Most dietary interventions with EPA and DHA have used capsules as the vehicle for the intervention and have aimed to deliver high doses of fatty acids. The use of capsules in the everyday situation has limitations as described in section 4.1. This study will investigate the influence of dietary interventions with foodstuffs enriched in either α-LNA or EPA and DHA on immune cell function.

5.1.1. Aims of This Chapter

It is thus apparent that further work is required to establish the effects of *n-3* PUFA on the human innate immune response in terms of the function of monocytes and neutrophils. The reader is referred to section 4.2. for details of the study design, information about the subject characteristics and dietary information.

The overall aims of this dietary intervention study are outlined in section 4.1.1. This chapter intends to investigate these aims in the context of the innate immune response.

- to investigate the immunological effects of EPA and DHA at levels that can be reasonably readily incorporated into the diet through foodstuffs
- to investigate the immunological effects of α-LNA at levels that can be reasonably readily incorporated into the diet through foodstuffs
- to ascertain a better estimate of the relationship between the amount of α-LNA and of EPA and/or DHA in the diet and innate immune function
- to establish if there are any differences due to gender in the immunomodulatory effects of α-LNA, EPA and DHA

Hypotheses:

- There will be dose-dependent effects of α-LNA and EPA+DHA on human innate immune cell function

The effects of dietary α-LNA on human innate immune cell function will be less marked than those of EPA+DHA

In this chapter the following measures of innate immune function will be examined:

- The number of monocytes and NK cells in peripheral blood
- The expression of cell surface markers on monocytes
- The oxidative burst activity of neutrophils and monocytes
- The phagocytic activity of neutrophils and monocytes
- The production of the cytokines TNF-α, IL-6 and IL-1β
- The production of the eicosanoid PGE_2

5.2. Methods

5.2.1. Chemicals

Formaldehyde, glutamine, HEPES-buffered RPMI medium (glutamine free), LPS, penicillin, streptomycin and sodium azide were all obtained from Sigma Chemical Co., Poole, UK. Cytokine concentrations were determined using ELISA kits, manufactured by Biosource, Belgium, and prostaglandin E_2 concentrations were determined using ELISA kits manufactured by Neogen. All kits were purchased from Appligene-Oncor-Lifescreen, Watford, UK. Oxidative burst and phagocytic activity were determined using kits obtained from Becton Dickinson, Oxford, UK. Fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies to human CD3

and CD14, and R. Phycoerythrin (RPE)-labelled monclonal antibodies to human CD11b, CD16 and CD54 were purchased from Serotec, Kidlington, UK.

5.2.2. Mononuclear cell preparation

Whole blood (40 ml) was layered onto an equivalent volume of Histopaque (density 1.077 g/ml) and peripheral blood mononuclear cells (PBMNC) were collected from the interface after centrifugation at room temperature (2000 rpm, 15 min). Plasma was collected from the top layer and some used for cell function experiments; the remainder was frozen at -20 °C for later fatty acid composition analysis (see section 4.2.5.).

The PBMNC were washed with Hepes-RPMI medium (containing penicillin, streptomycin and 0.75 mM glutamine) and then relayered onto an equal volume of Histopaque. The PBMNC were collected from the interface after centrifugation, and again washed with RPMI medium. Finally the PBMNC were resuspended in medium, counted using a Coulter cell counter (model Z1, Beckman Coulter, UK) and adjusted to the appropriate cell concentrations for use in cell function experiments. The remainder of cells not required for this purpose were centrifuged to a pellet (2000 rpm, 10 min) and frozen at -20 °C for fatty acid composition analysis (see section 4.2.5.).

5.2.3. Measurement of Mononuclear Cell Sub-sets

Flow cytometry was used to measure the presence of markers on the surface of mononuclear cells. Whole blood (100 µl) was doubly stained with antibodies (*Table 5.1.*); 10 µl FITC-labelled and 10µl RPE-labelled antibodies were used. The sample was incubated for 30 min at 4°C. Erythrocytes were then lysed using a lysing solution containing formaldehyde (37.5 ml/100 ml), diethylene glycol (45 ml/100 ml) and 0.2 mM Tris (17.5 ml/100 ml). The remaining cells were washed first with PBS and subsequently with modified PBS (mPBS = PBS containing BSA (0.5 g/500 ml) and sodium azide (0.325 g/500 ml)). The cells were then fixed in FACS Fix (PBS containing 2 ml/100 ml formaldehyde). The samples were then refrigerated (for

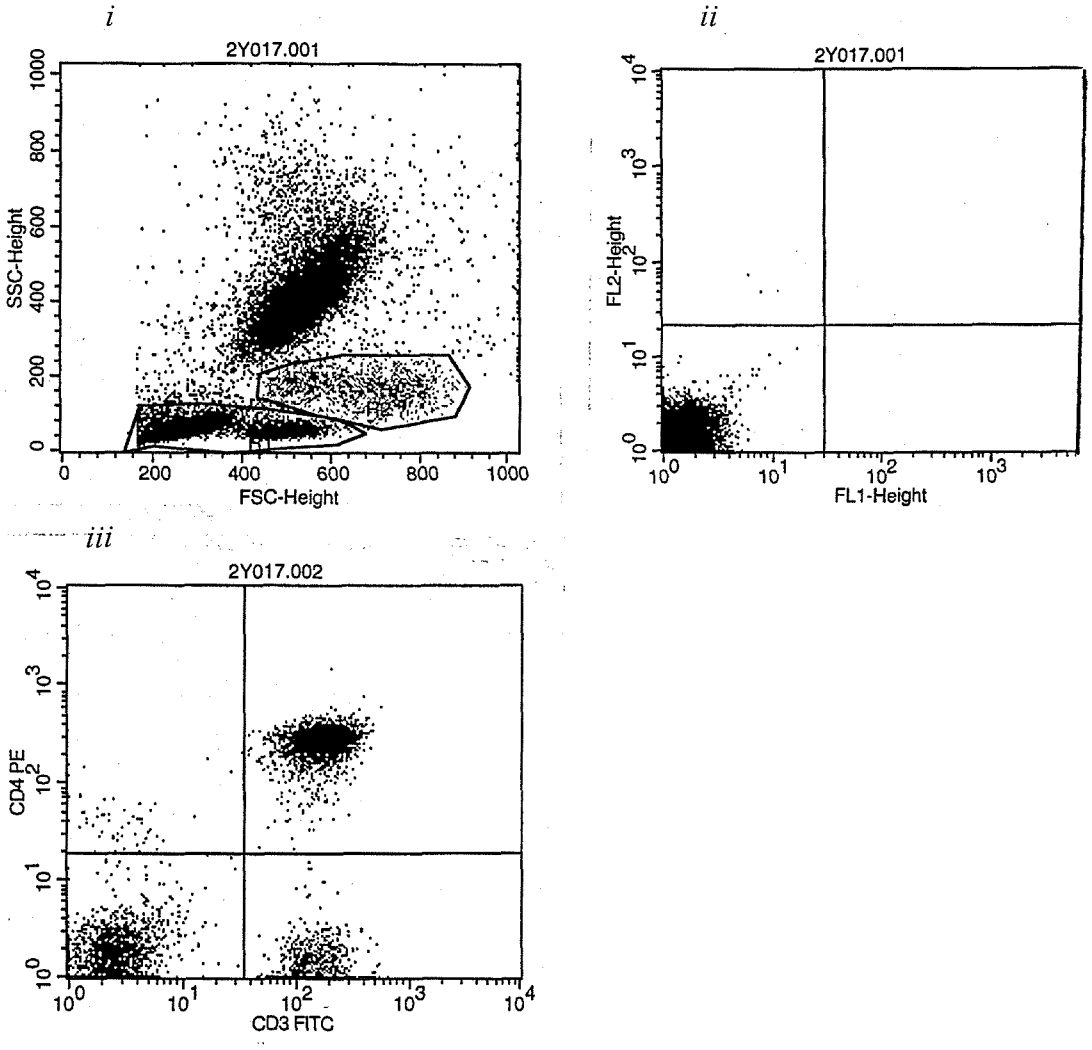
up to 24 h) until they could be examined for fluoresence using a Becton Dickinson FASCscalibur fluorescence-activated cell sorter. Fluorescence data were collected on 2 x 10^5 viable cells. Results were analysed using Cellquest software. Sample profiles are shown in *Figure 5.1,* and the identification (gating) of the different cell subtypes is also explained in *Figure 5.1.*. The % of NK cells in the 'lymphocyte' region and the % of monocytes in the 'monocyte' region were determined in terms of the percentage marker-positive cells. NK cells were identified as CD3-CD16+ and monocytes were identified as CD14+. CD54 and CD11b are adhesion molecules (ICAM-1 and Mac-1 respectively) and the expression of these on $CD14^+$ cells was determined as % positive cells and mean fluorescence intensity (a measure of the level of expression on the +ve cells).

Table 5.1. Stains used to identify the different cell subsets

Cell	**Stain 1**	**Stain 2**
NK cells	Anti CD3*	Anti $CD16^+$
ICAM-1 +ve monocyte	Anti CD14*	Anti $CD54^+$
Mac-1 +ve monocyte	Anti CD14*	Anti $CD11b^+$

* = FITC-labelled; $^+$ = RPE-labelled

Figure 5.1. Typical flow cytometry profiles showing identification and quantification of different mononuclear cell populations.



i. The profile from each sample is first displayed in a dot plot such that leukocytes in the sample can be grouped by their size (forward-scatter) and granularity (side-scatter) of the cells. The different cell types are then gated into regions to be further displayed in dot plots, so that the cell surface markers can be studied. Lymphocytes were gated into region 1 (R1) and monocytes into region 2 (R2).

ii. The auto-fluorescence of the cells was ascertained in this dot plot. This was established so that the background fluorescence of the cells could be taken into account in the analyses of the remaining samples.

iii. The gated lymphocytes stained for the cell surface markers, CD3 and CD16 were displayed in a dot plot. The dot plot was then divided into 'quadrants' such that the number of NK cells (i.e. cells that are stained for CD16 but not CD3 (top left-hand corner of the profile)) could be determined.

The monocytes in each sample were gated as shown above in *Figure 5.1.ii.* and displayed in dot plot profiles. The expression of CD14 was than examined in the same way as in *Figure 5.1.iii.*. and the % of monocytes was determined as the sum of the top right and bottom right quadrants i.e. all cells that were CD14+. The level of expression of the adhesion molecules, ICAM-1 and Mac-1 was then determined as the mean fluorescence intensity (MFI) of CD54+ and CD11b+ cells i.e. the MFI of the top right quadrant of monocyte profiles.

5.2.4. Cytokine and PGE_2 Production

PBMNC were cultured at 37°C in an air/CO_2 (19:1) atmosphere in a 24-well culture plate at a density of 2 x 10^6 cells/well and a total culture volume of 2 ml in RPMI medium supplemented with 0.75 mM glutamine, antibiotics (penicillin and streptomycin) and 5% (v/v) autologous plasma and 15 μg/ml LPS. After 24 h, the plate was centrifuged (1000 rpm, 5 min) and the supernatant collected and frozen at -20°C for later analysis. Cytokine and PGE_2 concentrations were determined using ELISA kits according to the manufacturers instructions. The samples were diluted as appropriate, and a standard curve was constructed to determine cytokine concentrations, with the range of detection 2 to 1690 pg/ml (IL-6), 2 to 1850 pg/ml (IL-1β), 3 to 1525 pg/ml (TNF-α) and 0.5 to 10.0 ng/ml (PGE_2). The percentage coefficient of variation for the cytokines measured was: for intra-assay 5.6 (IL-6), 3.4 (IL-1β) and 2.8 (TNF-α); or inter-assay 7.5 (IL-6), 4.4 (IL1-β) and 2.8 (TNF-α).

5.2.5. Measurement of leukocyte oxidative burst

Leukocyte oxidative burst was determined using Phagoburst kits supplied by Becton Dickinson. A sample of heparinized whole blood was stored at room temperature for approximately 2-3 h until the assay could be performed. Blood was cooled on ice for ten minutes. Blood was then vortexed and three aliquots (100 μl) were incubated in a preheated waterbath, (37°C) for 10 min with 20 μl of one of two activating substances: opsonized *E. coli* or phorbol myristic acteate (PMA) or with washing solution (control). The samples were then incubated for a further 10 min at 37°C with a substrate solution. This solution contains dihydrorhodamine (DHR) 123, which acts as a fluorogenic substrate. It is this substrate which is oxidised (from DHR 123 to rhodamine 123 which is fluorescent) by the reactive oxygen species produced by leukocytes upon stimulation. Lysing solution (2 ml) was added to stop the reaction and to lyse erythrocytes. Leukocytes were then fixed, and the DNA stained according to the manufacturer's instructions. Cell preparations were then analysed by flow cytometry on a Becton Dickinson FACScalibur fluorescence-activated cell sorter. Fluorescence data were collected on 2 x 10^5 viable cells. Results were analysed using Cellquest software. The percentage of neutrophils or monocytes

having produced reactive oxygen metabolites (recruitment) and the mean fluorescence intensity (MFI) (measure of oxidation quantity per cell) were determined. An index of activity was also calculated (% active cells x MFI) and gives a indication of the overall activity of the cell. An example of the profiles obtained is shown in *Figure 5.2.* and the procedure for gating and identifying active cells and their oxidative activity is also described. The intra-assay precision of this assay was determined on triplicate whole blood samples from healthy individuals and the percentage coefficient of variation was 0.9 (n=6) for neutrophils and 2.6 (n=6) for monocytes.

5.2.6. Measurement of leukocyte phagocytic activity

Leukocyte phagocytic activity was determined using Phagotest kits supplied by Becton Dickinson. A sample of heparinized blood was stored for between 2-3h at room temperature until 10 min prior to the start of the experiment, when it was put on ice. Blood (100 µl) was then incubated with 20 µl FITC-labelled, opsonized *E. coli* for 10 min either at 37°C (test) or 0°C (control). The samples were then removed and placed on ice to inhibit phagocytosis, and a quenching solution added to allow discrimination between internalised and surface-bound FITC-labelled *E. coli*. Erythrocytes were then lysed, the leukocytes fixed, and the DNA stained according to the manufacturer's instructions. Cell preparations were then analysed by flow cytometry on a Becton Dickinson FACScalibur fluorescence-activated cell sorter. Fluorescence data were collected on 2 x 10^5 viable cells. Results were analysed using Cellquest software. The percentage of cells having performed phagocytosis (gated neutrophils and monocytes), and the number of ingested bacteria per cell (mean fluorescence intensity (MFI)) were determined. The index of activity was then calculated (% active cells x MFI) which gives an indication of the overall index of activity. An example of the profiles obtained is displayed in *Figure 5.3.* and the procedure for gating and identifying active cells and their phagocytic activity is also described. The intra-assay precision of this assay was determined on triplicate whole blood samples from healthy individuals and the percentage coefficient of variation was 0.8 (n=6) for neutrophils and 5.0 (n=6) for monocytes.

Figure 5.2. Typical profiles obtained when measuring leukocyte oxidative burst activity.

Figure 5.2 a. A 'control' sample - whole blood incubated with no stimulant at 37°C.

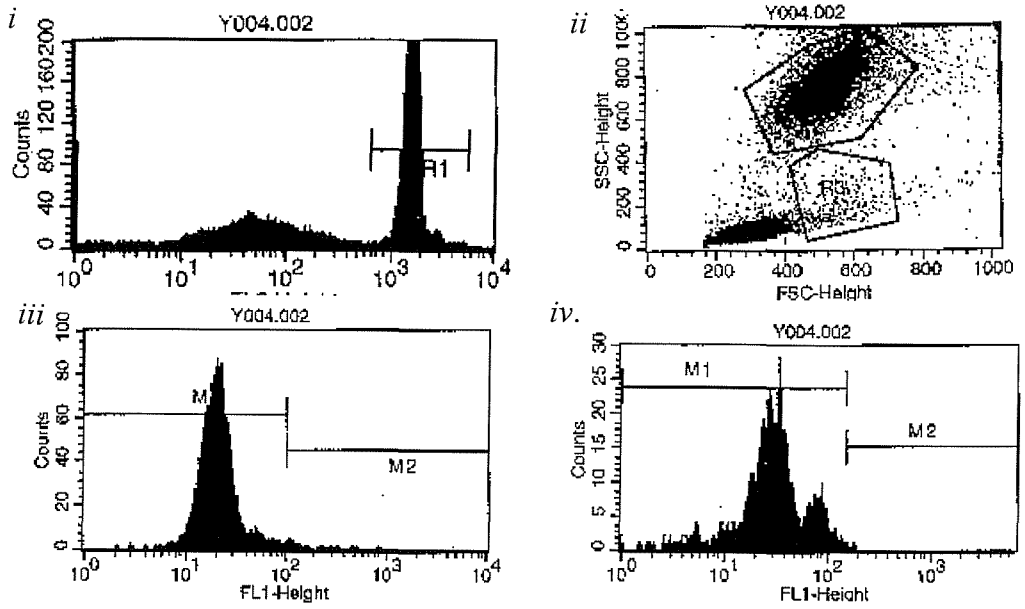


Figure 5.2 b. A 'test' sample - whole blood incubated with *E.coli* at 37°C.

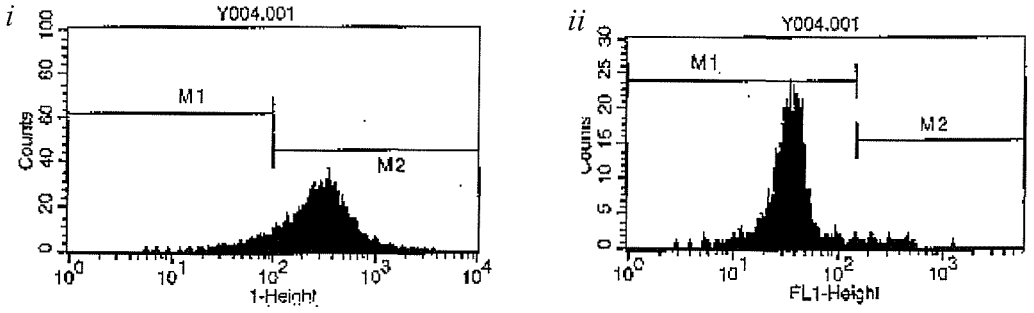


Figure 5.2 c. A 'test' sample - whole blood incubated with PMA at 37°C.

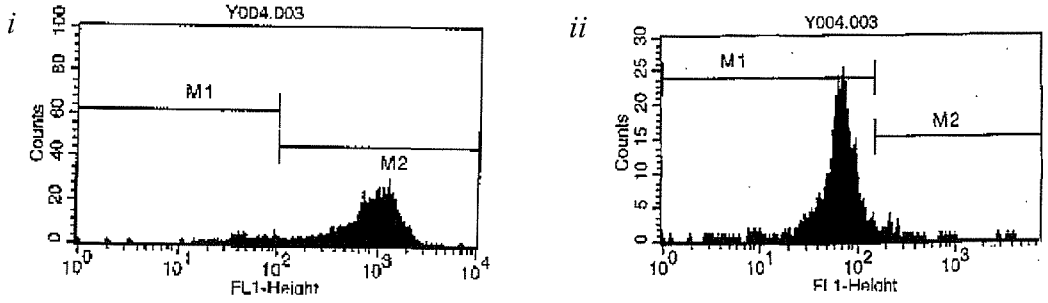


Figure 5.2. a. i. The profile from each sample was first displayed in a histogram such that leukocytes could be isolated (gated) from any other material in the sample as well as any bacteria which have not been ingested (if present). This is achieved since any diploid cells present will be stained with propidium iodide, which fluoresces, in the FL-2 channel.

Figure 5.2.a.ii. The gated leukocytes were then displayed in a dot plot such that they were grouped by the size (forward scatter) and granularity (side-scatter) of the cells. The different cell types were then gated into regions to be further displayed in histograms. Neutrophils were then gated into region 2 (R2), and monocytes into region 3 (R3).

Figure 5.2.a.iii. The neutrophils from the control sample were then displayed in a histogram which showed the absolute number of cells against the FITC-stain which fluoresces in the FL-1 channel. The use of 'markers' on the profile enabled the number of cells which had undergone oxidative burst to be set at 1-2 % of all gated cells (cells in marker 2 (region M2)).

Figure 5.2. a. iv. The monocytes from the control sample were displayed in a histogram as above. Markers were used as above with the number of cells having undergone oxidative burst again set at 1-2 % of all gated cells.

Figure 5.2.b.i. The neutrophils from the test sample, incubated with *E. coli* were displayed in a histogram (as for *Figure 5.2. a. iii.*). The use of 'markers' at the same position as for the control enabled the number of cells which had undergone oxidative burst to be established (cells in M2), as well as the quantity of reactive metabolites produced per granulocyte (mean fluorescence of cells in M2)

Figure 5.2.b.ii. The monocytes from the test sample, incubated with *E. coli* were displayed in a histogram (as for *Figure 5.2. a. iv.*). The use of 'markers' at the same position as for the control enabled the number of cells which had undergone oxidative burst to be established (cells in M2), as well as the quantity of reactive metabolites produced per monocyte (mean fluorescence of cells in M2)

Figure 5.2.c.i. The neutrophils from the test sample, incubated with PMA were displayed in a histogram (as for *Figure 5.2. a. iii.*). The use of 'markers' at the same

position as for the control enabled the number of cells which had undergone oxidative burst to be established (cells in M2), as well as the quantity of reactive metabolites produced per granulocyte (mean fluorescence of cells in M2)

Figure 5.2.c.ii. The monocytes from the test sample, incubated with *E. coli* were displayed in a histogram (as for *Figure 5.2. a. iv.*). The use of 'markers' at the same position as for the control enabled the number of cells which had undergone oxidative burst to be established (cells in M2), as well as the quantity of reactive metabolites produced per granulocyte (mean fluorescence of cells in M2)

Figure 5.3. Typical profiles obtained when measuring leukocyte phagocytic activity.

Figure 5.3 a. A 'control' when whole blood was incubated with stimulant at 0°C.

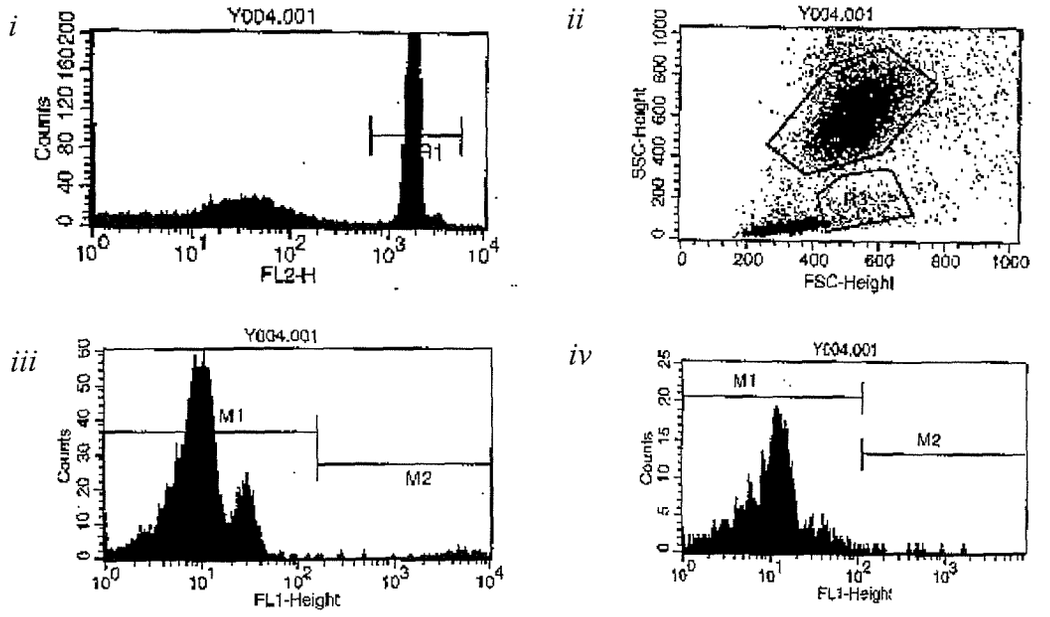


Figure 5.3 b. A 'test' sample when whole blood was incubated with *E. coli* at 37°C.

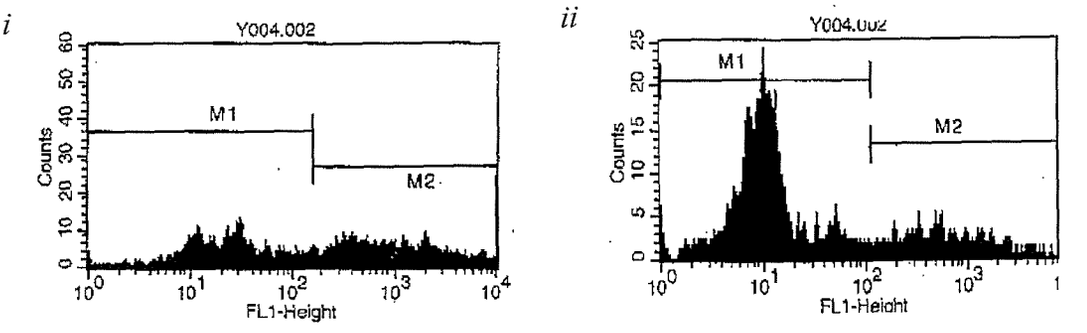


Figure 5.3. a. i. The profile from each sample was first displayed in a histogram such that leukocytes can be isolated (gated) from any other material in the sample as well as any bacteria which have not been ingested (if present). This is achieved since any diploid cells present will be stained with propidium iodide, which fluoresces, in the FL-2 channel.

Figure 5.3.a.ii. The gated leukocytes were then displayed in a dot plot such that they could be grouped by the size (forward scatter) and granularity (side-scatter) of the cells. The different cell types were then gated into regions to be further displayed in

histograms. Neutrophils were then gated into region 2 (R2), and monocytes into region 3 (R3).

Figure 5.3.a. iii. The neutrophils from the control sample were then displayed in a histogram which showed the absolute number of cells against the FITC-stain which fluoresced in the FL-1 channel. The use of 'markers' on the profile enabled the number of cells which had undergone oxidative burst to be set at 1-2 % of all gated cells (cells in marker 2 (region M2)).

Figure 5.3. a. iv. The monocytes from the control sample were displayed in a histogram as above. Markers were used as above with the number of cells having undergone phagocytosis again set at 1-2 % of all gated cells.

Figure 5.3. b.i. The neutrophils from the test sample, incubated with *E. coli* were displayed in a histogram (as for *Figure 5.3. a. iii.*). The use of 'markers' at the same position as for the control enabled the number of cells which had undergone phagocytosis to be established (cells in M2), as well as the quantity of bacteria engulfed per granulocyte (mean fluorescence of cells in M2)

Figure 5.3. b.ii. The monocytes from the test sample, incubated with *E. coli* were displayed in a histogram (as for *Figure 5.3. a. iv.*). The use of 'markers' at the same position as for the control enabled the number of cells which had undergone phagocytosis to be established (cells in M2), as well as the quantity of bacteria engulfed per monocyte (mean fluorescence of cells in M2)

5.2.7. Statistical Analysis

Data are shown as the mean ± SEM. For the cytokine and cell sub-set measurements, data were obtained for approximately 30 subjects per treatment group (see *Table 4.7.* for details), these being those subjects in all 3 cohorts of the study. The phagocytic activity and oxidative burst activity of neutrophils and monocytes were measured in approximately 20 subjects per treatment group, these being most subjects in cohorts 2 and 3. The variation in the number of measurements made for these innate immune parameters was due to logistic and financial constraints.

The statistical analyses were initially performed using a two-way analysis of variance to establish the effects of treatment and time and the interaction between them. Where treatment or time were significant one-way analysis of variance and a post-hoc least significance test with Bonferonni's correction were then performed to identify differences between groups at a given time point and differences within a treatment group over time. In all cases a value of $P<0.05$ was taken to indicate statistical significance and the statistical package SPSS Version 10.0 (SPSS Inc., Chicago, IL) was used.

Significant differences over time are expressed as:

a. Significantly different from 0 month
b. Significantly different from 3 month
c. Significantly different from 6 month

Significant differences between groups at 0, 3 or 6 months are expressed as:

A. Significantly different from the placebo treatment
B. Significantly different from the 5.0 g α-LNA treatment
C. Significantly different from the 10.0 g α-LNA treatment
D. Significantly different from the 0.7 g EPA+DHA treatment
E. Significantly different from the 1.5 g EPA+DHA treatment

Linear correlations were determined as Spearman's Rank correlations. r values indicated by # are significantly different at the $P<0.05$ level.

5.3. Results

The innate immune parameters measured were the proportions of NK cells and monocytes in the bloodstream, the expression of adhesion molecules on circulating monocytes, the phagocytic and oxidative burst activity of neutrophils and monocytes and the concentrations of the mediators TNF-α, IL-1β, IL-6, IL-10 and PGE_2 in culture media from PBMNC stimulated with LPS.

These measurements will be described firstly in terms of the baseline data to determine any differences in the parameters measured due to gender, age or the fatty acid composition of the cells involved (i.e. PBMNC PL) at the start of the intervention (see section 4.3.6. for details of the fatty acid composition). This will enable potential determinants of variation in innate immune cell function to be identified.

The effects of the dietary treatments on the immune parameters measured will then be examined. In addition, the effects of gender and age on the modulation of these parameters with the different treatments will be described. Correlations between the changes in fatty acid composition of PBMNC PL and innate immune cell function will also be described (see section 4.3.6. for details of the changes in fatty acid composition). The latter will allow the relationship between dietary fatty acid intake, and immune cell composition and function to be clarified. The investigation of this relationship will be made for the study group as a whole and for the 10.0 g α-LNA and 1.5 g EPA+DHA treatment groups. This is because the changes in fatty acid composition of PBMNC PL over the intervention period were only observed in these two treatment groups (see section 4.3.6.).

5.3.1. Analysis of NK Cell and Monocyte Sub-Sets – Cells of the Innate Immune System

The % of NK cells and monocytes was determined as well as the expression of the adhesion molecules ICAM-1 and Mac-1 on monocytes at 0, 3 and 6 months of intervention (*Table 5.2.*).

5.3.1.1. The % of NK cells and Monocytes in Peripheral Blood

There were no differences in the proportions of NK cells (expressed as a proportion of the lymphocyte population) or monocytes (expressed as a proportion of the 'monocyte' population) in the different treatment groups at baseline (*Table 5.2.*). NK cells comprised about 5% of the lymphocyte population and monocytes comprised about 40 to 50% of the population defined by R3 in *Figure 5.1.* The % of NK cells and monocytes were significantly different between males and females ($p<0.05$). The % of NK cells for males was 6.7 ±0.77 %, while for females it was 4.1 ±0.56%. There was no effect of age on the proportions of these cells at baseline.

Two-way ANOVA established that there was no effect of treatment on the % of NK cells or monocytes. However, there was a significant effect of time on the % of NK cells ($p<0.002$) and on the % of monocytes ($p<0.006$). These effects were investigated further. There were significant effects of the α-LNA treatments on the % of NK cells and of the 1.5 g EPA+DHA/day treatment on the % of monocytes when a one-way ANOVA was performed (*Table 5.2.*). The % NK cells tended to decrease in all treatment % NK cells was significant for both of the α-LNA groups, while in the EPA+DHA treatment groups the decrease did not reach significance (*Table 5.2.*). The % monocytes tended to increase in all groups, from 0 to 6 months (*Table 5.2.*). However, the only increase in % of monocytes which reached significance was in the 1.5 g EPA+DHA group (*Table 5.2.*). There were no differences in % NK cells or % monocytes between any of the treatment groups at any time during the intervention.

There were small differences with gender in the % of NK cells and monocytes in peripheral blood over the intervention period. There were differences in the proportions of monocytes, but not NK cells, between males and females at 3 months in the 1.5 g EPA+DHA/day group and both the α-LNA groups. Males had a greater % of monocytes then females, but this is probably a reflection of the differences at baseline rather than an effect of treatment. This gender difference was not seen at 6 months and no differences in the proportions of these cells were seen in the placebo or 0.7 g EPA+DHA/day groups over the intervention period. There was no effect of

age on the % NK cells or monocytes in peripheral blood in any of the treatment groups over the intervention period.

Table 5.2. The % of NK cells and monocytes in peripheral blood (n=30/group)

Treatment	Time (m)	NK cells (CD3-CD16+) (as a % of lymphocytes)	Monocytes (CD14+) (as a % of cells in region R3)
Placebo	0	5.21 ±0.97	41.3 ±3.81
	3	3.81 ±0.40	49.8 ±3.53
	6	4.80 ±1.00	50.0 ±4.62
5.0 g α-LNA	0	5.4 ±1.10 [b]	43.9 ±4.05
	3	2.8 ±0.36 [a]	48.0 ±3.74
	6	3.2 ±0.37	45.1 ±3.46
10.0 g α-LNA	0	5.1 ±0.98 [bc]	38.0 ±3.61
	3	2.6 ±0.39 [a]	47.9 ±4.20
	6	2.9 ±0.26 [a]	44.3 ±4.90
0.7g EPA+DHA	0	5.1 ±0.91	41.6 ±3.05
	3	4.9 ±1.96	50.3 ±4.17
	6	3.3 ±0.44	53.7 ±4.28
1.5 g EPA+DHA	0	7.3 ±1.63	34.2 ±2.86 [c]
	3	4.3 ±1.30	48.5 ±3.33
	6	4.2 ±0.65	53.9 ±4.45 [a]

Significant differences are expressed as:
a. Significantly different from 0 month
b. Significantly different from 3 months
c. Significantly different from 6 months

5.3.1.2 Expression of Adhesion Molecules on Circulating Monocytes

The proportion of monocytes also expressing CD54 (ICAM-1) or CD11b (Mac-1) was approximately 30 to 40% (data not shown). There was no difference in the proportion of monocytes expressing these molecules or on their level of expression of among the treatment groups at baseline. The expression of these adhesion molecules on monocytes was not different between males and females at baseline, and there was no effect of age on expression at baseline.

Two-way ANOVA established that there was no effect of treatment on the proportion of monocytes expressing CD54 or 11b on their level of expression.

However, there was a significant effect of time on the expression of Mac-1 by monocytes ($p<0.0001$), but not on the expression of ICAM-1. The effect of time was investigated further. A one-way ANOVA established that there was a significant decrease in the expression of Mac-1 on monocytes in all treatment group, including the placebo (*Table 5.3*). The expression of ICAM-1 was not significantly affected, except in the 1.5 g EPA+DHA/day group, where there was a significant from 3 to 6 months of intervention (*Table 5.3.*). There was no difference among the treatment groups in the expression of either of the adhesion molecules between males and females, or with age, over the 6 months of intervention.

Table 5.3. The level of expression of adhesion molecules on monocytes (n=30/group)

Treatment	Time (m)	ICAM +ve Monocytes ($CD14^+CD54^+$)	MAC +ve Monocytes ($CD14^+CD11b^+$)
Placebo	0	138.2 ±25.9	670.7±68.7 [bc]
	3	129.1 ±26.3	357.0 ±53.6 [a]
	6	115.8 ±50.4	400.7 ±52.0 [a]
5.0 g α-LNA	0	171.5 ±33.6	622.3 ±71.4 [b]
	3	168.7 ±33.8	316.6 ±45.8 [a]
	6	100.9 ±20.8	432.8 ±58.9
10.0 g α-LNA	0	156.6 ±27.5	657.5 ±74.3 [bc]
	3	201.6 ±53.1	355.2 ±44.3 [a]
	6	105.4 ±34.6	393.1 ±57.4 [a]
0.7g EPA+DHA	0	144.8 ±31.5	603.8 ±76.0 [bc]
	3	147.9 ±31.9	342.5 ±42.8 [a]
	6	197.2 ±79.6	370.9 ±54.4 [a]
1.5 g EPA+DHA	0	128.7 ±23.4	563.1 ±63.1 [b]
	3	150.7 ±36.3 [c]	377.5 ±43.8 [a]
	6	49.6 ±7.30 [b]	394.8 ±50.2

Significant differences are expressed as:
a. Significantly different from 0 month
b. Significantly different from 3 months
c. Significantly different from 6 months

5.3.2. Oxidative Burst Activity

The oxidative burst activity of neutrophils and monocytes was investigated in three ways. The % of neutrophils or monocytes performing oxidative burst (% cells; also termed recruitment), the production of oxidative species by these cells (MFI) and the

overall index of activity (% cells x MFI) were determined at 0, 3 and 6 months of intervention.

There was a significant increase in the recruitment of neutrophils when stimulated with both *E. coli* and PMA compared to the control samples (approx. 2% for control and >90% for stimulated) and recruitment was similar in response to both stimuli (approx. >90%). There was a significant increase in the activity (i.e. the production of oxidative species) when neutrophils were stimulated. The production of reactive species by neutrophils stimulated with PMA was approximately twice that observed with *E. coli* as the stimulus.

There was a significant increase in the recruitment of monocytes when stimulated with both *E. coli* and PMA compared to the control samples (approx. 2% control and 50-70% for stimulated) and again recruitment was similar for both stimuli (approx. 50 -70%; *Table 5.4.*). There was a significant increase in the activity (i.e. the production of oxidative species) when monocytes were stimulated. The production of reactive species by monocytes stimulated with PMA was similar to that observed with *E. coli* as the stimulus.

5.3.2.1. Analysis of Baseline Data

At baseline there were no significant differences between any of the treatment groups in the % of neutrophils or monocytes performing oxidative burst, in the production of oxidative species by these cells, or in the index of activity. This was the case for both stimuli.

There was no difference in any of these parameters between males and females or with age at baseline.

Relationship between the Fatty Acid Composition of PBMNC PL and Oxidative Burst Activity of Neutrophils and Monocytes at Baseline

The proportions of various fatty acids in PBMNC PL were correlated against the % of neutrophils and monocytes performing oxidative burst, the production of oxidative

species and the index of burst activity after stimulation with *E. coli* and PMA. The correlation coefficients for these relationships are shown in *Tables 5.4.* and *5.5.*

Stimulation of Neutrophils with E. coli.

The % of neutrophils performing oxidative burst, the production of reactive species, and the index of burst activity were all negatively correlated with the ratio of SFA to PUFA in PBMNC PL (*Table 5.4; Figure 5.4.*).

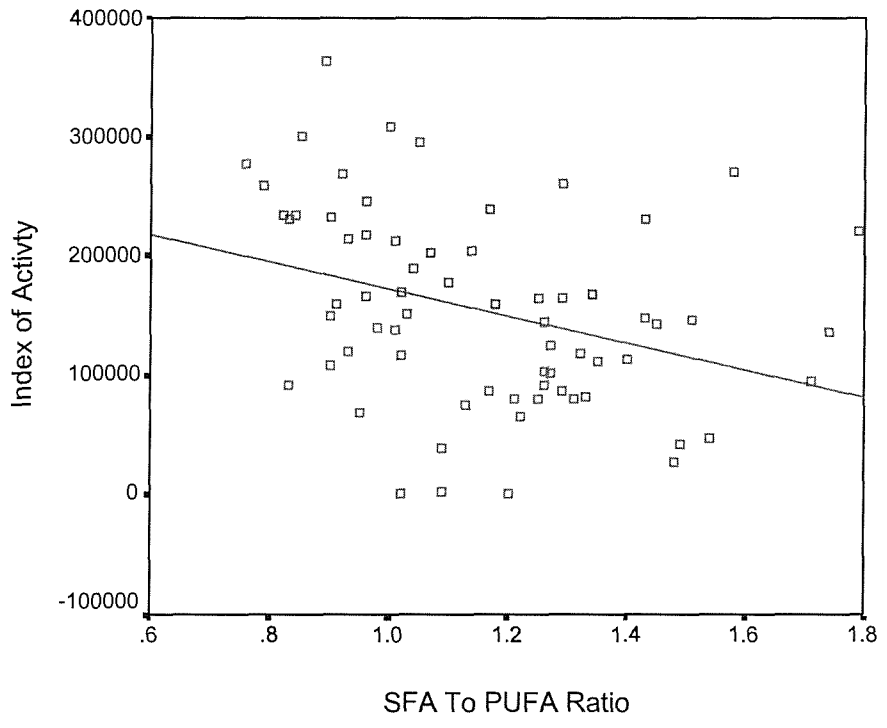
Table 5.4. Correlation coefficients (r) between the fatty acid composition of PBMNC PL and the oxidative burst activity of neutrophils after *E. coli* or PMA stimulation at baseline

	E. coli			**PMA**		
	% Cells	**MFI**	**Index of Activity**	**% Cells**	**MFI**	**Index of Activity**
SFA/PUFA	-0.23 #	-0.46 #	-0.45 #	-0.12	-0.31#	-0.06
Total *n*-6	0.27 #	0.48 #	0.49 #	0.10	0.31#	-0.08
Total *n-3*	0.45 #	0.53 #	0.55 #	0.21	0.39#	0.03
n-6/n-3	-0.38 #	-0.38 #	-0.39 #	-0.21	-0.32#	-0.13
α-LNA	0.36 #	0.62 #	0.62 #	0.23	0.46#	0.00
AA	0.27 #	0.53 #	0.53 #	0.17	0.36#	0.03
EPA	0.22	0.06	0.08	-0.09	0.02	-0.08
DHA	0.39 #	0.36 #	0.38 #	0.11	0.31#	-0.08

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=100

The proportions of individual PUFA (α-LNA, AA and DHA) and of total *n*-6 and *n*-3 PUFA were all positively correlated with the parameters of neutrophil oxidative burst investigated (*Table 5.4.*). Suprisingly, the proportion of EPA was not related to the oxidative burst activity of these cells, although there was a non-significant positive correlation between EPA content and the recruitment of neutrophils. The *n*-6 to *n*-3 PUFA ratio was negatively correlated with parameters of neutrophil respiratory burst (*Table 5.4.*).

Figure 5.4. Correlation between the SFA to PUFA ratio in PBMNC PL and the index of respiratory burst activity of neutrophils after *E. coli* stimulation at baseline



Stimulation of Neutrophils with PMA

In contrast to the findings using *E. coli* as the stimulus, there were no significant correlations between fatty acid composition of PBMNC PL and the recruitment of neutrophils for oxidative burst when they were stimulated with PMA (*Table 5.4.*). However, the relationships between the ratio of SFA to PUFA, and with the proportions total *n-6* and *n-3* PUFA and of individual PUFA and the production of reactive species (i.e. MFI) were the same as those observed using *E. coli* as the stimulant: i.e. as the proportion of SFA increased compared to PUFA there was a significant decrease in production of reactive species, the proportion of individual PUFA (both *n-3* and *n-6*) was positively correlated with the ability of neutrophils to produce reactive species, and the ratio of *n-6* to *n-3* PUFA was negatively correlated with the production of reactive species (*Table 5.4.*). As when *E. coli* was used as the stimulus, there were no significant relationships between of EPA content and measures of oxidative burst by neutrophils stimulated with PMA.

Despite the significant effects on MFI, the lack of significant effect of PBMNC PL fatty acids on recruitment meant that there was no significant relationship between fatty acid composition and the index of activity.

Stimulation of Monocytes with E. coli

There were significant correlations between the composition of the PBMNC PL and the parameters of monocyte oxidative burst (*Table 5.5.*). There was a negative correlation between the SFA to PUFA content and the % of monocytes undergoing respiratory burst. An increased ratio of these fatty acids was associated with an overall decrease in the ability of monocytes to mount an oxidative burst response (i.e. decreased index of activity) despite a compensatory increase in the production of reactive species by the active cells (*Table 5.4.*).

Table 5.5. Correlation coefficients (r) between the fatty acid composition of PBMNC PL and the oxidative burst function of monocytes after stimulation with *E. coli* or PMA at baseline

	E. coli			**PMA**		
	% Cells	**MFI**	**Index of Activity**	**% Cells**	**MFI**	**Index of Activity**
SFA/PUFA	-0.36 #	0.32 #	-0.28 #	-0.40#	0.27#	-0.38#
Total *n*-6	0.37 #	-0.44 #	-0.29 #	0.46#	-0.36#	0.39#
Total *n-3*	0.50 #	-0.45 #	0.38 #	0.52#	-0.36#	0.46#
n-6/n-3	-0.43 #	0.28 #	-0.32 #	-0.40#	0.24#	-0.36#
α-LNA	0.49 #	-0.50 #	0.43 #	0.48#	-0.45#	0.44#
AA	0.34 #	-0.34 #	0.34 #	0.46#	-0.28#	0.43#
EPA	0.16	-0.18	-0.01	0.09	-0.09	0.06
DHA	0.38 #	-0.43 #	0.30 #	0.40#	-0.30#	0.33#

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=100

The proportions of individual PUFA (α-LNA, AA and DHA) and of total *n*-6 and *n*-3 PUFA were all correlated with the measures of monocyte oxidative burst investigated (*Table 5.5.*). There were significant positive correlations between the content of α-LNA, AA, DHA, total *n*-6 PUFA and total *n*-3 PUFA and the recruitment of monocytes. In contrast to neutrophils, there were negative correlations between the proportion of these fatty acids and the capacity to produce reactive species. The overall index of activity was increased with an increase in the proportion of *n*-3 fatty acids. Again the proportion of EPA had little effect on the

oxidative burst activity of monocytes. The ratio of *n*-6 to *n*-3 PUFA was negatively correlated with the recruitment of monocytes, and with the index of activity of these cells, despite a positive relationship between the ratio of these PUFA and the MFI.

Stimulation of Monocytes with PMA

Similar associations between PBMNC PL fatty acid composition and monocyte respiratory burst in response to PMA were seen as were seen when *E. coli* was used as the stimulus (*Table 5.5.*).

5.3.2.2. The Effects of the Different Dietary Treatments on Oxidative Burst

Two-way ANOVA established that there was no significant effect of treatment on the oxidative burst activity of neutrophils or monocytes in terms of the % cells performing oxidative burst, the production of oxidative species or index of activity when either *E. coli* or PMA were the stimulus (*Tables 5.6.* and *5.7.*). However, there was an effect of time on some of these parameters. After *E. coli* stimulation there was no significant effect of time on the % neutrophils or monocytes performing oxidative burst, while there was a significant effect of time on production of reactive species by monocytes ($p<0.027$) but not neutrophils. After PMA stimulation there was no significant effect of time on the % neutrophils performing oxidative burst while for monocytes the effect of time was significant ($p<0.002$). The production of oxidative species by neutrophils or monocytes was not significantly effected by time.

Effect of the Different Treatments on Neutrophil Oxidative Burst Activity

When stimulated with *E. coli* the % of active neutrophils was unchanged over the 6 months of intervention in all treatment groups except the 0.7 g EPA+DHA/day group where there was a significant decrease form 0 to 6 months (*Table 5.6.*). There was a tendency for the production of reactive species by neutrophils to increase in the 1.5 g EPA+DHA/day and 10.0 g α-LNA/day treatment groups from 0 to 6 months, while the production of these species tended towards a decrease in the placebo, 0.7 g EPA+DHA and 5.0 g α-LNA treatment groups over the 6 month intervention period (*Table 5.6.*). These trends for changes in recruitment and reactive species production

resulted in an overall tendency for a decrease in the oxidative burst index of activity by neutrophils in the placebo, 0.7 g EPA+DHA/day and 5.0 g α-LNA/day treatment groups and increases in this parameter for the 1.5 g EPA+DHA/day and 10.0 g α-LNA/day treatment groups. Hence, the increases in recruitment of neutrophils in these latter groups were not adequate to offset the decrease in production of reactive species, and thus the overall activity of the neutrophils was decreased.

When stimulated with PMA there was no change in the recruitment of neutrophils in the placebo, 0.7 g EPA+DHA and 5.0 g α-LNA treatment groups, while in the 1.5 g EPA+DHA and 10.0 g α-LNA treatment groups there was a tendency for an increase in recruitment from 0 to 6 months. There was a tendency for the production of reactive species to increase in these latter treatment groups and to decrease in the 0.7 g EPA+DHA and 5.0g α-LNA treatment groups. The overall oxidative burst index of activity tended towards an increase in the 1.5 g EPA+DHA and 10.0 g α-LNA treatment groups and a decrease in the 0.7 g EPA+DHA and 5.0 g α-LNA treatment groups.

Thus it would seem that, although not significant, there are differential effects of different doses of the *n-3* PUFA, α-LNA, EPA and DHA on the ability of neutrophils to mount an oxidative burst response.

Effect of the Different Treatments on Monocyte Oxidative Burst Activity

There were no significant effects of the interventions on the recruitment of monocytes, the production of reactive species or on the index of activity (*Table 5.7*). When stimulated with *E. coli* , over the 6 month intervention period, there was a tendency for an increase in recruitment of monocytes in the 0.7 g EPA+DHA, 1.5 g EPA+DHA and 10.0 g α-LNA treatment groups. However, in the 5.0 g α-LNA treatment group there was no change in recruitment, and in the placebo group there was a tendency for a decrease in the % cell performing oxidative burst. In all groups there was a tendency for a decrease in monocyte oxidative burst index of activity from 0 to 6 months. These trends in changes in recruitment and production of oxidative burst reactive species resulted in a tendency for a decrease in monocyte index of activity in all groups except the 0.7 g EPA+DHA group. Thus, the decreases

(and increases) in the production of oxidative species were reflected in the index of activity.

When stimulated with PMA there was a tendency for an increase in recruitment of monocytes in all treatment groups over the intervention period. The production of reactive species was unchanged in the placebo and 1.5 g EPA+DHA treatment groups, and tended towards an increase in the 10.0 g α-LNA treatment group and a decrease in the 0.7 g EPA+DHA and 5.0 g α-LNA treatment groups. These trends for changes in recruitment and production of oxidative burst reactive species resulted in a tendency for an increase in the oxidative burst index of activity in the placebo and 1.5 g EPA+DHA treatment groups, and a decrease in the 10.0 g α-LNA treatment group. The index of activity was unchanged in the 0.7 g EPA+DHA and 5.0 g α-LNA treatment groups. Thus, the tendency for a decrease in the production of reactive species was reflected in the index of activity for the 0.7 g EPA+DHA and 5.0 g α-LNA since there was no increase in monocyte recruitment. In the 1.5 g EPA+DHA and 10.0 g α-LNA the tendency for an increase in recruitment and increase in the production of reactive species was reflected in a trend for an increase in the index of activity.

Table 5.6. The effect of the different treatment groups on the oxidative burst activity of neutrophils (n=20/group)

Treatment	Time (m)	% Cells			Median Fluorescence Intensity (MFI)		Index of Activity (x 10^3) (% Cell x MFI)	
		Control	*E. coli*	PMA	*E. coli*	PMA	*E. coli*	PMA
Placebo	0	1.4 ±0.2	91.2 ±1.7	93.6 ±0.8	645 ±75	1414 ±159	60 ±7	133 ±15
	3	1.9 ±0.4	92.2 ±1.4	93.6 ±1.4	922 ±99 [c]	1687 ±257	85 ±9 [c]	160 ±26
	6	2.7 ±0.7	87.9 ±4.4	93.1 ±1.4	594 ±55 [b]	1908 ±260	54 ±5 [b]	180 ±25
5.0 g α-LNA	0	2.7 ±0.5	93.8 ±1.1	91.1 ±4.5	764 ±95	1729 ±167	73 ±9	165 ±17
	3	1.8 ±0.4	88.7 ±3.6	89.9 ±3.1	888 ±99 [c]	1459 ±146	50 ±9	133 ±15
	6	2.3 ±0.5	91.2 ± 1.9	87.4 ±5.3	581 ±47 [b]	1373 ±179	53 ±4	131 ±19
10.0 g α-LNA	0	3.4 ±1.1	92.3 ±1.3	79.0 ±8.7	575 ±85 [b]	1354 ±232	53 ±8 [b]	126 ±24
	3	2.5 ±0.6	90.6 ±2.5	95.2 ±0.7	971 ±110 [a]	1652 ±167	90 ±11 [a]	158 ±16
	6	2.6 ±0.7	92.3 ±1.5	95.8 ±1.6	738 ±70	1925 ±274	69 ±7	187 ±27
0.7g EPA+DHA	0	1.9 ±0.4	91.6 ±1.8 [c]	90.3 ±2.9	759 ±109	1677 ±186	71 ±11	152 ±19
	3	2.0 ±0.4	93.7 ±1.3	93.1 ±1.3	1049 ±151	1751 ±261	99 ±15	165 ±25
	6	5.1 ±4.2	84.2 ±5.5 [a]	92.1 ±2.9	649 ±81	1456 ±197	59 ±7	136 ±20
1.5 g EPA+DHA	0	2.8 ±0.5	91.9 ±1.4	88.6 ±5.4	729 ±95	1629 ±188	69 ±9	157 ±19
	3	2.9 ±0.5	93.8 ±1.3	92.1 ±2.0	819 ±106	1547 ±189	77 ±10	145 ±19
	6	2.4 ±0.6	91.3 ±1.7	96.2 ±0.7	812 ±109	2045 ±240	75 ±11	197 ±23

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;

Table 5.7. The effect of the different treatment groups on the oxidative burst activity of monocytes (n=20/group)

Treatment	**Time (m)**	**% Cells**			**Median Fluorescence Intensity (MFI)**		**Index of Activity (x 10^3) (% Cell x MFI)**	
		Control	***E. coli***	**PMA**	***E. coli***	**PMA**	***E. coli***	**PMA**
Placebo	0	5.5 ±1.1	53.9 ±7.4	58.3 ±8.6	282 ±70	233 ±37	8 ±2	10 ±2
	3	4.0 ±1.1	46.8 ±6.5	64.4 ±7.7	437 ±126	280 ±60	13 ±4	14 ±3
	6	4.2 ±0.9	45.4 ±6.8	73.4 ±7.2	222 ±58	234 ±51	6 ±2	13 ±2
5.0 g α-LNA	0	6.8 ±1.4	46.1 ±6.9	54.7 ±9.3	310 ±103	372 ±133	7 ±1	10 ±2
	3	5.5 ±0.8	51.3 ±6.9	67.9 ±8.3	390 ±120	296 ±66	11 ±3	12 ±2
	6	8.8 ±2.9	46.2 ±7.3	63.0 ±7.9	265 ±76	222 ±83	9 ±3	10 ±2
10.0 g α-LNA	0	4.6 ±1.2	40.8 ±7.3	46.6 ±10.1	352 ±82	188 ±16	11 ±5	17 ±8
	3	5.1 ±0.8	52.7 ±7.4	69.7 ±8.8	438 ±136	352 ±94	14 ±4	22 ±9
	6	5.0 ±0.9	48.6 ±8.4	76.5 ±8.2	250 ±64	204 ±38	6 ±0.7	13 ±2
0.7g EPA+DHA	0	6.5 ±1.2	46.0 ±6.8	53.4 ±8.3	395 ±93	288 ±49	11 ±2	10 ±2
	3	4.4 ±0.8	56.2 ±7.5	70.2 ±8.2	361 ±117	227 ±40	9 ±2	12 ±2
	6	5.4 ±0.9	57.0 ±7.5	72.5 ±8.6	199 ±45	204 ±37	7 ±0.9	10 ±1
1.5 g EPA+DHA	0	4.9 ±1.1	43.5 ±6.7	56.0 ±9.2	309 ±66	238 ±38	8 ±6	11 ±7
	3	7.1 ±1.3	55.9 ±6.8	67.1 ±8.7	305 ±101	216 ±25	14 ±0.4	13 ±8
	6	4.1 ±0.8	49.2 ±7.1	77.5 ±6.8	207 ±48	241 ±46	6 ±3	15 ±10

Effects of Gender and Age on the Oxidative Burst Activity of Neutrophils and Monocytes with the Different Intervention Treatments

There were no significant effects of gender or age on the oxidative burst activity of neutrophils or monocytes at any time during the intervention in any of the treatment groups.

The Relationships between the Changes in the Fatty Acid Composition of PBMNC PL and Changes in the Oxidative Burst Activity of Monocytes and Neutrophils with the Different Treatments

The correlation coefficients between the fatty acid composition of PBMNC PL and the measures of respiratory burst activity at baseline suggest that the activity of neutrophils and monocytes is influenced by fatty acid composition (*Table 5.4.*; *Table 5.5.*). The relationship between the changes in fatty acid composition of PBMNC PL and the measures of respiratory burst activity, after stimulation with *E. coli* and PMA, were investigated for all subjects together (n=100), and for the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups (n=30/group). The relationship between changes in respiratory burst activity and changes in fatty acid composition were not investigated in the 5.0 g α-LNA/day and 0.7 g EPA+DHA/day groups since the changes in the composition of the PBMNC PL were not significant (see section 4.3.6.).

Relationships between the changes in fatty acid composition of PBMNC PL and respiratory burst activity of neutrophils stimulated with E. coli or PMA

There were no significant relationships between changes in fatty acid composition of PBMNC PL and changes in any measure of neutrophil respiratory burst activity with *E. coli* stimulation, when the dietary groups were considered as a whole or when the 10.0 g α-LNA/day group alone was considered (data not shown). However, there were significant relationships between the changes in measures of respiratory burst and changes in fatty acid composition when the 1.5 g EPA+DHA/day group was considered alone (*Table 5.8*). Changes in the proportion of total SFA and in the SFA to PUFA ratio were negatively correlated with the changes in the recruitment of

neutrophils and the production of oxidative species. Changes in the proportions of total PUFA, *n*-6 and *n*-3 PUFA and the individual PUFA (α-LNA, EPA and DHA) were all positively correlated with the recruitment of neutrophils and the production of reactive species. Changes in the ratio of *n*-6 to *n*-3 PUFA were also associated with changes in respiratory burst activity by neutrophils (*Table 5.8.*). As this ratio increased there was a decrease in both recruitment of cells and production of reactive species.

Table 5.8. Correlation Coefficients (r) between change in fatty acid composition of PBMNC PL and changes in measures of neutrophil respiratory burst activity in the 1.5 g EPA+DHA/day group (0 to 6 month) with *E. coli* stimulation

	% Cells	**MFI**
SFA	-0.829 #	-0.600
PUFA	0.486	0.900 #
SFA/PUFA	-0.714	-0.600 #
Total n-6	0.429	0.800 #
Total n-3	0.829 #	0.700 #
n-6/n-3	-0.943 #	-0.700 #
α-LNA	0.500 #	0.500
EPA	0.900 #	1.000 #
DHA	0.714	0.800 #

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=20

When stimulated with PMA there were significant relationships between changes in PBMNC PL and the recruitment of neutrophils and production of reactive species in all the groups considered as a whole, and in the 1.5 g EPA+DHA/day group. Changes in the respiratory burst activity of neutrophils with PMA stimulation was similarly related to the fatty acid composition of PBMNC PL in a similar way as when *E. coli* was the stimulus. There were no significant relationships between the changes in neutrophil recruitment and production of reactive species and changes in fatty acid composition of PBMNC PL in the 10.0 g α-LNA/day group.

Relationships between changes in fatty acid composition of PBMNC PL and respiratory burst activity of monocytes stimulated with E. coli or PMA

There were no significant relationships between changes in fatty acid composition of PBMNC PL and changes in any measure of monocyte respiratory burst activity, with *E. coli* stimulation, when the 10.0 g α-LNA/day group was considered alone.

However, there were significant relationships between changes in measures of respiratory burst and fatty acid composition when the 1.5 g EPA+DHA/day group was considered alone. In this group changes in the proportion of total SFA and the SFA to PUFA ratio were both negatively correlated with changes in recruitment of monocytes and index of monocyte respiratory burst activity (r=-0.657 and r= -0.600, respectively for recruitment of monocytes; r=-0.679 and r=-0.750, respectively for production of reactive species). Changes in total PUFA and *n*-6 PUFA were positively correlated to both of these parameters (r=0.543 and r= 0.486, respectively for recruitment of monocytes; r=0.893 and r= 0.857, respectively for index of monocyte respiratory burst activity). Changes in proportion of *n*-3 PUFA was positively correlated only with recruitment of monocytes (r=0.771). Changes in proportions of individual fatty acids (α-LNA, EPA and DHA) were positively correlated to changes in the recruitment of monocytes and the overall index of respiratory burst activity (r= 0.500, r=1.00 and r=0.771, respectively, for recruitment of monocytes; r=0.500, r=0.900 and r=0.321, respectively, for index of monocyte activity). There were no significant relationships between the changes in fatty acid composition of PBMNC PL and changes in monocyte recruitment or the index of monocyte respiratory burst activity when the groups were considered together as a whole. However, there were significant negative correlations between the changes in total proportion of PUFA and *n*-6 PUFA and changes in the production of reactive species (r=-0.359 and r=-0.344 respectively). Furthermore changes in the SFA to PUFA ratio were significantly positively associated with changes in production of reactive species by monocytes (r=0.319) in this group. There were no significant relationships between the changes in fatty acid composition of PBMNC PL and monocyte respiratory burst activity when PMA was used to elicit a response.

5.3.3. Phagocytic Activity

The phagocytic activity of neutrophils and monocytes was investigated in three ways. The % of neutrophils or monocytes performing phagocytosis (% cells or recruitment), the number of bacteria engulfed per active neutrophil or monocyte (MFI) and the overall index of activity (% cells x MFI) were determined at 0, 3 and 6 months of intervention.

There was a significant increase in the recruitment of neutrophils when stimulated with *E. coli* compared to the control cells not stimulated (approx. 2% for control and >70% for stimulated cells). There was a significant increase in the recruitment of monocytes when stimulated with *E. coli* compared to the control cells (approx. 2% control and >20% for stimulated cells). There was a significant increase in the activity, that is the number of bacteria engulfed by active cells, when neutrophils or monocytes were stimulated.

5.3.3.1. Analysis of Baseline Data

At baseline there were no significant differences between any of the treatment groups in the % of neutrophils or monocytes performing phagocytosis, in the number of bacteria engulfed by these active cells, or in the index of activity at baseline (*Tables 5.10* and *5.11*). There was no difference in any of these parameters between males and females or with age at baseline.

Relationship between Fatty Acid Composition of PBMNC PL and Phagocytic Activity at Baseline

The proportions of various fatty acids in PBMNC PL were correlated against the absolute % of neutrophils and monocytes performing phagocytosis, the number of bacteria engulfed by the active cells and the overall index of activity after stimulation with *E. coli* (*Table 5.9).*

Relationship between Fatty Acid Composition of PBMNC PL and Neutrophil Phagocytosis

The recruitment of neutrophils, their ability to engulf bacteria and the overall index of phagocytic activity were significantly negatively correlated to the SFA to PUFA ratio of PBMNC PL (*Table 5.9.*; *Figure 5.5.*). The proportions of individual PUFA (α-LNA, AA and DHA) and of total PUFA were positively correlated with recruitment of neutrophils, number of bacteria engulfed and the overall index of activity (*Table 5.9.*), while the ratio of *n*-6 to *n*-3 PUFA was negatively correlated with an increase in all parameters measured. There was little relationship between the

proportion of EPA and phagocytic activity, but the AA/EPA ratio correlated positively with the activity of these cells.

Figure 5.5. The relationship between the SFA to PUFA ratio in PBMNC PL and the index of phagocytic activity of neutrophils after *E. coli* stimulation at baseline

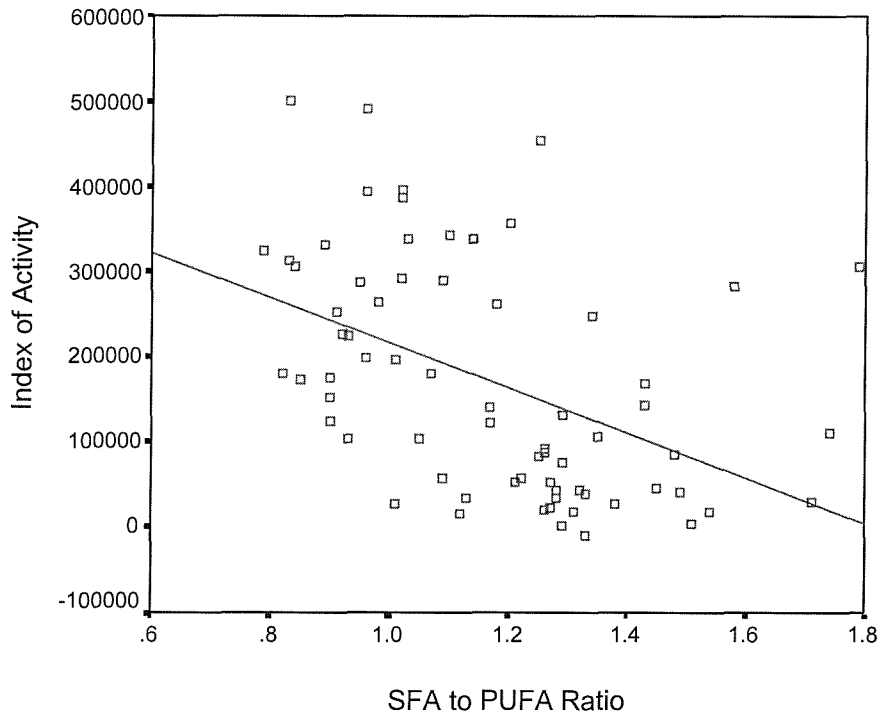


Table 5.9. The correlation coefficients (r) between the fatty acid composition of PBMNC PL and the phagocytic function of neutrophils after *E. coli* stimulation at baseline

	Monocytes			Neutrophils		
	% Cells	**MFI**	**Index of Activity**	**% Cells**	**MFI**	**Index of Activity**
SFA/PUFA	-0.40 #	-0.46 #	-0.50 #	-0.28 #	-0.52 #	-0.46 #
Total PUFA	0.34 #	0.20 #	0.24 #	0.28 #	0.18 #	0.31 #
n-6/n-3	-0.14	-0.25 #	-0.21	-0.06	-0.31 #	-0.21
α-LNA	0.67 #	0.63 #	0.72 #	0.41 #	0.60 #	0.63 #
AA	0.38 #	0.49 #	0.50 #	0.30 #	0.49 #	0.45 #
EPA	-0.13	0.03	-0.06	-0.12	0.03	-0.07
DHA	0.28 #	0.52 #	0.47 #	0.20	0.55 #	0.43 #
AA/EPA	0.31 #	0.20	0.29 #	0.22	0.19	0.25 #

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=100

Relationship between Fatty Acid Composition of PBMNC PL and Monocyte Phagocytosis

The recruitment of monocytes, their ability to engulf bacteria and the overall index of phagocytic activity were significantly negatively correlated with the SFA to PUFA ratio of PBMNC PL (*Table 5.9.*).

The proportions of individual PUFA (α-LNA, AA and DHA) and of total PUFA were positively correlated with recruitment of monocytes, the number of bacteria engulfed and the overall index of activity (*Table 5.9.*). There was little relationship between the proportion of EPA and phagocytic activity, but the AA/EPA ratio correlated positively with the activity of these cells.

5.3.3.2. The Effects of the Treatments on the Phagocytic Activity

Two-way ANOVA established that there were no significant effects of treatment on the % of neutrophils or monocytes performing phagocytosis or on the number of bacteria engulfed per active neutrophil or monocyte. There were however significant effects of time on the % of neutrophils performing phagocytosis ($p<0.010$) and the % of monocytes performing phagocytosis ($p<0.035$). The number of bacteria engulfed per active neutrophil was also significantly affected by time ($p<0.0001$), as was the number of bacteria engulfed per active monocyte ($p<0.0001$). These effects were investigated further.

Effect of Treatment on Neutrophil Phagocytosis

There were no significant effects of the treatments on the recruitment of neutrophils, although there was a tendency for an increase in recruitment from 0 to 6 months for all treatment groups, except the 5.0 g α-LNA/day group where there was tendency towards a decrease (*Table 5.10.*). The number of bacteria engulfed per active neutrophil tended to increase in all treatment groups from 0 to 6 months, and this increase was significant for the 1.5 g EPA+DHA/day group. The overall index of activity increased significantly in the 1.5 g EPA+DHA/day and 10.0 g α-LNA/day treatment groups, with a tendency towards an increase in the remaining groups (*Table 5.10.*).

Effect of Treatment on Monocyte Phagocytic Activity

There were no significant effects of the treatments on the recruitment of monocytes (*Table 5.11.*), although, there was a tendency for a decrease in all groups, receiving *n*-3 PUFA while in the placebo group recruitment tended to increase. There was a tendency for an increase in the number of bacteria engulfed per monocyte in all groups and this reached significance for the placebo and 1.5 g EPA+DHA/day groups after 6 months. The changes in recruitment and MFI resulted in an increase in the phagocytic index of activity for the placebo, 1.5 g EPA+DHA/day and 10.0 g α-LNA/day groups and a decrease in the index of activity in the 0.7 g EPA+DHA/day and 5.0 g α-LNA/day groups; however, these were not statistically significant changes.

Effects of Gender and Age on the Phagocytic Activity of Neutrophils and Monocytes with the Different Intervention Treatments

There were no effects of gender or age on the phagocytic activity of neutrophils or monocytes at any time during the intervention in any of the treatment groups.

Table 5.10. The effect of the different treatment groups on the phagocytic activity of neutrophils (n=20/group)

Treatment	**Time (m)**	**% Cells**		**Median Fluorescence Intensity (MFI)**	**Index of Activity (x 10^3)**
		Control	***E. coli***	***E. coli***	**% Cell x MFI**
Placebo	0	1.3 ±0.1	70.4 ±4.2	2106 ±332	171 ±31
	3	1.9 ±0.4	77.6 ±2.1	2259 ±241	179 ±21
	6	1.6 ±0.3	81.1 ±3.4	3253 ±494	253 ±38
5.0 g α-LNA	0	1.3 ±0.3	80.3 ±4.7	2517 ±313	222 ±31
	3	1.6 ±0.2	87.0 ±2.1	1970 ±170	176 ±17
	6	2.0 ±0.4	75.7 ±2.9	2787 ±402	209 ±29
10.0 g α-LNA	0	1.5 ±0.2	71.9 ±4.9	2394 ±457	172 ±31 [c]
	3	1.5 ±0.3	74.8 ±4.9	2746 ±243	196 ±18
	6	1.2 ±0.2	77.5 ±2.3	3565 ±528	288 ±45 [a]
0.7g EPA+DHA	0	1.7 ±0.3	71.1 ±6.0	2703 ±412	176 ±35
	3	1.6 ±0.3	82.0 ±2.8	2529 ±189	209 ±19
	6	1.6 ±0.3	77.5 ±5.5	3011 ±438	215 ±33
1.5 g EPA+DHA	0	1.6 ±0.2	67.3 ±5.5	1901 ±316 [c]	136 ±29 [c]
	3	1.8 ±0.6	75.7 ±3.2	2566 ±215	196 ±19
	6	3.2 ±0.6	80.9 ± 2.7	3106 ±396 [a]	253 ±34 [a]

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 months; c. Significantly different from 6 months

Table 5.11. The effect of the different treatment groups on the phagocytic activity of monocytes (n=20/group)

Treatment	**Time (m)**	**% Cells**		**Median Fluorescence Intensity (MFI)**	**Index of Activity (x 10^3)**
		Control	***E. coli***	***E. coli***	**% Cell x MFI**
Placebo	0	2.2 ±0.4	18.7 ±2.1	1585 ±236 [c]	36 ±7
	3	2.4 ±0.5	24.6 ±3.5	2151 ±261	46 ±7
	6	3.0 ±0.6	23.2 ±4.1	2589 ±301 [a]	60 ±14
5.0 g α-LNA	0	3.4 ±0.8	24.1 ±2.9	1896 ±238	51 ±9
	3	2.0 ±0.9	34.7 ±4.5 [c]	1663 ±176	62 ±12
	6	2.3 ±0.8	17.3 ±3.2 [b]	2182 ±419	36 ±11
10.0 g α-LNA	0	3.0 ±0.6	23.3 ±3.3	1660 ±260	45 ±12
	3	2.5 ±0.4	26.6 ±3.9	2270 ±176	56 ±8
	6	1.4 ±0.3	19.5 ±3.4	2816 ±582	58 ±22
0.7g EPA+DHA	0	4.6 ±0.9 [bc]	23.8 ±3.1	1844 ±264	43 ±9
	3	1.8 ±0.3 [a]	23.4 ±2.7	2270 ±217	56 ±7
	6	1.9 ±0.3 [a]	15.5 ±2.6	2868 ±655	32 ±7
1.5 g EPA+DHA	0	2.6 ±0.6	25.1 ±3.1	1265 ±220 [c]	36 ±8
	3	2.1 ±0.6	19.2 ±4.1	2151 ±238	36 ±7
	6	2.5 ±0.6	23.9 ±4.9	3335 ±604 [a]	81 ±21

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 months; c. Significantly different from 6 months

Relationships between Changes in the Fatty Acid Composition of PBMNC PL and Changes in Phagocytic Activity of Neutrophils and Monocytes after the Different Treatments

The correlation coefficients between the fatty acid composition of PBMNC PL and the measures of phagocytic activity at baseline suggest that the activity of neutrophils and monocytes is influenced by fatty acid composition (*Table 5.9*). The relationship between the changes in fatty acid composition of PBMNC PL and the measures of phagocytic activity, after stimulation with *E. coli*, were investigated for the dietary groups together (n=100), and for the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups alone (n=20/group). The relationship between changes in phagocytic activity and changes in fatty acid composition were not investigated in the 5.0 g α-LNA/day and 0.7 g EPA+DHA/day groups since the changes in the composition of the PBMNC PL were not significant (see section 4.3.6.).

There were no significant relationships between changes in the fatty acid composition of PBMNC PL and changes in the measures of phagocytic activity of monocytes in the 10.0 g α-LNA day group. However, there were significant relationships when the groups were considered together and in the 1.5 g EPA+DHA/day group alone. In this latter group changes in the proportion of SFA and the SFA to PUFA ratio were significantly, negatively correlated with both the changes in the number of monocytes performing phagocytosis and the index of monocyte phagocytic activity. There were no relationships between changes in the MFI (number of bacteria engulfed per monocyte) and the fatty acid composition of PBMNC PL (*Table 5.12.*). Changes in the proportions of total PUFA, *n*-6 and *n*-3 PUFA and of the individual fatty acids)AA, EPA and DHA) were all positively related to changes in the number of monocytes performing phagocytosis and the index of monocyte phagocytic activity (*Table 5.12.*). Changes in the ratio of *n*-6 to *n*-3 PUFA were negatively correlated to both these measures of phagocytic activity. There were no significant or strong correlations between the changes in numbers of bacteria engulfed per monocyte and the fatty acid composition of PBMNC PL in the 1.5 g EPA+DHA/day group

When all the groups were considered together there were similar relationships between changes in fatty acid composition of PBMNC PL and measures of phagocytic activity as seen in the 1.5 g EPA+DHA/day group. However, the only significant relationships observed were between the changes in proportions of total PUFA, *n*-6 PUFA and AA and the index of monocyte phagocytic activity where there were positive correlations (r= 0.391, r=0.376 and r=0.451 respectively), and also between the changes in the proportion of AA and the number of monocytes performing phagocytosis (r=0.302).

Table 5.12. Correlation Coefficients (r) between changes in fatty acid composition of PBMNC PL and changes in measures of monocyte phagocytic activity in the 1.5 g EPA+DHA/day group (0 to 6 months) with *E. coli* stimulation

	% Cells	**MFI**	**Index of Activity**
SFA	-0.900 #	-0.179	-0.886 #
PUFA	0.900 #	0.286	0.429
SFA/PUFA	-0.900 #	0.00	-0.886 #
Total *n*-6	0.900 #	0.00	0.886 #
Total *n*-3	0.800	0.029	0.800
n-6/*n*-3	-0.600	-0.179	-0.429
AA	0.700	-0.036	0.600
EPA	0.992 #	0.400	0.900 #
DHA	0.100	-0.214	0.257

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=20

There were no significant relationships between changes in the fatty acid composition of PBMNC PL and the measures of phagocytic activity of neutrophils in the 10.0 g α-LNA/day group. However there were significant relationships when the groups were considered together and in the 1.5 g EPA+DHA/day group alone. The correlation coefficients for the 1.5 g EPA+DHA/day group are shown in Table 5.13. The relationships between changes in neutrophil phagocytic activity and PBMNC PL fatty acid composition were very similar to those seen for monocytes in this dietary group (Table 5.12). When the groups were considered together the only significant relationships between changes in fatty acid composition of PBMNC PL and neutrophil phagocytic activity were that the changes in proportions of total PUFA, n-6 PUFA and the fatty acid AA were positively correlated with changes in the index of neutrophil phagocytic activity (r= 0.391, r=0.376 and r= 0.451, respectively).

Changes in the proportion of AA were also positively correlated to the number of neutrophils performing phagocytosis (r=0.302).

Table 5.13. Correlation Coefficients (r) between change in fatty acid composition of PBMNC PL and changes in measures of neutrophil phagocytic activity in the 1.5 g EPA+DHA/day group (0 to 6 months) with *E. coli* stimulation

	% Cells	MFI	Index of Activity
SFA	-0.943 #	0.036	-0.600
PUFA	0.029	0.393	0.829 #
SFA/PUFA	-0.943 #	0.179	-0.600
Total *n*-6	0.943 #	-0.143	0.600
Total *n*-3	0.700	0.029	0.800
n-6/*n*-3	-0.486	-0.036	-0.600
AA	0.771 #	-0.143	0.543
EPA	0.800	0.400	0.999 #
DHA	-0.200	-0.179	-0.257

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=20

5.3.4. LPS-Elicited Cytokine production by Cultured PBMNC

The concentrations of the cytokines TNF-α, IL-1β, IL-6 and IL-10 were measured in the media taken from PBMNC cultured with LPS at 0, 3 and 6 months of intervention.

There were significant increases in the concentrations of TNF-α, IL-1β, IL-6 and IL10 when the PBMNC were cultured with LPS compared to the control cells not cultured with LPS (approx. 1500 pg/ml, 9000 pg/ml, 400 pg/ml and 30 pg/ml for TNF-α, IL-6, IL-1β and IL-10 respectively for the control cells; > 7000 pg/ml, >25000 pg/ml, >5000 pg/ml and >400 pg/ml for TNF-α, IL-6, IL-1β and IL-10, respectively, for the LPS-stimulated cells).

5.3.4.1. Baseline data

There were no significant differences between the treatment groups in the concentrations of TNF-α, IL-1β, IL-6 and IL-10 in the culture media taken from PBMNC cultured with LPS at baseline. There were no significant differences in the concentrations of any of the cytokines measured between the different genders or with age at baseline.

Relationships between the Fatty Acid Composition of PBMNC PL and the Production of the Cytokines TNF-α, IL-1β IL-6 and IL-10 at Baseline

The correlation coefficients between the fatty acid composition of PBMNC PL at baseline and the concentrations of cytokines produced after LPS-stimulation of PBMNC suggest that the capacity of PBMNC to produce IL-1β, IL-6 and IL-10 (but not TNF-α) is influenced by their fatty acid composition (Table 5.14.).

The proportions of α-LNA, AA, total PUFA, and total *n*-6 PUFA, and the ratio of PUFA to SFA in the PBMNC PL were all negatively correlated with IL-6 and IL-10 produced by PBMNC stimulated with LPS (*Table 5.14.*). Total *n*-3 PUFA and DHA content were positively correlated with IL-1β production and negatively correlated with IL-10 production (*Table 5.14.*). The proportion of EPA in PBMNC PL was not significantly related to production of any of the cytokines measured.

Table 5.14. The correlation coefficients (r) between the fatty acid composition of PBMNC PL and the concentration of cytokines measured in medium taken from PBMNC cultured with LPS

	TNF-α	**IL-6**	**IL-1β**	**IL-10**
Total SFA	-0.084	0.232	0.012	-0.187
Total PUFA	-0.121	-0.365#	0.182	-0.441#
SFA/PUFA	0.063	0.176	-0.130	0.309#
Total *n*-6	-0.168	-0.513#	0.080	-0.505#
Total *n-3*	-0.006	-0.007	0.209#	-0.204#
n-6/n-3	0.006	-0.180	-0.154	-0.022
α-LNA	-0.06	-0.487#	0.177	-0.590#
AA	-0.163	-0.414#	0.084	-0.441#
EPA	-0.043	-0.124	0.055	-0.042
DHA	0.000	-0.090	0.255#	-0.238#
AA/EPA	-0.01	-0.090	-0.042	-0.161

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=150

5.3.4.2. The Effects of the Different Treatments on the Production of the Cytokines TNF-α, IL-1β, IL-6 and IL10

Two-way ANOVA established that there was no effect of treatment on the concentration of the cytokines TNF-α, IL-1β, IL-6 and IL-10 in culture medium from PBMNC stimulated with LPS. However, there was a significant effect of time

on concentrations of TNF-α ($p<0.007$), IL-1β ($p<0.0001$) and IL-6 ($p<0.001$). These effects were investigated further.

The concentrations of the cytokines measured during the intervention period are shown in *Table 5.15.* There was a trend in all treatment groups for the level of TNF-α to decrease from 0 to 3 months of intervention. The concentration of this cytokine then returned to near baseline levels by 6 months, except in the 5.0 g α-LNA/day group. The concentration of TNF-α at 3 and 6 months was significantly different in the placebo and 1.5 g EPA+DHA/day groups. Overall, there was no significant difference in TNF-α concentrations from 0 to 6 months in any of the groups (*Table 5.15.*).

The concentration of IL-6 also tended to decrease from 0 to 3 months and then return to near baseline concentrations in the placebo, 0.7 g EPA+DHA/day and 10.0 g α-LNA/day groups. IL-6 production was significantly different for the 0.7 g EPA+DHA/day group between 3 and 6 months. Overall, there was a non-significant tendency for an increase in the concentration of this cytokine in the both EPA+DHA groups with no change in either of the α-LNA groups from 0 to 6 months.

The concentration of IL-1β was increased in all treatment groups except the 10.0 g α-LNA/day group from 0 to 6 months. The increase in IL-1β production was significant in the placebo and both EPA+DHA groups.

The pattern of a decrease from 0 to 3 months and then a return to near baseline concentration at 6 months seen for TNF-α and IL-6 was also apparent for IL-10 in the placebo and EPA+DHA groups. The concentrations of IL-10 at 3 and 6 months were significantly different for the placebo and 1.5 g EPA+DHA groups. There was a tendency towards an increase in the concentration of IL-10 in both the α-LNA groups.

Table 5.15. Cytokine concentrations in the medium of monocytes cultured with and without LPS (n=30/group)

Treatment	Time (m)	TNF-α (pg/ml)		IL-6 (pg/ml)		IL-1β(pg/ml)		IL-10 (pg/ml)	
		- LPS	**+ LPS**	**- LPS**	**+ LPS**	**- LPS**	**+ LPS**	**- LPS**	**+ LPS**
Placebo	0	1710 ±345	11119 ±1337	17689 ±2950	37223 ±4479	924 ±197	5611 ±615 [c]	71 ±14	577 ±118
	3	1071 ±254	7301 ±1195 [c]	12320 ±2924	24208 ±4177	723 ±202	4954 ±888 [c]	44 ±10	352 ±97 [c]
	6	2368 ±606	12375 ±1630 [b]	19150 ±3808	28740 ±4444	1322 ±414	8616 ±864 [ab]	147 ±34	785 ±123 [b]
5.0 g α-LNA	0	1947 ±453	12266 ±1405	16033 ±3149	33403 ±3673	1088 ±213	5660 ±636	74 ±12	469 ±68
	3	2350 ±622	10632 ±2446	17434 ±3442	35807 ±4749	1461 ±577	5432 ±884	66 ±21	421 ±91
	6	1937 ±464	9635 ±1231	18210 ±3576	36714 ±4826	1259 ±293	6853 ±782	86 ±17	687 ±115
10.0 g α-LNA	0	2468 ±456	11521 ±1329	22626 ±3636	39421 ±4442	1262 ±306	5259 ±679	112 ±12	361 ±68
	3	1444 ±447	8148 ±1215	15228 ±3283	27365 ±4818	969 ±219	5162 ±847	53 ±21	332 ±91
	6	2087 ±640	10671 ±1480	17040 ±3561	37120 ±3404	1170 ±313	5611 ±721	92 ±17	573 ±115
0.7g EPA+DHA	0	1855 ±397	8417 ±1181	15778 ±3050	35528 ±4770	750 ±246	4678 ±772 [c]	69 ±13	490 ±96
	3	1164 ±269	6688 ±1046	9315 ±1826	22280 ±3901 [c]	689 ±127	3875 ±539 [c]	107 ±22	362 ±78
	6	1801 ±381	9492 ±1357	17780 ±3614	40158 ±4233 [b]	1211 ±297	7266 ±869 [ab]	171 ±31	622 ±84
1.5 g EPA+DHA	0	2016 ±467	10976 ±1784	14422 ±2608	31208 ±4144	562 ±94	4848 ±660	102 ±32	663 ±169
	3	1643 ±449	8804 ±1166	12895 ±2912	32798 ±4553	424 ±82	5085 ±989	34 ± 9	443 ±105 [c]
	6	2279 ±499	12195 ±1366	18775 ±3559	42595 ±4060	990 ±231	6728 ±722	167 ±41	800 ±107 [b]

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 months; c. Significantly different from 6 months

Effects of Gender and Age on the Production of the Cytokines TNF-α, IL-1β, Il-6 and IL-10 with the Different Treatments

There were no effects of the gender or age of the subjects on the concentrations of the cytokines measured in the media taken from PBMNC cultured with Con A.

Relationships between the Changes in the Fatty Acid Composition of PBMNC PL and Changes in the Production of the Cytokines TNF-α, IL-1β, Il-6 and IL-10 with the Different Treatments

The correlation coefficients between the fatty acid composition of PBMNC PL and the production of LPS-elicited cytokines at baseline suggest cytokine production by monocytes is influenced by their fatty acid composition (*Table 5.14.*). The relationship between the changes in fatty acid composition of PBMNC PL and the production of these cytokines were investigated for the dietary groups together (n=150), and for the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups (n=30/group). The relationship between changes in cytokine production and changes in fatty acid composition were not investigated in the 5.0 g α-LNA/day and 0.7 g EPA+DHA/day groups since the changes in the composition of the PBMNC PL were not significant (see section 4.3.6.).

There were no significant relationships between the changes in fatty acid composition of PBMNC PL and changes in production of TNF-α, IL-6, IL-1β and IL-10 by PBMNC in the 10.0 g α-LNA/day group. The only significant relationship in the groups when considered as a whole was in the production of IL-6 where changes in production were positively correlated to the proportion of AA in PBMNC PL (r=0.238). In the 1.5 g EPA+DHA/day group there were several significant relationships between changes in IL-6 production and changes in fatty acid composition of PBMNC PL. Changes in the proportions of total PUFA, *n*-6 PUFA, α-LNA, AA and DHA were significantly negatively correlated to the changes in production of IL-6 (r=-0.721, r=-0.758, r=-0.929, r=-0.704 and r=-0.709, respectively), while for total *n*-3 PUFA and EPA this negative correlation was found but did not reach significance (r= -0.539 and r=-0.583, respectively). In this dietary

group there was also a significant correlation between the changes in production of TNF-α and in the proportion of α-LNA in PBMNC PL (r=-0.618).

5.3.5. LPS-Elicited Eicosanoid Production

The concentration of the eicosanoid PGE_2 in media taken from PBMNC cultured with LPS was measured at 0, 3 and 6 months of intervention (*Table 5.16*).

There was a significant increase (about 4-fold) in the concentration of PGE_2 produced when the PBMNC were cultured with LPS compared to the control cells (*Table 5.16*).

5.3.5.1. Baseline data

There were no significant differences in the concentration of PGE_2 in the culture media taken from PBMNC stimulated with LPS between any of the treatment groups at baseline. There were no significant differences in the concentration of PGE_2 between the different genders or with age at baseline.

Relationship between the Fatty Acid Composition of PBMNC PL and the Production of PGE_2 at Baseline

There were no significant relationships between the fatty acid composition of PBMNC PL and the concentration of the LPS-elicited PGE_2 in PBMNC culture media.

5.3.5.2. The Effects of the Different Treatments on the Production of PGE_2

Two-way ANOVA established that there was no effect of treatment on the concentration of PGE_2. However, there was an effect of time (p=0.009). This effect was investigated further. A one-way ANOVA indicated that there was no difference in the concentration of PGE_2 at any time of the intervention period with any treatment over the 6 months of intervention (*Table 5.16.*). However, there was a

trend for a time-dependent decrease in the concentration of PGE_2 in all groups over the 6 months of intervention.

Table 5.16. PGE_2 concentration in the media taken from of PBMNC cultured with and without LPS

Treatment	Time (m)	PGE_2 (ng/ml)	
		- LPS	**+ LPS**
Placebo	0	14.1 ±4.9	40.6 ±11.2
	3	14.9 ±6.5	28.2 ±8.9
	6	8.40 ±3.2	17.5 ±4.0
5.0 g α-LNA	0	7.24 ±1.7	43.7 ±12.9
	3	18.5 ±5.9	37.5 ±10.7
	6	12.4 ±3.9	31.0 ±8.1
10.0 g α-LNA	0	11.9 ±4.7	36.9 ±8.3
	3	11.5 ±3.6	31.2 ±10.2
	6	5.22 ±1.3	17.4 ±8.9
0.7g EPA+DHA	0	10.3 ±2.6	49.3 ±11.3
	3	23.2 ±6.9	28.1 ±9.7
	6	17.2 ±5.8	26.7 ±8.8
1.5 g EPA+DHA	0	9.90 ±2.3	34.1 ±8.2
	3	11.4 ±4.9	32.6 ±9.1
	6	5.50 ±1.1	24.7 ±6.9

Relationships between Changes in the Fatty Acid Composition of PBMNC PL and Changes in the Production of PGE_2 with the Different Treatments

The relationship between the changes in fatty acid composition of PBMNC PL and the changes in concentration of PGE_2 measured in culture media taken from PBMNC stimulated with LPS were investigated. There were no significant relationships between the changes in fatty acid composition of PBMNC PL and the changes in the production of PGE_2.

5.4. Discussion

5.4.1. NK Cell and Monocyte Number and the Expression of Monocyte Adhesion Molecules

The proportion of NK cells (expressed as a % of total lymphocyte population) over the intervention period was decreased in all treatment groups except the placebo. This decrease was significant with 5.0 and 10.0 g α-LNA/day, but not for 0.7 or 1.5 g EPA+DHA/day. The proportion of monocytes increased in all groups, but this only reached significance in the 1.5 g EPA+DHA group/day. Few studies have been published report the proportions of NK cells or of monocytes with *n*-3 PUFA intervention, but in a recent study which provided 2.25 g EPA/day for 3 months to males aged 20 to 40 years, found that the % of NK cells (as a % of total lymphocytes) was significantly decreased (E.A. Miles and P.C. Calder, personal communication). A decresed number of NK cells might indicate a decreased ability of the host to respond to viral infections or to deal with tumour cells, since there are are the key roles of these cells. In the present study the level of expression of the adhesion molecule ICAM-1 on monocytes was decreased after 1.5 g EPA+DHA/day was consumed for 6 months. The decrease in ICAM-1 expression on monocytes concurs with a study where the same dose of EPA+DHA/day was provided but for the shorter time of 3 weeks (Hughes *et al.,* 1996). Decreased ICAM-1 expression on monocytes would hinder their ability to bind to endothelial cells and enter the sub-endothelial space. This could result in decreased ability of monocytes to move into sites of infection. On the other hand the reduced movememnt of monocytes to sites of inflammatory activity (e.g. atherosclerotic plaques and synovial joints) could contribute to the benefical anti inflammatory effects of fish oil in inflammatory disease.

5.4.2. Effect of Age and Gender on Measures of Innate Immune Function

Both experimental and clinical evidence has demonstrated that there are age-dependent changes within the immune system, and these are thought to be mainly in

the T-lymphocyte system (Makinodan and Kay, 1980; Miller, 1992). There were no effects of the age (or gender) on measures of innate immunity made in the present study. It is reported that macrophage functions such as phagocytosis and oxidative burst are preserved, or even in some cases enhanced with ageing (Lesourd and Mazari, 1999), with no change in IL-1 or IL-6 production (Doria *et al.,* 1988; Nafiger *et al.,* 1993). The results of the present study concur with pervious studies of lack of effect of ageing on monocyte function. The production of PGE_2 by macrophages has been shown to increase with age in mice (Hayek *et al.,* 1997) and this may contribute to the decrease in T-lymphocyte functions, in particular lymphocyte proliferation, observed with age (see section 6.4.2.).

5.4.3. Effects of the Dietary n*-3 PUFA on Innate Immune Function*

Oxidative Burst Activity

In the present study there were no significant modulatory effects of doses of 0.7 or 1.5 g EPA+DHA/day or of 5.0 or 10.0 g α-LNA/day on the ability of neutrophils to mount an oxidative burst response in response to *E. coli* and phorbol myristic aceteate (PMA). Likewise, there was no effect of any of the interventions on the ability of monocytes to mount an oxidative burst response. There are three previous studies that have investigated neutrophil oxidative burst after *n*-3 PUFA intervention. A dose of 2.16 g EPA/day was found to decrease the production of superoxide species after stimulation of neutrophils with PMA (Thompson *et al.,* 1991). However, doses of 0.27 to 2.25 g EPA+DHA/day or 4.1 g α-LNA/day did not significantly affect superoxide production in response to n-formyl-mat-leu-phe (fMLP) (Healy *et al.,* 2000). A dose of 0.55 g EPA+DHA/d had no significant effect on the production of hydrogen peroxide by neutrophils stimulated with PMA (Varming *et al.,*1995). Studies of monocyte oxidative burst response have shown that a decrease of between 30 % and 55 % in superoxide production can be achieved after consumption of 3.6 g EPA+ 2.8 g DHA/day for 6 weeks (Fisher *et al.,* 1990), while 3.8 g EPA or 3.8 g DHA/day (Halvorsen *et al.,* 1997) or 0.34 g EPA + 0.19 g

DHA/day (Schmidt *et al.,* 1996) had no effect on the production of reactive oxygen species by monocytes.

The contrasting results of *n*-3 PUFA intervention studies may be a result of several factors. These include the use of different stimuli e.g. *E. coli,* PMA, fMLP for neutrophils and *E. coli* and latex beads for monocytes, and differences in other experimental conditions. It has been shown in the present study that different stimuli elicit a response of different magnitude in neutrophils (but not monocytes) i.e. the production of reactive species by neutrophils with PMA stimulation was approximately twice that with *E. coli* stimulation. The different lengths of study duration and the dose of the *n*-3 PUFA may also contribute to the differences in outcome of these studies. The effect of this latter parameter is difficult to determine due to the variation in the other factors, but it appears that doses of EPA+DHA which elicit changes in neutrophil oxidative burst activity may be different to those which achieve changes in this function in monocytes. The present study examined the effect of *n*-3 PUFA intervention on the recruitment of neutrophils and monocytes, as well as the production of reactive species. The studies cited here do not give this information and the differences in oxidative burst activity will be influenced by the number of cells which are active. This could be important with respect to the outcomes of these studies and should be borne in mind when considering the results. Nevertheless, it appears that increasing consumption of long-chain *n*-3 PUFA to 1.5 g/day or of α-LNA to 10.0 g/day does not affect oxidative burst by neutrophils or monocytes. This is an important observation since it indicates that even a substantial increase in *n*-3 PUFA consumption will not impair this fundamental aspect of immune function.

Phagocytic Activity of Neutrophils and Monocytes

In the present study there was a significant increase in the index of activity of neutrophils in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/ day groups, while there was a tendency for the number of *E. coli* engulfed per neutrophil over the intervention period to increase in all groups and this was significant for the 1.5 EPA+DHA/day group. The recruitment of monocytes had a tendency to decrease over the intervention period for the *n*-3 PUFA intervention groups, while there was a

tendency for the number of bacteria engulfed per monocyte to increase in these groups, and this was significant for the 1.5 g EPA+DHA group. The index of activity tended to increase in both the 1.5 g EPA+DHA/day and 10.0 g α-LNA/day groups.

There is only one study in the literature that has investigated the effects of EPA+DHA intervention on monocyte phagocytic activity in humans, and no study has done this for α-LNA. There are no studies of this parameter in neutrophils after *n*-3 PUFA intervention. Halvorsen *et al.* (1997) found no effect of 3.8 g of EPA or DHA/day on the ability of monocytes to engulf unopsonised or opsonised *E. coli* after a 7-week intervention, although EPA tended to increase bacterial attachment. In this study the recruitment of monocytes was not reported. From this limited literature it would at first appear that even 3.8 g EPA or DHA cannot modulate human monocyte phagocytosis. However, the results from the present study suggest that although recruitment of monocytes tended to decrease with *n*-3 PUFA intervention there was a tendency for the number of bacteria engulfed per monocyte and the index of activity to increase, and this was significant for the 1.5 g EPA+DHA/day and 10.0 g α-LNA/day groups. There may be an increase by the active monocytes in their capacity to engulf bacteria, and thus to compensate for the lesser number of active cells, and this has been observed in animal studies (Chapter 3). The effects of these two intervention groups on neutrophil function were slightly different in that there was a tendency for an increase in all measures of neutrophil phagocytosis, and this indicates that there was no need for the compensation seen in monocytes. Taken together these observations suggest that increasing *n*-3 PUFA consumption enhances phagocytosis. The fatty acid composition of PBMNC PL only changed significantly in the two dietary groups where significant effects of intervention on phagocytic activity were observed. In these two groups the PL became enriched with *n*-3 PUFA It is thought that the fatty acid composition of the cell membrane, and the degree of unsaturation, may be important with respect to phagocytic activity and that membrane fluidity is an important factor in determining this cell function (see section 1.3 and 5.4.4.).

Cytokine Production

In the present study there was no significant effect of the 0.7 g or 1.5 g EPA+DHA/day or of 5.0 or 10.0 g α-LNA/day on the production of TNF-α, while the production of IL-6 was non-significantly increased in the EPA+DHA groups but not in the α-LNA groups. The production of IL-10 tended to increase only in the two α–LNA groups, while the production of IL-1β was significantly increased in the EPA+DHA groups, and unchanged in the α-LNA groups.

There have been many studies conducted which have investigated the effects of dietary EPA+DHA on LPS-elicited cytokine production, and only one which has investigated the effects of α-LNA on this parameter. The outcomes of these experiments have led to many conflicting results with regard to the effects of *n*-3 PUFA on the production of these cytokines. The conflicting results of these studies are due to several factors. These include the dose of EPA+DHA used. Those studies which use the highest doses of EPA+DHA (> 5.2g EPA+DHA/day) report inhibition of LPS-elicited cytokine production (Gallai *et al.,* 1993; Kelley *et al.,* 1999). A decrease of between 20 % to 30 % was found in TNF–α production, while IL-1β production was decreased by 25 % to 45 % in these studies. Those studies which have used the lowest doses (< 1 g EPA+DHA/day) report no effect on production of these cytokines (Schmidt *et al.,* 1996; Blok *et al.,* 1997). The nature of the sample used to assay the cytokine also appears to impact on the result of the study. Those studies which have combined cell lysate with supernatant report inhibition of doses of 1.24 to 4.7 g EPA+DHA/day on LPS-elicited cytokine production (Endres *et al.,* 1989; Meydani *et al.,* 1991; Meydani *et al.,* 1993; Caughey *et al.,* 1996). In these studies decreases of between 7 % and 85 % were observed in TNF-α production, while IL-1β production was decreased by 70 % to 90 %, after stimulation with varying agents including LPS and *S. epidermidis*.

Those studies which investigated doses of 1.9 g to 3.2 g EPA+DHA/day and assayed LPS-elicited cytokines in the cell culture supernatants only found no effect on the production of these cytokines (Cannon *et al.,* 1995; Blok *et al.,* 1997; Yaqoob *et al.,* 2000). This suggests that there are effects of *n*-3 PUFA on the intracellular rather than extracellular cytokine concentrations. Molvig *et al.* (1991) found that 1.6 or 3.2

g EPA+DHA/day had no effect on the concentrations of TNF-α or IL-1β in the supernatant of LPS-, phytahemagglutinin (PHA)- or purified protein derivative (PPD)-stimulated mononuclear cells or monocytes. However, significant decreases in the cellular concentration of IL-1β after LPS or PHA stimulation were observed with the 3.2 g EPA+DHA/day intervention. Thus, it may well be that *n*-3 PUFA intervention has differential effects on the intra- and extracellular concentration of cytokines.

A third key factor in the differences between these experiments which may lead to differences in the outcomes of *n*-3 PUFA interventions is the cell culture conditions used. Cooper et al. (1993) outlined differences in the production of IL-1 and IL-6 after intervention with 0.7 g to 0.95 g EPA + 0.45 g to 0.62 g DHA, with varying concentrations of LPS, used as the stimulus. If a concentration of < 1 ng/ml of LPS was used to stimulate cultures then the concentrations of IL-1 and IL-6 were decreased by the intervention, while if LPS was used at > 10 ng/ml no effect on the concentrations of these cytokines was found.

The only study to investigate the effect of α-LNA intervention found that 13.7 g/day of this fatty acid decreased TNF-α and IL-1β production by 27 % and 30 %, respectively (Caughey *et al.*, 1996). These cytokines were assayed in combined cell lysate and supernatant.

In the present study the measurement of TNF-α, IL-6 and IL-1β concentrations was made in the culture supernatant. In previous studies where supernatants were used to assay these cytokines no effect of much larger doses of EPA+DHA or α-LNA were found, while in those studies where cell lysate and supernatant were assayed, doses as small as 1.24 g EPA+DHA/day have been shown to decrease the production of LPS-elicited cytokines. Thus, it may be that the nature of the measurements in the present study may well account for the absence of any effect of the EPA+DHA interventions. There are however other confounding factors such gender and age which may contribute to the differences between the present study and previous studies with similar doses of *n*-3 PUFA. Meydani *et al.* (1991) suggested that the cells of older females were more sensitive to the reported inhibitory effects of 2.4 g EPA+DHA/day, with respect to the production of IL-1β and TNF-α. However, the

results of the present study and of other studies do not concur with the finding of Meydani *et al.* (1991). Many factors such as the health of the elderly and the lack of reporting of gender in these other studies may well account for these differences (see section 5.4.2 and section 6.4.2.). Other confounding factors include the level of fat in the diet (see section 6.4.3.) and the dose of fatty acid used.

5.4.4. Correlations between Innate Immune Function and Fatty Acid Composition of PBMNC PL

Membrane fluidity is determined by the unsaturation and chain length of the fatty acids in the cell membrane (Stubbs and Smith, 1984) (section 1.3.3.). As the number of double bonds in and chain length of the fatty acids which comprise the membrane increase the more unsaturated the membrane and the more fluid it is thought to become. This has been shown experimentally (Mahoney *et al.,* 1981; Calder *et al.,* 1994) (*Figure 1.18.*). Changing the fatty acid composition of the diet has been shown to change the fatty acid composition of immune cells (Chapter 4) and this may have ramifications for the functioning of membrane receptors and enzymes, and also for functions of the cell which intimately involve the cell membrane such as phagocytosis and perhaps cell proliferation. The effects of increasing the unsaturation of the membrane on macrophage phagocytosis have been shown *in vitro* (Calder *et al.,* 1990) (*Figure 1.19*). An increase in the PUFA content of the membrane of macrophages was shown to significantly increase phagocytosis compared to a no fatty acid control and compared to membranes containing greater proportions of saturated fatty acids (Calder *et al.,* 1990). Similar findings have been shown in animal feeding studies when EPA was fed at 2.2 and 4.4 g EPA/100g total fatty acids (Chapter 3).

Oxidative Burst Activity

In the present study there were significant relationships between the proportions of certain fatty acids and the measures of oxidative burst activity of both monocytes and neutrophils at baseline. The proportion of SFA, and the ratio of SFA to PUFA and of *n*-6 to *n*-3 PUFA were negatively correlated with the oxidative burst activity of

neutrophils, and with the recruitment of monocytes. However, the proportions of these fatty acids were positively correlated with the production of monocyte reactive species. The proportions of *n*-3 and *n*-6 PUFA and the individual PUFA, α-LNA, AA and DHA were positively correlated with the recruitment of neutrophils and monocytes, and the production of reactive species by neutrophils. However, the proportions of these fatty acids were negatively correlated with the production of reactive species by monocytes. There was no relationship between the proportion of EPA and the oxidative burst activity of either neutrophils or monocytes.

When EPA and DHA were supplemented in the diet there were significant relationships between the changes in fatty acid composition of PBMNC PL and the changes in the recruitment of neutrophils and the production of reactive species after both *E. coli* and PMA stimulation. There were significant negative correlations between the proportions of SFA and the SFA to PUFA and *n*-6 to *n*-3 ratios and recruitment of neutrophils and production of oxidative species. There were significant positive correlations between the proportions of total PUFA, *n*-3 and *n*-6 PUFA and the individual fatty acids α-LNA, AA and DHA. It is notable that a change in the proportion of EPA was also significantly correlated with a change in the recruitment of neutrophils and the production of reactive species. When EPA and DHA were supplemented in the diet a similar relationship between the change in the fatty acid composition of PBMNC PL and the change in the monocyte oxidative burst activity was observed as that for neutrophils.

Thus, it would seem that a more unsaturated membrane results in an increased oxidative burst response by neutrophils in response to both *E. coli* and PMA. An increase in the content of the total proportion of *n*-6 or *n-3* PUFA in the membrane significantly increased the recruitment of neutrophils, the capacity of these cells to produce reactive species and an overall increase in the index of activity. However, the ratio of these fatty acids is also important in determining oxidative burst activity since an increase in the proportion of *n*-6 to *n-3* PUFA resulted in a significant decrease in the recruitment of cells, production of reactive species and overall index of activity. Thus, the amount and type of PUFA in the cell membrane has an effect on the ability of neutrophils to perform oxidative burst.

The oxidative burst activity in response to different stimuli may be affected by a change in membrane composition. It is notable that in the present study the relationship between the fatty acid composition of PBMNC PL and the oxidative burst activity of neutrophils was not as significant when PMA was the stimulus compared to the response when *E. coli* was the stimulus. These two stimuli (i.e. *E. coli* and PMA) act in different ways to elicit an oxidative burst response by monocytes and neutrophils (see section 1.1.2.1., *Figure 1.2.*). *E. coli* must bind to a membrane receptor while PMA directly acts upon PKC to elicit a response. Thus different relationships between fatty acid composition and oxidative burst may reflect differences in the effects of membrane composition on a receptor versus down-stream signalling pathway activation of the oxidative burst response in neutrophils (see section 1.1.2.1 and *Figure 1.2.*).

The effects of fatty acid composition on the oxidative burst response of monocytes at baseline was slightly different to that of neutrophils in that there was a negative relationship between the proportions of PUFA and SFA in the PBMNC PL and the production of reactive species in monocytes, while in neutrophils this relationship was positive. There was no relationship between the proportion of EPA and this parameter. However, enrichment of PBMNC PL with EPA and DHA led to this difference in relationship between fatty acid composition and production of reactive species between the cell types being negated. This implies that the monocytes may not have had the optimal fatty acid composition to mount an oxidative burst response at baseline, and that enrichment in PUFA, and perhaps more specifically EPA, serves to increase their capacity to produce oxidative species when stimulated.

Phagocytic Activity

In the present study the proportions of SFA to PUFA and *n*-6 to *n*-3 were negatively correlated with the recruitment of both neutrophils and monocytes to perform phagocytosis. The proportions of these fatty acids were also negatively correlated with the number of *E. coli* engulfed per active cell. The proportions of total PUFA and the individual fatty acids α-LNA, AA and DHA were all positively correlated with the recruitment of neutrophils and monocytes and the number of *E. coli* engulfed per cell. There was no relationship between the proportion of EPA in

PBMNC PL and these measurements of phagocytic activity. Although the AA to EPA ratio was positively correlated with both the number of cells performing phagocytosis and the number of *E. coli* engulfed per neutrophil or monocyte.

When the PBMNC PL became enriched with EPA and DHA there were significant correlations between the changes in the proportions of PUFA and the recruitment of both neutrophils and monocytes. However the magnitude of these correlations was greater than at baseline. The relationship between changes in the proportion of EPA and the recruitment of cells and the number of bacteria engulfed per cell also became significant, while those with DHA became non-significant. This suggests that an increased proportion of PUFA in PBMNC PL may lead to an increase in the phagocytic activity of neutrophils and monocytes, and that an increase in EPA rather than DHA is related to increases in phagocytic activity. The positive relationship between AA and EPA in PBMNC PL and phagocytic activity at baseline also indicated that an increase in AA rather than EPA in PBMNC PL is more positively related to phagocytic activity. *In vitro* work has shown that enrichment of the macrophage membrane with PUFA results in an increased phagocytic activity of these cells (Calder *et al.,* 1990) (*Figure 1.19*). However, Calder *et al.* (1990) also showed in this experiment that the increases in EPA and DHA in macrophage membranes led to a smaller increase in phagocytic activity than enrichment with AA. Thus, the results of the present study agree with the results that have been obtained in *in vitro* work. The relationships between the fatty acid composition of the PBMNC PL and phagocytosis are thus quite complex, with a point at which the degree of unsaturation of the membrane perhaps starts to inhibit phagocytic activity.

Cytokine and Eicosanoid Production

There was no significant relationship between the fatty acid composition of PBMNC PL and the production of TNF-α at baseline. However, the proportions of α-LNA, AA and total PUFA and *n*-6 PUFA and the ratio of PUFA to SFA were all negatively correlated with the production of IL-6 and IL-10 at baseline. The proportions of total *n*-3 PUFA and of DHA were positively correlated with IL-1β production and negatively correlated with IL-10 production. There was no relationship between the production of any of the cytokines and the proportion of EPA. When the PBMNC PL

became enriched in EPA and DHA there were significant relationships between the changes in the same fatty acids and the production of IL-6 as were observed at baseline. In addition, the relationship between the change in total *n*-3 PUFA and EPA and IL-6 production became non-significantly, negatively correlated. There were however no other significant relationships between changes in fatty acid composition of PBMNC PL and the production of other LPS-elicited cytokines. There were negative correlations between the changes in the proportions total *n*-6 PUFA, AA and the AA to EPA ratio and the production of PGE_2.

The results of the present study suggest that an increase in the proportions of PUFA, and thus a more unsaturated membrane, would seem to decrease the production of IL-6. There is little effect of fatty acid composition on the production of either TNF-α or IL-1β. The mechanism behind the effect of the fatty acid composition upon IL-6 production has not been clearly defined. However, there are several possibilities and these include the effects of membrane fluidity on the stimulus receptor and thus differences in the level of stimulation achieved. Membrane composition and perhaps fluidity could also affect the production of signalling molecules within the cell, and perhaps also effect the release of the cytokine protein molecules into the extracellular environment. Evidence for this arises in the different concentrations measured in cell lysate and supernatant samples (Molvig *et al.,* 1991). Alterations in the production of the eicosanoid PGE_2 with changes in AA and the AA to EPA ratio, would be expected since AA is the precursor for the production of this lipid mediator. A decrease in AA in the PBMNC PL would lead to a decrease in the substrate for PGE_2 production. On the other hand EPA may also act as a precursor for the production of prostaglandins, but with a resultant production of PGE_3 rather than PGE_2. The ELISA used to assay PGE_2 in the present study is less than 0.01% cross reactive for PGE_3. Thus, any decrease in PGE_2 would be detected but any increase in PGE_3 was not investigated.

5.5. Conclusions

In the present study there were no significant effects on the oxidative burst activity of neutrophils or monocytes after intervention with 0.7 g or 1.5 g EPA+DHA/day or

with 5.0 g or 10.0 g α-LNA/day for 6 months. This was despite significant changes in the fatty acid composition of PBMNC PL in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups. The production of cytokines by LPS-stimulated PBMNC was predominately unaffected by the interventions. This would indicate that these fundamental aspects of innate immunity are not impaired by the consumption of *n*-3 PUFA at the levels given in this study. However, since some aspects of both monocyte and neutrophil phagocytosis were modulated by consumption of the higher levels of *n*-3 PUFA in this study it may be that innate immunity is enhanced by consumption of 1.5g EPA+DHA/day or 10.0 g α-LNA/day. This study suggests that not all aspects of innate immunity may be modulated to the same extent by the levels of *n*-3 PUFA consumed in this study

CHAPTER 6. THE EFFECTS OF DIETARY MODULATION WITII *n*-3 POLYUNSATURATED FATTY ACIDS ON ACQUIRED IMMUNITY

6.1. Introduction

Section 1.4 described how changes in the consumption of *n*-3 polyunsaturated fatty acids (PUFA) have been reported to modulate the function of lymphocytes which are intimately involved in the acquired immune response. It is the modulation of the fatty acid composition of the cell and the lymphocyte membranes in particular, which may result in the modification of lymphocyte function (see section 1.3.). The modulation of the fatty acid composition of peripheral blood mononuclear cells (PBMNC) PL has been investigated in the present study and is described in Chapter 4.

The Effects of Changing the Fatty Acid Composition of PBMNC is Ubiquitous To All Immune Cells

Modification of the fatty acid composition of PBMNC, of which lymphocytes comprise a large proportion, following increased dietary intake of *n*-3 PUFA has been demonstrated in a number of studies (see sections 4.4.3. and 5.1).

An important consideration regarding changes in fatty acid composition of PBMNC PL and changes in immune cell function, discussed later in this thesis, would be whether changes in the fatty acid composition of total PBMNC are reflected uniformly across the various types of immune cell e.g. lymphocytes, monocytes, NK cells and neutrophils. This has been shown to be the case when fish oil is added to the diet (Gibney and Hunter, 1993). The effects of α-linolenic acid (α-LNA) intervention on different cell types has not yet been investigated, but will be assumed to be similar to that seen after eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intervention. Thus, changes observed in PBMNC PL can be thought to occur in T-lymphocytes, monocytes and neutrophils.

Summary of the Changes in Fatty Acid Composition of PBMNC PL in the Present Study

There were significant changes in the composition of PBMNC PL with respect to certain fatty acids with the different interventions used here (see section 4.3.5.). There were marked changes in the content of arachidonic acid (AA), EPA and DHA. There was a significant decrease in AA in all groups. There was a significant increase in EPA in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups. There was no change in the content of α-LNA in any of the groups, while there was a decrease in DHA in the α-LNA groups and an increase in DHA in the 1.5 g EPA+DHA/day group. Changes in other types of fatty acids were also observed. In all groups the proportion of 16:0 increased, while there was no change in any other fatty acid in any groups except the 10.0 g α-LNA/day where there was a decrease in 18:2 *n*-6. The ratio of SFA to PUFA increased in all groups. The ratio of *n*-6 to *n*-3 PUFA decreased in both EPA+DHA groups but this was only significant for the 1.5 g EPA+DHA/day group. There was also a decrease in this ratio for the 10.0 g α-LNA/. There was a decrease in the ratio of AA to EPA in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups.

Summary of the Changes in Immune Function Observed with α-LNA, EPA and DHA Intervention

The modulation of lymphocyte functions after manipulation of dietary fatty acids have been discussed in Chapter 1 (section 1.4.). The overall outcome of these experiments is that increased consumption of α-LNA and the longer chain *n*-3 PUFA, EPA and DHA can have modulatory effects on the functioning of these cells. The fatty acids α-LNA, EPA and DHA have inhibitory effects on the proliferation of human lymphocytes (Kelley *et al.*, 1991; Molvig *et al.,* 1991; Meydani *et al.,* 1991; Meydani *et al.,* 1993). However, a study by Yaqoob *et al.,* (2000) showed no effect of a dose of 3.2 g EPA+DHA/day on lymphocyte proliferation.

The production of the cytokines IL-2 and IFN-γ was decreased by the consumption of EPA and DHA in humans (Meydani *et al.,* 1991; Endres *et al.,* 1993; Gallai *et al.,* 1993). However, again the study of Yaqoob *et al,* (2000) reported no effect of EPA+DHA intervention on the production of these cytokines or IL-10 with 3.2.g EPA+DHA/day. In contrast 1.23 g EPA+DHA/day has been shown to effect a decrease in the production of IL-2 when part of a low-fat, low-cholesterol diet (Meydani *et al.,* 1993). No study has investigated the impact of dietary α-LNA on the production of cytokines by human lymphocytes.

The effects of dietary *n*-3 PUFA on the delayed-type hypersensitivity response are also somewhat contradictory. DHA, at a dose of 6 g/day, was found to have no effect on the DTH response (Kelley *et al.,* 1998), while EPA+DHA, at a dose of 1.25 g/day resulted in a 45% decrease in the response (Meydani *et al.,* 1993). There is very little known about the effects of α-LNA on the DTH response in humans, although one study has shown that 18.0 g /day of α-LNA, as part of a linseed oil-rich diet, resulted in a non-significant 23% decrease in the DTH response (Kelley *et al.,* 1991).

The difference in the outcomes of these experiments probably results from differences in the experimental conditions used, as in similar studies of monocyte functions (see section 5.1.). These include differences in cell culture conditions used. For example, the stimulus, and the concentrations used, may differentially activate the cells under study e.g. Concanavalin A (Con A) (Yaqoob *et al.*, 2000) and phytohemagglutinin (PHA) (Meydani *et al.*, 1991), although both are considered to be T-cell mitogens they may have different effects on T-cell subsets. Factors such as age have been shown to influence T-lymphocyte functions such as lymphocyte proliferation. Meydani *et al.* (1991) demonstrated that the effects of fish oil intervention are more marked in older subjects compared to younger subjects with the respect to this function. This might explain some of the differences in outcomes between studies. Factors such as gender have also been shown to influence some aspects of the immune system. In the studies of T-lymphocyte function and *n*-3 PUFA cited here the subjects recruited were predominantly male (even in the study of Yaqoob *et al.* (2000) where a mixture of males and females were studied). The study of Meydani *et al.* (1991) stands apart, since the subjects in that study were all female. Thus, as with experimental conditions and doses, subject differences must be

considered carefully in order to account for the difference in outcomes between studies.

Despite the interest in fatty acids and immune function, relatively little is known about the relationship between the habitual intake of fatty acids (which is reflected in the fatty acid composition of cells of free-living individuals) and immune function. Caughey *et al.* (1996) demonstrated that were statistically significant inverse relationships between PBMNC EPA content and both TNF-α and IL-1β production after lipopolysaccharide (LPS) stimulation. TNF-α and IL-1β production decreased as the PBMNC EPA content increased to approximately 1% of total fatty acids, after which an increase in EPA had little effect on cytokine production. The current study provides an opportunity to investigate the relationships between intake of fatty acids, the fatty acid composition of PBMNC PL and immune cell function.

Most dietary interventions with EPA and DHA have used capsules as the vehicle for the intervention and have aimed to deliver high doses of fatty acids. The use of capsules in the everyday situation has limitations as described in section 4.1. This study will investigate the influence of dietary interventions with foodstuffs enriched in either α-LNA or EPA and DHA on acquired immune cell function.

6.1.1. Aims of This Chapter

Thus, it is apparent that further work is required to establish the effects of *n*-3 PUFA on the functioning of lymphocytes as discussed in section 1.5 where the overall aims of this dietary intervention study are outlined. This chapter intends to investigate these aims in the context of the acquired immune response. The reader is referred to section 4.2. for details of the study design, information about the subject characteristics and dietary information.

The overall aims of this dietary intervention study are outlined in section 4.1.1. This chapter intends to investigate these aims in the context of the acquired immune response.

- to investigate the immunological effects of EPA and DHA at levels that can be reasonably readily incorporated into the diet through foodstuffs
- to investigate the immunological effects of α-LNA at levels that can be reasonably readily incorporated into the diet through foodstuffs
- to ascertain a better estimate of the relationship between the amount of α-LNA and of EPA and/or DHA in the diet and acquired immune function
- to establish if there are any differences due to gender in the immunomodulatory effects of α-LNA, EPA and DHA

Hypotheses:

- There will be dose-dependent effects of α-LNA and EPA+DHA on human acquired immune function
- The effects of dietary α-LNA on human acquired immune function will be less marked than those of EPA+DHA

In this chapter the following will be examined:

- The number of lymphocytes and lymphocyte subsets in peripheral blood
- The production of cytokines IL-2, IFN-γ, IL-4 and IL-10
- The proliferative response of lymphocytes to Con A stimulation
- The delayed-type hypersensitivity response

6.2. Methods

6.2.1. Chemicals

Formaldehyde, glutamine, HEPES-buffered RPMI medium (glutamine free), Con A penicillin, streptomycin and sodium azide were all obtained from Sigma Chemical Co., Poole, UK Cytokine concentrations were determined using ELISA kits,

manufactured by Biosource, Belgium, and prostaglandin E_2 concentrations were determined using ELISA kits manufactured by Neogen. All kits purchased from Appligene-Oncor-Lifescreen, Watford, UK. [6-^{3}H] Thymidine (2 Ci/mmol) was obtained from Amersham International, Amersham, UK. Fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies to human CD3 and CD19, and the R. Phycoerythrin (RPE)-labelled monclonal antibodies to human CD4 and CD8 were purchased from Serotec, Kidlington, UK.

6.2.2. Mononuclear Cell Preparation

PBMNC were prepared as described in 5.5.2.

6.2.3. Analysis of Mononuclear Cell Sub-populations

Whole blood (100 µl) was doubly stained with antibodies (10 µl FITC-labelled and 10µl RPE-labelled) as shown in *Table 6.1.* Subsequent processing and flow cytometry was performed as described in section 5.2.3. T-cells were identified as CD3+ and B-cells were identified as CD19+. The proportions of the T cell subtypes, Th and Tc cells, were determined as CD3+CD4+ and CD3+CD8+, respectively, as described in *Figure 5.1.*

Sample profiles are shown in *Figure 5.1,* and the identification (gating) of the different cell subtypes in this chapter was performed in the same way as described in section 5.2.3. The % of T-cells and B-cells were determined in terms of the percentage marker-positive cells. T-cells were identified as total CD3+ cells (i.e. the sum of the top right and bottom right quadrants for this profile) and B-cells were identified as total CD19+ cells (i.e. the sum of the top right and bottom right quadrants for this profile). The % Th and Tc cells were determined as the double-positively stained cells as shown in *Table 6.1.*, and this corresponds to the top right hand quadrant of the appropriate profile.

Table 6.1. Stains used to identify the different mononuclear cell subsets

Cell	Stain 1	Stain 2
Helper T Cell	Anti CD3*	Anti $CD4^+$
Cytotoxic T Cell	Anti CD3*	Anti $CD8^+$
B-cells	Anti CD19*	-

* = FITC-labelled; $^+$ = RPE-labelled

6.2.4. Cytokine Concentration

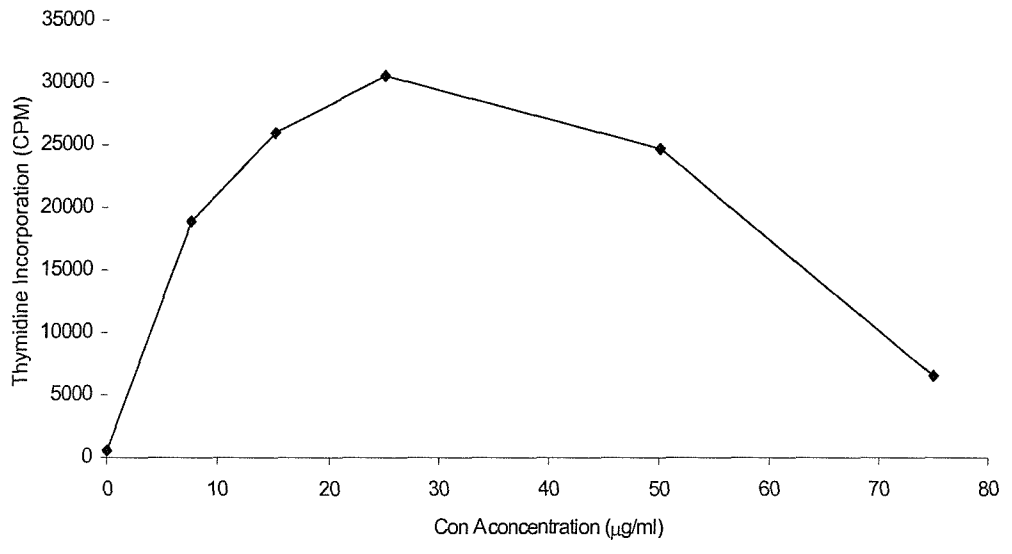
PBMNC were cultured at 37°C in an air/CO_2 (19:1) atmosphere in a 24-well culture plate at a density of 2 x 10^6 cells/well and a total culture volume of 2 ml in RPMI medium supplemented with 0.75 mM glutamine, antibiotics (penicillin and streptomycin) and 5% (v/v) autologous plasma and 25 μg/ml Con A. After 24 h, the plate was centrifuged (1000 rpm, 5 min) and the supernatant collected and frozen at -20°C for later analysis. Cytokine and PGE_2 concentrations were determined using ELISA kits according to the manufacturers instructions. The samples were diluted as appropriate, and a standard curve was constructed to determine cytokine concentrations, with the ranges of detection 0.002 to 35 IU/ml (IL-2), 0.03 to 30 IU/ml (IFN-γ), 0.05 to 1370 pg/ml (IL-4) and 0.5 to 10.0 ng/ml (PGE_2). The percentage coefficient of variation for the cytokines measured was: for intra-assay 5.7 (IL-2), 3.2 (IFN-γ) and 3.0 (IL-4); for inter-assay 7.5 (IL-2), 7.7 (IFN-γ) and 3.2 (IL-4).

6.2.5. Lymphocyte Proliferation

PBMNC were cultured, in triplicate, at 37°C in an air/CO_2 (19:1) atmosphere in a 96-well plate at a density of 2 x 10^5 cells/well. The total culture volume was 200 μl and comprised RPMI medium supplemented with 0.75 mM glutamine, antibiotics (penicillin and streptomycin) and 5% (v/v) autologous plasma and a range of concentrations of Con A. After 48 h of culture 20 μl [6-^{3}H] thymidine was added to each well (0.2 μ Ci/well) and the cells were incubated for a further 18 h. The cells were then harvested onto glass fiber filters and washed and dried using a Skatron Cell Harvester. Radioactive thymidine incorporation was determined by liquid

scintillation counting for 1 min. Data are expressed as peak thymidine incorporation and stimulation index = thymidine incorporation / thymidine incorporation in the absence of Con A. *Figure 6.1* shows a typical response of thymidine incorporation in relation to Con A concentration: the response forms a bell shaped curve which peaks at approximately 25 μg/ml of Con A.

Figure 6.1 Lymphocyte proliferation measured as thymidine incorporation response to a range of concentrations of Con A



6.2.6. Delayed-Type Hypersensitivity Skin Test

Delayed type hypersensitivity (DTH) is part of the cell-mediated immune reaction in which the ultimate effector cell is the activated mononuclear phagocyte (see section 1.1.4). By the application of a skin test an *in vivo* measure of cell-mediated immunity can be made. The DTH reaction evolves over 24-48 hr and results in the elimination of antigen.

The DTH skin response was assessed using the MultiTest-CMI kits manufactured by Merieux Institute Inc., Paris, France. The MultiTest-CMI rather than the conventional skin test was used to avoid the boosting effect observed with repeated

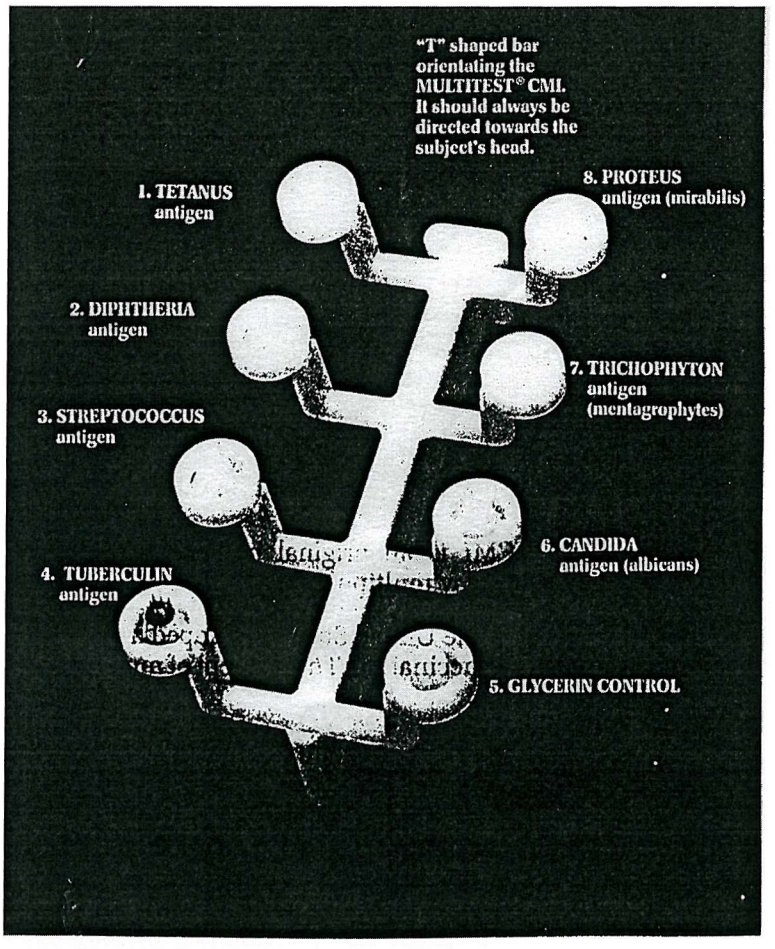
administration (Lesourd *et al.,* 1985). The test consisted of applying a single-use disposable applicator of acrylic resin with eight heads loaded with either glycerine control or with one of seven recall antigens (*Table 6.2; Figure 6.2*). The applicator was held to the skin for a period of 10 sec. The diameter of positive reactions was measured at 48 hr after administration of the test. An imprint of the test area was taken and transferred to an analysis grid.

The antigen score was calculated using a well-established image analysis apparatus at Unilever Research, Vlaardingen, The Netherlands. This instrument measures the area of each individual induration and then calculates an average diameter for each induration (diameter (mm) = 2 x $\sqrt{}$ (area (mm^2) /π). An induration of $\geq$ 2 mm^2 was considered positive. If a positive reaction to the glycerine control was observed, the diameter of its induration was subtracted from each of the other positive reactions. Results were interpreted as the total number of positive reactions and the cumulative score (calculated as the total diameter of induration of all positive reactions). The test was administered at the baseline and at the end of the intervention period i.e. 6 months.

Table 6.2. The recall antigens applied with the skin test applicator

Antigen Sample	**Sample No.**	**Antigen Sample**	**Sample No.**
Tetanus	1	Control	5
Diptheria	2	Candida albicans	6
Streptococcus	3	Trichophyton mentagrophytes	7
Tuberculin	4	Proteus mirabilis	8

Figure 6.2. DTH Applicator



6.2.7. Statistical Analysis

Data are the mean ± SEM. Cytokine production and lymphocyte proliferative response were measured in approximately 30 subjects per treatment group (i.e. most subjects in all cohorts). The DTH response was only measured in 20 subjects per treatment group due to logistic and financial constraints (i.e. most subjects in cohorts 2 and 3).

The statistical analyses were initially performed using a two-way analysis of variance to establish the effects of treatment and time and the interaction between them. Where treatment or time were significant one-way analysis of variance and a post-hoc least significance test with Bonferonni's correction were then performed to identify differences between groups at a given time point and differences within a treatment group over time. In all cases a value of $p<0.05$was taken to indicate statistical significance and the statistical package SPSS Version 10.0 (SPSS Inc., Chicago, IL) was used.

Significant differences over time are expressed as:

a. Significantly different from 6 month
b. Significantly different from 0 month
c. Significantly different from 6 month

Significant differences between groups at 0, 3 or 6 months are expressed as:

A. Significantly different from the placebo treatment
B. Significantly different from the 5.0 g α-LNA treatment
C. Significantly different from the 10.0 g α-LNA treatment
D. Significantly different from the 0.7 g EPA+DHA treatment
E. Significantly different from the 1.5 g EPA+DHA treatment

Linear correlations were determined as Spearman's Rank correlations. r values indicated by # are significantly different at the $P<0.05$ level.

Independent t-tests were also performed where a one–way analysis could not be e.g. to establish differences between males and females. Data with a $^{+}$ indicates significant differences between males and females.

6.3. Results

The parameters of acquired immune function investigated in this study were the % of B-cells and T-cells in peripheral blood, lymphocyte proliferation in response to a T-lymphocyte mitogen and the concentrations of cytokines produced by stimulated lymphocytes. The DTH response, an in vivo indication of cell-mediated immune function, was also investigated. These measurements will be described firstly in terms of the baseline data in order to describe any differences due to gender, age or the fatty acid composition of the cells involved (i.e. PBMNC) at the start of the intervention. The fatty acid composition of PBMNC PL was reported in section 4.3.6. This examination of baseline data will enable several of the potential determinants of variation in acquired immune cell function to be identified.

The effects of the treatments on the immune parameters measured will then be examined. In addition, the effects of gender and age on the modulation of these parameters with the different treatments will be described. Correlations between the changes in fatty acid composition of PBMNC PL and acquired immune cell functions will also be described (see section 4.3.6. for details of the changes in fatty acid composition). This will allow the relationship between dietary fatty acid intake, and immune cell composition and function to be clarified. The investigation of this relationship will be made for the study group as a whole and for the 10.0 g α-LNA and 1.5 g EPA+DHA treatment groups. This is because the changes in fatty acid composition of PBMNC PL over the intervention period were only observed in these two treatment groups see section 4.3.6.).

6.3.1. Lymphocyte Subsets

The % total T and B cells and the % of the T cell subsets, Th and Tc cells, were determined at 0, 3 and 6 months of intervention (*Table 6.3.*).

6.3.1.1. Baseline Data

There were no differences between the different treatment groups in the % of T- or B-cells, or in the % of Th or Tc cells in peripheral blood at baseline (*Table 6.3.*). T-

cells comprised about 60% of the lymphocyte population with Th cells and Tc cells comprising approximately 40% and 20%, respectively, of the T-cell population. B-cells comprised about 10% of the lymphocyte population. There were no differences in the proportions of these cell types between males and females at baseline.

6.3.1.2. Effects of the Treatments on Lymphocyte Subsets in Peripheral Blood

Two-way ANOVA established that there was no effect of treatment on the % of T-cells, B-cells, Th or Tc cells. However, there was a significant effect of time on the % of T-cells ($p<0.0001$), Th cells ($p<0.032$) and Tc cells ($p<0.0001$). These effects were investigated further.

One-way ANOVA established that there was no significant change in the % of T-cells, T-cell subsets or % of B-cells from 0 to 6 months of intervention (*Table 6.3.*). However, there was a small, but significant, decrease in the % of Tc cells in the 5.0 g α-LNA/day treatment group from 0 to 3 months of intervention. However, by 6 months the % of Tc cells in this group had almost returned to the baseline value. In the placebo group there was a small, significant decrease in the % B-cells from 0 to 3 months, but by 6 months the % of these cells had tended return to the baseline value.

Table 6.3. The % T-cells, Th cells, Tc cells and B-cells in peripheral blood (n=30/group)

Treatment	Time (m)	T-cells ($CD3^+$)	Th cells ($CD3^+CD4^+$)	Tc cells ($CD3^+CD8^+$)	B cells ($CD19^+$)
Placebo	0	62.5 ±1.9	37.6 ±2.0	21.0 ±1.4	12.1 ±1.8[b]
	3	53.7 ±3.0	34.4 ±2.4	16.5 ±1.3	7.90 ±0.7[a]
	6	56.5 ±2.8	36.1 ±2.0	18.8 ±1.3	8.70 ±0.8
5.0 g α-LNA	0	63.4 ±2.3	38.7 ±1.7	21.3 ±1.8[b]	10.5 ±0.8
	3	57.4 ±2.4	36.4 ±2.1	15.7 ±1.0[a]	8.80 ±0.8
	6	59.4 ±2.1	39.5 ±2.1	17.3 ±1.5	9.20 ±0.6
10.0 g α-LNA	0	63.3 ±3.0	38.9±2.1	22.4 ±1.6	9.90 ±0.8
	3	57.3 ±3.2	35.9 ±2.4	19.0 ±1.7	7.80 ±0.8
	6	58.6 ±3.1	37.0 ±2.3	20.5 ±1.7	12.7 ±2.2
0.7 g EPA+DHA	0	63.0 ±2.9	39.9 ±2.5	20.3 ±1.6	12.6 ±2.2
	3	56.0 ±3.1	36.9 ±2.3	15.2 ±1.8	11.2 ±3.5
	6	60.5 ±2.6	40.2 ±1.9	18.6 ±1.3	8.20 ±0.6
1.5 g EPA+DHA	0	60.3 ±2.5	37.9 ±2.8	18.6 ±1.2	8.50 ±0.6
	3	52.2 ±2.9	32.7 ±2.7	15.3 ±1.5	9.65 ±2.6
	6	59.4 ±2.3	39.0 ±2.2	18.4 ±1.0	9.39 ±0.8

Significant differences over time are expressed as:

a. Significantly different from 0 month

b. Significantly different from 3 months

6.3.2. Con A-Elicited Cytokine Production by Cultured PBMNC

The concentrations of the cytokines IL-2, IFN-γ, IL-4 and IL-10 were measured in the supernatants taken from PBMNC cultured with Con A at 0, 3 and 6 months of intervention (Table 4.4.).

There was a significant increase in the concentrations of IL-2, IFN-γ, IL-4 and IL-10 when the PBMNC were cultured with Con A compared to the control cells not cultured with Con A (approx. 0.25 IU/ml, 3 IU/ml, 1.5 IU/ml and 90 IU/ml for IL-2, IFN-γ, IL-4 and IL-10 respectively for the control cells; > 7 IU/ml, > 65 IU/ml, > 40 pg/ml and > 400 pg/ml for IL-2, IFN-γ, IL-4 and IL-10, respectively, for the Con A-stimulated cells) (*Table 6.4*).

6.3.2.1. Baseline Data

There were no significant differences between the treatment groupsin the concentrations of IL-2, IFN-γ, IL-4 and IL-10 in the culture media from PBMNC stimulated with Con A at baseline (*Table 6.4.*).

Effect of Age and Gender on the Concentrations of IL-2 IFN-γ, IL-4 and IL-10

There were significant differences in the concentration of IL-4 produced by cells from males and females at different ages. There was a decrease in the concentration of IL-4 produced by cells from females and an increase in concentration produced from males as the subjects increased in age (*Figure 6.3*). This difference in concentration between males and females was significant ($p<0.001$) for the cells from subjects aged 50-70 years. There was no effect of age and gender on production of any of the other cytokines except IL-2. The concentration of this cytokine produced by cells from males was greater than females in the age group 50-70 years (*Figure 6.4.*).

Table 6.4. Cytokine concentrations in the medium of lymphocytes cultured with and without Con A (n=30/group)

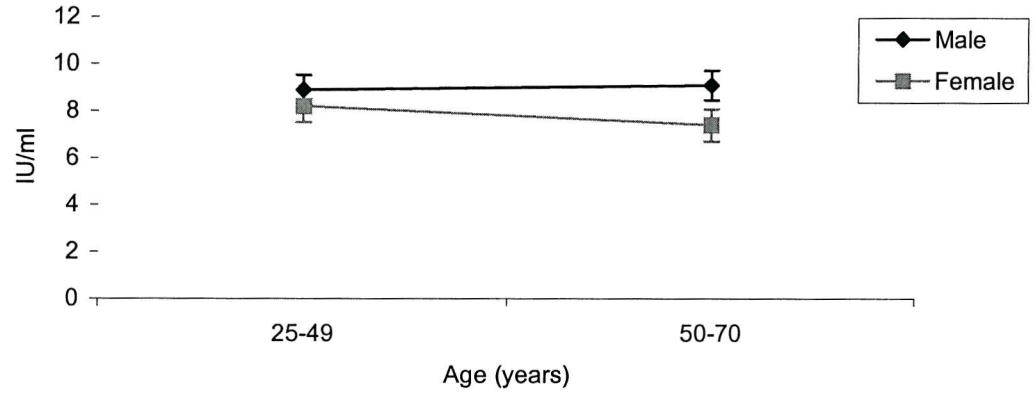
Treatment	Time (m)	IL-2 (IU/ml)		IFN-γ (IU/ml)		IL-4 (pg/ml)		IL-10 (pg/ml)		IFN-γ /IL-4
		- Con A	+ Con A	- Con A	+ Con A	- Con A	+ Con A	- Con A	+ Con A	
Placebo	0	0.23 ±0.02	8.89 ±1.02	3.36 ±0.98	137.47 ±27.7	1.55 ±0.22	56.38 ±10.64	71 ±14	442 ±83	3.2 ±0.7
	3	0.25 ±0.02	8.98 ±1.32	1.92 ±0.46	74.60 ±13.4	1.65 ±0.50	52.03 ±13.07	44 ±10	362 ±65	2.3 ±0.4 [c]
	6	0.26 ±0.02	9.42 ±1.46	6.31 ±2.24	167.19 ±33.2	1.44 ±0.21	44.60 ±10.69	147 ±34	473 ±84	5.7 ±0.9 [b]
5.0 g α-LNA	0	0.25 ±0.01	8.99 ±1.17	5.22 ±1.87	148.25 ±28.3	1.48 ±0.25	58.30 ±11.82	74 ±12	405 ±64	3.8 ±0.6
	3	0.27 ±0.02	8.08 ±1.33	3.37 ±1.03	96.70 ±27.9	1.72 ±0.27	45.01 ±10.56	66 ±21	362 ±54	2.8 ±0.5 [c]
	6	0.33 ±0.10	8.52 ±1.19	2.65 ±0.71	161.47 ±36.7	1.74 ±0.38	63.42 ±15.19	86 ±17	500 ±59	5.3 ±1.1 [b]
10.0 g α-LNA	0	0.22 ±0.01	8.27 ±1.69	4.76 ±2.34	98.12 ±17.8	1.52 ±0.17	46.26 ±9.25	112 ±12	252 ±48	3.9 ±0.9
	3	0.25 ±0.02	8.80 ±1.83	2.77 ±0.80	65.75 ±9.60	1.60 ±0.28	50.94 ±12.30	53 ±21	455 ±71	2.5 ±0.9
	6	0.26 ±0.01	7.20 ±1.08	2.21 ±0.54	125.76 ±24.1	1.62 ±0.24	45.60 ±11.34	92 ±17	340 ±57	3.3 ±0.7
0.7g EPA+DHA	0	0.27 ±0.03	7.84 ±0.97	8.24 ±2.77	117.12 ±24.5	1.39 ±0.19	77.30 ±22.20	69 ±13	362 ±75	2.5 ±0.5
	3	0.24 ±0.02	7.32 ±1.50	2.12 ±0.59	67.75 ±15.7	1.39 ±0.25	59.90 ±19.25	107 ±22	375 ±70	1.7 ±0.4 [c]
	6	0.28 ±0.04	10.56 ±1.96	4.03 ±1.74	133.25 ±18.8	1.43 ±0.18	50.10 ±9.80	171 ±31	484 ±81	4.5 ±1.0 [b]
1.5 g EPA+DHA	0	0.25 ±0.02	8.08 ±0.96	3.46 ±0.93	112.7 ±23.9	1.50 ±0.19	66.94 ±12.70	102 ±32	495 ±61	2.4 ±0.6
	3	0.25 ±0.02	7.30 ±1.04	1.70 ±0.51	77.06 ±24.6	1.17 ±0.18	72.99 ±20.38	34 ± 9	434 ±100	1.4 ±0.3
	6	0.26 ±0.02	8.45 ±1.22	2.99 ±0.77	155.18 ±40.1	1.36 ±0.24	57.15 ±11.39	167 ±41	485 ±87	4.1 ±1.1

Significant differences are expressed as: b. Significantly different from 3 months; c. Significantly different from 6 months

Figure 6.3. The effect of age and gender on the concentration of IL-4 produced by Con A-stimulated PBMNC (n=150)

+ Data are significantly different using an Independent t-test ($p < 0.05$)

Figure 6.4. The effect of age and gender on the concentration of IL-2 produced by Con A-stimulated PBMNC (n=150)



Relationship Between the PBMNC PL Fatty Acid Composition and the Production of Con A-Elicited Cytokines at Baseline

The correlation coefficients between the fatty acid composition of PBMNC PL at baseline and the concentrations of cytokines produced after Con A-stimulation of

PBMNC suggests that the capacity of PBMNC to produce IL-2, IFN-γ, IL-4 and IL-10 is related to their fatty acid composition (*Table 6.5.*).

The proportions of α-LNA, AA, DHA, total PUFA, total *n*-6 or *n*-3 PUFA and the ratio of PUFA to SFA, in the PBMNC PL were all positively correlated with the concentration of IFN-γ and IL-4 in the media taken from PBMNC stimulated with Con A (*Table 6.5.*). The proportions of each of these fatty acids in PBMNC PL were also positively associated with IL-2 production, except for the proportions of total *n*-3 PUFA and DHA which were not related to the production of this cytokine. The ratio of *n*-6 to *n*-3 PUFA was significantly negatively correlated with IFN-γ production, with no relationship to the production of IL-2 or IL-4. There were no significant relationships between fatty acid composition of PBMNC PL and the production of IL-10 by PBMNC stimulated with Con A.

Table 6.5. Correlation coefficients (r) between the fatty acid composition of PBMNC PL and the concentration of cytokines measured in medium taken from PBMNC cultured with Con A (n=150)

	IL-2	**IFN-γ**	**IL-4**	**IL-10**
Total SFA	0.062	0.112	0.258#	-0.044
Total PUFA	0.233#	0.491#	0.363#	0.072
SFA/PUFA	-0.108	-0.304#	-0.140	-0.028
Total *n*-6	0.332#	0.454#	0.453#	0.008
Total *n*-3	-0.006	0.421#	0.135	0.140
n-6/*n*-3	0.062	-0.265#	0.039	-0.165
α-LNA	0.262#	0.590#	0.660#	-0.118
AA	0.299#	0.397#	0.373#	0.000
EPA	-0.103	0.148	0.038	0.041
DHA	-0.009	0.377#	0.147	0.022
AA/EPA	0.277#	0.037	0.137	-0.041

Data are significant using a Spearman's Rank correlation ($p<0.05$)

6.3.2.2. The Effects of the Treatments on the Production of the Cytokines IL-2, IFN-γ, IL-4 and IL-10 by PBMNC

Two-way ANOVA established that there was no effect of treatment on the concentration of the cytokines IL-2, IFN-γ, IL-4 and IL-10 in culture medium from PBMNC stimulated with Con A. However, there was a significant effect of time on concentration of IFN-γ ($p<0.0001$). This effect was investigated further.

There was a tendency for an increase in IL-2 production in the 0.7 g EPA+DHA/day and placebo groups, while the production of IL-2 was unchanged in the 5.0 g α-LNA/day and 1.5 g EPA+DHA/day groups and tended to decrease in the 10.0 g α-LNA/day group (*Table 6.6*). The production of IFN-γ and IL-10 tended to increase in all groups from 0 to 6 months (*Table 6.6*). The production of IL-4 tended to decrease in both EPA+DHA groups and the placebo group, while there was no change in IL-4 production in the 10.0 g α-LNA/day group, and a tendency toward an increase in the 5.0 g α-LNA/day group (*Table 6.6*). There were differences in the ratio of production of IFN-γ to IL-4 over the intervention period (*Table 6.6.*). There was a trend toward an increase in this ratio in all groups from 0 to 6 months, except in the 10.0 g α-LNA/day group where there was no change. Between 0 and 3 months there was a trend for a decrease in the ratio of IFN-γ to IL-4, but between 3 and 6 months there were was a trend for an increase and this was significant in the placebo, 5.0 g α-LNA/day and 0.7 g EPA+DHA groups.

Effects of Gender and Age on the Concentrations of the Cytokines IL-2, IFN-γ, IL-4 and IL-10 with the Different Treatments

There were no effects of the gender or age of the subjects on the concentration of the cytokines measured in the media taken from PBMNC cultured with Con A.

The Relationship Between the Changes in the Fatty Acid Composition of PBMNC PL and Changes in the Production of the Cytokines IL-2, IFN-γ, IL-4 and IL-10 with the Different Treatments

The significant correlations between the fatty acid composition of PBMNC PL and the concentrations cytokines after Con A stimulation of PBMNC at baseline suggests that the capacity of PBMNC to produce IL-2, IFN-γ and IL-4 is influenced by their fatty acid composition (*Table 6.7.*). The relationships between the changes in fatty acid composition of PBMNC PL and the changes in concentrations of these cytokines from 0 to 6 months was investigated for the dietary groups together (n=150), and for the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups (n=30/group). The relationship between changes in cytokine production and changes

in fatty acid composition were not investigated in the 5.0 g α-LNA/day and 0.7 g EPA+DHA/day groups, since the changes in the composition of the PBMNC PL were not significant (see section 4.3.6.).

The changes in the proportions of AA, EPA and DHA were related to the changes in production of the cytokines IL-2, IFN-γ, IL-4 and IL-10. When all groups were considered together the change in the proportion of AA was significantly negatively correlated to the change in the production of IL-10 (r=-0.378), while the change in the proportion of DHA was significantly, positively correlated to the change in the production of IFN-γ (r=0.269). However, the relationships between the changes in the proportions of AA, EPA and DHA and the changes in cytokine production were stronger when the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups were considered alone. In the 10.0 g α-LNA/day group the change in the proportion of AA was significantly, negatively correlated to change in IL-10 production (r= -0.791), while the change in the proportion of EPA was positively correlated to change in IL-4 production (r= 0.488) and the change in the proportion of DHA significantly negatively correlated to change in IFN-γ production (r=0.518). In the 1.5 g EPA+DHA/day group the change in the proportion of EPA was significantly negatively related to change in IL-10 production (r=-0.786), while the change in the proportion of EPA was significantly positively correlated (r=0.720) to the change in IL-2 production in this group. There were no significant correlations between the change in the proportions of total SFA to total PUFA, total PUFA, total *n*-3 or *n*-6 PUFA or the ratio of n-6 PUFA to n-3 PUFA in PBMNC PL and the change in the production of Con A-elicited cytokines.

6.3.3. Modulation of Lymphocyte Proliferation

The proliferative response of lymphocytes to Con A stimulation was measured at 0, 3 and 6 months of intervention (*Table 6.7*).

6.3.3.1. Baseline data

There were no significant differences in the proliferation of lymphocytes in response to Con A between any of the treatment groups at baseline (*Table 6.6*). There were no significant effects of age or gender on the proliferation of lymphocytes at baseline.

Relationship between the Fatty Acid Composition of PBMNC PL and the Proliferation of Lymphocytes at Baseline

There were significant relationships between the fatty acid composition of the PBMNC PL and the ability of lymphocytes to proliferate at baseline (*Table 6.7.*). The proportion of the individual PUFA AA and DHA, the total PUFA and total *n*-3 or *n*-6 PUFA were all positively correlated with the proliferation of lymphocytes. The proportion of total SFA, the ratios of SFA to PUFA and of *n*-6 to *n*-3 PUFA were all negatively correlated with lymphocyte proliferation. There was no relationship between the proportion of α-LNA or ratio of AA to EPA and lymphocyte proliferation.

Table 6.6. The effect of treatment on the ability of lymphocytes to proliferate in response to Con A stimulation (n=30/group)

Treatment	Time (m)	- Con A	Peak Thymidine Incorporation	Stimulation Index
Placebo	0	740 ±126	29345 ±2880	72.04 ±9.99
	3	940 ±254	40526 ±4776	121.0 ± 24.6
	6	538 ±68	31288 ±1952	96.80 ±15.2
5.0 g α-LNA	0	691 ±118	33086 ±2669	75.00 ±8.55
	3	1305 ±303	39212 ±3394	100.1 ±18.4
	6	523 ±60	34637 ±1980	92.65 ±11.8
10.0 g α-LNA	0	863 ±142	31503 ±3402 [b]	76.77 ±13.5
	3	1670 ±531	45523 ±5126 [ac]	99.70 ±23.7
	6	574 ± 96	31659 ±2347 [b]	80.80 ±10.2
0.7g EPA+DHA	0	910 ±228	35768 ±2880	70.86 ±10.7
	3	1221 ±373	40553 ±2387	102.2 ±14.5
	6	588 ±91	34013 ±1797	84.04 ±8.87
1.5 g EPA+DHA	0	916 ±312	31230 ±2911	82.49 ±12.5
	3	761 ±300	33477 ±2479	118.6 ±17.7
	6	577 ±312	29637 ±1542	90.74 ±11.8

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month;

Table 6.7. The correlations between fatty acid composition and the lymphocyte proliferative response at baseline

	Peak Thymidine Incorporation
Total SFA	-0.259#
Total PUFA	0.326#
SFA/PUFA	-0.375#
Total *n*-6	0.185#
Total *n*-3	0.424#
n-6/*n*-3	-0.365#
α-LNA	-0.001
AA	0.203#
EPA	0.110
DHA	0.326#
AA/EPA	-0.040

Data are significant using a Spearman's Rank correlation ($p<0.05$); n=150

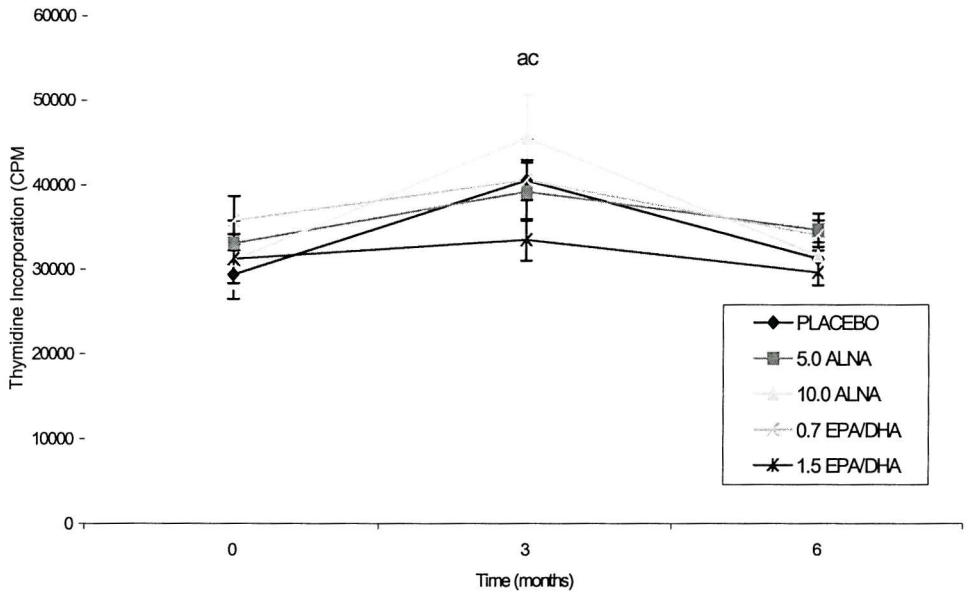
6.3.3.2. The Effects of the Different Treatments on the Absolute Proliferative Response of Lymphocytes

Two-way ANOVA established that there was no effect of treatment on peak thymidine incorporation into lymphocytes stimulated with a range of concentrations of Con A. However, there was a significant effect of time on peak thymidine incorporation ($p<0.0001$). This effect was investigated further.

Peak thymidine incorporation tended to be higher at 3 months compared to 0 and 6 months in all groups (*Table 6.6.*). This was significant in the 10.0 g α-LNA/day group (*Table 6.6.*). In addition, the 1.5 g EPA+DHA/day treatment group was different from the other dietary groups, since the change in peak thymidine incorporation at 3 months compared to 0 and 6 months was very small, compared to the other groups. This was not significant compared to the placebo group, or compared to the proliferative response observed at 0 and 6 months (*Figure 6.5.*). There were no significant differences in the peak thymidine incorporation between any of the groups at any time during the intervention period (*Table 6.6.*).

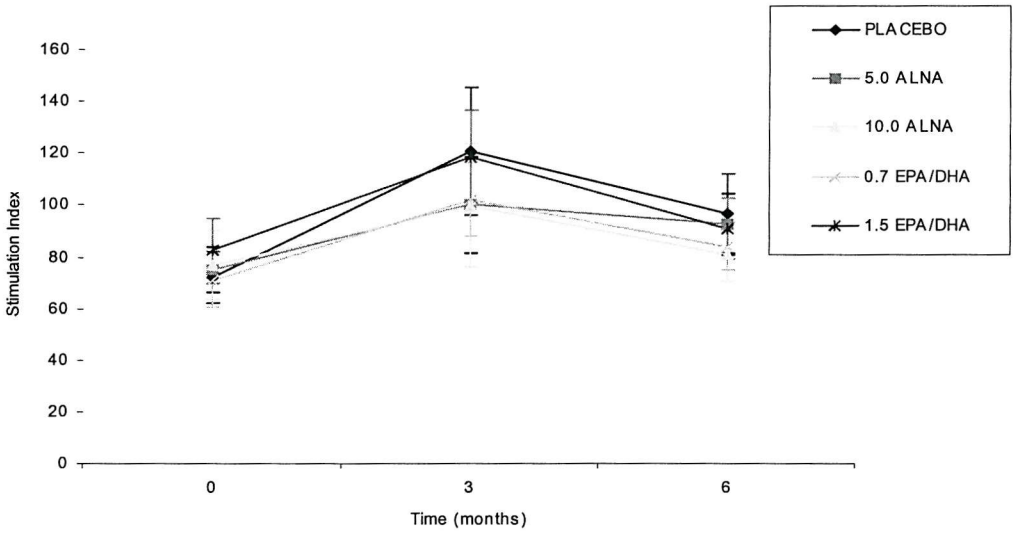
There were no significant effects of treatment on the stimulation index (thymidine incorporation / thymidine incorporation in the absence of Con A). However, this index tended to increase from 0 to 3 months in all groups and then to return toward baseline at 6 months (*Table 6.6*; *Figure 6.6*).

Figure 6.5. The effects of treatment on the peak thymidine incorporation of lymphocytes



Significant differences are expressed as: a. Significantly different from 0 month; c. Significantly different from 6 month;

Figure 6.6. The effect of treatment on the SI of lymphocytes



Relationships Between Changes in the Fatty Acid Composition of PBMNC PL and Changes in Proliferative Response of Lymphocytes after the Different Intervention Treatments

The correlation coefficients between the fatty acid composition of PBMNC PL and the proliferation of lymphocytes in response to Con A at baseline suggests that the capacity of PBMNC to proliferate was influenced by their fatty acid composition (*Table 6.5.*). The relationships between the changes in fatty acid composition of PBMNC PL and the changes in proliferative response were investigated for the dietary groups together (n=150), and for the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups alone (n=30/group). The relationships between changes in proliferation and changes in fatty acid composition were not investigated in the 5.0 g α-LNA/day and 0.7 g EPA+DHA/day groups since the changes in the composition of the PBMNC PL in these groups were not significant (see section 4.3.6.).

The changes in the proportions of total PUFA and of *n*-3 PUFA were significantly, positively correlated with changes in lymphocyte proliferation when all subjects were considered together (r=0.240 and r=0.301, respectively), and for the 1.5 g EPA+DHA/day group alone (r=0.692 and r=0.297). The change in the proportions of α-LNA, EPA and DHA in PBMNC PL of the 1.5 g EPA+DHA/day group were also significantly positively correlated with changes in the proliferation of lymphocytes (r=0.808, r=0.725 and r=0.571 respectively). There was a negative (but not significant) relationship between changes in the proportion of AA and lymphocyte proliferation (r=-0.489) for this group. There were no significant relationships between changes in fatty acid composition of PBMNC PL and changes in lymphocyte proliferation in the 10.0 g α-LNA/day group.

6.3.4. Relationship Between Changes in Cytokine Production and Lymphocyte Proliferation

The relationships between the concentrations of Con A-elicited cytokines (IL-2, IFN-γ and IL-4), the ratio of IFN-γ to IL-4 and the proliferation of lymphocytes were investigated. At baseline there was no significant correlation between the concentrations of the cytokines IL-2, IFN-γ and IL-4 and lymphocyte proliferation

(r=0.052, r=0.179 and r=–0.078, respectively). However, there was a significant correlation between the IFN-γ / IL-4 ratio and lymphocyte proliferation (r=0.223; $p<0.05$).

6.3.5. Modulation of the Delayed Type Hypersensitivity

There were no differences between the treatment groups at baseline in terms of the total area of the indurations at 48 hr after the application of the skin test, or in the number of positive indurations (*Table 6.8.*).

Two-way ANOVA established that there was an effect of treatment on the number of positive reactions (indurations) present at 48 hr after the application of the skin test ($p<0.02$), but not on the total area of the induration. There was no significant effect of time on either of these parameters. One-way ANOVAs were not able o identify any differences between the treatment groups (*Table 6.9.*). However, there was a non-significant tendency towards a decrease in the cumulative area in each of the treatments groups, including the placebo group except the 10.0 g α-LNA/day group..

Table 6.8. The effects of the treatments on the DTH response (n=20/group)

Treatment	Indurations (n)		Cumulative Area (mm^2)	
	0 Month	6 Months	0 Month	6 Months
Placebo	2.3 ±0.31	1.8 ±0.33	26.8 ±6.7	21.8 ±5.8
5.0 g α-LNA	1.2 ±0.21	1.2 ±0.20	19.2 ±4.5	14.5 ±4.9
10.0 g α-LNA	1.4 ±0.23	1.5 ±0.26	20.9 ±7.5	19.4 ±6.7
0.7 g EPA+DHA	1.6 ±0.28	1.6 ±0.31	22.6 ±8.8	18.6 ±5.2
1.5 g EPA+DHA	1.9 ±0.28	1.6 ±0.28	21.1 ±4.5	16.1 ±3.3

6.4. Discussion

6.4.1. Lymphocyte Subsets

Analysis of the lymphocyte populations revealed no significant changes in the % of T-, Th, Tc or B-lymphocytes over the intervention period. As discussed in section 1.4. few studies with dietary fish oil report lymphocyte populations and those that have, found no effect of increased fish oil in the diet on proportions of these cells. No

changes in the proportions of T-, Th, Tc or B-lymphocytes in rat spleen or thymus occurred following fish oil feeding (Yaqoob *et al.*, 1994a; Sanderson *et al.*, 1995). In humans, several studies have shown no effect of increasing the intake of EPA+DHA by up to 3.2 g/day on the proportion of total lymphocytes or the proportion of lymphocytes as T-lymphocytes, B-lymphocytes (Meydani *et al.*, 1993; Yaqoob *et al.*, 2000; Thies *et al.*, 2001b). However, in one study the proportion of lymphocytes which were CD4+ was decreased while the proportion of lymphocytes which were CD8+ increased (Meydani *et al.*, 1993). That study included fish oil in a low-fat, low-cholesterol diet. The level of fat in the diet and the age of the subjects used may contribute to the differences in the observed effects. The current study agrees with several earlier studies (Yaqoob *et al.*, 2000; Thies *et al.*, 2001b) and it is concluded that increasing consumption of n-3 PUFA will have little impact on circulating lymphocyte subsets.

6.4.2. Effect of Age and Gender on Measures of Acquired Immune Function

Both qualitative and functional age-dependent changes have been described in the immune system, and these are thought to be mainly in the T-lymphocyte system (Makinodan and Kay, 1980). A decrease in the proliferative response of human lymphocytes is associated with an increase in age (Murasko *et al.*, 1987; Nagel et al., 1988). Murasko *et al.* (1987) showed that proliferation in both males and females in response to Con A was lower by 50% in a group aged 60 to 69 years and lower by 72% in a group aged 80 to 89 years, compared to a group aged 20 to 29 years. In the present study no effect of age was found on the proliferation of lymphocytes in response to the same concentration of Con A as used by Murasko *et al.* (1987). Differences in other experimental conditions (for example serum or plasma type and length of culture) may well contribute to the differences in outcomes of these studies.

Other factors which might influence the results obtained, include the ages and health status of the subjects. Most studies which are designed to investigate the effects of age on immune function use the SENIEUR protocol for the selection of subjects (Lithtgart *et al.*, 1984), but even this approach has led to subjects with clinically ongoing and/or nutritional deficiency or other environmental factors influencing immune responses

(Lesourd *et al.*, 1994; Mazari and Lesourd, 1998). Thus, even apparently-healthy individuals may have underlying factors which could influence the results of age-dependent investigations of immune function.

Modulation of cytokine production with age has also been reported. The production of IL-2 has been found to decrease with age (Nagel *et al.*, 1988; Cakman *et al.*, 1996), while the production of IFN-γ is unchanged (Weifeng *et al.*, 1986) and the production of IL-10 increased (Cakman *et al.*, 1996). The decreased proliferative response of lymphocytes is not fully reconstituted by an increase in IL-2 *in vitro* (Rabinowich *et al.*, 1985). Thus, it is thought that age-dependent effects on lymphocyte proliferation are related to changes in IL-2 receptor expression and to impairment of endogenous IL-2 synthesis (Rabinowich *et al.*, 1985). The production of PGE_2 has been shown to increase with age in mice (Hayek et al., 1997) and this may contribute to the decrease in T-lymphocyte functions, in particular lymphocyte proliferation, observed with age. However, no effect of age on lipopolysaccharide (LPS)-elicited PGE_2 production was found in the present study and this may help to explain an absence of age-dependent changes in lymphocyte functions (see section 5.4.2.). It may be that the proportions of T-lymphocytes and their subsets may influence cytokine production since it is reported that the number of lymphocytes in peripheral blood decreases with age (Lesourd *et al.*, 1994; Huppert *et al.*, 1998). There is also evidence that the proportions of T-lymphocyte subsets alter with age. Ageing is associated with a decrease in the CD8+ subset, while the CD4+ subset remains unchanged in very-healthy elderly subjects (65 to 85 years) (Lesourd *et al.*, 1994). The absence of a decrease in T-lymphocyte number or change in subset composition in the present study, with age, may also explain the absence of a change in lymphocyte proliferative response or cytokine production which has been observed in other studies.

In the present study significant effects of age on the production of IL-4 were also observed when males and females of different age groups were compared. The production of IL-4 was slightly but not significantly increased in males with age while in females production was non-significantly decreased. A significant difference in the production of IL-4 was observed for females compared to males in subjects aged 50 to 70 years. This age-dependent pattern of production of IL-4 was also observed for IL-2. In the study of Murasko *et al.* (1987) it was also reported that IL-2 production and

lymphocyte proliferation decreased to a greater extent in females than males aged over 50 years. Most studies which report age-dependent changes in immune function do not report the gender of the subjects used and this may account for the different results obtained.

6.4.3. *Effects of the Dietary* n-*3 PUFA on Acquired Immune Function*

Cytokine Production

In the present study there were no significant effects of a dose of 0.7 g or 1.5 g EPA+DHA on the production of IL-2, IL-4, IFN-γ, IL-10 or the ratio of IL-4 to IFN-γ. The literature reports that increasing the consumption of EPA and DHA to 3.1 + 1.9 g/day results in a decrease in the production of IL-2 and IFN-γ (Gallai *et al.*, 1993), while 2.8 g EPA + 1.9 g DHA/day had no effect on IL-2 production (Endres *et al.*, 1993). A slightly lower dose of 3.2 g EPA+DHA/day also had no effect on IL-2, IFN-γ or IL-10 production (Yaqoob *et al.*, 2000). However, 1.23 g EPA+DHA/day has been shown to decrease IL-2 production (Meydani *et al.*, 1993). This dose of EPA+DHA was part of a low-fat diet (26% energy as fat) while in the present study the diet contained 36 % energy as fat. A low-fat diet is associated with an increase in lymphocyte proliferation (Kelley *et al.*, 1991), and thus may be associated with an increase in IL-2 production. In addition, a decrease in the total intake of fatty acids in the study of Meydani *et al.* (1993) will serve to increase the ratio of EPA+DHA to other fatty acids in the diet. These facts when considered together in a study may well mean that the effects of a dose of 1.23 g EPA+DHA/day on IL-2 production may be amplified.

A dose of 2.4 g EPA+DHA/day has also been observed to significantly decrease IL-2 production (Meydani *et al.*, 1991). However, this study was conducted in older women aged 51 to 68 years. As discussed in section 6.4.2. IL-2 production decreases with age, and it may be that the effects of EPA+DHA on this parameter are amplified with age, and thus the effects of similar doses are not seen in younger subjects such as those used in the study of Yaqoob *et al.* (2000) where the mean age was 40. The

difference in culture conditions used in these experiments may also contribute to differences in the results obtained. The nature of the sample in which the cytokine was assayed may impact on the results of the study. Meydani *et al.* (1991; 1993) measured Con A-elicited cytokine production in combined cell lysate and supernatant, while Yaqoob *et al.* (2000) and the present study measured cytokine concentrations in cell supernatant only. Thus, slightly different measurements are being made i.e. in some studies extracellular cytokine concentration are measured while in others a combination of intra- and extracellular cytokines concentrations are established. It has been shown that the concentrations of LPS-elicited IL-1β production is differentially affected by *n*-3 PUFA intervention in intra- and extracellular samples (Molvig *et al.*, 1991) (see section 5.4.3.).

The only study to investigate the effects of increasing dietary intake of α-LNA on Con A-elicited cytokines reported that 18 g α-LNA/day had no effect on production of IL-2 with Con A or phytohemmagglutinin (PHA) stimulation (Kelly *et al.*, 1991). The present study saw no effect of 5.0 or 10.0 g α-LNA/day on the production of IL-2, IL-4, IFN-γ or IL-10. Since a dose of 10.0 g α-LNA/day results in enrichment of EPA to the same extent as 1.5 g EPA+DHA/day, with no enrichment in DHA, and no effects of 1.5 g EPA+DHA/day on Con A-elicited cytokine production were observed, then a dose of 10.0 g α-LNA/day would also not be expected to have modulatory effects.

Lymphocyte Proliferation

The study of Kelley *et al.* (1991) measured lymphocyte proliferation in males aged 21 to 37 years, given 18 g α-LNA/day as part of a low-fat diet (29 % energy as fat). Lymphocyte proliferation in response to Con A was decreased by 65%. The present study included 5.0 g or 10.0 g α-LNA as part of a higher fat diet (>35% energy as fat) and found no significant effect of intervention of lymphocyte proliferation. Since a low-fat diet is associated with an increase in proliferation compared to a high fat diet (25 % compared to 40 % energy as fat) (Kelley *et al.*, 1991), and with an increase in the ratio of the fatty acid of interest compared to the remaining fatty acids in a low-fat diet there may well be an amplification of resultant modulatory effects of dietary α-

LNA. Differences in culture conditions may also contribute to any differences in the outcomes of these studies with α-LNA intervention.

In the present study no significant effects of 0.7 g or 1.5 g EPA+DHA were observed on the proliferation of lymphocytes in response to Con A stimulation. Although peak thymidine incorporation tended to be higher at 3 months compared to 0 and 6 months in all groups and this was significant in the 10.0 g α-LNA/day group. In addition, the 1.5 g EPA+DHA/day treatment group was different from the other dietary groups, since the change in peak thymidine incorporation at 3 months compared to 0 and 6 months was very small, compared to the other groups. This was not significant compared to the placebo group, or compared to the proliferative response observed at 0 and 6 months. There was no effect on lymphocyte proliferation of a dose of 2.8 g EPA + 1.9 g DHA in males with a mean age of 28 (Endres *et al.*, 1993), while 6 g DHA/day (as part of a low-fat diet) was also found to have no effect on this parameter in males with a mean age of 33 (Kelley *et al.*, 1998). However, Molvig *et al.* (1991) found that 1 g EPA + 0.6 g DHA or 2 g EPA + 1.2 g DHA dose-dependently decreased, by up to 30 %, the proliferative response of lymphocytes to PHA in males with a mean age of 26 years.

A dose of 1.23 g EPA+DHA/day has been shown to decrease lymphocyte proliferation in response to Con A and PHA by 24 % and 36 %, respectively (Meydani *et al.*, 1993). This dose of EPA+DHA was part of a low-fat diet (26% energy as fat) while in the present study the diet contained 36 % energy as fat. A low-fat compared to a high-fat diet (25 % to 40 % energy as fat) is associated with an increase in lymphocyte proliferation (Kelley *et al.*, 1991). In addition, the decrease in the total intake of fatty acids in the study of Meydani *et al.* (1991) will serve to increase the ratio of EPA+DHA to other fatty acids in the diet. Consideration of these facts together may well mean that the effects of a dose of 1.23 g EPA+DHA/day on lymphocyte proliferation may be amplified. A dose of 2.4 g EPA+DHA/day has also been observed to significantly decrease lymphocyte proliferation by 36 % (Meydani *et al.*, 1991). However, this was in older women aged 51 to 68 years. As discussed in section 6.4.2. lymphocyte proliferation decreases with age, and it may be that the effects of EPA+DHA on this parameter may be amplified with age, and thus not seen in younger subjects such as those used in the study of Yaqoob *et al.* (2000) where the

mean age was 40, and a dose of 2.1 g EPA + 1.1g DHA/day was consumed. The difference in culture conditions used in these experiments may well also contribute to differences in the results obtained. However, differences in the age and gender of the subjects may also be very influential in the results obtained and may contribute to the differences seen. In addition, the ratio of EPA to DHA used in this intervention was much lower than that used in many other EPA+DHA interventions. Since the study of Kelley *et al.* (1998) showed no effect of 6 g of DHA (in the absence of EPA), as part of a low-fat diet, while lower doses of EPA+DHA do exert effects, then it would appear that it is perhaps EPA rather than DHA is the fatty acid which exerts immunomodulatory effects, and this would help to explain the lack of modulation of lymphocyte proliferation seen in the current study.

The Delayed Type Hypersensitivity Response

In the present study there were no significant effects of any the dietary interventions on the DTH response. This accords with the lack of effect on lymphocyte proliferation and cytokine production. Kelley *et al.* (1991) showed that 18 g α-LNA/day (as part of a low-fat diet (23% energy as fat)) decreased the total induration diameter from 36 to 28 mm in males aged 21 to 37 years. The difference in fat content in the diet may well explain the differences between this and the present study. Likewise, 1.24 g EPA+DHA/day (as part of a low-fat diet (25 % energy as fat)) resulted in a 45 % decrease in the DTH total induration (Meydani *et al.*, 1993). Kelley *et al.* (1998) have shown no effect of 6 g DHA/day (as part of a low-fat diet (30% energy as fat)) on the DTH response. Thus, it appears that as part of a low-fat diet the DTH response can be modulated with 18 g α-LNA/day or 1.24 g EPA+DHA/day, while 5.0 or 10.0 g α-LNA/day or 0.7 g or 1.5 g EPA+DHA/day as part of a higher-fat diet as used in the present study has no effect. Whether the difference in outcomes of these experiments is due to subjects in the present study consuming a higher fat diet (>35 % energy as fat), or whether it is because the ratio of EPA to DHA was much less than that used in previous interventions is unclear. DHA seems to have little effect on the DTH response even at 6 g/day, with a low-fat versus high-fat diet (40 % to 25 % energy as fat) also not associated with any change in DTH response (Kelley *et al.*, 1992b). Thus, it would seem that the dose of EPA consumed, and perhaps its ratio to DHA, is important in the modulation of the DTH response. Since lymphocyte proliferation and

the production of IL-2 are fundamental in the DTH response, and age has been shown to modulate these T-lymphocyte functions, then the age of the subjects used may well also be important.

6.4.4. Correlations between Acquired Immune Function and Fatty Acid Composition of PBMNC PL

Membrane fluidity is determined by the unsaturation and chain length of the fatty acids in the cell membrane (section 5.4.4.). In the present study there were significant positive correlations between the proportion of PUFA in PBMNC PL and IL-2, IL-4 and IFN-γ production by Con A stimulated PBMNC at baseline. When α-LNA, EPA and DHA were supplemented in the diet there were correlations between the changes in fatty acid composition and the changes in production of some cytokines. A change in the proportion of EPA was positively correlated with the production of IL-4 and IL-2, while a change in EPA and DHA were negatively correlated with a change production of IL-10 and IFN-γ respectively. A change in the proportion of AA was negatively correlated with changed in IL-10 production. Thus, it would appear that the more unsaturated the membrane and thus more fluid the membrane, the greater the capacity to produce IL-2 and IL-4 in response to Con A stimulation, while the production of IL-10 and IFN-γ may be decreased. There may also be specific effects of the type of PUFA in which the membrane is enriched.

In the present study the proliferation of lymphocytes was found to be positively correlated with the proportion of PUFA, total *n*-6 or *n*-3, AA, EPA and DHA in PBMNC PL. The proportion of SFA and the SFA to PUFA ratio of PBMNC PL were negatively correlated with lymphocyte proliferation at baseline. The changes in fatty acid composition with the different interventions and the subsequent changes in total PUFA and *n*-3 PUFA were positively correlated with the proliferation of lymphocytes. Thus, as the unsaturation of the membrane increases the greater the ability of lymphocytes to proliferate.

The mechanisms of exactly how fatty acid composition of PBMNC PL could alter cytokine production have not yet been clearly defined. However, there are several possibilities for this mechanism and these include the effects of membrane fluidity on

the stimulus receptor and thus differences in the level of stimulation achieved. Membrane composition and perhaps fluidity could also affect the production of signalling molecules within the cell, and perhaps on the release of the cytokine protein molecules into the extracellular environment. Evidence for this arises in the different concentrations measured in cell lysate and supernatant samples (Molvig *et al.*, 1991).

6.5. Conclusion

In the present study there were no significant effects on the production of Con A-elicited cytokines or the proliferation of lymphocytes in response to Con A after intervention with 0.7 g or 1.5 g EPA+DHA/day or with 5.0 g or 10.0 g α-LNA/day for 6 months. The lack of effect on cellular functions was mirrored by a lack of effect on the *in vivo* DTH response. This was despite significant changes in the fatty acid composition of PBMNC PL in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day treatment groups.

The absence of effects on the parameters of acquired immune function would seem to concur with several other intervention studies. There is an indication that the doses of *n*-3 PUFA used in the present study and in several others, were not adequate to elicit changes in certain immune cell functions in healthy people consuming a high fat diet (>35 % energy as fat). When a lower fat diet, is used, it would seem that more marked effects on acquired immune cell functions are observed. Similarly when older subjects have been studied the effects of intervention with *n*-3 PUFA on acquired immune function would appear to be more pronounced. The DTH response was unaffected by the dietary interventions used in the present study and this indicates that all subjects were able to mount successful immune responses.

In conclusion, an increase in *n*-3 PUFA consumpation (as either α-LNA or long-chain *n*-3 PUFA) to the levels used here does not appear to alter acquired immunity. Since this aspect of the immune system is responsible for defence against microbes, for immune regulation and immunological memory, it would seem that the lack of effect of *n*-3 PUFA suggests no adverse immune effects. Nevertheless, at higher levels and in other groups there may be immunological effects (detrimental and beneficial) at

these levels of consumption. Taken even at these modest levels of intake there may be other benefical effects, such as TAG-lowering.

CHAPTER 7. DISCUSSION

This chapter aims to draw together the results described in this thesis in terms of the aims and hypotheses set out in the various chapters (see sections 1.5, 2.1,3.1,4.1,5.1 and 6.1).

7.1. Fatty acid composition

7.1.1. Animal Studies

There were significant dose-dependent increases in the content of *n*-3 PUFA in rat and murine spleen cells. In rats there was a significant dose-dependent increase in the proportion of EPA in spleen leukocyte phospholipids (PL) with the doses of 2.2 g and 4.4 g EPA/100 g fatty acids. However, in mice a dose of 4.4 g EPA/100 g fatty acids was required to achieve enrichment, although significant dose-dependent increases in DPA were observed in this species (this was also observed in rats). In neither species were there significant increases in the content of DHA after EPA feeding at a dose of 2.2 g/100g fatty acids. This may attributable to the inhibition of Δ6 destaurase as discussed in section 3.4.1. It was also apparent that there appeared to be a significant effect of the position of EPA within dietary TAG on its incorporation into the PL fraction in mouse spleen cells, with a significant increase in EPA content when fed in the *sn*-2 position. In rats this was only detected when the PC species were analysed. The enrichment of DHA in spleen cell PL, when fed at 2.2 g and 4.4g DHA/100g fatty acids, although dose-dependent, was not influenced by the position of DHA in dietary TAG in either rats or mice.

In rat plasma there were also significant changes in the composition of the different fractions (PL, triacylglycerol (TAG) and cholesterol ester (CE)). There were significant dose-dependent increases in EPA and DPA in plasma PL after feeding 2.2 g and 4.4 g EPA/100 g fatty acids with a change in the content of DHA only achieved at the higher dose of EPA. However, in plasma TAG and CE there were dose-dependent increases in EPA, DPA and DHA when EPA was fed. There were dose-dependent increases in DHA (and EPA) in both plasma PL and TAG with 2.2 g and

4.4 g DHA/100 g fatty acids while there was only an increase in EPA (and not DHA) in the plasma CE fraction. There was no effect of the position of EPA or DHA in dietary TAG on their incorporation into plasma lipid fractions.

Since the species of the plasma phospholipids were not determined it is not possible to say whether or not there was enrichment of EPA when fed in the *sn*-2 position of dietary TAG, as was detected in the PC species of spleen leukocyte PL. However, it may be that this was the case, making it possible to conclude that there is at least partial retention of EPA at the *sn*-2 position of dietary TAG from the point of ingestion through to the incorporation into certain cells of the body and that incorporation may be specific to certain (phospho)lipid species. The absence of an increase in spleen leukocyte PL DHA after feeding EPA, despite increases in the plasma PL, may be attributable to more than one factor. It may be that immune cells are not able to synthesise DHA as described in section 3.4.1, and this may be compounded by a requirement for the uptake of EPA compared to DHA into the membrane from passing lipoproteins, and then a requirement to maintain the membrane at a particular fluidity, thus inhibiting DHA uptake.

7.1.2. Human Study

The results of the human study show that the incorporation of α-LNA, EPA and DHA into a staple of the diet (margarine) can result in the enrichment of plasma PL in these fatty acids. There was dose-dependent enrichment of plasma PL in both EPA and DHA after 0.7 g or 1.5 g EPA+DHA/day, while there was maximal enrichment in α-LNA and EPA, but no change in DHA content after 5.0 g α–LNA intervention. There was no change in plasma PL arachidonic acid or α-LNA content in any of the intervention groups. With respect to PBMNC PL there was no change in α–LNA content after 5.0 g or 10.0 g α–LNA/day, while EPA content increased to the same extent after the higher doses of both α–LNA and EPA+DHA. DHA content increased after the higher dose of EPA+DHA. There was a significant decrease in AA in all treatment groups but this was not dose-dependent.

The occurrence of EPA in plasma PL after consuming increased levels of α-LNA, suggests that α-LNA can be converted to EPA in humans, presumably in the liver.

The elongation of EPA to DPA is also apparent with a rise in DPA for the 10.0 g α-LNA/day treatment, which was not as marked in the 5.0 g α-LNA/day group. There was no increase in DHA after increased intake of α-LNA suggesting that this last step in the elongation pathway is somehow inhibited. This could be due to inhibition (or low activity) of the enzyme Δ6 desaturase (see *Figure 1.10.*), which is involved in the elongation of DPA to DHA. An increase in EPA after increased α-LNA intake may be responsible for the inhibition of the Δ6 desaturase enzyme. The fact that there was no effect of the dose of α-LNA on the enrichment of plasma PL in EPA suggests that above a certain dose of α-LNA, there is an alternative route for α-LNA disposal rather than its elongation to EPA. It has been shown the oxidation of α-LNA might be slightly increased by diets rich in EPA and DHA (Vermunt *et al.,* 1999). Thus, the increase in EPA seen in plasma PL synthesised from dietary α-LNA may reach a certain level and may well than cause feedback inhibition on the conversion of α-LNA to EPA i.e. start to channel α-LNA through the oxidation pathway rather than the elongation pathway. It would appear that provision of even large amounts of α-LNA is not a useful way to enrich PBMNC in long-chain PUFA, and in fact it may even lead to depletion of DHA. It appears that there is limited conversion of DPA (and its precursors) to DHA. This raises questions about how DHA is synthesised in humans and whether or not DHA is in fact essential in the human diet. It also has implications for the inclusion of α-LNA into foods. It would seem that α-LNA or EPA cannot substitute for pre-formed DHA in the diet. However, further work is needed in this area to elucidate this satisfactorily.

7.2. Innate immune function

7.2.1. Animal Study

In terms of murine monocyte function there was no effect of dietary DHA while dietary EPA was found to dose-dependently decrease the number of monocytes performing phagocytosis. Thus, the effect of EPA on this aspect of innate immunity was greater than that of DHA. When EPA was fed in the *sn*-2 position there was an increased ability for active monocytes to engulf bacteria, while when fed in the *sn*-

1(3) position this was not seen. It is notable that the feeding of this diet also resulted in the greatest increase in the proportion of EPA in the spleen leukocyte phospholipids in mice and in the PC species of rat spleen leukocyte PL. The present study suggests that, although EPA in the *sn*-2 position of dietary TAG decreased the number of monocytes (and neutrophils) performing phagocytosis, there may be a compensatory increase in the capacity of the actively phagocytosing cells. Thus, overall there may be little effect of EPA-feeding (in the *sn*-2 position) on phagocytic activity. If however the EPA in fish oil is predominantly in the *sn*-1(3) position there is no such compensation by the phagocytosing monocytes and neutrophils, leading to an overall decrease in phagocytosis. A change in fatty acid composition is associated with change in phagocytic activity. This study has shown that the position of EPA but not DHA in dietary TAG has an effect on phagocytic activity. Therefore, maintenance of the position of EPA in 2-MAG, through digestion, absorption and incorporation into cell membranes might be a factor in determining the effect of dietary *n*-3 PUFA on phagocytosis. The effects were dose-dependent and were more profound for EPA in the *sn*-2 position. Feeding levels as low as 2.2 g EPA/100 g fatty acids in the diet can have modulatory effects on monocyte and neutrophil phagocytosis.

7.2.2. Human Study

In the human study there were no modulatory effects of doses of 0.7 or 1.5 g EPA+DHA/day or of 5.0 or 10.0 g α-LNA/day on the ability of neutrophils and monocytes to mount an oxidative burst in response to *E. coli* and phorbol myristic aceteate (PMA). There were no significant effects of the interventions on the ability of PBMNC to produce the cytokines when stimulated with LPS except IL-1β, where the production was significantly increased in the α-LNA groups.

In the human study there was a significant increase in the index of phagocytic activity of neutrophils in the 10.0 g α-LNA/day and 1.5 g EPA+DHA/day groups, while there was a tendency for the number of *E. coli* engulfed per neutrophil over the intervention period to increase in all groups and this was significant for the 1.5 g EPA+DHA/day group. The recruitment of monocytes had a tendency to decrease over the intervention period for the *n*-3 PUFA intervention groups, while there was a tendency for the number of bacteria engulfed per monocyte to increase in these groups, and this was

significant for the 1.5 g EPA+DHA group. The index of activity for monocytes tended to increase in both the 1.5 g EPA+DHA/day and 10.0 g α-LNA/day groups. Thus, it would appear that there was a similar effect of EPA supplementation on monocyte phagocytosis as observed in mice. Unfortunately the position of EPA (or DHA) within the dietary TAG molecule of the EPA+DHA interventions is not known for the preparations used for the human study. Thus, it is not possible to say whether or not there is an influence of the position of EPA on monocyte phagocytic activity, but it would appear that there is an increase in the number of bacteria engulfed by human monocytes to compensate for the decrease in the number of active cells, when the diet is supplemented with 1.5 g EPA+DHA/day. In addition, this increase may well be such that the phagocytic ability of monocytes is significantly enhanced when the diet is supplemented with this dose of EPA+DHA. This may be related to the significant increases in EPA content, which were seen in the PBMNC PL at a dose of 1.5 g EPA+DHA/day. This would concur with *in vitro* studies where the content of *n*-3 PUFA and the unsaturation of the membrane have been shown to influence the phagocytic activity of monocytes (see section 1.3).

7.3. Acquired immune function

7.3.1. Animal Studies

In terms of rat lymphocyte functions there was no effect of feeding either 2.2 g or 4.4 g EPA or DHA/100 g fatty acids on the production of cytokines, while lymphocyte proliferation was dose-dependently decreased when EPA was fed in the *sn*-2 position of dietary TAG compared to the *sn*-1(3) position. DHA had no effect on the proliferation of lymphocytes. It has previously been shown that EPA is more active in influencing human lymphocyte function than DHA (Thies *et al.*, 2001b), but in contrast the effects of these fatty acids were found to be equipotent in rats (Peterson *et al.*, 1998b). In the present study it was observed that EPA in different positions of dietary TAG resulted in different effects on spleen leukocyte PC molecular species. This may account for the different effects of EPA in the *sn*-2 versus *sn*-1(3) position on lymphocyte proliferation. This suggests that analysis of PL molecular species is important, since although there was no effect of the position of EPA in dietary TAG

on total PL enrichment in EPA, there was an effect on PC molecular species composition. These results also indicate that PC composition is important in the regulation of proliferation of lymphocytes as Jolly *et al.* (1997) suggest. Although the level of DHA was increased in PL and in PC it had little or no effect on lymphocyte function in the present study. This indicates that EPA rather than DHA is the important fatty acid in fish oil with regard to the modulation of lymphocyte function.

7.3.2. Human Studies

In the present study there were no significant effects of a dose of 0.7 g or 1.5 g EPA+DHA or 5.0 g or 10.0 g α-LNA on the production of IL-2, IL-4, IFN-γ, IL-10 or the ratio of IL-4 to IFN-γ. Furthermore, no significant effects of 0.7 g or 1.5 g EPA+DHA were observed on the proliferation of lymphocytes in response to Con A stimulation. However, there did appear to be a smaller increase in the peak thymidine incorporation in the 1.5 g EPA+DHA/day group at 3 months compared to the other groups, including the placebo, although this was not significant. Since similar lymphocyte proliferation results were seen in the rat feeding study and between the human and murine phagocytic activity in this thesis it may be that EPA was predominately in the *sn*-2 position of the TAG preparations used in the human study. The doses used in the rat and mouse studies were greater than those used in the human intervention and this may account for the absence of significant effects of the doses of EPA (+DHA) used in the human study.

The DTH response was unaffected by the dietary interventions in the present study and this indicated that all subjects were able to mount a successful immune response. Thus, at the doses of *n*-3 PUFA consumed in the present study no modulation of acquired immune function in healthy individuals was observed despite an increase in *n*-3 PUFA in the PBMNC PL. Since this aspect of the immune system is responsible for defence against microbes and immune regulation it would seem that the lack of effect of *n*-3 PUFA suggests no adverse immune effects.

7.4. Relationship between fatty acid composition of PBMNC PL and immune cell function

It would seem that the fatty acid composition has more profound effects on immune cell functions which directly involve the membrane such as phagocytosis. In the present study the phagocytic activity of human monocytes were found to be positively correlated with the proportion of PUFA in PBMNC PL, while the proportion of SFA and the SFA to PUFA ratio of PBMNC PL were negatively correlated with monocyte phagocytosis. Thus, as the unsaturation of the membrane increases the greater the ability of monocytes to perform certain aspects of phagocytosis. The murine study in this thesis also seems to suggest this but to go further and indicate that the *n*-3 PUFA EPA has the more modulatory effect of the two long chain PUFA EPA and DHA, on murine monocyte phagocytosis. These studies also suggest that the position of EPA with in the TAG molecule is important.

The results of the human study suggest that there is a much smaller influence of membrane unsaturation on the production of cytokines. It is interesting that the human study suggests that an increase in the proportions of PUFA and thus a more unsaturated membrane would seem to decrease the production of IL-6. An inhibitory effect of EPA feeding and subsequent enrichment in spleen cell PL was observed in the murine study. Thus the results seem to concur although the mechanism behind the effect of the fatty acid composition upon IL-6 production has not been clearly defined. However, there are several possibilities for this mechanism and these include the effects of membrane fluidity on the stimulus receptor and thus differences in the level of stimulation achieved. Membrane composition and perhaps fluidity could also affect the production of signalling molecules within the cell, and perhaps on the release of the cytokine protein molecules into the extracellular environment

7.5. Conclusions

Thus, in conclusion it would seem that although significant dose-dependent modulation of the fatty acid composition of plasma and immune cells in humans is possible at 0.7 g or 1.5 g EPA+DHA/day and with 5.0 g and 10.0 g α-LNA, there was little significant effect on the measures of immune function made in this study. Thus,

there was no dose-dependent effect of these fatty acids on human immune function, nor were the effects of α-LNA less significant than those of EPA+DHA. However, this study does show that introducing *n*-3 PUFA into the diet through the foodstuffs rather than capsules can result in significant modulation of the fatty acid composition of the lipid pools of the body, and since there were no effects on immune function this may be beneficial for groups of the population who which to take advantage of the other beneficial effects of *n*-3 PUFA, such as their blood lipid lowering effects.

This study also shows that there may be important considerations as to the source of *n*-3 PUFA in the diet. With respect α-LNA, this may not be a suitable source of the long chain *n*-3 PUFA DHA and this may have ramifications for certain sections of the population such as growing children and pregnant women. The source of fish oil consumed may also be important since, fish oils differ in their content of EPA and in its position in TAG, and this may well influence the effects of EPA on the immune system and on the incorporation into lipid pools.

CHAPTER 8. APPENDIX

8.1. The Additive Content of the Margarines

Table 8.1. The Emulsifier, Flavouring, Stabiliser Content

	Placebo	5.0 g α-LNA	10.0g α-LNA	0.7 g EPA+DHA	1.5 g EPA+DHA
Emulsifiers	0.20	0.20	0.20	0.20	0.20
β-carotene (0.4%)	0.10	0.10	0.10	0.10	0.10
Flavour	0.013	0.013	0.013	0.013	0.013
BHA	0.21	0.21	0.21	0.21	0.21
BHT	0.011	0.011	0.011	0.011	0.011
Propyl gallate	0.008	0.008	0.008	0.008	0.008
Potassium-sorbate	0.10	0.10	0.10	0.10	0.10
EDTA disodium	0.025	0.025	0.025	0.025	0.025

g/100 g margaine; BHA = Butylated hydroxyaldehyde; BHT = Butylated hydroxytoluene.
Data were supplied by Unilever, The Netherlands

8.1.1. The Analysis of Vitamin E Content of the Margarines

The amount of vitamin E present in the margarines was standardised based on the amount and type of PUFA present. Intake of PUFA increases the requirement for vitamin E in two ways (Muggli, 1994):

- an increased need for protection from peroxidation of cell membranes
- to offset the impairment of vitamin E absorption by a PUFA-rich diet

The RoPUFA supplied by Hoffman La Roche contained a relatively high amount of vitamin E and so the levels of this vitamin in the other treatment groups were increased accordingly. Calculations were based on the estimated minimum requirements of vitamin E needed according to Muggli (1994) (*Table 8.2.*).

The fatty acid profile and the amount of natural vitamin E already present in the fats and oils used was analysed. Using this information and the final product composition the requirement of vitamin E was calculated. Vitamin E was added to the treatments in the form of dl-α-tocopheryl acetate, and the unit which vitamin E is expressed as is

tocopherol equivalent (TE). There are 0.67 α-TE/mg to 1 IU/mg of dl-α-tocopheryl acetate.

The fatty acid analysis and calculation of required vitamin E of the individual fats and oils are shown in Appendix 1. Based on the final fat blend composition of each treatment the required vitamin E was calculated (Appendix 2). The amount of the different tocopherols and tocotrieneols already present in the individual oils and fats were converted to TE (Appendix 3). The assumption was made that during refining of the final fat blend the natural amount of vitamin E would decrease by 20%.

The final vitamin E contents are shown in *Table 8.3.* All data were supplied by Unilever, The Netherlands.

8.2. Fatty Acid Composition Analysis of Plasma Triacylglycerol and Cholesterol Ester

The data presented here are the fatty acid composition of plasma triacylglycerol and cholesterol esters determined after dietary modulation as described in chapter 4.

The statistical analysis has been conducted as in section 4.2.7.

Table 8.2. The vitamin E requirements for the fatty acids used in the manufacture of the test margarines

Double Bonds	**Fatty Acid**		**Vitamin E requirement (mg dl-α-tocopheryl acetate/g fatty acid)**
1	Oleic	18:1 n-9	0.13
1	Gadoleic	20:1 n-11	0.13
1	Erucic	22:1 n-9	0.13
1	Cetoleic	22:1 n-11	0.13
2	Linoleic	18:2 n-6	0.89
3	γ-linolenic	18:3 n-6	1.34
3	α-linolenic	18:3 n-3	1.34
3	Dihomo-γ-linolenic	20:3 n-3	1.34
4	Arachidonic	20:4 n-6	1.79
5	Eicosapentaenoic	20:5 n-3	2.24
6	Docosahexaenoic	22:6 n-3	2.68

Table 8.3. The vitamin E composition of the test margarine- calculated requirement and final measured amounts

Diet	**Requirement of vitamin E based on calculation according to Muggli, 1994. See Table 7.1. (TE/kg fat)7**	**Vitamin E present (based on tocopherol analysis* (TE/kg fat)**	**Surplus (+) or shortage (-) of vitamin E (TE/kg fat)**	**Vitamin E added (TE/kg fat)**	**dl-alpha-tocoperyl-acetate added (mg/kg product)***	**Final vitamin E content of margarines analysed by Unilever (TE/kg product)**	**Mean + range of actual vitamin E content of mararines analysed by Unilever (TE/kg product)**	**Excess to calculated vitamn E (TE/kg product)****
Placebo	343	415	+72	77	98	418	427 (315-467)	+9
5.0 g α-LNA	393	287	-106	255	324	460	516 (497-544)	+56
10.0 g α-LNA	465	80	-385	534	678	522	528 (473-633)	+6
0.7 g EPA +DHA	371	520	+149	0	0	503	531 (519-591)	+28
1.5 g EPA +DHA	371	520	-149	0	0	503	531 (519-591)	+28

*dl-alpha-tocopheryl-acetate contains 0.67 TE per mg, the product contains 85% fat

** surplus to target levels given in column 7 e.g. 418

Data were supplied by Unilever, The Netherlands.

Table 8.4. The fatty acid composition of plasma triacylglycerol

Diet	Time (m)	16:0	18:0	18:1 n-9	18:2 n-6	γ18:3 n-6	α18:3 n-3	20:0	20:1 n-6	20:2 n-6	20:3 n-6	20:4 n-6	20.5 n-3	22:5 n-3	22:6 n-3
Placebo	0	25.60	3.94	36.93	17.81	0.62	1.12	0.48	0.66^{bc}	0.552^{bc}	0.58	1.12	0.38	0.63	0.95
		±0.69	±0.30	±0.88	±0.89	±0.09	±0.15	±0.15	±0.06	±0.06	±0.12	±0.09	±0.06	±0.10	±0.15
	3	26.00	3.21	35.33	20.08	0.47	1.3	0.3	0.43	0.348	0.51	1.60	0.35	0.74	0.89
		±0.57	±0.14	±0.57	±0.78	±0.04	±0.09	7±0.08	±0.05	±0.04	±0.08	±0.09	±0.05	±0.13	±0.10
	6	26.60	3.43	36.47	19.67	^{B}0.46	0.14	0.31	0.43	0.371	0.49	1.12	0.54	0.62	1.18
		±1.17	±0.20	±1.26	±1.23	±0.04	±0.27	±0.06	±0.05	±0.04	±0.08	±0.10	±0.10	±0.06	±0.17
5.0 g α-LNA	0	26.41	3.46	35.73	19.35	0.44	0.89^{bc}	0.50	0.63^{bc}	0.50	0.56	0.95	0.44	0.84	0.88
		±0.55	±0.12	±0.84	±0.85	±0.08	±0.07	±0.14	±0.07	±0.07	±0.08	±0.06	±0.10	±0.08	±0.87
	3	25.96	3.55	33.67	17.71	0.39	^{AC}2.01	0.29	0.39	0.37	0.53	0.92	0.40	0.58	0.81
		±0.89	±0.18	±0.72	±1.09	±0.03	^{DE}±0.13	±0.04	±0.05	±0.04	±0.04	±0.09	±0.05	±0.06	±0.81
	6	26.65	0.42	35.50	17.94	0.27	1.84	0.28	0.39	0.33	0.42	1.02	0.50	0.53	0.85
		±0.57	±0.14	±0.65	±0.80	±0.03	±0.13	±0.04	±0.05	±0.05	±0.02	±0.08	±0.10	±0.05	±0.85
10.0 g α-LNA	0	25.30	4.01^{bc}	37.90	18.30	0.43	1.02^{bc}	0.28	0.63^{c}	0.51	0.43	1.02	0.31	0.48^{bc}	0.59^{bc}
		±0.61	±0.19	±0.57	±0.60	±0.04	±0.07	±0.04	±0.03	±0.04	±0.03	±0.07	±0.03	±0.04	±0.04
	3	25.30	3.29	^{BD}37.00	^{D}16.8	0.35	^{AB}3.65	0.30	0.48	0.42	0.38	0.89	0.49	0.73	0.80
		±0.75	±0.20	±0.80	±0.53	±0.05	^{DE}±0.30	±0.05	±0.06	±0.05	±0.03	±0.09	±0.06	±0.07	±0.07
	6	25.08	3.31	38.60	18.23	0.35	3.15	0.25	0.43	0.45	0.41	0.95	0.81^{ab}	0.72	0.87
		±0.85	±0.14	±1.23	±0.92	±0.07	±0.25	±0.05	±0.05	±0.06	±0.05	±0.11	±0.16	±0.07	±0.07
0.7g EPA +DHA	0	25.28	3.85^{b}	36.9	19.49	0.38	1.07	0.25	0.63^{bc}	0.48	0.46	1.04	0.29^{c}	0.49	0.72^{c}
		±0.74	±0.28	±0.71	±1.04	±0.06	±0.08	±0.04	±0.04	±0.03	±0.05	±0.06	±0.04	±0.05	±0.08
	3	26.68	3.47	34.8	19.11	0.38	1.23	0.37	0.41	0.32	0.41	1.01	0.41	0.54	1.00
		±0.78	±0.17	±0.54	±0.75	±0.04	±0.10	±0.07	±0.05	±0.04	±0.04	±0.09	±0.05	±0.06	±0.08
	6	24.98	3.05	35.97	20.08	0.42	1.23	0.27	0.40	0.42	0.43	1.09	0.55	0.56	1.13
		±0.63	±0.09	±0.61	±1.00	±0.05	±0.09	±0.46	±0.05	±0.10	±0.03	±0.06	±0.09	±0.04	±0.11

Table 8.4. The fatty acid composition of plasma triglyceride continued .....

Diet	Time (m)	16:0	18:0	18:1 n-9	18:2 n-6	γ18:3 n-6	α18:3 n-3	20:0	20:1 n-6	20:2 n-6	20:3 n-6	20:4 n-6	20.5 n-3	22:5 n-3	22:6 n-3
1.5 g EPA +DHA	0	26.06 ±0.80	3.91 ±0.19	36.40 ±0.95	20.01 ±0.84	0.49 ±0.06	1.10 ±0.11	0.48 ±0.11	0.63 ±0.03	0.49 ±0.04	0.47 ±0.05	1.07 ±0.08	0.31^{bc} ±0.05	0.57 ±0.05	0.80^{bc} ±0.07
	3	25.40 ±0.49	3.67 ±0.23	33.90 ±0.91	20.89 ±0.97	0.43 ±0.12	1.18 ±0.08	0.29 ±0.05	0.50 ±0.08	0.51 ±0.12	0.40 ±0.06	1.08 ±0.09	$^{ABD}0.68$ ±0.06	0.99 ±0.26	$^{AB}2.05$ ^{CD}±0.19
	6	25.60 ±0.69	$^{D}3.92$ ±0.34	33.15 ±0.80	19.57 ±0.59	0.35 ±0.03	1.37 ±0.09	0.32 ±0.05	0.43 ±0.06	0.38 ±0.05	0.39 ±0.03	1.16 ±0.08	0.68 ±0.06	0.73 ±0.04	$^{AB}2.01$ ^{CD}±0.19

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

Table 8. 5. The fatty acid composition of plasma cholesterol ester

Diet	Time (m)	16:0	18:0	18:1 n-9	18:2 n-6	γ18:3 n-6	α18:3 n-3	20:0	20:1 n-6	20:2 n-6	20:3 n-6	20:4 n-6	20.5 n-3	22:6 n-3
Placebo	0	10.81 ±0.32	1.22 ±0.19	16.01 ±0.42	48.45 ±1.08	1.07 ±0.09	0.61 ±0.05	0.13 ±0.04	0.33 ±0.04	0.31 ±0.07	0.85 ±0.35	5.14 [c] ±0.23	1.06 ±0.09	2.65 ±0.42
	3	11.12 ±0.31	1.30 ±0.15	15.59 ±0.37	50.64 ±1.08	[E]1.00 ±0.07	[BCDE] 0.76 ±0.08	0.40 ±0.09	0.25 ±0.03	0.33 ±0.54	0.83 ±0.57	[E] 5.73 ±0.26	[CE] 1.25 ±0.11	1.33 ±0.27
	6	10.33 ±0.36	1.64 ±0.42	14.54 ±0.64	51.50 ±1.45	1.06 ±0.12	[BCDE] 0.64 ±0.07	0.10 ±0.08	0.56 ±0.34	0.40 ±0.73	0.79 ±0.49	6.29 ±0.35	[CE] 1.64 ±0.29	3.61 [b] ±0.56
5.0 g ALNA	0	10.52 ±0.29	1.09 ±0.18	15.37 ±0.59	49.87 ±1.13	0.90 ±0.07	0.48 [bc] ±0.03	0.15 ±0.07	0.31 ±0.08	0.22 ±0.03	1.05 ±0.11	4.95 ±0.24	1.20 [c] ±0.22	1.61 ±0.30
	3	10.88 ±0.27	1.04 ±0.11	15.63 ±0.39	50.41 ±0.98	0.83 ±0.06	1.07 ±0.06	0.10 ±0.03	0.20 ±0.02	0.30 ±0.06	1.47 ±0.66	4.62 [c] ±0.24	1.50 ±0.20	1.56 ±0.27
	6	9.95 ±0.25	1.01 ±0.11	15.27 ±0.27	51.35 ±1.24	1.03 ±0.12	1.03 ±0.11	0.13 ±0.03	0.33 ±0.10	0.34 ±0.05	0.82 ±0.10	5.86 ±0.36	2.08 ±0.29	3.54 [ab] ±0.61
10.0 g ALNA	0	10.74 ±0.25	1.19 ±0.14	15.90 ±0.46	48.78 ±2.04	0.98 ±0.09	0.50 [bc] ±0.03	0.46 ±0.25	0.36 [bc] ±0.05	0.21 ±0.02	0.97 ±0.12	5.12 ±0.25	1.08 [bc] ±0.22	2.31 ±0.36
	3	10.71 ±0.25	1.01 ±0.11	16.49 ±0.51	48.40 ±1.19	0.74 ±0.07	2.25 [a] ±0.20	[D] 0.10 ±0.03	0.21 ±0.02	0.17 ±0.03	0.70 ±0.03	4.85 [c] ±0.19	1.87 ±0.19	1.32 ±0.27
	6	10.32 ±0.43	1.24 ±0.19	16.79 ±0.84	50.39 ±2.10	1.00 ±0.12	2.10 [a] ±0.31	0.27 ±0.16	0.20 ±0.04	0.97 ±0.45	0.81 ±0.06	6.03 ±0.47	2.58 ±0.38	4.52 [ab] ±0.68
0.7g EPA +DHA	0	10.37 ±0.21	0.92 ±0.10	15.85 ±0.40	51.04 ±1.13	0.90 ±0.06	0.52 ±0.04	0.22 ±0.07	0.34 ±0.05	0.26 ±0.03	0.90 ±0.07	5.19 ±0.24	0.83 [bc] ±0.07	1.89 ±0.32
	3	11.13 ±0.41	1.87 ±0.58	15.29 ±0.46	49.69 ±0.129	0.88 ±0.08	0.60 ±0.06	0.61 ±0.29	0.28 ±0.06	0.31 ±0.06	1.03 [c] ±0.11	5.40 ±0.25	1.61 ±0.28	1.28 ±0.17
	6	9.80 ±0.22	1.00 ±0.11	15.36 ±0.66	52.80 ±1.35	1.10 ±0.23	0.72 ±0.13	0.70 ±0.02	0.19 ±0.02	0.40 ±0.09	0.75 ±0.02	5.82 ±0.27	1.82 ±0.26	3.69 [ab] ±0.34

Table 8. 5. The fatty acid composition of plasma cholesterol ester continued .....

Diet	Time (m)	16:0	18:0	18:1 n-9	18:2 n-6	γ18:3 n-6	α18:3 n-3	20:0	20:1 n-6	20:2 n-6	20:3 n-6	20:4 n-6	20.5 n-3	22:6 n-3
1.5 g EPA +DHA	0	10.51 ±0.18	1.34 ±0.18	15.29 ±0.31	51.79 ±0.94	0.88 ±0.08	0.58 ±0.05	0.23 ±0.05	0.46 ±0.06	0.41 ±0.12	0.96 ±0.09	5.18 [bc] ±0.21	1.05 [bc] ±0.13	1.84 [bc] ±0.28
	3	11.29 ±0.65	1.63 ±0.35	15.43 ±0.55	49.90 ±2.05	0.72 ±0.08	0.61 ±0.07	0.22 ±0.08	0.34 ±0.13	0.31 ±0.07	1.58 ±0.76	4.64 [a] ±0.24	2.37 [a] ±0.18	1.69 [a] ±0.26
	6	10.13 ±0.31	1.12 ±0.13	14.77 ±0.42	52.14 ±1.22	0.88 ±0.14	0.64 ±0.06	0.15 ±0.05	0.21 ±0.02	0.33 ±0.05	0.75 ±0.03	1.99 [a] ±0.23	3.17 [a] ±0.37	3.38 [a] ±0.45

Significant differences are expressed as: a. Significantly different from 0 month; b. Significantly different from 3 month; c. Significantly different from 6 month; A. Significantly different from the placebo treatment; B. Significantly different from the 5.0 g ALNA treatment; C. Significantly different from the 10.0 g ALNA treatment; D. Significantly different from the 0.7 g EPA+DHA treatment; E. Significantly different from the 1.5 g EPA+DHA treatment

CHAPTER 9. REFERENCES

Abbas AK, Lichtman AH and Pober JS (1994) Cellular and Molecular Immunology (2nd Edition). WB Saunders Company.

Alam R, Yatsu RM, Tsui L, Alam S (1989) Receptor-mediated uptake and 'retroendocytosis' of high-density lipoproteins by cholesterol-loaded human monocyte-derived macrophages; a possible role in enhancing reverse cholesterol transport. *Biochimica et Biophysica Acta* **1004**, 292-299.

Allain CA, Poon LS, Chan, CSG, Richmond W, Fu PC (1974) Enzymatic determination of total serum cholesterol. *Clinical Chemistry* **20**, 470.

Anel A, Naval J, Gonzalez B, Torres JM, Mishal Z, Uriel J, Pineiro A (1990) Fatty acid metabolism in human lymphocytes. I Time-course changes in fatty acid composition and membrane fluidity during blastic transformation of peripheral blood lymphocytes. *Biochimica et Biophysica Acta* **1044**, 323-331.

Aoyama T, Fukui K, Taniguichi K, Nagaoka S, Yamaoto T, Hashimoto Y (1996) Absorption and metabolism of lipids in rats depend on fatty acid isomeric position. *Journal of Nutrition* **126**, 225-231.

Ardawi MS and Newsholme EA (1984) Metabolism of ketone bodies, oleate and glucose in lymphocytes of the rat *Biochemical Journal* **221**, 255-260.

Babu US, Bunning VK, Wiesenfeld P, Raybourne RB, O'Donnell M (1997) Effect of dietary flaxseed of fatty acid composition, superoxide, nitric oxide generation and antilisterial activity of peritoneal macrophages from female Sprague-Dawley rats. *Life Sciences* **60**, 545-554.

Bach (1975) Regulation of theta-antigen expression by agents altering cyclic AMP level and by thymic factor. *Annals New York Academy of Science* **249**, 316-320.

Bang HO & Dyerberg J (1972) Plasam lipids and lipoproteins patterns in Greenlandic West Coast Eskimos. *Acta medica Scandanavia* **192**, 85-94.

Bang HO, Dyerberg J, Sinclair HM (1980) The composition of the Eskimo food in north western Greenland. *The American Journal of Clinical Nutrition* **33** 2657-2661.

Barone J, Herbert JR, Reddy MM (1989) Dietary fat and Natural Killer cell activity. *The American Journal of Clinical Nutrition* **50** 861.

Bechoua S, Dubois M, Nemoz G, Largarde M & Prigent AF (1998) Docosahexaenoic acid lowers phosphatidate levels in human activated lymphocytes despite phospholipase D activation. *Journal of Lipid Research* **39**, 873-883.

Bell MV and Sargent JR (1987) Effects of the fatty acid composition of phosphatidylserine and diacylglycerol on the *in vitro* activity of protein kinase C from rat spleen: influences of (n-3) and (n-6) polyunsaturated fatty acids. *Comparative Biochemistry and Physiology* **86B**, 227-232.

Belluzzi A, Miglio F (1998) N-3 fatty acids in the treatment of Crohn's disease. In: Kremer J, editor. Medicinal fatty acids in inflammation. Basel: Birkhauser Verlag, 1998, p. 91-101.

Bendich A (1993) Vitamin E and human immune functions. In: Nutrition and Immunology, pp. 217-228 [DM Klurfeld, editor]. New York & London: Plenum Press.

Benyon S (1998) Metabolism and Nutrition. Mosby.

Berry and Nishizuka (1990) Protein kinase C and T-cell activation *European Journal of Biochemistry* **189**, 205-214.

Berger A, German JB, Chiang BL, Ansari AA, Keen CL, Fletcher MP, Gershwin MR (1993) Influence of feeding unsaturated fats on growth and immune status of mice. *Journal of Nutrition* **123**, 225-233.

Betz M and Fox BS (1991) Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *Journal of Immunology* **146**, 108-113.

Billiar T, Bankey P, Svingen B, Curran RD, West MA, Holman RT, Simmons RL, Cerra FB (1988) Fatty acid uptake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. *Surgery* **104**, 343-349.

Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911-917.

Blok WL, de Bruijn MFTR, Leenan PJM, Elinh WMC, van Rooijen N, Stanley ER, Buurman WA, van der Meer JWM (1996) Dietary n-3 fatty acids increase spleen size and postendotoxin circulating TNF in mice; Role of macrophages, macrophages precursors, and colony-stimulating-factor-1. *Journal of Immunology* **157**, 5569-5573.

Blok WL, Deslypere JP, Demacker PNM, Van der Ven-Jongekrijg J, Hectors MPC, Van der Meer JWM, Katan MB (1997) Pro-and anti-inflammatory cytokines in healthy volunteers fed various doses of fish oil for 1 year. *European Journal of Clinical Investigation* **27,** 1003-1008.

Blonk MC, Bilo HJG, Nauta JJP, Poppsnijders C, Mulder C, Donker AJM (1990) Dose-response effects of fish oil supplementation in healthy volunteers. *American Journal of Clinical Nutrition* **52,** 120-127.

Borofsky MA, Zurier RB, Rosenbaum H, Weiner DB, Williams WV (1992) Effects of polyunsaturated fatty-acids on Interleukin –2- dependent T-cell growth. *Immunologic Research* **11** 154-164

Bray MA, Gordon D, Marle J (1986) Leukotrienes in inflammation *Agents and Actions* **6**, 171-175.

Braun J, Severson D (1992) Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochemical Journal* **287**, 337-347.

Breittmayer JP, Pelassey C, Cousin JL, Bernard A, Aussel C (1993) The inhibition of fatty acids of receptor-mediated calcium movements in Jurkat T-cells is due to increased calcium extrusion. *Journal of Biological Chemistry* **268**, 20812-20817.

Bretscher MS (1972) Asymmetrical lipid bilayer structure for biological membranes *National New Biologist* **61**, 11-12.

Brenner (1984) Effect of unsaturated acids on membrane structure and enzyme kinetics *Progress in Lipid Research* **23** 69-96.

Brenner and Peluffo (1966) Effect of saturated and unsaturated fatty acids in the desaturation *in vitro* of palmitic, stearic, oleic, linoleic and linolenic acid. *Journal of Biological Chemistry* **241** 5213.

Brink EJ, Haddeman E, Defouw NJ, Weststrate JA (1995) Positional distribution of stearic acid and oleic acid in a triacylglycerol and dietary calcium concentration determines the apparent absorption of these fatty acids in rats. *Journal of Nuitrition***125** 2379-2387

British Nutrition Foundation's Task Force. Unsaturated Fatty Acids: Nutritional and Physiological Significance. London: Chapman & Hall, 1992.

British Nutrition Foundation. n-3 Fatty Acids and Health. London: British Nutrition Foundation, 1999.

Brouard C & Pascal M (1990) Effects of moderate dietary supplementation's with n-3 fatty acid on macrophage and lymphocyte phospholipid and macrophage eicosanoid synthesis in the rat. *Biochemica et Biophysica Acta* **1047**, 19-28.

Brouard C & Pascal M (1993) Modulation of rat and human lymphocyte function by n-6 and n-3 polyunsaturated fatty acids and acetylsalicylic acid. *Annals of Nutrition and Metabolism* **37** 146-159.

Broughton KJ, Johnston CS, Pace BK, Liebman M, Kleppingen KM (1997) Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. *American Journal of Clinical Nutrition* **65** 1011-1077.

Budowski P, Crawford MA (1985) α-linolenic acid as a regulator of the metabolism of arachidonic acid: dietary implications of the ratio n-6:n-3 fatty acids. Proceedings of the Nutrition Society 44, 221-229.

Burr ML, Sweetnam PM & Fehily AM (1994) Diet and reinfarction. *European Heart Journal* **15**, 1152-1153.

Buttke TM (1984) Inhibition of lymphocyte proliferation by free fatty acids I. Differential effects on mouse B-lymphocyte and T-lymphocyte. *Immunology* **53** 235-242.

Cakman I, Rohwer J, Schutz RM, Kirchner H, Rink L (1996) Dysregulation between Th1 and TH2 cell sub-populations in the elderly. *Mechanisms of Aging and Development* **87** 197-209.

Calder PC and Yaqoob P (2000) The level of protein and fat in the diet of pregnant rats both affect lymphocyte function in the offspring. *Nutrition Research* **20** (7) 995-1005.

Calder PC (1995) Fatty acids, dietary lipids and lymphocyte functions. *Biochemical Society Transactions* **23** 302-309.

Calder PC (1998) Dietary fatty acids and lymphocyte functions. *Proceedings of the Nutrition Society* **57** (4) 487-502.

Calder PC (1997) n-3 Polyunsaturated Fatty Acids and Cytokine Production in Health and Disease. *Annals of Nutrition and Metabolism* **41** 203-234.

Calder PC, Yaqoob P, Harvey DJ, Watts A, Newsholme EA (1994a) Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. *Biochemical Journal* **300**, 509-518.

Calder PC, Yaqoob P and Newsholme EA (1994b) Triacylglycerol metabolism by lymphocytes and the effect of triacylglycerols on lymphocyte proliferation. *Biochemical Journal* **298**, 605-611.

Calder PC & Newsholme EA (1992a) Polyunsaturated fatty acids suppress human peripheral blood lymphocyte proliferation and interleukin-2 production. *Clinical Science* **82**, 695-700.

Calder PC & Newsholme EA (1992b) Unsaturated fatty acids suppress IL-2 production and transferrin receptor expression by Con-A stimulated rat lymphocytes. *Mediators of Inflammation* **1**, 107-112.

Calder PC, Bond JA, Bevan SJ, Hunt SV, Newsholme EA (1991) Effect of fatty acids on the proliferation of concanavalin A-stimulated rat lymph node lymphocytes. *International Journal of Biochemistry* **23**, 579-588.

Calder PC, Bond JA, Harvey DJ, Gordon S, Newsholme EA (1990) Uptake of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochemical Journal* **269**,807-814.

Callard RE (1989) Cytokine regulation of B-cell growth and differentiation. *British Medical Bulletin* **45** 371-388.

Carnielli VP, Luijendijk IHT, van Beek RHT, Boerma GJM, Degenhert HJ, Sauer PJJ (1995) Effect of dietary triacylglycerol fatty acid positional distribution on plasma lipid classes and their fatty acid composition in preterm infants. *American Journal of Nutrition* **62**, 776-781

Cannon GW, Hibbs JB, Hoidal JR, Griffiths MM (1995) Nitric oxide production during adjuvant induced arthritis and collagen induced arthritis. *Arthritis and Rheumatism* **38**, 840.

Cashel KM, Greenfield H (1994) Principal sources of dietary fat in Australia – evidence from apparent consumption data and the national dietary survey. British *Journal of Nutrition* **71** 753-773

Cathcart MK, Li Q, Chisholm GM (1995) Lipoprotein receptor interactions are not required for monocyte oxidation of LDL. *Journal of Lipid Research* **36**,1857-1865.

Caughey GE, Mantzioris E, Gibson RA, Cleland LG and James MJ (1996) The effect on human tumour factor αand interleukin 1β production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. *American Journal of Clinical Nutrition* **63**, 116-122.

Chait A, Iverius P-H and Brunzell (1982) Lipoprotein lipase secretion by human monocyte-derived macrophages. *Journal of Clinical Investigation* **69**, 490-493.

Chandra RK (1983) Numerical and functional deficiency in T helper cells in protein-energy malnutrition. *Clinical and Experimental Immunology* **51**, 126-132.

Chang HR, Arsenijevic D, Pechere JC, Piguet PF, Mensi N, Girardier L, Dulloo Ag (1992) Dietary supplementation with fish oil enhances *in vivo* synthesis of tumour necrosis factor. *Immunology Letters* **34**, 13-18.

Chapkin RS, Akoh CC, Lewis RE (1992) Dietary fish oil modulation of in vivo peritoneal macrophage leukotriene production and phagocytosis. *Journal of Nutrition and Biochemistry* **3**, 599-604.

Chapkin RS, Fowler KH, McMurray DN, Fan YY, Aukema HM (1993) Purified dietary N-3 polyunsaturated fatty acids alter diacylglycerol mass and molecular species composition in concanavalin A stimulated murine splenocytes. *Biochimica et Biophysica Acta* **1210** 89-96.

Chapkin RS, Ziboh VA, McCullough JL (1987) Dietary replacement with fish oil did not reverse epidermal hyperproliferation of EFA deficient guinea pig skin. *Clinical Research* **35**, A248- .

Chapkin RS, Miller CC (1990) Chain elongation of eicosapentaenoic acid in the macrophage. *Biochimica et Biophysica Acta* **1042**, 265-267

Chow SC, Ansotegui IJ & Jondal M (1990) Inhibition of receptor-mediated calcium influx in T cells by unsaturated and non-esterified fatty acids. *Biochemical Journal* **267**, 727-732.

Clark WF, Parbtani A, Huff MW, Reid B, Holub BJ, Falardeau P (1989) Omega-3 fatty acid dietary supplementation in systemic lupus erythematosus. *Kidney International* **36**, 653-660.

Clarke SD, Thullier P, Baillie RA, Shaw X (1999) Peroxisome proliferator activated receptor: in a family of lipid activated transcription factors. *American Journal of Clinical Nutrition* **70**, 566-571.

Cooper Al, Gibbons L, Horan MA, Little RA, Rothwell NJ (1993) Effect of dietary fish oil supplementation on fever and cytokine production in human volunteers. *Clinical Nutrition* **12** 321-328.

Crabtree GR and Clipstone NA (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357** 695-697

Crevel RWR, Friend JV, Goodwin BFJ & Parish WE (1992) High fat diets and the immune response of C57 Bl mice. *British Journal of Nutrition* **67**, 17-26.

Davies MJ, Gordon JL, gearing AJH, Rigont R, Neville N, Katz D, Kyriakopoulos (1993) Expression of adhesion molecules ICAM-1, VCAM-1, PECAM, and E-Selectin in human atherosclerosis. *Journal of Pathology* **171,** 223-229.

Daviglus ML, Stamler J, Orencia AJ et al (1997) Fish consumption and the 30-year risk of fatal myocardial infarction. *The New England Journal of Medicine* **336**, 1046-1052.

D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trichieri G (1993) Interleukin 10 (IL-10) inhibits human leukocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *Journal Of Experimental Medicine* **178**, 1041-1048.

D'Ambola JB, Aeberhard EE, Trang N, Graffar S, Barrett CT, Sherman MP (1991) Effect of dietary (n-3) and (n-6) fatty acids on in vivo pulmonary bacterial clearance by neonatal rabbits. *Journal of Nutrition* **121**, 1262-1269.

De Deckere Eam, Krover O, Vershuren PM & Katan MB (1998) Health aspects of fish and n-3 polyunsaturated fatty acids from plant and marine origin. *European Journal of Clinical Nutrition* **52**, 749-753.

De Lorgeril M, Renaud S, Mamelle N et al (1994) Mediterranean alpha-linolenic acid rich diet in secondary prevention of coronary heart disease. *Lancet* **343**, 1454-1459.

Dept. of Health and Social Security (1991). Dietary reference values for food energy and nutrients for the U.K. Report of the cardiovascular committee on medical and food policy in London: HMSO (Report on heart and social subjects 41).

De Sanctis JB, Blanca I, Adzioch D and Bianco NE (1994) Lipoprotein lipase expression in natural killer cells and its role in their cytotoxic activity. *Immunology* **83**, 232-239.

Devi MA & Das UN (1994) Antiproliferative effect of polyunsaturated fatty acids and interleukin-2 on normal and abnormal human lymphocytes. *Experentia* **50** 489-492

Dira G (1988) Immunoregulation in aging. *International Journal of Medicine* **4** 83-85.

Dittmer (1961) Blood and Other Body Fluids: Biological Handbook (1961). Federation of American Society for Experimental Biology.

Dolecek TA (1992) Epidemiological evidence of relationships between dietary polyunsaturated fatty acids and mortality in the multiple risk factor intervention trial. *Proceedings of the Society of experimental Biology and Medicine* **331**, 1194-1199.

Dumont JE, Jauniaux JC, Roger PP (1989) The cyclic AMP-mediated stimulation of cell proliferation. *Trends in Biological Science* **14** 67-71.

Dyerberg J, Bang HO, Hjφrne N (1975) fatty acid composition of the plasma lipids in Greenland Eskimos. *Acta medica Scandanavia* **200,** 69.

Eicher SD, McVey DS (1995) Dietary modulation of Kupffer cell and splenocyte function during *Salmonella typhimurium* challenge in mice. *Journal of Leukocyte Biology* **58**, 32-39.

Elsbach P and Weiss J (1988) Phagocytosis of bacteria and phospholipid degradation. *Bichimica et Biophysica Acta* **947**, 29-52.

Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Potter AA (1993) CD36 is a receptor for oxidized low density lipoprotein. *Journal of Biological Chemistry* **268**, 1811-1816.

Endres S, Meydani SN, Ghorbani R, Schindler R & Dinarello CA (1993) Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. *Journal of Leukocyte Biology* **54**, 599-603.

Endres S, Ghorbani r, Kelley VE, Georgilis K, Lonnemann G, van der Meer JMW, Cannon JG, Rogers TS, Klempner MS, Weber PC, Schaeffer EJ, Wolff SM, Dinarello CA (1989) The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumour necrosis factor by mononuclear cells. *New England Journal of Medicine* **312**, 1217-1224.

Erickson KL, McNeill CJ, Gershwin ME, Ossmann JB (1980) Influence of dietary fat concentration and saturation on immune ontogeny in mice. *Journal of Nutrition* **110**, 1555-1572.

Ertel W, Morrion MH, Ayala A, Chaudry IH (1993) Modulation of a macrophage membrane phospholipid by n-3 polyunsaturated fatty acids increases interleukin 1 release and prevents suppression of cellular immunity following haemorrhagic shock. *Archives of Surgery* **128**,15-21.

Farrell D (1998) Enrichment of hen eggs with n-3 long chain fatty acids and evaluation of enriched eggs in humans. *American Journal of Clinical Nutrition* **68**, 538-544.

Fernandes G, Troyer DA, Jolly CA (1998) The effects of dietary lipid on gene expression and apoptosis. *Proceedings of the Nutrition Society* **57**, 543-550.

Fisher M, Levine PH, Weiner BH, Johnson MH, Doyle EM, Ellis PA, Hoogasian JJ (1990) Dietary n-3 fatty acid supplementation reduces superoxide production and chemiluminescence in monocyte enriched preparation of leukocytes. *American Journal of Clinical Nutrition* **51**, 804-808.

Fitzgerald L (1988) Exercise and the immune system. *Immunology* **9**, 337-339.

Folch J, Lees M, Sloane – Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry* **226** 497-509.

Fowler KH, McMurray DN, Fan YY, Aukema HM, Chapkin RS (1993) Purified dietary n-3 polyunsaturated fatty acids alter diacylglycerol mass and molecular species composition in concanavalin A-stimulated murine splenocytes. *Biochimica et Biophysica Acta* **1210**, 89-96.

Frayn K (1996) Metabolic regulation : a human prospective. Portland Press London.

Friend JV, Lock SO, Gurr MI & Parish WE (1980) Effect of different dietary lipids on the immune responses of Hartley strain guinea pigs. *International Archives of Allergy and Applied Immunology* **62**, 292-301.

Fritsche KL, Cassity NA, Huang SC (1991) Effect of dietary fats on the fatty acid compositions of serum and immune tissues in chickens. *Poultry Science* **70** 1213-1222.

Fujikawa M, Yamashita N, Yamazaki K, Sugiyama E, Suzuki H & Hamazaki T (1992). Eicosapentaenoic acid inhibits antigen-presenting cell function of murine splenocytes. *Immunology* **75**, 330-335.

Gallai V, Sarchielli P, Trequattrini A, Franceschini M, Floidi A, Firenze C, Alberti A, Di Benedetto D & Stragliotto (1993) Cytokine secretion and eicosanoid production in the peripheral blood mononuclear cells of MS patients undergoing dietary supplementation with n-3 polyunsaturated fatty acids. *Journal of Neuroimmunology* **56**, 143-153.

Gibney MJ, Hunter B (1993) The effects of short term and long term supplementation with fish oil on the incorporation of N-3 polyunsaturated fatty acids into cells of the immune system in healthy volunteers. *European Journal of Clinical Nutrition* **47** 255-259.

Gibson RA, James MJ, Neumann MA, Hawkes JS, Cleland LG (1992) Incorporation of dietary oleate, linoleate, α-linolenate and eicospentaenoate into the plasma lipid fractions of four strains of rat. *Biochimica et Biophysica Acta* **1126** 49-52.

Gregory J, Foster K, Tyler H and Wiseman M (1990) The dietary and Nutritional Survey of British Adults. London; HMSO.

Goldyne ME, Stobo JD (1982) Human monocytes synthesize eicosanoids from T lymphocyte-derived arachidonic acid Prostaglandins 24, 623.

Goldyne ME, Stobo JD (1981) Immunoregulatory role of prostaglandins and related lipids. *Critical Reviews in Immunology* **2**, 189-223.

Goodwin JS, Messner RP & Peake GT (1974) Prostaglandin suppression of mitogen stimulated leukocytes in culture. *Journal of Clinical Investigation* **4,** 368-378.

Goodwin JS & Cueppens J (1983) Regulation of the immune response by prostaglandins. *Journal of Clinical Immunology* **3**, 295-315.

Grimm H, Tibell A, Norrlind B, Blecher C, Wilker S, Schwemmle K (1994) Immunoregulation by parental lipids: impact on the n-3 to n-6 fatty acid ratio. *Journal of Parenteral and Enteral Nutrition* **18**, 417-421.

Groth L (1976) Acta Dermato- venereologica **197**, 8-61.

Halvorsen DA, Hansen J-B, Grimsgaard S, Bonna KH, Kierulf P, Norday A (1997) The effect of highly purified eicosapentaenoic and docosahexaenoic acids on monocyte phagocytosis in man. *Lipids* **32**, 935-942.

Han SN & Meydani SN (1999) Vitamin E and infectious disease in the aged. *Proceedings of the Nutrition Society* **58**, 697-705.

Hannsson GK (1997) Cell-mediated immunity in atherosclerosis. *Current Opinion in Lipidology* **8**, 301-311.

Hardardoittir I, Kinsella JE (1992) Increasing the dietary n-3 to n-6 polyunsaturated fatty acid ratio increase tumour necrosis factor production by murine resident peritoneal macrophages without an effect on elicited peritoneal macrophages. *Journal of Nutrition* **122**, 1942-1951.

Harris WS (1996) N-3 fatty acids and lipoproteins comparison of results for human and animal studies. *Lipids* **31** 243-252.

Hayek GM, Mura C, Wu D, Beharka AA, Han SN, Paulson E, Hwang D, Meydani SN (1997) Enhanced expression of inducible cyclooxygenase with age in murine macrophages. *Journal of Immunology* **159** 2445-2451.

Healy DA, Wallace FA, Miles EA, Calder PC, Newsholme P (2000) Effect of low to moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids* **35** 763-768.

Homan van der Heide JJ, Bilo HJG, Donker, JM, Wilmink JM, Tegzess AM (1993)Effect of dietary fish oil on renal function and rejection in cyclosporine-treated recipients of renal transplants. *New England Journal of Medicine* **329**, 769-773.

Hsu, K-HL, Ghanta VK and Hiramoto RN (1981) Immuno-suppressive effect of mouse serum lipoproteins. *Journal of Immunology* **126**, 1909- 1913.

Huang L and Pagano RE (1975) Interaction of phospholipid vesicles with cultured mamalial cells. I., Characteristics of uptake. *Journal of Cell Biology* **67**, 49-60.

Huang S-C, Misfeldt ML, Fritsche KL (1992) Dietary fat influences Ia antigen expression and immune cell populations in the murine peritoneum and spleen. *Journal of Nutrition* **122**, 1219-1231.

Hubbard NE, Somers SD, Erickon KL (1994) Effect of dietary fish oil on development and selected functions of murine inflammatory macrophages. *Journal of Leukocyte Biology* **49**, 592-598.

Hubbard NE, Chapkin JB, Erickon KL (1994) Effect of dietary linseed oil on tumoricidal activity and eicosanoid production in murine macrophages. *Lipids* **29**, 651-655.

Hughes DA, Pinder AC, Piper Z, Johnson IT & Lund EK (1996) Fish oil supplementation inhibits the expression of major histocompatability complex class II molecules and adhesion molecules on human monocytes. *American Journal of Clinical Nutrition* **63**, 267-272.

Hughes DA & Pinder AC (1997) N-3 polyunsaturated fatty acids modulate the expression of functionally associated molecules on human monocytes and inhibit antigen presentation *in vitro*. *Clinical and Experimental Immunology* **110**, 516-523.

Hughes DA (1998) *In vitro* and *in vivo* effects of n-3 polyunsaturated fatty acids on human monocyte function. *Proceedings of the Nutrition Society* **57**, 5521-525.

Huppert FA, Solomou W, O'Connor S, Morgan K, Sussans P, Brayne C (1998) Aging and lymphocyte populations: whole blood analysis of immune markers in a large population sample of healthy elderly individuals. *Experimental Immunology* **33** 593-600.

Illingworth DR, Portman OW, Robertson AL, JR and WA Maygar (1973) The exchange of phospholipids between plasma lipoproteins and rapidly dividing human cells grown in tissue culture. *Biochimica et Biophysica Acta* **306**, 422-436.

Innis SM, Dyer R, Quinlan P, Diersenschade D (1995) Palmitic acid is absorbed as SN-2 monopalmitin from milk and formula with rearranged triacylglycerols and results in increased plasma triglyceride SN-2 and cholesteryl ester palmitate in piglets. *Journal of Nutrition* **125** 73-81.

Jacob Co, Fronek Z, Lewis GD, Koo M, Hansen JA, Mcdevitt HO (1990) Heritable major histocompatibilty complex class II-associated differences in production of tumour necrosis factor α:

Relevance to genetic predisposition to systemic lupus erythematousus. *Proceedings of the National Academy of Science* **87**, 1233-1237.

Jackson SK, Darmani H, Stark JM, Harwood JL (1992) Interferon-gamma increases macrophage phospholipid polyunsaturation: a possible mechanism of endotoxin sensitivity. *International Journal of Experimental Pathology* **73**, 783-791.

Janeway CA (1983) Immune response genes, the problem of the non-responder, a commentary on the 1A molecule of the antigen presenting cell plays a critical role in immune response gene regulation of T-cell activation. *Journal of Molecular and Cellular Immunology.* **1,** 15-18.

Jeffery NM, Sanderson P, Newsholme EA & Calder PC (1997a) Effects of varying the type of saturated fatty acid in the rat diet upon serum lipid levels and spleen lymphocyte functions. *Biochimica et Biophysica Acta* **1345**, 223-236.

Jeffery NM, Cortina M, Newsholme EA & Calder PC (1997b) Effects of variations in the proportions of saturated, monounsaturated and polyunsaturated fatty acids in the rat diet on spleen lymphocyte functions. *British Journal of Nutrition* **77**, 805-823.

Jeffery NM, Newsholme EA & Calder PC (1997c) the level of polyunsaturated fatty acids and the n-6 to n-3 polyunsaturated fatty acid ratio in the rat diet both affect serum lipid levels and lymphocyte functions. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **57**, 149-160.

Jeffery NM, Sanderson P, Sherrington EJ, Newsholme EA & Calder PC (1996) The ratio of *n*-6 to *n*-3 polyunsaturated fatty acids in the rat diet alters serum lipid levels and lymphocyte functions. *Lipids* **31**, 737-745.

Joe B, Lokesh BR (1994) Role of capsaicin, curcumin and dietary n-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages. *Biochimica et Biophysica Acta* **1224**, 255-263.

Jolly CA, Jiang Y-H, Chapkin RS & McMurray DN (1997) Dietary n-3 polyunsaturated fatty acids suppress murine lympho-proliferation, interleukin-2 secretion and the formation of diacylglycerol and ceramide. *Journal of Nutrition* **127**, 37-43.

Jorgensen MH, Hernell O, Hughse EL, Michaelson KF (2001) Is there a relationship between docosahexaenoic acid concentration in mothers milk and visual development in term infants? *Journal of Paediatric Gastroenterology and Nutrition* **32** 293-296.

Kammer GM (1988) The adenylate cyclase-cAMP- protein kinase A pathway and regulation of the immune response. *Immunology Today* **9**, 222-229.

Kelly JP & Parker CW (1979) Effect of arachidonic acid and other unsaturated fatty acids on mitogenesis in human lymphocytes. *Journal of Immunology* **122**, 1556-1562.

Kelley VE, Ferritti A, Izui S, Strom TB (1985) A fish oil diet rich in eicosapentaenoic acid reduces cyclooxygenase metabolites and suppresses lupus in MRL-lpr mice. *Journal of Immunology* **134**, 1914-1919.

Kelley DS, Nelson GJ, Serrato CM, Schmidt PC, Branch LB (1988) Effects of type of dietary fat on indices of immune status in rabbits. *Journal of Nutrition* **118**, 1376-84.

Kelley DS, Branch LB, Love JE, Taylor PC, Rivera YM & Iacono JM (1991) Dietary alpha-linolenic acid and immunocompetance in humans. *American Journal of Clinical Nutrition* **53**, 40-46.

Kelley DS, Nelson GJ, Taylor PC, Schmidt PC, Mackey B (1992a) Dietary Alpha-linolenic acid and the fatty acid composition of human peripheral blood mononuclear cells. *FASEB Journal* **6**, A1213.

Kelley DS, Taylor PC, Nelson GJ, Schmidt PC, Ferretti A, Erickson KL, Yu R, Chandra RK (1999) Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. *Lipids* **34** 317-324.

Kelley DS, Dougherty RM, Branch LB, Taylor PC & Iacono JM (1992b) Concentration of dietary n-6 polyunsaturated fatty acids and human immune status. *Clinical Immunology and Immunopathology* **62**, 240-244.

Kelley DS, Nelson GJ, Love JE, Branch LB, Taylor PC, Schmidt PC, Mackey BE, Iacono JM (1993) Dietary α-linolenic acid alters tissue fatty acid composition, but not blood lipids, lipoproteins or coagulation status in humans. *Lipids* **28**, 533-537.

Khair-El-Din TA, Sicher SC, Vazquez MA, Wright WJ, Lu CY (1995) Docosahexaenoic acid, a major constituent of fetal serum and fish oil diets, inhibits IFN-γ-induced Ia-expression by murine macrophages *in vitro*. *Journal of Immunology* **154**, 1296-1306.

Khalfoun B, Thibault G, Bardos P & Lebranchu Y (1996a) Docosahexaenoic and eicosapentaenoic acids inhibit in vitro human lymphocyte-endothelial cell adhesion. *Transplantation* **62**, 1649-1657.

Khalfoun B, Thibault G, Lacord M, Gruel Y, Bardos P & Lebranchu Y (1996b) Docosahexaenoic and eicosapentaenoic acids inhibit human lymphoproliferative responses in vitro but not the expression of T cell surface activation markers. *Scandanavian Journal of Immunology* **43**, 248-256.

Khoo JC, Mahoney EM and Wiztum JL (1981) Secretion of lipoprotein lipase by macrophages in culture. *Journal of Biological Chemistry* **256** 7105-7108.

Kinsella JE, Lokesh B, Broughton S & Whelan J (1990) Dietary polyunsaturated fatty acids and eicosanoids; potential effects on the modulation of the inflammatory and immune cells: An overview. *Nutrition* **6**, 24-44.

Kishmoto A, Takai Y, Mori U, Nishizuka Y (1980) *Journal of Biological Chemistry* **255** 2273-2276.

Kollmorgen GM, Sansing WA, Lehman AA, Fischer G, Longley RE, Alexander SS Jr, King MM, McCay PB (1979) Inhibition of lymphocyte function in rats fed higher-fat diets *Cancer Research* **39**, 3458-3462.

Kook AI and Rubeinstein D (1970) The role of serum lipoproteins in the release of phospholipids by rat liver slices. *Biochimica et Biophysica Acta* **202**, 396-398.

Kremer JM, Lawrence DA, Jubiz w, Giacomo R, Rynes K, Bartholomew LE, Sherman M (1990) Dietary fish oil and olive oil supplementation in patients with Rheumatoid arthritis. *Arthritis and Rheumatism* **33**, 810-820.`

Krieger M and Hertz J (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor related protein (LRP). *Annual Review Biochemistry* **63**, 601-637.

Kromhout D, Bosschieter EB, Coulander CD (1985) The inverse relation between fish consumption and 20 year mortality from coronary heart disease. *New England Journal of Medicine* **312,** 1205-1209.

Kromann N and Green A (1980) Epidemiological studies in the Upernavik District, Greenland. *Acta Medica Scandanavia* **208**, 401-406.

Kuby J (1997) Immunology (3rd Edition). Freeman Publishers.

Kunkel SL, Remick DG, Spengher M, Chensue SW (1982) *Advances in Prostaglandin, Thromboxanes and Leukotrienes* **9** 331-339.

Leaf DA, Connor WE, Barstad L, Sexton G (1995) Incorporation of dietary n-3 fatty acids into the fatty acids of human adipose tissue and plasma lipid classes. *American Journal of Clinical Nutrition* **62**, 68-73.

Lee JH, Ikeda I, Sugano M (1992) Effects of dietary n-6/n-3 polyunsaturated faaty acid balance on tissue lipid levels, fatty acid patterns, and eicosanoid production in rats. *Nutrition* **8**, 162-166.

Lee TH, Hoover RL, Williams JD, et al. (1985) Effects of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *New England Journal of Medicine* **312,** 1217-1224.

Leskanich CO, Matthews KR, Wkrup CC, Noble RC, Hazeldine M (1997) The effect of dietary oil containing n-3 fatty acids o the fatty acid physiochemical and organoleptic characteristics of pig meat and fat. *Journal of Animal Science* **75**, 673-683.

Lesourd B and Marzari L (1999) Nutrition and the elderly. The proceedings of the Nutrition Society **58**, 685-695.

Lesourd B, Laisney C, Salvatore R, Meaume S, Moulias R (1994) Decreased maturation of T cell population factors on the appearance of double negative CD4-, CD8-, CD2+ cells. *Archives of Gerontology and Geriatrics* **4** S139-S154.

Lesourd BM, Wang A, Moulias R (1985) Serial delayed cutaneous hypersensitivity skin testing with multiple recall antigens in healthy volunteers: booster effect study. *American Allergy* **55**, 725-735.

Levy JA, Ibraim AB, Shirai T, Ohta K, Nagasawa R, Yoshida H, Estes J, Gardner M (1982) Dietary fat affects immune response, production of antiviral factors, and immune complex disease in NZB/NZW mice. *Proceedings of the National Academy of Science USA* **79**, 1974-1978.

Lewis GP (1983) Immunoregulatory activity of metabolites of arachidonic acid and their role in inflammation. *British Medical Bulletin* **39**, 243-248.

Li D, Sinclair A, Wilson A, Nakkote S, Kelly F, Abedin L, Mann N, Turner A (1999) Effect of dietary α-linolenic acid on thrombotic risk factors in vegetarian men. *American Journal of Clinical Nutrition* **69**, 872-882.

Liao F, Shin HS, Rhee SG (1993) *In vitro* tyrosine phosphorylation of PLC Gamma 1 and PLC Gamma 2 by SRC family protein tyrosine kinases. *Biochemical and Biophysical Research Communications.***191**, 1028-1033.

Lithtgart GJ, Corberand JX, Fournier C, Galanaud P, Hijmans W, Kennes B, Muller-Hermelink HK, Steinmann GG (19840 Admission criteria for immunogerontological studies in man: the Seniuer protocol. *Mechanisms of Aging and Development* **28** 47-55.

Locniskar M, Nauss KM, Newberne PM (1983) The effect of quality and quantity of dietary fat on the immune system. *Journal of Nutrition* **113**, 951-961.

Lokesh BR, Sayers TJ, Kinsella JE (1990) Interleukin-1 and tumour necrosis factor synthesis by mouse peritoneal macrophages is enhanced by dietary n-3 polyunsaturated fatty acids. *Immunology Letters* **23,** 281-286.

Lokesh BR, Hseih HL, Kinsella JE (1986) Peritoneal macrophages from mice fed dietary (n-3) polyunsaturated fatty acids secrete low levels of prostaglandins. *Journal of Nutrition* **116**, 2547-2552.

Lokesh BR, Wrann M (1984) Incorporation of palmitic acid or oleic acid into macrophage membrane lipids exerts differential effects on the functions of normal mouse peritoneal macrophages. *Biochimica et Biophysica Acta* **792**, 141-148.

Lokesh BR, Black JM, German JB, Kinsella JE (1988) Docosahexaenoic acid and other dietary omega-3 polyunsaturated fatty acids suppress leukotriene synthesis by mouse peritoneal macrophages. *Lipids* **23**, 968-972

Lovegrove JA, Brooks CN, Murphy MC, Gould BJ, Williams CM (1997) Use of manufactured foods enriched with fish oils as a means of increasing long chain n-3 polyunsaturated fatty acid intake. *British Journal of Nutrition* **78**, 223-236.

MAFF (Ministry of Agriculture Fisheries and Food) Food Information. Surveillance Sheet 127. Dietary intake of iodine and fatty acids. London: MAFF 1997.

Magrum LJ, Johnston PV (1983) Modulation of prostaglandin synthesis in rat peritoneal macrophages with ω-3 fatty acids. *Lipids* **18**, 514-521.

Mahoney EM, Scott WA, Landsberger FR, Hamill Al, Cohn ZA (1980) Influence of fatty acyl substitution on the composition and function of macrophage membranes. *Journal of Biological Chemistry* **255**, 4910-4917.

Mahoney EM, Khoo JC, Steinberg D (1982) Lipoprotein lipase secretion by human monocytes and rabbit alveolar macrophages in culture. *Proceedings National Academy of Science USA* **74** *4895-4899.*

Mukaida N, Okamoto S.I., Ishikawa K (19940 Molecular mechanisms of IL-8 gene expression. *Journal of Leukocyte Biology* **56**, 554-558.

Makinodan T, Kay MMB (1980) Age influence on the immune system. *Advances in Immunology* **29** 287-330.

Maniongui C, Blond JP, Ulmann L, Durand G, Poisson JP & Bezard J (1993) Age-related changes in Δ6 and Δ5 desaturase activities in rat liver microsomes. *Lipids* **28**, 291-297.

Mann NJ, Johnson LG, Warrick GE, Sinclair AJ (1995) The arachidonic acid content of the Australian diet is lower than previously estimated. *Journal of Nutrition* **125** 2528-2535.

Mantzioris E, Cleland LG, Gibson RA, Neumann MA, Demasi M, James MJ (2000) Biochemical effects of a diet containing foods enriched with n-3 fatty acids. *American Journal of Clinical Nutrition* **72**, 42-48.

Mantzioris E, James MJ, Gibson RA, Cleland LG (1995) Differences exist in the relationships between dietary linoleic and α-linolenic acids and their respective long chain metabolites. *American Journal of Clinical Nutrition* **61**, 320-324.

Mantzioris E, James MJ, Gibson RA, Cleland LG (1994) Dietary substitution with α-linolenic acid rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. *American Journal of Clinical Nutrition* **59**, 1304-1309.

Marangoni F, Angeli MT, Colli S, Eligini S, Tremoli E, Sirtori CR & Galli C (1993) Changes of n-3 and n-6 fatty acids in plasma and circulating cells of normal subjects, after prolonged administration of

20:5 (EPA) and 22:6 (DHA) ethyl esters and prolonged washout. *Biochimica et Biophysica Acta* **1210,** 55-62.

Marignani PA, Sebaldt RJ (1995) Formation of second messenger diradylglycerol in murine peritoneal macrophages is altered after in vivo (n-3) polyunsaturated fatty acid supplementation. *Journal of Nutrition* **125**, 3030-3040.

Marshall LA, Johnstone PV (1985) The influence of dietary essential fatty acids on rat immunocompetent cell prostaglandin synthesis and mitogen-induced blastogensis. *Journal of Nutrition* **115**, 1572-1580.

May CL, Southworth AJ & Calder PC (1993) Inhibition of lymphocyte protein kinase C by unsaturated fatty acids. *Biochemical and Biophysical Research Communications* **195**, 823-828.

Mazari L, Lesourd B (1998) Nutritional influences on immune response in healthy aged persons. *Mechanisms of Aging and Development* **104** 25-40.

Mertin J, Hughes D, (1975) Specific inhibitive action of PUFA in lymphocyte transformation induced by PHA and PPD. *International Archive of Allergy and Applied Information.* **48,** 203-210.

McGowan MW, Artiss JD, Strandbergh DR, Zak N (1983) A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clinical Chemistry* **29**, 538.

Meydani SN, Yogeeswaran G, Liu S, Baskar S, Meydani M (1988) Fish oil and tocopherol induced changes in natural killer cell mediated cytotoxicity and PGE2 synthesis in young and old mice. *Journal of Nutrition* **118**, 1245-1252.

Meydani SN, Endres S, Woods MM, Goldin BR, Soo C, Morril-Labrode A, Dinarello C & Gorbach SL (1991) Oral (n-3) fatty acid supplementation suppresses cytokine and lymphocyte proliferation: comparison between young and older women. *Journal of Nutrition* **121,** 547-555.

Meydani SN, Lichtenstein Ah, Cornwall S, Meydani M, Goldin BR, Rasmussen H, Dinarello CA & Schaffer EJ (1993) Immunologic effects of national cholesterol education step-2 diets with and without fish-derived n-3 fatty acid enrichment. *Journal of Clinical Investigation* **92**, 105-113.

Miller RA (1992) Aging and immune function. *International Review of Immunology* **124** 890-904.

Miles EA and Calder PC (1998) Modulation of immune function by dietary fatty acids. *Proceedings of the Nutrition Society* **57**, 277-292.

Molvig J, Pociot F, Worsaae H, Wogensen LD, Baek L, Christensen P, Mandruppoulsen T, Andersen K, Madsen P, Dyerberg J, Nerup J (1991) Dietary supplementation with omega 3 polyunsaturated fatty acids decreases mononuclear cell proliferation and interleukin 1 beta content but not monokine

secretion in healthy and insulin dependent diabetic individuals. *Scandinavian Journal of Immunology* **34**, 399-410.

Moriguchi T, Greiner RS, Salem N (2000) Behavioural deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *Journal of Neurochemistry* **75** 2563-2573.

Morrhuauer H, Holman RT (1963) Effect of linolenic acid upon the metabolism of linoleic acid. *Journal of Nutrition* **81,** 67-74.

Mosmann TR, Coffman RL (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Reveiws in Immunology* **7**, 145-173.

Morrow WJ, Ohashi Y, Hall J, Pribnow J, Hirose J, Shirai T, Levy JA (1985) Dietary fat and immune function. I. Antibody responses, lymphocyte and accessory cell function in (NZB x NZW) F1 mice. *Journal of Immunology* **135**, 3857-3863.

Mosquera J, Rodriguez-Itrube B, Parra G (1990) Fish oil dietary supplementation reduces Ia expression in rat and mouse peritoneal macrophages. *Clinical Immunology and Immunopathology* **56**, 124-129.

Muggli R (1994) Polyunsaturated fatty acids and requirements of vitamin E . *Fett Wissenschaft Technologie-Fat Science Technology* **96**, 17-19.

Muller U, Jongeneel CV, Nedospasov SA, Fischer, Undahl K, Steinmetz M (1987) Tumour necrosis factor and lymphotoxin genes close to H-2D in the mouse major histocompatibility complex. *Nature* **3,** 265-267.

Murasko DM, Weiner P, Kaynes D (1987) Decline in mitogen induced proliferation of lymphocytes with increasing age. *Clinical and Experimental Immunology* **70** 440-448.

Murphy MG (1990) Dietary fatty acids and membrane protein function. *Journal of Nutritional Biochemistry* **1** 68-79.

Nafiger J, Bessege JP, Guillosson JJ, Damais C, Lesourd B (1993) Decreased capacity of Il-1 production by monocytes of infected elderly patients. *Aging, Immunology and Infectious Diseases* **4** 25-34.

Nash HL (1987) Can exercise make us immune to disease? *Physics and Sports Medicine* **1**,250-253.

Newsholme P, Newsholme EA (1989) Rates of utilisation of glucose, glutamine and oleate and formation of end products by mouse peritoneal macrophages in culture. *Biochemical Journal* **261**, 211-218.

Novak TJ, Rothenberg EV (1990) cAMP inhibits induction of interleukin 2 but not of interleukin 4 in T cells. *Proceedings of National Academy of Science USA* **87**, 9353-9357.

Opmeer FA, Adolfs MJ, Bonta IL (1984) Regulation of prostaglandin E_2 receptors in vivo by dietary fatty acids in peritoneal macrophages from rats. *Journal of Lipid Research* **25**, 262-268.

Ossman JB, Erickson KL, (1980) *Nutrition Research International* **22**, 279-284.

Paliogianni F, Kincaid RL, Boumpas DT (1993) Prostaglandin E2 and other cyclic AMP elecating agents inhibit interleukin 2 gene transcription by counteracting calcinuerin-dependent pathways. *Journal of Experimental Medicine* **178**, 1813-1817.

Panyani GS (1999) Targetting cells involved in the pathogenesis of rheumatoid arthritis. *Rheumatology* **38 (S2)** 8-10.

Parker CW (1987) Lipid mediators produced through the lipoxygenase pathway. *Annual Reviews in Immunology* **5**, 65-84.

Peterson LD, Thies F, Sanderson P, Newsholme EA, Calder PC (1998a) Low levels of eicosapentaenoic and docosahexanoic acids mimic the effects of fish oil upon rat lymphocytes. *Life Sciences* **62**, 2209-2217.

Peterson LD, Jeffrey NM, Thies F, Sanderson P, Newsholme EA, Calder PC (1998b) Eicosapentaenoic and docosahexaenoic acids alter rat spleen leukocyte fatty acid composition and prostaglandin E_2 production but have different effects on lymphocyte functions and cell-mediated immunity. *Lipids* **33**, 171-180.

Poon R, Richards JM, Clark R (1981) The relationship between plasma lipid composition and physical-chemical properties. II. Effect of phospholipid fatty acid modulation on plasma membrane physical properties and enzymatic activities. *Biochimica et Biophysica Acta* **649** 58-66.

Pufal DA, Quinlan PT, Salter AM (1995) Effect of dietary triacylglycerol structure on lipoprotein metabolism: A comparison of the effects of dioleoylpalmitoylglycerol in which palmitate is esterified to the 2- or 1 (3)-position of the glycerol. *Biochimica et Biophysica Acta* **1258**, 41-48.

Purasiri P, McKechnie A, Heys SD & Eremin O (1997) Modulation in vitro of human natural cytotoxicity, lymphocyte proliferative response to mitogens and cytokine production by essential fatty acids. *Immunology* **92**, 166-172.

Rao A (1991) Signalling mechanisma in T cells. *Critical Reveiws in Immunology* **10** 495-519.

Rabinowich H, Goses Y, reshef T, Klajman A (1985) Interleukin 2 production and activity in aged humans. *Mechanisms of Aging and Development* **32** 213-226.

Redgrave TG, Kodali DR, Small DM (1988) The effect of triacyl-sn-glycerol structure on the metabolism of chylomicrons and triacylglycerol-rich emulsions in the rat. *Journal of Biological Chemistry* **263**, 5118-5123.

Reed CF (1968) Phospholipid exchange between plasma and erythrocytes in man and the dog. *Journal of Clinical Investigation* **47**, 749-760.

Renier G, Skamene E, de Sanctis J, Radzioch D (1993) Dietary n-3 polyunsaturated fatty acids prevent the development of atherosclerotic lesions in mice: Modulation of macrophage secretory activities. *Arterio Thombosis* **13**, 1515-1524.

Richieri GV, Kleinfeld AM (1990) Free fatty acids inhibit cytotoxic T lymphocyte –mediated lysis of allogenic target cells. *Journal of Immunology* **145**, 1074-1077.

Rodgers JB (1998) N-3 fatty acids in the treatment of ulcerative colitis. In Kremer J, editor. Medicinal fatty acids in inflammation. Basel: Birkhauser Verlag, 1998. P. 103-109.

Roitt I (1997) Essential Immunology (9th Edition). Blackwell Science Publications.

Romagani S (2000) The role of lymphocytes in allergic disease. *Journal of Allergy and Clinical Immunology* **105** 399.408.

Roper RL and Phipps RP (1994) Prostaglandin E2 regulation of the immune response. *Advances in Prostaglandin Thromboxane Leukotriene Research* **22**, 101-111.

Roschlau P, Bernt E, Gruber W (1974) Enzymatische bestimmung des gesamt-cholesterins im serum. *Z. Klin. Chem. Klin Biochem.* **12**, 403-407.

Ross G, Stier J (1999) Lifetime risk of developing coronary heart disease. *Lancet* **353**, 924.

Rotondo D, Earl CR, Laing KJ, Kaimakamis D (1994) Inhibition of cytokine-stimulated thymic lymphocyte proliferation by fatty acids: the role of eicosanoids. *Biochimica et Biophysica Acta* **1223**, 185-194.

Sanderson P and Calder PC (1998) Dietary fish oil appears to prevent the activation of phospholipase C-gamma in lymphocytes. *Bichimica et Biophysica Acta* **1392**, 300-308.

Sanderson P, Yaqoob P & Calder PC (1995) Effects of dietary manipulation upon rat spleen lymphocyte functions and the expression of lymphocyte surface molecules. *Journal of Nutritional and Environmental medicine* **5**, 119-132.

Sanderson P & Calder PC (1998) Dietary fish oil diminishes lymphocyte adhesion to macrophage and endothelial cell monolayers. *Immunology* **94**, 79-87.

Santoli D, O'Connor R, Cesano A, Phillips P, Colt TL, Lange B, Clark SC, Rovera G (1990) Synergistic and antagonistic effects of IL 1 Alpha and IL 4, respectively, on the IL 2 dependent growth of a T cell receptor gamma, delta and human T leukaemia cell line. *Journal of Immunology* **144**, 4703-4711.

Schmidt EB, Varming K, Pederson JO, Lervang HH, Grunnet N, Jersild C & Dyerberg J (1992) Long-term supplementation with *n*-3 fatty acids, II: Effect on neutrophil and monocyte chemotaxis. *Scandanavian Journal of Clinical Laboratory Investigation* **52**, 229-236.

Schmidt EB, Pedersen JO, Varming K, Ernst E, Jersild C, Grunnet N, Dyerberg J (1991) N3 Fatty acids and leukocyte chemotaxis – effects in hyperlipidemia and dose response studies in healthy men. *Arteriosclerosis and Thrombosis* **11**, 429-435.

Schmidt EB, Kristensen SD, Decaterina R, Illingworth DR (1993) The effects of N3 fatty acids on plasma lipids and lipoproteins and other cardiovascular risk factors in patients with hyperlipidemia. *Atherosclerosis* **103**, 107-121.

Schmidt EB, Sorensen PJ, Pedersen JO, Jersild C, Ditzel J, Grunnet N, Dyerberg J (1989) The effect of N3 polyunsaturated fatty acids on lipids, hemostasis, neutrophil and monocyte chemotaxis in insulin dependent diabetes mellitus. *Journal of Internal Medicine* **225**, 201-206.

Schmidt EB, Varming K, Moller JM, Bulow Pederson I, Madsen P, Dyerberg J (1996) No effect of very low dose n-3 fatty acids on monocyte function in healthy humans. *Scandinavian Journal of Clinical Investigation* **56**, 87-92.

Schroit AJ, Gailly R (1979) Macrophage fatty acid composition and phagocytosis: Effect of unsaturation on cellular phagocytic activity. *Immunology* **35**, 199-205.

Serhan CN (1996) Lipid mediator networks in cell signalling *FASEB* **10** 1147-1158.

Shankar AH and Prasad AS (1998) Zinc and immune function: the biological basis of altered resistance to infection. *American Journal of Clinical Nutrition* **68**, 447S-463S.

Sherman AR and Spear AT (1993) Iron and immunity. In: Nutrition and Immunology, pp 285-307 [DM Klurfield, editor]. New York & London: Plenum Press.

Sherrington EJ, Harvey DJ, Calder PC (1995a) The effects of dietary lipid manipulation on macrophage phospholipid fatty acid composition and membrane fluidity. *Proceedings of the Nutrition Society* **54**, 121A.

Sherrington EJ, Snaderson P, Calder PC (1995b) The effect of dietary lipid manipulation on macrophage cell surface molecule expression. *Biochemical Society Trans* **23**, 272S.

Shimuzu S *et al.*(1980) *Analytical Biochemistry* **107**, 193-198.

Singer SJ and Nicolson GL (1972) *Science* **175** 720-731.

Simpolous AP (1991) Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition 52*, 438-463.

Sipka S, Dey I, Buda C, Csongor J, Szegedi G, Farkas T (1996) The mechanism of inhibitory effect of eicosapentaenoic acid on phagocytic activity and chemotaxis of human neutrophil granulocytes. *Clinical Immunology and Immunopathology*. **79**, 224-228

Small DM (1991) The effects of glyceride structure on absorption and metabolism. *Annual Reveiws in Nutrition* **11** 413-434.

Snyder SH, Bredt DS (1992) Biological roles of nitric oxide. *Scientific American* **66**, 68.

Snyder DS, Beller DI, Unanue ER (1982) Prostaglandins modulate macrophage Ia expression. *Nature* **299**, 163-165.

Somers SD, Erickson KL (1994) Alteration of tumour necrosis factor-α production by macrophages from mice fed diets high in eicosapentaenoic and docosahexaenoic acids. *Cellular Immunology* **153**, 287-297.

Soyland E, Nenster MS, Braathen L & Devron CA (1993) Very long chain *n*-3 and *n*-6 polyunsaturated fatty acids inhibit proliferation of human T lymphocytes *in vitro*. *European Journal of Clinical Investigation* **23**, 112-121.

Soyland E, Lea T, Sandstad B & Drevon CA (1994) Dietary supplementation with very long chain n-3 fatty acids in man decreases the expression of the interleukin-2 receptor (CD25) on mitogen-stimulated lymphovcytes from from patients with inflammatory skin diseases. *European Journal of Clinical Investigaiton* **24**, 236-242.

Spector AA, Mathur SN, Kaduce TL, Hyman BT (1981) Lipid nutrition and metabolism of cultured mammalian cells. *Progress of Lipid Research* **19**, 155-186.

Spector AA, Steinberg D, Tanake A (1965) *Journal of Biological Chemistry* **240**, 1032-1041.

Sperling RI, Benincaso AI, Knoell CT, Larkin JK, Austen KF, Robinson DR (1993) Dietary omega 3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. *Journal of Clinical Investigation* **91**, 651-660.

Spolarics Z, Lang CH, Bagby G and Speizer JJ (1991) Glutamine and fatty acid oxidation are the main sources of energy in Kuppfer and endothelial cells. *American Journal of Physiology* **261**, G185-G190.

Springer TA (1990) Adhesion receptors and the immune system. *Nature* **346**, 425-433.

Stoolman LM (1989) Adhesion molecules controlling lymphocyte migration. *Cell* **56** 907-910.

Stray N, Letnes H and Blomhoff JP (1990) Intracellular regulation of lipoprotein lipase in human monocyte-derived macrophages. *Biochemica and Biophysica Acta* **1045**,280-284.

Stremmel W (1988) Uptake of fatty acids by jejunal mucosal cells is mediated by a fatty acid binding membrane protein. *Journal of Clinical Investigation* **82**, 2001-2010.

Stremmel W, Kleinert H, Fitscher BA, Gunawan J, Klaassen Schulter C, Moller K, Wegener M (1992) Mechanisms of cellular fatty acid uptake. *Biochemical Society Transaction* **20**, 814-817.

Stubbs CD and Smith AD (1984) The modification of mammalian membrane polyunsaturated fatty acid composition in relation t membrane fluidity and function. *Biochimica et Biophysica Acta* **779** 89-137.

Sumida C, Graber R, Nunez E (1993) Role of fatty acids in signal transduction: modulators and messengers. *Prostaglandins, Leukotrienes, Essential Fatty Acids* **48**, 117-122.

Summers LKM, Fielding BA, Ilic V, Quinlan PT, Frayn KN (1998) The effect of triacyl-fatty acid positional distribution on postprandial metabolism in subcutaneous adipose tissue. *British Journal of Nutrition* **79**, 141-147.

Surette ME, Whelan J, Lu GP, Hardardottir I, Kinsella JE (1995) Dietary N 3 polyunsaturated fatty acids modify syrian hamster platelet and macrophage phospholipid fatty acyl composition and eicosanoid synthesis – a controlled study. *Biochimica et Biophysica Acta – Lipids and Lipid Metabolism* **1255**, 185-191.

Suziki K, Hara M and Kitani A (1990) Augmentation of LDL receptor activities on lymphocytes by interleukin-2 and anti CD3 antibody: a flow cytometric analysis. *Biocemica and Biophysica Acta* **1042**, 352-363.

Suziki H, Kawarabayashi Y, Kando J, Abe T, Nishiwaka K, Kimura S, Hashimoto T, Yamamoto T (1990) *Journal of Biological Chemistry* **265** 8681-8685.

Takahashi S, Kawarbayasi Y, Nakai T, Sakai J, Yammato T (1992) Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. *Proceedings National Academy of Science* **89**, 9252-9256.

Tappia PS & Grimble RF (1994) Complex modulation of cytokine induction by endotoxin and tumour necrosis factor from peritoneal macrophages of rats by diets containing fats of different saturated, monounsaturated and polyunsaturated fatty acid composition. *Clinical Science* **87**, 173-178.

Tappia PS, Troughton KL, Langley-Evans SL and Grimble RF (1995) Cigarette smoking influences cytokine production and antioxidant defences. **88**, 485-489.

Tappia PS, Man WJ, Grimble RF (1995) Influence of unsaturated fatty acids on the production of tumour necrosis factor and interleukin-6 by rat peritoneal macrophages. *Molecular and Cellular Biochemistry* **143**, 89-98.

Tappia PS, Ladha S, Clark DC, Grimble RF (1997) The influence of membrane fluidity, TNF receptor binding, cAMP production and GTPase activity on macrophage cytokine production in rats fed a variety of fat diets. *Molecular Cell Biochemistry* **166**, 135-143.

Thies F, Peterson LD, Powell JR, Nebe-von-Caron G, Hurst TL, Matthews KR, Newsholme EA, Calder PC (1999) Manipulation of the type of fat consumed by growing pigs affects plasma and mononuclear cell fatty acid compositions and lymphocyte and phagocyte functions. *Journal of Animal Science* **77**, 137-147.

Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC (2001a) Dietary supplementation with eicosapentaenoic acid, but not with other long chain n 3 or n 6 polyunsaturated fatty acids, decreases natural killer cell activity in healthy subjects aged >55 y. *American Journal of Clinical Nutrition* **73**, 539-548.

Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC (2001b) Dietary supplementation with gamma linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans. *Journal of Nutrition* **131**, 1918-1927.

Thompson PJ, Misso NLA, Passarelli M, Phillips MJ (1991) The effect of eicosapentaenoic acid consumption on human neutrophil chemiluminescence. *Lipids* **26**, 1223-1226.

Trotter J, Flesch I, Schmidt B, Ferber E (1982) Acyltransferase-catalyzed cleavage of arachidonic acid from phospholipids and transfer to lysophosphatides in lymphocytes and macrophages. *Journal of Biological Chemistry* **257** 1816-1823.

Tsang WM, Weyman C, Smith AD (1977) *Biochemical Society Transactions* **5**, 153-154.

Turek JJ, Schoenlein IA, Bottoms GD (1991) The effect of dietary n-3 and n-6 fattya cids on tumour necrosis factor-α production and leucine aminopeptidase levels in rat peritoneal macrophages. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **43**, 141-149.

Turek JJ, Schoenlein IA, Clark LK & van Alstine WG (1994) Dietary polyunsaturated fatty acids effects on immune cells of the porcine lung. *Journal of Leukocyte Biology* **56**, 599-604.

Ulbricht TLV & Southgate DAT (1991) Coronary heart disease: seven dietary factors. *The Lancet* **338**, 985-962.

Valge VE, Wong JG, Datlof BM, Sinskey AJ, Rao A (1988) Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell* **55**, 101-112.

Varming K, Schmidt EB, Svaneborg N, Moller JM, Lervang HH, Grunnet N, Jersild C, Dyerberg J (1995) The effect of n 3 fatty acids on neutrophil chemiluminescence. *Scandinavian Journal of Clinical and Laboratory Investigation* **55**, 47-52.

Veillette (1988) The CD 4 and CD8 T cell surface antigens are associated with the internal mambrnae tyrosine-protein kinase p56lck. *Cell* **55** 301.308.

Vermunt SHF, Mensink RP, Simonis AMG & Hornstra G (1999) Effects of age and dietary n-3 fatty acids on the metabolism of [^{13}C]-α-linolenic acid. *Lipids* **34**, S127.

Virella G, Kilpatrick JM, Rugeles MT, Hyman B, Russel R (1989) Depression of humoral responses and phagocytic functions in vivo and in vitro by fish oil and eicosapentaenoic acid. *Clinical immunology Immunopathology* **52**, 257-270.

Virella G, Fourspring K, Hyman B, Haskill-Stroud R, Long L, Virella I, La Via M, Gross AJ & Lopes-Virella M (1991) Immunosuppressive effects of fish oil in normal human volunteers: Correlation of the in vitro effects of eicosapentaenoic acid on human lymphocytes. *Clinical immunology Immunopathology* **61**, 161-176.

Volker D, Garg M (1996) Dietary n-3 fatty acid supplementation in rheumatoid arthritis- Mechanisms, clinical outcomes, controversies, and future directions. *Journal Clinical Biochemical Nutrition* **20**, 83-97.

Wallace FA, Miles EA, Calder PC (2000a) Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages. *Cytokine* **12** (9) 1374-1379.

Wallace FA, Miles EA, Evans C, Stock TE, Yaqoob P, Calder PC (2001) Dietary fatty acids influence the production of Th1, but not Th2, type cytokines. *Journal of Leukocyte Biology* **69**, 449-457.

Wallace JMW, McCabe AJ, Robson PJ, Keogh MK, Murray CA, Kelly PM, Marquez-Ruiz G, McGlynn H, Gilmore WS, Strain JJ (2000b) Bioavailability of n-3 polyunsaturated fatty acids (PUFA) in foods enriched with microencapsulated fish oil. *Annals of Nutrition and Metabolism* **44**, 157-162.

Wander RC, Hall JA, Gradin JL, Du S, Jewell DE (1996) The ratio of (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs. *Journal of Nutrition* **127**, 1198-1205.

Wander RC, Hall JA, Gradin JL, Du S-H & Jewell (1997) The ratio of dietary (n-6) to (n-3) fatty acids and antioxidants on immunoglobulin production by mesenteric lymph nodes lymphocytes of Sprague-Dawly rats. *Journal of Biochemistry* **120**, 138-144.

Wang CS, Kuksis A, Manganaro F (1982) Studies on the substrate specificity of purified human milk lipoprotein lipase. *Lipids* **17** 278-284.

Watanabe S, Hayashi H, Onozaki K, Ojuyama H (1991) Effect of dietary α-linolenate/linoleate balance on lipopolysaccharide-induced tumour necrosis production in mouse macrophages. *Life Sciences* **48**, 2013-2020.

Weyman C, Mugant SJ, Bilkin , Smith AD (1977) *Biochimica et Biophysica Acta* **496**, 155-166.

Wolf M Droege W (1982) Inhibition of cytotoxic responses by prostaglandin E2 in the presence of interleukin 2. *Cell Immunology* **72**, 286-293.

Wu D & Meydani SN (1998) n-3 Polyunsaturated fatty acids and immune function. *Proceedings of the Nutrition Society* **57**, 503-509.

Wu D, Meydani SN, Meydani M, Hayek MG, Huth P & Nicolosi RJ (1996) Immunologic effects of marine- and plant- derived n-3 polyunsaturated fatty acids in nonhuaman primates. *American Journal of Clinical Nutrition* **63**, 273-280.

Wu DY, Meydani M, Leka LS, Nightingale Z, Handelman GJ, Blumberg JB, Meydani SN (1999) Effect of dietary supplementation with blackcurrant seed oil on the immune response of healthy elderly subjects. *American Journal of Clinical Nutrition* **70**, 536-543.

Yam D, Eliraz A, Berry EM (1996) Diet and Disease - The Israeli paradox: Possible dangers of a high omega-6 polyunsaturated fatty acid diet. *Israeli Journal of Medical Science* **32**, 1134-1143.

Yamada K, Hung P, Yoshimura K, Taniguchi S, Lim BO & Sugano M (1996) Effect of unsaturated fatty acids and antioxidants on immunoglobulin production by mesenteric lymph-node lymphocytes of Sprague-Dawley rats. *Journal of Biochemistry* **120**, 138-144.

Yamashita N, Yokoyama A, Hamazaki T, Yano S (1986) Inhibition of natural killer cell activity of human lymphocytes by eicosapentaenoic acid.. *Biochimica et Biophysica Research Communications* **138**, 1058-1067.

Yamashita N, Sugiyama E, Hamazaki T, Yano S (1988) Inhibition of natural killer cell activity by eicosapentaenoic acid *in vivo* and *in vitro*. *Biochimica et Biophysica Research Communications* **150**, 497-505.

Yamashita N, Maruyama M, Yamazaki K, Hamazaki (1991) Effect of eicosapentaenoic and docosahexaenoic acid on natural killer cell activity in human peripheral blood lymphocytes. *Clinical Immunology and Immunopathology* **59**, 335-345.

Yaqoob P, Newsholme EA & Calder PC (1994a) The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. *Immunology* **82**, 603-610.

Yaqoob P, Newsholme EA, Calder PC (1994b) Inhibition of natural killer cell activity by dietary lipids. *Immunology Letters* **41**, 241-247

Yaqoob P, Newsholme EA & Calder PC (1995a) Influence of cell culture conditions on diet-induced changes in lymphocyte fatty acid composition. *Biochimica et Biophysica Acta* **1255**, 333-340.

Yaqoob P, Newsholme EA & Calder PC (1995b) The effect of fatty acids on leukocyte subsets and proliferation in rat whole blood. *Nutrition Research* **15**, 2, 279-287.

Yaqoob P & Calder PC (1995a) The effects of dietary lipid manipulation on the production of murine T-cell-derived cytokines. *Cytokine* **7**, 548-553.

Yaqoob P & Calder PC (1995b) Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. *Cellular immunology* **163**, 120-128.

Yaqoob P, Knapper J, Webb, D, Williams C, Newsholme EA & Calder PC (1998) Effect of olive oil on immune function in middle-aged men. *American Journal of Clinical Nutrition* **67**, 603-610.

Yaqoob P, Pala HS, Cortina-Borja M, Newsholme EA, Calder PC (2000) Encapsulated fish oil enriched in α–tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. *European Journal of Clinical Investigation* **30**, 260-274.

Yi , PI, Beck G and Zucker S (1981) Rat plasma lipoprotein inhibitor of lymphocyte proliferation: specific membrane receptor for very low density lipoproteins. *International Archives Allergy and Applied Immunology* **65**, 8-14.

Yoshida H, Mawatari M, Ikeda I, Imaizumi K, Seto A, Tsuji H (1999) Effect of dietary seal and fish oils on triacylglycerol metabolism in rats. *Journal of Nutritional Science and Vitaminology* **45**, 411-421.

Yoshino S & Ellis EF (1987) Effect of fish oil –supplemented diet on inflammation and immunological processes in rats. *International Archives of Allergy and Applied Immunology* **84**, 233-240.

Ziboh VA. (1998) The role of n-3 fatty acids in psoriasis. In: Kremer J, editor. Medicinal fatty acids in inflammation.Basel: Birkhauser Verlag, 1998. P. 45-53.